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Research Article | Araştırma Makalesi

ESTABLISHMENT OF ACQUIRED SORAFENIB RESISTANCE IN HEPATOCELLULAR CARCINOMA CELL LINE AND EXAMINATION OF EMT

HEPATOSELÜLER KARSİNOM HÜCRE HATTINDA EDİNİLMİŞ SORAFENİB DİRENCİNİN BELİRLENMESİ VE EMT'NİN İNCELENMESİ

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ABSTRACT

Objectives: Sorafenib is one of the major drugs used in Hepatocellular carcinomas (HCC) treatments, but its usage can be limited by acquired drug resistance. The aim of the study is to examine the activity of Epithelial mesenchymal transition (EMT) process in the formation of sorafenib resistance in HCC cells.

Methods: In this study, we established sorafenib-resistant HCC cell lines and characterized them using cell viability assays (WST-1). Expression levels EMT-related genes were determined by RT-PCR.

Results: As a result of increasing doses of sorafenib, HEPG2-SR cells were formed. When the EMT process in resistant cells was examined, it was determined that CDH2 and TWIST expression increased during the resistance acquisition process ($p<0.001$).

Conclusion: Sorafenib is one of the most important treatment options, especially in HCCs who relapse after transplantation. However, since sorafenib resistance may develop in patients with high TWIST expression, new treatment options are needed in these patients

Keywords: Hepatocellular carcinoma, drug-resistance, EMT, Sorafenib

ÖZ

Amaç: Sorafenib, Hepatoselüler karsinoma (HCC) tedavilerinde kullanılan başlıca ilaçlardan biridir, ancak kazanılmış ilaç direnci nedeniyle kullanımı sınırlıdır. Çalışmada HCC hücrelerinde sorafenib direnci oluşum aşamasında epitelyal mezenkimal geçiş (EMT) sürecinin aktivitesinin incelenmesi amaçlanmaktadır.

Yöntem: HCC hücre hattı olan HEPG2'ye artan dozda sorafenib uygulanarak sorafenib'e dirençli HCC hücre hatları oluşturuldu (HEPG2-SR), kontrolü hücre proliferasyon testi olan WST-1 ile sağlandı. Direnç aşamasında RT-PCR analiz ile EMT ile ilgili genlerin ekspresyon profilleri incelendi.

Bulgular: Artan sorafenib dozları sonucunda HEPG2-SR hücreleri oluştu. Dirençli hücrelerde EMT süreci incelendiğinde, direnç kazanma sürecinde CDH2 ve TWIST ekspresyonunun arttığı belirlendi ($p<0.001$).

Sonuç: Sorafenib, özellikle transplantasyon sonrası nüks gösteren HCC'lerde en önemli tedavi seçeneklerinden biridir. Ancak TWIST ekspresyonu yüksek olan hastalarda sorafenib direnci gelişebileceğinden bu hastalarda yeni tedavi seçeneklerine ihtiyaç duyulmaktadır.

Anahtar Kelimeler: Hepatoselüler karsinom, ilaç direnci, EMT, Sorafenib

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Introduction

Hepatocellular carcinoma (HCC) is one of the most frequently observed neoplasms worldwide and ranks third in cancer-related deaths.[1] HCC mostly develops due to cirrhosis resulting from chronic Hepatitits B virus (HBV) and Hepatitis C virus (HCV) infection.[2] In the treatment of this disease, different approaches such as tumor resection, radiofrequency ablation, chemoembolization, radioembolization or orthotopic liver transplantation (orthotopic liver transplantation: OLT) are applied.[3] OLT is an increasingly common curative treatment option, especially for HCC patients with cirrhosis and no local or distant metastases.[4] However, recurrence after transplantation is one of the most important factors reducing the success of the treatment in patients receiving this treatment.[5] Sorafenib is a multikinase inhibitor approved by the Food and Drug Administration (FDA) for the treatment of HCC.[6] However, HCC patients may develop drug resistance during chemotherapy with Sorafenib.[7]

In recent studies, it has been emphasized that the Epithelial Mesenchymal Transition (EMT) is migratory in the progression of solid tumors and the development of drug resistance.[8,9] The EMT is a multistep process whereby epithelial cells change in plasticity by transient de-differentiation into a mesenchymal phenotype. In carcinoma progression, EMT plays a crucial role in early steps of metastasis when cells lose cell-cell contacts due to ablation of E-cadherin and acquire increased motility to spread into surrounding or distant tissues and drug resistance.[10-12]

ZEB (ZEB1/2), basic helix loop helix protein 38 (TWIST), and Snail (SNAIL1/2), have been reported as factors that can mediate gene expression and regulate EMT. Among these, ZEB1 may be particularly important as a key transcription factor for EMT: in the earliest stages of EMT, TWIST is induced by TGF-β signaling, a critical cellular initiator of EMT. These transcription factors are correlated with resistance to chemotherapy in cancers and disrupts the epithelial phenotype.[13-15]

In studies conducted in HCCs, a limited number of studies have shown that this mechanism plays a role in the development of recurrence and poor prognosis after treatment.[9] However, the relationship between Sorefenib resistance in HCCs and EMT is unknown.

In the current study, it is aimed to determine the role of the EMT mechanism in the HCC cell line that has become Sorefenib resistant.

Methods

Cell Culture

Human HCC HepG2 cells were obtained from the American Type Culture Collection (ATCC, Manassas, VA, USA). Cells were incubated in Dulbecco's modified Eagle's medium (DMEM) (Gibco BRL, Grand Island, NY, USA) supplemented with 10% fetal bovine serum at 37°C and 5% CO2. Sorafenib (Sigma-Aldrich, St. Louis, MO, USA) was dissolved in dimethyl sulfoxide (DMSO) as a 100 mM stock solution.

Establishment of Sorafenib Resistance

Initially, sorafenib was administered at a dose range of 2.5 µM-25 µM to determine the lethal dose of Sorefenib in HEPG2 cells (IC50: killing more than 50% of cells).[16] Cell proliferation was analyzed with the WST-1 kit at 24, 48 and 72 hours.

HEPG2 cells were exposed to sorafenib at a low dose (2.5 µM) and higher dose (5 µM, 7.5 µM and 10 µM) when cells grew stably (reached 80% occupancy). The medium containing sorafenib was changed every 2 days for 3 months. Eventually, cells were observed to be able to proliferate in medium containing 10 µM sorafenib (a clinically relevant dose). HepG2 cells rendered resistant to sorefenib were named HEPG2-SR.

RNA Isolation, cDNA Synthesis and RT-PCR Analysis

Total RNA was isolated from HEPG2 and HEPG2S cells using E.Z.N.A.® Total RNA Kit I (Omega BioTek Inc., Norcross, GA, USA). All RNAs were checked for quality and quantity using a spectrophotometer (Beckman Coulter, Inc., Fullerton, CA, USA). Approximately 1 µg of RNA was used for cDNA synthesis with a high capacity cDNA Reverse Transcription Kit (Applied Biosystems, Foster City, CA, USA). Then, based on TaqMan™ Gene Expression Assays technology, CDH1 (E-Kaderin, Hs01023895_m1), SNAI1 (Snail, Hs00195591_m1) and CDH2 (N-cadherin, Hs00983056_m1), TWIST (Hs04989912_s1) and MMP9 (Hs00957562_m1) on AbiStepOnePlus™ instrument (Applied Biosystems, Foster City, CA, USA) -PCR analysis was performed. Gene expression levels were normalized to (Actin beta, Hs01060665_g1) expression.[10]

Statistical Analysis

For statistics, GraphPad Prism 6 (La Jolla, CA, USA) was used in all experiments. All analyzed values were expressed as mean ± standard deviation. Statistics of gene expressions were used to compare gene expression differences between HEPG2 and HEPG2S, using the $2^{-\Delta\Delta Ct}$ method.

Results

Chronic exposure of HepG2 cells to increasing concentrations of sorafenib resulted in sorafenib-resistant cell lines termed HepG2-SR. Incubation of sorafenib with HepG2 cells reduced their viability in a concentration-dependent manner (Figure 1). However, HepG2-SR cells proved resistant to sorafenib when exposed to the same concentration of sorafenib as their viability was significantly higher than that of the respective parent cells (Figure 1). In the presence of 20 µM sorafenib, HepG2-SR a was 36.3%, while the main cells were almost completely nonviable (Figure 1). While the IC50 dose of Sorafenib for HEPG2 cells was determined as 8 µM, it was observed that this dose increased above 25 µM in HEPG2-SR cells ($P< 0.001$).

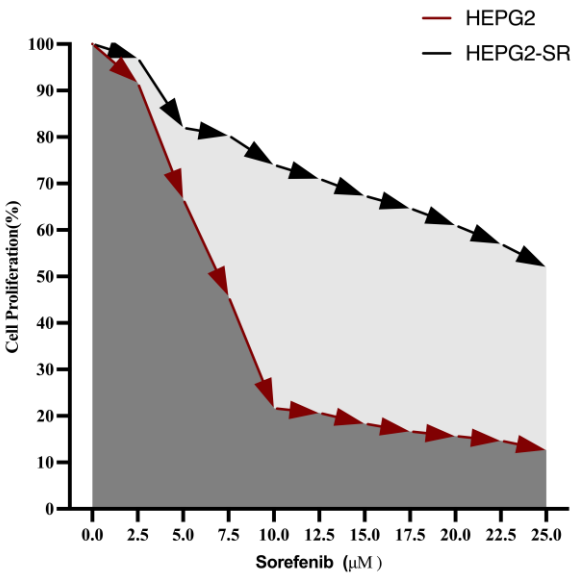

Figure 1. HepG2-SR exhibited elevated IC50 for sorafenib compared with its matched maternal cell line.

To evaluate EMT activity in sorafenib-resistant HEPG2-SR cells, EMT markers CDH1, CDH2, SNAIL, TWIST and MMP9 were analyzed at the mRNA level. Compared to HEPG2 cells, there was no statistically significant expression difference in CDH1, SNAIL and MMP9 mRNAs in HEPG2-SR cells (Figure 2A), while CDH2mand TWIST were found to be 3.2 and 3.6 times higher expressed in HEPG2-SR cells, respectively (Figure 2B and Figure 2C, p<0.001).

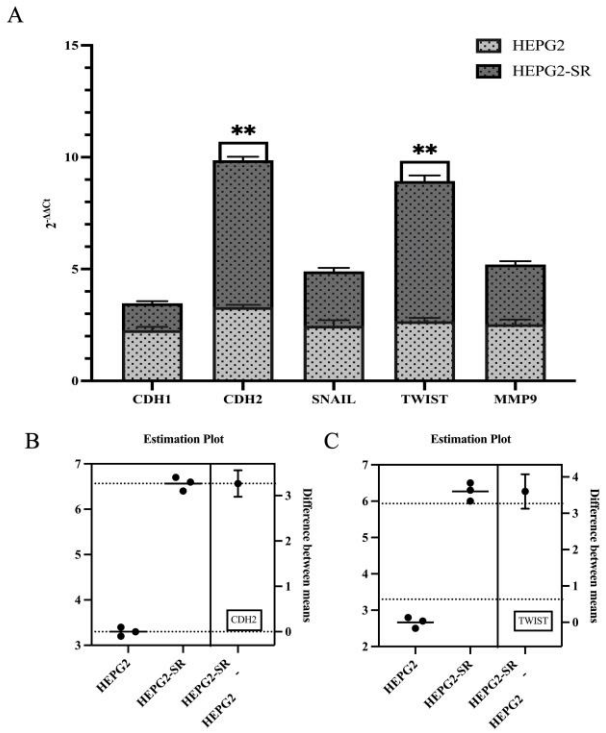

Figure 2. The expression profiles of EMT markers in HEPG2-SR compared to HEPG2 cells. (A) CDH2 was 3.2-fold up regulated in HEPG2-SR, (B) TWIST was found to be 3.6-fold higher exoressed in HEPG2-SR cells compared to HEPG2 cells.

Discussion

Sorafenib remains the globally accepted systemic first-line treatment for advanced HCC.[5] Even though it only has modest improvement in over-all median survival, its approval in 2007 is one of the hallmarks of HCC treatment.[6] Sorafenib is a multikinase inhibitor with antiangiogenic and antiproliferative effects and is the only clinically approved drug for patients with advanced HCC.[3] The main target of sorafenib is the serine/threonine kinase Raf-1, which is involved in the Raf/mitogen-activated protein kinase (MAPK)/extracellular signal-regulated kinase (ERK) pathway.[4] Sorafenib shows potent inhibitory activity against cell proliferation, invasion, metastasis and multi-drug resistance (MDR) by inhibiting MAPK signaling in HCC. However, this promising treatment has shown limited survival benefits (2.8 months) with very low response rates (2-3%).[4]

Recently published studies highlight that EMT plays a role in chemoresistance as well as shorter disease-free survival in HCC.[6,7] EMT, a developmental process involving loss of epithelial cell markers and acquisition of mesenchymal cell characteristics, is thought to have important roles in the development of the invasive and metastatic potential of HCC.[8] The characteristic down-regulation of E-cadherin is expressed as the essential step of EMT and the zinc finger transcriptional repressors Snail, Slug and Twist are considered to bind to the E-boxes of the E-cadherin promoter and suppress its transcription in response to the upstream signal. These transcription factors are the most prominent repressors of E-cadherin transcription.[9] In addition, the SNAIL transcription factor plays a crucial role in the expression of mesenchymal markers such as Vimentin and matrix metalloproteinases (MMP-2, 9) in HCC cells.[11] These studies suggest that SNAIL expression is an important step leading to invasion, metastasis, and HCC progression. In a previous report, sorafenib was shown to exert potent inhibitory activity against EMT by inhibiting SNAIL expression in HCC cells via the MAPK signaling pathway.[11] However, the association between EMT and MDR in sorafenib-resistant HCC cell lines has been rarely reported.

In our study, we analyzed the activation of the EMT mechanism in sorafenib-resistant HEPG2-SR cells. We determined that CDH2 (N-cadherin) and TWIST expressions were at least 3 times higher in HEPG2-SR cells than in HEPG2 cells. TWIST, which is a member of the basic helix-loop-helix class of proteins, is known to induce EMT and promote metastasis in many solid tumor.[14-16] Lee et al. associated high TWIST expression with poor prognosis in their study on HCC tissues and HCC cell lines.17 Recent studies have reported that EMT is associated with chemoresistance in cancer.[17-19]TWIST overexpression is also correlated with chemotherapy resistance in various types of cancer and leads to a poorer prognosis.[20]Therefore, TWIST may be considered a novel therapeutic target in overcoming MDR in liver cancer.

However, there is no study showing the effectiveness of TWIST on sorafenib resistance.

Sorafenib is one of the most preferred treatment options, especially in HCC patients with recurrence. However, drug resistance is an important problem. In our study, we investigated the activity of the EMT mechanism in the resistance acquisition process of the cells by creating sorafenib resistance in HEPG2 cells, and we determined that TWIST increased with the development of resistance in this process. Understanding the EMT as might contribute to enlighten new treatment strategies to overcome drug resistance.

Compliance with Ethical Standards

Ethical committee approval is not required for this study.

Conflict of Interest

The authors declare there are no conflicts of interest—financial or otherwise—related to the material presented herein.

Author Contribution

Authors contributed equally to this work.

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