MTDH Inhibits K48-Linked Degradation of TAK1 and Promotes Sorafenib Resistance in Hepatocellular Carcinoma

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Research

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Abstract

Background

Identifying novel and actionable targets in hepatocellular carcinoma (HCC) remains an unmet medical need. TAK1 was originally identified as a TGF- β -activated kinase and was further proved to phosphorylate and activate numerous downstream targets and promote cancer progression. Although TAK1 depletion leads to early onset of hepatocarcinogenesis in mice, the role of TAK1 in developed HCC progression and targeted therapy resistance is poorly understood.

Methods

The expression of TAK1 and MTDH in HCC cell lines and patients and sorafenib-resistant models was analyzed by in silico analysis, quantitative real-time PCR, western blotting and immunohistochemistry. In vivo and In vitro experiments were introduced to examine the function of TAK1 or MTDH in HCC and sorafenib resistance using small interfering RNA and pharmacological inhibitors in combination with or without sorafenib. Co-immunoprecipitation and RNA immunoprecipitation was carried out to determine the binding between TAK1 and FBXW2 or between MTDH and FBXW2 RNA.

Results

Our findings unraveled the clinical significance of TAK1 in promoting HCC and sorafenib resistance. We identified a novel E3 ubiquitin ligase, FBXW2, targeting TAK1 for K48-linked polyubiquitylation and subsequent degradation. We also found that MTDH contributes to TAK1 upregulation in HCC and sorafenib resistance, through binding to FBXW2 mRNA and accelerate its degradation. Moreover, combination of TAK1 inhibitor and sorafenib suppressed the growth of sorafenib-resistant HCCLM3 xenograft in mouse models.

Conclusion

These results revealed novel mechanism underlying TAK1 protein degradation and highlighted the therapeutic value of targeting TAK1 in suppressing HCC and overcoming sorafenib resistance.

Background

Liver cancer is among the most commonly diagnosed cancers and the leading causes of cancer deaths. Hepatocellular carcinoma (HCC) comprises 75%-85% of liver cancer cases. [1] HCC is of great tumor heterogeneity, which is the major cause of tumor progression and treatment failures and could be the result of diverse mutations that had been identified by next-generation-sequencing-based studies through multi-omics approaches.[2] Sorafenib is the first targeted drug, however, provided limited improvement in survival due to the low frequency of sorafenib-targeting mutations and the early occurrence of secondary drug resistance in HCC patients.[3] As we previously reviewed, the underlying mechanisms of sorafenib resistance involves various cellular processes, complex regulatory signaling network, genetic and

epigenetic regulations, and tumor microenvironment.[4] Based on these researches, numerous drugs including small molecule agonists or inhibitors, epigenetic, metabolic, or microenvironmental modulators, stemness inhibitors, oxidative stress inducers, and even nucleic acid therapies were being explored in combination with sorafenib to improve HCC patients outcomes.[4-6] However, none of them had been applied in clinical use. Hence, identifying targets especially those with multiple signals convergence or divergence, to overcome sorafenib resistance is of great clinical significance.

Transforming growth factor- β (TGF- β) - activated kinase 1 (TAK1, also known as MAP3K7) can be activated by a diverse set of intra- and extra-cellular stressors, and is known to regulate both nuclear factor- κ B (NF- κ B), TGF- β , and mitogen-activated protein kinase (MAPK) signaling pathways. Accumulating studies demonstrated that TAK1 mediates pro-survival activities, tumor progression, and chemoresistance in cancers.[7] However, as a hub of cellular homeostasis, the activity of TAK1 is context-dependent and cancer type-dependent, which is mainly attributed to the dual effect of TAK1-mediated NF- κ B and TGF- β signaling in cancers.[8] The role of TAK1 in liver is diverse. Early studies presented that TAK1 deletion in hepatocytes caused severe cell death, compensatory proliferation, hepatic inflammation, liver fibrosis, and early-onset of hepatocarcinogenesis.[9, 10] However, TAK1 deletion is rarely found in HCC patients according to The Cancer Genome Atlas (TCGA) database. Moreover, recent studies also proved that TAK1 was associated with poor survival of HCC patients and promoted hepatic steatosis, EMT phenotypes, drug resistance, and cancer metastasis.[11-13] These studies indicated that TAK1 might exert pro-survival function in transformed malignant hepatocytes. In this context, the role of TAK1 in HCC progression and therapy resistance requires further elucidation.

TAK1 activity is regulated by its binding partners TAB1 and TAB2/TAB3 and relies on post translational modifications including ubiquitination and phosphorylation.[14] Several E3 ligases, deubiquitinating enzymes, and phosphatases had been previously identified mediating K63-linked poly-ubiquitylation or deubiquitylation and dephosphorylation of TAK1, leading to TAK1 activation or inactivation. [7, 11, 15-17] Yet, the E3 candidates targeting TAK1 for K48-linked poly-ubiquitylation and degradation are largely unknown. MTDH has already been proved to be a master regulator in several crucial aspects of tumor progression, including transformation, evasion of apoptosis, invasion, metastasis, and chemoresistance. TAK1 has a large overlap with MTDH in terms of downstream pathways and biological functions.[18, 19] However, whether MTDH and TAK1 regulate with each other remains unknown and their role in sorafenib resistance have not been well studied.

Here, we demonstrated that both MTDH and TAK1 contributes to HCC progression and sorafenib resistance, with the combination of the *in vitro*, *in vivo* studies and clinical data analysis from HCC patients. We identified FBXW2 as a novel E3 ubiquitin ligase targeting TAK1 for K48-linked poly-ubiquitination and degradation, and MTDH functions as an upstream effector of TAK1 at post-translational level through binding to FBXW2 mRNA and promoting its degradation. Our results highlighted the key roles and the molecular mechanisms of MTDH/TAK1 axis in HCC and sorafenib resistance, thus providing novel targets for clinical intervention.

Methods

Cell culture and transfections: Human embryonic kidney 293 (HEK293) cells, Hepatocellular carcinoma Huh7, HepG2, SK-hep-1, HCCLM3, HLF, SMMC7721, JHH7, PLC/PRF/5, and Ha22T cells, normal liver L02 and Chang cells, were obtained from the American Type Culture Collection and were maintained in Dulbecco's Modified Eagle's Medium (DMEM) containing 10% (v/v) fetal bovine serum (FBS) at 37°C in 5% CO₂ condition. All cell lines were routinely tested to be negative for mycoplasma contamination. Transfection of siRNA and plasmids was performed using Lipofectamine 3000 Reagents (Thermo Fisher) following the manufacturer's instructions.

In vivo antitumor study: Four- to five-week-old BALB/c athymic nude mice (nu/nu, male) were purchased from Shanghai SLAC Laboratory Animal Centre. Mice were fed with a regular diet (RD) and had free access to water and food. All mice procedures were approved by the Sir Run-Run Shaw Hospital Committee on Use and Care of Animals. The cell-line-derived xenograft models was constructed as described below: Around 5×10^6 HCCLM3-SR cells were mixed with PBS in a total volume of 0.2 mL and were then subcutaneously injected into both flanks of mice. When the tumours reached a volume of ~ 500 mm³, the mice were then sacrificed and the tumors were cut into small pieces evenly and re-planted into one flank of mice. When the tumors reached ~ 100 mm³, mice were then randomized into 4 groups (6 mice per group). Sorafenib (25 mg/kg, p.o.) was given once a day; 5z-7-oxozeaenol (20 mg/kg, i.p.) was administered once every two days; mice of the control group received only dimethyl sulfoxide as the vehicle control. The growth of tumors was measured at the indicated time points and average tumor volumes were calculated according to the equation, $\text{volume} = (\text{length} \times \text{width} \times \text{width})/2$. After 2 weeks, mice were killed and all the tumour tissues were collected, fixed and sectioned.

Human specimens and immunohistochemical staining: Human HCC tumor tissue microarrays data were purchased from Shanghai Xinchao Biotech, China. For immune-histochemical staining, the sections were deparaffinized in xylene and rehydrated through graded ethanol. Antigen retrieval was performed for 20 min at 100 °C with 0.1% sodium citrate buffer (pH 6.0). After quenching of endogenous peroxidase activity with 3% H₂O₂·dH₂O and blocking of non-specific binding with 5% bovine serum albumin buffer, sections were incubated overnight at 4 °C with indicated antibodies. Following three times washes of PBS, the sections were treated with HRP conjugated secondary antibody for 30 min at room temperature, and stained with 0.05% 3, 3-diaminobenzidine tetrahydrochloride (DAB). Slides were photographed with Virtual slide microscope. Quantification of the immune-histochemical staining was conducted based on the ratio and intensity of the staining.

Statistical analysis: Results are expressed as mean $\pm$ SEM. The Student's t-test or one-way analysis of variance, if appropriate, were used for comparisons between groups. For analysis of tissue microarrays, the Fisher's exact test was used. The level of significance was $P < 0.05$ (using the abbreviations 'ns' for not significant, '*' for $P < 0.05$, '**' for $P < 0.01$, and '***' for $P < 0.001$). The number of independent experiments was $n \geq 3$ (if not depicted otherwise). Calculations were performed using the GraphPad Prism Software.

Results

TAK1 deletion is rare and TAK1 expression is upregulated in developed HCC patients

We first analyzed the mutation status and expression of TAK1 *in silico* using The Cancer Genome Atlas (TCGA) database, Gene Expression Omnibus (GEO) dataset [GSE14520[20]], and our own data set [Sir Run-Run Shaw Hospital (SRRSH)]. We observed that TAK1 deletion is rarely found in HCC patients (6/366) not to mention missense mutation (3/366) with unknown function in TCGA dataset (Fig.1A). But messenger RNA (mRNA) expression of TAK1 was significantly elevated in human HCC tissues as compared with non-tumorous livers (Fig.1B). Moreover, analysis of GSE6764[21] dataset revealed that TAK1 expression was already upregulated in very early HCC and tended to increase with HCC progression (Fig.1C). We also observed ranging TAK1 protein level among different HCC cell lines, almost all of which exhibited higher TAK1 expression than that in normal liver cells (Figure 1D). Survival analysis of TCGA dataset showed that TAK1 expression was negatively correlated with overall survival (OS) and disease-free survival (DFS) of HCC patients (Fig.1E). Moreover, SRRSH set analysis revealed the similar results (Fig. 1E). To address the role of TAK1 in HCC patients, we carried out Gene Set Enrichment Analysis (GSEA) using TCGA data and revealed that high TAK1 expression was positively correlated with a series of upregulated gene sets in HCC and was negatively correlated with those downregulated in HCC (Fig. 1F). Taken together, these data suggested that although artificial TAK1 deletion leads to HCC initial, TAK1 functions as a tumor promoter in developed HCC and is a potential prognostic marker of HCC patients.

TAK1 suppression inhibits clonogenicity, proliferation and oncogenic signaling in HCC

To better address the functions of TAK1 in HCC, we carried out experiments to determine the functional impact of TAK1 in Huh7 and HCCLM3 cell lines. Genetic knockdown of TAK1 by specific small interfering RNA (siRNA) and pharmacological inhibition of phospho-TAK1 by 5z-7-oxozeaenol (OXO)[22, 23] both strongly induced apoptosis in these two cell lines, with increased Annexin V-stained apoptotic cells and accumulated floating cell fragments(Fig.2A; Supplementary Fig.1A-C). In addition, TAK1 suppression also caused cell cycle arrest in G1 phase and inhibited clonogenicity of HCC cells (Fig.2B-C). Moreover, TAK1 suppression inhibited cell proliferation in most HCC cell lines (Fig.2D). TAK1 was known to boost NF- κ B phosphorylation and its transcriptional activity[12], which mediates the transcription of numerous pro-survival and anti-apoptosis signals in HCC.[24] Therefore, we tested the mRNA expression of NF- κ B targets through quantitative polymerase chain reaction (qPCR) and the results showed lower expression of Bcl-2, an anti-apoptosis regulator, and higher expression of GADD45B, a DNA damage inducer, upon OXO treatment (Fig.2E). TAK1 activates multiple oncogenic signals, which were known to be critical players in HCC tumorigenesis. Indeed, OXO treatment or TAK1 knockdown significantly reduced the protein expression of phosphorylated ERK, phosphorylated AKT, phosphorylated mTOR, phosphorylated Rb, and cyclin D1 but not cyclin B1 (Fig.2F-G; Supplementary Fig.1D). Altogether, TAK1 suppression induced cell cycle arrest and cell apoptosis in HCC cells and inhibited clonogenicity, proliferation and oncogenic signaling *in vitro*, indicating TAK1 a potential target for developed HCC.

TAK1 expression can be reduced by targeted drugs and is retained in sorafenib-resistant cell lines

To unravel the role of TAK1 in sorafenib resistance, we established stable sorafenib-resistant (SR) models of normal HCC cell lines (Huh7, SK-hep-1, HepG2, and HCCLM3), following the instruction in previous study.[6, 25] The sorafenib resistance was determined by that the IC50 shift towards a higher concentration in all the resistant cell lines compared to the parental cell lines (Fig. 3A; supplementary Fig.2A). The colony formation assays also showed that SR cells are more proliferative under sorafenib treatment (Fig.3B). Both primary and second drug resistance are mainly attributed to tumor heterogeneity.[26, 27] In many tumor entities including HCC, therapy-induced evolving are achieved through epithelial-to-mesenchymal transition (EMT) and cancer stemness acquisition.[28-30] Therefore, we first characterized the mesenchymal state and stemness features in SR cell line models. As expected, we observed profound morphological changes in SR cells transforming into spindle-like shape under an electron microscope (Fig.3C), indicating that SR cells were undergoing EMT process, which was one of the well-known underlying mechanisms of sorafenib resistance.[31] The EMT status in SR cells was further characterized by loss of ZO1 and gain of mesenchymal markers such as N-cadherin, although the expression of another epithelial marker E-cadherin was not consistently changed among four SR cell lines (Fig.3D; supplementary Fig.2B). Unlike other studies,[25] the SR cells we build tend to cluster together rather than being separated from each other, especially under sorafenib treatment, and this might be attributed to the retain of E-cadherin to maintain cell-to-cell contacts (supplementary Fig 2C). These features might also be explained by the existence of intermediate EMT states and multifunction of E-cadherin according to recent advances in research.[32, 33] Given that gain of stemness improves drug tolerance of normal tumor cells,[26] we examined the stem features of SR cells by CD90 staining, a stemness marker. Expectedly, CD90 positive SR cells were enriched in the center of normal SR cell clusters (supplementary Fig 2D), suggesting that some of SR cells acquired stemness traits and became the origin of sorafenib resistance. Oncogenic pathways activation and intracellular signaling compensation upon drug treatment are important layers of HCC progression and sorafenib resistance.[34] Indeed, we revealed higher pERK, pAKT and β -catenin expression in SR cells, indicating activation of MAPK, PI3K/AKT, and Wnt pathways in sorafenib resistance (Fig.3D-E).

Next, we detected the TAK1 expression in SR cells compared to the parental cells, and observed significant higher protein level of TAK1 in SR cells (Fig 3F). We also stained the TAK1 protein in tumor samples from 59 HCC patients that received surgery prior to sorafenib treatment. Further Kaplan–Meier survival analysis revealed that HCC patients with higher level of TAK1 expression had a shorter OS and DFS (Fig.3G). Taken together, these data indicated that TAK1 was involved in the development of sorafenib resistance and its level predicts sorafenib response in HCC patients. Interestingly, upon sorafenib treatment, the protein level of TAK1 decreases as the concentration increases in the parental HCC cells but decreases more slowly in SR cells (Fig.3H). Such TAK1 protein decreases could also be observed upon Regorafenib or Lenvatinib treatment (supplementary Fig 2E). These suggested TAK1 a direct or indirect target of targeted drugs. Indeed, one study reported that TAK1 mediated sorafenib-induced hand-foot skin reaction. [35] More importantly, it also indicated that unknown mechanism mitigated the inhibitory effect of sorafenib on TAK1 expression in SR cells.

FBXW2 E3 ubiquitin ligase targets TAK1 for ubiquitination and degradation

To uncover the regulatory mechanism of TAK1 expression in SR cells, we first detected mRNA level of TAK1 and observed no consistently significant changes between SR cell lines and their parental cells (Fig.4A). Further, chlorhexidine (CHX) assay indicated that the TAK1 protein degradation was retarded in SR cells (Fig.4B). As discussed above, K48-linked poly-ubiquitination and degradation of TAK1 are largely unknown. F-box proteins are components of SKP1–cullin 1–F-box protein (SCF) E3 ligase complexes and have pivotal roles in multiple cellular activities.[36] However, none of F-box proteins have been proved to mediate TAK1 degradation. F-box proteins bind short, defined degradation motifs in substrates.[37] Regarding FBXW2 of which the substrates remains largely unknown, the consensus degron sequences defined as TSXXXS was required for its binding to substrates such as SKP2.[38, 39] Interestingly, we identified such motif in TAK1 which is evolutionarily conserved (Fig.4C), suggesting TAK1 a potential substrate of FBXW2. Notably, it was found that FBXW2 protein expression was downregulated in SR cells compared to parental HCC cells (Fig.4D). Following this lead, we first used immunoprecipitation (IP) pull down and identified *in vitro* interaction between FBXW2 and TAK1 (Fig.4E). We also ectopically expressed a variety of F-box proteins including FBXW2 followed by IP assay and found that FBXW2 is not the only F-box proteins that could bind to TAK1 but has the highest immunoprecipitation affinity of TAK1 (supplementary Fig 3A-B). Next, we assessed the effect of FBXW2 on TAK1 level. Indeed, FBXW2 knockdown increased the levels of total TAK1 protein, while FBXW2 overexpression reduced them, which could be reversed by MG132 or MLN4924, a NEDD8-activating enzyme inhibitor that inhibits the neddylation of the Cullin subunits of Cullin RING E3 ligases (Fig.4F; supplementary Fig 3C-D). These data together with the existence of SKP1 and CUL1 protein in TAK1 immunoprecipitants (supplementary Fig 3E), suggested that FBXW2 regulated TAK1 protein level through SKP1-CUL1-FBXW2 complex. Indeed, FBXW2 promoted TAK1 K48-linked polyubiquitylation and shortened its protein half-life (Fig.4G-H). Immunofluorescence analysis suggested that TAK1 and FBXW2 were colocalized in cytoplasm (Fig.4I). Taken together, we identified FBXW2 as a new E3 ubiquitin ligase targeting TAK1 for K48-linked polyubiquitylation and degradation.

MTDH regulates TAK1 at protein level through promoting FBXW2 mRNA degradation

MTDH has been regarded as a diver oncogene in HCC.[40] TAK1 and MTDH function similarly in tumor progression and therapy resistance, activate common downstream pathways, and response to similar intra- or extra cellular stimuli according to recent researches. [7, 19] However, the relationship between MTDH and TAK1 is unknown. Gene Set Enrichment Analysis (GSEA) using TCGA data revealed that MTDH expression was closely related to the regulation of transcription factor activity, cell cycle, apoptosis, mTOR pathways, TGFβ pathway, and so on (Supplementary Table 1; supplementary Fig.3G), consistent with previous studies.[41-43] It is possible that TAK1 could be regulated by MTDH in the HCC progression and sorafenib resistance. We first altered the MTDH levels to examine its effect on TAK1 level. MTDH knockdown significantly reduced TAK1 protein expression, while the mRNA levels of TAK1 were not consistent among four HCC cell lines (Fig.5A-B), indicating that post-translational regulations were involved. Indeed, further mechanistic dissection demonstrated that MTDH knockdown could accelerate the protein degradation of TAK1 (Fig.5C).

Importantly, the Top 3 GSEA defined gene sets presented that MTDH was highly involved in RNA degradation and ubiquitin-mediated proteolysis (Fig.5D). Several researches had implied that MTDH acted as an RNA-binding protein (RBP).[44-46] Also illustrated was the decreased mRNAs that could bind to MTDH including a large amount of E2 and E3 ubiquitin ligases such as FBXW2 upon a dual PI3K/mTOR inhibitor treatment.[44] This indicates that MTDH could accelerate the degradation of these mRNAs in response to drug stimuli. Yet, the role of MTDH in RNA stability or translation are not known. We thus detected the mRNA expression of those E2 and E3 ligases upon MTDH knockdown and found that the decrease of MTDH expression increased their mRNA level (Fig.5E). Regarding FBXW2, MTDH knockdown also increased its protein level in HCC cells. (Fig.5F). Thus, we hypothesized that MTDH regulates K48-linked poly-ubiquitination and degradation of TAK1 through downregulating its E3 ubiquitin ligase FBXW2. We found that MTDH overexpression partially rescued TAK1 protein level and reduced K48-polyubiquitylation upon FBXW2 transfection (Fig.5G), suggesting FBXW2-mediated TAK1 degradation could be mitigated by MTDH. RNA-immunoprecipitation (RIP) revealed much higher MTDH-bound FBXW2 mRNA compared to IgG, suggesting the specific association of FBXW2 mRNA and MTDH (Fig.5H). Immunofluorescence and fluorescence in situ hybridization (FISH) assay for MTDH protein and FBXW2 mRNA also showed that FBXW2 mRNA overlapped with those MTDH localized at cytoplasm (Figure.5I).

Tumor necrosis factor alpha (TNF- α)/NF- κ B pathway plays important role in the development of inflammation-driven HCC including viral hepatitis-related and NASH- or NAFLD-related HCC.[47] Recent study also indicated that TNF- α might serve as a predictor of sorafenib response in HCC patients.[48] Given that MTDH and TAK1 both activate NF- κ B signaling, we treated HCC cells with TNF- α . Results showed TNF- α treatment increased MTDH and TAK1 protein expression, as well as decreased FBXW2 protein level in a time course and a dose-dependent manner (supplementary Fig.4H). These findings indicate that MTDH/FBXW2/TAK1 might mediate TNF- α -promoted HCC progression and sorafenib resistance. In summary, MTDH regulates TAK1 protein level through binding to and subsequently degrade FBXW2 mRNA.

MTDH promotes HCC and sorafenib resistance

Next, we aim to elucidate the function of MTDH in HCC and sorafenib resistance and whether TAK1 mediates these functions. Similarly, in silico analysis revealed elevated mRNA expression of MTDH in human HCC tissues as compared with non-tumorous livers (Fig.6A), which was attributed to amplified somatic copy numbers of MTDH that were consistent with the protein level according to IHC scoring in HCC patients (Fig.6B-C).[40] In addition, MTDH mRNA was increasing from very early HCC to very advanced HCC according to GSE6764 (n=75) dataset (Fig.6D), suggesting that MTDH was involved in progression and aggressive phenotype of HCC. We also observed higher MTDH mRNA and protein expression in most HCC cells than that in normal liver cells (Supplementary Fig.4A). Survival analysis based on MTDH mRNA level in GSE14520 and SRRSH dataset indicated that high expression of MTDH predicts worse OS and DFS or RFS (Fig.6E; Supplementary Fig.4B). MTDH has been widely proved to be involved in chemotherapeutics resistance,[19] however, its role in targeted therapy sensitivity remains unknown. We then detect the expression of MTDH in our SR cell models and observed significantly higher

protein and mRNA levels of MTDH in SR cells (Fig.6F). Of note, among the HCC patients with sorafenib treatment after surgery, those with higher MTDH expression had significantly worse OS and DFS compared to those with lower MTDH expression (Fig.6G), indicating an important role of MTDH in sorafenib resistance of HCC.

TAK1 mediates MTDH-induced sorafenib resistance

To verify the role of MTDH in HCC and targeted-therapy resistance, we carried out functional experiments and revealed that MTDH knockdown by three siRNAs inhibited proliferation of HCC cells and the third siRNA performed best according to the decrease in cell viability, as well as the knockdown efficiency (Supplementary Fig.5A-B). Knockdown of MTDH also induced apoptosis and cell cycle arrest in G1 phase of HCC cells (Supplementary Fig.5C-D). Mechanistically, MTDH suppression reduced classical oncogenic signaling such as pAKT, p-mTOR, and NF- κ B p105 (supplementary Fig.6E). We overexpressed MTDH in HCC cell lines and assessed the relationship between MTDH and the half maximal inhibitory concentration (IC50) of several targeted drugs including Sorafenib, Regorafenib, and Lenvatinib. The results showed that IC50 of these three drugs, especially sorafenib, were higher in HCC cells that stably expressing MTDH (Fig.7A). We then combined MTDH knockdown and sorafenib treatment in SR cells to further evaluate the function of MTDH in sorafenib resistance. Results showed that MTDH knockdown re-sensitized SR cells to sorafenib-induced apoptosis and inhibitory of proliferation (Fig.7B). Regarding the EMT transformation and mesenchymal state in SR cells, we found that the expression of EMT markers were significant reduced and the migratory potential of SR cells were inhibited upon MTDH suppression (Supplementary Fig.6F-G). Furthermore, phalloidin staining analysis revealed that MTDH knockdown curbed SR cell stretching via inhibiting actin remodeling and subsequent cell-to-cell contacts (Supplementary Fig.6H).

As presented above, we found that MTDH could regulate TAK1 protein level through binding to and subsequently degrade FBXW2 mRNA. Next, we aim to explore whether TAK1 is involved in MTDH-mediated sorafenib resistance. Consistent with previous findings, HCC cells with ectopically expressed MTDH became more resistant to sorafenib, and such resistance could be reversed by sorafenib in combination with OXO or NG-25, another TAK1 inhibitor (Fig.7C-D). These data suggested that MTDH functions as a promoter of HCC progression and sorafenib resistance and targeting TAK1 could overcome MTDH-mediated sorafenib resistance.

Targeting TAK1 overcomes sorafenib resistance in vitro and in vivo

Since there are no drugs available for MTDH inhibition, targeting TAK1 might be an alternative approach to overcome MTDH-dependent or independent sorafenib resistance. Indeed, combination of sorafenib and OXO strongly inhibited its clonogenicity and proliferation compared to sorafenib or OXO treatment alone in SR cell models (Fig.7E; supplementary Fig.6A). Flow cytometry assays showed that OXO in combination with sorafenib largely increased proportion of apoptotic HCC cells and re-sensitized SR cells to sorafenib-induced apoptosis, while both sorafenib and OXO alone induced apoptosis in parental cells (Fig.7F; supplementary Fig.6B). Particularly, we used an *in vivo* xenograft tumor model to evaluate the

combined anticancer effect of sorafenib and OXO by inoculating subcutaneously the HCCLM3-SR cells into the flank sides of nude mice, followed by treatment with vehicle control, OXO, or sorafenib alone or in combination. When used non-toxic concentrations (Supplementary Fig.6C), sorafenib or OXO alone did not suppress the tumor growth *in vivo*, however, significant suppression was observed in combination group in terms of tumor volumes, tumor weights, and Ki67-staining, as well as downstream oncogenic proteins (Fig.7G-H; supplementary Fig.6D-E). Thus, TAK1 inhibitors sensitized HCC SR cells to sorafenib as tested in both *in vitro* cell-culture and *in vivo* xenograft models.

Discussion

Most HCC patients are diagnosed at an advanced stage, when surgical approaches and locoregional therapies are no longer feasible. Improvements in patients outcomes from most systematic therapies have been modest, although several targeted drugs and immune checkpoint inhibitors have been proved effective in the last decade [49] and more combinational strategies are being explored [50]. The underlying mechanisms of sorafenib resistance remains complex and largely unknown. To better address this critical problem, we had established acquired sorafenib-resistant HCC cell models by treating HCC cells with increasing concentrations of sorafenib in culture media over time.⁵⁰ Based on this models, our previous studies had proved the crucial role of androgen receptor signals and several non-coding RNAs in sorafenib resistance, highlighting the epigenetic regulation of driver oncogenes in sorafenib resistance.[5, 6, 51-53] However, therapy-induced tumour cell evolving is comprehensive and systematic in terms of cellular and molecular alteration. Thus, identifying other novel targets still remains an unmet medical need, requiring new insights into underlying molecular mechanisms that support hepato-carcinogenesis, HCC progression, and drug resistance based on genomic, transcriptomic, and epigenomic studies.

Recently, in most other tumour entities, TAK1 has been considered a robust therapeutic target especially in lung, colon, and pancreatic cancers, most of which exhibit frequent KRAS mutations.[54, 55] Although artificial TAK1-deletion leads to early onset of hepatocarcinogenesis in liver, HCC patients in real world rarely harbour TAK1-deletion mutations according to TCGA database. Moreover, we observed that HCC specimens harbour higher TAK1 mRNA expression which was increasing from early-stage to advanced-stage HCC patients. Metabolic syndrome together with alcohol abuse and hepatitis C infection contribute to the increasing incidence and death rate of liver cancers.[56] TNF- α signalling is involved in inflammation-driven HCC including hepatic steatosis and virus infection above. Indeed, TAK1, which is activated in response to TNF- α , promoted non-alcoholic steatohepatitis (NASH) and non-alcoholic fatty liver disease (NAFLD).[15-17, 57] TNF- α was also indicated to be potential target to overcome sorafenib resistance.[48] These clues promoted us to explore the role of TAK1 in developed HCC, especially in HCC progression and therapy resistance. Indeed, we found elevated protein level of TAK1 in HCC patients, HCC cell lines and sorafenib-resistant cell models, and its expression predicts poor patient outcomes. *In vivo* and *in vitro* assay also suggested that genetic knockdown and pharmacological inhibition of TAK1 using inhibitors OXO and NG-25 significantly suppressed cell proliferation and tumour growth, and overcame sorafenib resistance.

On the other hand, the regulation of TAK1 expression remains largely unknown. We observed that sorafenib treatment reduced total protein of TAK1 in HCC cells, suggesting TAK1 a direct or indirect target of sorafenib. However, TAK1 protein but not mRNA expression was elevated in SR cells and was retained even under sorafenib treatment. While most studies focus on K63-polyubiquitylation-dependent activation of TAK1, [11, 17, 58] these data lead us to explore which E3 ligase mediates K48-linked degradation of TAK1. F-box proteins mediate the degradation of a large number of regulatory proteins involved in diverse processes and have emerging roles in cancer and drug resistance.[36, 59] Previous study identified FBXW5 as a functional suppressor rather than an expression regulator for TAK1.[60] We used IP assays and identified FBXW2 a novel E3 ligase targeting TAK1 for K48-linked ubiquitylation and subsequent degradation through specific degron sequences. Our results also revealed that FBXW2 is not the only F-box protein that could bind to TAK1, regardless nonspecific binding to tag beads. It is not surprising that other F-box protein also regulate TAK1 activation and protein degradation, given that TAK1 is a gatekeeper of cellular homeostasis and must be under tight regulation. Yet, how other F-box proteins regulate TAK1 needs further investigation. Moreover, some issues remain unexplored in this study including how FBXW2 recruits the substrate TAK1 and whether such recruitments depend on degron phosphorylation and which kinases are responsible for the phosphorylation.

MTDH has been identified as a master and actional gene in HCC as well as other tumour entities. It functions as a downstream mediator of the transforming activity of oncogenic Ha-Ras and c-Myc and activates MAPK, PI3K/Akt, nuclear factor kappa B (NF- κ B), and Wnt/ β -catenin signaling pathways.[61] Several studies have demonstrated that MTDH is an RNA-binding protein and could bind to many E3 or E2 mRNAs including FBXW2, and regulate their stability or translation.[44, 46] Furthermore, MTDH and TAK1 function similarly in tumour progression and therapy resistance, respond to overlapped stimuli and activate common downstream pathways.[7, 19] Our expression analysis and functional experiments also proved that MTDH is essential for HCC development and sorafenib resistance. Above findings and clues suggested that MTDH might be a potential upstream regulator for TAK1 at post-translational level. Indeed, our findings suggested that MTDH regulates TAK1 protein level through binding to FBXW2 mRNA and promoting its degradation. However, pharmacological suppression of MTDH based on neither druggable structure nor bioactive compounds remains achievable. This study also proved that targeting TAK1 reversed MTDH-dependent and -independent sorafenib resistance *in vitro* and *in vivo*. In summary, MTDH overexpression in SR cells retarded the degradation of TAK1 protein and thus mitigated the inhibitory effect from sorafenib on TAK1. However, how long-term sorafenib treatment resulted in upregulation of MTDH is still unknown and has not been elucidated in this study. Taken together, presenting study identified the MTDH/FBXW2/TAK1 axis (Fig.8) which is important in HCC progression and sorafenib resistance, and provides evidence for TAK1 a robust therapeutic target in HCC and MTDH-dependent or -independent sorafenib resistance, rendering further preclinical and clinical studies to explore.

Conclusion

In the current study, our findings indicated that TAK1 upregulated in developed HCC and relative to poor progression and sorafenib resistance. Overexpression of TAK1 promotes proliferation, inhibits apoptosis, and induce sorafenib resistance. Mechanistically, MTDH promotes the TAK1 expression through binding to and accelerate the gradation of mRNA of FBXW2, which is identified as a novel E3 ligase of TAK1 in this study. Our study also demonstrated TAK1 inhibitors sensitized HCC SR cells to sorafenib in both *in vitro* cell-culture and *in vivo* xenograft models, indicating TAK1 inhibition a promising therapy for HCC suppression and improving sorafenib sensitivity.

Abbreviations

HCC: Hepatocellular Carcinoma

TCGA: The Cancer Genome Atlas

EMT: Epithelial-mesenchymal transition

GEO: Gene Expression Omnibus

SRRSH: Sir Run-Run Shaw Hospital

OS: Overall survival

DFS: Disease-free survival

siRNA: Small interfering RNA

OXO: 5z-7-oxozeaenol

qPCR: quantitative polymerase chain reaction

SR: Sorafenib resistance

CHX: Chlorhexidine

SCF: SKP1–cullin 1–F-box protein complex

GSEA: Gene Set Enrichment Analysis

RBP: RNA-binding protein

RIP: RNA-immunoprecipitation

FISH: Fluorescence in situ hybridization

NASH: non-alcoholic steatohepatitis

Declarations

Availability of data and materials

All data generated and analyzed during this study are included in this published article and its additional file.

Author contributions

SJ Xia, JJ Xu and XJ Cai provided the idea of the manuscript. SJ Xia, L Ji and Z Wan performed the experiments and wrote the manuscript. Liye Tao assisted in western blotting and immunohistochemical staining. Y Pan, ZJ Lin and J Zhao assisted in animal studies and bioinformatic analysis. Haoqi Pan and Liuxin Cai assisted in plasmids construction. JJ Xu and XJ Cai reviewed and edited the manuscript before submission. JJ Xu and XJ Cai also made substantial contributions to the discussion of content.

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Ethics approval and consent to participate

All mice procedures were approved by the Sir Run-Run Shaw Hospital Committee on Use and Care of Animals.

Consent for publication

Not applicable.

Competing interests

The authors declare no competing interests.

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Figures

Figure 1 TAK1 expression in HCC

Figure 1

TAK1 expression in HCC (A) MAP3K7 (encoding TAK1 protein) status in TCGA database (B) mRNA expression of TAK1 in human HCC tissues as compared with non-tumorous livers from TCGA, GSE14520, and SRRSH dataset. (C) mRNA expression of TAK1 in different stages of HCC from GSE6764 dataset. (D) Protein level of TAK1 among different HCC cell lines compared to normal liver cell lines (L02 and Chang). (E) Kaplan-Meier survival curves of patients with low or high TAK1 mRNA expression based on TCGA and

SRRSH datasets. Significance was determined using Kaplan-Meier analyses. Survival analysis was performed using log-rank test. (F) Gene sets associated with TAK1 expression using TCGA data and Gene Set Enrichment Analysis (GSEA) analysis.

Figure 2 Function of TAK1 in HCC

Figure 2

The function of TAK1 in HCC (A) Proportion of Annexin V stained apoptotic cells analysed by Flow Cytometry in Huh7 or HCCLM3 cells transfected with TAK1 siRNA or 5Z-7-Oxozeaenol (OXO) treatment, a

TAK1 inhibitor. Three biological repeats per group. (B) Cell cycle analysis and representative results of HCCLM3 cells using Flow Cytometry upon OXO treatment. Three biological repeats per group. (C) Colony formation of HCCLM3 cells treated with different concentration of OXO. Colony numbers were measured by Image J software. Three biological repeats per group. (D) Colony formation of different HCC cell lines upon OXO treatment. Three biological repeats per group. (E) Real-time quantitative PCR (RT-qPCR) quantification of Bcl-2 and GADD45 β mRNA in HCCLM3 cells treated with OXO or not. (F-G) Western blotting analysis of TAK1 downstream proteins levels upon genetic knockdown or pharmacological inhibition of TAK1 in Huh7 and HCCLM3 cells.

Figure 3 TAK1 expression in sorafenib-resistant HCC cell models

Figure 3

TAK1 expression in sorafenib-resistant HCC cell models (A) Relative cell viability of Huh7-SR and HCCLM3-SR cells upon sorafenib treatment with increasing dosage compared to their parental cells. Relative cell viability was measured by cell counting kit 8 (CCK8) reagent and normalized to 0 μ M. (B) Colony formation assay was performed in HCCLM3 wild type and SR cells treated with sorafenib (7 μ M). Colony numbers were measured by Image J software. Three biological repeats per group. (C)

Morphological changes of Hepg2-SR and HCCLM3-SR cells compared to their parental cells. (D) Western blotting analysis of EMT-related markers and proteins in HCCLM3-SR and Huh7-SR cells compared to their parental cells. (E) Western blotting analysis of FAK/AKT/mTOR pathways in HCCLM3-SR and Huh7-SR cells compared to their parental cells. (F) Western blotting analysis of TAK1 protein level in four SR cells compared to their parental cells. (G) IHC staining of TAK1 in HCC patients received sorafenib treatment prior to surgery and Kaplan-Meier survival curves of patients with low (n=32) or high TAK1 (n=27) scores. Significance was determined using Kaplan-Meier analyses. Survival analysis was performed using log-rank test. (H) Western blotting analysis of TAK1 protein level in HCCLM3-SR cells compared to their parental cells in response to sorafenib treatment.

Figure 4 FBXW2 E3 ubiquitin ligase targets TAK1 for ubiquitination.

Figure 4

FBXW2 E3 ubiquitin ligase targets TAK1 for ubiquitination (A) RT-qPCR quantification of TAK1 mRNA expression in four SR cells compared to their parental cells. (B) Chlorhexidine (CHX) assay was performed in HCCLM3-SR cells and their parental cells for indicated time periods and TAK1 protein was measured by Western blotting analysis. (C) Evolutionary conservation of FBXW2 degron motif on TAK1. (D) Western blotting analysis of FBXW2 expression in HCCLM3-SR and its parental cells. (E) HEK293 cells

were transfected with indicated plasmids, followed by IP with anti-Flag or anti-Myc (F) Western blotting analysis of TAK1 expression in Huh7 cells with FBXW2 knockdown or overexpression, treated with MG132 or MLN4924. (G) Half-life of TAK1 protein in HCCLM3 cells transfected with Myc-FBXW2 or vector plasmid as control. (H) Immunoblotting analysis of Myc-Ub in Flag tag pull-down and input derived from HEK293T cells transfected with indicated constructs. (I) Immunofluorescence analysis of the TAK1 and Myc-FBXW2 location in Huh7 cells. Green, TAK1; Red, Myc-FBXW2; Blue, DAPI.

Fig 5 MTDH regulates TAK1 at protein level

Figure 5

MTDH regulates TAK1 at protein level through promoting FBXW2 mRNA degradation (A) Western blotting analysis of TAK1 and expression in HCCLM3, Huh7, SK-hep-1, and HepG2 cells transfected with MTDH siRNA or scramble control. (B) RT-qPCR quantification of TAK1 mRNA expression in four HCC cells transfected with MTDH siRNA or scramble control. (C) Half-life of TAK1 protein in HCCLM3 cells transfected with MTDH siRNA or scramble control. (D) KEGG pathways significantly enriched in HCC patients with high expression of MTDH using TCGA data. (E) RT-qPCR quantification of E3 mRNAs expression in HCCLM3-sr cells transfected with MTDH siRNA or scramble control. (F) Western blotting analysis of MTDH and FBXW2 protein expression in HCCLM3 cells transfected with MTDH siRNA or scramble control. (G) Immunoblotting analysis of TAK1 protein expression in Huh7 cell lysates transfected with indicated constructs. (H) RNA-IP and RT-qPCR analysis of FBXW2 mRNA binding to MTDH protein compared to IgG. (I) Immunofluorescence analysis of the MTDH and FBXW2 mRNA location in HCCLM3 cells. Green, MTDH; Red, FBXW2 mRNA; Blue, DAPI.

Figure 6 The role of MTDH in HCC and Sorafenib resistance

Figure 6

The role of MTDH in HCC and Sorafenib resistance (A) mRNA expression of MTDH in human HCC tissues as compared with non-tumorous livers from TCGA, GSE14520, and SRRSH dataset. (B-C) Somatic copy numbers of MTDH in TCGA and GSE38323 datasets, and its associations with MTDH staining in HCC patients according to GSE38323 dataset. (D) mRNA expression of MTDH in different stages of HCC from GSE6764 dataset. (E) Kaplan-Meier survival curves of patients with low or high MTDH mRNA expression

based on GSE14520 and SRRSH datasets. Significance was determined using Kaplan-Meier analyses. Survival analysis was performed using log-rank test. (F) Western blotting analysis of MTDH protein level in four SR cells compared to their parental cells. (G) MTDH staining in HCC patients receiving sorafenib prior to surgery and Kaplan-Meier survival curves of these patients with low or high TAK1 staining. Significance was determined using Kaplan-Meier analyses. Survival analysis was performed using log-rank test.

Figure 7. TAK1 mediates the function of MTDH in HCC and sorafenib resistance *in vivo* and *in vitro*

Figure 7

Targeting TAK1 to overcome sorafenib resistance in vitro and in vivo (A) Relative cell viability of HCCLM3 with stably expression MTDH or not treated with increasing dosage of Sorafenib, Regorafenib, or Lenvatinib. (B) Relative cell viability and proportion of apoptotic cells in HCCLM3 or Huh7 cells transfected with MTDH siRNA or scramble control in combination with sorafenib or DMSO. Three biological repeats per group. (C) Relative cell viability of HCCLM3 with stably expression MTDH or not treat with sorafenib and OXO alone or in combination for 48 hours. (D) Relative cell viability of HCCLM3 with stably expression MTDH or not treated with OXO or NG25 alone or in combination sorafenib of increasing dosage or for indicated time periods. Three biological repeats per group. (E) Colony formation assay was performed in HCCLM3-SR cells treated with sorafenib alone or in combination with OXO. Colony numbers were measured by Image J software. Three biological repeats per group. (F) Proportion of Annexin V stained apoptotic cells analysed by Flow Cytometry in HCCLM3-SR or its parental cells treated with sorafenib alone or in combination with OXO. Three biological repeats per group. (G) Gross view of tumors in mice treated sorafenib alone or in combination with OXO. (H) Total volume or weight of tumour bear in mice treated sorafenib alone or in combination with OXO in an indicated time period.

Figure 8 The schematic diagram of MTDH/FBXW2/TAK1 in HCC and sorafenib resistance

Figure 8

The schematic diagram of MTDH/FBXW2/TAK1 in HCC and sorafenib resistance.

Supplementary Files

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