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Targeting ODC1 inhibits tumor growth through reduction of lipid metabolism in human hepatocellular carcinoma

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ABSTRACT

Ornithine decarboxylase 1 (ODC1), a metabolic enzyme critically involved in the polyamine biosynthesis, is commonly upregulated in hepatocellular carcinoma (HCC). Despite its altered expression in human HCC tissues, the molecular mechanism by which ODC1 alters the course of HCC progression and functions in HCC cell survival is unknown. Here we identified that silencing of ODC1 expression with small interfering (si) RNA causes inhibition of HCC cell growth through blockade of cell cycle progression and induction of apoptosis. Next, to obtain insights into the molecular changes in response to ODC1 knockdown, global changes in gene expression were examined using RNA sequencing. It revealed that 119 genes show same directional regulation (76 up- and 43 down-regulated) in both Huh1 and Huh7 cells and were considered as a common ODC1 knockdown signature. Particularly, we found through a network analysis that KLF2, which is known to inhibit PPARγ expression and adipogenesis, was commonly up-regulated. Subsequent Western blotting affirmed that the downregulation of ODC1 was accompanied by a decrease in the levels of PPARγ as well as of PARP-1, cyclin E1 and pro-caspase 9 delaying cell cycle progression and accelerating apoptotic signaling. Following the down-regulation of PPARγ expression, ODC1 silencing resulted in a strong inhibition in the expression of important regulators of glucose transport and lipid biogenesis, and caused a marked decrease in lipid droplet accumulation. In addition, ODC1 silencing significantly inhibited the growth of human HCC xenografts in nude mice. These findings indicate that the function of ODC1 is correlated with HCC lipogenesis and suggest that targeting ODC1 could be an attractive option for molecular therapy of HCC.

1. Introduction

Hepatocellular carcinoma (HCC) is the sixth most commonly diagnosed and the third most lethal neoplasm, causing an estimated 700,000 deaths worldwide annually [1]. HCC occurs predominantly due to risk factors such as hepatitis B virus and hepatitis C virus infections, alcohol abuse, obesity, and type 2 diabetes, and its occurrence varies geographically [2]. About 75% of HCCs are closely associated with chronic inflammation caused by viral hepatitis in which continuous inflammation and hepatocyte regeneration occur [3]. This chronic condition may include the accumulation of genetic and/or epigenetic changes that activate signaling pathways, altering cell metabolism and the tumor

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microenvironment, and generating liver cancer stem cells (CSCs) [4,5]. Overall, only 30–40% of HCC patients are diagnosed as eligible for current curative treatments because of late diagnosis, underlying liver disease, and lack of effective treatment options [6,7]. Currently, Sorafenib is the only recommended molecular targeted therapy for patients with advanced HCC (Barcelona clinic liver cancer stage C) having invasive or extrahepatic tumors. However, the standard of care for advanced liver cancer can extend the median overall survival for only about one year. Therefore, the need to develop a novel molecular targeted therapy is urgent. HCCs are phenotypically and genetically heterogeneous tumors driven by diverse molecular mechanisms [7]. However, HCCs exhibit certain common traits selected through genomic and epigenetic alterations [8,9]. Identification of common genomic alterations may provide an opportunity for anti-cancer treatment through targeted therapy [10].

Previous studies of gene expression signatures as predictors of survival classes of HCC patients found that expression patterns of a limited number of genes were significantly associated with disease prognosis [11]. We confirmed these findings by therapeutically targeting the identified "survival" genes, including COP1, CSN5, and HDAC2 using small interfering RNA (siRNA) [12–14]. Overexpression of one survival gene ODC1, was significantly correlated with poor survival of HCC patients [11]. ODC1 is the first rate-limiting enzyme in the polyamine biosynthesis pathway, which catalyzes the decarboxylation and conversion of ornithine to putrescine (primary type of polyamine) [15]. In terms of functional involvement in tumor progression, ODC1 was defined as a critical downstream arbiter of MYCN-driven oncogenesis in embryos [16]. Furthermore, it has been reported that the expressions of ODC and c-myc in both mRNA and protein levels have progressively increased from adjacent normal tissue to cirrhosis, to well-differentiated HCC, to poorly-differentiated HCC [17]. Despite the functional significance of ODC1 in cell proliferation and its altered expression in human HCC tissues, the molecular mechanism by which ODC1 alters the course of HCC progression and functions in HCC cell survival is unknown. In this study, we demonstrated that siRNA silencing of ODC1 gene expression caused a blockade of cell cycle progression and induction of apoptosis through reduction of HCC lipid metabolism.

2. Materials and methods

2.1. Cell culture and reagents

Human HCC cell line Huh1 was purchased from the Japanese Collection of Research Bioresources Cell Bank (JCRB Cell Bank; Osaka, Japan), and Huh7, PLC/PRF/5 and Hep3B were from the Korean Cell Line Bank (KCLB; Seoul, Korea). The cells were maintained in Dulbecco's Modified Eagle's Medium (DMEM) supplemented with 10% fetal bovine serum (HyClone, South Logan, UT, USA) and 1% penicillin/streptomycin (HyClone) at 37 °C with 5% CO_2.

2.2. siRNA transfection

ODC1 siRNA duplexes were chemically synthesized by Ambion (Austin, TX, USA; ODC1 siRNA: ID#4390824). Silencer Negative Control #1 siRNA (NCsiRNA) (Ambion) was used for control experiments and does not target any endogenous transcript. For an MTT and apoptosis assay, cells were plated in 96-well plates at 30% density 24 h before transfection. 0.25 μl of Lipofectamine 2000 (Invitrogen, Carlsbad, CA, USA) was mixed with siRNA molecules in a volume of 50 μl Opti-MEM I (Invitrogen) to adjust the final concentration of siRNA to 15 nM. The medium was replaced 6 h after transfection. NCsiRNA was used in the same quantity and transfected to the cells simultaneously. This optimized condition was scaled up for different assays that use 24-well or 6-well plates or 10-cm diameter culture dishes.

2.3. Measurement of cell proliferation and apoptotic cell death

For the measurement of cell proliferation, log phase cells were seeded at 30% confluence in 96-well plates in 100 μl of culture media without antibiotics one day before transfection. After 24 h, cells were transfected as described above. The growth inhibitory effects of target siRNA were assayed using the CellTiter 96 Aqueous One Solution Cell Proliferation Assay Kit (Promega, Madison, WI, USA) as recommended by the manufacturer. The colored formazan products were determined by measuring the absorbance at 490 nm using a VersaMax microplate reader (Molecular Device, Sunnyvale, CA, USA). The percentage of viable cells was calculated by comparing the optical density using the following formula: (1 − absorbance of a target siRNA-treated well)/absorbance of a non-treated or control siRNA-treated well × 100). Cell cycle analysis was performed using a fluorescence-activated cell sorter (FACS) after propidium iodide (PI)/RNase staining. Apoptotic cells were stained using FITC Annexin V Apoptosis Detection Kit I (BD Pharmingen, San Jose, CA, USA) following the manufacturer's instruction. Cell death was measured by FACSVerse flow cytometry (BD Bioscience) and quantified by the FlowJo software program.

2.4. RNA sequencing

Total RNA was extracted using the RNeasy mini kit (Qiagen, Valencia, CA, USA). The quantity and quality of the total RNA were evaluated by using RNA electropherograms (Bio-Rad Experion, Hercules, CA, USA) and assessing the RNA quality indicator (RQI). The total RNA from each sample with an RQI value over 8.0 was used. The resulting mRNA samples were processed for the sequencing libraries using the Illumina TruSeq Stranded mRNA sample preparation kit (Illumina, San Diego, CA, USA) following the manufacturer's protocols. RNA sequencing was performed by the Illumina HiSeq 2500 to generate non-directional, paired-end 100-base-pair reads. Quality-filtered reads were mapped to the human reference genome sequences, hg19 (UCSC Genome Bioinformatics, https://genome.ucsc.edu) using tophat2 (http://ccb.jhu.edu/software/tophat). The relative transcript abundance was estimated by counting the fragments per kilobase of exon model per million mapped sequence reads (FPKM), and differentially expressed genes were evaluated using the cufflinks package (http://cole-trapnell-lab.github.io/cufflinks/). All sequencing data were submitted to the Gene Expression Omnibus database with the accession number GSE73268. The significantly overlapping pathways and Gene Ontology categories with differentially expressed genes were analyzed using DAVID (http://david.abcc.ncifcrf.gov).

2.5. Western blot analysis

Total cell lysates were prepared by lysing the cells in RIPA buffer (Thermo Scientific, Rockford, IL, USA), which contains protease and phosphatase inhibitor cocktail (Thermo Scientific). Equal amounts (100 μg) of total proteins were fractionated by SDS-PAGE on a 6%–12% gel and transferred to Polyvinylidene difluoride (PVDF) membranes (Roche, Basel, Switzerland). The membrane was blocked with 5% milk/Tris-buffered saline plus Tween 20 (TBST) and incubated with primary antibodies against human cyclin E1, PPARγ, and PARP-1 (sc-247, sc-7196, and sc-8007, respectively; Santa Cruz Biotechnology, Santa Cruz, CA, USA). Primary antibodies against human ODC1 and Caspase-9 were purchased from Abcam