Original Article

Thyroid hormone T3 augments the cytotoxicity of sorafenib in Huh7 hepatocellular carcinoma cells by suppressing AKT expression

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ABSTRACT

Background and Objectives: Hepatocellular carcinoma (HCC) is a primary cancer that poorly responds to treatment. Molecular cancer studies led to the development of kinase inhibitors, among which sorafenib stands out as a multi-kinase inhibitor approved by FDA for first line use in HCC patients. However, the efficiency of sorafenib was shown to be counteracted by numerous subcellular pathways involving the effector kinase AKT, causing resistance and limiting its survival benefit. On the way of breaking such resistance mechanisms and increase the efficiency of sorafenib, deeper understanding of hepatocellular physiology is essential. Thyroid hormones were shown to be metabolized in liver and inevitably affect the molecular behaviour of hepatocytes. Interestingly, thyroid hormone T3 was also demonstrated to be potentially influential in liver regeneration and treatment with this hormone reportedly led to a decrease in HCC tumor growths. In this study, we aimed to uncover the impact of T3 hormone on the cytotoxic response to sorafenib in HCC in vitro.

Materials and Methods: We pre-treated the HCC cell line Huh-7 with T3 prior to sorafenib exposure both in 2D and 3D culture. We checked cell viability with MTT assay in 2D culture and measured the sizes of 3D spheroids with bright-field microscopy followed by a surface analysis with ImageJ. We also performed scratch assay to measure cell migration as well as western blot and qPCR to uncover affected pathways.

Results: We observed an additive effect to sorafenib's cytotoxicity both in 2D and 3D culture. Cell migration assay also confirmed our finding and pointed out a benefit of T3 hormone in HCC cell migration. Western blot experiments showed that T3 exerts its additive effect by suppressing AKT expression upon sorafenib treatment both at protein and gene expression levels.

Conclusion: Our results open a promising new avenue in increasing sorafenib's cytotoxicity where thyroid hormone T3 is utilized to modulate AKT expression to combat resistance, and warrant further studies in the field.

KEY WORDS: AKT, HCC, hepatocellular carcinoma, liver cancer, sorafenib, T3, thyroid hormone

INTRODUCTION

Hepatocellular carcinoma (HCC) is the most common form of liver cancer. This devastating disease occurs when hepatocytes transform into malignant tumor cells with the influence of factors including obesity, type-2 diabetes, cirrhosis, alcohol-induced liver diseases, or virus infections.[1] Despite its increasing incidence, current HCC treatment options are insufficient, mostly due to the complexity of its molecular pathogenesis. The carcinogenesis of the liver is a multi-step biological process involving a plethora of pathways. The key players are known to involve the abnormal activation of signaling pathways such as PI3K/AKT/mTOR, MAPK/ERK, JAK/STAT, Wnt/ß-Catenin; disturbed balance between oncogenes and tumor suppressors, as well as genomic instability.[2] Sorafenib, a multi-kinase inhibitor, stands out as an FDA-approved first-line treatment option currently being used in clinics.[3] It works by inhibiting the Ras/Raf/MEK/ERK signaling pathways a that drive tumor growth.[4] However, there is only limited therapeutic response to this agent. Resistance to sorafenib arises due to multiple

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factors, including but not limited to the overexpression of AKT and Epidermal growth factor receptor (EGFR), as well as the activation of autophagy.[5]

In addition to main signaling cascades within the cell, the last decade also witnessed valuable studies that put forward the impact of physiological factors in HCC development. Thyroid hormones 3,5,3'-triiodo-L-thyronine (T3) and 3,5,3',5'-tetraiodo-L-thyronine (T4) were linked to HCC development, and interestingly, an increased association between hypothyroidism and HCC was reported.[6–8] Thyroid gland secretes 80% of the inactive thyroid hormone T4 and 20% of the active T3 hormone. Because the T4 hormone is inactive, it needs to be converted to the active version, T3, to perform its functions.[9] Roughly 60% of this conversion occurs in the liver with the help of deiodinase enzymes, which remove one iodine atom from the T4 hormone. One of the deiodinase enzymes that convert T4 to T3, iodothyronine deiodinase 1, is primarily expressed in the liver and kidney.[10] One of the receptors of thyroid hormone, TRß1, is also predominantly expressed in the liver.[11] In previous literature studies, it was emphasized that the TRß1 receptor has a tumor suppressor function, and oncogenic transformation is observed in the absence of TRß1.[12–14] Studies on HCC cells revealed that the T3 hormone itself upregulated the tumor suppressor p21 through endoglin and downregulated the oncogenes CDK2, cyclin E, and phospho-Rb.[14,15] T3 also downregulated ELF2, the transcription factor linked to tumor growth and cell proliferation, and increased DKK4, which inhibits cell invasion and metastatic spread by reducing matrix metalloproteases.[16–18] In line with this, Kowalik *et al*. observed that T3 itself may be a potential HCC antitumor agent by inducing a differentiation program and a metabolic switch.[19] Moreover, the thyroid hormone axis was reported many times as crucial for liver regeneration in liver disease thanks to its effect on hepatocyte differentiation.[20]

Due to the poor prognosis and limited therapeutic options of HCC, it is essential to come up with new treatment strategies which could reach clinics easily. In light of the information given above, we wondered whether the cytotoxic effect of the FDA-approved HCC drug sorafenib[21] could be enhanced in the presence of thyroid hormone T3 treatment, which is widely used in clinics to treat hypothyroidism.[22] To test this hypothesis *in vitro*, we utilized the HCC cell line Huh7. We pre-treated Huh7 cells with T3, followed by continuous sorafenib exposure in 2D and 3D cultures. We then analyzed cell migration and documented phosphorylation/expression of key HCC proteins, with valuable implications on the molecular insights in augmenting the cancer cell death effect of sorafenib in the presence of T3.

MATERIALS AND METHODS

2D Cell culture

Human HCC cell line Huh7 was cultured with Dulbecco's Modified Eagle's Medium (DMEM) containing 10% fetal bovine serum (FBS) and 1% penicillin-streptomycin with incubation conditions of 5% CO_2 and 37°C. Cells were trypsinized and collected when they reached 90% confluence. When the cells were in expansion, they were seeded onto new 10 cm round culture dishes with a 20% confluence level. Passaging was performed every 2–3 days.

3D spheroid culture

3D Spheroids were formed using the hanging drop method. Huh7 cell line was seeded on 10-cm culture plates at 2×10^5 per plate and incubated for 24 hours. Cells were then pre-treated with 0.1 μM T3 or dimethyl sulfoxide (DMSO) as control and incubated for 48 hours. After the incubation, cells were collected with trypsin, and a cell concentration of 1×10^3 cells per 30 μL DMEM + FBS was prepared. Cells were seeded as 30 μL droplets to the interior of a 10-cm plate lid, which then was inverted and closed onto the culture plate. The plate was incubated for 24 hours while continuing the pre-treatment with T3. Sorafenib or DMSO was then carefully added to the drops for the next 72 hours. Bright-field photos were taken each day to observe the formation and growth of the spheroids. The areas of the spheroids were calculated using ImageJ software (NIH, USA).[23]

Treatments

T3 (Liothyronine sodium; T3 analog) and sorafenib were purchased from Abcam (ab145834; ab141966, respectively). Drugs were dissolved in DMSO, aliquoted, and stored at −20°C. Fresh aliquots were used each time. Cell lines were pre-treated with either DMSO or T3 (liothyronine sodium) in concentrations of 0.001 μM, 0.01 μM, and 0.1 μM. Sorafenib treatments were applied either as 5 μM or 10 μM.

MTT Assay

Cells were seeded onto 10-cm round culture dishes at 2×10^5 per plate. They were cultured in DMEM with 10% FBS and 1% P/S for 24 hours. As a pretreatment, each plate was treated with a different dose of T3, the control group was given media containing DMSO in the same amount as T3, and all culture dishes were incubated for 48 hours. Cells were then collected with trypsin and seeded into 96-well plates using 6×10^5 cells per well. Pretreatment continued for a further 24 hours. Cells that received a total of 72 hours pre-treatment were then subjected to subsequent drug treatment (sorafenib or DMSO) in the absence and presence of T3, followed by incubation for an additional 72 hours. After incubation, 3-(4,5-dimethylthiazol-2-yl)-2,5 diphenyltetrazolium bromide (MTT) (BioBasic, T0793) test was performed according to the protocol that had been published previously.[24] Optical density (OD) was read at 570 nm by a microplate reader. Cell viability was calculated using the formula (OD value of treated samples – blank)/(OD value of untreated control samples – blank). All experiments were performed in triplicates.

Migration assay

Cells were seeded onto 10 cm Petri dishes with DMEM medium containing 10% FBS and 1% P/S and were allowed to attach for 24 hours. The next day, the medium was replaced with DMEM containing 0.1 μM T3 or DMSO and incubated for 48 hours. Cells were then trypsinized and seeded onto 12-well plates (3×10^5 cells per well) to continue pre-treatment in DMEM media containing 0.1 μM T3 or DMSO. The medium was then discarded, and a scratch was made along the mid-axis of the well plates using a sterile pipette tip. Pre-treated cells then received the second treatment as 5 μM sorafenib or DMSO. Cell migration pictures were captured under a microscope immediately after scratching and 24, 72, and 120 hours later. Gap-filling images were analyzed with the Image J software (NIH, USA).[23]

RNA Isolation and qPCR

For qPCR analysis, 2×10^5 cells were seeded onto 10-cm dishes and were allowed to attach for 24 hours. Cells were then pre-treated with 0.1 μM T3 or DMSO for 72 hours. Following pre-treatment, they received 5 μM sorafenib or DMSO for 24 hours. After washing with PBS, total RNAs were extracted using Quick-RNA™ MiniPrep Kit (Zymo Research, R1054) according to manufacturer's instructions. For cDNA synthesis, 0.5-1 μg of isolated total RNA was reverse transcribed using VitaScript™ FirstStrand cDNA Synthesis Kit (Procomcure Biotech, PCCSKU1301) with oligo dTs and the qPCR reaction was performed in 96-well plates using A.B.T. ™ 2X qPCR SYBR-Green MasterMix (Atlas Biotechnology, Q03-01-05). The relative gene expression levels were determined using CFX Connect Real-Time PCR Detection System (Bio-Rad Laboratories) and calculated with the Livak method.[25] Each experiment was performed in triplicates.

Western blot

2×10^5 cells were incubated in 10-cm dishes for 24 hours. Later, they were treated with DMSO, 0.1 μM T3, 5 μM Sorafenib, or 0.1 μM T3 + 5 μM Sorafenib for 72 hours. Cells were then washed with PBS and lysed in lysis buffer. Cellular protein lysates were extracted and quantified with the DC™ Protein Assay (Bio-Rad Laboratories, 500-0112). The concentrations of the samples from the different treatments were equalized and loaded into SDS-PAGE. The separated proteins were transferred onto a polyvinylidene fluoride membrane using standard procedures. The membrane was blocked with 5% milk powder in 1X Tris-buffered saline and 0.1% Tween-20 (TBST) and probed with primary antibodies overnight at 4°C. Once washed with TBST, blots were incubated at room temperature with HRP-conjugate rabbit or mouse secondary antibodies for 1 hour. Membranes were washed with TBST and detected using Clarity™ Western ECL Substrate (Bio-Rad Laboratories, 170-5060). Images were captured with Vilber Fusion Solo S optical system.

Antibodies

The following antibodies were used in Western Blot analysis: PARP (Cell Signaling Technology, 46D11, 1:1000), phospho-β-catenin (Cell Signaling Technology, S552,1:1000), β catenin (BD Biosciences, 610153, 1:1000), phospho-AKT (Ser473) (Cell Signaling Technology, D9E, 1:2000), pan-AKT (Cell Signaling Technology, C67E7, 1:2000), Phospho-p44/42 MAPK (Erk1/2) (Thr202/Tyr204) (Cell Signaling Technology, 9101S, 1:2000), p44/42 MAPK (Erk1/2) (Cell Signaling Technology, 137F5, 1:2000), β-actin (Cell Signaling Technology, 13E5, 1:1000), Anti-rabbit IgG HRP-linked secondary antibody (Cell Signaling Technology, 7074S, 1:5000), Goat-anti-mouse IgG (H + L), HRP conjugate (Advansta, R-05071-500, 1:5000).

Statistical analysis

Data were expressed as mean ± standard error. Analysis of significance was performed with two-way Analysis of variance (ANOVA) using the GraphPad Prism 8.0 software for Windows (GraphPad Software, La Jolla California USA, www.graphpad.com).

RESULTS

T3 pre-treatment improves sorafenib's cytotoxicity in Huh7 cells in 2D culture

To understand the effect of thyroid hormone T3 on the cytotoxicity of sorafenib on hepatocellular carcinoma cells, we employed Huh7 cells, which are known to exhibit epithelial-like hepatocellular characteristics.[26–28] As it takes a few days for the effects of T3 to manifest on hepatocytes,[29,30] we utilized a pre-treatment plan where cells were incubated with T3 for three days, followed by the continuation of T3 dosing along with sorafenib treatment at varying concentrations [Figure 1a]. To determine the effective dose of T3 and sorafenib, we did a dose titration on Huh7 cells in line with previously published studies,[31,32] where they were exposed to DMSO or 0.001μM, 0.01 μM and 0.1 μM T3 as a three days pre-treatment followed by another three days in the presence and absence of 1 μM and 5 μM sorafenib [Figure 1b]. The cell viability was then assessed with an MTT assay.[33] T3 treatment alone did not cause a significant change in the proliferation of Huh7 cells, and sorafenib treatment alone caused a decrease in proliferation only at 5 μM. We observed a significant additive effect on sorafenib's cytotoxicity at 0.1 μM of T3 concentration [Figure 1b].

T3 pre-treatment improves sorafenib's cytotoxicity in Huh7 cells in 3D culture

2D monolayer culture often lacks key morphological and physiological tissue properties and thereby fails to recapitulate *in situ* drug response.[34] To overcome this, the hanging drop technique[35] was used to generate 3D spheroid cultures. Huh7 cells were treated with 0.1 μM T3 or DMSO for two days and then seeded as droplets onto culture plate lids. Pre-treatment was continued for one day while cells formed spheroids, and the pre-treatment was completed as outlined in Figure 1a. For the next three days, spheroids were treated with

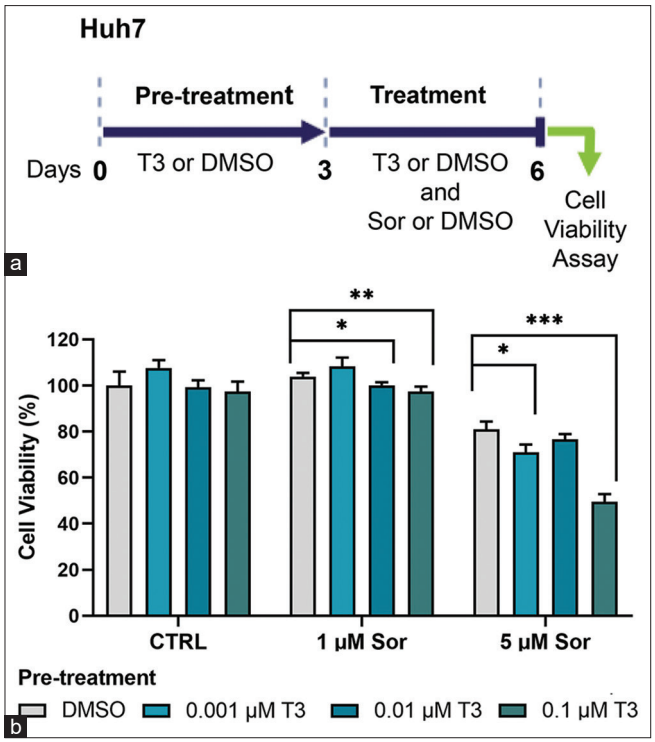

Figure 1: Effects of T3 pre-treatment on sorafenib's cytotoxicity on Huh7 cells in 2D culture. (a) Time-course of the treatment scheme (Sor: sorafenib). (b) Cell viability as demonstrated by MTT assay. DMSO (vehicle) concentrations were equalized in volume for all groups, including sorafenib-treated groups, to account for the artifactual DMSO toxicity. (Two-way ANOVA: $^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$, error bars = SEM)

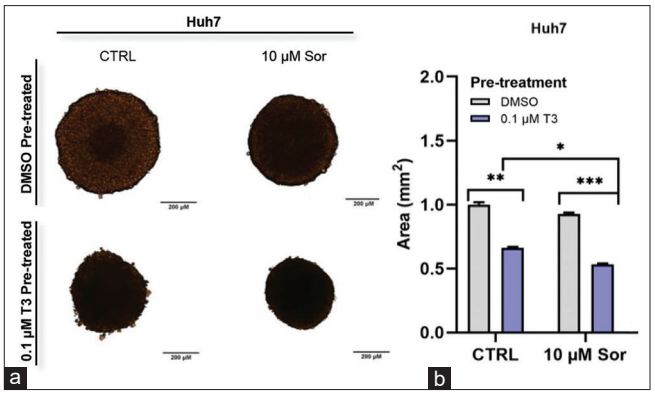

Figure 2: Effects of T3 pre-treatment on sorafenib's cytotoxicity on Huh7 cells in 3D culture. (a) Representative Huh7 spheroids were pre-treated with either 0.1 µM T3 or DMSO before 10 µM sorafenib treatment. Scale Bar: 200 µm. (b) Spheroid area comparisons as calculated by Image J (Two-way ANOVA: $^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$, error bars = SEM)

sorafenib or DMSO in the absence or presence of T3. Because of the increased resistance in spheroids (3D) compared to 2D cell culture and the expected low drug penetration rates, the dose of sorafenib this time was increased to 10 µM in line with the study of Karabiçici *et al.*[36] as the sorafenib exerts its effect at higher concentrations in 3D conditions. T3 treatment alone or in combination with sorafenib significantly reduced the spheroid size, and T3 pre-treatment brought an additive effect for sorafenib response in 3D [Figure 2a and b].

T3 treatment slows down Huh7 cell migration

To further elucidate the effects of T3 treatment on Huh7 hepatocellular carcinoma cells, a migration assay was performed with the help of the scratch method.[37] Cells were pre-treated with 0.1 µM T3 for three days [as Figure 1a outlined], followed by scratching. Next, cells were treated with 5 µM sorafenib or DMSO in the continuum of T3 or DMSO treatment. The gap closure in the scratched wound was visualized and assessed with a bright-field microscope every 24 hours for five days [Figure 3a], and images were analyzed with Image J using a wound-healing analysis pipeline [Figure 3b].[38] Although statistically insignificant, a trend of impaired migration was realized in the presence of T3 for the first 24 hours. Gap closure difference reached statistical significance in three days (72 hours) for T3 pre-treated cells, and there was an insignificant trend of impairment in cell migration with T3 pre-treatment for the sorafenib treatment group. Altogether, T3 treatment slowed down Huh7 cell migration, and it seems to exert its effect under the influence of sorafenib as well, even though the latter was statistically insignificant.

T3 pre-treatment modulates the expression and/or phosphorylation statuses of key HCC proteins with an emphasis on AKT down-regulation

To gain a deeper mechanistic insight into the effects of T3 in the previous results presented here, we treated Huh7 cells (in 2D) for 24 hours with DMSO (CTRL), 5 µM sorafenib, 0.1 µM T3 and 5 µM sorafenib + 0.1 µM T3 and observed the expression/phosphorylation changes of crucial signaling and apoptosis proteins involved in HCC pathogenesis.[39] This time, we limited the treatment window to a shorter period to be able to see the initial response upon the treatments before potential pathway rewiring takes place. We verified the increase in sorafenib's cytotoxicity in the presence of T3 by observing an elevation in the levels of cleaved PARP, which is an indicator of apoptotic onset[40] [Figure 4a]. A reduction of β-catenin phosphorylation was also documented with standalone T3 treatment, but this was mostly attributed to a decrease in overall β-catenin levels, which reportedly drives HCC pathogenesis and resistance.[41] Moreover, we checked AKT phosphorylation (S473p), which was reported to be elevated as a resistance response not only to sorafenib within the context of HCC[42,43] but also to other kinase inhibitors used in various other types of cancers.[44] Our result pointed out an increase in the phosphorylation levels of AKT in Huh7 cells in response to sorafenib treatment, which was in agreement with previous studies.[42,43] Interestingly, T3 pre-treatment helped Huh7 cells maintain AKT phosphorylation at levels closer to untreated cells in response to sorafenib treatment. Standalone T3 treatment, on the other hand, caused a marked decrease in total AKT levels, which seem also mildly elevated in response to standalone sorafenib treatment. When we examined gene expression levels of AKT for all three AKT

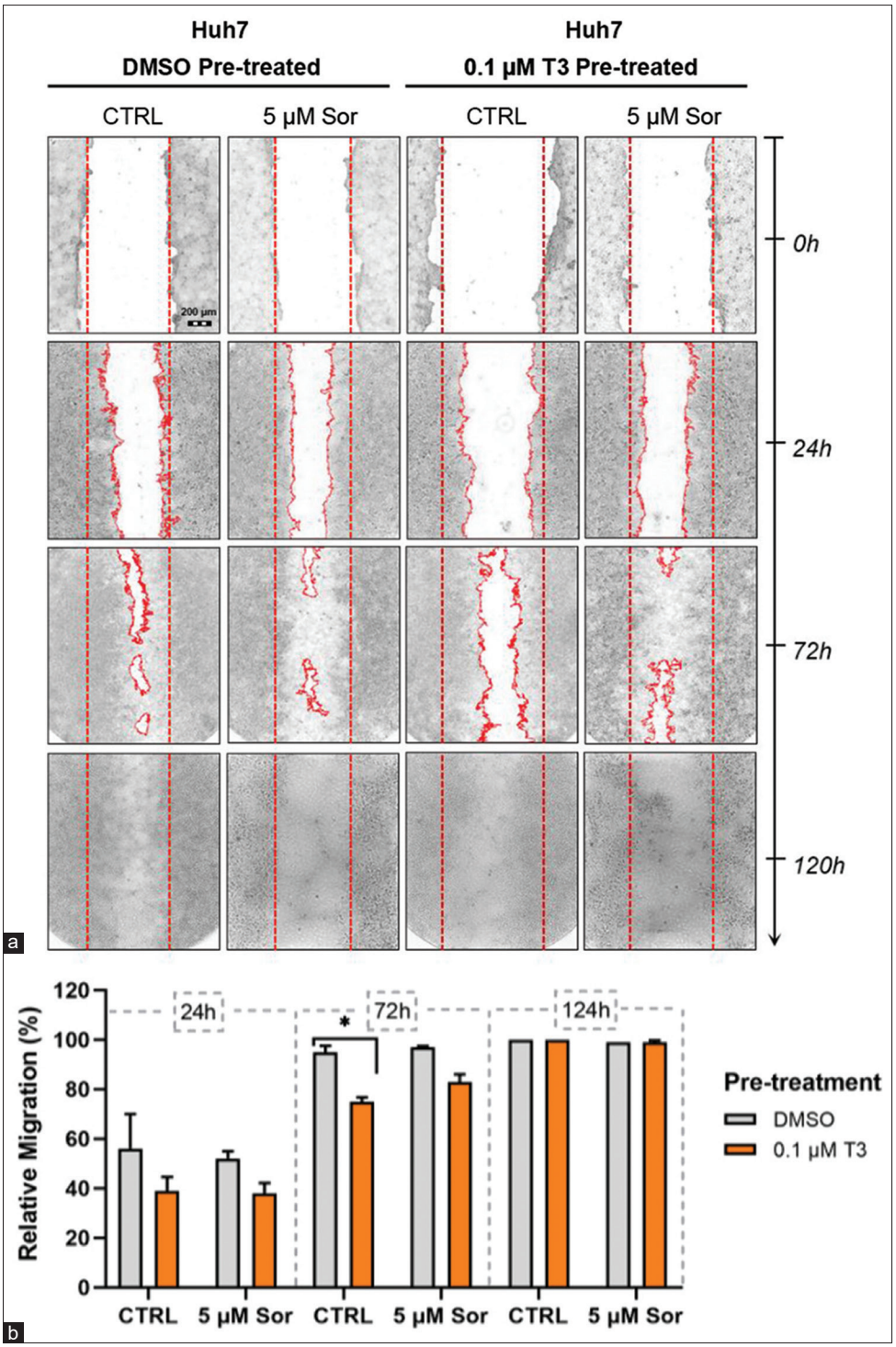

Figure 3: Effects of T3 on Huh7 cell migration in the presence and absence of sorafenib. (a) Representative bright-field microscopy images of gap closures of Huh7 cells upon DMSO or T3 pre-treatment followed by DMSO or sorafenib treatment. (b) Analysis of relative migration with Image J. (Two-way ANOVA, **P* < 0.05, error bars = SEM)

isoforms with quantitative real-time PCR (Q-PCR), we realized a dramatic increase in the expressions of all isoforms as a response to sorafenib [Figure 4b]. Importantly, this possibly resistance-related increase was rescued with T3 pre-treatment followed by sorafenib treatment. Still, the effects on AKT did not seem to be resonated at the phosphorylation levels of ERK [Figure 4a], which is a downstream effector kinase of the RAS/RAF pathway.[45]

DISCUSSION

The multitude of physiological functions exerted by the thyroid

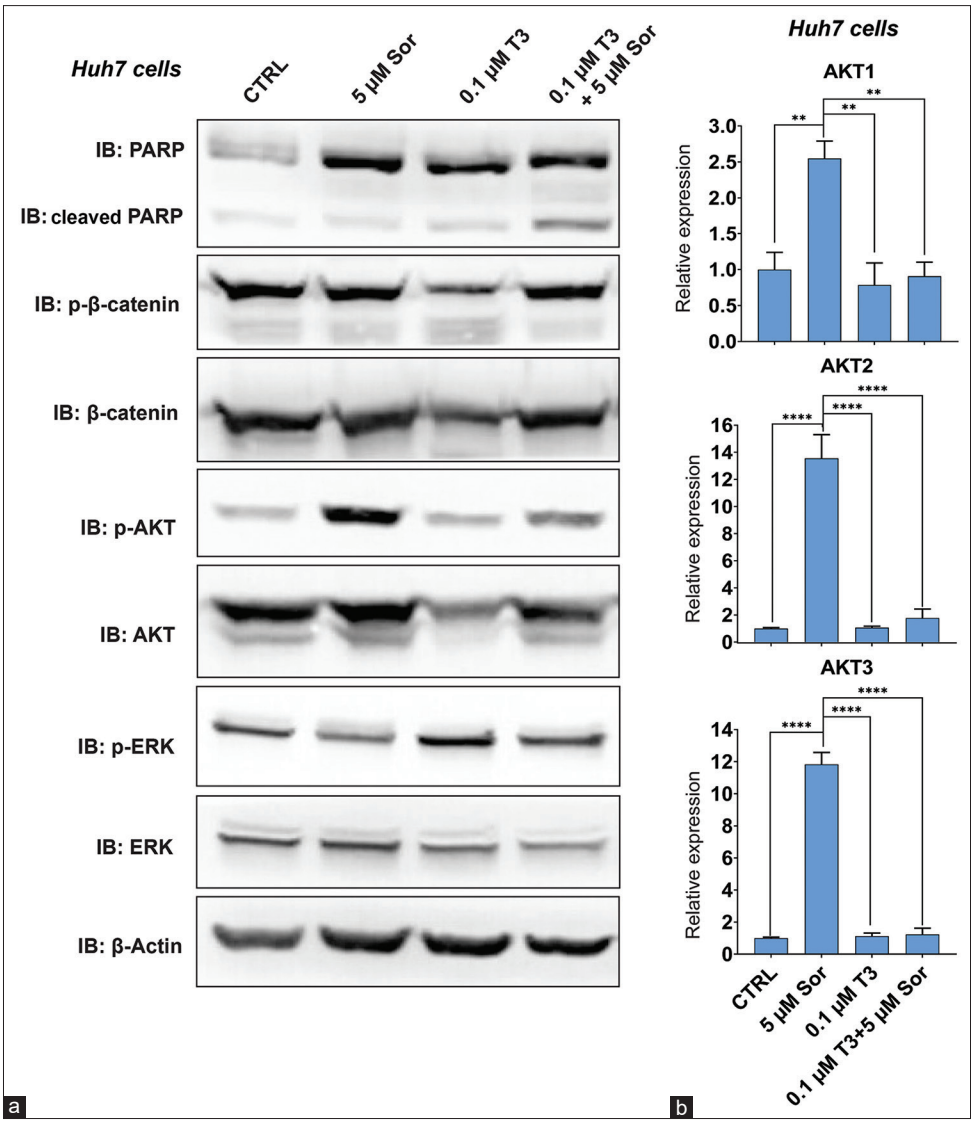

Figure 4: Phosphorylation and expression levels of key hepatocellular carcinoma proteins. (a) Western blot image of total protein lysates obtained after DMSO (CTRL), 5 μM sorafenib, 0.1 μM T3, and 0.1 μM T3 + 5 μM sorafenib treatment for 24 hours. Immuno-blotted proteins were indicated on the left panel of the image. (b) Quantitative real-time PCR result obtained after treatments for three main AKT isoforms. Gene expression levels were shown relative to β-actin. (Two-way ANOVA: **P* < 0.05, ***P* < 0.01, ****P* < 0.001, error bars = SEM)

hormone in crucial processes, including development, growth, and metabolism, makes it no wonder that it was also associated with cancer.[18] Hepatocellular carcinoma comes across as a type of cancer where T3 activity was shown to inhibit the progression through modulating the metabolic pathways and inducing re-differentiation of neoplastic cells into a benign phenotype suggesting T3 could potentially be a therapeutic agent for patients with HCC.[19] In this study, we combined the FDA-approved first-line treatment sorafenib with T3 hormone pre-treatment to investigate whether supporting therapy with thyroid hormone could increase efficacy in comparison to standalone treatment. We tested this on Huh7 hepatocellular carcinoma cells *in vitro*, both in 2D and 3D conditions, and observed that T3 treatment brings an additive effect to sorafenib response. Cell migration assay also was in line with this observation, and western blots, as well as Q-PCR, confirmed that the impact of T3 on HCC is likely to be arisen by its down-regulating effect on AKT expression and phosphorylation.

Recent studies suggested that drug sensitivity in HCC is associated with differentiation status.[27,36,46] Huh7 cell line exhibits epithelial-like characteristics,[28] and the β-catenin prompts mutations, which makes these cells sensitive to β-catenin inhibition.[47,48] We documented a small decrease in phosphorylated β-catenin levels with T3 treatment. Even though this effect seems to be reversed when cells were also exposed to sorafenib, it could explain a potential dimension where T3 activity was influential in HCC. The most remarkable effect of T3 in this study was on the AKT pathway, where AKT levels were significantly impaired in the presence of T3. Interestingly, a correlation between the differentiation status of tumor nodules and p-AKT expression levels has been

found in HCC, where increased p-AKT levels indicate a highly differentiated tumor.[49] More importantly, AKT levels were shown to serve as a prognostic marker in HCC patients,[50] and acquired resistance to sorafenib treatment was associated with increased AKT expression and phosphorylation.[42,43] Here, we confirmed that sorafenib treatment indeed increases AKT levels, which was surprisingly suppressed in the presence of thyroid hormone T3. This observation possibly explains the beneficial additive effect of T3 in sorafenib treatment, as documented with our MTT, spheroid, and cell migration assays.

In conclusion, our findings provide new insights into how T3 hormone modulates the molecular behavior of HCC cells, indicating a promising new avenue where T3 treatment could be considered as a combinatory agent to increase the effect of FDA-approved sorafenib. As newer studies are warranted with more HCC cell lines and *in vivo* models to further elaborate the results presented here, transcriptomic and phosphoproteomic approaches would be useful to understand unknown dimensions of thyroid hormone function in HCC. Considering the fact that thyroid hormone treatment is already widely used in clinics for various other health conditions, the phenomena explained in this research article has the potential to bring immediate benefit to HCC patients.

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Conflicts of interest

There are no conflicts of interest.

REFERENCES

1. Marengo A, Rosso C, Bugianesi E. Liver cancer: Connections with obesity, fatty liver, and cirrhosis. Annu Rev Med 2016;67:103–17.
2. Chen C, Wang G. Mechanisms of hepatocellular carcinoma and challenges and opportunities for molecular targeted therapy. World J Hepatol 2015;7:1964–70.
3. Méndez-Sánchez N, Vásquez-Fernández F, Zamora-Valdés D, Uribe M. Sorafenib, a systemic therapy for hepatocellular carcinoma. Ann Hepatol 2008;7:46–51.
4. Shaaban S, Negm A, Ibrahim EE, Elrazak AA. chemotherapeutic agents for the treatment of hepatocellular carcinoma: Efficacy and mode of action. Oncol Rev 2014;8:246.
5. Zhu YJ, Zheng B, Wang HY, Chen L. New knowledge of the mechanisms of sorafenib resistance in liver cancer. Acta Pharmacol Sin 2017;38:614–22.
6. Cable EE, Finn PD, Stebbins JW, Hou J, Ito BR, van Poelje PD, *et al*. Reduction of hepatic steatosis in rats and mice after treatment with a liver-targeted thyroid hormone receptor agonist. Hepatology 2009;49:407–17.
7. Hassan MM, Kaseb A, Li D, Patt YZ, Vauthey JN, Thomas MB, *et al*. Association between hypothyroidism and hepatocellular carcinoma: A case-control study in the United States. Hepatology 2009;49:1563–70.
8. Reddy A, Dash C, Leerapun A, Mettler TA, Stadheim LM, Lazaridis KN, *et al*. Hypothyroidism: A possible risk factor for liver cancer in patients with no known underlying cause of liver disease. Clin gastroenterol hepatol 2007;5:118–23.
9. Pirahanchi Y, Toro F, Jialal I. Physiology, Thyroid Stimulating Hormone. In: StatPearls. Treasure Island (FL): StatPearls Publishing; 2021. Available from: http://www.ncbi.nlm.nih.gov/books/NBK499850/. [Last accessed on 2021 Mar 24]
10. Visser TJ, Kaptein E, Terpstra OT, Krenning EP. Deiodination of thyroid hormone by human liver. J Clin Endocrinol Metab 1988;67:17–24.
11. Chi HC, Tsai CY, Tsai MM, Yeh CT, Lin KH. Molecular functions and clinical impact of thyroid hormone-triggered autophagy in liver-related diseases. J Biomed Sci 2019;26:24.
12. Gionfra F, De Vito P, Pallottini V, Lin HY, Davis PJ, Pedersen JZ, *et al*. The role of thyroid hormones in hepatocyte proliferation and liver cancer. Front Endocrinol 2019;10. Available from: https://www.frontiersin.org/articles/10.3389/fendo0.2019.00532/full. [Last accessed on 2021 Mar 24]
13. Peng X, Zhou Y, Sun Y, Song W, Meng X, Zhao C, *et al*. Overexpression of modified human TRβ1 suppresses the growth of hepatocarcinoma SK-hep1 cells *in vitro* and in xenograft models. Mol Cell Biochem 2018;449:207–18.
14. Yen CC, Huang YH, Liao CY, Liao CJ, Cheng WL, Chen WJ, *et al*. Mediation of the inhibitory effect of thyroid hormone on proliferation of hepatoma cells by transforming growth factor-beta. J Mol Endocrinol 2006;36:9–21.
15. Lin YH, Huang YH, Wu MH, Wu SM, Chi HC, Liao CJ, *et al*. Thyroid hormone suppresses cell proliferation through endoglin-mediated promotion of p21 stability. Oncogene 2013;32:3904–14.
16. Chi HC, Liao CH, Huang YH, Wu SM, Tsai CY, Liao CJ, *et al*. Thyroid hormone receptor inhibits hepatoma cell migration through transcriptional activation of Dickkopf 4. Biochem Biophys Res Commun 2013;439:60–5.
17. Chung IH, Liu H, Lin YH, Chi HC, Huang YH, Yang CC, *et al*. ChIP-on-chip analysis of thyroid hormone-regulated genes and their physiological significance. Oncotarget 2016;7:22448–59.
18. Krashin E, Piekiełko-Witkowska A, Ellis M, Ashur-Fabian O. Thyroid hormones and cancer: A comprehensive review of preclinical and clinical studies. Front Endocrinol 2019;10:59.
19. Kowalik MA, Puliga E, Cabras L, Sulas P, Petrelli A, Perra A, *et al*. Thyroid hormone inhibits hepatocellular carcinoma progression via induction of differentiation and metabolic reprogramming. J Hepatol 2020;72:1159–69.
20. Tang Q, Zeng M, Chen L, Fu N. Targeting thyroid hormone/thyroid hormone receptor axis: An attractive therapy strategy in liver diseases. Front Pharmacol 2022;13:871100.
21. Keating GM. Sorafenib: A review in hepatocellular carcinoma. Target Oncol 2017;12:243–53.
22. Salvatore D, Porcelli T, Ettleson MD, Bianco AC. The relevance of T3 in the management of hypothyroidism. Lancet Diabetes Endocrinol 2022;10:366–72.
23. Schneider CA, Rasband WS, Eliceiri KW. NIH Image to ImageJ: 25 years of image analysis. Nat Methods 2012;9:671–5.
24. Zhang Y, Ma L, Lu E, Huang W. Atorvastatin Upregulates microRNA-186 and Inhibits the TLR4-mediated MAPKs/NF-κB pathway to relieve steroid-induced avascular necrosis of the femoral head. Front Pharmacol 2021;12:583975.
25. Livak KJ, Schmittgen TD. Analysis of relative gene expression data using real-time quantitative PCR and the $2-\Delta\Delta CT$ method. Methods 2001;25:402–8.
26. Buontempo F, Ersahin T, Missiroli S, Senturk S, Etro D, Ozturk M, *et al*. Inhibition of Akt signaling in hepatoma cells induces apoptotic cell death independent of Akt activation status. Invest New Drugs 2011;29:1303–13.

27. Caruso S, Calatayud AL, Pilet J, La Bella T, Rekik S, Imbeaud S, *et al*. Analysis of liver cancer cell lines identifies agents with likely efficacy against hepatocellular carcinoma and markers of response. Gastroenterology 2019;157:760–76.
28. Yuzugullu H, Benhaj K, Ozturk N, Senturk S, Celik E, Toylu A, *et al*. Canonical Wnt signaling is antagonized by noncanonical Wnt5a in hepatocellular carcinoma cells. Mol Cancer 2009;8:90.
29. Alvarado TF, Puliga E, Preziosi M, Poddar M, Singh S, Columbano A, *et al*. Thyroid hormone receptor β agonist induces β-catenin-dependent hepatocyte proliferation in mice: Implications in hepatic regeneration. Gene Expr 2016;17:19–34.
30. Gnoni GV, Rochira A, Leone A, Damiano F, Marsigliante S, Siculella L. 3,5,3'triiodo-L-thyronine induces SREBP-1 expression by non-genomic actions in human HEP G2 cells. J Cell Physiol 2012;227:2388–97.
31. Garten A, Grohmann T, Kluckova K, Lavery GG, Kiess W, Penke M. Sorafenib-induced apoptosis in hepatocellular carcinoma is reversed by SIRT1. Int J Mol Sci 2019;20:4048.
32. Huang PS, Lin YH, Chi HC, Chen PY, Huang YH, Yeh CT, *et al*. Thyroid hormone inhibits growth of hepatoma cells through induction of miR-214. Sci Rep 2017;7:14868.
33. van Meerloo J, Kaspers GJ, Cloos J. Cell sensitivity assays: The MTT assay. Methods Mol Biol Clifton NJ 2011;731:237–45.
34. Takai A, Fako V, Dang H, Forgues M, Yu Z, Budhu A, *et al*. Three-dimensional organotypic culture models of human hepatocellular carcinoma. Sci Rep 2016;6:21174.
35. Ayvaz I, Sunay D, Sariyar E, Erdal E, Karagonlar ZF. Three-dimensional cell culture models of hepatocellular carcinoma - A Review. J Gastrointest Cancer 2021;52:1294–308.
36. Karabicici M, Azbazdar Y, Ozhan G, Senturk S, Firtina Karagonlar Z, Erdal E. Changes in Wnt and TGF-β signaling mediate the development of regorafenib resistance in hepatocellular carcinoma cell line HuH7. Front Cell Dev Biol 2021;9:639779.
37. Liang CC, Park AY, Guan JL. *In vitro* scratch assay: A convenient and inexpensive method for analysis of cell migration in vitro. Nat Protoc 2007;2:329–33.
38. Venter C, Niesler CU. Rapid quantification of cellular proliferation and migration using ImageJ. BioTechniques 2019;66:99–102.
39. Deldar Abad Paskeh M, Mirzaei S, Ashrafizadeh M, Zarrabi A, Sethi G. Wnt/β-catenin signaling as a driver of hepatocellular carcinoma progression: An emphasis on molecular pathways. J Hepatocell Carcinoma 2021;8:1415–44.
40. Weaver AN, Yang ES. Beyond DNA repair: Additional functions of PARP-1 in cancer. Front Oncol 2013;3:290.
41. Xu C, Xu Z, Zhang Y, Evert M, Calvisi DF, Chen X. β-Catenin signaling in hepatocellular carcinoma. J Clin Invest 2022;132:e154515.
42. Bort A, Spínola E, Rodríguez-Henche N, Díaz-Laviada I. Capsaicin exerts synergistic antitumor effect with sorafenib in hepatocellular carcinoma cells through AMPK activation. Oncotarget 2017;8:87684–98.
43. Chen KF, Chen HL, Tai WT, Feng WC, Hsu CH, Chen PJ, *et al*. Activation of phosphatidylinositol 3-kinase/Akt signaling pathway mediates acquired resistance to sorafenib in hepatocellular carcinoma cells. J Pharmacol Exp Ther 2011;337:155–61.
44. Rascio F, Spadaccino F, Rocchetti MT, Castellano G, Stallone G, Netti GS, *et al*. The pathogenic role of PI3K/AKT pathway in cancer onset and drug resistance: An updated review. Cancers (Basel) 2021;13:3949.
45. Lavoie H, Gagnon J, Therrien M. ERK signalling: A master regulator of cell behaviour, life and fate. Nat Rev Mol Cell Biol 2020;21:607–32.
46. Rebouissou S, Nault JC. Advances in molecular classification and precision oncology in hepatocellular carcinoma. J Hepatol 2020;72:215–29.
47. Cagatay T, Ozturk M. p53 mutation as a source of aberrant β-catenin accumulation in cancer cells. Oncogene 2002;21:7971–80.
48. Ding Z, Shi C, Jiang L, Tolstykh T, Cao H, Bangari DS, *et al*. Oncogenic dependency on β-catenin in liver cancer cell lines correlates with pathway activation. Oncotarget 2017;8:114526–39.
49. Kunter I, Erdal E, Nart D, Yilmaz F, Karademir S, Sagol O, *et al*. Active form of AKT controls cell proliferation and response to apoptosis in hepatocellular carcinoma. Oncol Rep 2014;31:573–80.
50. Zhang Y, Guo X, Yang M, Yu L, Li Z, Lin N. Identification of AKT kinases as unfavorable prognostic factors for hepatocellular carcinoma by a combination of expression profile, interaction network analysis and clinical validation. Mol Biosyst 2014;10:215–22.