Abstract. Development of novel targeted therapy holds promise for conquering chemotherapy resistance, one of major hurdles in current liver cancer treatment. We found that long non-coding RNA TUC338 is involved in the development of hepatocellular carcinoma (HCC) and sorafenib resistance. HCC cell lines were transfected with siTUC338, then cell proliferation and invasion ability were investigated by MTT and Transwell assay. Sorafenib resistance HepG2 cells were generated to test the role of TUC338 in sorafenib sensitivity. Intratumoral delivering of siTUC338 was used to analyze the sorafenib treatment response in HepG2/Sor xenografts in vivo. Higher levels of TUC338 were found both in HCC tissues and cell lines, knockdown of TUC338 was accompanied with increased expression of RASAL1 in HCC cell line with increased proliferation and invasion ability, knockdown of TUC338 could activate the RASAL1 pathway and inhibit tumor growth genes by directly targeting RASAL1 3'-UTR. Furthermore, knockdown of TUC338 in HepG2 sorafenib sensitized its reaction to the treatment of sorafenib, which was accompanied by increased expression RASAL1; intratumoral delivering of siTUC338 could also restore sorafenib treatment response in HepG2/Sor xenografts in vivo. These findings provide direct evidence that the TUC338/RASAL1 axis might play an essential role in sorafenib-resistance of liver cancer cells, suggesting the signaling cohort could serve as a novel therapeutic target for the treatment of chemotherapy resistant liver cancer.

Introduction

Hepatocellular carcinoma (HCC) is the most common primary liver tumor and the third most frequent cause of cancer-related death worldwide (1). Surgical resection and traditional chemotherapy are typical forms of treatment for patients with HCC (2). Sorafenib (Nexavar) is the first and only targeted therapy to clinically improve overall survival in patients with advanced HCC, and has given hope to researchers for effective agents to combat HCC (3), but the clinical response is seriously limited by drug resistance. To develop optimum strategy for overcoming sorafenib treatment resistance is one of the major concerns in currently liver cancer treatment. Long-lasting endeavors have been made in identifying genes inducing chemoresistances in the past decades (4,5). However, the link between specific gene and sorafenib treatment resistance in liver cancer therapy remains unclear.

Non-coding RNAs (ncRNA) have recently been implicated in hepatocarcinogenesis and tumor progression, represent promising targets for cancer (6), ncRNAs include short and long non-coding RNAs (7). Short ncRNAs have a length of under 200 nucleotides (nt) while long ncRNAs (IncRNAs) are more than 200 nt in length, frequently ranging up to 100 kb (8). Recent studies estimated that the number of IncRNAs in humans is approximately 15,000 and found that most IncRNAs displayed tissue-specific expression patterns (9). Increasing evidence identified that IncRNAs epigenetically regulate the expression of genes involved in fundamental cellular processes such as cell proliferation, apoptosis, differentiation, and migration, suggesting their potential oncogenic or tumor suppressing roles in cancer development (10). The TUC338 (transcribed ultra-conserved region 338) gene was first identified as uc.338, along with 480 other ultra-conserved elements in the human genome. Expression of this RNA gene has been found to markedly increase in hepatocellular carcinoma (HCC) cells (11). Moreover, TUC338 expression correlates with disease progression, and raises the possibility that it may be involved in malignant transformation (12). It is also...
reported that lost restoration of TUC338 in liver cancer cells inhibit their cell proliferation and invasion (13), indicating a functional role in liver cancer cell growth.

RAS proteins control many cellular processes, including cell migration, proliferation, differentiation, and survival. The RAS GTPase-activating protein (RasGAP) gene RASAL1 has been demonstrated as a tumor suppressor gene which act as a negative modulator of the RAS signaling pathway by catalyzing RAS inactivation (14). RASAL1 expression is decreased in many tumors, including colorectal tumor (15), thyroid cancer (16), gastric cancer (17), prostatic cancer and bladder cancer (18). However, no data have been reported on its association with chemoresistance. RASAL1 is stimulated also possibly in the development of resistance of tumor cells to chemotherapy and to ionizing radiation (20), RASAL1 might inhibit tumor development and sensitivity to drug-therapy by negatively regulating RAS activation.

Based on findings that the TUC338 and RASAL1 functionally regulate the tumor progression, we postulated that TUC338 might play a role in the chemotherapy resistance of liver cancer cells, which might be mediated by its regulation of the expression of RASAL1. In the present study, we explored the functional significance of TUC338/RASAL1 axis in the sorafenib-resistance in liver cancer cells by in vitro and in vivo studies.

Our results showed that elevated expression of TUC338 was found in HCC tissues and cell lines, and knockdown TUC338 in liver cancer cells inhibit the cell proliferation and invasion ability accompanied with the overexpression of RASAL1. Furthermore, knockdown TUC338 inhibited the RASAL1 pathway and decreased the tumor growth genes, by increase the RASAL1 transcription. Silencing of TUC338 in HepG2/Sor cells could also decrease the cell proliferation and eradicated its resistance to sorafenib in vitro and in vivo. These findings suggest that TUC338 might promote sorafenib-resistance in liver cancer, which is mediated via its regulation of RASAL1 and this might be a novel therapeutic target for sorafenib-resistant liver cancer therapy.

Materials and methods

Samples. Twelve pairs of HCC samples and adjacent non-tumor tissues were obtained from surgical specimens at department of hepatobiliary surgery, the first affiliated hospital of Wenzhou Medical University (Wenzhou, China) after informed consent. Clear hepatocellular carcinoma was diagnosed histopathologically. All these specimens were snap-frozen in liquid nitrogen after excision. The study methodologies conformed to the standards set by the Declaration of Helsinki. Informed consent was obtained from each participant, collection and usage of all specimens were approved by the local ethics committee.

Cell culture and reagents. The human hepatoma cell lines, HepG2, SMMC-7721, BEK-7402, Hep3B, and Huh-7, and the liver cell line L02 were purchased from the cell bank of Type Culture Collection at the Chinese Academy of Sciences (Shanghai, China). The cells were maintained in RPMI-1640 or DMEM supplemented with 10% fetal bovine serum (FBS), 100 U/ml penicillin and 100 µg/ml streptomycin at 37°C in 5% CO2, incubation. Sorafenib (BAY 43-9006) was purchased from MedChem Express (Princeton, NJ, USA) and dissolved in DMSO. The final DMSO concentration was <0.1%.

siRNA and transfection. For TUC338 knockdown experiments, siRNA was designed and synthesized by GenePharma Co., Ltd (Shanghai, China), and the sequences were as follows: si-TUC338: SenseSeq: 5'-CCCCAGGACAGGUACAGC ATT-3', AntiSeq: 5'-UCAGUUCAGGCGUCATT-3'; si-NC: SenseSeq: 5'-UUCUGCCAACGUAGCUTT-3', AntiSeq: 5'-ACGUGACACGUCCAGAATT-3'. HepG2, SMMC-7721 and Huh-7 cells were plated at a density of 2x10^3 cells/well in 6-well plates, overnight culture, cells were transfected with si-NC or si-TUC338 by Lipo2000 (11668019, Life Technology) using 100 nM/well siRNA in a 6-well plate following the manufacturer's instructions. At 48 h after transfection, total protein and RNA samples were prepared, proliferation assay, invasion assay, luciferase assay were carried as described below.

Real-time PCR. Total RNA was extracted using TRIZol® reagent (Invitrogen). DNaseI-treated RNA was used for first strand cDNA synthesis using M-MLV reverse transcriptase (Promega) and oligo (dT) 15 according to the manufacturer's protocols and 1 µl cDNA samples were used for conventional PCR amplification. Real-time quantitative PCR analysis was performed in a real-time PCR system (StepOne, Applied Biosystems) and the expression levels of TUC338 were normalized to 18s determined by a SYBR Green-based comparative cycle threshold CT method. Real-time PCR primers were: TUC338-F: 5'-GGTGGGAGGGGATGGTGTCAGT-3', TUC338-R: 5'-TGGTTGAATAGGGTTGGGGG-3'; RASAL1-F: 5'-TGATTTCTCTTCTTGGCATCTT-3', RASAL1-R: 5'-GATTTGCGTAATCGAGCTGAA-3'; VEGF-F: 5'-AGGGCAGAATCATCACGAAGT-3', VEGF-R: 5'-AGGGTCTCGATTGGATGGCA-3'; GLUT-1-F: 5'-GGCATGTGTA-3', GLUT-1-R: 5'-ACGCTTCCCTACCTGGTGAT-3'; 18s-F: 5'-AGGGTCTCGATTGGATGGCA-3', 18s-R: 5'-AGGGTCTCGATTGGATGGCA-3'.

Western blotting. Total proteins were extracted with RIPA buffer containing proteinase/phosphatase inhibitors (Thermo Fisher Scientific, Cambridge, MA, USA). Thirty micrograms of cell lysate and tumor tissue lysate were separated on 12% sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) gels and then transferred to Immobilon-P transfer membrane (Millipore). Specific anti-RASAL1 (ab170711, 1:1000 dilution), anti-VEGF (ab1316, 1:1000 dilution), anti-GLUT-1 (ab115730, 1:1000 dilution), anti-P-glycoprotein (ab170904, 1:1000 dilution), anti-β-actin (ab3280, 1:3000 dilution) primary antibodies (Abcam, USA) were used, and HRP conjugated immunoglobulin was used as a secondary antibody (Zen BioScience, Chengdu, China). Immuno blot analysis was performed with the indicated antibodies and visualized with Immobilon Western (Millipore), which were quantified using...
Cells were seeded at 5x10^3 cells/well and cultured seeded according to their doubling time.

In vitro cell growth assays

**Cell counting.** Viable cells were counted as previously described (21). When the cells were cultured to 70-80% confluence, sorafenib was added into the medium for different concentrations at different time periods. All counts were performed on triplicate wells and repeated in three independent experiments. The results were reproduced in three independent experiments. The results are expressed as mean ± SEM of three independent experiments. **P<0.001** compared to LO2 group. Data are shown as mean ± SEM of three independent experiments.

Figure 1. Upregulation of TUC338 in human hepatocarcinoma (HCC) tissues and cell lines. (A) Real-time PCR analysis of TUC338 expression in 12 HCC specimens compared to their pair-matched adjacent non-tumor tissues (NTT). Expression levels were normalized to 18s and expressed as relative change compared to NTT. (B) Real-time PCR analysis of TUC338 expression in the liver cell line L02 and HCC cell lines HepG2, SMMC-7721, BEL-7402, Hep3B, and Huh-7. Expression levels were normalized to 18s and expressed as relative change compared to L02. Data are shown as mean ± SEM of three independent experiments. The results are expressed as mean ± SEM of three independent experiments. **P<0.01, ***P<0.001 compared to L02 group. Data are shown as mean ± SEM of three independent experiments.

**MTT assay.** Cells were seeded at 5x10^3 cells/well and cultured under various concentration of sorafenib for different time periods. Viable cells were determined by MTT assay as previously described (22).

**In vivo tumor study.** Male nude mice (4-6 weeks, 18-20 g) were purchased from the Model Animal Research Center of Nanjing University. The animals were housed in a temperature- and humidity-controlled room with a 12-h on-off light cycle and given free access to food and water. To establish the xenograft mouse model, HepG2/Sor sorafenib-resistant cancer cells were diluted with phosphate-buffer saline (PBS) at a concentration of 2x10^7 cells/ml, and 200 µl HepG2/Sor cells were injected subcutaneously into the left back limb of each mouse. Tumor volumes were determined every five days after injection and calculated as previously described (23). Mice with xenograft volume >500 mm^3 were treated with 4 mg/kg sorafenib (Sor) intraperitoneally (i.p.) combined with intratumoral injection of physiological saline twice a week (Sor+saline group), siNC (Sor+siNC group) and siTUC338 (Sor+siTUC338 group) for four weeks. Mice were sacrificed and tumors were dissected for real-time PCR and western blot analysis. The animal study was carried out in strict accordance with the recommendations in the guide for the care and use of laboratory animals of the First Affiliated Hospital of Wenzhou Medical University and Research Institute Animal Care and Use Committee. All the protocols were approved by the First Affiliated Hospital of Wenzhou Medical University and Research Institute Animal Care and Use Committee (approval number 21040608). Surgery was performed under sodium pentobarbital anesthesia, and all efforts were made to minimize suffering.

**Plasmid construction and luciferase assay.** To determine whether the entire human RASAL1 3'-untranslated region (UTR) segment was amplified by PCR homo-genomic DNA was used as a template. The PCR products were inserted into the pGL3-Basic plasmid (Ambion). For luciferase reporter assays, 1 µg of firefly luciferase reporter plasmid, 0.5 µg of β-galactosidase expression vector (Ambion), and equal amounts (200 pmol) of siNC or siTUC338 were transfected into cells in 6-well plates. The β-galactosidase vector was used as a transfection control. At 48 h after transfection, cells were assayed using luciferase assay kits (Promega).

**Statistical analysis.** The results are expressed as mean ± SEM from at least three independent experiments. Significance analysis of normally distributed data were performed using two-tail Student's t-test and P-values of <0.05 and 0.01 were considered significant and highly significant, respectively.

**Results**

**TUC338 expression is significantly increased in HCC tissues and cell lines.** To determine whether TUC338 was involved in regulation of human HCC tumorigenesis, we first detected TUC338 levels in HCC tumor and adjacent non-tumor tissues, using real-time PCR (n=12). As shown in Fig. 1A, TUC338 expression was significantly upregulated in HCC tissues compared to normal adjacent liver tissue. We also analyzed TUC338 expression in liver cell line L02 and five human HCC cell lines (HepG2, SMMC-7721, BEK-7402, Hep3B, and Huh-7). Consistently, TUC338 was increased in all HCC cell lines examined compared to L02 cells (Fig. 1B). Taken together, these findings suggested that TUC338 is upregulated in human HCC.

**Inhibition of TUC338 decreases cell proliferation and invasion by activation of RASAL1.** Functional role of TUC338 in liver cancer cells was explored by siRNA knockdown TUC338...
in HepG2, SMMC7721 and Huh-7 cells which showed higher basal expression levels of TUC338. It was observed that the expression of TUC338 was significantly downregulated 48 h after transfection with siTUC338 by Lipo2000 in mRNA levels as compared with the siNC control (Fig. 2A). Moreover, the western blot assay showed that transduction of siTUC338 in HCC cells could increase the mRNA and protein levels of RASAL1 (Fig. 2B). These findings provide evidence that TUC338 regulated the expression of RASAL1, regarded as tumor suppressor in many tumors (14,24).

To establish whether siTUC338 plays a suppressing role in HCC tumorigenesis, we analyzed cell viability using MTT assay and invasion ability using Transwell assay in HCC cells transfected with siTUC338. As shown in the left panel of Fig. 2C, knockdown of TUC338 markedly reduced HCC cell viability in HepG2, SMMC7721 and Huh-7 cell lines. Similarly, siTUC338 also significantly decreased invasion ability of human HCC cells (Fig. 2C right panel) in the three cell lines.

siTUC338 activates RASAL1 signaling pathway and inhibits tumor growth genes by directly targeting RASAL1. We next used real-time PCR and western blot to examine whether TUC338 knockdown results in activation of RASAL1 signaling pathway. The immunoblot assay showed an increase...
in the phospho-MEK1/2, phospho-ERK1/2 and phospho-Akt in TUC338 knockdown HCC cells (Fig. 3A). Downregulated expression of tumor growth genes vascular endothelial growth factor (VEGF), glucose transporter 1 (GLUT-1), and multidrug resistance gene (MDR1), produces P-glycoprotein; (P-gp) at the transcriptional and the translational levels were also detected after TUC338 knockdown (Fig. 3B and C). Moreover, to determine whether TUC338 could affect the transcriptional activity of RASAL1, we co-transfected RASAL1 luciferase reporter plasmid with siNC or siTUC338 into HepG2, SMMC7721 and Huh-7 cells. Of note, siTUC338 increased the relative luciferase activity in a dose-dependent manner (Fig. 3D). Collectively, our data demonstrate the functional link between TUC338 and the RASAL1 signaling pathway in human HCC cells.

Knockdown of TUC338 sensitizes HCC cells to sorafenib. The sorafenib resistant subline HepG2/Sor was generated by intermittent exposure of sensitive parental HepG2 cells to 30 ng/ml sorafenib in DMEM culture medium. We treated HepG2, HepG2/Sor and siTUC338 or siNC transfected HepG2/Sor cells with sorafenib and measured cell viability and counted the cell number. Cell viability and cell number results showed that transfected siNC HepG2/Sor cells are highly resistant to sorafenib.
sorafenib and that transfection with siTUC338 significantly reduced sorafenib resistance (Fig. 4A). Next, we investigated the mRNA and protein levels of TUC338 and RASAL1 in HepG2/Sor cells using real-time PCR and western blot assay. As shown in Fig. 4B and C, TUC338 was overexpressed and RASAL1 was significantly downregulated both in mRNA and protein levels in HepG2/Sor. Our data revealed that knockdown of TUC338, an lncRNA which was elevated in HCC tissues and cell lines, could sensitize HCC cells to sorafenib by upregulation of RASAL1, a major tumor suppressor.

**Knockdown TUC338 helps to increase sorafenib treatment response in sorafenib-resistant in vivo via increasing the expression of RASAL1.** Since IncRNA-TUC338 is capable of regulating RASAL1 expression in vitro, we next used the xenograft tumor nude mouse model to investigate the effect of endogenous TUC338 on RASAL1. After 4 weeks of treatment, the mean tumor volume in the Sor+siTUC338 group was statistically lower compared to that in Sor+siNC group and Sor group (Fig. 5A).

The TUC338 levels in tumor tissues were evaluated by real-time PCR. After intratumoral injection with siTUC338, the TUC338 was significantly decreased in the Sor+siTUC338 group compared with the siNC and physiological saline control group (Fig. 5B).

To understand the functions of TUC338 on the sorafenib treatment by regulating the expression of RASAL1, RASAL1 mRNA and protein levels were valued. Real-time PCR and western blots showed that the expression of RASAL1 both in mRNA and protein levels were upregulated after intratumoral injection with siTUC338 (Fig. 5C).

**Discussion**

Resistance to the treatment of sorafenib is one of the major obstacles in current metastatic liver cancer chemotherapy (25,26). Increasing evidence indicates that targeting IncRNA is a promising strategy to improve the efficacy of treatment (27). Although great endeavors have been made, more and more IncRNAs have been discovered as effective chemotherapy inducers or inhibitor, which may be attributed to the complexity of the development of chemotherapy resistance.

To this end, we focused on TUC338, an IncRNA which is overexpressed in liver cancer and may act as a tumor inducer, to illustrate the function of IncRNA in the development process of chemoresistance in liver cancer in vitro and in vivo. Small interfering RNA (siRNA) was used to decrease the expression of TUC338 in HCC cell lines, and its inhibition effect in the proliferation and invasion ability was observed, consistent with previous studies. Noteworthy, the downregulation of
TUC338 was accompanied with the upregulation of RASAL1, the activation of RASAL1 pathway and the inhibition of tumor growth genes and proteins. Luciferase assay showed that siTUC338 increased the relative RASAL1 promoter luciferase activity in a dose-dependent manner, and demonstrated the direct functional link between TUC338 and the RASAL1.

As a tumor suppressor, RASAL1 was found to inhibit cancer progression and as a negative modulator of the RAS signaling pathway by catalyzing RAS inactivation. It was evidenced that enforced expression of RASAL1 in gastric cancer cells could suppress cell proliferation and the transformation ability through RAS/ERK signaling pathway (17,24), while activated RAS is involved not only in tumor progression but also possibly in the development of resistance of tumor cells to chemotherapy and ionizing radiation, suggesting targeting this oncogenic protein may be a potential strategy for the treatment of chemoresistance. To this end, a sorafenib-resistant liver cancer cell line, HepG2/Sor, was established, which could simplify and better mimic the development process of chemoresistance in liver cancer in vivo. We observed decreased expression of RASAL1 in HepG2/Sor at both mRNA and protein level as compared to that in its parental cell line. More interestingly, this downregulation is concomitant with increased expression of TUC338, which is evidenced as a direct repressor of RASAL1.

Based on in vitro evidence that TUC338 and RASAL1 might be involved in sorafenib-resistance in liver cancer cells, the in vivo efficacy of inhibition of TUC338 in sorafenib treatment were further explored. Our findings that enhanced tumor growth inhibition efficacy induced by sorafenib combined with intratumoral delivering siTUC338 accompanied with overexpression of RASAL1 in tumor xenografts indicates that the TUC338/RASAL1 axis could be a potential therapeutic target for current liver cancer therapy.

The data obtained in the present study suggest that the TUC338/RASAL1 axis might play an essential role in sorafenib-resistance of liver cancer cells, siTUC338 was able to inhibit the RASAL1 pathway and tumor growth genes by directly targeting RASAL1 3'-UTR. Knockdown of TUC338 in HepG2/Sor could sensitize its reaction to the treatment of sorafenib, which was accompanied by the increased expression of RASAL1 both in vivo and in vitro. Suggesting the signaling cohort could serve as a novel therapeutic target for the treatment of chemotherapy resistant liver cancer.

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References


