

Brief Communication

Differences of ferroptosis-related genes between White and Asian patients with liver cancer

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Abstract: Ferroptosis results from metabolic dysregulation and is closely linked to liver cancer. Although a ferroptosis-related gene signature in liver cancer has been established, the precise regulatory mechanism is still unclear. To identify shared pathogenic genes linked to ferroptosis across liver cancer patients from diverse racial backgrounds, we evaluated various ferroptosis-related genes, constructing a signature for both Asian and White patients using The Cancer Genome Atlas (TCGA) database. Based on the differential expression and functionality of ferroptosis-associated genes, we selected Farnesyl diphosphate farnesyl transferase 1 (FDFT1), Acyl-CoA synthetase long-chain 4 (ACSL4) and Endoplasmic reticulum membrane protein complex 2 (EMC2) for further study in liver cancer cells. FDFT1, ACSL4 and EMC2 induced ferroptosis of liver cancer cells through upregulation of reactive oxygen species (ROS) levels and downregulation of glutathione peroxidase (GPX4). Current data indicate no notable influence of racial differences on the functionality of ferroptosis-related genes. Our data suggests potential novel therapeutic avenues for liver cancer treatment.

Keywords: Ferroptosis, liver cancer, SLC1A5, GPX4, ROS

Introduction

Primary liver cancer is a common malignant tumor worldwide characterized by high recurrence and mortality rates [1]. Despite the utilization of various treatment modalities for liver cancer patients - including surgery, radiofrequency ablation, radiotherapy, and chemotherapy - challenges persist, with metastasis and recurrence remaining prevalent [2]. Consequently, targeted therapy has emerged as a potential breakthrough in treating liver cancer [3]. Given that prolonged targeted therapy can inadvertently promote tumor cell anti-apoptosis, treatments pivoting on ferroptosis present promising avenues of application [4].

Ferroptosis is a ferric ion dependent, non-apoptotic cell death caused by the accumulation of lipid peroxides [5]. The molecular mechanism of ferroptosis mainly relies on two mutually competitive biochemical processes within cells, namely the production and elimination of

lipid peroxidation [5]. When cells are unable to eliminate excess reactive oxygen species (ROS) through antioxidant mechanisms, the accumulated oxidative lipids can induce ferroptosis [5]. The liver, as a pivotal organ for iron storage and metabolism, holds a significant role in maintaining physiological balance in the human body, with iron balance being particularly crucial [6]. Hepcidin, produced by the liver, helps regulate iron levels and maintain equilibrium by managing the absorption, export, utilization, and storage of intracellular iron [7]. However, different lifestyle habits may lead to an overload of iron content in the body, such as consuming large amounts of meat or cooking in iron pots [8]. Excessive iron deposition can detrimentally affect the liver, potentially causing liver damage and leading to hepatic lobular fibrosis, cirrhosis, and even liver cancer [9]. Numerous regulatory factors have been identified for ferroptosis in liver cancer cells [10]. System xc- is an important intracellular antioxidant system composed of two subunits, SLC7A11 and SLC3A2 [10].

SLC7A11 is responsible for the main transport activity, with high specificity for cystine and glutamate, while SLC3A2 serves as a chaperone protein [10]. Cystine is converted into glutathione (GSH) via the catalysis of glutamate cysteine ligase (GCL) and glutathione synthase (GSS) [10]. GSH acts as a reducing cofactor for glutathione peroxidase (GPX4), a membrane lipid repair enzyme [11]. Inhibiting the activity of System xc- may suppress the absorption of cysteine, affect GSH synthesis, and consequently result in a decline in the activity of membrane lipid repair enzyme GPX4, reduced cellular antioxidant capacity, and thus promote ferroptosis [11].

Despite preliminary investigations into the molecular mechanism of ferroptosis in liver cancer cells, to our knowledge, no prior studies have discerned differences in ferroptosis-related genes between Asian and White patients with liver cancer. In this study, we aim to identify shared pathogenic genes associated with ferroptosis across diverse racial backgrounds by screening for differential genes between Asian and White liver cancer patients, using The Cancer Genome Atlas (TCGA) database. Additionally, we evaluated the prognostic roles of ferroptosis-related genes in Asian and White liver cancer patients, respectively, to ascertain their clinical relevance. Finally, the functions and molecular mechanisms of these genes were examined in liver cancer cells derived from both Asian and White patients.

Materials and methods

Bioinformatics analysis

Liver hepatocellular carcinoma (LIHC) mRNA data (HTSeq FPKM) and corresponding clinical data were downloaded from TCGA database (<http://cancergenome.nih.gov/>). R programming “ggplot2” and “pheatmap” packages were used to illustrate differential expression levels of ferroptosis-related genes between Asian and White liver cancer patients. The “Survival package” in R was utilized to assess the correlation between ferroptosis-related genes and patient prognosis.

Cell culture and plasmid transfection

HCC cell lines SNU-449 cells (derived from Korean HCC patients) and HepG2 cells (derived

from White HCC patients) were obtained from the American Type Culture Collection (Manassas, VA, USA). Cells were maintained at 37°C in a 5% CO₂ incubator in Dulbecco's Modified Eagle's Medium (DMEM) (Sigma-Aldrich, Shanghai, China) containing 10% heat-inactivated fetal bovine serum (FBS) and 1% penicillin-streptomycin (100 IU/ml). SNU-449 and HepG2 cells were transfected with shFDFT1 plasmid (sc-61610-SH, Santa Cruz Biotechnology, Shanghai, China), shACSL4 plasmid (sc-60619-SH, Santa Cruz), or shEMC2 plasmid (sc-77588-SH, Santa Cruz) using Lipofectamine 2000 (Invitrogen, Shanghai, China) following manufacturer's protocol.

Real-time reverse transcription PCR

Total RNA was isolated from cells by using an RNeasy Mini Kit (Beyotime Biotechnology, Shanghai, China). Primers for Farnesyl diphosphate farnesyltransferase 1 (FDFT1) were 5'-TGTGACCTCTGAACAGGAGTGG-3' (sense) and 5'-GCCCATAGAGTTGGCACGTTCT-3' (antisense). Primers for Acyl-CoA synthetase long-chain 4 (ACSL4) were 5'-GCTATCTCCTCAGACACACCGA-3' (sense) and 5'-AGGTGCTCCAACCTCTGCCAGTA-3' (antisense). Primers for endoplasmic reticulum membrane protein complex 2 (EMC2) were 5'-AGAAAGCGTAAGATTGCCATTCGA-3' (sense) and 5'-AGTTCATGCCAGGCTTCTTGTC-3' (antisense). GAPDH was used as an internal control for normalization with primers 5'-AATGGACAACCTGGTCGTGGAC-3' (sense) and 5'-CCCTCCAGGGGATCTGTTTG-3' (antisense).

MTT assay

Cytotoxicity Assay Kit was purchased from Beyotime Biotechnology (Shanghai, China). After cells (1×10^3 cells/well) attached on 96-well plates 48 h, 20 μ l of MTT solution was added to each well. After 4 hours, absorbance was measured at 570 nm using the TECAN microplate reader (Tecan Trading AG, Switzerland).

Detection of ROS

Intracellular ROS concentrations were quantified using the ROS detection kit (Beyotime) as per the manufacturer's guidelines. Cells were washed with PBS and incubated with DCFH-DA (1:1000) in 37°C. After which, cells were ana-

lyzed by flow cytometry (Becton, Dickinson and Company, Shanghai, China).

Detection of malondialdehyde (MDA)

MDA concentrations were determined using the Lipid Peroxidation MDA Assay Kit (Beyotime) in accordance with the manufacturer's protocol. Absorbance was recorded at 532 nm utilizing the TECAN microplate reader (Tecan Trading AG).

Glutamine uptake assay

Cells were incubated with [3H]-L-Gln (200 nmol/l) in Gln-free medium at 37°C for 20 min, followed by harvesting for Gln measurements using a liquid scintillation counter (PerkinElmer, Shanghai, China).

Western blot

Cells were lysed in RIPA lysis buffer (Beyotime) containing a protease inhibitor cocktail (Sigma-Aldrich). Protein samples (30 µg/lane) were separated by 10% SDS-polyacrylamide gel electrophoresis and transferred to a nitrocellulose (NC) filter membrane (Beyotime). Primary antibodies included FDFT1 (sc-271602; Santa Cruz Biotechnology, Shanghai, China), ACSL4 (sc-365230; Santa Cruz), EMC2 (sc-166011; Santa Cruz), FANCD2 (sc-20022; Santa Cruz), SLC1A5 (8057; Cell Signaling Technology, Shanghai, China), SLC7A11 (98051; Cell Signaling), and GAPDH (sc-47724; Santa Cruz). After secondary antibody incubation (Beyotime), proteins were detected by chemiluminescence using the ECL Western blotting detection system (Beyotime).

Statistical analysis

All data are presented as the mean ± SD from three independent experiments, each conducted in triplicate. Inter-group variances were determined using an unpaired, two-tailed Student's t-test. A *P*-value < 0.05 was considered statistically significant.

Results

Identification of the distinct genes related with ferroptosis in White and Asian HCC patients

The heatmap highlighted differential expressions of ferroptosis-related genes in White and

Asian HCC patients (**Figure 1A**). Statistically significant variations in the expression of HSPB1, RPL8, EMC2, FANCD2, ALT1, NFEF2L2, FDFT1, and CDKN1A were observed between the two patient groups (**Figure 1A**, *P* < 0.05). In tumor tissues of White and Asian HCC patients, CDKN1A, NFEF2L2, and ALT1 were more prominently expressed in White patients than in Asian patients (**Figure 1B**, *P* < 0.05). Conversely, higher expressions of HSPB1, RPL8, EMC2, FANCD2, and FDFT1 were observed in Asian patients compared to White patients (**Figure 1B**, *P* < 0.05). Kaplan-Meier plots showed that high FDFT1 expression correlated with improved disease-free survival (DFS) for Asian patients (**Figure 1C**, *P* = 0.0178). Conversely, White patients exhibiting low ECM2 expression had a favorable DFS compared to those with high expression (**Figure 1D**, *P* = 0.00261). However, these genes did not significantly impact the overall survival (OS) in either Asian or White patients (**Supplementary Figure 1**, *P* > 0.05). No significance of ACSL4 expression nor its prognostic role was found in White and Asian HCC patients (**Figure 1B** and **Supplementary Figure 2**, *P* > 0.05). Additionally, gene correlations were explored. As shown in **Figure 1E**, positive correlations were found among FDFT1, ACSL4, and GPX4; these genes were further negatively correlated with ECM2, SLC7A11, SLC1A5 in the tissues from Asian patients. In White patients, ACSL4, FDFT1, SLC7A11, SLC1A5, and EMC2 were negatively correlated with GPX4 (**Figure 1F**).

The function and mechanisms of EMC2, FDFT1, and ACSL4 in ferroptosis

Bioinformatic analyses revealed that EMC2 expression varied between Asian and White patients and was consistently negatively correlated with GPX4. ACSL4 expression was consistent across both races, but its correlation with GPX4 was reversed. The expression and correlation of FDFT1 were consistent in both races. Based on these observations, we focused on EMC2, FDFT1, and ACSL4 to understand the varying functions and mechanisms across different racial groups in ferroptosis. For an accurate representation, post expression analysis of the aforementioned genes (**Supplementary Figures 3** and **4**), HepG2 cells (derived from White HCC patients) and SNU-449 cells (from Asian HCC patients) were chosen for further study.

Ferroptosis in liver cancer

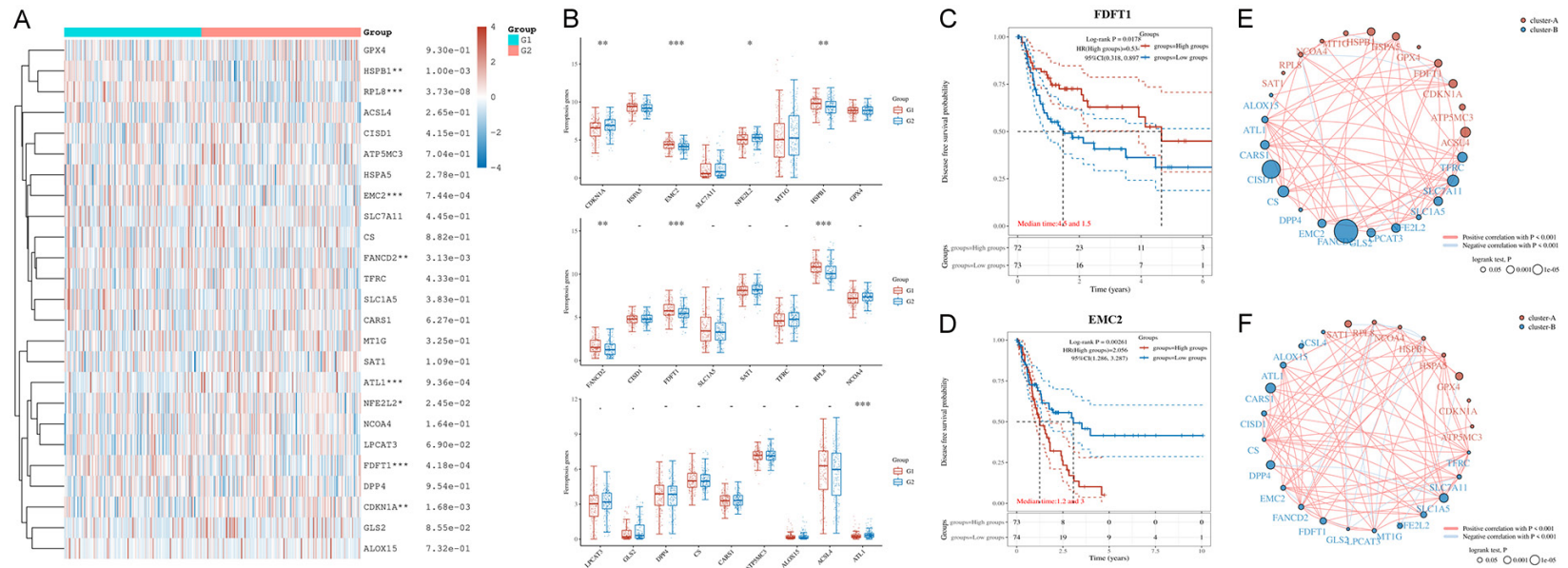


Figure 1. Differentially ferroptosis-related genes in liver cancer tissues of Asian patients and White patients. Heatmap (A) and column chart (B) of differentially ferroptosis-related genes in Asian patients and White patients with liver cancer. G1: Asian patients; G2: White patients. Kaplan-Meier analysis for the prognostic roles of FDFT1 (C) and EMC2 (D) in the diseases-free survival of patients with liver cancer. Circles represent ferroptosis-related mRNA, the line represents the relationship between genes in Asian patients (E) and White patients (F).

Following FDFT1 knockdown, both HepG2 and SNU-449 cells exhibited increased proliferation compared to their respective parental cells (**Figure 2A**, $P < 0.05$). In FDFT1 knockdown cells, both ROS and MDA levels were downregulated when compared to the matched cells (**Figure 2B** and **2C**, $P < 0.05$). Higher glutamine (Gln) consumption was observed in FDFT1 knockdown cells compared with the parental cells (**Figure 2D**, $P < 0.05$). Notably, ACSL4 or EMC2 knockdown similarly enhanced the proliferation of HepG2 and SNU-449 cells while suppressing ROS and MDA production (**Figure 2**, $P < 0.05$). FDFT1 knockdown upregulated SLC7A11, SLC1A5, FANCD2, and GPX4 protein levels in HCC cells (**Figure 3A**, $P < 0.05$). EMC2 knockdown induced SLC1A5 and GPX4 expression in HCC cells (**Figure 3B**, $P < 0.05$). ACSL4 knockdown induced FANCD2 and GPX4 expression in HCC cells (**Figure 3C**, $P < 0.05$). These findings suggest that while the functional roles of these genes in ferroptosis across racial groups are comparable, the underlying molecular mechanisms differ.

Discussion

In this study, we identified distinct genes associated with ferroptosis in both Asian and White HCC patients. Among these genes, the expression and function of FDFT1 was similar in both racial groups. While ACSL4 expression was not significantly different between the groups, its function varied. EMC2 displayed differences in expression, but its functional role remained unchanged across the two groups. These observations prompted us to further investigate the differential mechanisms of ferroptosis across racial groups with liver cancer.

Disorders in iron metabolism are a key feature that differentiates ferroptosis from other mechanisms of cell death [4]. Iron participates in mitochondrial oxidative phosphorylation, promotes lipid peroxidation and ROS aggregation, induces oxidative stress, and causes cell death [12]. Numerous studies have established a link between HCC and ferroptosis, which is predominantly regulated by System xc- and GPX4 in liver cancer [13-15]. Ferroptosis inducers, such as erastin and sorafenib, stimulate liver cancer cell ferroptosis by influencing the activities of System xc- or GPX4 [13-15]. ACSL4 acts as a

positive regulator of ferroptosis. Its downregulation curbs erastin-induced ferroptosis in liver cancer cells, an effect associated with ACSL4-mediated 5-hydroxyeicosatetraenoic acid lipotoxicity [16]. Furthermore, ACSL4 promotes the progression of liver cancer by fatty acid metabolism reprogramming through the c-Myc/SREBP1 pathway [17]. Consistent with prior research, we observed that ACSL4 knockdown reduced ROS and MDA levels in HCC cells. Both bioinformatic and in vitro evidence suggested a race-independent inhibitory effect of ACSL4 knockdown on ferroptosis.

Bioinformatic analysis revealed differing EMC2 expression levels between Asian and White HCC patients. The endoplasmic reticulum membrane protein complex (EMC) family influences protein transport, endoplasmic reticulum stress, and lipid homeostasis [18]. EMC2, alternatively known as KIAA0103 or TTC35, was more susceptible to erastin and identified as a ferroptosis-related gene in breast cancer and esophageal adenocarcinoma [19, 20]. Due to limited research on EMC2 in liver cancer, we assessed its impact on ferroptosis in HCC cells. Our findings confirmed that EMC2 knockdown reduced ROS levels, thus protecting HCC cells from ferroptosis. Mechanistically, EMC2 knockdown also suppressed ferroptosis by upregulating SLC7A11 and GPX4 expression in HCC cells.

Bioinformatic analyses depicted FDFT1 as a positive regulator of ferroptosis in Asian HCC patients, whereas it negatively regulated ferroptosis in White patients. FDFT1, also known as squalene synthase (SQS), is involved in cholesterol synthesis [21]. FDFT1 is highly expressed in prostate cancer, colorectal cancer, and esophageal cancer, while it shows comparatively low expression in clear cell renal cell carcinoma [22-25]. We verified that FDFT1 knockdown elevated the expression of SLC7A11 and SLC1A5 in HCC cells. In addition, FANCD2 was also upregulated in HCC cells with FDFT1 knockdown. FANCD2 has been demonstrated to regulate tumorigenesis, apoptosis, and metastasis of cancer cells [26]. It has been reported to inhibit ferroptosis by managing the JAK2/STAT3 pathway in osteosarcoma [27]. Although our findings, in line with previous research, underscored the positive roles of FDFT1 in ferroptosis, we observed no functional disparities

Ferroptosis in liver cancer

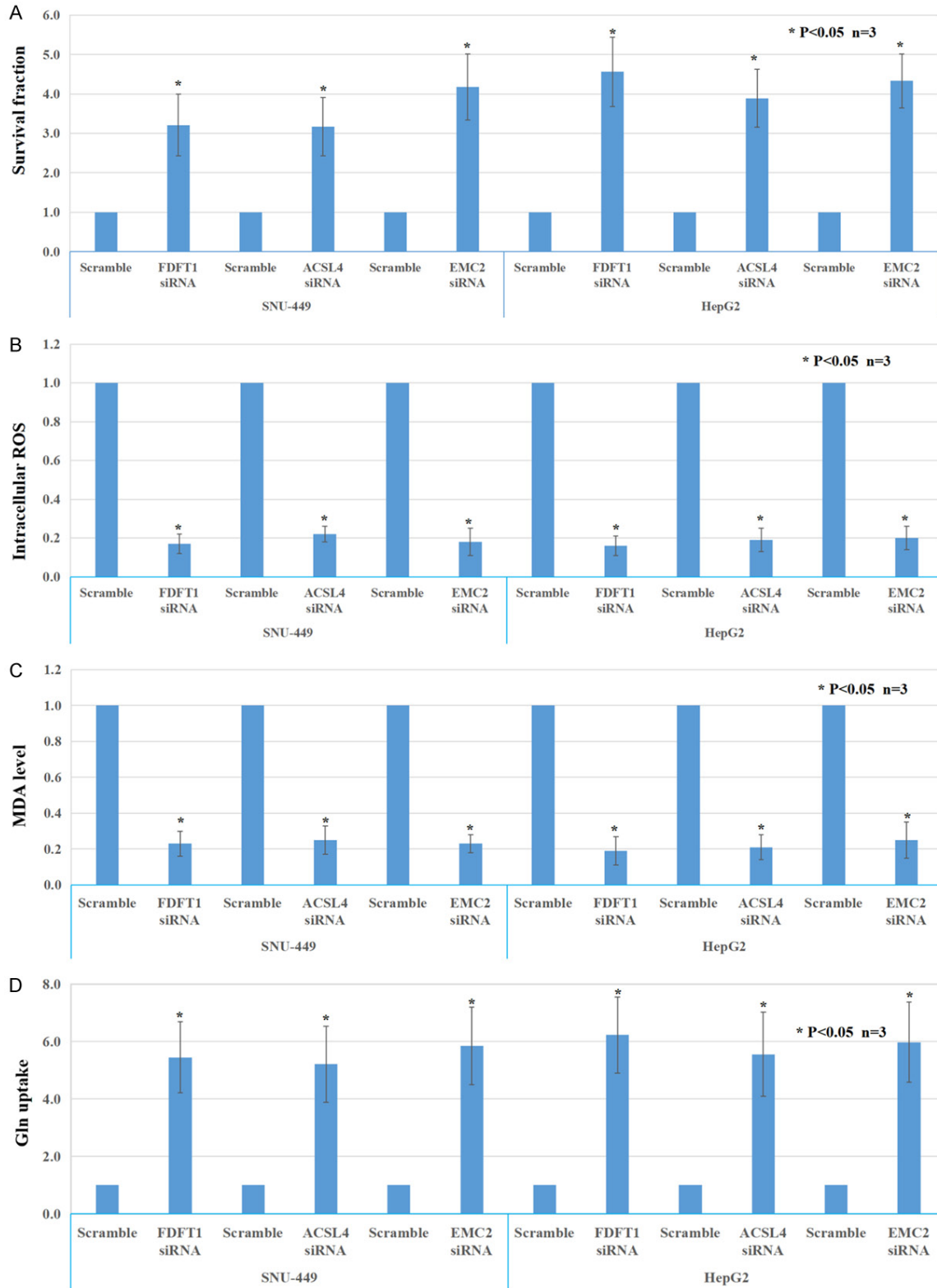


Figure 2. The roles of FDFT1, ACSL4 and EMC2 in ferroptosis of liver cancer cells. (A) Cell viability was assayed by MTT of HepG2 and SNU-449 cells after FDFT1, ACSL4 or EMC2 knockdown juxtaposed to parental cells. (B) Intracellular ROS, (C) MDA formation, and (D) Gln uptake activities of HepG2 and SNU-449 cells after FDFT1, ACSL4 or EMC2 knockdown juxtaposed to parental cells.

Ferroptosis in liver cancer

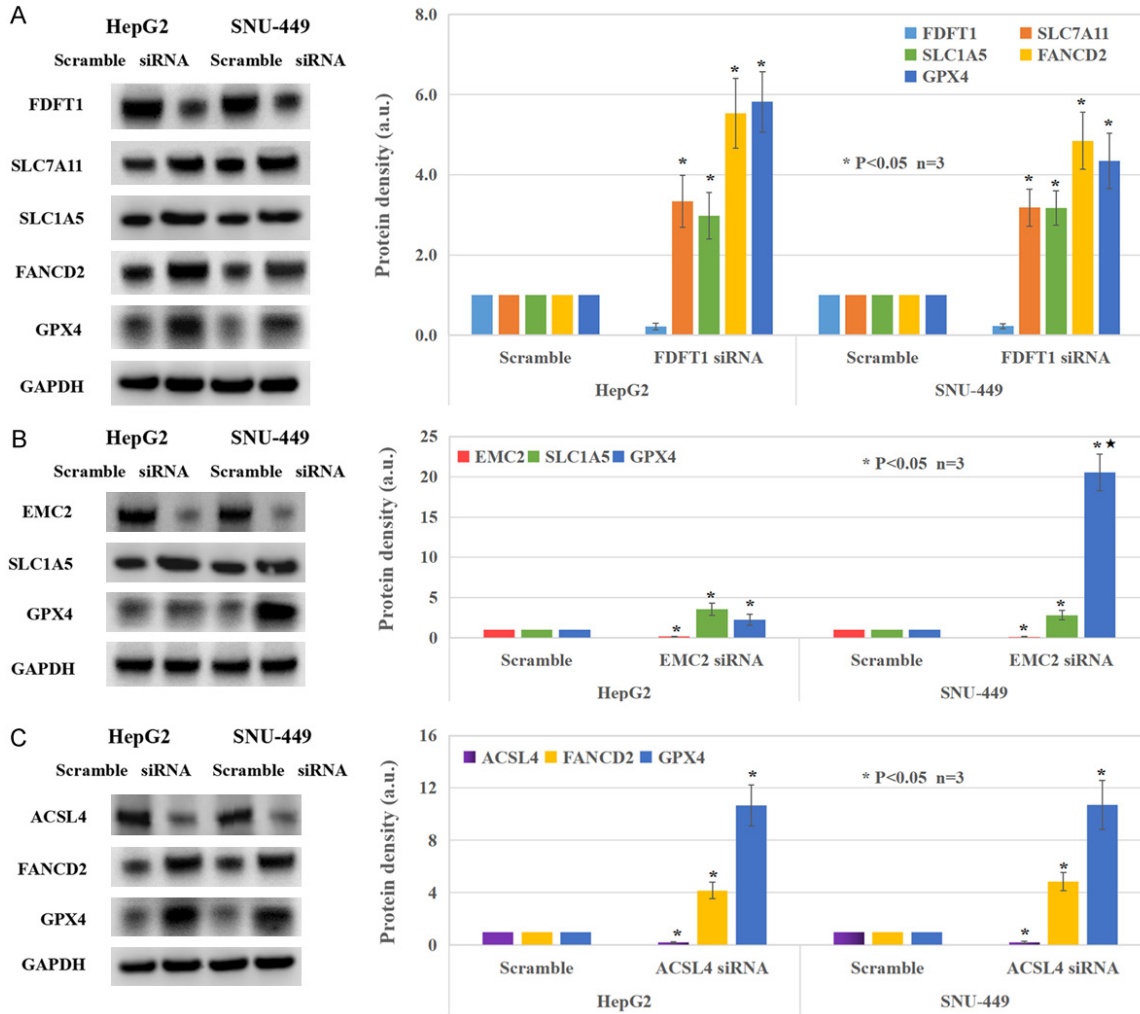


Figure 3. The molecular mechanisms of FDFT1, ACSL4 and EMC2 in ferroptosis of liver cancer cells. A. SLC7A11, SLC1A5, FANCD2 and GPX4 protein expression in FDFT1 knockdown HepG2, SNU-449, and parental cells by western blotting. B. SLC1A5 and GPX4 protein expression in EMC2 knockdown HepG2, SNU-449, and parental cells by western blotting. C. FANCD2 and GPX4 protein expression in ACSL4 knockdown HepG2, and SNU-449, and parental cells by western blotting.

for FDFT1 between HCC cells derived from Asian and White patients.

This study delineated a ferroptosis-associated gene signature in both Asian and White patients (Figure 4). Based on varied expression and functionality of these genes, we selected FDFT1, ACSL4, and EMC2 for in vitro validation. All three genes positively regulated HCC cell ferroptosis primarily through the upregulation of GPX4 expression. However, based on the current data, the reason behind the differential effects of FDFT1 on ferroptosis, as revealed by bioinformatics, between Asian and White HCC patients remains elusive. Racial differences were found to not significantly impact the functionality of ferroptosis-related genes. Never-

theless, these findings should be validated in vivo for more comprehensive insights.

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Disclosure of conflict of interest

None.

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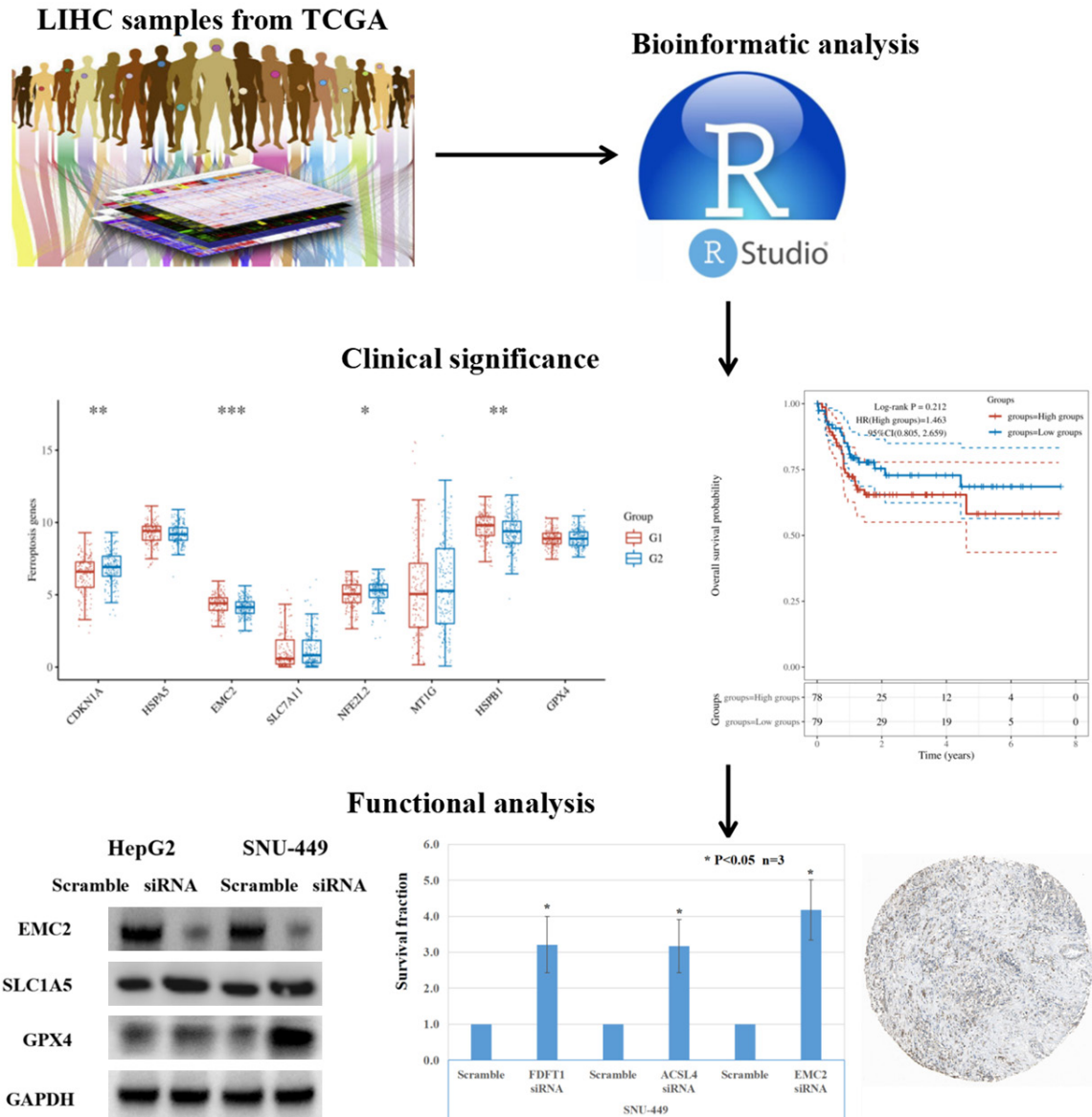


Figure 4. Flow chart of bioinformatic analysis and functional validation of DEGs related with ferroptosis from the Asian and White patient cohort in TCGA database.

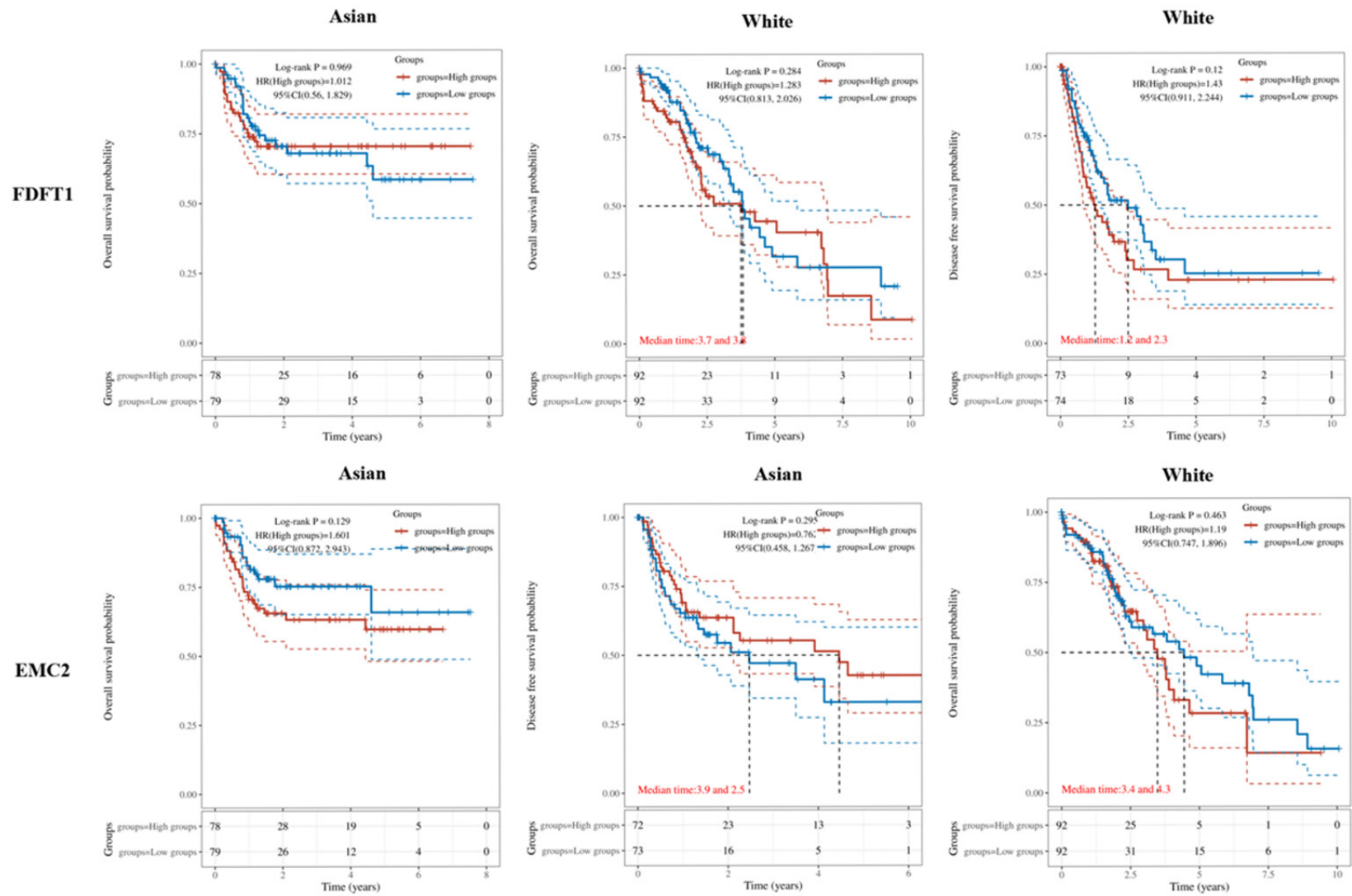
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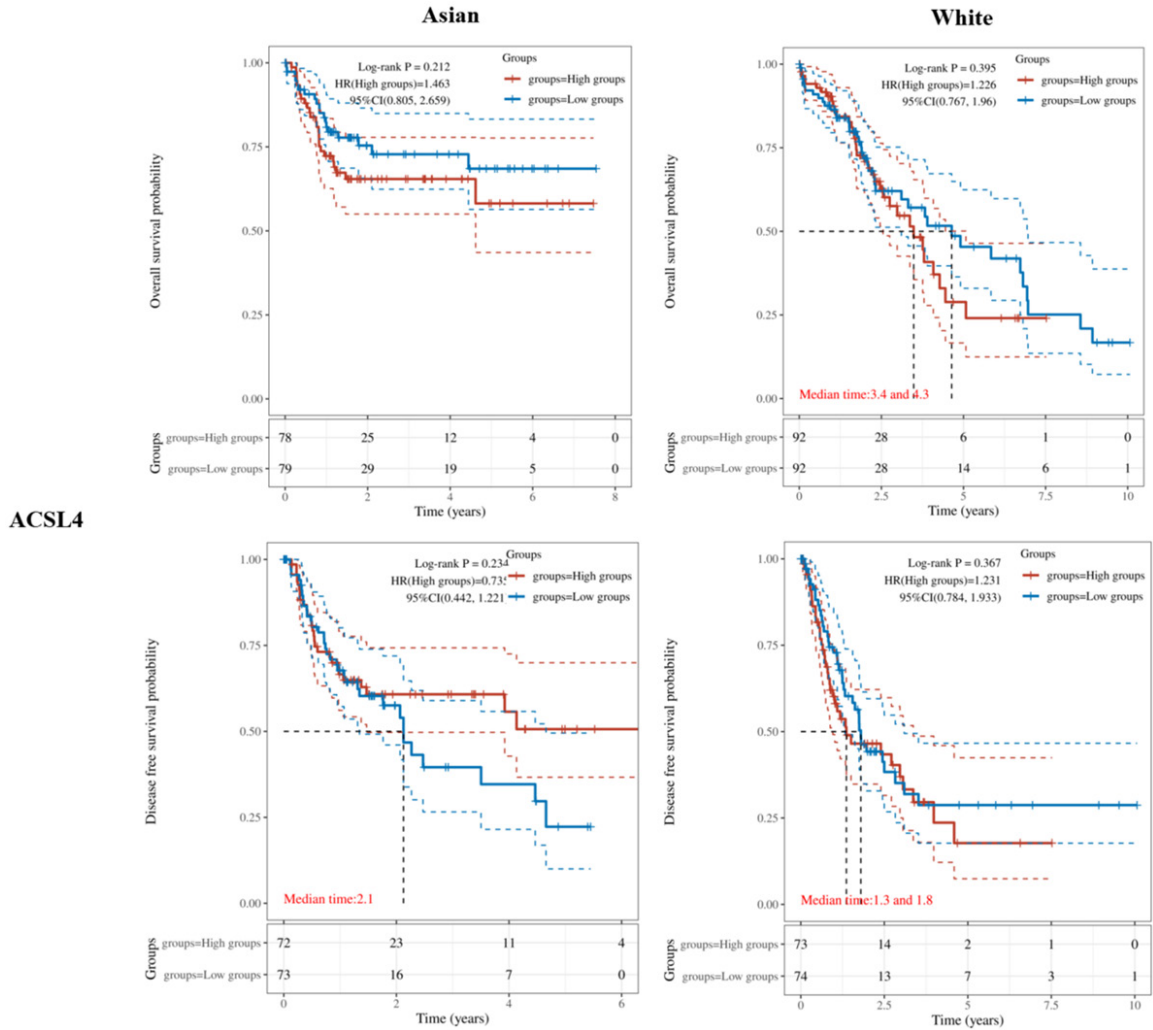
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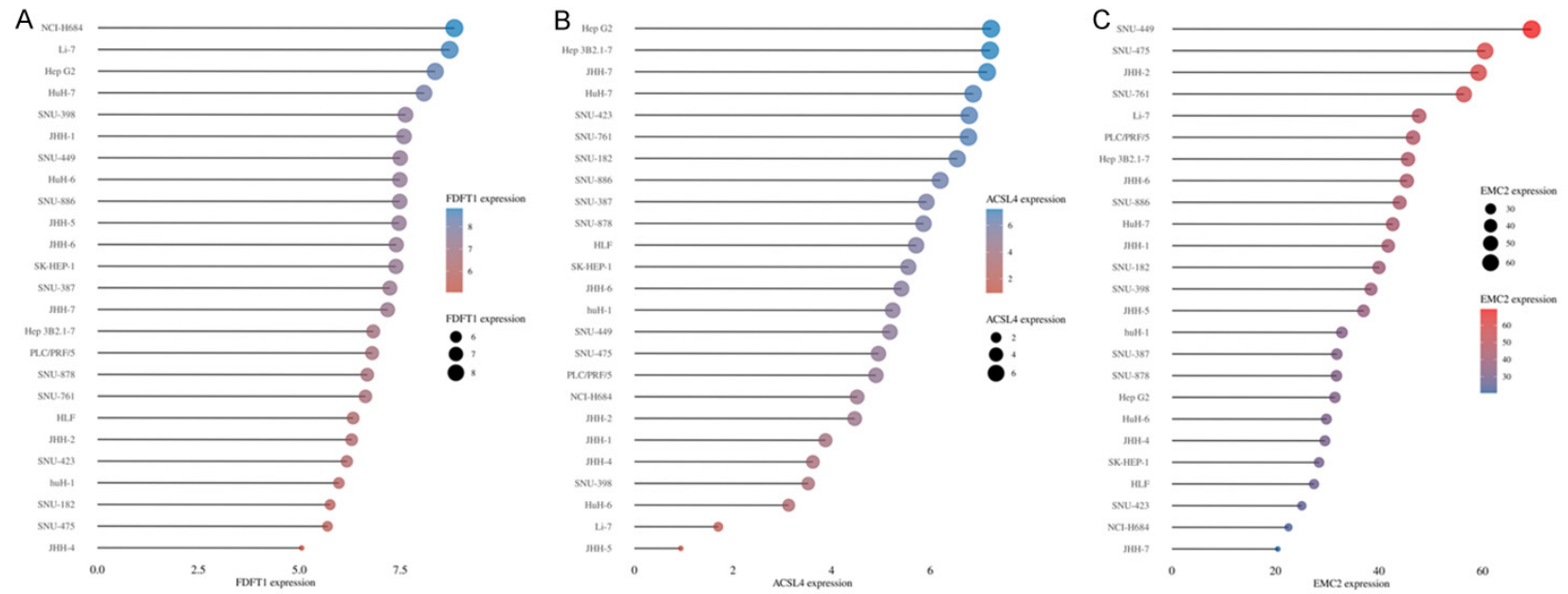
Supplementary Figure 1. Kaplan-Meier analysis for the prognostic roles of FDFT1 and EMC2 in the overall survival and the diseases-free survival of the Asian and White patients with liver cancer.

Ferroptosis in liver cancer



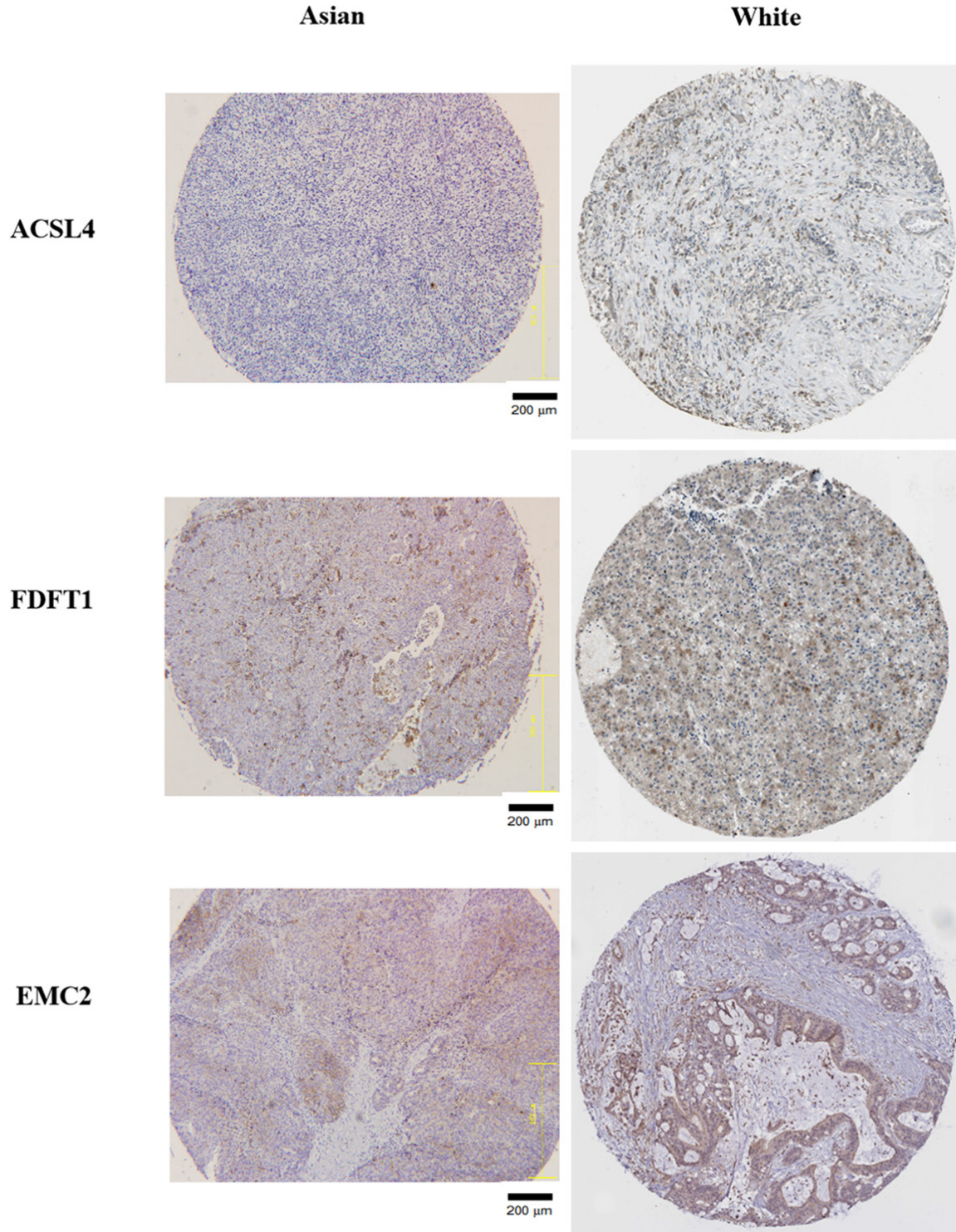
Supplementary Figure 2. Kaplan-Meier analysis for the prognostic roles of ACSL4 in the overall survival and the diseases-free survival of the Asian and White patients with liver cancer.

Ferroptosis in liver cancer



Supplementary Figure 3. FDFT1, ACSL4 and EMC2 mRNA levels in liver cancer cells.

Ferroptosis in liver cancer



Supplementary Figure 4. FDFT1, ACSL4 and EMC2 protein levels in the Asian and White patients with liver cancer. The data of White patients were obtained from Human Protein Atlas database (<https://www.proteinatlas.org/>). Tissue microarray of Asian HCC tissues was purchased from OUTDO Biotech (Shanghai, China).