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Original Research

Nuclear localization dictates hepatocarcinogenesis suppression by glycine N-methyltransferase

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ABSTRACT

Background: GNMT (glycine N-methyltransferase) is a tumor suppressor gene, but the mechanisms mediating its suppressive activity are not entirely known.
Methods: We investigated the oncosuppressive mechanisms of GNMT in human hepatocellular carcinoma (HCC). GNMT mRNA and protein levels were evaluated by quantitative RT-PCR and immunoblotting. GNMT effect in HCC cell lines was modulated through GNMT cDNA induced overexpression or anti-GNMT siRNA transfection.
Results: GNMT was expressed at low level in human HCCs with a better prognosis (HCCB) while it was almost absent in fast-growing tumors (HCCP). In HCCB, the nuclear localization of the GNMT protein was much more pronounced than in HCCP. In Huh7 and HepG2 cell lines, GNMT forced expression inhibited the proliferation and promoted apoptosis. At the molecular level, GNMT overexpression inhibited the expression of *CYP1A* (Cytochrome p450, aromatic compound-inducible), *PREX2* (Phosphatidylinositol-3,4,5-trisphosphate-dependent Rac exchange factor 2), *PARP1* [Poly (ADP-ribose) polymerase 1], and *NFKB* (nuclear factor-kB) genes. By chromatin immunoprecipitation, we found GNMT binding to the promoters of *CYP1A1, PREX2, PARP1,* and *NFKB* genes resulting in their strong inhibition. These genes are implicated in hepatocarcinogenesis, and are involved in the *GNMT* oncosuppressive action.
Conclusion: Overall, the present data indicate that GNMT exerts a multifaceted suppressive action by interacting with various cancer-related genes and inhibiting their expression.

Introduction

Glycine methyltransferase (GNMT) contributes to the regulation of cellular S-adenosylmethionine/S-adenosylhomocysteine ratio (SAM/SAH) and of SAM-dependent methylation reactions [1]. GNMT has a relatively high Km value for SAM and is weakly inhibited by SAH [2]. Therefore, it exhibits appreciable activity at physiological SAM (0.1–0.2 μmol/g of liver) and SAH (0.02–0.06 μmol/g of liver) concentrations. GNMT binds to and may be inhibited by methyltetrahydrofolate (MTHF) [3]. High SAM concentrations inhibit MTHF reductase, with consequent decrease in the transformation of 5,10-methylenetetrahydrofolate to MTHF and dissociation of GNMT-MTHF complex [4]. GNMT release regulates cells' folate content and MTHFR-dependent remethylation of homocysteine to methionine.

GNMT is an HCC suppressor gene whose expression is downregulated in cirrhotic liver and HCV-related HCC [5]. Gnmt-KO mice, lacking the 1–5 exons of the *Gnmt* gene, exhibit a consistent rise of free methionine and SAM, respectively, and a decrease in SAH level. HCC develop prevalently in female mice aged 14–24 months [6]. High serum aminotransferase, methionine, and SAM levels, associated with liver steatosis and fibrosis occur in a male Gnmt-KO mouse lacking *Gnmt* exon 1 in which HCCs develop at the age of 8 months [7]. These mice exhibit increased activity of LKB1 and RAS [8] and high susceptibility to aflatoxin B1-related HCC [9]. In contrast, the GNMT inducer 1,2,3,4, 6-penta-O-galloyl-β-D-glucopyranoside has anti-HCC effects *in vitro* and *in vivo* [10].

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Decreased/absent *GNMT* expression occur in fetal rabbit liver and fast-growing HCCs compared to normal adult rat liver [11]. *GNMT* gene insertion/deletion and promoter region polymorphism are early events in human HCC [12], suggesting that *GNMT* is a susceptibility gene in HCC. *GNMT* was also hypothesized as a susceptibility gene in prostate cancer [13]. In this respect, it is interesting to note that *BHMT* and *GNMT* genes are connected to the genetic susceptibility to rat liver cancer [14].

Global DNA hypomethylation and aberrant expression of DNA methyltransferases 1 and 3b occur from early to late HCC development stages in Gnmt-KO mice [15]. These mice exhibit activation of *Gadd45a, Pak1, Mapk3*, and *Dsup3* genes of MAPK pathway and upregulation of *β-catenin, cyclin D1*, and *c-Myc* genes related to the Wnt pathway [15]. These findings suggest that *GNMT* is a tumor suppressor gene for HCC and might be associated with gender disparity in liver cancer susceptibility. Furthermore, JAK-STAT (Janus kinase and signal transducer and activator of transcription) genes are associated with HCC development in Gnmt-KO mice [8,15], and GNMT contributes to the regulation of AKT signaling by interacting with the mTOR (mammalian target of rapamycin)-inhibitor DEPTOR (DEP domain-containing MTOR-interacting protein) [16], and enhancing proteasomal degradation of the PTEN inhibitor PREX2 [16]. In Gnmt KO-mice, AKT is activated in a PREX2-dependent manner [17].

GNMT is a predominantly cytoplasmic protein; however, it translocates into the nucleus upon its transfection in cancer cells [18,19] or after binding to benzo[*a*]pyrene (BaP) [19,20]. Nuclear GNMT binds to chromatin, and functions independently of its catalytic activity [21]. In the present work, we evaluated whether GNMT multifaced behavior is linked to the genetic predisposition to liver cancer and investigated the nuclear mechanisms responsible for the GNMT suppressive activity.

Results

GNMT expression in HCC prognostic subgroups

The relationship between GNMT expression and HCC prognosis were evaluated in two groups of 46 patients each, defined HCCB [HCC with better prognosis (survival > 3 years)] and HCCP [HCC with poorer prognosis (survival < 3 years)] (Table 1). No significant differences between the two groups occurred as concerns patients' sex, etiology, and presence of cirrhosis. Significantly larger tumor size, Edmondson-Steiner grade, alpha-fetoprotein secretion, proliferation index (Ki67 expression), and *Midkine* expression (as index of poor differentiation) [22], occurred in HCCP than in HCCB.

GNMT mRNA expression was much lower in HCCs, compared to the corresponding surrounding livers, and ~1.8 folds lower in HCCP than in HCCB (Fig. 1A). These results were confirmed at the protein level, showing a pronounced GNMT decrease in HCCs and GNMT levels ~4 folds lower in HCCP than HCCB (Fig. 1B). For the determination of the subcellular distribution of GNMT in HCCs, the nuclei were separated from cytoplasm by diffrential centrifugation [23] and due to the precence of b-actin in both cytoplasms and nuclei [24], GAPDH and Histone 3 were used as reference genes for the cytoplasmc and nuclear GNMT distribution, respectively. In HCCs with different prognosis, GNMT levels were 2–4 folds higher in the cytosol of both HCCs and SLs compared to the respective nuclear levels (Fig. 2). The smallest GNMT nuclear amount was detected in HCCP, where GNMT was ~7 folds lower than the cytosolic counterpart.

Immunohistochemical analysis of GNMT localization (Fig. 3) revealed a GNMT positivity in HCCB of ~55%, ~15% and 25% in the cytosol, nuclei and nuclei plus cytosol, respectively. In HCCP, GNMT cytosolic positivity was 70% against a positivity of ~2.78% in the nuclei and 4.1% in the nuclei plus cytosol. Altogether, the data in Figs. 3 and 4 indicate that higher HCC aggressiveness is associated with an overall reduction of GNMT levels and a decrease of the protein's nuclear localization.

Table 1
Clinicopathological features of HCC patients.

	HCCB	HCCP
No. of patients		
Males	25	24
Females	21	22
Age (mean ± SD)	71 ± 6	68 ± 5
Etiology		
HBV	30	35
HCV	11	5
Ethanol	5	6
Cirrhosis		
+	35	39
–	11	7
Tumor size[a]		
>5 cm	25	30
<5 cm	21	16
Edmondson/Steiner grade		
I	3	1
II	37	10
III	5	28
IV	1	7
Alpha-fetoprotein secretion[b]		
>300 ng/ml of serum	18	30
<300 ng/ml of serum	28	16
Proliferation index (x 10^3)[c]	8.4 ± 2.6	16.3 ± 1.9
Midkine expression (x 10^3)[d]	3.2 ± 0.7	42.2 ± 6.4
Survival after partial liver resection (months). Mean ± SD[e]	68 ± 9	24 ± 8

HCCB, HCC with better prognosis (survival > 3 years). HCCP, HCC with poorer prognosis (survival.
< 3 years).
[a] HCCB vs HCCP $P < 0.001$.
[b] HCCB vs HCCP < 0.001.
[c] HCCB vs HCCP $P < 0.001$.
[d] HCCB vs HCCP $P < 0.0001$.
[e] HCCB vs HCCP $P < 0.0001$.

GNMT expression is under genetic control in rat HCC

Thirty -two weeks after initiation by diethylnitrosamine, a lower number of dysplastic nodules was present in the liver of genetically resistant BN rats than in the liver of susceptible F344 rats [25] and nodule volume was 3.7–5 folds lower in BN than in F344 rats. Fifty-four/six weeks after initiation, moderately/poorly differentiated HCCs developed in all F344 rats, while well-differentiated HCCs were present only in the 23% of BN rats [25]. GNMT expression, evaluated in normal liver, nodules and HCCs of both rat strains (Fig. 4), was about 2 and 3.3 folds lower in nodules and HCCs of F344 rats with respect to normal liver. Interestingly, in the normal BN rat liver, GNMT expression was 1.4 folds higher than in the F344 rat liver and remained elevated in nodules and HCCs.

CYP1A1 expression

CYP1A1 polymorphism is an important modulator of hepatocarcinogenesis [26]. GNMT binds to and is inhibited by BaP-induced Cyp1A1 protein [27,28]. Due to the variation of GNMT protein expression in human (Fig. 1) and rat (Fig. 4) HCCs, Cyp1A1 protein levels were also evaluated in these lesions. We used for these experiments a laboratory collection of human HCCs and livers, and nodules and HCCs from BN and F344 rats. Although some variability depending from different availability of the tissue used, the results in Fig. 5A showed the absence of significant changes in CYP1A1 amount between HCCB and HCCP, while CYP1A1 level of both tumors was 1.5–2 fold lower than that of the correspondent surrounding livers (Fig. 5A). In BN rats, Cyp1A1 underwent a small but significant decrease in nodules and HCCs with respect to surrounding liver, while in F344 rats, Cyp1A1

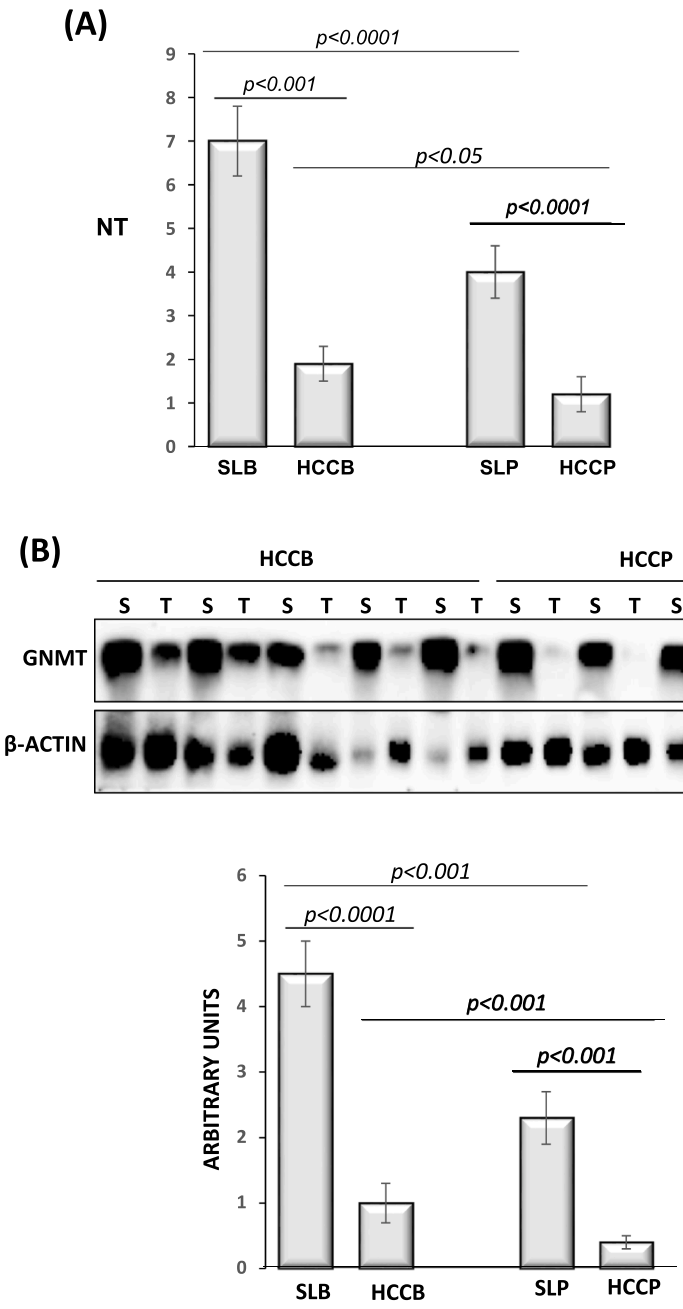

Fig. 1. Expression of GNMT in human hepatocellular carcinoma (HCC) with better (HCCB) and poorer (HCCP) prognosis and corresponding surrounding livers (SL). (A) GNMT mRNA: N Target (NT) = $2^{-\Delta Ct}$; ΔCt = Ct RNR18S-Ct target gene. Data are means (SD) of 42 HCCB and 42 HCCP and corresponding non-neoplastic surrounding livers (SL). (B) Representative Western blots and chemiluminescence analysis of GNMT in HCC and corresponding SL. Optical densities were normalized to β-actin levels. Data are means (± SD) of 46 HCCB and 46 HCCP and corresponding SLs.

levels were 6 and 45 folds lower in nodules and HCCs, respectively, than in the correspondent surrounding liver (Fig. 5B).

Effects of GNMT transfection

GNMT transfection in Huh7 and HepG2 liver tumor cells induced a sharp restraint in cell viability (Fig. 6A) and cell migration (Fig. 6B), whith respect to controls. Hydrogen peroxide triggered a significant rise of apoptosis in HuH7 and HepG2 cells transfected with GNMT, as evaluated by ELISA (Fig. 7A). These results were roughly confirmed by FACS analysis, which showed high apoptogenic properties of GNMT, especially at the higher hydrogen peroxide concentration (Fig. 7B).

The determination of the subcellular distribution of GNMT (Fig. 8) revealed a predominant distribution in the cytoplasm alone of both Huh7 and HepG2 cells, almost always asscіated with nuclear GNMT localization with cytoplasmatic GNMT. Forced GNMT expression triggered a sharp rise of GNMT in the cytoplasm and in the nucleus of Huh7 cells and a rise of GNMT in cytoplasm alone in HepG2 cells in which the basal level of GNMT was about 5-fold higher than in Huh7 cells. However, in both cell types the total basal GNMT positivity (nuclear with/without cytoplasmic positivity) was lower than the cytoplasmic positivity and increased significantly in GNMT-transfected cells.

To analyze the mechanism of the GNMT suppressive action, we evaluated the GNMT effect on the expression of *CYP1A1* [26] and of other, functionally related, supposed GNMT target genes, such as *PREX2* [17], *PARP1* [29], and *NFkB* [30]. ChIP analysis (Fig. 9A) showed that in GNMT transfected Huh7 and HepG2 cells, GNMT protein was bound to *CYP1A1, PREX2, PARP1,* and *NFKB* gene promoters. This binding strongly inhibited the four genes' expression in both cell lines (Fig. 9B) and multiple regression analysis showed zero-order correlation coefficients for the four genes (p varying from −0.1408 to 0.1847), indicating that none of these genes was an independent factor influencing GNMT expression. Furthermore, the levels of these genes were more elevated in human HCCs than in corresponding surrounding

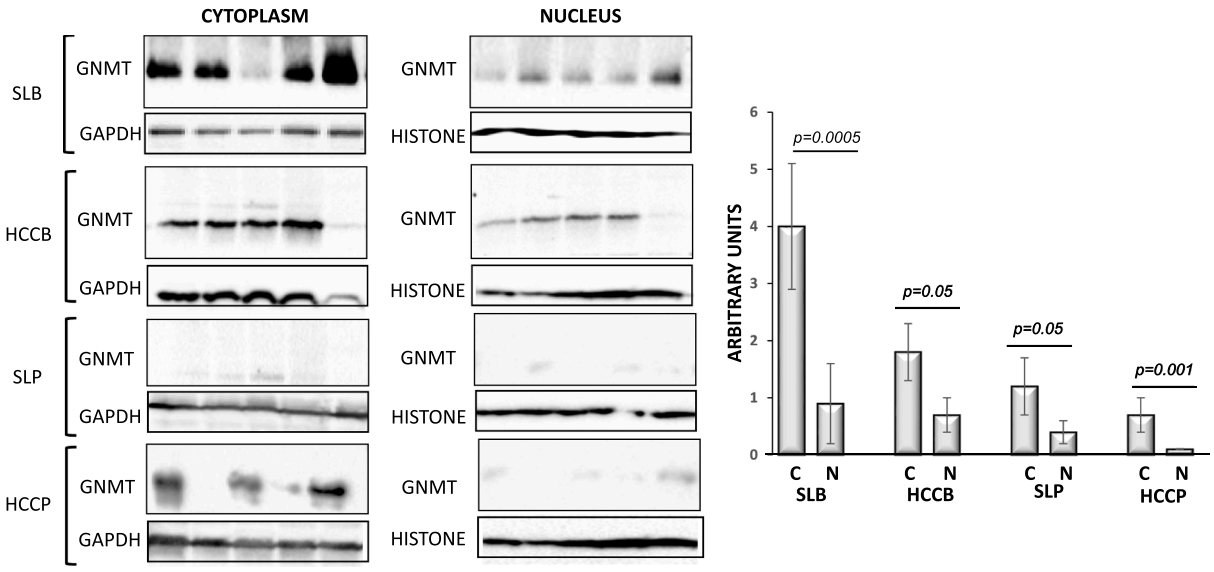

Fig. 2. Distribution of GNMT in the cytoplasm (C) and nucleus (N) of HCC with better (HCCB) and poorer (HCCP) prognosis and corresponding surrounding liver (SL).

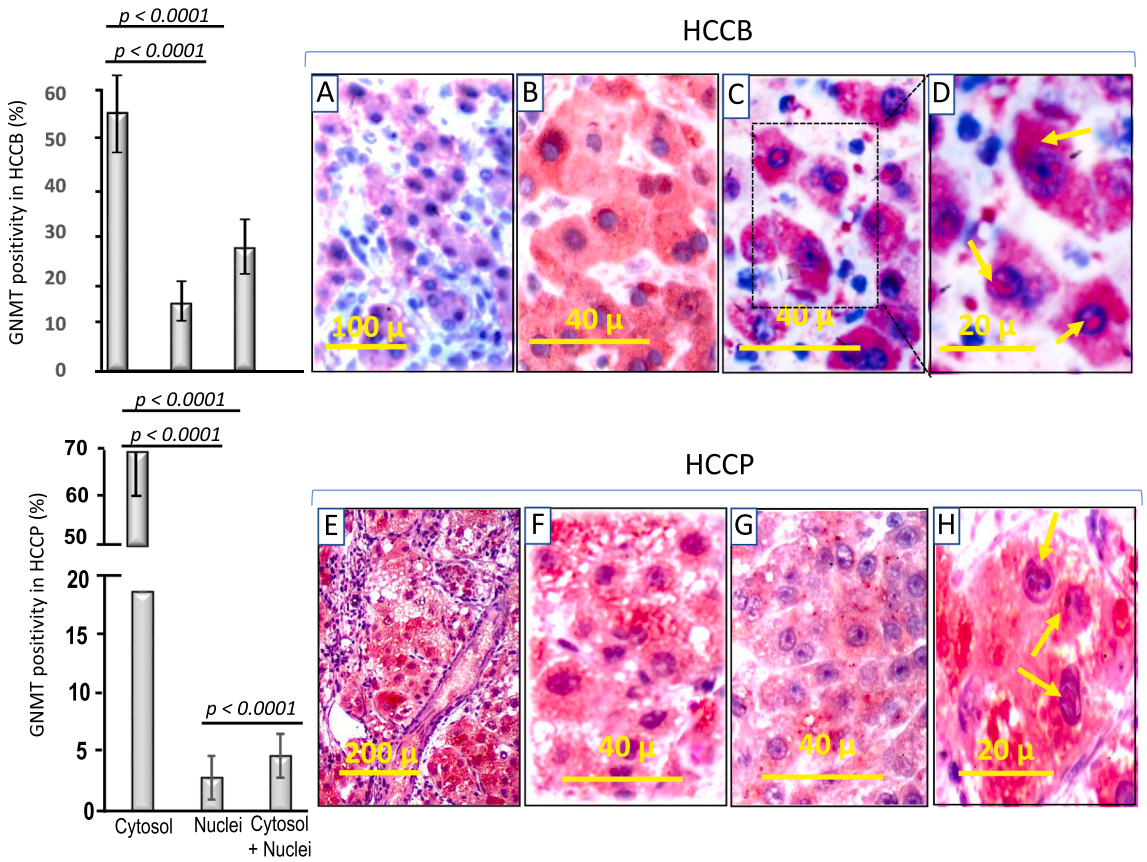

Fig. 3. Quantitative analysis of positivity of immunoistochemical (IHC) staining of GNMT in HCC with better (HCCB) and poorer (HCCP) prognosis, and representative pictures of IHC staining. Arrows indicate the nuclear positivity of IHC staining. Data are means (SD) of 46 HCCB and 46 HCCP.

non-neoplastic livers, and in HCCP than in HCCB (Fig. 10).

Discussion

Following the observastion of the presence of GNMT in the mammalian liver [3,4], where it is prevalently localized in the cytoplasm [21], previous findings showed that GNMT expression is downregulated in cirrhotic liver and HCC induced by hepatitis C virus [11,12]. Accumulating evidences suggest that *GNMT* gene inactivation might contribute to liver tumorigenesis initiation and progression [5]. Our observations, showing a higher decrease of *GNMT* expression in fast-growing human and rat HCCs than in slower-growing human and rat tumors, suggest a role of GNMT as an inhibitor of HCC progression.

Previous studies on the function of GNMT in hepatocarcinogenesis

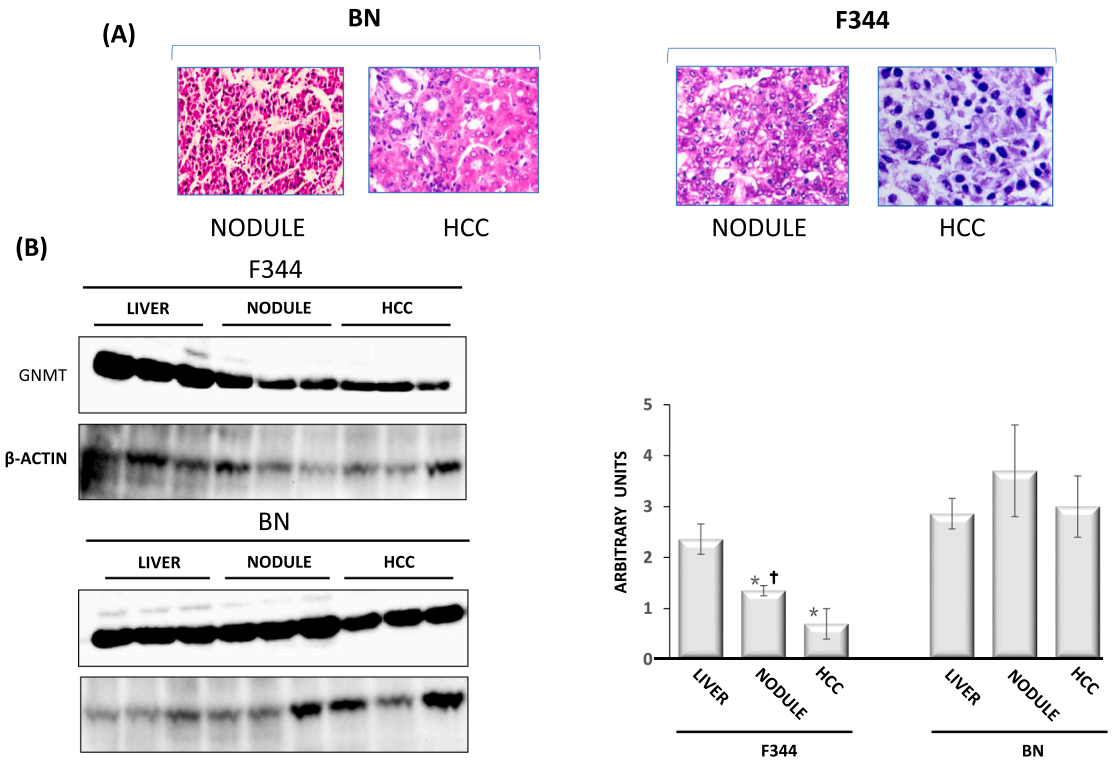

Fig. 4. Expression GNMT in normal liver, preneoplastic nodules, and HCCs of F344 and BN rats. Representative Western blots and chemiluminescence analysis of GNMT in normal liver, preneoplastic nodules, and HCCs of F344 and BN rats. Optical densities were normalized to β-actin levels and expressed in arbitrary units. Data are means (SD) of 5 normal livers, nodules, and HCCs of F344 and • BN rats. Different from normal liver for $p < 0.0001$.
†Different from HCC for $P = 0.0299$.

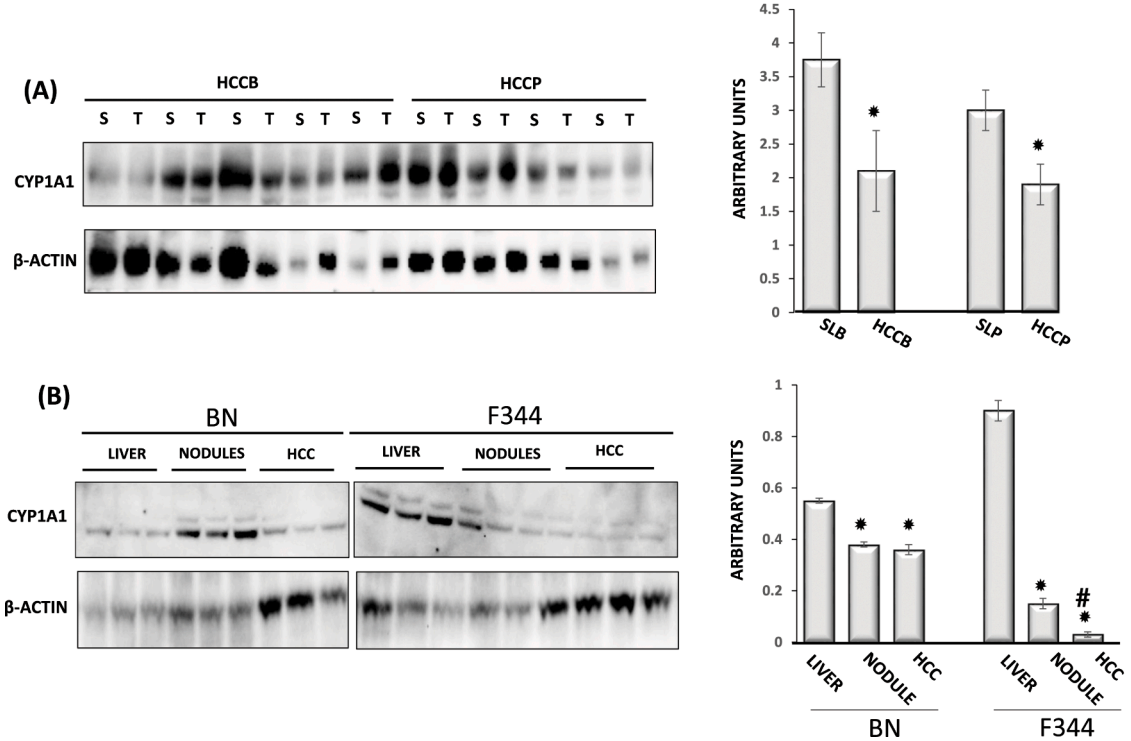

Fig. 5. (A) Expression of CYP1A1 in the non-tumorous surrounding liver (S) and tumor tissue (T) of human HCC with better (HCCB) and poorer (HCCP) prognosis, and (B) expression of CYP1A1 in normal liver, nodules (12 weeks after initiation) and HCC in BN and F344 rats. Representative Western blots and chemiluminescence analysis of CYP1A1 are shown. Optical densities were normalized to β-actin levels. (A) Data are means (SD) of 6 HCCB and 6 HCCP and corresponding surrounding livers (SLB, SLP); *HCCP different from HCCB for $p < 0.001$.
#HCCB/P different from SLB/P for $p < 0.001$. (B) *Different from normal liver for $p < 0.001$. Different from nodules for $p = 0.001$.

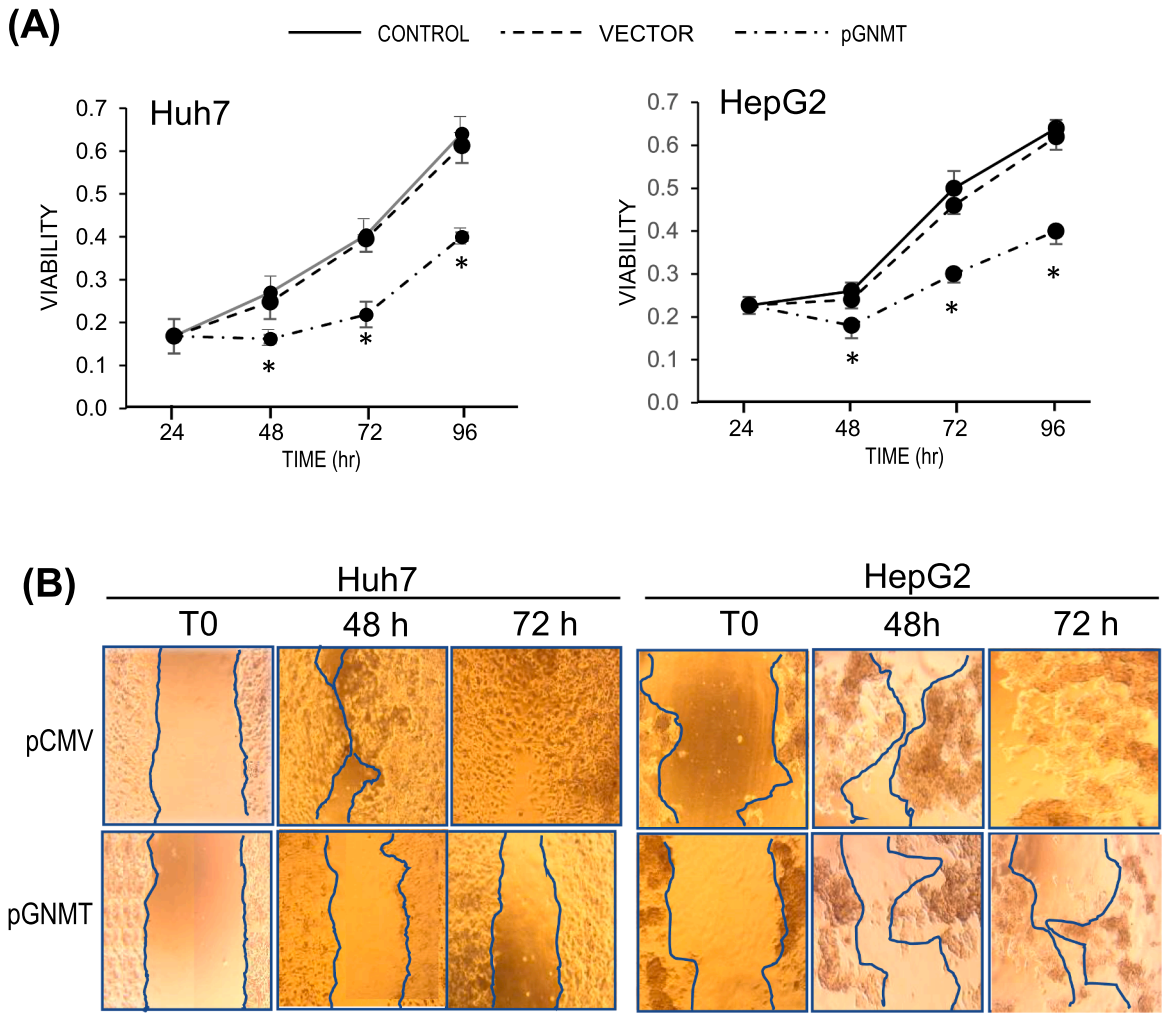

Fig. 6. Effect of GNMT transfection in Huh7 and HepG2 cells. (A) Cell viability. Data are means (SD) of 3 experiments. Asterisks: GNMT significantly different from Control and Vector for at least $p < 0.001$. (B) Representative image of the migration ability of cells transfected with the pGNMT plasmid. The restriction of the wounded area was evaluated at the times indicated after wounding (zero-time). Three independent analyses of cell migration *in vitro* showed the same variations of the wounded area restriction at the different times.

showed the development of HCC in female Gnmt-KO mice wih disrupted 1–5 Gnmt exons [7,15]. This genotype was associated with global DNA hypomethylation, aberrant expression of DNA methyltransferases 1 and 3b, and activation of the MAPK pathway genes [15]. In male Gnmt-KO mice with disrupted exon 1, high methionine and SAM levels and liver steatosis occur, and HCC develops at 8 months [7]. In these mice the Ras and JAK/STAT (Janus kinase/signal transducer and activator of transcription) pathways are activated coincidently with the suppression of the RASSF (Ras inhibitors Ras-association domain family/tumor suppressor) 1 and 4 and the SOCS (JAK/STAT inhibitors suppressor of cytokine signaling) 1–3 and cytokine-inducible SH2-protein. [7].

Our results show that GNMT influences human HCC outcome, being significantly more expressed in HCCs with better prognosis, compared to HCCP. In HCCB, GNMT nuclear localization was much higher than in HCCs with poorer prognosis. In rats differently susceptible to HCC, comparative functional genetic experiments showed that neoplastic lesions of genetically resistant rat strains cluster together with human HCCs with better prognosis, whereas HCCs of susceptible F344 rats cluster with human HCCs with poorer prognosis [14,31]. Notably a signifcantly higher *Gnmt* expression was found in preneoplastic lesions of the genetically resistant BN rats compared to liver lesions of susceptible F344 rats, indicating the existence of genetic control over the GNMT suppressive action. Functional experiments showed that forced *GNMT* overexpression induces a potent inhibition of viability and migration of both Huh7 hepatocarcinoma cells and HepG2 hepatoblastoma cells, and of colony formation of the latter cells. This was associated with a significant increase in apoptosis induced by hydrogen peroxide, thus confirming the suppressive properties of the *GNMT* gene. Different mechanisms support the suppressive action of GNMT. GNMT (which has been shown to be multifunctional) and B[*a*]P are involved in the induction of CYP1A1 and of GNMT as a potential role in the modulation of hypoxia inducible factor-1 function [32,33]. Previous work demonstrated the interaction of GNMT with DEPTOR, a mTORC1 binding protein overexpressed in HCC [34]. The consequent decrease in DEPTOR availability was associated with a negative feedback loop from S6K to PI3K leading to a decrease of AKT signaling [35,36]. Furthermore, the exposure to BaP and aflatoxin B1 induces GNMT nuclear translocation [37,38]. Nuclear GNMT inhibits the expression of the *CYP1A1* gene and its activation by BaP, contributing to the decrease of BaP- and AFB1-DNA adducts [39]. Furthermore, GNMT is a polycyclic aromatic hydrocarbon (PAH)-binding protein [40] that can mediate the induction of CYP1A1 by PAHs [41,42]. The mechanisms determining the GNMT distribution in the different intracellular compartments are presently unknown and need further analysis. In the present work, we demonstrated that GNMT binds to the promoters of *CYP1A1, PREX2, PARP1,* and *NFKB* genes and sharpy inhibits their expression, thus strongly inhibiting their carcinogenic effect (Suppl. Fig. 1). Indeed, different studies have shown the association of Cyp1A1 polymorphisms

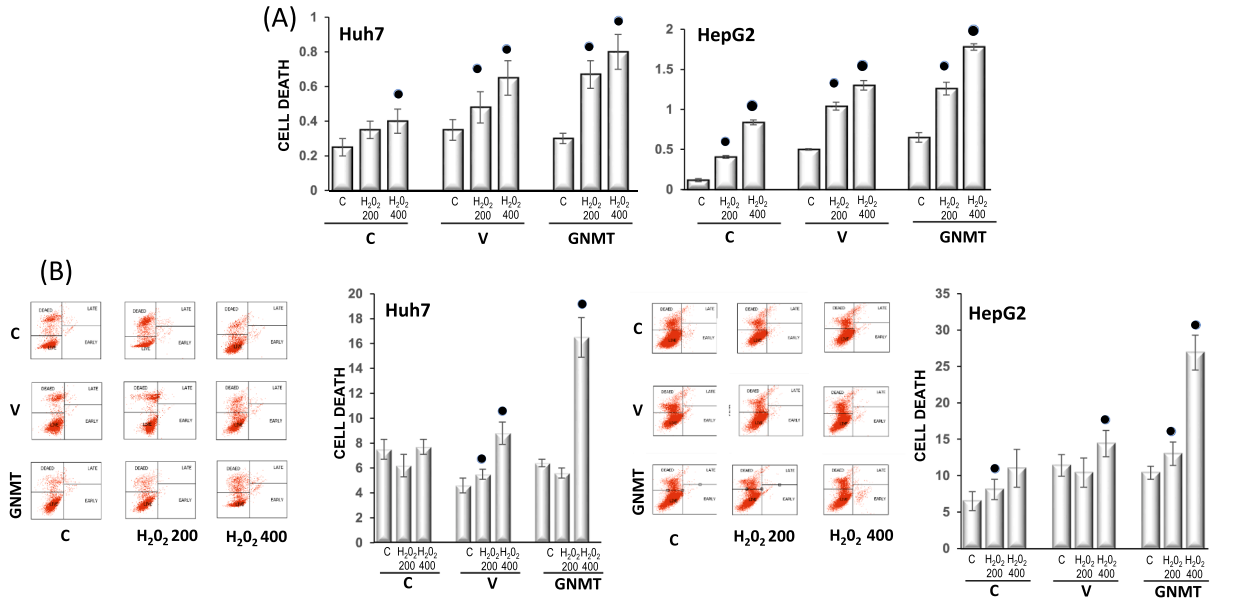

Fig. 7. Effect of GNMT transfection on the apoptosis induced by hydrogen peroxide in Huh7 and HepG2 cells. (A) * Different from control (C) for at least $p < 0.002$; • different from C for $p < 0.0002$; (B) • different from C for at least $p < 0.05$.

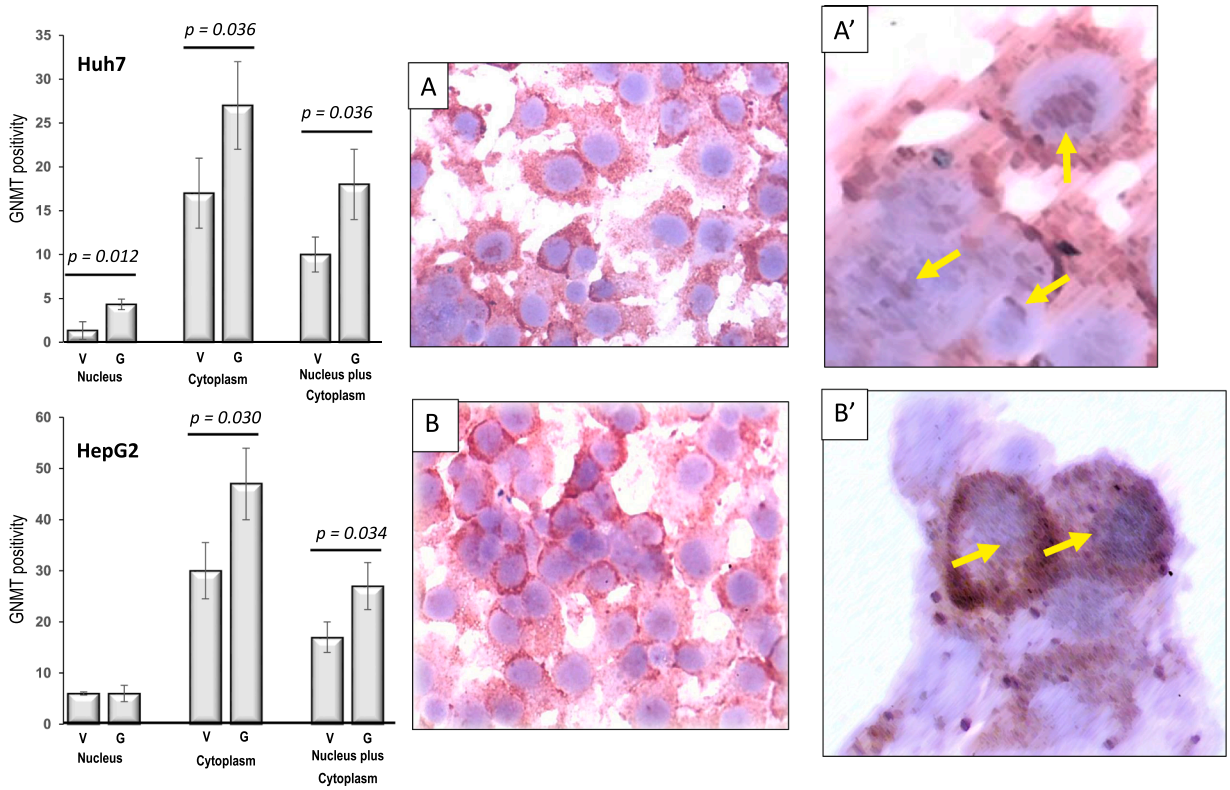

Fig. 8. Quantitative analysis of GNMT staining positivity in Huh7 and HepG2 cells, as assessed by immunohistochemistry. Data are means (SD) of 5 experiments. GNMT (G) vs. vector (V) at least $P < 0.01$. The arrows indicate nuclear positivity. Panels A/A' IHC staining of GNMT in Huh7 cells; panels B/B' IHC staining of GNMT in HepG2 cells at low and high magnification. The arrows indicate nuclear positivity.

with the risk of developing breast [43], liver [44], and lung [45,46], and Tomcod [47] cancers. Studies in humans suggest that variations in *CYP1A1* cDNA influences the genetic susceptibility to neoplasia [48]. Therefore, the inhibition of *CYP1A1* expression by GNMT may suppress the development of tumors associated with *Cyp1A* polymorphism. PREX2 is a regulator of the small guanosine triphosphatase Rac, that inhibits PTEN (Phosphatase and tensin homolog) activity, thus upregulating the PI3K/AKT signaling pathway [43,44,49]. Different studies decribed the beneficial therapeutic effect of PI3K/AKT inhibitors especially if associated with RAS/MEK/ERK inhibitors [49]. PARP1 protein is an enzyme linking covalently polymers of poly(ADP-ribose) (PAR) to histones. DNA activated by PARylation is a scaffold to recruit and coordinate the XRCC1 repair proteins. [50]. The decrease of *PARP1* expression impairs these repair mechanisms and the consequent

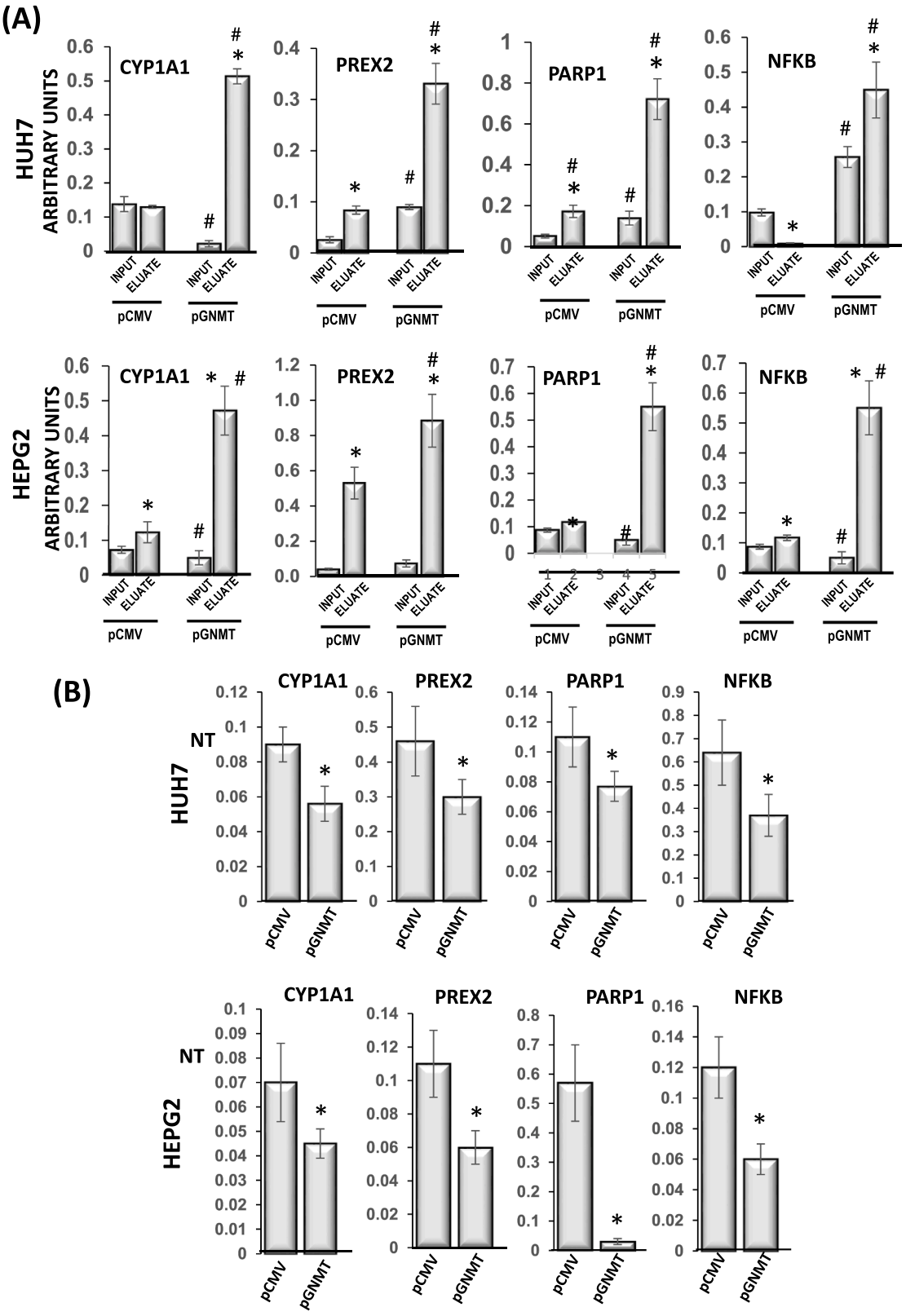

Fig. 9. GNMT binding to CYP1A1, PREX2, PARP1, and NF-KB genes in Huh7 and HepG2 cell lines. (A) Chromatin immunoprecipitation by GNMT antibody in Huh7 and HepG2 cells. (B) CYP1A1, PREX2, PARP1, and NF-KB expression in normal and GNMT-transfected Huh7 and HepG2 cells: N Target (NT)=$2^{-\Delta Ct}$; ΔCt = Ct RNR18S-Ct target gene. Data are means (SD) of 5 experiments. Statistical analysis: (A) input vs. eluate: * at least $p < 0.05$; pCMV vs. pGNMT: # at least $P < 0.05$. (B) pCMV vs. pGNMT: *, at least $P < 0.05$.

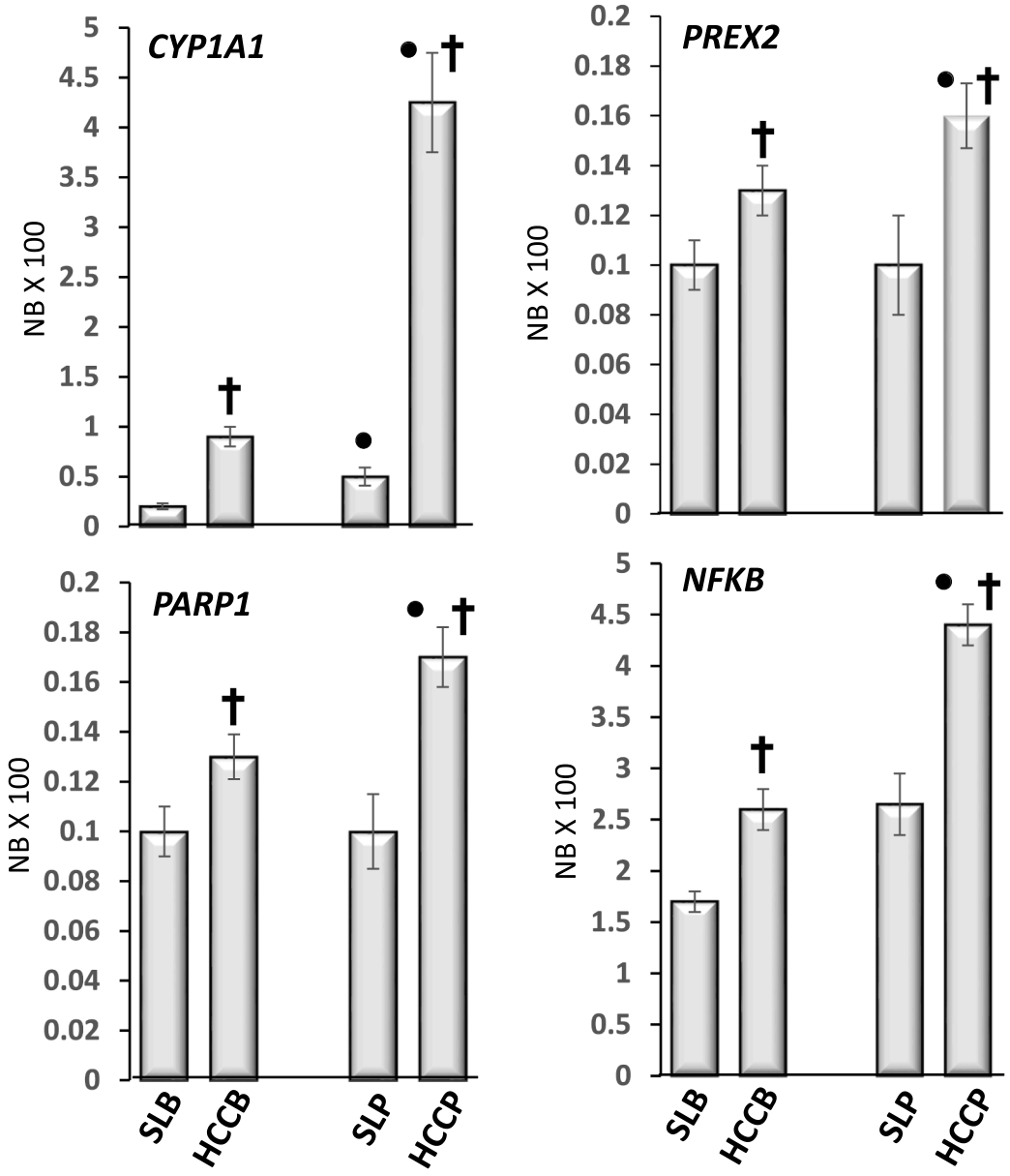

Fig. 10. Expression of CYP1A1, PREX2, PARP1, and NF-KB in human HCCB and HCCP and respective surroundings (S). N Target (NT) = $2^{-\Delta Ct}$; ΔCt = Ct RNR18S-Ct target gene. Data are means (SD) of 10 HCCB and 10 HCCP and corresponding surrounding liver. Statistical analysis: • different from surrounding for $P < 0.001$; † different from HCCB for at least $P < 0.01$.

genomic instability is incompatible with cancer growth [50]. According to this hypothesis, PARP1 inhibitors have been recently tested in the therapy of breast, ovarian, prostate, and pancreatic cancers [51]. NF-kB regulates different biological responses, such as the immune response and inflammation and liver oncogenesis [52]. *NF-kB* activation in malignancy increments the expression of genes involved in the proliferation and migration of cancer cells. Activation of NF-kB, AP-1, and STAT transcription factors is a frequent early event in human HCC [53] and *NF-kB* gene is considered a therapeutic target for cancer treatment [54].

In conclusion, our result show that the inhibition of *CYP1A1, PREX2, PARP1,* and *NFKB* genes is implicated in the *GNMT* oncosuppressive activity and contributes to the genetic predisposition to HCC and to the prognosis of human HCCs, being *GNMT* more expressed in HCCB than HCCP and differently located subcellularly.

Materials and methods

Human tissue samples

Ninty-two HCCs and corresponding surrounding non tumorous livers (SLs) were used. Table 1 shows patients' clinicopathological features. Liver tissues were archival samples kindly provided by the Department of Surgery "Pietro Valdoni", University of Rome "La Sapienza", and the Department of Surgery, University of Sassari. Informed patients'consent and Institutional Review Board approval was obtained at these Departments.

Animals and treatments

F344 and BN rats were treated according to the ''resistant hepatocyte'' protocol. Diethylnitrosamine (DENA) dissolved in saline (100 mg/ml) was injected intraperitoneally to 54 rats at a dose of 200 mg/kg.

Dysplastic nodules (DNs) and HCCs we collected at 30–32 and 64–56 weeks, receptively. Cross images have been included in Fig. 4 [54]. Study protocols were in compliance with our institution's guidelines for the use of laboratory animals.

Cell lines and treatments

Certified Huh7 human HCC and HepG2 human hepatoblastoma cell lines (ATCC) were cultured in Dulbecco's modified Eagle medium containing 10% FBS at 37 °C. Cells (0.8×10^6 in 6 cm dishes) were transfected with pCMV6 empty vector or pCMV6-GNMT expression vector (400 ng of GNMT cDNA). Apoptosis was induced by 200/400 μM H_2O_2, 6 h after GNMT transfection. Cells were used 24 h after transfection. Proliferation and progression indices of human HCCs were evaluated by determining Ki-67 and MDK (Midkine) expression, respectively. Cell viability of cell lines was determined by the MTT test (Sigma, St. Louis, USA). For the wound-healing assay, Huh7 and HepG2 cells monolayers in 6-well plates, transfected with GNMT, were wounded with sterile pipette tips. Pictures were acquired by an EclipseTE-300 Nikon microscope.

Quantitative real-time RT-PCR

Real-Time PCR reactions were conducted with 75–300 ng of cDNA, obtained accordingly to High Capacity c-DNA Reverse Transcription Kit (Applied Biosystem, CA, USA), using a Quantitect SYBR Green PCR kit & Quantitect Primer Assay (Qiagen Gmbh, Hilden, Germany) [14].

Western blot

Hepatic tissue samples and cultured cancer cells were homogenized in lysis buffer [30 mM Tris (pH 7.5), 150 mM NaCl, 1% NP-40, 0.5% Na deoxycholate, 0.1% SDS, 10% glycerol, and 2 mM EDTA] containing the Complete Protease Inhibitor Cocktail (Roche Molecular Biochemicals, Indianapolis, IN) and sonicated. Protein concentrations were determined with the Lowry-Folin assay (Sigma, St. Louis). Primary antibody reactions were followed by 1 hr incubation with horseradish peroxidase secondary antibody diluted 1:5000 and revealed with the Chemiluminescence Substrate Kit (Pierce Chemical Co., NY). Potein densities were calculated by the Image-Quant 5.1 software (GE Healthcare, Piscataway, NJ), and normalized to β-Actin (Santa Cruz Biotechnology). For determation of the localization of cytoplasmatic and nuclear GNMT the purity of the subcellular fractions was analyzed by GAPDH (glyceraldehyde dehydrogenase) and Histone 4, respectively.

Apoptosis

After PBS washing, cells were stained with Annexin V-APC Apoptosis Kit (BD Pharmingen™ Cat no 556,547) and propidium iodide. Stained cells, acquired by FACS Canto (Becton Dickinson, San Jose, USA) flow cytometry were analyzed by Diva Software 6.3. For each experimental point, 30.000 total events were acquired. The percentage of early, late apoptotic, and necrotic cells was analyzed using Microsoft Office Excel 2017 and GraphPad Prism 7.0 software. Apoptosis was also quantified with the "Cell Death detection ELISA kit" (Roche).

Chromatin-DNA immunoprecipitation (ChIP) analysis

Following 1% formaldehyde-assisted chromatin fixation, tissues were homogenized by handheld TissueRuptor (Qiagen). Nuclei release was achieved by Dounce homogenization using a tight pestle. Chromatin was sheared by sonication, and Input-DNA was collected. Sheared chromatin (200–1000 bp) was precleared (using Salmon Sperm DNA/Protein A Agarose-50% slurry for 30 min at 4 °C with shaking, then 3% BSA), and immunoprecipitated by 4 μg of GNMT-rabbit polyclonal antibody (Proteintech, USA) for 16 h at 4 °C. A negative control was obtained from chromatin immunoprecipitated with IgG normal control in the absence of specific GNMT-rabbit polyclonal antibodies. Input-DNA and antibody-chromatin complexes were washed, extracted, treated with proteinase K, and heated for 2.5 h to reverse cross-links, and purified to perform Chip-real Time PCR detection. DNA concentration was determined spectrophotometrically, and diluted aliquots of each sample were electrophoresed to assess chromatin shearing efficacy. Selected nested specific ChiP-primer sets were used to amplify the promoter region of the genes of interest, ensuring that the Ct values generated measured the real quantity of DNA (Table 2, supplementary materials). Assays were carried out on the Thermal Cycle 7500 Fast Real-time PCR System (AB-Applied BioSystems) with SYBR Green Real-time PCR kit and LNA-enhanced primers, generated by LNA oligonucleotide design software (Qiagen Gmbh, Hilden, Germany). As a control, RT-PCR of the glyceraldehyde 3-phosphate dehydrogenase (Gapdh) promoter was performed. Real-time PCR Ct values were normalized to Input-DNA Ct, according to the formula: "target site binding: $2^{-\Delta Ct}$, where ΔCt = Ct of immunoprecipitated DNA - Ct of Input DNA [55].

Histology and immunohistochemistry (IHC)

Formalin-fixed liver and HCC tissues embedded in paraffin were cut and stained with hematoxylin and eosin (H&E) for routine histology. IHC staining of GNMT protein was performed using anti-GNMT polyclonal Ab (Proteintek cod. 18,790–1-AP), diluted 1:100 with R.T.U. VECTASTAIN KIT (cod. PK-7800, VECTOR Laboratories) and Vector® *NovaRED*™ Substrate Kit for Peroxidase (Cat. N°: SK-4800). The control with normal mouse IgG showed no staining (not shown).

Statistics

Data are expressed as means ± SD. GraphPad Prism 9.0 (www.graphpad.com) was used to evaluate the significance of differences between means.

CRediT authorship contribution statement

Maria M. Simile: Investigation. **Antonio Cigliano:** Investigation. **Panagiotis Paliogiannis:** Investigation. **Lucia Daino:** Investigation. **Roberto Manetti:** Formal analysis. **Claudio F. Feo:** Formal analysis. **Diego F. Calvisi:** Funding acquisition, Supervision, Writing – original draft. **Francesco Feo:** Funding acquisition, Supervision, Writing – original draft. **Rosa M. Pascale:** Funding acquisition, Supervision, Writing – original draft.

Declaration of Competing Interest

The authors 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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Supplementary materials

Supplementary material associated with this article can be found, in the online version, at doi:10.1016/j.tranon.2021.101239.

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