INTRODUCTION

Hepatocellular carcinoma (HCC) refers to one of the most prevalent malignancies worldwide, ranked as the second leading cause of cancer-induced death in China (1). The root reason for HCC’s high mortality is because of poor prognosis, drug resistance, metastasis, and lack of satisfactory curative medication (2). Sorafenib (Sor), a small multi-kinase inhibitor, is FDA of the United States food and drug administration (FDA) of the United States approved drug-based treatment for terminal primary liver cancer (3). Sor treatment can induce cell apoptosis via inactivating several tyrosine protein kinases, which may suppress tumor growth (4). However, detailed pharmacological mechanism of Sor against HCC is limited. In biology, ER stress presents a group of pathophysiological processes, and it has been great interest in controlling this pathway for the treatment of clinical diseases. In addition, apoptosis is closely interconnected with ER stress via disorder protein folding in cell (5, 6). Therefore, a better understanding of biological mechanism regarding the cell fate that regulates the development of HCC may identify therapeutic target for Sor-treated advanced HCC patients. In current study, we collected respective samples of sera, non-treated and Sor-treated HCC patients for conducting a series of biochemical analyses and immunoassays, as well as the underlying molecular mechanism would be discussed correspondingly.

MATERIALS AND METHODS

Human design

A total of ten patients were medically diagnosed with advance HCC through biochemical assays and pathological diagnosis before taking Sor therapy. As a non-treated control, sera form respective HCC patients were collected for biochemical tests. After being treated with 200 mg of Sor twice a day for around 12 months, samples of sera were prepared through centrifugation with 3,000 rpm at 4°C for 15 min, followed by storing at –20°C until use. In addition, some of liver samples
were fixed with 4% paraformaldehyde for further immunostaining. Other parts were stored instantly at -80°C for subsequent immunoblotting assay. In a statement, human ethical guidelines were followed by the Declaration of Helsinki as described elsewhere (7).

Determination of serous parameters

All serological parameters were calculated by use of an automatic biochemical analyser (Hitachi, Japan) from clinical laboratory of Guigang City People’s Hospital. Inflammatory cytokines contents and other lipids-associated molecules in sera between grouped stages were calculated using commercial enzyme linked immunosorbent assay kits (Shanghai Elisa Biotech, China), and final data were produced from a standard curve (8).

Immunohistochemical protocols

Briefly, paraffin-embedded staged liver cancer samples were prepared as 5 µm sections for further rehydration and permeabilization. Followed by being blocked with 5% bovine serum albumin buffer, the sections were incubated with diluted primary antibodies of PCNA, CK19 (1:100, Bioss, China) at 4°C overnight. And then relevant ready-to-use SABC-POD kit (Boster, China) were subjected to antigen-antibody complex for 1 hour. Then, 3, 3’-diaminobenzidine (Boster, China) was assessed when aP < 0.05. Student’s t test for grouped comparison. Statistical significance was assessed when  < 0.05.

RESULTS

Baseline data in hospitalized Sor-treated hepatocellular carcinoma patients

As results, human plasma levels of liver functional molecules (ALT and AST) and the concentrations of AFP, FGF21 were decreased in Sor-treated HCC patients when compared to those in non-treated controls (P < 0.05). In addition, serological levels of TG, T-CHOL, IFN-γ, and TNF-α levels in Sor-treated HCC patients were lowered (P < 0.05), while the IL-10 content was increased (P < 0.05) (Table 1).

Hepatocellular immunophenotype-positive cells in Sor-treated hepatocellular carcinoma

To characterize intrahepatic immunophenotype-labelled cells in Sor-treated liver cancer cells, immunohistochemical and immunofluorescent immunostainings were conducted respectively. As results, proliferative biomarker for PCNA and

Table 1. Clinical parameters of Sor-treated patients with hepatocellular carcinoma (HCC).

<table>
<thead>
<tr>
<th>Clinical parameters</th>
<th>Sor-treated HCC</th>
<th>Non-treated HCC</th>
</tr>
</thead>
<tbody>
<tr>
<td>ALT (U/L)</td>
<td>87.80 ± 8.92 a</td>
<td>390.70 ± 82.32</td>
</tr>
<tr>
<td>AST (U/L)</td>
<td>68.60 ± 9.16 a</td>
<td>261.60 ± 39.98</td>
</tr>
<tr>
<td>AFP (ng/mL)</td>
<td>54.26 ± 9.03 a</td>
<td>141.26 ± 34.45</td>
</tr>
<tr>
<td>FGF21 (ng/mL)</td>
<td>2.30 ± 0.23 a</td>
<td>6.04 ± 0.37</td>
</tr>
<tr>
<td>TG (mmol/L)</td>
<td>1.23 ± 0.18 a</td>
<td>3.22 ± 0.45</td>
</tr>
<tr>
<td>T-CHOL (mmol/L)</td>
<td>2.94 ± 0.45 a</td>
<td>6.47 ± 1.03</td>
</tr>
<tr>
<td>IFN-γ (pg/ml)</td>
<td>77.65 ± 6.43</td>
<td>95.12 ± 7.15</td>
</tr>
<tr>
<td>TNF-α (pg/ml)</td>
<td>206.07 ± 25.37 e</td>
<td>653.83 ± 66.41</td>
</tr>
<tr>
<td>IL-10 (pg/ml)</td>
<td>779.51 ± 70.15 e</td>
<td>345.57 ± 33.48</td>
</tr>
</tbody>
</table>

The comparable data with „a” label were considered to be statistically significant.

Abbreviations: ALT, alanine transaminase; AST, aspartate transaminase; AFP, alpha-fetoprotein; FGF21, fibroblast growth factor 21; TG, triglyceride; T-CHOL, total cholesterol; IFN-γ, IL-10, interferon gamma; interleukin-10; TNF-α, tumor necrosis factor alpha.
metastasized biomarker for CK19 in non-treated liver cancer tissues showed strong expression in respective cytoblasts and epithelia when compared to these in Sor-treated cells. In immunofluorescence staining, PCNA- and CK19-labelled cells in Sor-treated HCC tissue were detected positively, accompanied with the positive outcome of immunohistochemical assay. The positive cell counts in Sor-treated group were less than those in non-treated samples ($P < 0.05$) (Fig. 1).

Characterization of endoplasmic reticulum stress-based regulatory proteins in Sor-treated hepatocellular carcinoma

In order to further assess biological characteristics of ER stress-associated effectors in Sor-treated HCC cells, immunostaining immunoblotting tests and were performed respectively. As results, key regulatory proteins related to ER stress of ATF6, eIF2a, GRP78, XBP1 in Sor-treated cells were increased, resulting in visibly elevated fluorescence-labelled cells when compared to those in non-treated HCC cells. To validate the morphology-positive outcomes, quantitative data from Western blot revealed that upregulated expressions of endogenous ATF6, eIF2a, GRP78, XBP1 in Sor-treated cells were observed, which changed expressions were greater than these in non-treated controls ($P < 0.05$) (Fig. 2).

DISCUSSION

Primary hepatic cancer (HCC) represents a fatal tumor that starts in the liver, which makes up 80% of hepatic cancer cases (14). The diagnosis of HCC can be clinically identified by blood tests (serological markers), medical imaging and relevant tissue biopsy (15). A majority of cancer cells mainly produce necessary energy via a high rate of glycolysis, inducing proliferation of cells (16). Lipids metabolism is responsible for energy supply for uncontrolled growth of cancer cells (17). Inflammation gradually constructs the microenvironment around tumors, benefiting to cancerous survival, proliferation and migration (18). Fibroblast growth factor 21 (FGF21), a hepatokine produced by hepatocytes, regulates tumor growth and invasion, sugar intake and energy homeostasis (19). Chronically, the elevation in FGF21 expression in the liver seems to protect against liver injury (20). Cytokeratin-19 (CK-19) is commonly used as a biomarker to differentiate epithelial cells origin from cancer cells, functioning on carcinomatous metastasis (21). Cytokines exert a key role in the progression of hepatic fibrosis, a diseased condition before cancerization. Further, in intrahepatic disturbed lymph flow, TNF-$\alpha$ may function as an antifibrogenic effect in hepatic fibrogenesis (22). In this study, Sor-pre-treated livers showed markedly increased blood contents of ALT, AST, AFP, preliminarily meaning that these early diagnostic HCC patients were identified prior to being prescribed with Sor. Moreover, serological levels of TG, T-CHOL, and IFN-$\gamma$, and TNF-$\alpha$ levels in Sor-treated HCC patients were lowered, while the IL-10 content was increased. Thus, inhibited lipids-based and inflammatory stresses induced by Sor might be one of molecular mechanism for pharmacological anti-HCC. In addition, reduced serologic level of FGF21 in Sor-treated HCC patients was observed, speculated that hepatocellular deficit of FGF21-regulated lipids metabolism might be responsible for one of potential anti-proliferative mechanisms in Sor-treated HCC. Further, both PCNA and CK19-labelled cell counts in Sor-treated HCC livers were reduced. These findings indicated that Sor suppressed neoplastic proliferation and resultant metastasis in malignant liver cancer cells.

ER stress, derived from abnormal protein misfolding, plays biological effect on cancerous fate. Mounting evidence shows that activation of protein misfolding is required for oncogenic transformation upon ER stress (23). Basically, the tumors
microenvironment and induction of ER stress are regulated predominantly by functional regulatory molecules, such as ATF6, eIF2α, GRP78, XBP1 (24). In an outstanding study the pulsed electromagnetic fields reduced ER stress triggered by tunicamycin in human liver carcinoma (HepG2) cells (25). Thus, induction of ER stress occurred in tumor cells may be an effect of method to fight against advanced HCC. In current experiments, Sor-treated terminal HCC cells showed elevated expressions of ATF6, eIF2α, GRP78, XBP1 molecules, as identified in immunoblotting validation. On the basis of current data, we reasoned that suppressed ER stress in advanced HCC cells aided in promoting the progression of tumorigenesis. Beneficially, Sor could inhibit abnormal ER stress in liver cancer cells, gradually regulation of microenvironment in liver tumor and suppression of cancerogenesis. Taken together, we conclude that Sor blocks tumor growth in advanced HCC cells through activating hepatocellular ER stress.

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