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Subcellular distribution and Nrf2/Keap1-interacting properties of Glutathione S-transferase P in hepatocellular carcinoma

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ABSTRACT

The oncogene and drug metabolism enzyme glutathione S-transferase P (GSTP) is also a GSH-dependent chaperone of signal transduction and transcriptional proteins with key role in liver carcinogenesis. In this study, we explored this role of GSTP in hepatocellular carcinoma (HCC) investigating the possible interaction of this protein with one of its transcription factor and metronome of the cancer cell redox, namely the nuclear factor erythroid 2-related factor 2 (Nrf2).

Expression, cellular distribution, and function as glutathionylation factor of GSTP1-1 isoform were investigated in the mouse model of N-nitrosodiethylamine (DEN)-induced HCC and in vitro in human HCC cell lines. The physical and functional interaction of GSTP protein with Nrf2 and Keap1 were investigated by immunoprecipitation and gene manipulation experiments.

GSTP protein increased its liver expression, enzymatic activity and nuclear levels during DEN-induced tumor development in mice; protein glutathionylation (PSSG) was increased in the tumor masses. Higher levels and a preferential nuclear localization of GSTP protein were also observed in HepG2 and Huh-7 hepatocarcinoma cells compared to HepaRG non-cancerous cells, along with increased basal and Ebselen-stimulated levels of free GSH and PSSG. GSTP activity inhibition with the GSH analogue EZT induced apoptotic cell death in HCC cells. Hepatic Nrf2 and c-Jun, two transcription factors involved in GSTP expression and GSH biosynthesis, were induced in DEN-HCC compared to control animals; the Nrf2 inhibitory proteins Keap1 and β -TrCP also increased and oligomeric forms of GSTP co-immunoprecipitated with both Nrf2 and Keap1. Nrf2 nuclear translocation and β -TrCP expression also increased in HCC cells, and GSTP transfection in HepaRG cells induced Nrf2 activation.

In conclusion, GSTP expression and subcellular distribution are modified in HCC cells and apparently contribute to the GSH-dependent reprogramming of the cellular redox in this type of cancer directly influencing the transcriptional system Nrf2/Keap1.

1. Introduction

Hepatocellular carcinoma (HCC) is characterized by high prevalence, morbidity, and mortality; it represents between the 75% and 85% of primary liver cancers and the second most common cause of cancer-

related death [1,2]. Chronic liver diseases are all significant risk factors for HCC, including chronic hepatitis C and B, aflatoxin-contaminated foods, non-alcoholic fatty liver disease (NAFLD), excessive drinking, genetic factors, smoking, obesity and type 2 diabetes [3,4].

Treatments applied at different stages of HCC include liver resection,

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percutaneous thermal ablation, radiotherapy, chemotherapy, immunotherapy and liver transplantation, but there are still many limitations to existing therapy protocols for advanced HCC thus leading to the poor survival [5]. The reasons for this negative outcome are that HCC is a malignant liver tumor characterized by high molecular heterogeneity, which has hampered the development of targeted therapies [6]. Late diagnosis and the development of a multidrug resistance phenotype are other important aspects [7–12] that combine with the lack of early evident clinical symptoms and specific biomarkers that may tackle the rapid progression of the disease. Therefore, a better characterization of the carcinogenetic process and the identification of new therapeutic targets are urgent clinical needs in HCC.

The human enzyme glutathione S-transferase P (GSTP) has been identified as an important oncogene in different types of human cancers [13–15]. This is a highly inducible and relatively abundant GST isoform of several cell types, including the liver cell (reviewed in Ref. [14]), and its expression is increased in HCC [16,17] in which it is proposed to sustain carcinogenesis [18]. Besides phase II drug metabolism function (corresponding to a GSH conjugation reaction to neutralize electrophilic metabolites and xenobiotics), GSTP can act as a glutathionylase to promote the post-translational S-glutathionylation of redox-sensitive cysteines in low pKa environments of proteins in the cytosol as well as in the nucleus and some organelles such as mitochondria and the endoplasmic reticulum (see Ref. [19] and references therein). In this respect, GSTP can act as a chaperon of several proteins in which glutathionylation influences their stability and function in a dynamic and reversible regulation cycle that is under the influence of the cellular redox. Along with its abundance, the propensity of this protein to physically interact with a number of cellular proteins, suggests for this glutathionylase a key role in the coordination of different and functionally related proteins involved in either physiological or pathological processes, including cancer [20]. For example, protein glutathionylation has been demonstrated to intervene in carcinogenesis, resistance to apoptosis, immune surveillance, metabolic reprogramming of cancer cells and multidrug resistance mechanisms [21–23]. Preliminary work by our group demonstrated that GSTP expression influences the cellular metabolism of GSH [24,25] and GSTP induction by seleno-organic compounds, a group of alkylating agents with marked reactivity against cellular thiols, was demonstrated to produce a physical and functional interaction of this protein with Nrf2 in human HCC cells [26]. Nrf2 is a transcriptional factor important in the stress response to cellular electrophiles as well as in the control of GSTP expression during hepatocarcinogenesis [27]. GSTP has also been described to physically interact with the Nrf2 inhibitory protein Keap1, promoting its glutathionylation on Cys residues critical for the formation of the Nrf2/Keap1 inhibitory complex [28].

These pieces of evidence suggest the hypothesis that GSTP induction in HCC may create the conditions for a cancer-specific interaction with the Nrf2/Keap1 transcriptional system; this may contribute to reprogram the metabolism and glutathionylation function of cellular GSH during carcinogenesis. This hypothesis will be investigated for the first time in vivo using the mouse model of DEN-induced HCC and mechanistic aspects of this possible interaction will be further explored in human HCC cell lines in which the activity an expression of GSTP will be modified through pharmacological and gene manipulation methods.

2. Materials and methods

2.1. Cell models and culture conditions

Hepatocellular carcinoma cell lines HepG2 and Huh7 were purchased from ATCC (Maryland, USA) and maintained in culture at 37 °C in an atmosphere of 5% CO₂; Huh7 cells were cultured in Dulbecco's Modified Eagle's Medium (DMEM, Gibco™, USA) supplemented with 10% Fetal bovine serum (FBS, Gibco™), 100 U/mL penicillin, and 100 mg/mL streptomycin (Invitrogen, CA, USA) whereas HepG2 cells were

maintained in Modified Eagle's Medium (MEM, Gibco™, USA) supplemented with 10% FBS (Gibco), 1% Non-essential amino-acids (NEAA, Gibco™, USA), 1% Ultra-glutamine (Lonza), 100 U/mL penicillin, and 100 mg/mL streptomycin (Invitrogen, CA, USA) at 37 °C and the presence of 5% CO₂.

Undifferentiated HepaRG cells were obtained from Thermo Fisher Scientific and were maintained at 37 °C in an atmosphere of 5% CO₂ using a William's E medium (Gibco™) containing 10% FBS (Gibco™), 1% Glutamax (Gibco™), 5 µg/mL insulin (Sigma-Aldrich, Saint-Louis, MO, USA), 50 µM hydrocortisone hemi-succinate (Sigma-Aldrich, Saint-Louis, MO, USA) and 100 IU/mL penicillin and 100 µg/mL streptomycin (growth medium). The cell monolayers were maintained at confluence changing the culture medium every 3 days and before treatment the cells were transferred for 14 days in differentiation medium (Thermo Fisher Scientific) which allows changing the phenotype of this undifferentiated lineage to resemble that of primary human hepatocytes.

2.2. HCC animal model

All experimental procedures involving animals were performed in accordance with animal protocols approved by the Institutional Committee on the Ethics of Animal Experiments of the Marche Polytechnic University (Ancona, Italy), and conducted in conformity with EU Directive 2010/63/EU for animal experiments (authorization number 411/2016-PR). Wild-type C57BL/6J mice were purchased from Charles River Laboratories International, Inc., Wilmington, MA, USA.

To induce HCC, male mice (n = 6 for each time-point) were injected intraperitoneally (i.p.) with 25 mg/kg DEN (N0258-1G; Sigma, St Louis, MO, USA) at the age of 14 days, while the control group (n = 6) was injected with PBS. At the end of treatment, the animals were anesthetized, sacrificed and analyzed for HCC formation 3, 7 or 9 months after DEN or PBS administration according to institutional guidelines to collect liver tissue for histology and biochemistry tests. At the end of the DEN treatment protocol (9-month timepoint) all the animals developed tumors with a median number of masses of 7.0 (ranging from 4 to 13), and tumor development was also confirmed by HepPar-1 tumor marker analysis using both immunohistochemistry and RT-PCR methods (Supplementary Fig. S1). All animal experiments were approved by the Ethics Committee of the Polytechnic University of Marche.

2.3. Protein extraction

For immunoblots and immunoprecipitation of murine tissues, samples were weighed and resuspended in a buffer consisting of PBS at pH 7.5, NP40 (1:50) and a cocktail of protease inhibitors (Roche). Then, after preliminary homogenization in a 2-ml potter, the samples steadily maintained in ice were further processed by sonication for 3 times (15–20 s each with intervals of 1 min). Then, the samples left in ice for 1 h were centrifuged twice at 12,000 rpm for 20 min at 4 °C to recover the total protein extract in the supernatant that was maintained at –80 °C until use.

Liver cell lines were lysed in ice-cold Cell Lysis Buffer containing 20 mM Tris-HCl (pH 7.5), 150 mM NaCl, 1 mM Na₂EDTA, 1 mM ethyleneglycol-tetra acetic acid (EGTA), 1% Triton, 2.5 mM sodium pyrophosphate, 1 mM β-glycerophosphate, 1 mM Na₃VO₄, 1 µg/ml leupeptin (Cell Signaling Technology, CST), and 20 µl/ml protease inhibitor cocktail (Roche). Debris were removed by centrifugation at 14,000 g for 40 min at 4 °C.

Total protein concentrations were determined using BCA Protein assay (Euroclone) using bovine serum albumin (BSA) as an external standard.

2.4. Immunoblot analysis

Protein extracts obtained from liver tissue or cell samples (10–20 µg

of total proteins) were heat-treated in loading sample buffer containing β -mercaptoethanol and processed for protein separation by 4–12% sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE). In some experiments, non-reducing conditions were applied to sample preparation for PAGE by omitting β -mercaptoethanol thus preserving intra and intermolecular disulfide bridges of protein structures. Proteins of slab gels were then transferred to nitrocellulose membrane (Hybond-ECL, Amersham, GE healthcare LifeScience, Pittsburgh, PA, USA). Membranes were incubated for 1 h with blocking buffer (TBS containing 5% dry milk and 0.05% Tween-20) and then overnight with primary antibodies that included anti-c-Jun (#2315; 1:1000, Cell Signaling Technology), anti-GSTP1 (1:1000, ALX-804-510, Enzo Life Sciences), anti-Keap1 (1:1000, #8047, Cell Signaling Technology), anti-Nrf2 (1:800, #12721, Cell Signaling Technology), anti- β -TrCP (1:1000; #4394, Cell Signaling Technology) or a mouse anti-actin antibody (1:1000, Cell Signaling Technology) diluted in blocking buffer (1/500 and 1/2000 dilution, respectively). After several washes in TBS containing 0.05% Tween-20, a horseradish peroxidase-conjugated anti-mouse (#7076) or anti-rabbit (#7074) secondary antibody (Cell Signaling Technology, CST) was added (dilution 1:2000 in blocking buffer) and detection was performed using an enhanced luminol-based chemiluminescent (ECL) detection system (ECL Clarity BioRad).

2.5. GST activity and pharmacological inhibition

GST activity was measured in HepG2 and HepaRG cells as previously described in Ref. [24] using 5 mM GSH (Sigma-Aldrich, St. Louis, MO) and 0.5 mM CDNB (Merck, Darmstadt, Germany) as second substrate in 0.1 M potassium phosphate buffer pH 6.5 at room temperature. The kinetics of GSH conjugation reaction was measured with a spectrophotometer (Thermo Fisher Scientific) following the absorbance of CDNB at 340 nm. The molar extinction coefficient used for CDNB conjugation was $9.6 \text{ mM}^{-1}\text{cm}^{-1}$.

GSTP activity inhibition experiments were performed utilizing the peptidomimetic inhibitor of GSTP1-1 ezatiostat (EZT or TLK199) (gamma-glutamyl-S-(benzyl)cysteinylyl-R-phenyl glycine diethyl ester) from MCE. This selective inhibitor of GSTP indirectly influences JNK and MAPK-ERK activity of cancer cells and hematopoietic cells as described in more details in Ref. [22].

2.6. Protein glutathionylation (PSSG) analysis

The levels of total PSSG in the protein extract of the liver tissue or cell samples was utilized as a surrogate measure of GSTP function as GSH-dependent chaperone of cellular proteins. In fact, GSTP can act as a glutathionylase in the post-translational S-glutathionylation of redox-sensitive cysteines in low pKa environments of proteins, including protein kinases, transcription factors, and endoplasmic reticulum and UPR proteins, thus forming a dynamic and reversible cycle of regulation for several cell processes (see Ref. [19] and references therein). After alkylation of Cys residues with NEM (Sigma-Aldrich), cellular proteins (10–20 μg) were fractionated under non-reducing conditions in 4–12% gradient SDS-PAGE and then transferred to a nitrocellulose membrane (Hybond-ECL, Amersham, GE healthcare LifeScience, Pittsburgh, PA, US) for immunoblot analysis utilizing an anti-GSH primary antibody (1:1000, ab19534, Abcam) and an anti-mouse IgG HRP-linked secondary antibody (1:2000, #7076, Cell Signaling Technology, CST). To produce reliable qualitative and semiquantitative results in the cell tests, PSSG determination was also carried out by FACS-Scan analysis using the Cayman's Glutathionylated protein detection kit (Cayman Chemical, Item No.10010721) on an Attune NxT Acoustic Focusing Cytometer (Thermo Fisher Scientific). The method allows a direct measurement of S-glutathionylated proteins in intact (permeabilized) cells.

2.7. Immunohistochemistry (IHC)

The 4 μm sections were mounted on uncoated glass slides and were deparaffinized and rehydrated in graded alcohol. Endogenous peroxidase blockage was carried out using 3% H_2O_2 . Thereafter, antigen repair was performed using 10 mM of citrate buffer and heating at 95°C . Subsequently, sections were incubated with the primary antibody anti-GSTP1 (HPA019869, Atlas Antibodies, 1:1000) overnight at 4°C , then incubated with the HRP-labelled secondary rabbit antibody at room temperature for 2 h. A 3,3'-diaminobenzidine (DAB) detection system (Dako, Denmark) was used to detect primary antibodies according to manufacturer's protocol. One slide was stained with hematoxylin and eosin (Sigma-Aldrich) and examined to confirm the presence of liver tumor.

2.8. Immunoprecipitation experiments

Pooled liver samples of DEN-HCC and CTL groups were prepared to obtain sufficient protein material for the immunoprecipitation (IP) experiments. Considering the possible influence of the oligomerization pattern of GSTP and on its chaperon function [22,23,25], the pools were prepared after preliminary screening of the monomeric and oligomeric forms of GSTP protein by immunoblot in each animal. Two pools were prepared that were low or high in the 24 kDa band of GSTP and their extracts (0.5 mg of proteins) were incubated with 50 μl of Dynabeads Protein G (Invitrogen) and primary antibody (anti-rabbit-Nrf2 or anti-rabbit-Keap1, diluted 1:50 vol/vol; Cell Signaling Technology, CST) for 20 min at room temperature under constant shaking. The immunoprecipitates were washed three times with cold PBS, and the mixture was boiled with SDS sample buffer. Immunoprecipitates were separated on a 10%SDS-PAGE and GSTP was recognized by immunoblotting (GSTP1 mouse mAb, 1:1000, ALX-804-510, Enzo Life Sciences).

2.9. GSTP transfection in human HepaRG cells

HepaRG cells were transfected with Lipofectamine 2000 (Invitrogen) according to manufacturer's instructions. Briefly, 2.5×10^7 cells seeded into a 150-cm^2 tissue culture flask using complete William's medium, were incubated for 30 min at 37°C in the presence of 12 ml of Accutase I solution (Lonza) for detachment. The cell suspension transferred to 15 mL tubes were centrifuged at $300 \times g$ for 5 min at room temperature to remove Accutase I and resuspended in 1 ml of medium for final counting with trypan blue. For the transfection protocol, a suspension of 2.5×10^6 cells was centrifuged for 10 min at $250 \times g$ at room temperature and transferred to 75 ml flask containing 10 ml complete William's medium. The plasmids diluted in nuclease free water were mixed with 30 μl Lipofectamine 2000 in 1.0 ml Opti MEM reduced serum medium (ThermoFisher) and after incubation for 15 min at room temperature, the mixture was added to the HepaRG cells. After 48 h, the transfected cells were seeded into 6-well or 96-well plates (200000 and 10000 cells/well, respectively) and incubated in a humidified incubator and then treated with Ebselen for 24 h. Transfection efficiency was verified by immunoblot using a mouse anti-GSTP antibody.

2.10. Statistical analysis

All data are presented as the mean \pm SD. Statistical analysis was performed using GraphPad Prism 5 software (GraphPad Software, San Diego, CA, USA). Unpaired Student's t-tests or One-way ANOVA were used and $p < 0.05$ was considered statistically significant.

3. Results

3.1. Changes in the expression and subcellular localization of GSTP during HCC development

GSTP protein expression (assessed as monomeric form, i.e. 24 kDa band, by low-resolution PAGE and reducing conditions) and activity linearly increased during tumor development in DEN-HCC mice (Fig. 1 A and 1B, respectively). Also, the subcellular distribution of GSTP protein changed during the preneoplastic phase of liver tissue transformation (i. e., between month 3 and 7 of the observation period) with a significant migration of this protein into the nucleus observed at month 7 (Fig. 1C).

GSTP expression, enzymatic activity and subcellular localization were also modified in HepG2 and especially in Huh-7 hepatocarcinoma cells (Fig. 2), thus recapitulating the conditions observed in vivo in the DEN-HCC model (Fig. 1). These changes were absent in the non-tumoral cell line HepaRG (Fig. 2). Moreover, the selective inhibitor of GSTP enzymatic activity EZT, in HCC cells reduced cell viability inducing apoptosis (Fig. 2E). Worth of note, the apparent activity of EZT as pro-apoptotic agent was superior than, and synergized with, that of the antitumoral drug sorafenib, pointing out a specific role of GSTP inhibition in the apoptotic signaling of HCC cells, for example by the EZT-induced dissociation of GSTP from its inhibitory complex with JNK, a

protein kinase with key role in regulating the apoptotic and proliferation signaling of the cancer cell (mechanism described in more details in Ref. [22]).

3.2. The levels of protein glutathionylation (PSSG) and cellular GSH are increased in HCC

The modified expression and subcellular localization of GSTP protein, shown earlier in 3.1, were associated with increased levels of PSSG both in the liver tissue of DEN-HCC mice during tumor development (Fig. 1D) and in HCC cell lines (Fig. 2C), suggesting increased glutathionylase activity of GSTP (reviewed in Ref. [20]) during tumor development.

Also, HepG2 hepatocarcinoma cells showed higher cellular levels and reduced efflux of GSH compared to non-tumoral HepaRG cells (Fig. 2E), which was observed either under baseline conditions or after exposure to Ebselen, a seleno-organic drug with alkylating function and GSTP/Nrf2 activating properties [25]. These findings indicate a cancer-specific mechanism of sequestration for this GSTP substrate that may eventually sustain its increased glutathionylase activity in HCC.

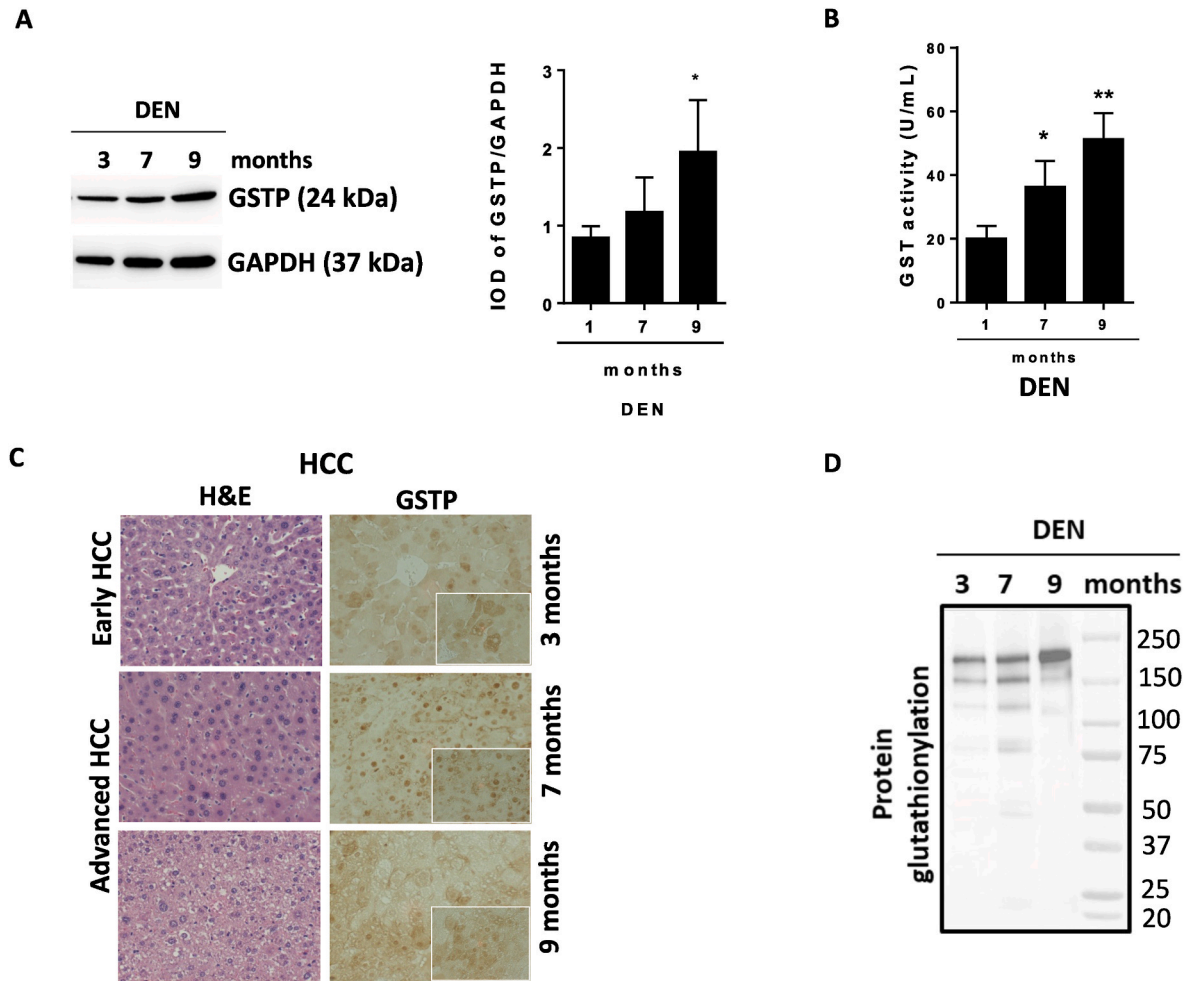


Fig. 1. Liver expression of GSTP and protein glutathionylation during tumor development in DEN-HCC mice. DEN-HCC mice were investigated after 3, 7 and 9 months of treatment with DEN or its vehicle (PBS) and tumor development was studied using the liver tumor marker HepPAR1 (Suppl. Figs. S1 and S2). (A) GSTP protein expression was studied by low-resolution SDS-PAGE separation and immunoblot in 100 µg of liver homogenate proteins exposed to complete denaturation by reducing agents and heating as described in the section Methods. (B) The same samples were also studied for GSTP enzymatic activity. (C) The tissue distribution of GSTP protein was investigated by immunohistochemistry and (D) protein glutathionylation was investigated in liver homogenate samples by immunoblot. Data were expressed as mean and standard deviation (SD) of experiments run in triplicate. T-test: *p < 0.05; **p < 0.001.

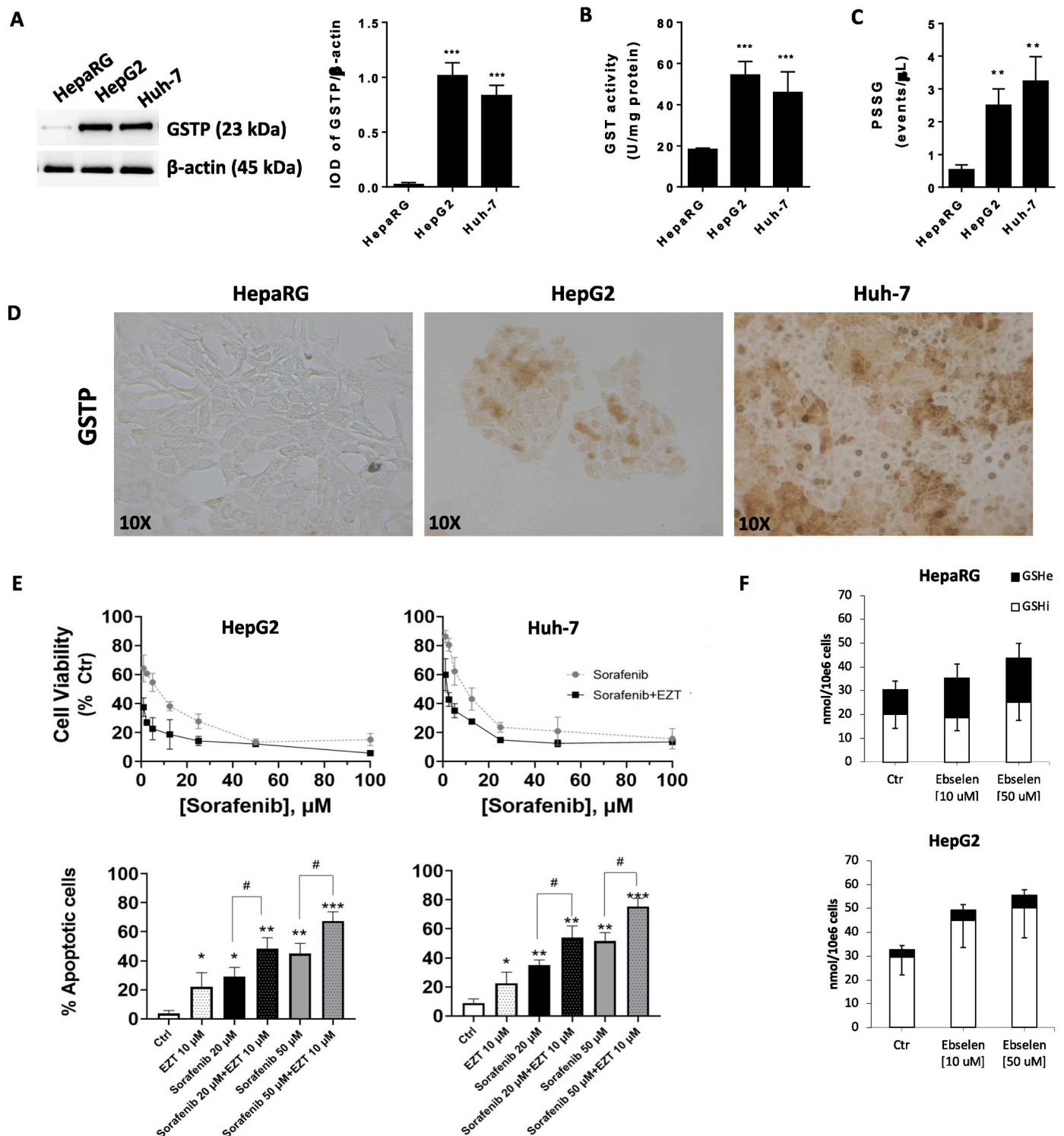


Fig. 2. GSTP expression, subcellular localization, and activity in human hepatocarcinoma cells (HepG2 and Huh-7) and in the non-tumoral liver cell line HepaRG. (A) GSTP protein expression, (B) enzymatic activity and (C) protein glutathionylation levels (a surrogate indicator of GSTP function as GSH-dependent chaperone of cellular proteins). (D) subcellular localization of GSTP as assessed by immunohistochemistry. (E) Effect of ezatiostat (EZT), a selective inhibitor of GSTP activity, and the anticancer drug sorafenib on cell viability and apoptosis of HepG2 and Huh-7 human hepatocarcinoma cells; treatments lasted for 72 h. (F) Cellular and extracellular levels of GSH (as GSHi and GSHe, respectively) were measured by HPLC in HepaRG and HepG2 cells at baseline and after treatment with Ebselen, a seleno-organic compound with alkylating properties and GSTP induction effects (E). Immunoblot and immunohistochemistry data were representative of three independent experiments. Bar chart data were as mean values \pm SD of three independent experiments. T-test or One way ANOVA: Ctr vs all treatment (* p < 0.05; ** p < 0.01; *** p < 0.0001); Sorafenib vs Sorafenib + EZT # p < 0.01.

3.3. HCC is associated with increased levels of expression of the Nrf2 transcription factor and its regulatory proteins

GSTP expression depends on the activity of different transcription factors, including Nrf2, the AP-1 complex and NFkB [14,19], which investigated in this study. Compared to control animals, DEN-HCC animals showed higher levels of hepatic Nrf2 (Fig. 3A) and GSTP (Figs. 3B and 1A), as well as of the Nrf2 inhibitory proteins Keap1 and β -TrCP (Fig. 3A). Worth of note, β -TrCP is a protease active in the glycogen synthase kinase 3 (Gsk3)-mediated and Keap1-independent degradation of Nrf2 [29], thus indicating a generalized activation, but limited efficacy, of the negative feedback systems for this transcriptional protein in DEN-induced HCC.

Nrf2 protein expression and nuclear translocation were also increased in the hepatocarcinoma cell lines HepG2 and Huh-7 compared to HepaRG cells, and the same was observed for the Nrf2 protease β -TrCP (Fig. 4A and B).

Also, the liver extracts of DEN-HCC mice showed increased levels of c-Jun protein (Fig. 3A), a component of the transcriptional complex AP-1 which is known to bind a specific consensus sequence of GSTP promoter region thus activating the expression of this gene during the exposure to alkylating agents [13,25]. The same was for NFkB transcription factor (Fig. 5) that is at the same time involved in GSTP transcription and modulated by the redox chaperone function of GSTP (reviewed in Ref. [23]).

3.4. GSTP co-immunoprecipitates with Nrf2 and Keap1 in the liver extract of DEN-HCC mice

Previous studies demonstrated that GSTP expression influences Nrf2 activity in different cell models [24–26]; these include HepG2 HCC cells in which co-immunoprecipitation experiments indicated that such an effect may depend on a physical (protein-protein) interaction between the chaperon GSTP and Nrf2; in fact, the co-immunoprecipitation was enhanced by the exposure to alkylating Se-organic compounds and GSTP protein oligomerization [26]. In this study we investigated whether such physical interaction can be revealed by the same immunoprecipitation strategy in vivo in the mouse liver during HCC development. The results demonstrate that GSTP coimmunoprecipitates with Nrf2 in the liver extract of both the control and DEN-HCC mice (Fig. 3B), and important enough, PAGE separation of GSTP protein carried out under reducing conditions, revealed the presence of both the monomeric and dimeric

forms of this protein in the co-immunoprecipitate as well as in the unbound fraction of the pooled liver extracts (Fig. 3B).

GSTP oligomerization appears to play a central role in its chaperone function [22]. Consequently, we further explored the physical state of GSTP during its interaction with Nrf2. To this end, non-reducing (native) conditions were utilized to prepare the proteins of the immunoprecipitation fractions for high-resolution gradient PAGE and immunoblot analysis (Fig. 5). For these immunoprecipitation experiments, the liver homogenates of DEN-HCC animals obtained at 9-month time point were pooled in subgroups considering the levels of the monomeric form of GSTP (24 kDa) observed in individual animals (Fig. 3A); this identified two subgroups labelled in Fig. 5 as low-GSTP and high-GSTP, respectively. The different expression of this monomeric form suggested the presence of some interindividual variability in the oligomerization pattern of this protein during HCC development that were confirmed by the immunoblot analysis performed under reducing conditions (Fig. 5A). In the co-immunoprecipitation fraction of HCC animals with high levels of monomeric (24 kDa) GSTP, the dimeric form was present at lower levels compared to control animals, whereas the low-GSTP subgroup showed an intermediate situation. The latter subgroup also presented a minor band corresponding to an oligomeric form resistant to the β -mercaptoethanol induced reduction of intermolecular bondings, with MW of approx. 75–100 kDa, which is compatible with the tetrameric form of GSTP subunits. This form was also present in the control group. The monomeric and dimeric forms largely predominate in the unbound fraction of high-GSTP animals being scarce in both the low-GSTP subgroup and control group.

The implementation of non-reducing conditions (Fig. 5C and D) demonstrated the involvement of oligomeric and multimeric forms of GSTP (assessed in Fig. 5C) in the interaction with Nrf2 (shown in Fig. 5D). These were observed as bands localized in the MW range between 75 and 100 kDa – apparently corresponding to the tetrameric form that show higher levels in DEN-HCC compared to control group – and at MW \geq 245 kDa which is compatible with a multimeric state of the protein present at the interface between the separating and stacking gel (Fig. 5C). Other oligomeric forms represented by two bands localized in the MW range 120–150 kDa were detected in the unbound fraction; these were more represented in the high-GSTP subgroup compared to other groups.

These findings demonstrate that the oligomerization state of GSTP can vary in vivo during HCC development and that only specific oligomers or multimers of GSTP co-immunoprecipitate with Nrf2 in DEN-

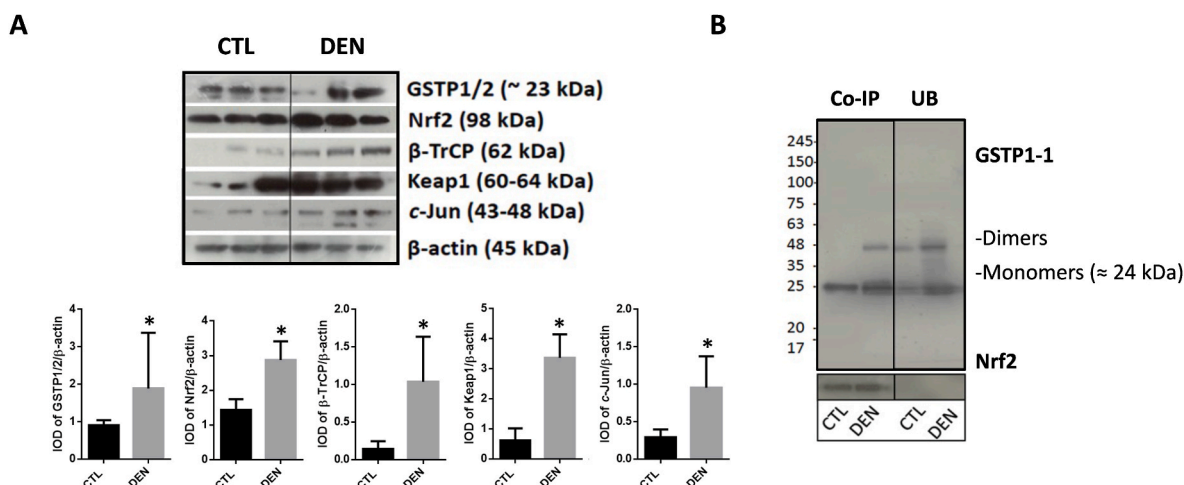


Fig. 3. Liver expression of Nrf2-related proteins and c-Jun transcription factor, and Nrf2-GSTP coimmunoprecipitation in DEN-HCC mice. (A) immunoblot analysis of Nrf2, its inhibitory proteins Keap1 and β -TrCP, and the transcription factor c-Jun. (B) Nrf2 immunoprecipitation was carried out to assess GSTP co-immunoprecipitation (Co-IP). The protein extracts were prepared from tumor masses isolated from DEN-HCC mice at 9-month time point and studied by immunoblot after complete denaturation and separation by gradient SDS-PAGE as described in the section Methods. UB = unbound fraction collected after IP. Bar chart data were mean values \pm SD of experiments run in triplicate. T-test: CTL vs DEN, * p < 0.05.

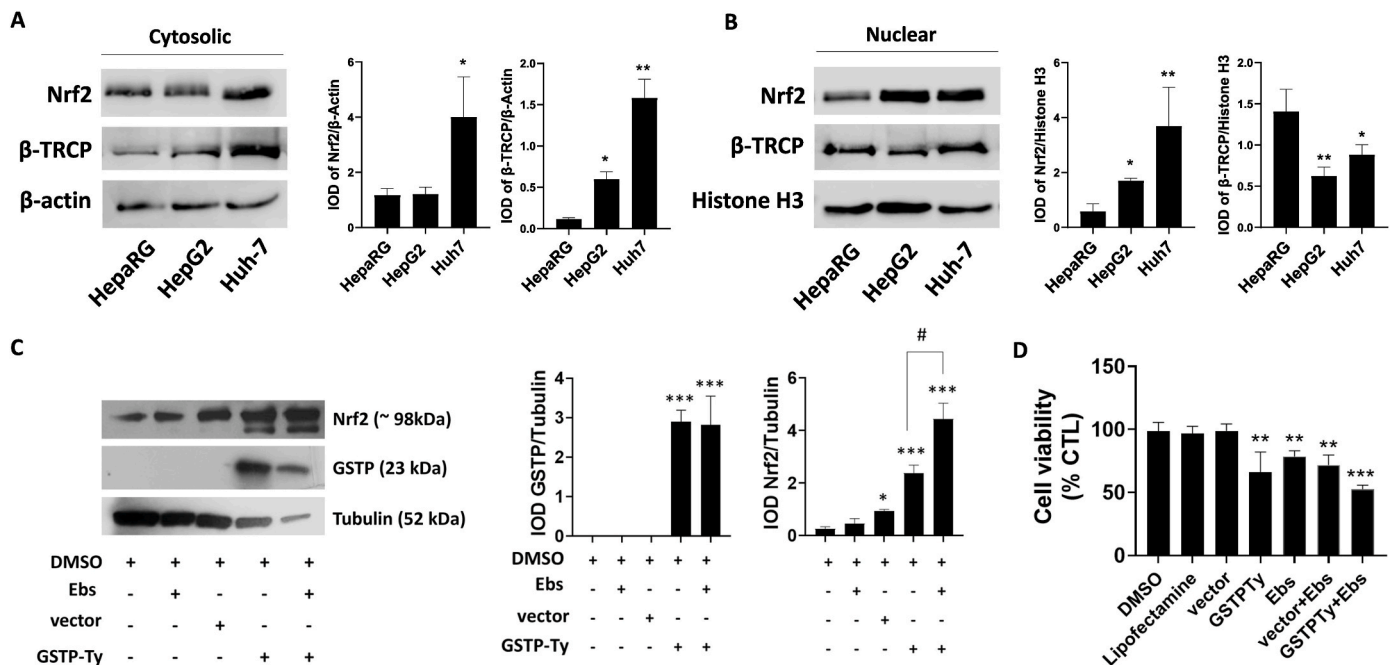


Fig. 4. Cytosolic and nuclear expression of Nrf2 and β -TRCP in HCC cells and effect of GSTP transfection on Nrf2 expression in nontumoral HepaRG cells. Nrf2 and β -TRCP expression were studied by immunoblot in HepG2 and Huh-7 human hepatocarcinoma cells and in non-tumoral HepaRG cells. Protein expression was assessed in both the cytosolic (A) and nuclear (B) fractions of the cell lines as described in the section Methods. Human GSTP1-1 gene was transfected by Lipofectamine 2000 in HepaRG cells and total Nrf2 expression (C) and cell viability (D) were studied by immunoblot and MTT test, respectively. Data were mean \pm SD of three independent experiments. T-test: HepaRG vs hepatocarcinoma cells, * $p < 0.05$; ** $p < 0.001$.

HCC and control animals.

It is worth considering the possibility that the observed interindividual variability in the forms of GSTP oligomerization of HCC animals may depend on the heterogeneity of response to DEN treatment (Suppl. Fig. S1B). However, these differences did not affect tumorigenesis and the trend of GSTP protein to oligomerize that were both confirmed to occur in all the DEN-treated animals (discussed in the section Methods), as it was for the changes of the other molecular markers investigated in this study, including Nrf2, its inhibitory proteins and c-Jun transcription factor (Fig. 3A).

Also, GSTP was found to co-immunoprecipitate with the Nrf2 inhibitory protein Keap1 (Fig. 5B). This protein-protein interaction was already described in literature as a potential Nrf2 activation mechanism by GSTP-mediated Keap1 protein glutathionylation and dissociation from the Nrf2/Keap1 inhibitory complex [28]. Unfortunately we studied this coimmunoprecipitation under reducing conditions only, and within such conditions GSTP was found in the coimmunoprecipitate of both the experimental groups mainly in its monomeric form, while the dimeric form of GSTP was retrieved in the unbound fraction.

3.5. GSTP gene transfection increases Nrf2 expression in HepaRG cells

Besides physical interactions with Nrf2 and its inhibitory protein Keap1 presented earlier, a functional interaction of GSTP with Nrf2 activity was documented through both GSTP gene ablation and pharmacological induction studies and it was demonstrated to involve the nuclear translocation of Nrf2, its self-transcription response and the induction of Nrf2-dependent genes including those of Cys/GSH metabolism [25]. In the present study, the functional response to a modification of GSTP expression was investigated for the first time in human liver cells stably transfected with human GSTP1-1 gene; such transfection resulted in a marked increase of both GSTP and Nrf2 protein expression (Fig. 4C), confirming the increased activity of this transcription factor. In GSTP transfected cells, Nrf2 protein expression was further enhanced by the treatment with Ebselen, and important enough

is the observation that the presence of an extra copy of GSTP in these cells synergized with this alkylating agent in reducing cell viability (Fig. 4D). Unfortunately, we cannot study the transfection of GSTP in HepG2 or Huh-7 cells since such gene manipulation was not tolerated by these HCC cells (not shown).

4. Discussion

GSTP is a GSH-dependent drug metabolism (phase 2) enzyme and an oncogene with proposed role in hepatocarcinogenesis [16–18]. Its induction in HCC and other forms of cancer is highly regulated and our data indicate that such a regulation can be replicated in the DEN-induced model of liver carcinogenesis [30].

Accordingly, we demonstrate that GSTP induction in the mouse liver affects GSTP protein expression, that linearly increased during DEN-induced carcinogenesis (Fig. 1A–B), while the subcellular distribution of this protein changes to a prevalent nuclear localization during the pre-neoplastic phase of mouse liver transformation (7-month time point, Fig. 1C). Increased nuclear levels of GSTP protein were also observed in HCC cell lines and particularly in Huh-7 cells that express high levels of GSTP (Fig. 2). In these cells the selective GSTP inhibitor EZT synergized with the anticancer drug sorafenib to induced apoptotic cell death, thus confirming the important role of GSTP overexpression in HCC survival.

These findings indicate that the induction and cellular redistribution of GSTP protein are involved in the carcinogenesis program of the liver cell.

GSTP gene induction in HCC could involve different transcription factors including Nrf2 [27]. Other transcription factors with consensus sequences in the promoter region of GSTP include the AP-1 complex [13, 25] and NFkB [31]. These transcriptional proteins coordinate the expression of this GSH-dependent gene with that of many other genes with important role in many physiological processes as well as in sustaining the tumor microenvironment ([14,19] and references therein). According to this multifactorial regulation, we found that all these GSTP-related transcription factors were upregulated in the liver of

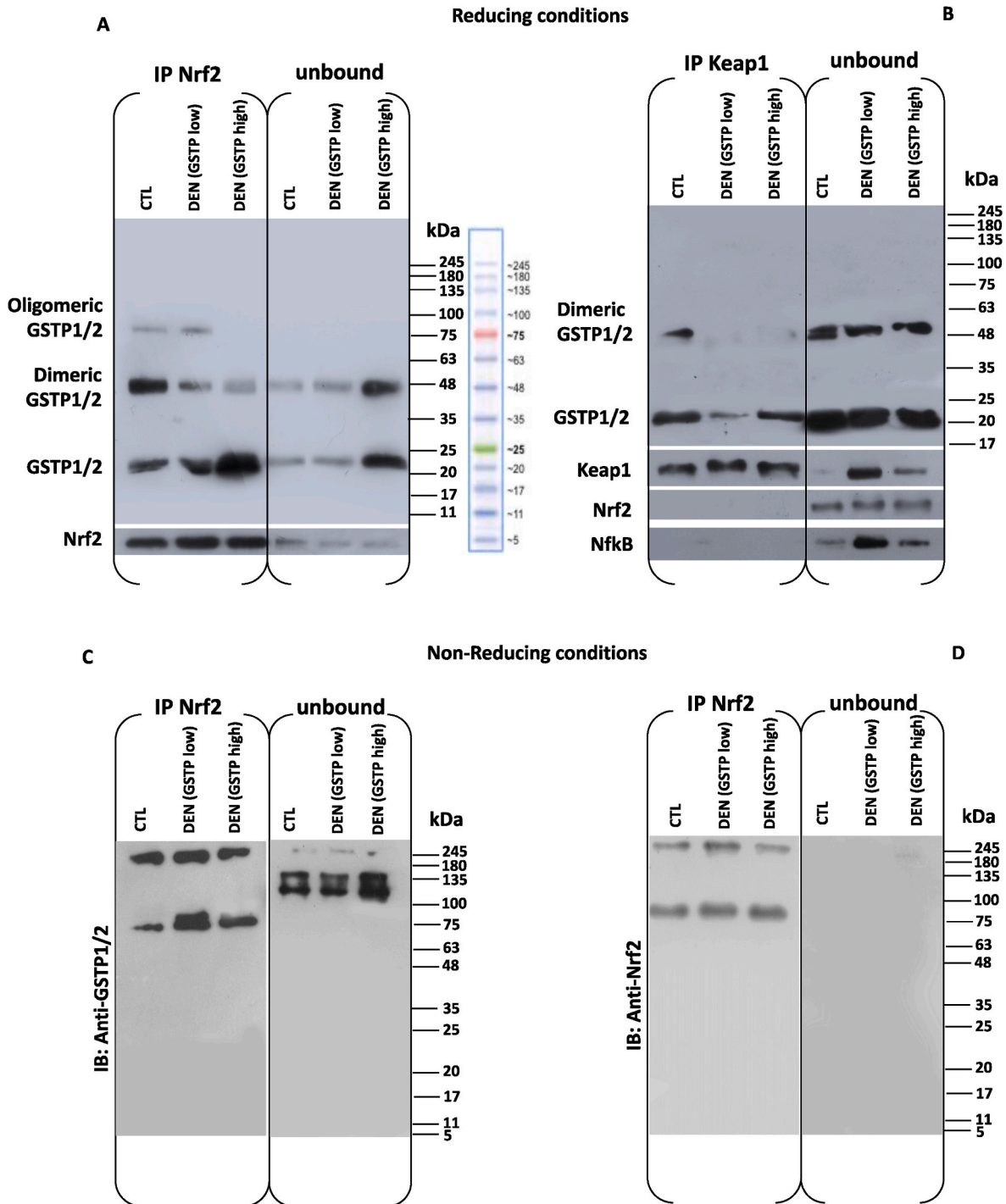


Fig. 5. Immunoblot of coimmunoprecipitate and unbound fractions of Nrf2 (A) and Keap1 (B) immunoprecipitation experiments carried out in the liver of DEN-HCC mice. Proteins were studied either under reducing (panels A and B) or non-reducing conditions (C and D), i.e. by omitting β -mercaptoethanol from the protein preparation buffer utilized for PAGE separation. Liver extracts of DEN-HCC mice obtained at 9-month timepoint were processed for immunoprecipitation after pooling samples in two subgroups according with the levels of the monomeric form of GSTP (identified with GSTP low and GSTP high labels). 20 μ g of proteins from the coimmunoprecipitation and unbound fractions were separated in large slab-gels and transblotted to nitrocellulose sheets for immunoblot analysis of (A, C and D) GSTP and Nrf2 in Nrf2 immunoprecipitation experiments (IP Nrf2), and (B) GSTP, Keap1, Nrf2 and NfκB in Keap1 immunoprecipitation experiments (IP Keap1).

DEN-HCC mice.

Since these transcription factors migrate into the nucleus to exert their activity, the observed nuclear translocation of GSTP during carcinogenesis may suggest a role for this protein in coordinating their response to transcriptional stimuli, including carcinogens with pro-oxidant properties as DEN and Ebselen that were investigated in this study. The chaperone function of GSTP protein can justify this role at the

mechanistic level; binary interactions of GSTP have been documented for the Nrf2 inhibitory protein Keap1 [28], and for the NFκB inhibitory proteins IκBα and IKKβ [31], as well as for a number of other proteins involved in the cancer-specific changes of the cell cycle regulation, ER stress response and cell death mechanisms, and of inflammatory and multidrug resistance mechanisms stimulated by alkylating agents, some of which are utilized in the chemotherapy of HCC [21–23]. In these

protein-protein interactions, GSTP can influence the activity of other proteins acting as a simple physical interactor, but in most of them, a role as redox chaperone has been documented for this protein. Its glutathionylase activity promotes the glutathionylation of critical Cys residues on several proteins under conditions of oxidative and nitrosative stress as well as during carcinogenesis to influence important cellular functions as energy metabolism, proliferation, and death programs [20, 22]. Accordingly, the levels of hepatic PSSG increased in a time-dependent manner during DEN-induced carcinogenesis thus following the same trend of GSTP protein expression and activity, and the same association between increased levels of PSSG and GSTP expression was observed in vitro in HCC cell lines (Figs. 1 and 2, respectively).

Previous work by some of us suggested that *GSTP* gene and protein expression influence the transcriptional activity of Nrf2 in different cell types and that GSTP and Nrf2 coimmunoprecipitate in HepG2 human HCC cells [26]. These findings together suggested that a direct physical interaction could stand behind a reciprocal (bidirectional) regulation mechanism of these proteins [25,26]. Present results appear to confirm such a mechanism. The transfection of non-tumoral HepaRG cells with *hGSTP1-1* gene (to our knowledge, the first gene manipulation procedure of this type reported in human hepatocytes) resulted in increased levels of expression for both GSTP and Nrf2 proteins (Fig. 4), thus

demonstrating the role of *GSTP* induction in the transcriptional activation of Nrf2 in the liver cell; important enough, the presence of a GSTP extracopy and its effect on Nrf2 activation reduced cell viability in these cells with an effect that synergized with that of the alkylating (thiol-reacting) agent Ebselen.

Previously, we also described that the ablation of *Nrf2* gene only partially affects GSTP expression, being compensated by other transcription factors such as c-Jun, an AP-1 complex component with key role in carcinogenesis, cancer cell metabolism and multi-drug resistance [13]. In DEN-HCC, both Nrf2 and c-Jun proteins showed increased levels of expression thus suggesting converging effects of these stress response transcription factors in the induction of *GSTP* and other cancer genes important for the HCC microenvironment and its redox reprogramming (Fig. 6a). Also, these aspects suggest a prevalent position of *GSTP* gene regulation and GSTP chaperone function in the peaking-order of oncogenic events that cause and maintain these changes of the cellular redox, including Nrf2 transcriptional activation.

Mechanistically, the binary interaction with Nrf2 protein is a main requisite to confirm this chaperone function of GSTP in HCC; here we demonstrate that the coimmunoprecipitation of GSTP with Nrf2 protein, previously observed in HCC cells [26], also occurs in vivo in the mouse liver, with some differences that characterized the control and DEN-HCC groups (Figs. 3B and 5A); immunoblot data indicate that oligomeric and

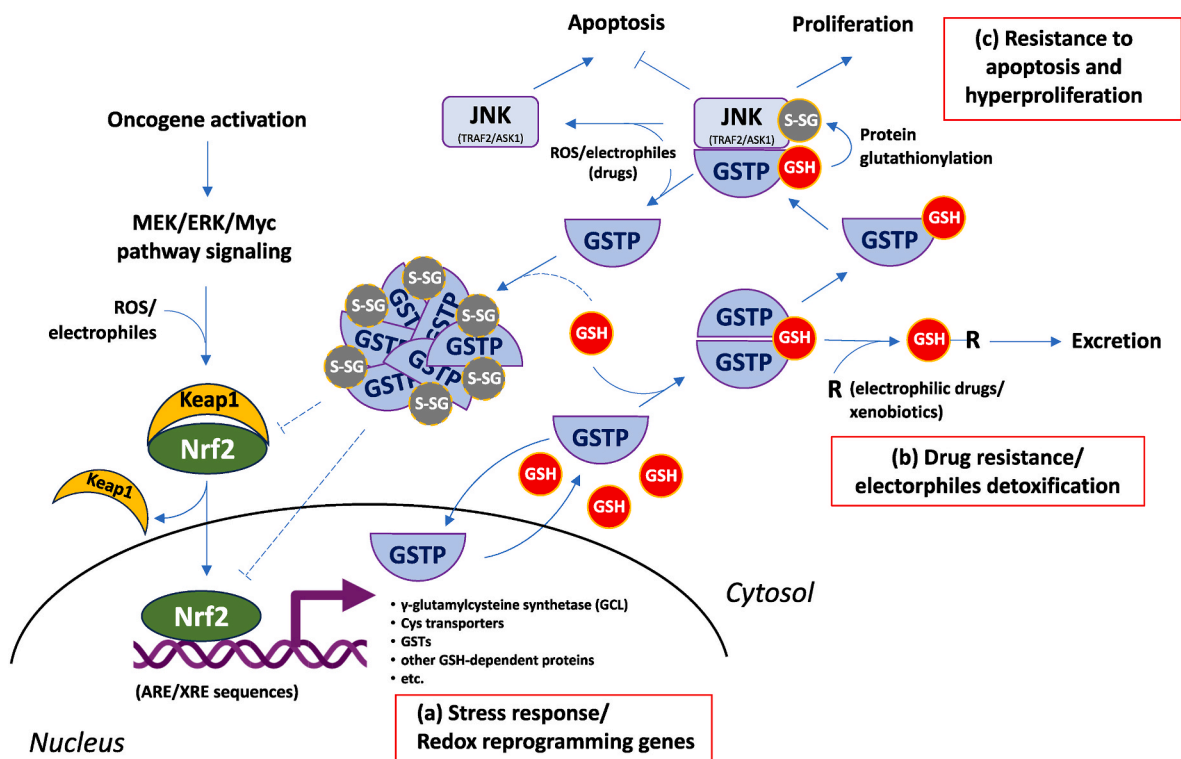


Fig. 6. Overexpression, oligomerization and interaction function of GSTP in HCC. GSTP expression is increased in HCC by the enhanced activity of Nrf2 and other transcription factors (for example c-Jun) that respond to oncogene activation and increased signaling of pro-survival protein kinases such as MEK/ERK. GSH biosynthesis is also induced by this cancer-dependent Nrf2 activation effect, ultimately leading to (a) a redox reprogramming effect and increased capability to respond to cellular stressors and electrophilic drugs through different antioxidant and detoxification pathways. (b) These include the enzymatic conjugation to GSH of cellular metabolites and xenobiotic drugs with electrophilic properties. This is a phase 2 drug metabolism reaction process catalyzed by this other GST isoenzymes, important to reduce electrophiles toxicity while increasing their solubility and excretion. (c) Also, GSTP overexpression increases the cellular availability of this protein to exert its chaperone function and glutathionylase activity toward several proteins with important cancer promoting activity; these include for example protein kinases as JNK and ASK1 with key role in hyperproliferation and resistance to apoptotic cell death, transcription factors as STAT3, NFkB/IKK and the Nrf2/Keap1 system described in this study, antioxidant proteins as Prx6, unfolded protein response and ER stress proteins and many others [reviewed in 14,19,22,23,28]. The high cellular levels of GSH observed in the HCC cell maintain a reduced environment with high levels of monomeric GSTP loaded with GSH to be transferred to partner proteins during complex formation. This explains the higher levels of glutathionylation observed for the cellular proteins of HCC cells and DEN-HCC liver. During the exposure to pro-oxidant stimuli, such as ROS or electrophilic anticancer drugs, for example Se-organic alkylating agents [26], and in vivo in the mouse liver (present study) GSTP oligomerization is observed as a process that favors the coimmunoprecipitation of this protein with Nrf2, suggesting a chaperone function also for the oligomeric forms of GSTP. The observed nuclear translocation of GSTP in HCC suggests that this chaperone function and the other roles of the overexpressed GSTP may also occur in the nucleus as important events in HCC progression.

multimeric forms of GSTP are primarily involvement in this protein-protein interaction. Interesting enough, the levels of the monomeric and oligomeric forms of GSTP found in the liver extracts of the different DEN-HCC animals can vary together with the MW of the oligomers/multimers identified in the immunoprecipitate studied under non-reducing conditions, i.e. omitting β -mercaptoethanol during protein samples preparation for PAGE separation and transblotting, and only some oligomeric/multimeric forms of GSTP co-immunoprecipitate with Nrf2, namely those in the MW range between 75 and 100 kDa and others with MW \geq 250 kDa, whereas oligomers in the MW range 100–135 kDa did not (Fig. 5C). This suggests that GSTP oligomerization and interaction with Nrf2 are specific and regulated process in HCC.

In this respect, GSTP oligomerization has been proposed to be part of its chaperone and glutathionylase function in cancer cells (reviewed in Refs. [20,22]); for example, in cancer cells high levels of GSTP1 and other GST isoforms, such as GST-A1 and GST-M1, may serve the purpose of enhancing JNK sequestration into multimolecular complexes that maintain this pro-apoptotic kinase in its inactive form. This may represent a mechanism of resistance to apoptotic cell death in which GSTP1 and other GSTs directly interacts with JNK to inhibit its kinase function or indirectly modulate the JNK pathway interacting with upstream kinases such as TRAF2 and ASK1 [22]. The GSTP-JNK inhibitory complex can be disrupted by the exposure of cancer cells to oxidative stress or drugs that alter the cellular redox, leading to GSTP oligomerization and consequent activation of JNK signaling (Fig. 6).

Apparently, the monomeric form of GSTP is primarily involved in JNK binding and inhibition [32] as well as in binary interactions with other cellular proteins to promote their glutathionylation [20], and this forms remains in equilibrium with the dimeric form that is catalytically active and responsible for the GSH-dependent detoxification of xenobiotics and cellular electrophiles (Fig. 6b); whereas, tetramers and forms of higher complexity are produced during the exposure to conditions of cellular oxidative stress, producing at the same time GSTP enzyme inactivation and the redox-dependent dissociation of the GSTP-JNK complex [32]. The propensity of GSTP monomers to oligomerize under pro-oxidant conditions appears to be an intrinsic feature of this protein confirmed both in cell free experiments and during the exposure of cancer cells to different types of electrophiles, including some prostanoids [33] and alkylating drugs [26]. A pro-oxidant environment may also increase GSTP protein glutathionylation [22 and references therein] on cys47 and cys101, which acts as an oligomer switch enhancing protein multimerization while impairing tiolase activity [34]. Recently, GSTP protein overexpression and oligomerization have also been demonstrated to occur in vivo in human leukocytes of patients affected by end-stage renal disease in the presence of increased levels of cellular oxidative stress and reduced Nrf2 protein expression [35].

Therefore, available data suggest that the monomeric and dimeric (reduced and active) forms of GSTP, and possibly of other GSTs, address its chaperone and enzymatic function to pathways different from those of the oligomeric and multimeric (inactive and oxidized) forms. Depending on the cellular redox, GSTP can distribute between the dimeric form which can dissociate to monomeric to heterodimerize and transfer a GSH molecule to other proteins that beside JNK include other stress response proteins with key role in the control of cell proliferation and death mechanisms (Fig. 6c), such as peroxiredoxin 6, TRAF2, ASK1 and many others [14,20,22,36], whereas the oligomeric or multimeric (inactive) forms produced under pro-oxidant conditions, for example during Se-compound treatment [26], appear to interact with Nrf2 and its inhibitory protein Keap1 (Fig. 5 and [28]).

Consistent with this interpretation, in HepG2 hepatocarcinoma cells the upregulation of GSTP and Nrf2 proteins, and the increased formation of PSSG (discussed earlier), are associated with an increased biosynthesis and cellular sequestration of GSH (Fig. 2E); this effect on cellular GSH was further enhanced by the treatment with Ebselen, an alkylating agent with GSTP/Nrf2 activating properties [25]. Such conditions are expected to influence not only the glutathionylase activity of GSTP, but

also the capacity of GSTP to oligomerize and physically interact with Nrf2 to modulate its transcriptional activity and redox-reprogramming effects in HCC cells. In this respect we hypothesize that the interacting properties of GSTP on the Nrf2/Keap1 system may have the role of driving the cancer cell to higher levels of resistance to cellular stressors, including anticancer therapies [37], while avoiding reductive stress and allostatic load effects of an excess of Nrf2 activation [38] (Fig. 6).

5. Conclusions

GSTP overexpression and nuclear translocation are both observed as characteristic events of DEN-induced carcinogenesis and are confirmed to occur in human HCC cells in association with significant changes of the cellular redox by increased levels of GSH and PSSG. Our results demonstrate that these changes promote a physical and functional interaction between GSTP and Nrf2. This transcription factor important in liver carcinogenesis, was activated by hGSTP1-1 transfection in human hepatocytes and co-immunoprecipitated with GSTP protein in the liver of DEN-HCC mice. The Nrf2 inhibitory protein Keap1, which is confined in the cytosol, is also involved in this interactome and apparently the conditions that promote such interactions in DEN-HCC and in HCC cells [26], are the induction of GSTP protein expression and its molecular reorganization to form specific oligomers and multimers.

Although these findings cannot straightforwardly be extended to human HCC, they may represent a starting point to shed more light on the molecular aspects and carcinogenic role of GSTP induction in this liver cancer.

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Institutional review board statement

The animal study protocol was approved by the Institutional Committee on the Ethics of Animal Experiments of the Marche Polytechnic University, Ancona, Italy (authorization number 411/2016-PR).

Informed consent statement

“Not applicable.”

CRediT authorship contribution statement

Desirée Bartolini: Writing – original draft, Methodology, Investigation, Formal analysis, Data curation, Conceptualization. **Anna Maria Stabile:** Investigation, Formal analysis. **Anna Migni:** Investigation, Formal analysis. **Fabio Gurrado:** Writing – original draft, Formal analysis, Data curation. **Gessica Lioci:** Writing – original draft, Formal analysis, Data curation. **Francesca De Franco:** Investigation, Formal analysis, Conceptualization. **Martina Mandarano:** Writing – original draft, Formal analysis, Data curation. **Gianluca Svegliati-Baroni:** Writing – original draft, Visualization, Supervision, Investigation, Conceptualization. **Manlio Di Cristina:** Investigation, Formal analysis, Conceptualization. **Guido Bellezza:** Methodology, Investigation, Formal analysis, Conceptualization. **Mario Rende:** Visualization, Supervision, Conceptualization. **Francesco Galli:** Writing – review & editing, Writing – original draft, Validation, Supervision, Project administration, Methodology, Data curation, Conceptualization.

Declaration of competing interest

The authors declare the following financial interests/personal relationships which may be considered as potential competing interests: Francesca De Franco is employee of TES Pharma, Srl, but her contribution to this work was independent from this company's projects and

business, being only based on personal collaboration with this group of academic authors to coordinate cell biology and molecular biology experiments of this study. 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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Abbreviations

ANOVA	Analysis of Variance
AP-1	Activator protein 1
BSA	bovine serum albumin
CDNB	2,4-Dinitrochlorobenzene
c-Jun	Jun proto-oncogene, a component of the heteromeric AP-1 transcription factor complex
DAB	3,3'-diaminobenzidine
DEN	N-nitrosodiethylamine
DMEM	Dulbecco's Modified Eagle's Medium
EZT	ezatiostat
FBS	Fetal bovine serum
GSH	glutathione
GSK3	glycogen synthase kinase 3
GSTP	Glutathione S-transferase P
HCC	hepatocellular carcinoma
JNK	c-Jun N-terminal kinase
Keap1	Kelch-like ECH-associated protein 1
MAPK-ERK	mitogen-activated protein kinase - extracellular signal-regulated kinase
MEM	Modified Eagle's Medium
MW	Molecular weight
NADPH	nicotinamide adenine dinucleotide phosphate hydrogen
NAFLD	non-alcoholic fatty liver disease
NEAA	Non-essential amino-acids
NFkB	Nuclear factor kappa B
Nrf2	nuclear factor erythroid 2-related factor 2
PSSG	protein glutathionylation
SDS	sodium dodecyl sulphate
β-TrCP	beta-Transducin repeat-containing protein

Appendix A. Supplementary data

Supplementary data to this article can be found online at <https://doi.org/10.1016/j.abb.2024.110043>.

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