Epigenetic upregulation and functional role of the mitochondrial aspartate/glutamate carrier isoform 1 in hepatocellular carcinoma

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ABSTRACT

Metabolic reprogramming is a common hallmark of cancer cells. Although some biochemical features have been clarified, there is still much to learn about cancer cell metabolism and its regulation. Aspartate-glutamate carrier isoform 1 (AGC1), encoded by SLC25A12 gene, catalyzes an exchange between intramitochondrial aspartate and cytosolic glutamate plus a proton across the mitochondrial membrane, so supplying aspartate to the cytosol. SLC25A12, expressed in brain, heart, and skeletal muscle, is silenced in normal liver. Here, we demonstrate that SLC25A12 gene is reactivated in hepatocellular carcinoma (HCC) HepG2 cell line through histone acetylation and CREB recruitment. Furthermore, SLC25A12 knockdown by small interfering RNA, impairs HepG2 cell proliferation by inducing cell cycle arrest. AGC1 sustains HCC cell growth by supplying cytosolic aspartate for nucleotide biosynthesis. In addition, SLC25A12-silenced HCC cells show a strong reduction of cell migration. Overall, we have provided evidence for molecular mechanisms controlling SLC25A12 gene expression in liver and pointing to an important role for AGC1 in HCC.

Keywords: Aspartate-glutamate carrier isoform 1 (AGC1); SLC25A12; hepatocellular carcinoma (HCC); epigenetics; gene expression; cancer metabolism.

1. INTRODUCTION

Hepatocellular carcinoma (HCC) is the fifth cause of cancer and a primary cause of cancer-linked death worldwide [1]. Despite the progress in therapy and advanced screening of high-risk patients, the prognosis has not had a significant improvement over recent years [2]. Among the main obstacles to successful treatment of HCC there are chemoresistance mechanisms, not always effective chemotherapeutic agents and a delayed diagnosis [3]. Therefore, at present, HCC represents an important challenge since there is no definitive cure beyond a liver transplant. The pathogenic mechanisms that lead to the onset of this tumor are very complex and heterogeneous including aberrant angiogenesis, cell cycle dysregulation, genetic and epigenetic aberrations [4]. Dysregulated metabolism and mitochondrial dysfunction are well-established hallmarks of cancer cells too [5, 6]. Over the past years, several mitochondrial alterations have been identified in human cancers, including HCC [7, 8]. Currently, many studies are focusing on new HCC molecular markers as well as therapeutic targets by analyzing genes and/or pathways involved in HCC progression.

The mitochondrial aspartate-glutamate carrier isoform 1 (AGC1) belongs to the mitochondrial carrier family: proteins localized in the inner membrane of mitochondria which catalyze the translocation of a variety of metabolites, nucleotides and cofactors across the membrane thus providing a link

between mitochondria and cytosol [9]. Therefore, mitochondrial carriers function has a pivotal link between cytosolic and mitochondrial metabolism. For this reason, mitochondrial carrier gene expression is tightly regulated in different tissues, in physiological and pathological conditions [10-16].

In humans there are two AGC isoforms: AGC1 and AGC2 encoded by SLC25A12 and SLC25A13, respectively. Both proteins catalyze a 1:1 exchange of aspartate for glutamate and are an essential component of the malate/aspartate shuttle [17, 18]. This shuttle transfers the reducing equivalents of NADH plus H^+ from cytosol to mitochondria and is therefore indispensable for glycolysis. AGC proteins are also important to supply aspartate to the cytosol. Cytosolic aspartate is required for gluconeogenesis from lactate and for urea, protein, purine, and pyrimidine synthesis [9]. Furthermore, AGC1 is abundant in brain, heart, and skeletal muscle, whereas AGC2 has been found in several tissues but most abundantly in liver where AGC1 is almost absent [19]. Despite AGC1 and AGC2 show the same substrate specificity, previous studies have highlighted some distinctive features of each isoform in normal and pathological conditions. In the liver, a main role of AGC2 is to provide cytosolic aspartate which together with citrulline is condensed to argininosuccinate via argininosuccinate synthase (ASS1), a cytosolic enzyme of the urea cycle. Therefore, SLC25A13 gene mutations cause type II citrullinemia (CTLN2), an autosomal recessive disease affecting liver and characterized by citrullinemia, hypoproteinemia,

and recurrent episodes of hyperammonemia often leading to rapid death [20]. Growing evidence suggests a relationship between CTLN2 and HCC. Indeed, many case reports describing the onset of HCC in patients affected by CTLN2 can be found in the literature [21-24]. In a more recent and extensive study, through a screening of subjects with non-viral HCC, Chang et al. have identified SLC25A13 different mutations in two HCC patients [25]. Other studies reported the involvement of AGC1 in neuronal development by supplying aspartate for N-acetylaspartate production [26]. However all the above mentioned papers lack metabolic analysis and gene expression data. In this context nothing is known about a possible role of AGC1, as well as the SLC25A12/SLC25A13 gene regulation and which mechanisms cause a differential expression of each isoform in various tissues.

Here we provide evidence, for the first time, that SLC25A12 upregulation in HCC cell lines is essential to promote HCC cell growth. Our findings highlight a key function of ACG1 in HCC suggesting a new target in the growing field of liver cancer metabolism.

2. MATERIALS AND METHODS

2.1 HCC tissue samples

Tumoral and peritumoral HCC tissue samples from 16 patients showing a alpha-fetoprotein value higher than 15 ng/ml were obtained from National Institute of Gastroenterology “S. De Bellis” Research Hospital, Castellana

Grotte, Italy in accordance with the Committee on Human Research approved procedures. Peritumoral samples were collected at least at 4–5 cm by the limit of the lesion. All of the samples were obtained with informed consent of the patients and were clinically confirmed.

2.2 Cell culture, treatments, transient transfection and RNA interference

HepG2, HuH7, and HLF cells (Sigma–Aldrich, St Louis, MO, USA) were maintained in high glucose DMEM (Dulbecco's modified Eagle's medium) containing 10% (v/v) fetal bovine serum, 2 mM L-glutamine, 100 U penicillin, and 100 µg/ml streptomycin at 37°C in 5% CO_2. Primary human hepatocytes (PHH, Lonza, Walkersville, MD, USA) were grown in hepatocyte culture medium (Lonza) following the manufacturer's instructions. Alexander cells (Sigma–Aldrich) were grown as reported previously [27]. Where indicated, human hepatocytes were incubated with 500 ng/ml Trichostatin A (TSA, Sigma–Aldrich) or 50 µM 5-aza-2'-deoxycytidine (AzaC, Sigma–Aldrich), either separately or together, for 12 hours. Subsequently, TSA and/or AzaC-treated cells were lysed and tested for AGC1 expression levels. HepG2 cells were treated with 500 µM hydroxycitrate (HCA, Sigma–Aldrich) for up to 24 hours. In some experiments, HepG2, HuH7, and HLF cells were treated with 10 mM aspartate (Sigma–Aldrich), 40 µM deoxynucleotides (dNTP, Sigma–Aldrich) and 10 µM 5-fluorouracil (5FU, Sigma–Aldrich). In RNA interference experiments, specific small interfering RNAs (siRNAs) targeting human

CREB1 ORF (s3489, Life Technologies, Paisley, UK), human CREB1 3'-UTR (hs.Ri.CREB1.13.1, Integrated DNA Technologies, Leuven, Belgium) human SLC25A12 (s16363, Life Technologies), SLC25A13 (s532443, Life Technologies) or control scramble siRNA were transfected in HepG2 cells using siPORT^{TM} NeoFX^{TM} Transfection Agent (Life Technologies). A siRNA (C6A-0126, Life Technologies) with no significant similarity to human, mouse, or rat gene sequences was used as negative control. HepG2 cells were transiently transfected as described previously [28] using pcDNA3-CREB1 and pcDNA3-SLC25A12 vectors [29].

2.3 Chromatin immunoprecipitation assay

Chromatin immunoprecipitation (ChIP) experiments were performed as previously reported [30] with slight modification. Briefly, 2 x 10^7 of HepG2 cells, treated with or without HCA (500 μM) for up to 24 hours or C646 (20 μM) for 6 hours, were fixed by 1% formaldehyde at 37°C for 10 minutes; afterwards, the cells were lysed and sheared by sonication in a 1% SDS lysis buffer to generate cellular chromatin fragments of 300–400 bp. The chromatin was immunoprecipitated for two hours at 4°C using protein G MicroBeads (MACS Miltenyi Biotec, Bergisch Gladbach, Germany) on a rocking platform with H_3Ac (P-2010-24, acetylated on lysines 9 and 14, Epigentek, Brooklyn, NY, USA), H_4Ac (P-2011-24, acetylated on lysines 5, 8, 12 and 16, Millipore) or CREB (PA1-850, Thermo Fisher Scientific, San Jose, CA, USA) specific

antibodies. Input (total chromatin extract) and mock (immunoprecipitation without antibody) samples were recovered and then used for PCR analysis. Magnetically labeled protein/DNA complexes were retained and washed by using microcolumns (MACS Miltenyi Biotec). After reverse cross-linking, DNA was purified and analyzed by PCR using a forward primer (5'-CGTCCCATGCCAATTTAGGAGCAT-3') and a reverse primer (5'-AGGTTCCGACGGATCAAAGAGCAC-3') that amplify a region of about 200 bp in length on the SLC25A12 gene promoter.

2.4 Cell proliferation assay

HepG2, HuH7, Alexander and HLF cells plated in 12-well plates were transfected with siRNA targeting human SLC25A12, SLC25A13 or control scramble siRNA, treated as required and grown up to 96 hours after transfection. Afterward, cells were trypsinized and cell counting of viable cells was performed by the Scepter™ 2.0 handheld automated cell counter (Millipore) according to the instructions. Assays were performed in three wells for each time-point and/or conditions and all experiments were repeated at least three times. Cell proliferation was also evaluated by the CellTiter 96® Non-Radioactive Cell Proliferation Assay (Promega) [27]. In brief, cells were seeded onto 96-well microtiter plates, transfected with siRNA targeting SLC25A12 or with scramble siRNA and then, 15 µl of the Dye solution was added to each well and the cells were incubated for 4 hours at 37°C.

Subsequently, solubilization solution/stop mix (100 µl) was added to each well and the cells were incubated for 1 hour at 37°C to promote the solubilization of formazan crystals. The level of MTT formazan was determined by measuring its absorbance at 570 nm using the GloMax plate reader (Promega).

2.5 Cell cycle analysis

For cell-cycle analysis, 1x 10^6 HepG2 cells transfected with or without siRNA targeting SLC25A12 up to 48 hours were fixed in 70% ethanol, incubated for 30 minutes at 4°C, treated with RNase A (500 µg/ml), and stained with propidium iodide 0.46 µM (Sigma-Aldrich) for 30 minutes at 37°C. Cell cycle analysis was performed on a flow cytometer (EPICS® Elite; Backman Coulter Miami, FL, USA).

2.6 Real-time PCR, SDS–PAGE and western blotting

Total RNA was extracted from 1 x 10^6 HepG2, HuH7, Alexander, HLF cells, or primary human hepatocytes treated as indicated, and reverse transcription was performed as reported [11]. RNA was extracted from liver samples after homogenization by using the RNeasy Mini Kit (Qiagen, Valencia, CA, USA). Real-time PCR was performed as previously described [31]. Assay-on-demand for human CREB (Hs00231713_m1), SLC25A12 (Hs00186535_m1), SLC25A13 (Hs01573625_m1), human MKI67 (Hs01032435_g1) and human

β-actin (4326315E) were purchased from Life Technologies™. Transcript levels were normalized against the β-actin expression levels. SDS-PAGE experiments were carried-out as reported previously [32]. For Western blot analysis, proteins were electroblotted onto nitrocellulose membranes (Bio-Rad, Hercules, CA, USA) subsequently treated with anti-AGC1 (sc-271056, Santa Cruz Biotechnology, Santa Cruz, CA), anti-AGC2 (sc-100937, Santa Cruz Biotechnology) and anti-β-actin (sc-47778, Santa Cruz Biotechnology) antibodies. The immunoreactions were detected by the Immobilon western ECL system (Millipore). Densitometry analysis of immunolabeled protein bands was performed using ImageJ quantitative software (NIH, Bethesda, MD, USA) and normalized to β-actin levels.

2.7 Transwell migration assay

Transwell migration assay was performed as previously described [33]. Briefly, 6.5 mm transwells with 8.0 µm pore polycarbonate membrane were used (3422, Corning Incorporated, USA). The lower side of membranes was coated with fibronectin (FC010, Millipore). Complete 10% FBS DMEM (400 µl) was then added in the lower chamber and 200 µl of DMEM serum free suspended cells (6 x 10^4 cells) were seeded into the upper chamber. Cells were left to migrate for 16 hours at 37°C, 5% CO_2, then the membranes were fixed for 10 minutes with 4% paraformaldehyde, and stained for 10 minutes with crystal violet. Unmigrated cells (upper side) were removed with a cotton

bud, whereas migrated cells (lower side) were counted under microscope. Four or five microscopic fields / transwell were analyzed.

2.8 Quantification of aspartate, UTP and RNA

The concentration of aspartate and UTP in SLC25A12- and SLC25A13-silenced HepG2 cells were assessed using specific kits from Sigma–Aldrich (MAK095-1KT) and MyBioSource (MBS2025220), respectively, according to the manufacturer's instructions. Cellular total RNA levels were determined via Multiscan GO 3.2 (Thermo Fisher Scientific). Aspartate, UTP and RNA levels were normalized to cell number.

2.9 Statistical analysis

Statistical significance of difference was determined using one-way analysis of variance. Results are presented as means ± SD of, at least, four independent experiments. Differences were considered as significant (*$p < 0.05$), very significant (**$p < 0.01$) and highly significant (***$p < 0.001$).

3. RESULTS

3.1 Expression of SLC25A12 and SLC25A13 in liver and hepatic cancer cells

We first evaluated SLC25A12 mRNA levels in tumoral and peritumoral samples from 16 HCC patients. We observed that SLC25A12 mRNA levels

were significantly higher in 11 (69 %) HCC than in peritumoral samples (Fig. 1A). Instead, only 4 HCC samples (25 %) showed an elevated SLC25A13 RNA content when compared to control ones (Fig. 1B). Furthermore, we measured - in primary human hepatocytes (PHH) and HepG2 hepatocarcinoma cancer cells - SLC25A12 mRNA and AGC1 protein basal levels by real-time PCR and western-blot analysis, respectively. Fig. 1C shows high levels of AGC1 expression in HepG2 cells compared to PHH where it is almost absent. As it is known that SLC25A13 is the predominant mitochondrial aspartate-glutamate carrier isoform in liver, we checked its expression pattern in PHH and HepG2 cells. As expected a great amount of both mRNA and protein was found in PHH whereas a reduction of SLC25A13 expression was observed in liver cancer cells (Fig. 1C). In light of the above results, it was surprising to find a strong SLC25A12 reactivation in HepG2 cells - where SLC25A13 is downregulated – according to SLC25A12 mRNA levels found in HCC samples. Thus, our interest was focused on SLC25A12 gene expression regulation in liver cells. In order to investigate the molecular mechanisms underlying the differential expression in normal and cancer cells, we evaluated the effect of epigenetic changes on SLC25A12 expression. First, PHH were treated with the demethylating agent 5-aza-2'-deoxycytidine (AzaC) and the histone deacetylase inhibitor trichostatin A (TSA), alone or in combination, for a period of 12 hours. Since this cell type is short-lived, we did not test the drugs for long periods. We found that SLC25A12 gene

expression was highly induced following TSA addition when compared to untreated cells (Fig. 1D). On the contrary, AzaC treatment did not affect SLC25A12 gene expression. When a combination of AzaC and TSA was used, we did not find a synergistic effect over SLC25A12 expression, and the observed increase was probably the result of TSA treatment alone (Fig. 1D). AGC1 protein levels are consistent with SLC25A12 mRNA expression pattern (Fig. 1D). These data suggest that SLC25A12 is epigenetically silenced in PHH by mechanisms involving histone acetylation but not DNA methylation.

3.2 SLC25A12 gene is epigenetically regulated through histone acetylation

Due to the short lifespan of PHH, we further investigated the relationship between SLC25A12 gene regulation and epigenetic mechanisms in HCC cell lines. Firstly, we analyzed both SLC25A12 and MKI67 – a gene known to be overexpressed in HCC [34] - mRNAs in HepG2, HuH7, Alexander and HLF liver cancer cell lines. The expression levels of SLC25A12 and MKI67 in HepG2 cells were used as reference for the other cell lines (Fig. 2A, B). Both SLC25A12 and MKI67 mRNA levels were higher in Alexander and lower in HLF than in HepG2 cells (Fig. 2A, B). In HuH7 we observed a decrease of the SLC25A12 and an increase of the MKI67 when compared to the mRNA reference level of each gene in HepG2 cells (Fig. 2A, B). We chose to use for our subsequent experiments HepG2 cells. Our approach was to block histone

acetylation process by reducing nuclear acetyl-CoA amount in liver cancer cell lines where we found high AGC1 expression levels. It is known that in mammalian cells ATP-citrate lyase (ACLY) activity is the main source of acetyl-CoA available for nuclear histone acetylation in physiological and pathological conditions [35-37]. Thus, we analyzed AGC1 time course expression upon ACLY inhibition by using hydroxycitric acid (HCA), a specific inhibitor. A marked decrease of about 3-fold SLC25A12 mRNA was evident at 6 and 12 hours followed by an evident increase within 24 hours after HCA addition compared to untreated cells (Fig. 2C). To better define the changes in SLC25A12 gene expression, we carried-out western blotting experiments as well as mRNA analysis and we confirmed a severe reduction of AGC1 at 6 and 12 hours after HCA treatment (Fig. 2C). A ChIP analysis from HepG2 cells pretreated with HCA was used to measure the levels of H3 and H4 histones bound to the endogenous SLC25A12 promoter. As shown in Fig. 2D, E, F, a strong decrease of the acetylated form of both H3 and H4 histones was associated with the SLC25A12 promoter at 6 hours in response to HCA. Twelve and twenty-four hours after the beginning of the ACLY inhibition, a progressive rise of the acetylated form of both H3 and H4 histones linked to the SLC25A12 promoter was observed (Fig. 2D, E, F). Our experiments clearly demonstrate a central role of histone acetylation status in controlling SLC25A12 gene expression in HepG2 cells.

3.3 Acetylation affects CREB-dependent SLC25A12 transcriptional regulation

In silico analysis identified a CREB binding site at position −525/−517 bp upstream the ATG translational codon and it has been reported that CREB transcriptional factor is a leading regulator of the SLC25A12 gene expression in neuronal cells [29].

We investigated whether CREB controls SLC25A12 gene transcription in HepG2 cells too. To this end, SLC25A12 expression levels were evaluated in CREB-silenced HepG2 cells by using a siRNA against CREB1 mRNA, after testing that its efficiency to silence the target mRNA was of about 75% (Fig. S1A). As shown in Fig. 3A CREB gene silencing caused a decrease of about 50% in SLC25A12 mRNA with respect to control cells. AGC1 protein was also found to be lower in CREB-silenced than in unsilenced HepG2 cells (Fig. 3A). These data were confirmed by using another siRNA targeting the 3'-UTR of CREB1 mRNA, able to efficiently silence CREB1 gene expression (Fig. S1B). To validate the 3' UTR-targeting siRNA specificity, a functional rescue experiment was carried out by overexpressing CREB1, as well as **SCL**25A12 in CREB1-silenced cells. Exogenous CREB1 and even more SLC25A12 overexpression significantly revert CREB1-silencing phenotype by increasing **SCL**25A12 mRNA and protein levels (Fig. S1C).

It is known that CREB function is regulated by acetylation [38]. To determine whether CREB-induced SLC25A12 gene expression in HepG2 cells involves

acetylation events, ChIP experiments were performed in the presence or absence of HCA treatment. Fig. 3B shows a significant decrease of CREB binding to SLC25A12 promoter within 6 hours after HCA exposure, when compared to untreated cells. At 12 and 24 hours after HCA addition a progressive increase of CREB binding was evident (Fig. 3B). Thus it appears that SLC25A12 transcription regulation through CREB protein is affected by acetylation events.

3.4 SLC25A12 gene silencing impairs cell proliferation

The data presented above indicate that SLC25A12 upregulation in HepG2 cells compared to primary hepatocytes was induced, at least in part, by acetylation mechanism. We therefore wondered what meaning might have high SLC25A12 expression levels in HepG2 cells. Firstly, we measured cell proliferation in SLC25A12-silenced HepG2 cells. SLC25A12 gene silencing efficiency was of about 80% with respect to untreated cells (Fig. S1D). As shown in Fig. 4A, SLC25A12 gene silencing induces a progressive decrease of cell growth up to 96 hours after transfection, when the cells number is reduced to about 30% compared to the unsilenced cells. Then HepG2 cells were incubated with siRNA against SLC25A13 obtaining a gene silencing efficiency of about 75% (Fig. S2A, B). Notably, HepG2 cell proliferation was affected by SLC25A12 and not by SLC25A13 gene silencing (Fig. S2C, D). Next, we investigated the effect of AGC1 on cell cycle progression. To

achieve this goal, flow cytometry analysis was performed in SLC25A12 knockdown cells. Twenty-four hours after SLC25A12 gene silencing we observed a significant cell number increase in G1 phase by about 50% and a strong decrease in S phase when compared to control cells (Fig. 4B). The same assay carried out 24 hours later confirmed similar results (Fig. 4B). These findings were in accordance with those of HepG2 cell counting, displayed in Fig. 4A. Thus, SLC25A12-silenced HepG2 cells are blocked in G1 phase when compared to unsilenced control cells. To strengthen the role of AGC1 in liver tumor cells we carried out SLC25A12 gene silencing in more cell lines such as HuH7, Alexander and HLF. Interestingly, all investigated cells showed a cell number reduction after SLC25A12 gene silencing (Fig. 4C). Ninety-six hours after gene silencing HuH7, Alexander as well as HLF displayed less than 50% of cells when compared to unsilenced cells (Fig. 4C). A similar trend of cell viability was obtained for each liver tumor cell line by performing MTT assay (Fig. S3). Altogether, the above results prove a relationship between SLC25A12 gene expression and liver cancer cell line proliferation.

3.5 AGC1 sustains cytosolic aspartate for de novo pyrimidine biosynthesis in HCC cell lines

Cytosolic aspartate provides nitrogen for the production of the pyrimidine and purine bases and carbon for de novo pyrimidine biosynthesis. Recent reports

identified aspartate as a key metabolite for nucleotide biosynthesis in dividing and cancer cells [39, 40]. Aspartate exits the mitochondria via AGC mitochondrial transporters. Building on these observations, to gain mechanistic insight into why AGC1 is required for cell proliferation, we investigated its involvement in nucleotide biosynthesis. In SLC25A12-silenced HepG2 cells, aspartate was added alone or in the presence of thymidylate synthase inhibitor 5-fluorouracil (5FU) and cell counting assay was performed. Of note, supplementing the medium with aspartate restored the proliferation of HepG2 SLC25A12-silenced cells to a similar level as the unsilenced cells (Fig. 5A). Conversely, when aspartate was added together with 5FU, aspartate was not able to rescue cell proliferation (Fig. 5A). Importantly, aspartate treatment reversed the effect of SLC25A12 gene silencing in HuH7 and HLF liver cancer cells too (Fig. 5B, C). Furthermore - under the same experimental conditions - we demonstrated that deoxynucleotides supplementation restored, at least in part, cell proliferation (Fig. 5A, B, C). Finally, a significant decrease of aspartate, UTP and total RNA levels in SLC25A12-silenced HepG2 cells (Fig. 5D, E, F) strongly strengthens the role of AGC1 in de novo pyrimidine biosynthesis. Remarkably, SLC25A13 gene silencing does not affect the amount of aspartate as well as UTP and RNA (Fig. 5D, E, F). These findings clearly highlight the specific function of AGC1 in sustaining cytosolic aspartate levels for nucleotide biosynthesis in HCC cell lines.

3.6 AGC1 affects liver cancer cell migration

Given the strong inhibition of cell proliferation and the consequent impaired nucleotide biosynthesis in HCC cell lines transfected with siRNA targeted against SLC25A12, we sought to determine the effect of AGC1 in cell migration. Using an *in vitro* transwell migration assay, gene silencing of SLC25A12 strongly reduced growth factor-induced directional migration of the HepG2 cells by about 50% (Fig. 6). Inhibition of migration in **SCL**25A12-deficient cells was not due to reduced cell viability, because cells transfected with either a scrambled control or SLC25A12 siRNA were equally viable over the 16 hours. These results suggest that AGC1 promotes migration in HepG2 cells.

4. DISCUSSION

A complex metabolic and epigenetic reprogramming occurs in cancer cells to respond to the new cellular demands - protein, nucleotide and fatty acid biosynthesis, etc. - in order to take a selective advantage in survival and growth [41, 42]. These changes affect gene regulation leading to a differential expression pattern during tumorigenesis. In this context we analyzed the expression of both asparate/glutamate mitochondrial carrier genes, *SLC25A12* and *SLC25A13*, in hepatocarcinoma cells. Focusing on *SLC25A12* gene, we show, for the first time, its upregulation by epigenetic mechanisms consisting in the histone hyperacetilation in HCC HepG2 cell

line. On the contrary, *SLC25A13* is downregulated in the same cell line. A completely different view from that of normal liver cells.

SLC25A12 gene expression appears to be strongly dependent on the amount of acetyl-CoA, as ACLY inhibition produces a reduction of H3 and H4 acetylated histones at the promoter and consequently a decrease of both mRNA and protein. Furthermore, acetylation status affects CREB-dependent SLC25A12 gene expression in HCC HepG2 cells. By contrast, DNA methylation changes seem not involved in SLC25A12 gene regulation (unpublished data).

These events promote SLC25A12 overexpression that strongly impacts cell proliferation since SLC25A12 gene silencing leads to a G1 cell cycle arrest of HCC HepG2 cell line. Maybe epigenetic upregulation of SLC25A12 could be part of the epigenetic reprogramming aimed to the liver cancer cell survival and growth [43, 44]. Noteworthy, Oncomine (https://www.oncomine.org) and Human Proteome Atlas (https://www.proteinatlas.org) databases used for an in-depth analysis of SLC25A12 mRNA and AGC1 protein levels respectively, reveal a strong upregulation of this gene in different types of tumors [45]. An updating of the Human Proteome Atlas analysis performed by Amodeo et al. [45] indicates SLC25A12 overexpression in liver cancer, as compared to non-cancer tissue. Significantly, by using immunohistochemistry, most samples of HCC show strong staining (https://www.proteinatlas.org/ENSG00000115840-SLC25A12/pathology).

Importantly, gene silencing of the SLC25A13 – the gene encoding for the mitochondrial aspartate-glutamate carrier isoform 2 (AGC2) - does not impair HCC cell line proliferation indicating a specific role of AGC1 in HCC. Unexpectedly, different glucose concentrations do not affect HCC HepG2 cell line proliferation under SLC25A12 gene silencing (Fig. S4). These outcomes uncover that beyond of the malate aspartate shuttle, AGC proteins might be differentially involved in specific cellular processes even when both transporters are present into the mitochondria at the same time. Our results show that HCC cell proliferation needs selectively AGC1 protein, at expense of AGC2 decrease.

Indeed, AGC1 increase is much higher than AGC2 decrease in HepG2 cell line when compared to normal hepatic ones. The high overexpression of AGC1 suggests that the aspartate transport to the cytosol plays a dual role of maintaining NADH/NAD^+ homeostasis: to provide NADH to the mitochondria through the malate/aspartate shuttle, and to support the increased metabolic biosynthesis for HCC cell proliferation. In fact, aspartate as well as deoxynucleotide treatment reverses cell proliferation of HCC SLC25A12-silenced HepG2 cells, but only in the absence of thymidylate synthase inhibition. Aspartate, UTP and RNA decrease in SLC25A12- and not in SLC25A13-silenced HepG2 cells strengthens the specific role of AGC1 in these cells.

Recently, aspartate has been described as a limiting metabolite for cancer cell growth [46, 47]. Since aspartate has poor cell permeability [47], the aspartate export by energized mitochondria is very important for de novo nucleotide synthesis. Of note, metabolic rewiring and genetic/epigenetic changes can impair the mitochondrial inner membrane electrochemical potential in tumor cells [48, 49], but a functional electron transport chain is needed for aspartate biosynthesis in proliferating cells [39, 50].

In this context, increased levels of AGC1 maintain the cell redox balance by moving NADH from cytosol to mitochondria thus fostering mitochondria energization and in turn promoting aspartate export in HCC cells. A specific function for AGC1 in redox homeostasis of cancer cells has been also underlined by Amodeo et al. [45]. These issues suggest that HCC cell proliferation needs selectively AGC1 protein overexpression to guarantee a threshold aspartate level necessary for redox homeostasis and for cellular proliferation.

Our hypothesis could be consistent with the kinetic characteristics valued for the recombinant AGC1 and AGC2 reconstituted in proteoliposomes. Indeed, while the half-saturation constants (K_m) are identical for both proteins, the maximum transport rate (V_{max}) of AGC1 is much lower than that of AGC2 [17]. It is possible that both AGC1 and AGC2 are implicated in malate/aspartate shuttle, while AGC1 could be specifically involved in nucleotide biosynthesis for HCC cell proliferation. Our results are in line with recent reports on the

metabolic transformation of cancer cells identifying aspartate derived from several metabolic pathways as a critical intermediate for nucleotide biosynthesis in dividing cells [40, 51]. Interestingly, aspartate is responsible for increased cell proliferation in citrullinaemia type I (CTLN I) caused by a deficiency of ASS1 as well as in several tumors where ASS1 expression is downregulated [52]. In both these conditions, Rabinovich et al. observe a diversion of aspartate toward de novo pyrimidine synthesis supported by carbamoyl-phosphate synthetase 2, aspartate transcarbamylase, and dihydroorotase (CAD) upregulation [52].

Of note, we also found a suppression of cell migration in SLC25A12-silenced HCC HepG2 cell line. It is not extraordinarily to uncover a metabolic enzyme involved in tumor cell migration and invasion as the metabolic switch, known as the Warburg effect, affects both cancer cell proliferation and cancer cell migration [53]. Recent studies suggest that not only critical enzymes regulate cancer metabolism but also their metabolic products strongly affect tumor cell migration and invasion [54].

In summary, our study demonstrates that SLC25A12 gene, little or not expressed in adult liver, is upregulated in HCC cell lines by epigenetic mechanisms and AGC1 is involved in HCC cell growth and migration by sustaining cytosolic aspartate levels for nucleotide biosynthesis. These findings provide a basis for further investigation of AGC1 and its potential diagnostic and/or therapeutic value in HCC.

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CONFLICT OF INTEREST

The authors declare no conflict of interest.

FIGURE LEGENDS

Fig. 1 SLC25A12 expression levels in liver samples and hepatic cell lines. (A and B) Total RNA extracted from 16 HCC tissues was used to quantify SLC25A12 and SLC25A13 mRNAs, respectively. mRNA from each tumoral (T) tissue was normalized to the relative peritumoral (PT) expression level (line). (C) Total RNA extracted from primary human hepatocytes (PHH) (gray bars) and HepG2 (black bars) cells was used to quantify SLC25A12 and SLC25A13 mRNAs (upper panels). AGC1, AGC2 and ß-actin proteins from PHH and HepG2 cells were detected by specific antibodies (lower panels). (D) Total RNA was extracted from primary human hepatocytes untreated (C) or treated with 5-aza-2'-deoxycytidine (AzaC) and the histone deacetylase inhibitor trichostatin A (TSA) alone or in combination (AzaC + TSA) and used to quantify SLC25A12 mRNA (upper panel). AGC1 and ß-actin proteins from PHH cells, treated as for the upper panel, were detected

by specific antibodies (lower panel). In (C) and (D) western blotting data are representative of three independent experiments. The intensities of immunolabeled protein bands were measured by quantitative software and normalized to β-actin. In (A), (B), (C), and (D) means ± S.D. of four replicate independent real-time PCR experiments are shown; where indicated differences between samples and relative controls (*$p < 0.05$, **$p < 0.01$, ***$p < 0.001$) were significant.

Fig. 2 SLC25A12 expression levels and regulation by histone acetylation in HCC cell lines. Total RNA extracted from HepG2, HuH7, Alexander and HLF cells was used to quantify SLC25A12 (A) and MKI67 (B) mRNAs, respectively. (C) HepG2 cells were treated with 500 μM hydroxycitrate (HCA) or vehicle for 6, 12, and 24 hours. Then, cells were lysed and used to quantify SLC25A12 mRNA (upper panel) and protein (lower panel) levels. SLC25A12 mRNA levels of induced cells at each time point were normalized against vehicle-treated controls. Real-time PCR values represent mean ± S.D. ($N = 5$). *** $p < 0.001$ versus time 0. Western blot data are representative of three independent experiments with similar results. (D) HepG2 cells treated as in (C) were used to carry out ChIP analysis with anti-H3 (H_3ac) and anti-H4 (H_4ac) acetylated histones antibodies. Lane Mock, PCR of immunoprecipitates without antibody; lane I, PCR of input DNA. ChIP agarose gel results representative of three independent experiments are

shown. qPCR-ChIP data obtained by using anti-H3 acetylated histone (E) and anti-H4 acetylated histone (F) antibodies are expressed as fold change versus control. qPCR values represent mean ± S.D. (N = 3); where indicated differences between samples and relative controls (*p < 0.05, **p < 0.01, ***p < 0.001) were significant.

Fig. 3 Effect of acetylation status on CREB-dependent SLC25A12 transcriptional regulation. (A) HepG2 cells transfected with siRNA targeting human CREB (siCREB) or control scramble siRNA (C) were assayed for SLC25A12 mRNA and AGC1 protein. Western blot data are representative of three independent experiments with similar results. The intensities of immunolabeled protein bands were measured by quantitative software and normalized to β-actin. (B) HepG2 cells treated with 500 μM hydroxycitrate (HCA) or vehicle for 6, 12, and 24 hours were assayed for ChIP analysis with anti-CREB antibody. Lane I, PCR of input DNA, lane Mock, PCR of immunoprecipitates without antibody. ChIP agarose gel results representative of three independent experiments are shown. qPCR-ChIP data obtained by using anti-CREB antibody are expressed as fold change versus control. qPCR values represent mean ± S.D. (N = 3); In (A), mRNA values represent mean ± S.D. (N = 5). In (A) and (B), where indicated, differences between samples and relative controls (**p < 0.01, ***p < 0.001) were significant.

Fig. 4 Cell proliferation and cell cycle analysis in SLC25A12-silenced HCC cell lines. (A) HepG2 cells were transfected with siRNA targeting human SLC25A12 (siSLC25A12) or control scramble siRNA (C) and used for cell counting 24, 48, 72 and 96 hours after transfection. (B) In HepG2 cells, transfected as described in (A), cell cycle distribution measurements by flow cytometry were carried out 24 and 48 hours after transfection. G1, S and G2/M phases are indicated. Images are representative of four independent experiments with similar results (left panel). On the right panel, data are shown as means ± SD of four duplicate independent experiments. Differences between C and siSLC25A12 samples in G1 or S phase were significant at 24 as well as at 48 hours after transfection (**$p < 0.01$). (C) HuH7, Alexander and HLF cells were transfected as described in (A) and used for cell counting 24, 48, 72 and 96 hours after transfection.

Fig. 5 AGC1 role in *de novo* pyrimidine biosynthesis. HepG2 (A), HuH7 (B) and HLF (C) cells were transfected with siRNA targeting human SLC25A12 (siSLC25A12) or control scramble siRNA (C). Forty-four hours later, cells were treated for 24 hours with 40 μM deoxynucleotides (dNTP) or 10 mM aspartate alone (Asp) or in combination with 10 μM thymidylate synthase inhibitor 5-fluorouracil (Asp+5FU) and then used to analyze cell number and viability. (D) Aspartate, (E) UTP and (F) total RNA levels were quantified in HepG2 cells transfected with siRNA targeting human SLC25A12 (siSLC25A12), human SLC25A13 (siSLC25A13) or control scramble siRNA

(C). Where indicated, differences between samples and relative controls (*p < 0.05, **p < 0.01, ***p < 0.001) were significant.

Fig. 6 Effect of SLC25A12 gene silencing on HCC cell migration. HepG2 cells were transfected with siRNA targeting human SLC25A12 (siSLC25A12) or control scramble siRNA (C). Forty-four hours later, cells were seeded in the transwell upper chamber. Cells were left to migrate for 16 hours at 37°C, 5% CO_2, then the membranes were fixed for 10 minutes with 4% paraformaldehyde, and stained. Migrated cells were counted under microscope. Differences between samples and relative controls (***p < 0.001) were significant (upper panel). The images of the microscopic fields (lower panel) are representative of four or five microscopic fields / transwell analyzed with similar results.

LEGENDS TO SUPPLEMENTARY FIGURES

Fig. S1 CREB1 and SLC25A12 gene silencing efficiency and specificity. HepG2 cells were transfected with siRNA targeting ORF of human CREB1 (siCREB), 3'UTR of CREB1 (siCREB 3'UTR), SLC25A12 (siSLC25A12), or control scramble siRNA (C) and used to quantify CREB1 (A, B) and SLC25A12 (C, D) mRNAs and respective proteins. (C) HepG2 cells transfected with a siRNA targeting human 3'UTR of CREB1 alone or together with pcDNA3-CREB1 or pcDNA-SLC25A12 were employed for SLC25A12 mRNA and protein quantification. (D) HepG2 cells transfected with a siRNA

targeting human SLC25A12 were used to quantify SLC25A12 mRNA and AGC1 protein. (C, D) Western blot data are representative of three independent experiments with similar results. The intensities of immunolabeled protein bands were measured by quantitative software and normalized to β-actin. Means ± S.D. of four replicate independent real-time PCR experiments are shown. Differences between samples and relative controls (**p < 0.01, ***p < 0.001) were significant.

Fig. S2 SLC25A13 gene silencing in HepG2 cells. HepG2 cells were transfected with siRNA targeting human SLC25A13 (siSLC25A13) or control scramble siRNA (C) and used to quantify SLC25A13 mRNA (A) and AGC2 protein (B) and for cell counting (C) and MTT assay (D) 72 hours after transfection. Means ± S.D. of four replicate independent real-time PCR experiments are shown. In (B) western blot data are representative of three independent experiments with similar results. The intensities of immunolabeled protein bands were measured by quantitative software and normalized to β-actin. Means ± S.D. of four triplicate independent cell counting experiments are shown.

Fig. S3 MTT assay in SLC25A12-silenced HCC cell lines. HLF, Alexander, HuH7 and HepG2 cells were seeded onto 96-well microtiter plates and transfected with siRNA targeting human SLC25A12 (gray bars) or control scramble siRNA (black bars). Seventy-two hours after transfection, MTT

assay was performed to evaluate cell viability. Means ± S.D. of four triplicate independent MTT assays are shown. Differences between samples and relative controls (***p < 0.001) were significant.

Fig. S4 Cell proliferation of SLC25A12-silenced HepG2 cells grown in low and high glucose media. HepG2 cells were seeded onto 24-well microtiter plates and transfected with siRNA targeting human SLC25A12 (gray bars) or control scramble siRNA (black bars) up to 72 hours in culture medium containing 3 mM (Low) or 11 mM (High) glucose. After incubation, trypsinized cells were investigated for cell viability. Means ± S.D. of four triplicate independent experiments are shown. Differences between samples and relative controls (**p < 0.01, ***p < 0.001) were significant.

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