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γ-Glutamylcysteine synthetase (γ-GCS) as a target for overcoming chemo- and radio-resistance of human hepatocellular carcinoma cells

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ABSTRACT

Aims: This study uncovered that the genetically endowed intracellular glutathione contents (iGSH) regulated by the catalytic subunit of γ-glutamylcysteine synthetase heavy chain (γ-GCSh) as a prime target for overcoming both the inherited and stimuli-activated chemo- and radio-resistance of hepatocellular carcinoma (HCC) cells.
Main methods: Reactive oxygen species (ROS) production and mitochondrial membrane potential (Δψm) were determined by the probe-based flow cytometry. The TUNEL assay was used as an index of radio-sensitivity and the MTT assay was used as an index of chemo-sensitivity against various anti-cancer agents. iGSH and γ-GCSh activity were measured by HPLC methods. γ-GCSh-overexpressing GCS30 cell line was established by tetracycline-controlled Tet-OFF gene expression system in SK-Hep-1 cells.
Key findings: The relative radio-sensitivities of a panel of five HCC cells were found to be correlated negatively with both the contents of iGSH and their corresponding γ-GCSh activities with an order of abundance being Hep G2 > Hep 3B > J5 > Mahlavu > SK-Hep-1, respectively. Similarly, the cytotoxicity response patterns of these HCC cells against arsenic trioxide (ATO), a ROS-producing anti-cancer drug, were exactly identical to the order of ranking instigated by the radiotherapy (RT) treatment. Next, γ-GCSh-overexpressing GCS30 cells were found to possess excellent ability to profoundly mitigate both the drop of Δψm and apoptotic TUNEL-positive cell population engendered by ATO, cisplatin, doxorubicin, and RT treatments.
Significance: Our data unequivocally demonstrate that γ-GCSh may represent a prime target for overcoming anti-cancer drugs and RT resistance for HCC cells.

1. Introduction

Hepatocellular carcinoma (HCC) is the most common form of primary liver cancer (PLC) which ranks fifth among the most prevalent deadly cancers in the world and the third largest cause of cancer-related death in the world annually [1–4]. Globally, the incidence of HCC is expected to rise owing to the increasing prevalence of hepatitis C virus (HCV) in Europe, North America and Japan. Additionally, the risk for HCC development is now known to be age-dependent. Thus, there will be increasing cases of elderly HCC patients owing to the extension of longevity of the population.

Thus far, surgery including transplantation remains the only potentially curative modality and yet recurrence rate are high and poor long-term survival [5,6]. Furthermore, radiotherapy (RT) is also an

Abbreviations: HCC, hepatocellular carcinoma; ONS, oxidative-nitrosative stress; ROS, reactive oxygen species; γ-GCSh, γ-glutamylcysteine synthetase heavy chain; γ-GC, γ-glutamylcysteine; GCS 30, γ-GCSh-overexpressing cell line; GSH, glutathione; iGSH, intracellular glutathione; ATO, arsenic trioxide; Δψm, mitochondrial membrane potential; PLC, primary liver cancer; RT, radiotherapy; DTPA, diethylenetriaminepentaacetic acid; MBB, monobromobimane; DMEM, Dulbecco's modified Eagle's medium; MSA, methane sulfonic acid; DTPA, diethylenetriaminepentaacetic acid; TUNEL, TdT-mediated dUTP-biotin nick end labeling; DCFH-DA, 2′, 7′-dichlorodihydrofluorescein diacetate

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important treatment modality to treat hepatoma patients with portal vein thrombosis or tumor that is too close to the dome of the diaphragm or too close to large blood vessels to have good trans-arterial embolization effect [7]. However, RT treatment in these HCC patients also suffered from recurrence problem due to radio-resistance acquisition. Therefore, radio-resistance problem also remains a serious impediment to successful treatment of HCC patients [8]. Under these premises, it is important to search for a potential target for overcoming RT- and chemo-resistance in order to improve the efficaciousness of both treatment modalities in the clinical settings.

With the availability of a panel of five HCC subclone variants with distinct disparity in differentiation status to serve as experimental cell models, we sought to address the following important issues: (1) To identify which HCC subclone variant is being genetically endowed with both chemo- and RT-resistance independent of stimuli-acquired mechanisms; (2) To delineate whether or not the genetically acquired chemo- or RT-resistance has a bearing with a coordinately regulated defense mechanism against oxidative stress instigated by anti-cancer drugs or RT treatments. Specifically, we will focus on the relationship between the constitutively expressed glutathione (GSH) contents and its rate-limiting catalytic enzyme γ-glutamylcysteine synthetase heavy chain (γ-GCSh) activity and the genetically acquired tolerability against ROS-generating anti-cancer drugs and RT treatments; (3) To obtain evidence that γ-GCSh is a prime target for overcoming genetically endowed chemo- and radio-resistance in HCC cells. Specifically, we will manipulate the intracellular GSH contents (iGSH) by transfecting SK-Hep-1 subclone variant, a constitutively low GSH-expressing subline, with p-Rev-TRE-control plasmid containing cDNA of human γ-GCSh. Through this manipulation, we hope to attest that the relative tolerability against either ROS-producing anti-cancer drugs or RT treatment of the newly created HCC subline could overwhelmingly be enhanced. Our data indicate that γ-GCSh is indeed a prime target for overcoming resistances of chemo- and RT- treatment modalities in HCC cells.

2. Materials and methods

2.1. Reagents

Sodium methane sulfonate, diethylenetriaminepentaacetic acid (DTPA), monobromobimane (MBB), methane sulfonic acid, γ-glutamylcysteine (γ-GC), reduced glutathione, sulfosalicyclic acid and β-actin antibody were all purchased from Sigma Aldrich (St Louis, MO). HPLC-grade acetonitrile and glacial acetic acid were purchased from Merck (Kilsyth, Australia). The APS-2 Hypersil LC column (150 mm × 4.6 mm i.d., particle size 5 μm) was obtained from Thermo Hypersil-Keystone (Bellefonte, PA). Polyclonal anti-γ-GCSh antibody was purchased from Santa Cruz Biotechnology (Santa Cruz, CA, USA).

2.2. Cell line and cell culture

Human hepatocellular carcinoma (HCC) cell lines (Hep G2, Hep 3B, J5, Mahlavu and SK-Hep-1 cells) were obtained from the Food Industry Research and Development Institute (Hsinchu, Taiwan). The stable γ-GCSh-overexpressing cell line (GCS 30) was developed by treating SK-Hep-1 cells with p-Rev-Tet-off and p-Rev-TRE/γ-GCSh plasmids as described below and maintained in medium containing G418 (400 μg/ml) and hygromycin B (1 μg/ml). These cells were maintained in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% tetracycline-free FBS, 1% penicillin/streptomycin in 5% CO_2 under a humidified environment. Cell viability was determined by MTT assay. Briefly, cells (1×10^6) were cultured in 60 mm tissue culture dishes for 24 h. The culture medium was replaced with new medium and then exposed to various concentrations of anti-cancer drug [arsenic trioxide (ATO)] to estimate IC_{50} values for each HCC subclone variants. After the indicated treatments, cells were incubated for 4 h with 5 mg/ml of MTT reagent and lysed with DMSO. Absorbance was measured at 595 nm in a microplate reader.

2.3. Irradiation technique

All five HCC subclone variants were sub-cultured in glass cover-slip and the cells were allowed to grow for three days before irradiation. The culture media was then replaced with HESS buffer before the cells were treated with a linear accelerator (Varian Medical System, Palo Alto, CA, USA) using 6 MV of energy with a source-to-target distance of 100 cm. The cells were placed on a 1-cm bolus and treated with a posterior-anterior direction portal to allow a 1-cm radiation buildup. A radiation absorption doses from 10 and 20 Gy per single fraction or a fractionation protocol (5Gy × 4) were delivered to cells. The treated cells were then fixed with 3.7% paraformaldehyde in medium for 6 h after irradiation, stained for apoptosis using TUNEL assay.

2.4. Construction of p-Rev-TRE-control/γ-GCSh plasmid

The full-length cDNA of human γ-GCSh was prepared from Hep G2 cells by RT-PCR using a set of primers (forward: 5′-ACGAGGCTGGTC CGTCTC-3′; reverse: 5′-AGGCATGGTACTGTAGCCAGT-3′; corresponding to a 2058-bp region of γ-GCSh). The cDNA of γ-GCSh was cloned into T-overhang vector (Bayou Biolabs, Harahan, LA) and confirmed by DNA sequencing. The cDNA of γ-GCSh inserted in T-overhang vector was released by double digestion with *Sal*I and *Kpn*I restriction enzymes and ligated into the *SalI/KpnI* sites of pQE-30 plasmid (Qiagen, Germany). The resulting recombinant plasmid was then doubly digested with *Sal*I and *Sph*I, and γ-GCSh cDNA was subcloned into the *SalI/Sph*I sites of p-Rev-TRE-control plasmid (BD Bioscience Clontech, Palo Alto, CA, USA).

2.5. Measurement of glutathione

For determination of GSH, cells were collected and homogenized in ice-cold 200 mM methane sulfonic acid (MSA). After centrifugation at 12,000 rpm for 5 min at 4 °C, 100 μl aliquot of the supernatant was taken, and 80 μl of 1 M HEPES buffer (pH 8.5) containing 5 mM diethylenetriaminepentaacetic acid (DTPA) and 120 μl of 50 mM monobromobimane was added. The reaction mixture was incubated at room temperature in the dark for 20 min and terminated by the addition of 100 μl of 0.4 M MSA. The supernatant was resolved on an APS-2 Hypersil HPLC column with an isocratic mobile phase of 4% (v/v) acetonitrile and 0.25% (v/v) acetic acid in water at a flow rate of 1.0 ml/min. Products were detected by a Waters Model 470 scanning fluorescence detector with excitation and emission settings of 394 and 480 nm, respectively.

2.6. Establishment of doxycycline-controlled γ-GCSh-overexpressing cell line (designated as GCS 30)

To establish a doxycycline-controlled γ-GCSh-overexpressing cell line, SK-Hep-1 cells were firstly transfected with p-Rev-Tet-off plasmid (BD Biosciences Clontech, Palo Alto, CA) and selected with media containing 1 mg/ml G418. For doxycycline-controlled cell lines selection, the above G418-resistant subclones were further transfected with p-Rev-TRE-Luc plasmid. Then, we established a doxycycline-controlled cell line, named SK-OFF. For γ-GCSh-overexpressing cell line establishment, SK-OFF cells were further transfected with p-Rev-TRE-control/γ-GCSh plasmids, and selected with media containing 1 μg/ml hygromycin B. Finally, the stable γ-GCSh-overexpressing cell line was confirmed by γ-GCSh activity assay and immunoblotting, and designated as GCS30.

2.7. Apoptosis assay

TdT-mediated dUTP-biotin nick end labeling (TUNEL) assay was