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Impact of zinc on hepatocellular carcinoma cell behavior and metallothionein expression: Insights from preclinical models

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ABSTRACT

Background: Zinc (Zn) is an essential trace element involved in a wide variety of cellular processes and is vital for optimal liver function. Our objective was to elucidate the potential therapeutic role of Zn in hepatocellular carcinoma (HCC), the third leading cause of cancer-related death and the first cause of death in patients with cirrhosis.

Methods: The impact of Zn supplementation on proliferation, invasion, migration, cell cycle, and apoptosis was conducted on four HCC cell lines as well as in a xenograft mouse model of HCC from which tumor gene expression profiles were also analyzed. Gene deregulation and protein expression were validated in human HCC tissues. Finally, Zn and MT1 (Metallothionein 1) levels were quantified in plasma from patients with HCC.

Results: Zn supplementation significantly modulated proliferation, invasion, and migration in HCC cell lines and induced apoptosis in a dose-dependent manner. Although Zn did not exhibit a significant increase in survival, Zn supplementation significantly altered the expression of MT genes. Specifically, MT1G and MT1H expression were notably suppressed in HCC tissues from mice and these results were validated in human HCC samples. Overall, gene and protein MTs expression was significantly lower in HCC areas compared to adjacent liver tissue and plasma Zn levels exhibited substantial variation across different stages of the liver disease.

Conclusion: Zn supplementation influences key cellular behaviors in a dose-dependent manner and upregulates the expression of MT family genes, which may have tumor-suppressive properties, *in vitro* an *in vivo* models. Future research should investigate the prognostic implications of Zn supplementation as part of a comprehensive therapeutic strategy for HCC patients.

Abbreviations: aHCC, advanced Hepatocellular Carcinoma; eHCC, early Hepatocellular Carcinoma; HCC, Hepatocellular Carcinoma; MT, Metallothionein; SF, Sorafenib; ZAC, Zinc Acexamate; Zn, Zinc.

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1. Introduction

Hepatocellular carcinoma (HCC) represents over 90 % of primary liver cancer cases with its incidence rising globally [1,2]. The development of HCC is closely linked to advanced liver fibrosis or cirrhosis, associated with more than 80 % of HCC cases [3]. Liver cirrhosis develops in well-established etiological contexts, such as chronic infections with hepatitis B or C viruses, excessive alcohol consumption, or metabolic dysfunction-associated steatotic liver disease. Furthermore, trace elements, notably copper, selenium, and zinc (Zn), appear to play pivotal roles in liver carcinogenesis [4,5].

Zinc is an essential trace element crucial for the structure and function of numerous proteins [6], with a direct impact on fundamental cellular processes such as proliferation and differentiation [7]. After iron, Zn is the second most abundant metal in the human body, and its deficiency is associated with liver diseases, including chronic hepatitis and cirrhosis [8–10]. Recent studies indicate that Zn supplementation in patients with chronic liver disease not only ameliorates liver pathology but also reduces HCC incidence [11–14].

The specific role of Zn in liver carcinogenesis has garnered particular attention in recent years. Zn concentrations in HCC tissues are markedly lower compared to the surrounding non-HCC hepatic parenchyma [15,16]. Zn may influence tumor development by modulating diverse molecular structures, including kinases, caspases, phosphatases, transcription factors, and receptors such as Zip14 [17].

In addition, Zn directly regulates metallothioneins (MTs), which are low molecular weight and cysteine-rich intracellular proteins capable of binding both essential and toxic metals [18]. MTs play a role in a broad array of processes, including metal homeostasis and detoxification, oxidative stress response, and cell proliferation [19]. Recently, the gene expression and methylation status of *MT1* isoforms have been proposed as potential biomarkers in various human cancers, including HCC [20–22].

Zinc Acexamate (ZAC) is a pharmaceutical agent available in several countries, used primarily in the treatment of peptic ulcers and osteoporosis, and it serves as a Zn source, providing related Zn effects.

The primary aim of the present study was to investigate the potential role of Zn as a therapeutic agent in HCC by administering ZAC and examining its molecular effects in different HCC cell lines as well as in a mouse model of HCC. Additional objectives included analyzing the expression levels of MT1 isoforms in human HCC tissues and assessing their potential as HCC biomarkers.

2. Material and methods

2.1. Human samples

Patients were recruited at the Liver Unit, Hospital Universitari Vall d'Hebron. Plasma samples from 23 patients with confirmed cirrhosis and 46 patients with confirmed HCC, either with noninvasive radiological criteria or histological confirmation, were prospectively collected. The study was conducted in accordance with the Declaration of Helsinki. The institutional ethical review board approved the protocol (PR(AG)194/2015), and all patients gave written informed consent before inclusion.

Peripheral venous blood was collected in a lithium heparin tube (BD Biosciences, USA) and processed within 4 h of collection. Plasma was collected after a first centrifugation at 1500 ×g for 15 min at 4 °C and then was further centrifuged at 16,000 ×g for 10 min at 4 °C and was immediately stored at -80 Cº. Liver specimens were collected prospectively and were processed with the help of an expert pathologist for specific tissue sampling and immediately stored at −80 °C.

2.2. Cell culture

HCC cell lines (Hep3B, HepG2, HuH7 and SNU423) were purchased from the American Type Culture Collection (ATCC, Manassas, VA, USA), and were maintained in DMEM (Hep3B, HepG2, HuH7) or RPMI-1640 medium (SNU423) supplemented with 10 % FBS, 100 U/mL penicillin and 100 μg/mL streptomycin. HCC cell lines were cultured at 37°C in a humidified atmosphere containing 5 % carbon dioxide.

The normal adult liver epithelial cell line THLE-3 was purchased from the American Type Culture Collection (ATCC, Manassas, VA, USA). THLE-3 cells were maintained at 37 °C in a humidified atmosphere containing 5 % CO_2 in BEGM media with an extra 5 ng/mL EGF, 70 ng/mL Phosphoethanolamine and 10 % fetal bovine serum. THLE-3 cells were seeded in a precoated plates with a mixture of 0.01 mg/mL fibronectin, 0.03 mg/mL bovine collagen type I and 0.01 mg/mL bovine serum albumin dissolved in BEGM medium.

Before beginning the experiments, all cell lines were authenticated by a STR profile (Applied Biosystems).

2.3. Cell viability assay

Cell lines (Hep 3B, HepG2, HuH7, SNU423 and THLE-3) were seeded in 96-well plates (1000 cells/well) and treated with fresh medium containing from 0 to 300 μM of ZAC. After 24,48 and 72 hours of treatment, the effect of ZAC on cell viability was measured using the MTT assay (Merck KGaA, Darmstadt, Alemania). Absorbance was measured at optical density (OD) of 590/650 nm using a microplate reader (BioTek, Winooski, VT, USA).

2.4. Wound healing assay

Hep 3B, HepG2, HuH7 and SNU423 cells were seeded in 6-well plates at 80 % of confluence. Once the cells reached 90–100 % confluency, a wound area was carefully created by scraping the cell monolayer with a sterile 10 μl pipette tip. Cells were treated with 100, 150 and 250 μM ZAC and incubated at 37°C in 5 % CO_2. The width of the wound area was monitored with an inverted microscope at 24, 48 and 72 h. The fraction of the area free of cells was calculated using ImageJ software.

2.5. Clonogenic assay

Hep 3B, HepG2, HuH7 and SNU423 cells treated with 100, 150 and 250 μM ZAC were seeded in six-well plates at 500 cells per well and incubated at 37°C incubator for 15 days. Cell colonies were fixed with 95 % ethanol and stained with 0.1 % crystal violet. Colonies were counted, and surviving fraction was calculated.

2.6. Transwell invasion and migration assay

For the invasion assay, Extracellular matrix (ECM) (Merck KGaA, Darmstadt, Alemania) was added to the upper compartment of the 8 μM pore insert. Hep 3B, HepG2, HuH7 and SNU423 cells were seeded at density of 2×10^5 cells. Otherwise, in the case of migration assay, each cell line was plated in upper part of transwell assay plates without ECM. 10 % of FBS was added to the medium in the lower chamber, as a chemoattractant. Hep 3B, HepG2, HuH7 and SNU423 were treated with 100, 150 and 250 μM ZAC. After 48 h, cells attached at the bottom of the transwell membrane were fixed with 5 % glutaraldehyde and later stained with 0.5 % crystal violet.

Finally, 10 images of each transwell were captured using a bright-field microscope at 10x and the total number of migrated cells was quantified using ImageJ software to estimate the percentage of stained area.

2.7. Cell cycle assay

Hep 3B, HepG2, HuH7 and SNU423 cells were plated at a density of 1×10^5 cells/well and treated with 0, 100, 150 and 250 μM of ZAC. After 24 and 48 hours, cells were collected and fixed with ice-cold 70 %

ethanol. After fixation, cells were incubated with 1 % Rnase A and DNA content were stained with 1 mg/mL of propidium iodide (PI) (Sigma-Aldrich). DNA content was analyzed with FACSCalibur (BD Biosciences) and quantified with the FloJo Software.

2.8. Apoptosis

Hep 3B, HepG2, HuH7 and SNU423 cells were plated and treated with 0, 100, 150 and 250 μM of ZAC. After 24 and 48 hours, cells were collected. Apoptosis were assessed by the Annexin V-FITC Apoptosis Detection Kit (Thermo Fisher Scientific, Waltham, MA USA). Finally, cells were analyzed by BD LSR Fortesa flow cytometry analyzer (BD Biosciences, Madrid, Spain), measuring the emission at 530 nm and 575 nm.

2.9. Animal model

Animal procedure was conducted in accordance with the European Union Guidelines for Ethical Care of Experimental Animals (EC Directive 86/609/EEC for animal experiments) and approved (Number of Resolution: 11720) by the Animal Care Committee of the Vall d'Hebron Institut de Recerca (VHIR, Barcelona, Spain). NUDE Swiss Crl:NU(Ico)-Foxn1^{nu} female mice were injected subcutaneously with 5 x 10^6 HuH7 cells for tumor development. When tumors reached 100 mm^3 volume, mice were randomly assigned to 4 groups: treated with ZAC (Laboratorios Viñas, Barcelona, Spain) (150 mg/kg) (n = 6), sorafenib (SF) (Merck KGaA, Darmstadt, Alemania) (15 mg/kg) (n = 6), ZAC+sorafenib (n = 5) or vehicle (n = 5). Mice were treated by oral gavage every 24 h. We defined survival as the time comprised between randomization and euthanasia. According to institutional ethical guidelines, mice were euthanized when tumors reached 10 % body weight (~2000 mm3) or mice showed discomfort. One hour after the last dose of treatment, animals were euthanized, and tumors collected and frozen for RNA extraction or fixed for immunohistochemically analysis.

2.10. Gene expression profiling

Total RNA was extracted from tumors of the vehicle mice (n = 5), ZAC treated mice (n = 6) and HuH7 cells treated with 0, 100, 150 and 250 μM of ZAC. Expression profiling was conducted using human Clariom™ S Assay (Thermo Fisher Scientific, Waltham, MA USA). Microarrays data was preprocessed using the RMA method [23,24] and the selection of differentially expressed genes were performed as previously described [25,26]. Gene Set Enrichment Analysis (GSEA) was performed based on Gene Ontology (Biological Process category, GO-BP), using direct gene annotations. Finally, an overrepresentation analysis (ORA) was also performed over GO-BP terms databases with the lists of up-/down-regulated genes found. Microarray data from samples included in this article are available via GEO (Accession number GSE277019)

2.11. Quantitative real-time RT—PCR

Total RNA was converted to cDNA using the High-Capacity cDNA Reverse Transcription Kit (Applied Biosystems, ThermoFisher Waltham, MA USA). qRT-PCR was performed using pre-designed probes (Thermo Fisher Scientific, Waltham, MA USA): *MT1E* (Hs01938284_g1), *MT1F* (Hs00744661_sH), *MT1G* (Hs04401199_s1), *MT1H* (Hs00823168_g1), *MT1M* (Hs00828387_g1) and *MT1X* (Hs00745167_sH). Relative mRNA levels of target genes were tested in triplicate and normalized with respect to that of *18S* (Hs99999901_s1), using the comparative (2-ΔCT) method.

2.12. Immunohistochemistry

Antibodies against MT1G (Epigentek, Farmingdale, NY, USA) and MT1H (Biorbyt, Wuhan, China) were used for immunohistochemistry staining according to the manufacturer's protocol. An liver pathologist defined the overall immunoreactive score of each sample as the product of the staining intensity and positive rate (0–300 %). The staining intensity was divided into four stages (no staining=0, weak staining=1, moderate staining=2, strong staining=3).

2.13. Quantification of Zn and Metallothionein 1 H in human plasma

Zn levels were quantified in plasma human samples by a colorimetric Zn determination test (Merck KGaA, Darmstadt, Alemania).

Metallothionein 1 H plasma levesls were measured with an ELISA (Enzyme-Linked ImmunoSorbent Assay) kit with higher sensitivity (MyBiosource,San Diego, CA, USA).

Each sample were tested in duplicate and data were acquired with a Varioskan Lux Reader (Thermo Fisher Scientific, Waltham, MA USA).

2.14. Statistical analysis

All the results from cell culture model were obtained from, at least, three independent experiments with three technical replicates in each one.

To compare means of two groups, umpaired t test was performed. Tests with p values below 0.05 were considered statistically significant.

To compare means of different groups, One-Way or Two-way ANOVA test was performed, followed by post-hoc Tukey's multiple comparisons test. Tests with p values below 0.05 were considered statistically significant.

Data analysis was performed with GraphPad 10.2.0 Software, LLC.

All authors had access to the study data and had reviewed and approved the final manuscript.

3. Results

3.1. Effects of ZAC on viability, migration, invasiveness and clonality in human HCC cell lines

Antiproliferative activity of serial concentrations of ZAC (25–300 μM) was examined in four HCC cell lines: Hep3B, HepG2, SNU423 and HuH7 and in a normal liver epithelial cell line, THLE3, using the MTT assay. We observed that ZAC treatment dose-dependently inhibited survival of HCC cells. All of HCC line cells showed significant sensitivity on ZAC treatment at 100 μM and higher ranging from 13 % to 75 %, 12–41 %, 31–76 % and 11–49 % in Hep3B, HepG2, SNU423 and HuH7 cell lines respectively **(**Fig. 1**A)** whereas the normal liver epithelial cell line THL3 only presented a significant decrease of 34 % after a 300 μM of ZAC exposure **(**Fig. 1**A)**. The IC_{50} values for Hep3B, HepG2, SNU423 and HuH7 after 48 h of exposure to ZAC were 179.1, 116.8, 116.3 and 364.7 μM while the IC_{50} for the normal epithelial liver cell line THLE3 was 973.1 μM. Additional data on viability and IC_{50} at 24 h and 72 h is available in Supplementary Table 1.

We next evaluated the effect of ZAC on HCC cell line migration. Migration through transwells decreased a range of 32–77 %, 20–59 %, 29–59 % and 39–71 % in Hep3B, HepG2, SNU423 and HuH7 cell lines respectively after 48 h of 100, 150 and 250 μM exposure of ZAC **(**Fig. 1**B and C)**. Wound healing assay showed that ZAC effectively inhibited migration of HCC cells in a dose-dependent manner when compared with their respective control groups at 24, 48 and 72 h **(**Fig. 2**A and B)**.

We also determined the effect of ZAC on invasion capacity, decreasing from 24 % to 70 %, 34–62 %, 22–80 % and 40–92 % in Hep3B, HepG2, SNU423 and HuH7 cell lines respectively after 48hof exposure at100, 150 and 250 μM of ZAC **(**Fig. 1**D and E)**. We next performed colony formation assays to investigate the effect of ZAC on

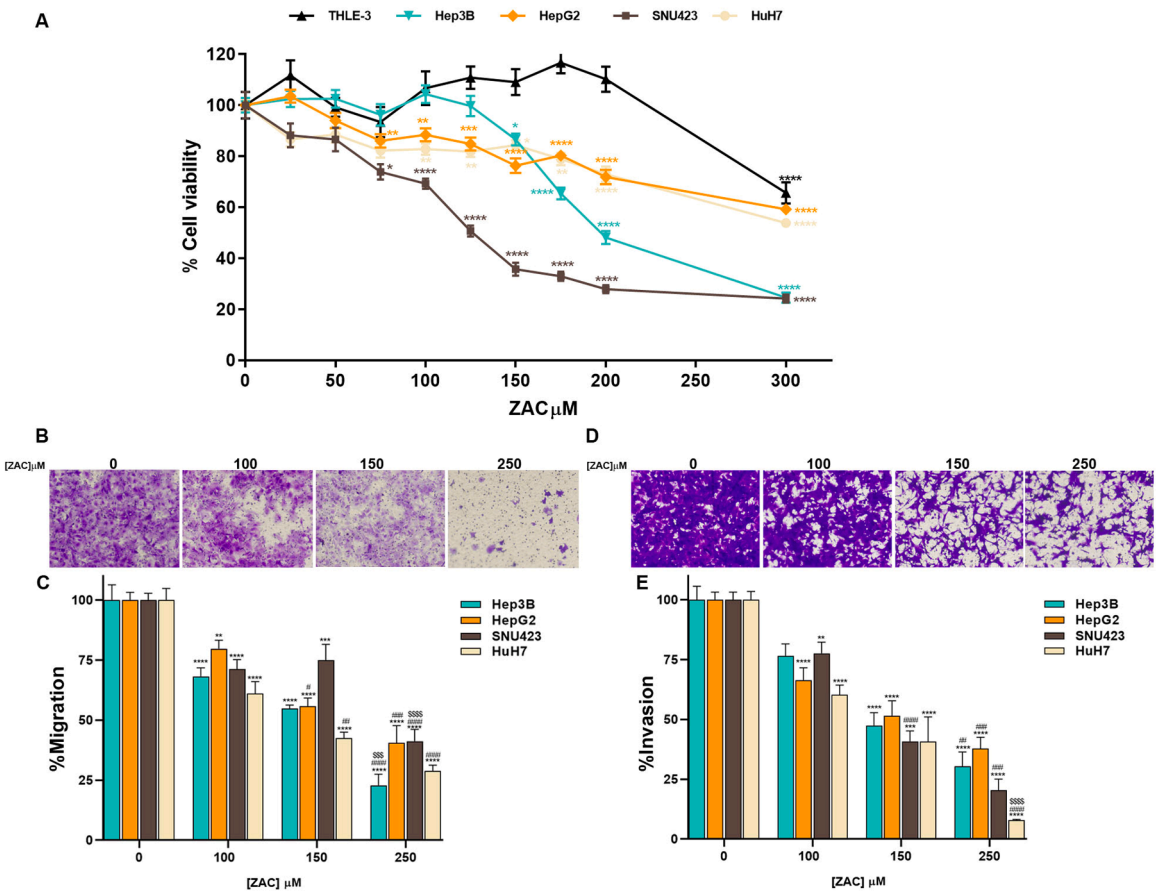

Fig. 1. ZAC addition reduces the viability, migration and invasiveness of HCC cells in vitro. **A:** Cell viability after 48 h of ZAC exposure in a normal liver cell line (THLE-3) and four HCC cell lines (Hep3B, HepG2, SNU423 and HuH7) * $p < 0.05$, * ** $p < 0.01$, * ** * $p < 0.001$, * ** * * $p < 0.0001$ indicates differences versus the control group. **B:** Representative images of crystal violet staining showing migration capabilities in Hep3B, HepG2, SNU423 and HuH7 after 48 h of treatment with 0, 100, 150 and 250 μM of ZAC. Magnification 4x **C:** Percentage of migrated Hep3B, HepG2, SNU423 and HuH7 cell lines after 48 h treated with 100, 150 or 250 μM of ZAC and without treatment. Bar graphs represent mean±SEM. * * $p < 0.01$, * ** $p < 0.001$, * ** * $p < 0.0001$ indicates differences versus the control group. # $p < 0.05$, ## $p < 0.01$, ### $p < 0.001$, #### $p < 0.0001$ indicates differences versus the 100 μM group. $$$ $p < 0.001$, $$$$ $p < 0.0001$ indicates differences versus the 150 μM group. **D:** Representative images of crystal violet staining showing invasion ability in Hep3B, HepG2, SNU423 and HuH7 cell lines after 48 h of treatment with 0, 100, 150 and 250 μM of ZAC. Magnification 4x. **E:** Percent invasive cells from Hep3B, HepG2, SNU423 and HuH7 cell lines after 48 h treated with 0, 100, 150 or 250 μM of ZAC. Bar graphs represent mean±SEM. * * $p < 0.01$, * ** $p < 0.001$, * ** * $p < 0.0001$ indicates differences versus the control group. ## $p < 0.01$, ### $p < 0.001$, #### $p < 0.0001$ indicates differences versus the 100 μM group and $$$$ $p < 0.0001$ indicates differences versus the 150 μM group. ZAC: Zinc Acexamate.

clonogenic potential of HCC cells. Hep3B, HepG2, SNU423 and HuH7 cells were treated with ZAC during 24, 48 and 72 h and the number of colonies was evaluated after 2 weeks. Our results showed that ZAC did not affect HCC cell lines similarly, showing Hep3B and SNU423 a greater sensitivity, reducing significantly the number of colonies formed after 48 h of exposure to 100 μM and higher concentrations of ZAC compared with their controls (Fig. 2 **C and D**).

3.2. *Effects of ZAC on cell cycle and apoptosis*

To investigate the effect of ZAC on the growth of HCC cells, cell cycle analysis was performed by flow cytometry. Hep3B, HepG2, SNU423, and HuH7 cells were treated with 100, 150, and 250 μM for 48 h and analyzed for DNA content. The results showed that following 48 h of exposure, ZAC did not significantly affect the cell cycle compared to the vehicle-treated group (Supplementary Figure 1).

Considering the antiproliferative activity of ZAC, we evaluated its possible apoptotic effects. ZAC treatment of Hep3B, HepG2, SNU423 and HuH7 was performed for 48 h. Exposure to 250 μM ZAC resulted in a significant increase in early apoptosis in all HCC cell lines compared to control cells (Mean±Standard deviation (SD)) (11.80 % ± 1 vs. 19.57 % ± 3.25, 3.46 % ± 1.35 vs. 34.90 % ± 2, 3.28 % ± 0.43 vs. 19.52 % ± 3.06 and 9.62 % ± 4.76 vs. 40.37 % ± 10.76 % in Hep3B, HepG2, SNU423 and HuH7 respectively, control vs 250 μM ZAC) (Fig. 3).

3.3. *Effects of ZAC in a cancer cell-derived xenograft tumors in immunodeficient mice*

We further examined the *in vivo* effect of ZAC using HuH7 xenograft model in nude mice. No significant differences in body weight were observed between ZAC-treated and control (vehicle) animals (Supplementary Figure 2**A**), suggesting that ZAC causes low host toxicity at the selected therapeutic dose. After 20 days, the average tumor volume of HuH7 xenografts treated with vehicle, ZAC, SF or ZAC+SF was 2080 ± 526, 1718 ± 437, 1070 ± 617 and 1553 ± 550 mm^3 respectively (Fig. 4**A**). ZAC treated mice exhibited a median survival 13.8 [9–19] days vs 9 days [3–18] for control mice. However this difference was not statistically significant ($p = 0.38$) (Fig. 4**B**). The median survival of SF treated mice was 22.8 [19–28] days ($p = 0.0017$ *vs* control and $p = 0.005$ *vs ZAC*) and SF+ZAC treated mice presented a median survival of 17 [10–26] days (Fig. 4**B**).

Although ZAC did not have an effect on tumor volume in the model, we investigated the intratumor molecular effects and gene expression

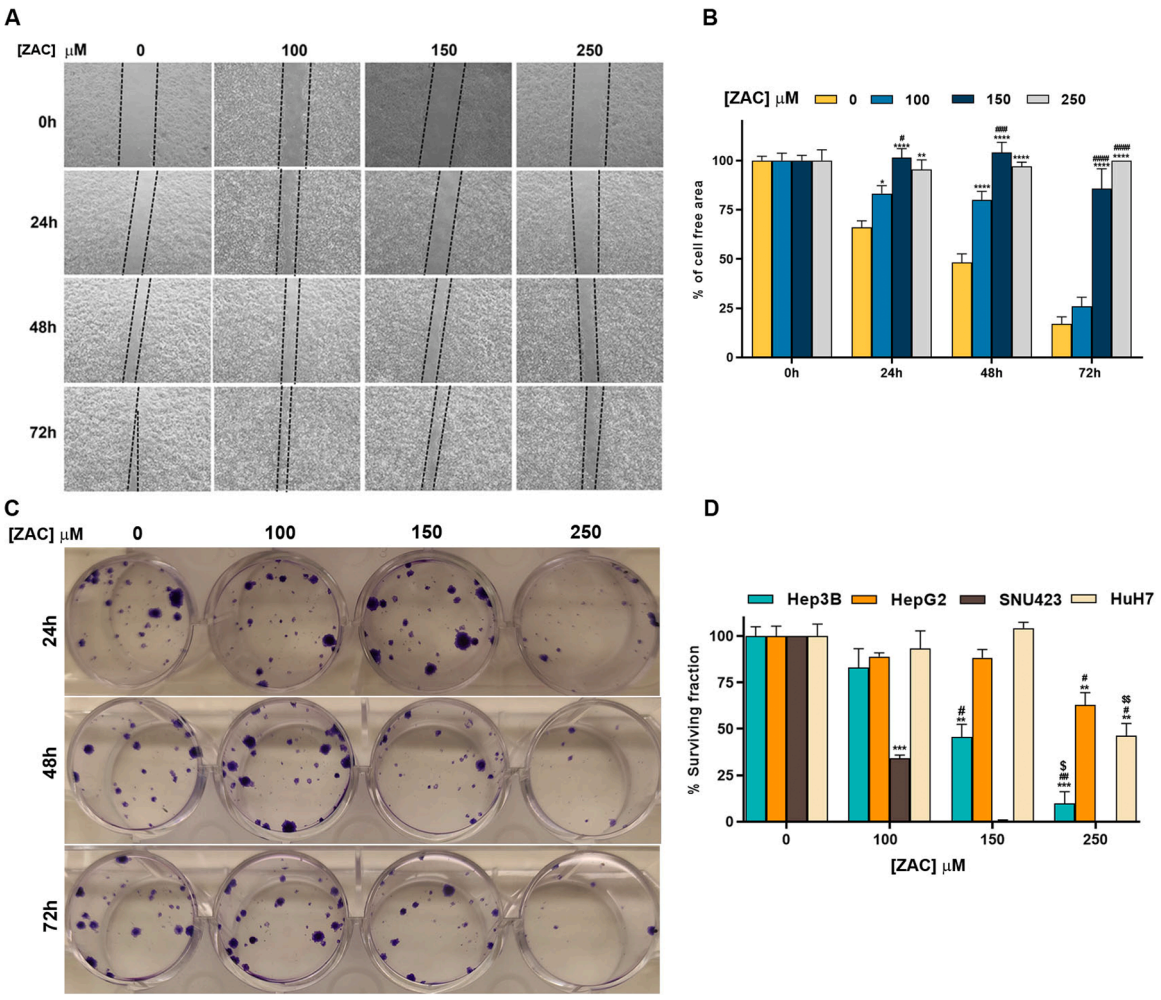

Fig. 2. ZAC affects migration and clonogenic capabilities in HCC cells. **A:** Representative images of wound-healing assay after 0, 24, 48 and 72 h of treatment with 0, 100, 150 and 250 μM of ZAC. Magnification 4x. **B:** Percentage of cell free area in Hep3B, HepG2, SNU423 and HuH7 cell lines after 0, 24, 48 and 72 h of treatment with 100, 150 and 250 μM of ZAC and without treatment. Bar graphs represent mean±SEM. * $p < 0.05$, * * $p < 0.01$, * ** * $p < 0.0001$ indicates differences versus the control group. # $p < 0.05$, #### $p < 0.0001$ indicates differences versus the 100 μM group. **C:** Representative images of cell colony formation after 24, 48 and 72 h of treatment with 0, 100, 150 and 250 μM of ZAC. **D:** Percentage of cell surviving fraction in Hep3B, HepG2, SNU423 and HuH7 cell lines after 2, 48 and 72 h of treatment with 100, 150 and 250 μM of ZAC and without treatment. Bar graphs represent mean±SEM. * * $p < 0.01$, * ** $p < 0.001$ indicates differences versus the control group. # $p < 0.05$, ## $p < 0.01$ indicates differences versus the 100 μM group and $ $p < 0.05$, $$ $p < 0.01$ indicates differences versus the 150 μM group. ZAC: Zinc Acexamate.

alterations caused by ZAC on cancer cells using microarrays. Tumoral tissue from mice treated with ZAC presented a change in the expression of 97 genes compared with tumoral tissue from vehicle treated mice, with 20 up-regulated and 77 down-regulated genes **(**Supplementary Table 2**).** Unsupervised hierarchical clustering of tumoral vehicle and ZAC samples based on the expression levels of the 97 differential expressed genes revealed a different expression pattern **(**Fig. 4**C).** Fig. 4D shows the network diagram for the five most enriched terms after the enrichment analysis performed (Gene Ontology (GO)). The comparison between tumors from control mice and ZAC-treated mice showed that the most affected downstream pathways due to the activation of MTs are the pathways involved in cellular stress responses, as well as the pathways associated with the detoxification of heavy metals and inorganic compounds. In addition to these effects, DNA synthesis pathways were also affected, with pathways involved in lipid and cholesterol metabolism being significantly suppressed **(**Supplementary Figure 2B **and C).** From the most significantly enriched GO-BP terms, the identified genes belonging to the *MT1* family (*MT1M*, *MT1E*, *MT1X*, *MT1H*, *MT1F*, and *MT1G*) are highly involved in cellular response to Zn ion and stress response to metal ion **(**Fig. 4**D).** Additionally, the expression from the *MT1* gene family was also significantly up-regulated in tumor tissue from ZAC-treated mice **(**Fig. 4**C,** Supplementary Table 2**).** HuH7 cells treated with ZAC showed a similar gene expression pattern **(**Supplementary Table 3**).**

3.4. *MT1 family gene expression in HCC*

Over-expressed genes included in the *MT1* gene family (*MT1M*, *MT1E*, *MT1X*, *MT1H*, *MT1F* and *MT1G*) were also examined by Q-PCR. Significant overexpression of *MT1G*, *MT1H* and *MT1M* genes were found in ZAC-treated mice ($p = 0.0137$, $p = 0.0364$ and $p = 0.0399$ respectively), which was consistent with the microarrays data **(**Supplementary Figure 3 **A-F).**

HuH7 cells exposed to ZAC presented significant overexpression in all genes studied from MT1 gene family, in a time- and dose-dependently **(**Supplementary Figure 3 **G-L).**

Finally, MT1G and MT1H expression were analyzed by q-PCR in a cohort of human tissue samples from early HCC patients (eHCC). Clinical and demographic parameters of these patients are summarized in Table 1. The median age of HCC patients was 62 years, 87 % were male, and all of them presented liver cirrhosis. Underlying etiology was viral in 56.6 %, alcohol-related in 17.4 % and 13 % presented metabolic

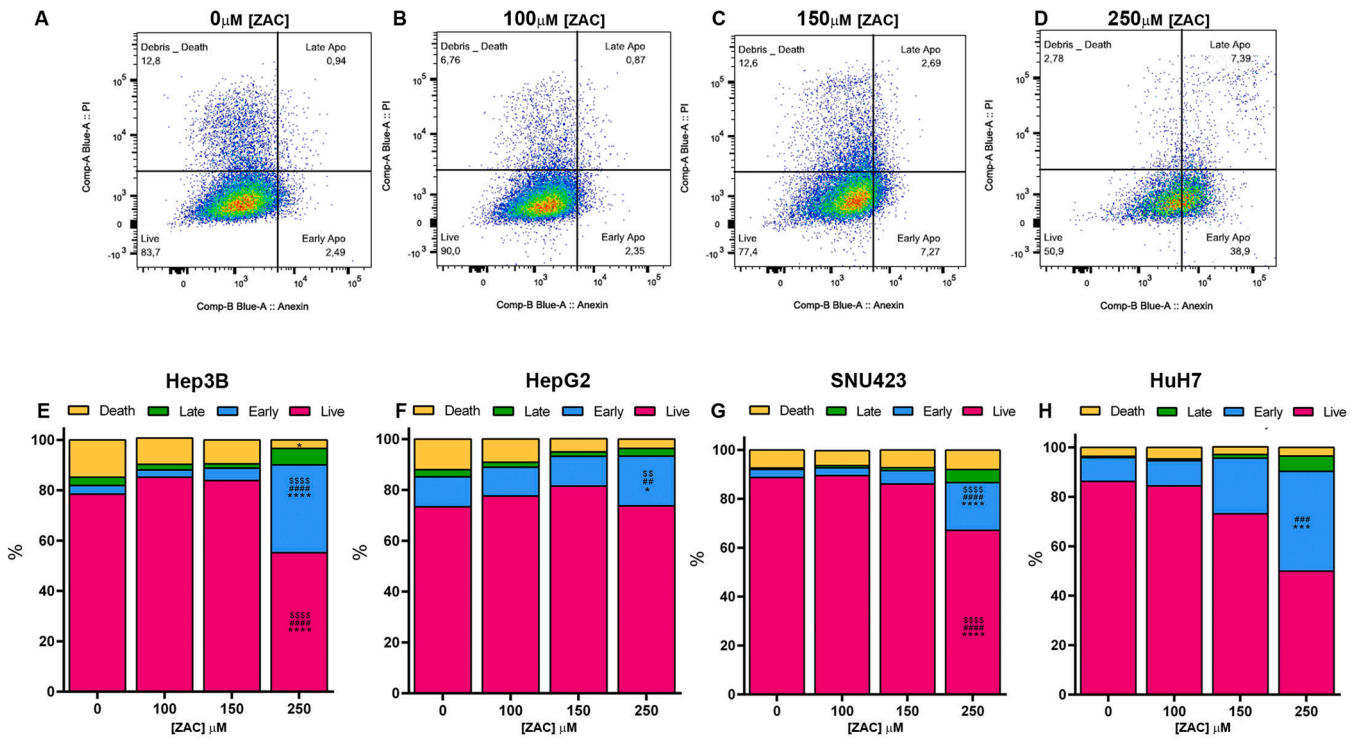

Fig. 3. ZAC addition induces apoptosis in HCC cells in vitro. A, B, C and D: Representative Scatter Plot showing cells in early/late apoptosis or death/alive after treatment with 0, 100, 150 or 250 μM of ZAC. **E, F, G and H:** Hep3B, HepG2, SNU423 and HuH7 cells respectively, treated with 0, 100, 150 and 250 μM of ZAC. * $p < 0.05$, * ** $p < 0.001$, * ** * $p < 0.0001$ indicates differences versus the control group. ## $p < 0.01$, ### $p < 0.001$, #### $p < 0.0001$ indicates differences versus the 100 μM group. $$ $p < 0.01$, $$$$ $p < 0.0001$ indicates differences versus the 150 μM group. ZAC: Zinc Acexamate.

dysfunction-associated steatotic liver disease. The average diameter of the largest tumor was 3.8 cm (range, 0.3–6.5 cm), while microvascular invasion (mVI) was present in 39 % of patients.

Regarding the expression of *MT1* gene family, fold-changes in *MT1G* expression was significantly suppressed in HCC tissue (-4.04 ± 2.8 [-9.70–4.40]) compared with adjacent tissue (0 ± 1.27 [-2.73–2.60]) ($p < 0.0001$) **(**Fig. 5**A)**. Similarly, *MT1H* expression (fold change) in HCC tissue was also significantly lower (-4.78 ± 3.03 [-10.36–5.28] than in adjacent tissue (0 ± 2.23 [-4.47–3.54]) (Mean±SD [Range]) ($p < 0.0001$) **(**Fig. 5**B)**.

3.5. MT1 family protein expression in HCC

We further detected the expression of MT1G and MT1H protein using immunohistochemistry on eHCC tissue. Our results showed that expression levels of MT1G and MT1H protein were significantly lower in tumor samples than in their paired non tumoral adjacent tissues. MT1G presented 109.5 %± 52.26 of staining in HCC tissue with 200 % of expression found in adjacent non tumoral tissue (mean±SD) ($p = 0.0001$) **(**Fig. 5**C)**. MT1H staining values were 121 % ± 44.86 in HCC tissue *vs* 200 % of staining present in adjacent tissue (mean ± SD) ($p = 0.0001$) **(**Fig. 5**D)**.

3.6. Zn and MT1 plasma levels in HCC

Zn content was determined by colorimetric test in plasma samples from control, liver cirrhosis, eHCC and aHCC patients. Clinical and demographic parameters of these patients are summarized in Table 1**.**

Plasma Zn levels were significantly higher in patients without liver disease (control) (0.81 ± 0.19) compared with patients with liver cirrhosis (0.62 ± 0.05 ng/μL) ($p = 0.02$) or patients with eHCC and aHCC (0.48 ± 0.17and 0.27 ± 0.07 ng/μL, respectively) ($p < 0.0001$) **(**Fig. 5**E)**. Plasma levels of Zn between patients with liver cirrhosis were also significantly higher from patients with eHCC or aHCC ($p = 0.02$ and $p < 0.0001$, respectively). Furthermore, patients with eHCC exhibited higher levels of Zn compared to those with aHCC ($p = 0.016$).

Plasma levels of MT1H and MT1G were also evaluated and plasma from patients with liver cirrhosis presented significant higher levels of circulating MT1H than in patients with aHCC (125 ± 14.70 *vs* 90.63 ± 5.01 pg/Ml, respectively) ($p = 0.03$). MT1H plasma levels between eHCC and aHCC did not show significant differences ($p = 0.9$). Plasmatic levels of MT1G were undetectable with the enzymatic assay (data not shown). **(Figure F)**.

4. Discussion

Previous literature indicates that Zn levels in HCC tissue are lower compared to surrounding hepatic parenchyma [27,28], in addition, there is marked hypozincemia observed in patients with chronic liver diseases [29,30]. However, the role and mechanisms of Zn in liver carcinogenesis has not been described.

Recent scientific evidence highlights Zn's involvement in the development of various cancers [31,32]. Zn dysregulation is recognized as a significant factor in cancer initiation and progression, often linked to Zn deficiency. This condition can be a result from factors such as downregulation of Zn transporter genes and mutations in Zn importer and transporter genes, disrupting cellular zinc homeostasis [33,34].

In this study, we investigated the role of ZAC in liver carcinogenesis and HCC development. Our objective was to assess ZAC's capacity to influence the carcinogenic abilities in HCC preclinical models. In addition, we treated four HCC cell lines and a healthy liver epithelial cell line with ZAC, observing an inhibition in cell proliferation, migration and invasiveness. ZAC induced a significant dose-response effect in HCC cell lines, with a reduction in viability of 13–49 % when treated with 100 μM of Zn whereas normal liver epithelial cells only exhibited significant decrease in viability at higher doses. ZAC treatment also inhibited migration (22–77 %) and invasiveness (22–92 %) in a dose-dependent manner. Furthermore, ZAC impacted colony formation in HCC cell

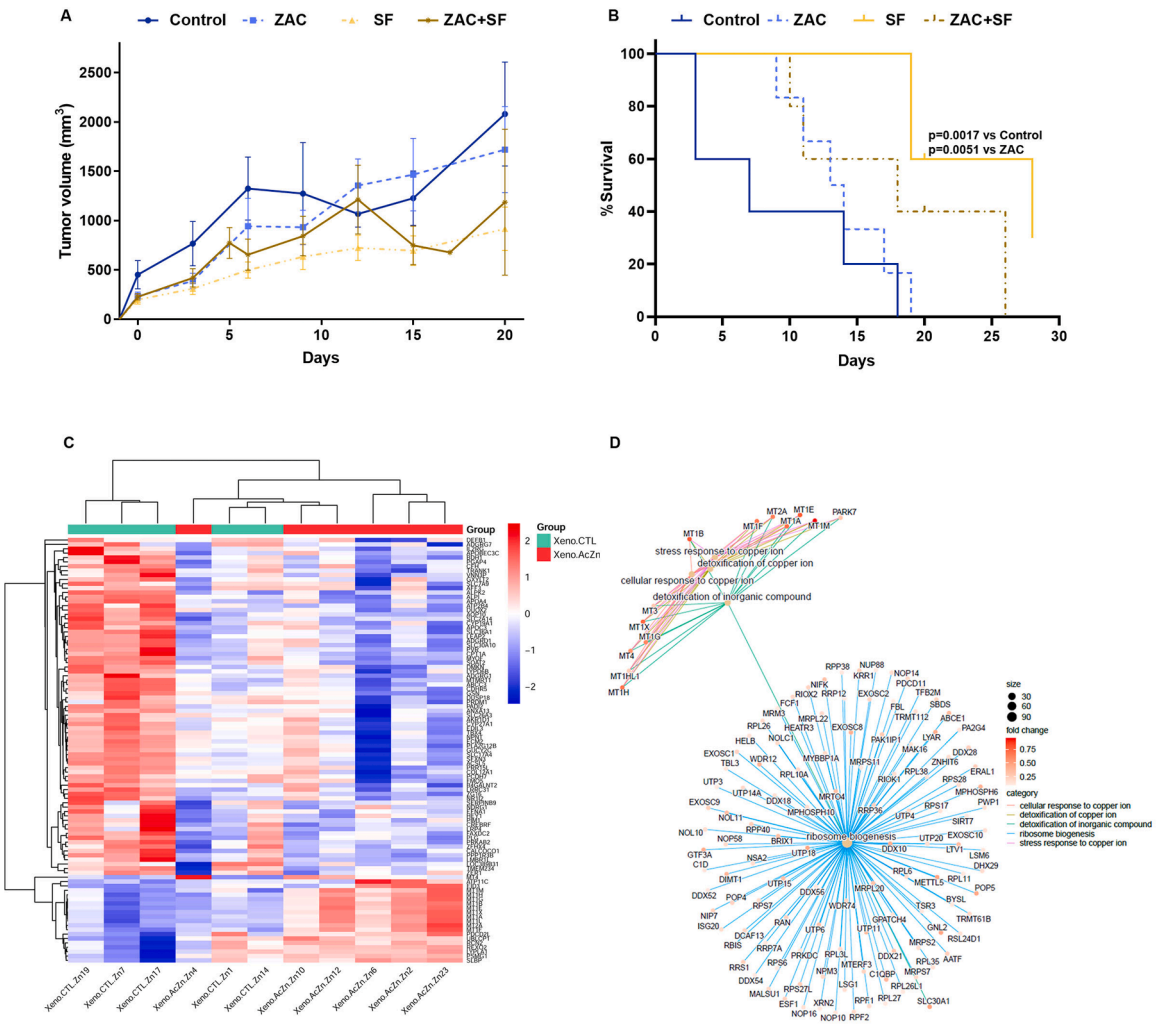

Fig. 4. Effect of ZAC in a xenograft model of HCC. A: Tumor volume (mm^3) in xenografts treated with ZAC, sorafenib and combination of both compared to vehicle. **B:** Kaplan-Meier curves in ZAC, sorafenib and combination treated versus vehicle mice. **C:** Heat map showing 97 differentially expressed genes between ZAC and vehicle treated tumors ($p < 0.05$, abs(logFC)> 0.5). The highest upregulated (red) and downregulated (blue) genes in ZAC tumours are listed. **D:** Network plot of the top 5 enriched GO terms of BP for comparison between ZAC and vehicle mice. ZAC: Zinc Acexamate, GO: Gene ontology, BP: Biological Processes.

lines. Similarly, Polaprezinc, a Zn complex used for treating gastric ulcers, has shown antifibrotic and antiproliferative properties in HCC cell lines [35–37].

Although Zn's role in apoptosis regulation has been documented [38], the associated mechanisms are diverse, complex, and cell-specific [39]. We also explored ZAC's role in apoptosis *in vitro*, finding that high doses of ZAC after 48 hours increased early apoptosis in all HCC cell lines compared to untreated cells.

In the animal HCC xenograft model, there was no significant reduction in tumor growth following Zn treatment. The short-term nature of the model, requiring euthanasia when tumor volume reaches 1700–2000 mm^3 , could limit the observation of significant differences. The restricted treatment duration may have impeded observable survival benefits from Zn supplementation. Furthermore, administering a single trace element, like Zn, is unlikely to substantially suppress tumor growth alone, given the complex processes governing tumor growth and progression. ZAC may have greater efficacy when combined with other therapeutic agents targeting these pathways synergistically. Future studies could explore various dosage regimens with extended treatment timelines to clearly elucidate the therapeutic effects of ZAC. Additionally, investigating Zn supplementation in combination with other anticancer therapies could reveal potential synergistic effects and refine experimental models to overcome current limitations.

Despite this lack of impact on tumor growth in the model, we analyzed ZACs effects on HCC tumors at the molecular level. Gene expression analysis showed positive regulation of MTs following ZAC treatment in both mouse tumors and HCC cell lines. MTs regulate cellular homeostasis of zinc and copper, crucial for cell proliferation and differentiation, and function as antioxidants protecting cells from free radicals and oxidative stress induced by mutagens, anticancer drugs, and radiation [40,41]. MTs also bind to heavy metals, shielding cells from toxicity [42]. Fig. 4D provides a network diagram comparing control mice tumors with those treated with ZAC, highlighting primary affected downstream pathways, including cellular stress responses and heavy metal detoxification. Pathways related to DNA synthesis and lipid and cholesterol metabolism, essential for cell replication, are impacted. Our results show that ZAC-treated HuH7 cells lead to an overexpression in the *MT1* gene family, affecting viability and apoptosis in a dose-dependent manner. The *MT*1 family has aberrant expression patterns described in several cancers, including HCC [21,43].

The silencing mechanism of the *MT1G* gene is primarily due to promoter hypermethylation [44–46]. Other mechanisms potentially contributing to MT-associated carcinogenesis include allelic loss on chromosome 16q12.1-q23.1 [47].

MT1G's role in tumor cell differentiation has been illustrated in colon cancer cell lines, primarily through the Notch signaling pathway

Table 1
Clinicopathological characteristics of patients with liver cirrhosis (n = 23), HCC (n = 66) and control patients (16).

Demographics		Cases
Clinicopathological characteristics		
Gender, n (%)	Male	
Cirrhosis		12 (52 %)
eHCC		44 (84 %)
aHCC		17 (74 %)
Age, median (range)		
Cirrhosis		64 [42–71]
eHCC		65 [33–82]
aHCC		63 [56–88]
Race/Ethnicity, n (%)	Caucasian	
Cirrhosis		20 (87 %)
eHCC		21 (95 %)
aHCC		20 (87 %)
Etiology, n (%)		
Cirrhosis	HCV	0 (0 %)
	ALD	12 (52 %)
	MASLD	7 (30 %)
eHCC	HCV	14 (32 %)
	ALD	6 (14 %)
	VHC+OH	6 (14 %)
	MASLD	6 (14 %)
	Others	12 (26 %)
aHCC	HCV	11 (48 %)
	ALD	2 (9 %)
	MASLD	3 (13 %)
	Others	7 (30 %)
Fibrosis (n (%)		
Cirrhosis	F4	18 (78 %)
	F3	2 (9 %)
eHCC	F4	24 (48 %)
	F3	13 (30 %)
	F2	5 (11 %)
aHCC	F4	12 (52 %)
	F3	1 (4 %)
Tumor size (cm), median (range)		
eHCC		3.2 [0.3–11]
aHCC		n.m
Single tumor (%)		
eHCC	Yes	40 (91 %)
aHCC	Yes	3 (13 %)
Events (%)		
eHCC	Recurrence	12 (27 %)
	Death	10 (23 %)
aHCC	Death	9 (39 %)
Follow-up, median months (range)		
Cirrhosis		6 [1–14]
eHCC		29 [1–87]
aHCC		15 [3-32]
Laboratory values		
		median (range)
Cirrhosis	AFP (mg/dL),	3.8 [1.30–11.1]
	Bilirubin (mg/dL),	0.9 [0.29–3.43]
	Albumin (g/L),)	4.3 [3.50–4.90]
	Platelet count	120 [47−299]
eHCC	AFP (mg/dL),	4 [1.1–838]
	Bilirubin (mg/dL),	0.65 [0.27–1.63]
	Albumin (g/L),)	4.3 [2.70–5.1]
	Platelet count	191 [91−364]
aHCC	AFP (mg/dL),	17.9 [1.8–41180]
	Bilirubin (mg/dL),	0.82 [0.32–3.04]
	Albumin (g/L),)	4.1 [4.6–3.1]
	Platelet count	195 [464−67]
Controls		**Cases**
Gender, n (%)	Male	43.75 %
Age, median [range]		42 [31–66]

eHCC: early Hepatocellular Carcinoma, aHCC: advanced Hepatocellular Carcinoma, AFP: Alpha-Fetoprotein

and Zn chelation and redistribution [48].

A similar mechanism may exist in HCC, where MT1G modulates cellular differentiation and impacts tumor development. MT1G is also a key regulator of sorafenib resistance in HCC, with sorafenib elevating *MT1G* mRNA and protein expression via *NRF2* activation. Inhibition of *MT1G* enhances sorafenib's anticancer efficacy *in vitro* and in tumor xenograft models [49]. *MT1G* isoform reactivation by sorafenib likely inhibits DNA methyltransferase activity, restoring the *MT1* promoter region [50,51]. Previous studies have shown *MT1* promoter hypermethylation and gene expression silencing in HCC tumors.

Fu et al. elucidated MT1G's molecular mechanisms, including inhibiting cell growth and invasion, and inducing cell cycle arrest and apoptosis via downregulation of AKT and RB phosphorylation, implicating PI3K/AKT and RB/E2F pathways [52].

MT proteins' prognostic significance has been suggested, with lower MT1 and MT2 expression associated with tumor differentiation grade, vascular invasion, recurrence, and poor long-term survival in HCC patients [53–55], however, our cohort's limited sample size likely prevented any validation.

MT1G and MT1H expression in HCC and adjacent tissues showed notable decreases in HCC tissue compared to non-tumoral tissue. MTs, involved in Zn homeostasis, are associated with HCC prognosis. Udali et al. observed *MT1G* and *MT1H* expression reduction in HCC tissue, attributed to decreased Zn content and promoter hypermethylation, resulting in transcriptional repression [20]. MT1 deletion in 44.3 % of an Asian cohort was associated with poor HCC prognosis, likely due to its influence on other genes regulating proliferation and metabolism, proposed as an independent prognostic factor [22].

MT1H has been described as a tumor suppressor by engaging with *EHMT1*, enhancing its methyltransferase activity on histone 3, suggesting a role in epigenetic gene expression regulation [56]. Zheng et al. found that *MT1H* inhibits tumor progression by suppressing the Wnt/β-catenin pathway, reducing cell proliferation, invasion, and migration [57].

Recent integrative analyses identified MT1H as a candidate prognostic biomarker in HCC [21,50]. Tamai et al. reported significant serum MT level increases in HCC patients treated with lenvatinib, suggesting MTs as treatment response biomarkers [17]. Our findings showed elevated circulating MT1H plasma levels in subjects without liver disease compared to those with cirrhosis or HCC. We hypothesize that reactivating *MT1* G and H isoforms with exogenous Zn could improve therapeutic interventions in HCC patients.

Zn addition downregulated *MT1* gene family expression in HCC cells and xenograft tumor samples from mice treated with ZAC. The diminished *MT1G* and *MT1H* expression in human HCC suggests MT1's role in modifying HCC's evolutionary course. Our human sample cohort demonstrated hypozincemia prevalence in HCC patients compared to those with cirrhosis, consistent with Zn deficiency in chronic liver disease patients [29,30,58]. Hosui et al. showed Zn administration improved liver function and reduced adverse events and HCC development incidence in chronic liver disease patients during long-term follow-up [11,12].

Oral Zn supplementation has been reported to directly inhibit progression by reducing serum type IV collagen levels, decreasing HCC development risk [59]. The precise mechanisms by which Zn suppresses cancer development remain unclear; one hypothesis is declining Zn levels in fibrosis leading to superoxide dismutase inactivation, while Zn administration restores superoxide dismutase activity, reducing reactive oxygen species (ROS) and potentially preventing carcinogenesis-driving epigenetic alterations [11].

The understanding of the cellular metabolism and signaling of Zn is evolving, requiring future investigations. In HCC treatment, Zn shows potential as a therapeutic co-agent but may have limited efficacy in isolation. The complex nature of cancer pathogenesis suggests that Zn is most effective when combined with other therapeutic strategies. Future research should elucidate the molecular mechanisms by which Zn and

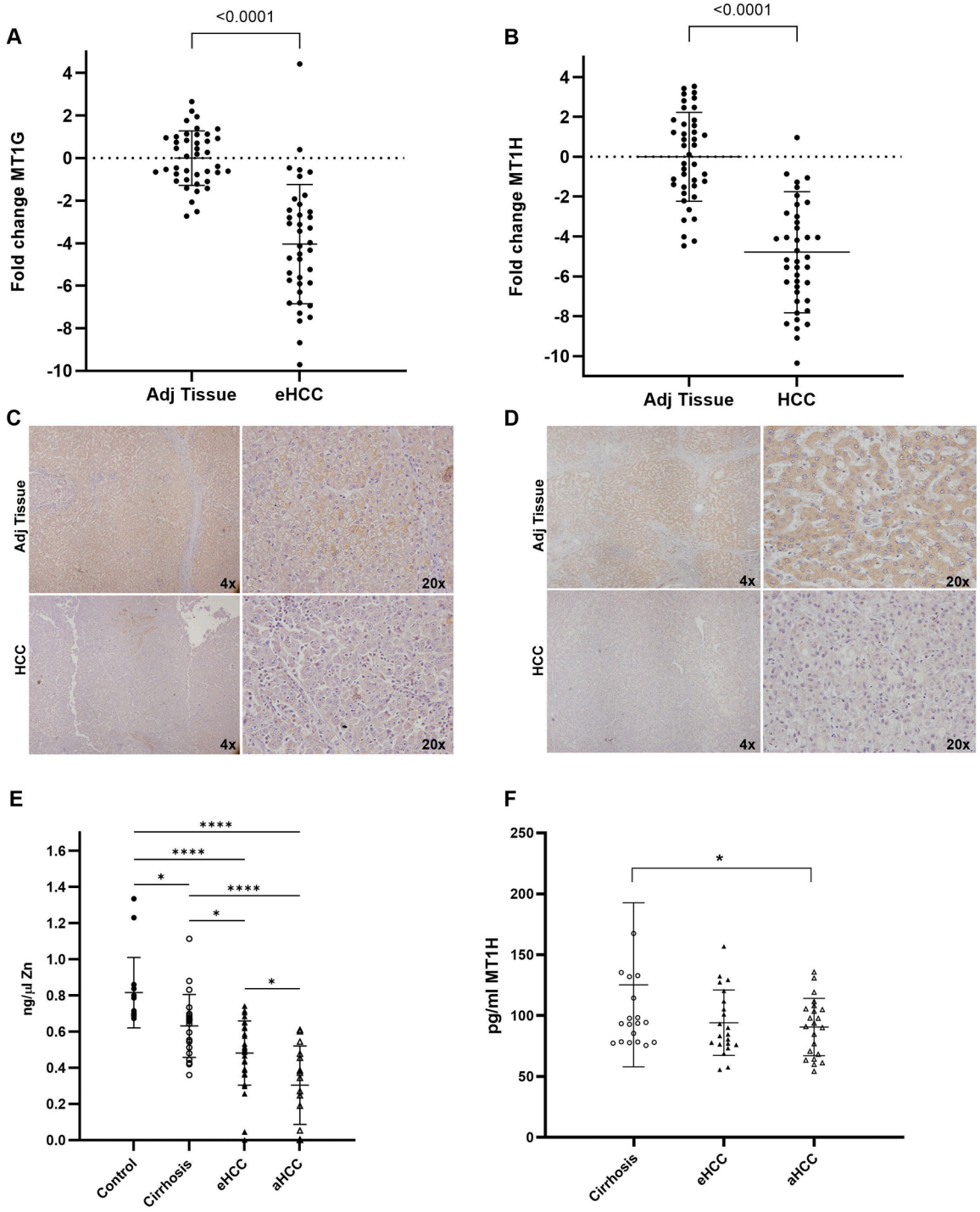

Fig. 5. MT1 family expression. A and B: MT1G and MT1H gene expression in human HCC and adjacent liver tissue. **C and D:** Representative immunohistochemical staining of MT1G and MT1H proteins in HCC tumors and paired adjacent tissue (Magnification 4x and 20x). **E and F:** Zn and MT1H plasma levels in patients with cirrhosis, eHCC and aHCC. eHCC: early HCC, aHCC: advanced HCC. * $p < 0.05$, * ** *$p < 0.0001$.

MT family genes influence HCC, potentially enhancing Zn's therapeutic benefit. Studies should consider optimal dosage, treatment duration, and combination with other therapies to realize Zn's full potential in HCC management.

Our results suggest ZAC as a promising co-treatment candidate for clinical trial evaluations. Its potential synergistic benefits with onco-specific therapies warrant comprehensive investigation in HCC patients.

CRediT authorship contribution statement

Higuera Mónica: Writing – review & editing, Writing – original draft, Validation, Supervision, Methodology, Investigation, Formal analysis, Data curation, Conceptualization. **Elena Vargas-Accarino:** Writing – review & editing, Investigation. **Torrens Maria:** Writing – review & editing, Investigation. **Bermúdez-Ramos María:** Writing – review & editing, Investigation. **Soriano-Varela Agnes:** Writing – review & editing, Investigation. **Salcedo María Teresa:** Writing – review & editing, Investigation. **Minguez Beatriz:** Writing – review & editing,

Writing – original draft, Validation, Supervision, Resources, Project administration, Methodology, Investigation, Funding acquisition, Formal analysis, Data curation, Conceptualization.

Declaration of Competing Interest

The authors declare the following financial interests/personal relationships which may be considered as potential competing interests: Beatriz Mínguez reports financial support and equipment, drugs, or supplies were provided by Laboratorios Viñas S.L. Beatriz Mínguez reports competitive financial support provided by Instituto de Salud Carlos III (ISCIII). Beatriz Mínguez reports a relationship with Bayer Shering Pharma that includes: consulting or advisory. Beatriz Mínguez reports a relationship with Eisai-Merck that includes: consulting or advisory. Beatriz Mínguez reports a relationship with AstraZeneca Pharmaceuticals LP that includes: consulting or advisory. Beatriz Mínguez reports a relationship with Roche Diagnostics GmbH that includes: consulting or advisory. The funders had no role in the design of the study; in the collection, analyses, or interpretation of data; in the writing of the manuscript, or in the decision to publish the results. If there are other authors, they declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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Authors' contribution

Conception and design of the work: MH and BM, Data collection: MH, EV, MT, MBR, ASV, BM, Data analysis and interpretation: MH, EV, MTS, BM, drafting the article: MH, BM, Critical revision of the article and final approval of the version to be published: MH, EV, MT, MBR, ASV, MTS, BM

Appendix A. Supporting information

Supplementary data associated with this article can be found in the online version at doi:10.1016/j.biopha.2025.117918.

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