The Leloir Pathway of Galactose Metabolism – A Novel Therapeutic Target for Hepatocellular Carcinoma

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Abstract. *Hepatocellular carcinoma (HCC) is one of the most lethal types of cancer worldwide, with poor prognosis and limited treatments. In order to identify novel therapeutic targets that will lead to development of effective therapies with manageable side effects, we tested the hypothesis that knocking-down galactokinase (GALK1) or galactose-1 phosphate uridylyltransferase (GALT) gene expression would control the growth of cultured hepatoma cells. Our results showed small interfering RNA (siRNA) against GALK1 or GALT inhibited the growth of HepG2 cells in culture. Western blot analysis revealed simultaneous down-regulation of multiple players of the phosphatidylinositol 3-kinase (PI3K)/protein kinase B (AKT) growth signaling pathway, as well as heat-shock protein 90 (HSP90) and poly ADP ribose polymerase (PARP). Reverse transcription-polymerase chain reaction (RT-PCR) data, however, showed no significant mRNA reduction of the encoded genes. Our study thus not only supports GALK1 and GALT as being possible novel targets for treating HCC, but also uncovers new post-transcriptional regulatory mechanisms that link the galactose metabolic pathway to protein expression of the PI3K/AKT pathway in hepatoma.*

Cancer is one of the leading causes of mortality and morbidity in both developing and developed nations. The American Cancer Society estimates that in 2016, there will be 1,685,210 new cancer cases diagnosed and 595,690 cancer deaths in the U.S. alone (1). Despite significant progress in anticancer therapies, many types of cancer, such as liver cancer, hepatocellular carcinoma (HCC) in particular,

continue to have poor prognosis (2). Moreover, the deleterious side-effects associated with even the most advanced types of cytotoxic chemotherapies often take a toll on the quality of life of the patients. Recent research efforts have switched from broad-based chemotherapy to targeted therapy in an attempt to minimize treatment toxicities (3); and emerging research shows that altered cellular metabolism in cancer cells can serve as new sites for targeted intervention (4-6).

In humans, metabolism of galactose absorbed from digested foodstuffs, as well as that produced endogenously, begins with the phosphorylation of hexose by the enzyme galactokinase (GALK1) to form galactose-1 phosphate (gal-1P) (Figure 1). In the presence of another enzyme, galactose-1 phosphate uridylyltransferase (GALT), gal-1P will then react with uridine diphosphate glucose (UDP-glucose) to form glucose-1 phosphate (glu-1P) and uridine diphosphate galactose (UDP-galactose) (Figure 1). UDP-galactose is a galactosyl donor for the biosynthesis of glycoproteins and glycolipids, which are crucial for many cancer cellular functions such as cell-cell recognition, metastasis and receptor-mediated signaling (7-11). UDP-galactose can also be formed directly from UDP-glucose *via* the action of UDP-galactose 4'-epimerase (GALE) (Figure 1), but this reaction alone is unlikely to be efficient enough to meet the cell's demand for UDP-galactose.

When compared to normal liver tissues, there is significant up-regulation of *GALK1* and *GALT* genes in human liver cancer tissues, indicating the higher demand for the products of the GALK1 and GALT enzymes by these cancer cells. Barretina and co-workers reported that the *GALK1* gene was up-regulated by at least six-fold in 28 different human liver cancer cell lines, while the *GALT* gene was up-regulated by eight-fold or more (12). Given the genetic heterogeneity of liver cancer (13), the up-regulation seen in 28 different cell lines examined is highly significant. In addition, our recent work on the mouse model of human GALT deficiency revealed a positive regulatory role of *GALT* gene function in the expression of the phosphatidylinositol 3-kinase (PI3K)/protein kinase B (AKT) growth signaling pathway

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Figure 1. The Leloir pathway of galactose metabolism. Under normal conditions, exogenous and endogenously-produced galactose is phosphorylated by galactokinase (GALK1) to form galactose-1-phosphate (gal-1P). In the presence of galactose-1-phosphate uridylyltransferase (GALT), gal-1P reacts with UDP-glucose to form glucose-1-phosphate and UDP-galactose. UDP-galactose can also be formed from UDP-glucose via the UDP galactose-4'-epimerase (GALE) reaction.

(14), which in turn plays a significant role in the growth and progression of human HCC (15). However, no GALK1-deficient mouse models are available for similar studies.

Deficiencies of GALK1 and GALT enzymatic functions have relatively benign and manageable consequences to normal, non-cancerous cells, which is crucial for preserving the quality of life for the patients under treatment. For example, patients with inherited deficiency of GALK1 (type II galactosemia) have increased incidence of pre-senile cataracts, but no life-threatening or other serious consequences (16). Cataract formation often self-resolves when the patient is put on a galactose-restricted diet. Some patients with inherited severe GALT deficiency (type I galactosemia, 0% GALT activity) might develop long-term complications such as ataxia (20% incidence) and primary ovarian insufficiency (92% incidence) (17, 18), but these complications are unlikely to pose serious concerns for older patients with terminal liver cancer.

In this study, we applied small interfering RNA (siRNA) targeting *GALK1* and *GALT* genes in the HCC model cell line HepG2 to test the hypothesis that knocking-down expression of *GAL* genes would have negative impact on the growth of the cultured hepatoma cells. Successful validation of these targets will pave the way for formal development of therapies.

Materials and Methods

Materials. HepG2 cell line was obtained from the American Type Culture Collection (Manassas, VA USA) and maintained in Dulbecco's modified Eagle's medium (DMEM) supplemented with

10% fetal bovine serum and penicillin/streptomycin. Antibody to glyceraldehyde 3-phosphate dehydrogenase (GAPDH) was purchased from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA, USA). Antibodies against phosphorylated AKT (Ser473) (p-AKT Ser473), phosphorylated AKT(Thr308) (p-AKT Thr308), total AKT (pan AKT), phosphorylated phosphatase and tensin homolog (Ser380) (p-PTEN Ser380), phosphorylated phosphoinositide-dependent kinase 1 (Ser241) (p-PDK1 Ser241), phosphorylated glycogen synthase kinase 3β (Ser9) (p-GSK3β Ser9), heat-shock protein 90 (HSP90) and poly ADP ribose polymerase (PARP) were purchased from Cell Signaling Technology, Inc. (Danvers, MA, USA). Fluorescently labeled secondary antibodies were purchased from Li-Cor Biosciences (Lincoln, NE, USA). Lipofectamin RNAiMax, RNeasy mini kit, Superscript RT kit, polyT primer, Taqman 2× Master buffer, primers and probes for *AKT*, *PDK1*, *GAPDH*, *PARP*, *GALT* and *GALK1* were purchased from Invitrogen (Carlsbad, CA, USA). siRNAs were also synthesized by Invitrogen.

Sequences for siRNAs. *GALK1*: GGGCCUUCUAUGUCAA GGGAGUGAU; *GALT*: GUCUCCACGCGCAGUACAGUUGC UU; Control: GUCUCCACGCGCAGUACAGUUGCUU.

siRNA transfection and growth monitoring of cultured HepG2 cells. HepG2 cells were plated in replicates in 12-well plates at a density of 5×10^3 cells/well at day 0. Soon after the cells settled, siRNAs against *GALK1*, *GALT* and control siRNA were transfected into the cells in multiple wells with Lipofectamin RNAiMax at 30 nM according to the manufacturer's protocol on the same day. The cells were then trypsinized from the respective wells and counted with a hemocytometer every other day. At day 4, another transfection was carried out in the same way as the first transfection.

Western blot analysis. HepG2 cells were collected at day 8 in lysis buffer [25 mM HEPES (pH 7.5), 120 mM NaCl, 1 mM EDTA,

Figure 2. Inhibition of HepG2 cell growth by siRNAs against both galactokinase (*GALK1*) and galactose-1-phosphate uridylyltransferase (*GALT*) genes. HepG2 cells were seeded in multiple wells at 5×10^3 per well in a 12-well plate at day 0. After the cells settled, different siRNA treatments were applied. Cell growth was monitored by counting cells every other day.

10 mM pyrophosphate, 10 mM glycerophosphate, 50 mM NaF, 0.5 mM orthovanadate, and EDTA-free protease inhibitors; Roche Applied Science, Indianapolis, IN, USA]. After centrifugation at $13,000 \times g$ for 10 min, samples containing 20–50 μ g of protein were resolved by sodium dodecyl sulfate-polyacrylamide gel electrophoresis, and proteins were transferred to nitrocellulose membrane (Bio-Rad Laboratories, Hercules, CA, USA), blocked in 5% non-fat dried milk, and incubated with the primary antibodies for p-AKT Ser473, p-AKT Thr308, pan AKT, p-PDK1 Ser241, p-PTEN Ser308, p-GSK3 β Ser9, HSP90, PARP and GAPDH overnight at 4°C. The blots were washed with wash buffer and secondary antibodies were applied for 1 h at room temperature. Then the blots were scanned with Odyssey scanner and the analysis was performed on Image Studio Lite (both Li-Cor Biosciences).

Reverse transcription polymerase chain reaction (RT-PCR). RNA extraction, reverse transcription for the same samples used above were carried out according to the manufacturer's protocol. The cDNA was mixed with 2 $\times$ TaqMan Master mix and primer/probe mix for *AKT*, *PDK1*, *PARP*, *GALK1*, *GALT* and *GAPDH* individually in 384-well PCR plate, and the RT-PCR was carried out in a 7900HT RT-PCR system from Applied Biosystems (Carlsbad, CA, USA), the data was imported to QuantStudio 12K Flex Software from Applied Biosystems (Carlsbad, CA, USA) for analysis.

Results

Knock-down of *GALK1* and *GALT* genes by siRNA inhibited the growth of cultured HepG2 cells. The growth of HepG2 cells treated with either the control or siRNAs against the two *GAL* genes had no apparent difference at the early stage of the experiment (day 1–5). But at day 6, growth of HepG2 cells treated with siRNAs targeting *GALK1* and *GALT* genes plateaued and the cell numbers started to decrease gradually, while cells of the control groups grew aggressively and reached 40×10^4 cells at the same time frame, as shown in Figure 2. Growth inhibition continued for another 4 days.

siRNAs against *GALK1* and *GALT* genes down-regulated PI3K/AKT signaling pathway. Our previous study of the skin fibroblasts derived from a mouse model of classic galactosemia revealed that *GALT* deficiency resulted in impaired PI3K/AKT signaling pathway in these cells (14). We investigated whether the same would hold true for cultured HepG2 cells. As shown in Figure 3a, treatment with *GALT* siRNA also induced the attenuation of PI3K/AKT

Figure 3. Knock-down of galactokinase (*GALK1*) and galactose-1-phosphate uridylyltransferase (*GALT*) gene expression led to down-regulation of the phosphatidylinositol 3-kinase (PI3K)/protein kinase B (AKT) signaling pathway. Western blot analyses of HepG2 cells at day 8 after treatment with *GALT* siRNA (a) and with *GALK1* siRNA (b).

signaling pathway. Abundances for both forms of phosphorylated AKT decreased when the HepG2 cells were treated with *GALT* siRNA, as well as the total AKT level, which in turn caused the down-regulation of phosphorylated GSK3 β . Both the positive and negative players of PI3K/AKT signaling pathway, pPDK1 and pPTEN, respectively, were also down-regulated. In addition, the levels of HSP90 and PARP also decreased.

Similarly, when the HepG2 cells were treated with *GALK1* siRNA, the levels of all the players of PI3K/AKT signaling pathway, except pPTEN, decreased (Figure 3b). The expression level of HSP90 and PARP were also down-regulated.

siRNAs targeting GALT and GALK1 down-regulated AKT pathway at the post-transcriptional level. In order to confirm the efficiency of siRNAs, we performed RT-PCR assays on the same samples used above. As expected, mRNA levels for both *GALK1* and *GALT* were down-regulated by over 80% by their respective siRNAs (Figure 4). Interestingly, the mRNA levels for *AKT*, *PDK1* and *PARP* did not change significantly upon treatment with *GALT* siRNA nor *GALK1* siRNA, which suggests the attenuation of the AKT pathway by these two siRNAs is through post-transcriptional mechanisms.

Discussion

HCC is a leading cause of cancer death worldwide (GLOBOCAN 2012, <http://globocan.iarc.fr>), and is the most rapidly growing cause of cancer-related mortality in the U.S. In contrast to developing countries, in which hepatitis B

virus infection is the major cause of HCC, chronic infection with hepatitis C virus is the leading risk factor for the increasing incidence in developed countries (19). In both scenarios, abnormal activation of the PI3K/AKT pathway has been found in tumor cells (20, 21). Studies have also shown that chronic activation of the PI3K/AKT pathway due to loss of PTEN leads to HCC in transgenic mouse models (22), and inhibition of this pathway by sorafenib or rapamycin greatly slowed down HCC progression in animal models (23).

In this study, we demonstrated that disruption of the galactose metabolism pathway by siRNAs against both *GALT*, and *GALK1* inhibited the growth of HepG2 cells in culture (Figure 2). Further analysis revealed that one of the most important cellular growth signal pathways, the PI3K/AKT pathway, was greatly attenuated in siRNA-treated cells. Our findings are significant because of multiple reasons. Firstly, we uncovered the link between the galactose metabolic pathway and the PI3K/AKT signaling pathway in these liver-specific cancerous cells. Secondly, the discordance between the reduced protein abundance of the members of the PI3K/AKT pathway (*e.g.* AKT, pPDK1 and pGSK-3 β) (Figure 3) and the unchanged mRNA levels of their encoding genes (Figure 4) revealed a novel post-transcriptional regulatory mechanism for these protein members.

Thirdly, since the abundance of HSP90 also decreased upon treatment with *GALT* or *GALK1* siRNA (Figure 4), we hypothesize that the post-transcriptional down-regulation of PI3K/AKT pathway is through affecting the abundance of HSP90, as AKT, pPDK1 and pGSK-3 β are all clients of

Figure 4. Reverse transcription-polymerase chain reaction (RT-PCR) assays for HepG2 cells treated with galactose-1-phosphate uridylyltransferase (GALT) (a) and galactokinase (GALK1) (b) siRNAs. HepG2 cells were harvested at day 8 after siRNA treatment and mRNA levels of protein kinase B (AKT), phosphoinositide-dependent kinase 1 (PDK1), poly ADP ribose polymerase (PARP), GALK1 and GALT were detected through RT-PCR.

HSP90, and the correct folding and maturation of these proteins are dependent on the proper functioning of HSP90. HSP90 is the most abundant chaperone protein in cells, being constitutively expressed, and can be further induced upon heat shock or other stress stimuli. It accounts for 1-2% of the total protein in unstressed cells, which increases to 4-6% under stress (24). As a chaperone, HSP90 assists folding of more than 200 client proteins (25), including many protein kinases, transcription factors, such as AKT, PDK1, mechanistic target of rapamycin (mTOR) and GSK-3 β (<http://www.picard.ch/downloads/Hsp90interactors.pdf>). HSP90 plays a crucial role in almost all hallmark aspects of oncogenesis, such as self-sufficiency in growth signaling,

evasion of death signals and angiogenesis (26-28). Thus inhibition of HSP90 functions has been regarded as a broader anticancer therapy approach rather than antagonists against a single signaling pathway. Not surprisingly, HSP90 inhibitors showed great potential in HCC in many studies (29-31). If our hypothesis is correct, we have found a new regulatory link between the galactose metabolic pathway and abundance of HSP90 in cancer cells. This link can be exploited to complement other existing anti-HSP90 therapies.

Fourthly, we discovered that one of the key DNA damage-repair proteins, PARP, was also down-regulated upon treatment with both *GALK1*, and *GALT* siRNA. PARP binds to DNA strand breaks and nicks upon DNA damage and

mediates the synthesis of PAR polymers from NAD, causing poly(ADP)-ribosylation of itself and other proteins to recruit other factors in DNA-damage repair (32-34). Its role in sensing DNA damage and initiating base excision repair of single-stranded breaks makes it an attractive anticancer target. PARP inhibitors have been investigated as potentiators of chemotherapy and radiation therapy for a large variety of solid malignancies, including breast, pancreatic, glioma, non-small cell lung, and melanoma (35, 36). PARP is not a client of HSP90, and we know of no reported case that these two proteins were down-regulated simultaneously in any biological event so far. Our results demonstrate, as far as we are aware for the first time, that targeting galactose metabolism pathway through *GALK1* and *GALT* siRNAs can simultaneously down-regulate HSP90 and PARP, which play key roles in homeostasis of cancer cells at both protein and DNA levels. It is unlikely that this finding was caused by off-target effect of siRNAs, as two different siRNAs against two different targets generated the same results.

As to the mechanism of how knocking-down *GALT* and *GALK1* have such profound effects on HepG2 cells, we believe that protein glycosylation might play an important role. As cancer cells grow at a much faster pace than normal cells, the demand for protein synthesis and protein glycosylation are much higher than normal cells. Early studies showed that in patients with classic galactosemia who are deficient in *GALT*, the substrates for protein glycosylation, UDP-glucose and UDP-galactose, are significantly reduced (37). Other studies have shown that interrupting protein glycosylation caused down-regulation of the PI3K/AKT pathway and cell growth arrest (38, 39). However, we cannot preclude possibilities of other mechanisms which may lead to the down-regulation of PI3K/AKT, HSP90 and PARP in cancer cells upon knocking-down *GALK1* and *GALT*.

Currently, sorafenib is the only selective chemotherapy for HCC and is one of the standard treatments for advance-stage HCC. Yet sorafenib still has many debilitating side-effects, including fatigue, rash, loss of appetite, diarrhea, high blood pressure, and redness, pain, swelling, or blisters on the palms of the hands or soles of the feet (40). Our observations validate *GALK1* and *GALT* as candidate targets for novel treatment for HCC. We expect that knocking-down these two targets will have minimum, manageable side-effects based on the studies of different types of galactosemia. In classic galactosemia in which patients have complete *GALT* deficiency, primary ovarian insufficiency and ataxia are major concerns (17, 18). But as we have shown in this study, we did not need to eliminate *GALT* expression entirely with *GALT* siRNA in order to achieve an effect. In fact, with only about 80% knock-down, we induced a profound impact on the PI3K/AKT pathway, HSP90 and PARP in HepG2 cells (Figures 3 and 4). Regarding the possibly side-effect of *GALK1* deficiency, the only concern is cataracts, which can

easily be removed through surgery (16). Based on these observations and facts, we believe that *GALK1* and *GALT* are novel and safer therapeutic targets for HCC.

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