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CELLULAR AND MOLECULAR GASTROENTEROLOGY AND HEPATOLOGY

ORIGINAL RESEARCH

Nrf2 Mutation/Activation Is Dispensable for the Development of Chemically Induced Mouse HCC

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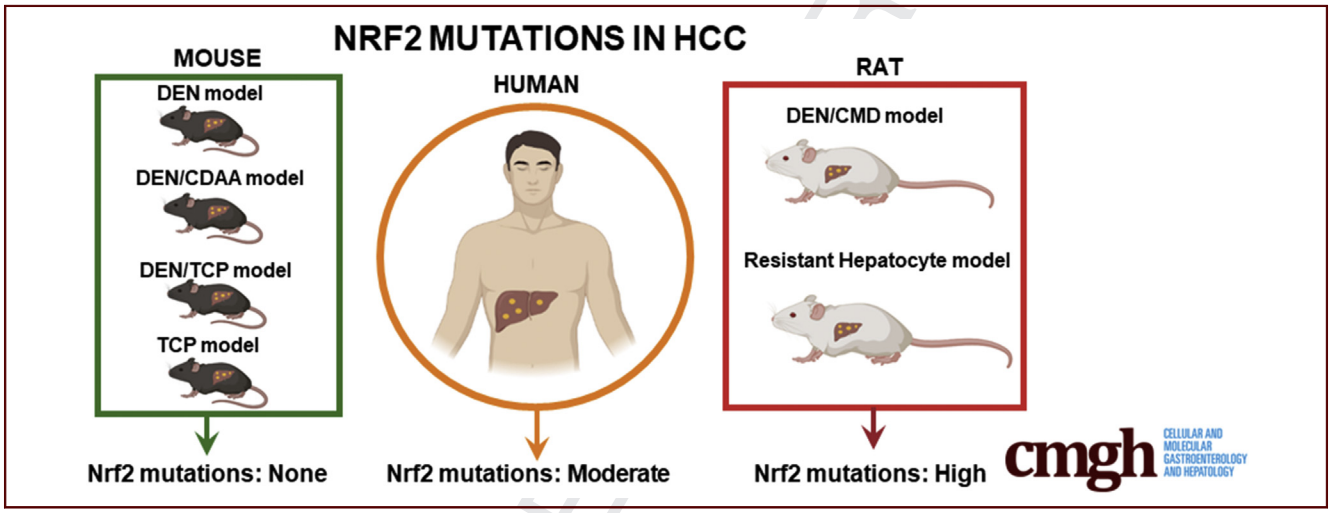

SUMMARY

Unlike human and rat hepatocellular carcinoma, chemically induced mouse hepatocellular carcinomas do not show *Nrf2* mutation/activation. Furthermore, metabolic reprogramming of neoplastic cells is absent as well. The results suggest that the mouse is not the ideal model to investigate the role of nuclear factor (erythroid-derived 2)-like 2 in hepatocarcinogenesis.

BACKGROUND & AIMS: Activation of the kelch-like ECH-associated protein 1 (Keap1)–nuclear factor (erythroid-derived 2)-like 2 (Nrf2) pathway has been associated with metabolic reprogramming in many tumors, including hepatocellular carcinoma (HCC). However, the contribution of *Nrf2* mutations in this process remains elusive. Here, we investigated the occurrence of *Nrf2* mutations in distinct models of mouse hepatocarcinogenesis.

METHODS: HCCs were generated by experimental protocols consisting of the following: (1) a single dose of diethylnitrosamine (DEN), followed by repeated treatments with the nuclear-receptor agonist 1,4-bis-[2-(3,5-dichloropyridyloxy)] benzene; (2) repeated treatments with 1,4-bis-[2-(3,5-dichloropyridyloxy)]benzene alone; (3) a single dose of DEN followed by exposure to a choline-deficient L-amino acid–defined diet; and (4) a single dose of DEN with no further treatment. All of these protocols led to HCC development within 28–42 weeks. Activation of the Keap1-Nrf2 pathway was investigated by analyzing the presence of *Nrf2* gene mutations, and the expression of *Nrf2* target genes. Metabolic reprogramming was assessed characterized by evaluating the expression of genes involved in glycolysis, the pentose phosphate pathway, and glutaminolysis.

RESULTS: No *Nrf2* mutations were found in any of the models of hepatocarcinogenesis analyzed. Intriguingly, despite the described cooperation between β-catenin and the Nrf2 pathway, we found no evidence of Nrf2 activation in both early dysplastic nodules and HCCs, characterized by the presence of up to 80%–90% β-catenin mutations. No HCC metabolic reprogramming was observed either.

CONCLUSIONS: These results show that, unlike rat hepatocarcinogenesis, *Nrf2* mutations do not occur in 4 distinct models of chemically induced mouse HCC. Interestingly, in the same models, metabolic reprogramming also was minimal or absent, supporting the concept that Nrf2 activation is critical for the switch from oxidative to glycolytic metabolism. *(Cell Mol*

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Keywords: Nrf2; Ctnnb1; Gene Mutation; Metabolic Reprogramming; HCC.

The knowledge of the genetic/epigenetic alterations implicated in hepatocellular carcinoma (HCC), one of the leading causes of cancer-related deaths worldwide,[1] is still fragmentary. Among the several modifications involved in HCC development, those involving *NRF2* (encoded by *NFE2L2*) are of particular interest, because the role of the kelch-like ECH-associated protein 1–nuclear factor (erythroid-derived 2)-like 2 (KEAP1–NRF2) pathway in cancer progression has provided conflicting results.[2–4] NRF2 is a master transcriptional activator of genes encoding enzymes that protect cells from oxidative stress and xenobiotics, and of various drug efflux pump members of the multidrug resistance protein family.[5] NRF2 is negatively regulated and targeted to proteasomal degradation by KEAP1.[6–9] Several studies have reported that point mutations in *KEAP1* or *NRF2* genes often are present in primary tumors.[10–15] Regarding human HCC, 2 studies performing whole-exome sequencing have shown mutations of either *NRF2* (6.4%) or *KEAP1* (8%), suggesting that the dysregulation of this pathway may play a relevant role in a subset of human HCCs.[16–18] Notably, increased levels of *NRF2* messenger RNA (mRNA) have been found to be associated with poor prognosis in human HCC.[19]

Although activation of this pathway has been shown in a variety of models of rodent hepatocarcinogenesis, the impact of Nrf2 mutations on this process remains elusive. Indeed, although a striking incidence of gene mutations has been found in preneoplastic and neoplastic rat hepatocytes,[20–22] in mouse models the oncogenic role of Nrf2 was instead attributed to its increased nuclear translocation and transcriptional activity, as a consequence of the accumulation of p62 it is binding to Keap1, and consequent release of Nrf2.[23–26]

This study aimed to investigate whether Nrf2 mutation/activation occurs in distinct mouse models of hepatocarcinogenesis. In addition, because it recently was reported that in human HCCs characterized by β-catenin mutations the KEAP1–NRF2 pathway is activated independently from mutations of these genes,[27,28] we also wished to determine whether *Nrf2* mutations could co-occur in β-catenin mutated HCC. Finally, we also investigated whether metabolic reprogramming, a hallmark of cancer cells, takes place in these experimental models. The results showed the complete absence of *Nrf2* mutations in all the examined murine models of hepatocarcinogenesis. They also indicated that the Keap1–Nrf2 pathway is not activated in *Ctnnb1* mutated HCCs, as well as in other models of mouse HCC. Finally, the analysis of the expression of genes involved in glycolysis, pentose phosphate pathway (PPP), and glutamine metabolism did not show any clear evidence of metabolic reprogramming.

Results

Nrf2 Mutations Are Absent in HCCs Generated by Diethylnitrosamine + 1,4-Bis-[2-(3,5-Dichloropyridyloxy)]Benzene or 1,4-Bis-[2-(3,5-Dichloropyridyloxy)]Benzene Alone

Previous studies have shown that a single treatment with diethylnitrosamine (DEN) followed by repeated treatments with 1,4-bis-[2-(3,5-dichloropyridyloxy)]benzene (TCPOBOP), the most potent agonist of constitutive androstane receptor and a nongenotoxic mouse hepatocarcinogen, or TCPOBOP alone induced HCC development in 100% of mice and that 81 and 90% of HCCs, respectively, showed *Ctnnb1* mutations.[29] Chronic treatment with TCPOBOP is associated with liver dysplasia and enhanced oxidative stress, although the proteins responsible for the increased oxidative stress have yet to be identified.[30] Because activation of Nrf2 represents one of the major antioxidant defense mechanisms leading to increased transcription of several cytoprotective enzymes and enhanced cell survival,[6,8,9] we investigated whether activation of the Keap1–Nrf2 pathway could be involved in HCC development in mice.

Our previous studies have shown that rat preneoplastic and neoplastic lesions induced by protocols of chemical hepatocarcinogenesis have a very high incidence of Nrf2 mutations.[20–22] Therefore, we initially investigated the presence of gene mutations in laser-microdissected mouse HCCs developed 28 weeks after a single dose of DEN followed by repeated treatments with TCPOBOP (experimental protocol 1). Microscopically, these tumors showed features typical of HCC such as atypic nuclei, cellular pleomorphism, and mitoses, as well as cell death (Figure 1*A*). Selected areas of livers from mice treated with TCPOBOP alone or untreated mice also were included in the analysis as control groups. Because published works indicate that most of the *Nrf2* mutations occur in exon 2,[31] we sequenced this exon in 20 HCCs using Sanger fluorescence–based sequence analysis. Quite unexpectedly, no mutation of *Nrf2* was observed in any of the 20 examined HCCs generated by the treatment consisting of DEN + TCPOBOP (Table 1), as well as in control livers or livers from mice treated with TCPOBOP alone for 28 weeks.

Next, we analyzed Nrf2 mutations in HCCs generated after 42 weeks of repeated treatment with TCPOBOP in the absence of DEN (experimental protocol 2). Similar to what was observed in HCCs obtained with the DEN + TCPOBOP protocol, these tumors were characterized by nuclear

Abbreviations used in this paper: **CDAA, choline-deficient L-amino acid–defined diet; cDNA, complementary DNA; DEN, diethylnitrosamine; HCC, hepatocellular carcinoma; Keap1, kelch-like ECH-associated protein 1; mRNA, messenger RNA; Nrf2, nuclear factor (erythroid-derived 2)-like 2; PPP, pentose phosphate pathway; qRT-PCR, quantitative reverse-transcriptase polymerase chain reaction; TCPOBOP, 1,4-bis-[2-(3,5-dichloropyridyloxy)]benzene.**

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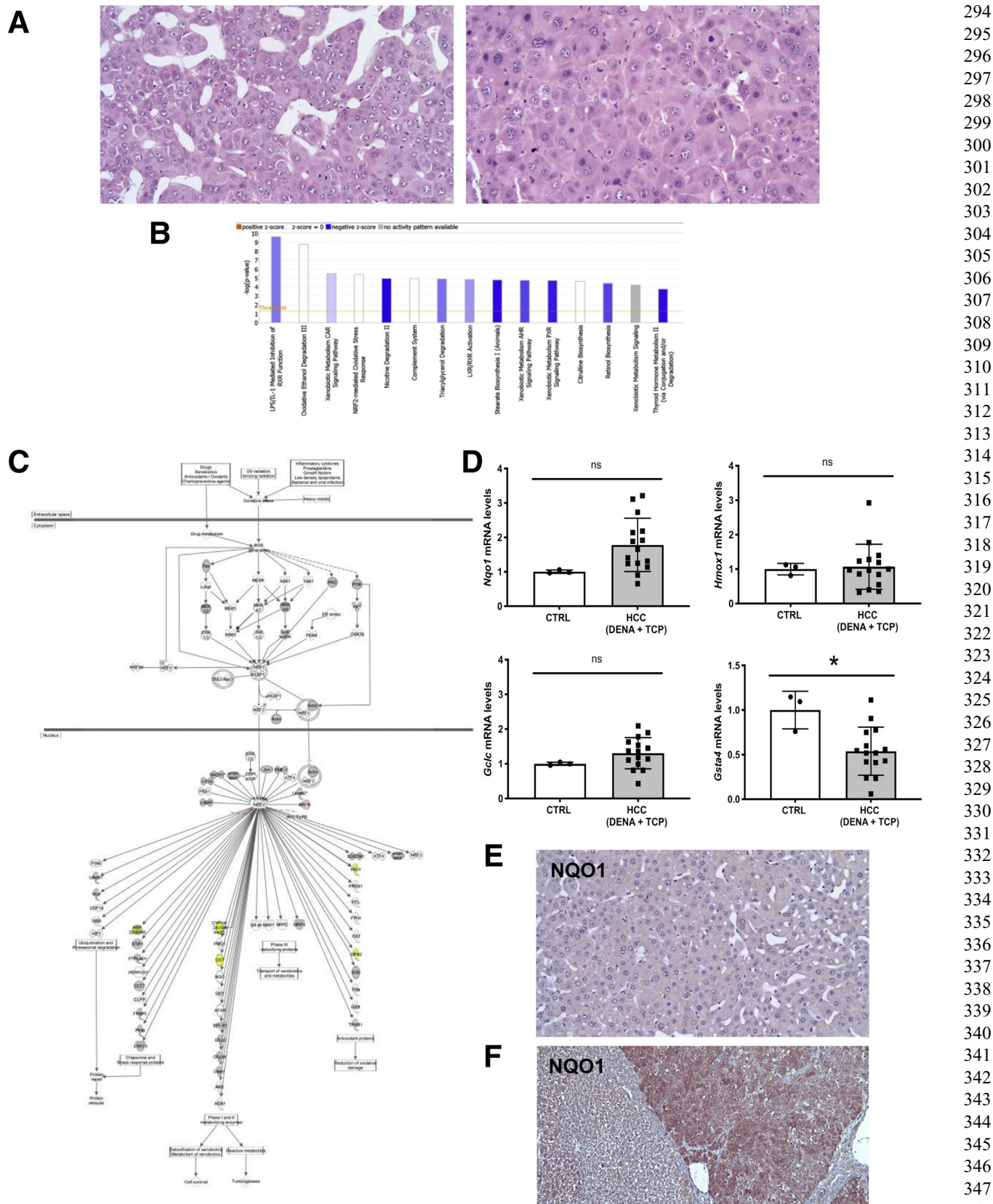
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LPS/IL-1 Mediated Inhibition of RXR Function
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Nicotine Degradation III
Complement System
Triacylglycerol Degradation
LXR/RXR Activation
Stearate Biosynthesis I (Animals)
Xenobiotic Metabolism AHR Signaling Pathway
Xenobiotic Metabolism PXR Signaling Pathway
Citrulline Biosynthesis
Retinol Biosynthesis
Xenobiotic Metabolism Signaling
Thyroid Hormone Metabolism II (via Conjugation and/or Degradation)
C
D
Nqo1 mRNA levels
Hmox1 mRNA levels
Gclc mRNA levels
Gsta4 mRNA levels
ns
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CTRL
HCC (DENA + TCP)
E
NQO1
F
NQO1

Table 1. Analysis of Nrf2 Mutations in Different Experimental Settings

Treatment	Strain	Sex	Time of death	Tumors n,	*Nrf2* mutations, N	*Ctnnb1* mutations, N
DEN + TCPOBOP	C3H	F	28 weeks	20	0	81% (Ref. 29)
TCPOBOP	C3H	F	42 weeks	11	0	90.9% (Ref. 29)
DEN + CDAA	C57BL	M	25 weeks	10	0	ND
DEN	B6C3F1	F	40 weeks	11	0	ND

CDAA, choline-deficient L-amino acid–defined diet; DEN, diethylnitrosamine; TCPOBOP, 1,4-bis-[2-(3,5-dichloropyridyloxy)] benzene.

atypia, pleomorphism, and increased mitotic activity (Figure 2*A*). As shown in Table 1, no Nrf2 mutation was found in these HCCs.

These results indicate that unlike preneoplastic and neoplastic lesions developed in rat livers after DEN treatment, mouse HCCs generated by the 2 aforementioned protocols are completely devoid of *Nrf2* mutation.

The Keap1–Nrf2 Pathway Is Not Activated in β-Catenin Mutated HCCs

Recent work on the mouse and human HCCs characterized by β-catenin mutations has shown that the Keap1–Nrf2 pathway is activated independently from *NRF2* mutations, suggesting cooperation between the oncogenic β-catenin and Nrf2 pathways in *Ctnnb1*-mediated HCC tumorigenesis.[27,28] Because HCCs generated by DEN + TCPOBOP or TCPOBOP alone display from 80% to 90% of *Ctnnb1* mutations,[29] we wished to assess whether activation of the Keap1–Nrf2 pathway could occur concomitantly with β-catenin mutation, but independently from *Nrf2* mutation.

Because sustained activation of the Keap1–Nrf2 pathway in the absence of NRF2 mutations already has been reported in animal models of rat hepatocarcinogenesis,[20,22] we examined such a possibility in tumors induced by DEN + TCPOBOP or TCPOBOP alone. To verify the status of the Keap1–Nrf2 pathway, we analyzed the mRNA profiling of these HCCs and their respective controls. As previously reported,[29] hierarchical clustering analysis of gene expression patterns performed on these HCCs did not identify any significant difference between HCCs generated by a genotoxic agent DEN and those caused by repeated administration of TCPOBOP alone. With regard to the Keap1–Nrf2 pathway, functional analysis of differentially expressed genes did not identify the Nrf2-mediated oxidative stress response among the most affected pathways. Moreover, when IPA was applied to investigate the Nrf2 target genes, the results showed that the expression of most of the Nrf2 target genes were not affected or even down-regulated (Figures 1*B* and *C* and 2*B* and *C*).

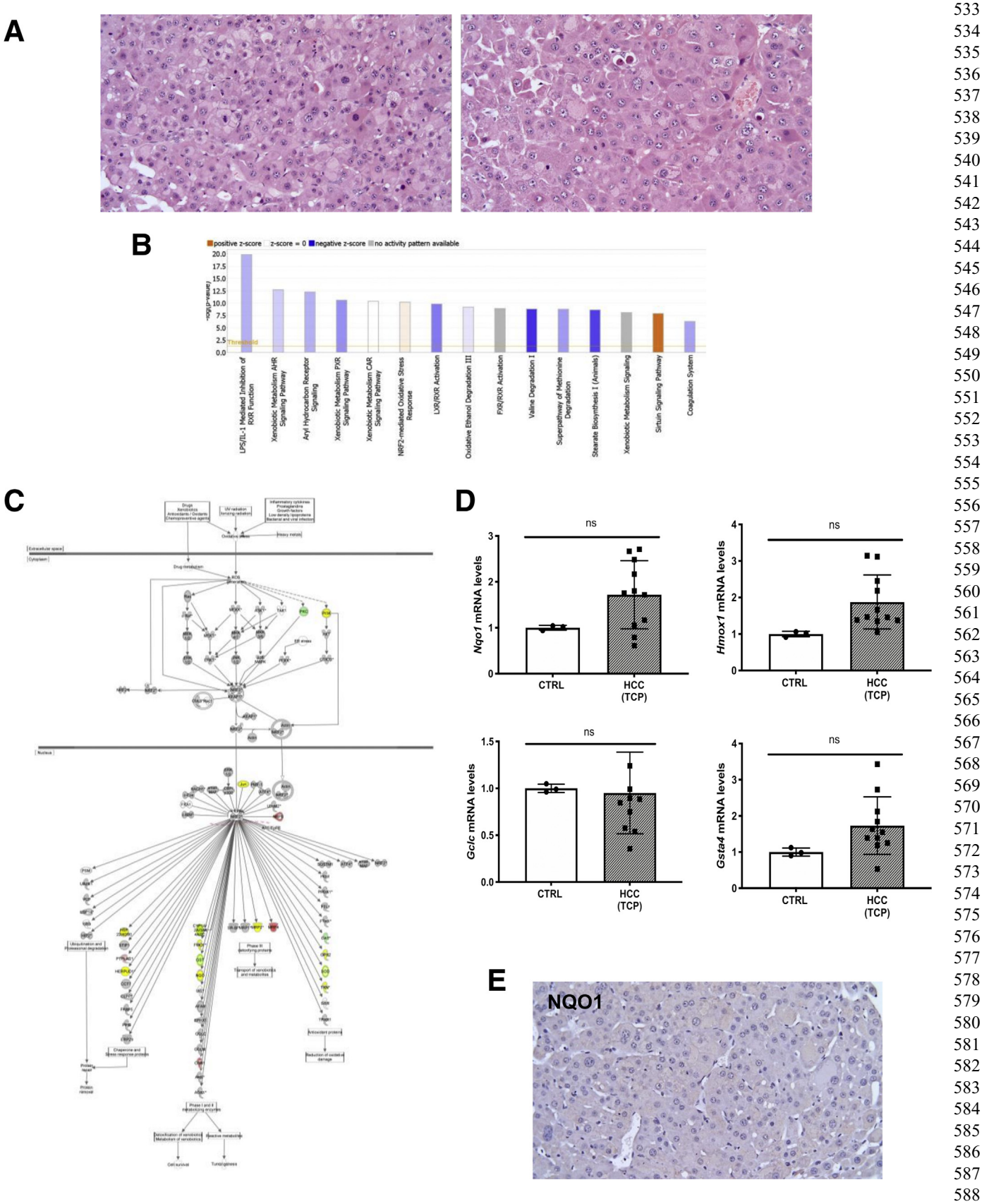
Next, to validate the transcriptomic profiling data we performed quantitative reverse-transcriptase polymerase chain reaction (qRT-PCR) analysis on the same complementary DNA (cDNA) used for gene sequencing. The results showed that among the examined Nrf2 target genes (*Nqo1*, *Hmox1*, *Gclc*, *Gsta4*), *Gsta4* was the only one significantly up-regulated in tumors compared with control livers (Figure 1*D*). Along the same line, no significant change in the expression of any of the same Nrf2 target genes was observed in HCCs generated by repeated treatment with TCPOBOP in the absence of DEN (Figure 2*D*). Lack of Nrf2 activation in mouse HCCs was confirmed by immunohistochemical analysis of NQO1, the best known target gene of Nrf2, showing that neoplastic mouse hepatocytes were completely negative (Figures 1*E* and 2*E*). Conversely, intense NQO1 immunostaining was observed in rat hepatocytes from nodules previously shown to carry Nrf2 mutation and activation of the Keap1–Nrf2 pathway (Figure 1*F*).[20]

The Keap1–Nrf2 Pathway Is Not Activated in β-Catenin Mutated Dysplastic Nodules

Our previous studies reported that activation of the Keap1–Nrf2 pathway occurs as early as in preneoplastic nodules induced in rats by protocols of chemical

Figure 1. (***See previous page***). **Histologic and molecular analyses of HCCs of DEN + TCPOBOP-treated mice.** (*A*) Microphotographs showing HCCs generated in mice treated with a single dose of DEN, followed by repeated treatment with TCPOBOP and killed 28 weeks thereafter (H&E: left, ×20; right, ×40). (*B*) Enriched functional pathways in HCCs vs TCP-treated livers. *P* values were determined using the Ingenuity scoring system. (*C*) Nrf2-mediated oxidative stress response pathway. Red, up-regulation; white, not modified; grey, no expression; yellow, genes whose expression was modified but did not pass the *P* value .05. (*D*) qRT-PCR analysis of *Nqo1*, *Hmox1*, *Gclc*, and *Gsta4* mRNA levels in 15 HCCs of mice treated as in panel *A*. In the HCC group, each *dot* corresponds to 1 tumor analyzed. Gene expression is reported as fold change compared with controls. The endogenous control gene mouse *Gapdh* was used for normalization. The results are expressed as means + SD. **P* < .05. (*E*) Representative image of a mouse HCC induced as in panel *A*. (*F*) Representative image of a rat preneoplastic nodule generated by the R-H model,[21] both stained with antibody anti-NQO1 (left, ×20; right, ×5). AHR, _; AKT, __; ASK1, ___; CAR, ____; CTRL, control; DENA, _____; ERK, _____; IL, interleukin; LPS, lipopolysaccharide; LXR, _____; MAPK, _____; MEK, _____; MEKK, _____; PI3K, _____; PKC, _____; PXR, _____; Ras, _____; ROS, reactive oxygen species; RXR, _____; TAK1, _____; TCP, TCPOBOP (1,4-bis-[2-(3,5-dichloropyridyloxy)]benzene).

hepatocarcinogenesis.[32] To rule out the possibility that the Nrf2 signaling pathway is activated during the early phase of tumorigenesis in mice, we investigated the Keap1–Nrf2 pathway in low- and high-grade dysplastic nodules generated 21 weeks after a single dose of DEN followed by weekly injections of TCPOBOP. At this time point, the



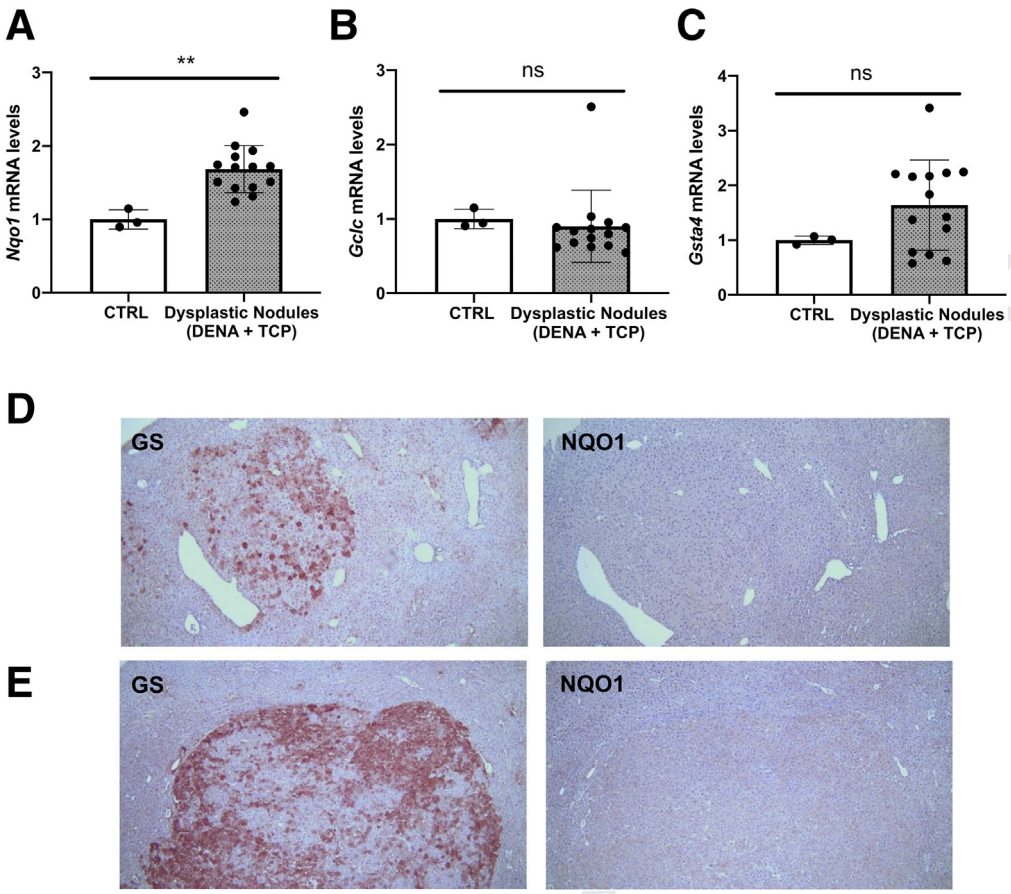


Figure 3. qRT-PCR analysis of *Nqo1*, *Gclc*, and *Gst4a* in dysplastic nodules. (*A–C*) qRT-PCR analysis of *Nqo1*, *Gclc*, and *Gst4a* in 14 dysplastic nodules of mice subjected to a single dose of DEN, followed by repeated treatments with TCPOBOP and killed 21 weeks thereafter. In the dysplastic nodules group, each *dot* corresponds to 1 nodule analyzed. Gene expression is reported as fold change relative to livers from untreated mice. Results are expressed as means ± SD. $^{**}P < .01$. (*D* and *E*). Serial sections of mouse liver showing low-grade (*upper panels*) and high-grade (*lower panels*) dysplastic nodules positive for glutamine synthetase (GS) and negative for NQO1 immunostaining (×5). CTRL, control; TCP, TCPOBOP (1,4-bis-[2-(3,5-dichloropyridyloxy)] benzene).

earliest neoplastic lesions are identifiable in the liver of mice subjected to this protocol.[29] For this purpose, we analyzed the expression of 3 well-known Nrf2 target genes (*Nqo1*, *Gclc*, and *Gsta4*). As already reported, 13 of 16 (81%) of these dysplastic nodules showed *Ctnnb1* mutation.[29] The results of qRT-PCR analysis did not show any clear evidence of activation of the Nrf2 pathway. Indeed, although Nqo1 expression was enhanced compared with control liver, albeit at a very low level, no significant change was observed for Gst4a and Gclc (Figure 3*A–C*). The lack of activation of the Nrf2 pathway was confirmed further by immunohistochemistry analysis, which showed a lack of Nqo1 staining in both low- and high-grade dysplastic nodules identified by their positivity to glutamine synthetase (Figure 3*D* and *E*).

Metabolic Reprogramming Does Not Occur in Mouse HCCs Subjected to DEN + TCPOBOP or TCPOBOP Alone

Nrf2 not only maintains redox homeostasis in quiescent cells, but also stimulates glucose consumption, PPP, and promotes the glutamine-utilizing reactions in the post–ribose-5-phosphate steps,[33] all suggestive of the metabolic reprogramming often associated with cancer cells.[34] However, whether Nrf2 activation is a sine qua non condition for metabolic reprogramming is unknown. Therefore, we wished to determine whether metabolic reprogramming was associated with neoplastic development in tumors lacking Nrf2 activation. Because enhanced glycolysis and activation of PPP are a common feature of cancer cells, we

Figure 2. (***See previous page***). **Histologic and molecular analyses of HCCs of mice subjected to repeated treatments with TCPOBOP alone.** (*A*) Microphotographs showing HCCs generated in the liver of mice exposed to repeated treatments with TCPOBOP and killed 42 weeks thereafter (H&E: left, ×20; right, ×40). (*B*) Enriched functional pathways in HCCs vs TCP-treated livers. *P* values were determined using the Ingenuity scoring system. (*C*) Nrf2-mediated oxidative stress response pathway. Red, up-regulation; green, down-regulation; white, not modified; grey, no expression; yellow, genes whose expression was modified but did not pass the *P* value .05. (*D*) qRT-PCR analysis of *Nqo1*, *Hmox1*, *Gclc*, and *Gsta4* mRNA levels in 11 HCCs of mice treated as in panel *A*. In the HCC group, each *dot* corresponds to 1 tumor analyzed. Gene expression is reported as fold change relative to livers from untreated mice. Results are expressed as means ± SD. (*E*) Representative image of a mouse HCC negative for Nqo1 immunostaining (×20). CTRL, control; TCP, TCPOBOP (1,4-bis-[2-(3,5-dichloropyridyloxy)]benzene).

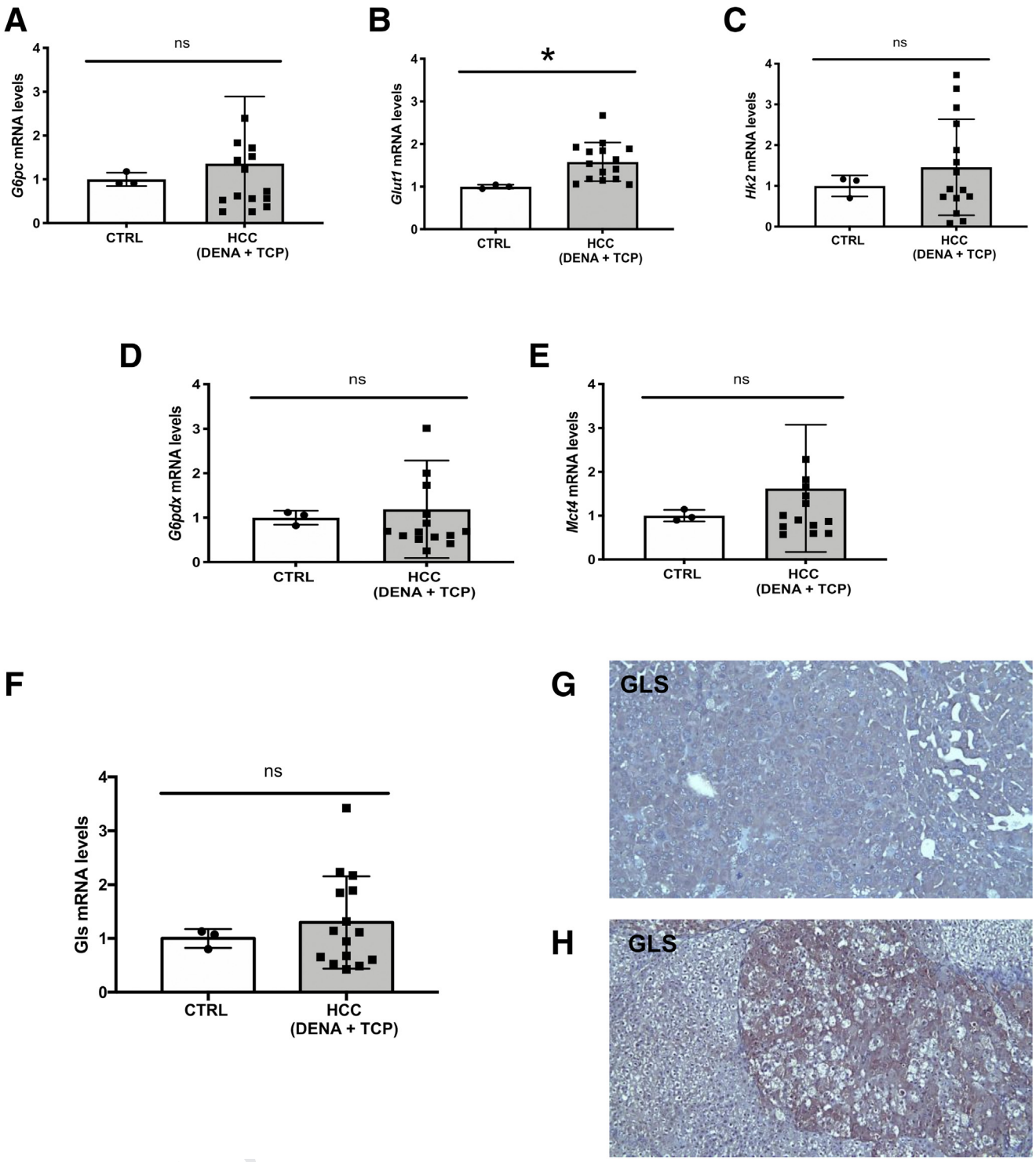


Figure 4. qRT-PCR analysis of *G6pc*, *Glut1*, *Hk2*, *G6pdx*, *Mct4*, and *Gls* in HCCs of mice treated with DEN and TCPOBOP. (*A–E*) qRT-PCR analysis of *G6pc*, *Glut1*, *Hk2*, *G6pdx*, and *Mct4* in 15 HCCs of mice treated with a single dose of DEN, followed by repeated treatment with TCPOBOP and killed 28 weeks after DEN treatment. In the HCC group, each *dot* corresponds to 1 tumor analyzed. Gene expression is reported as fold change relative to livers from untreated mice. Results are expressed as means ± SD. *$P < .05$. (*F*) qRT-PCR analysis of *Gls* in HCCs from mice treated as in panel *A*. Gene expression is reported as fold change relative to livers from untreated mice. Results are expressed as means ± SD. (*G*) Immunohistochemical analysis of a mouse HCC. (*H*) Immunohistochemical analysis of a rat preneoplastic nodule stained with an antibody antiglutaminase (left, ×20; right, ×5). CTRL, control; DENA, _____; GLS, _____; TCP, TCPOBOP (1,4-bis-[2-(3,5-dichloropyridyloxy)]benzene).

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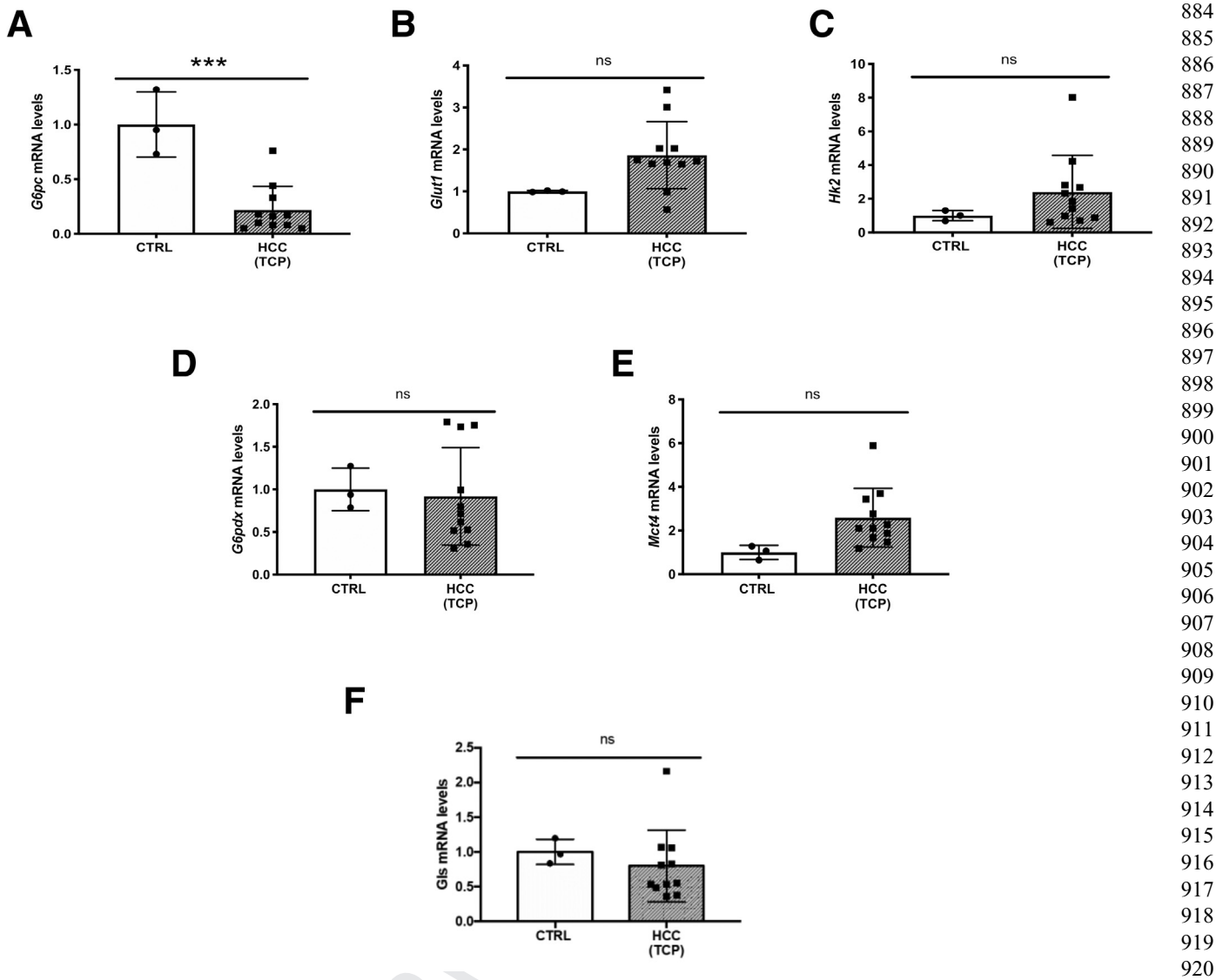

Figure 5. qRT-PCR analysis of *G6pc*, *Glut1*, *Hk2*, *G6pdx*, and *Mct4* in HCCs of mice treated with TCPOBOP alone. (*A–E*) qRT-PCR analysis of *G6pc*, *Glut1*, *Hk2*, *G6pdx*, and *Mct4* in 11 HCCs of mice subjected to repeated treatments with TCPOBOP and killed 42 weeks thereafter. Gene expression is reported as fold change relative to livers from untreated mice. Results are expressed as means ± SD. ***$P < .001$. (*F*) qRT-PCR analysis of *Gls* in 11 HCCs of mice treated as in panel *A*. In the HCC group, each *dot* corresponds to 1 tumor analyzed. Gene expression is reported as fold change relative to livers from untreated mice. Results are expressed as means ± SD. CTRL, control; TCP, TCPOBOP (1,4-bis-[2-(3,5-dichloropyridyloxy)]benzene).

investigated whether the expression of the glucose transporter 1 (Glut1), hexokinase 2 (Hk2), glucose-6 phosphate dehydrogenase (G6pdx) (the key limiting enzyme of the oxidative branch of PPP), and Mct4 (Slc16a3, responsible for lactate extrusion) were altered in HCCs induced by DEN + TCPOBOP. Because Nrf2 also is involved in glutamine metabolism and glutaminolysis,[33] we determined the expression levels of glutaminase (Gls). Finally, we also examined the expression of G6pc, the enzyme that catalyzes the hydrolysis of glucose-6-phosphate to glucose in the terminal step of glycogenolysis and whose deficiency leads to increased glycolysis. As shown in Figure 4*A–E*, only the expression of *Glut1*, but not that of *Hk2*, *G6pdx*, *G6pc*, or *Mct4*, was up-regulated significantly in HCCs generated by DEN + TCPOBOP. No change in the mRNA levels of *Glut1*, *Hk2*, and *Mct4* or *G6pdx* was found in tumors treated with TCPOBOP alone, although a significant decrease of G6pc mRNA levels was observed (Figure 5*A–E*).

Regarding glutaminolysis, we found that despite the increased expression of glutamine synthase previously found in HCC that developed in mice treated with DEN + TCPOBOP or TCPOBOP alone,[29] no change in the expression of mRNA levels of *Gls*, the enzyme generating glutamate from glutamine, was observed in HCCs generated by both of these experimental protocols (Figures 4*F* and 5*F*). Accordingly, no enhanced protein expression was found in neoplastic mouse hepatocytes by immunohistochemistry (Figure 4*G*), whereas Gls was strongly enhanced in rat preneoplastic hepatocytes (Figure 4*H*).

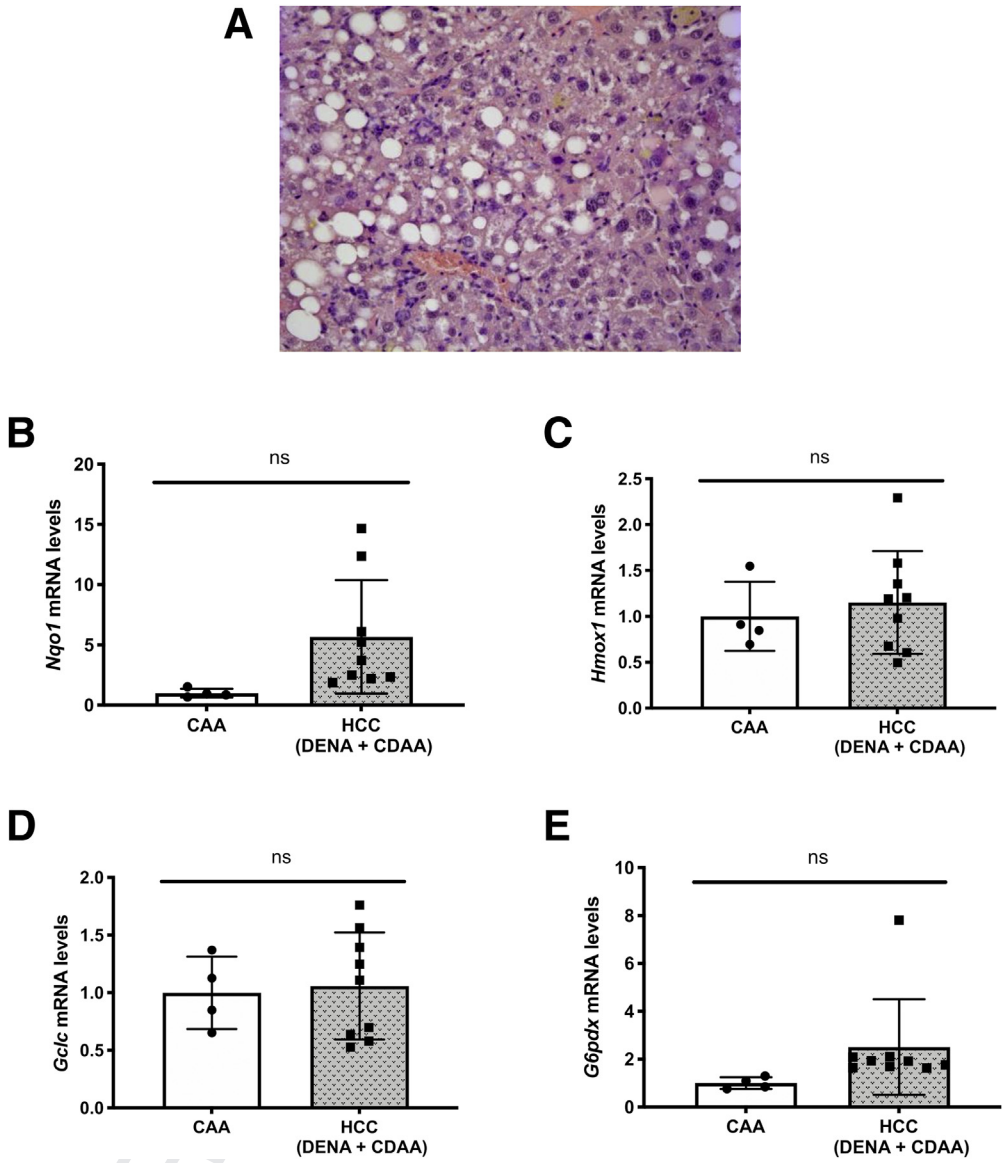

Figure 6. qRT-PCR analysis of *Nrf2* target genes in HCCs of mice treated with DEN and fed a CDAA diet. (*A*) Microphotograph showing a HCC generated in the liver of a mouse treated with a single dose of DEN, followed by a CDAA diet for 30 weeks (H&E, ×20). (*B–E*) qRT-PCR analysis of *Nqo1*, *Hmox1*, *Gclc*, and *G6pdx* mRNA levels in 9 HCCs of mice treated as in panel *A*. In the HCC group, each *dot* corresponds to 1 tumor analyzed. Gene expression is reported as fold change relative to livers from untreated mice. Results are expressed as means ± SD. CAA, _____; DENA, _____.

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Overall, these results indicate that metabolic reprogramming involving a shift toward glycolysis, PPP activation, and glutaminolysis does not take place in mouse HCCs developed by these animal models.

Nrf2 Mutations Are Absent in HCCs Generated by DEN + Choline-Deficient L-Amino Acid–Defined Diet

We envisaged the possibility that the lack of *Nrf2* mutations observed in mouse HCCs treated with TCPOBOP could be owing to some as yet unknown inhibitory effect of TCPOBOP on Nrf2-mutated hepatocytes. To test this hypothesis, we scored the presence of Nrf2 mutations in 10 HCCs generated by a single dose of DEN followed by feeding a choline-deficient L-amino acid–defined diet (CDAA) for 25 weeks (experimental protocol 3); notably, in rats the same regimen led to a high frequency of *Nrf2* mutations, ranging from 90% at the early stage of tumorigenesis to 25% in HCCs.[22] However, unlike rat HCCs, no *Nrf2* mutations could be observed in any of the examined murine tumors generated by a single dose of DEN followed by feeding a CDAA diet (Table 1).

The Keap1–Nrf2 Pathway Is Not Activated in HCCs From Mice Subjected to DEN + CDAA

To investigate whether Nrf2 activation still could take place in mouse HCCs generated by this protocol of hepatocarcinogenesis (Figure 6*A*), we analyzed the expression of Nrf2-target genes. As shown in Figure 6*B–E*, no change of the expression of the 4 examined Nrf2 target genes (*Nqo1*, *Hmox1*, *Gclc*, or *G6pdx*) was detected in the tumors, suggesting that the Keap1–Nrf2 pathway is not activated and therefore it does not exert a pivotal role in chemically induced mouse HCC.

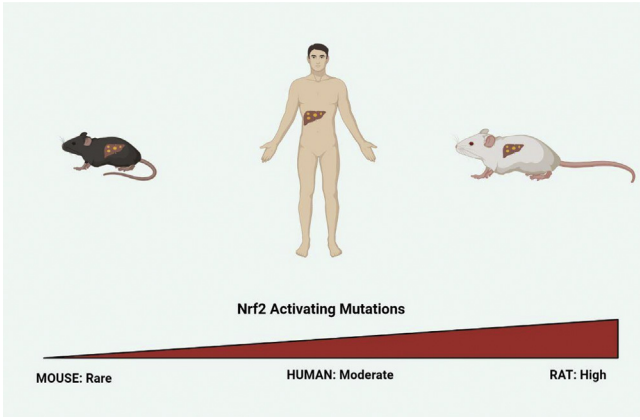

Figure 7. Frequency of Nrf2 mutations in human beings, rats, and mice.

Nrf2 Mutations Are Absent in HCCs Obtained by a Single Dose of DEN

It was reported that although *Nrf2* KO mice are completely refractory to DEN-induced HCC, tumors developed in wild-type mice by a single dose of DEN in the absence of any promoting procedure alone display 100% *Nrf2* mutation.[35] To rule out that promoting procedures of a different nature (TCPOBOP or CDAA) could somehow interfere with the expansion of *Nrf2* mutated cells, we scored mutations of this gene in mice in which HCC was induced by a single dose of DEN to weanling mice, without any further treatment (experimental protocol 4). However, none of the 11 HCCs induced by this protocol showed *Nrf2* gene mutations (Table 1).

Discussion

The Keap1–Nrf2 axis is a redox-sensitive signaling system, regulating up to 10% of human genes.[36] Activation of NRF2 not only protects cells from oxidative stress and DNA-damaging electrophiles, but it also confers cytoprotection against high levels of reactive oxygen species, leading to enhanced survival and resistance of cancer cells to chemotherapy.[37] The findings that NRF2 overexpression and/or somatic mutations of this gene take place in many human cancers, including HCC,[16,18] and that cancers with high NRF2 levels are associated with poor prognosis,[12,19] have unveiled an oncogenic role of NRF2. Further support to the oncogenic role of Nrf2 stems from the discovery that the genetic inactivation of *Nrf2* is sufficient to impair liver tumorigenesis in rats and mice.[21,35]

Among the several mechanisms responsible for Nrf2 activation, gene mutations have been described in approximately 6% of human HCCs, and in rats, since very early steps of the hepatocarcinogenic process, at a very high incidence,[16,18,20–22] suggesting their critical role in HCC development. Notably, in human HCC, *NRF2* mutations have been reported to occur concomitantly with β-catenin mutations.[16] In support of cooperation between the Keap1–Nrf2 pathway and β-catenin signaling, a very recent article reported an enrichment of the NRF2 program in human HCCs with CTNNB1 mutations, largely independent of NRF2 or KEAP1 mutations.[27] The same study also showed that mice with hepatocyte-specific oncogenic β-catenin activation increased Nrf2 activation, most likely to protect β-catenin–activated hepatocytes from oxidative damage, thus favoring tumor development, and proposed cooperation between oncogenic β-catenin signaling and the NRF2 pathway in CTNNB1-mediated HCC.[27] Furthermore, an independent work showed co-activation of β-catenin and NRF2 in 9% of all human HCCs, and discovered that co-expression of mutated *CTNNB1* with mutant *NRF2*, but not wild-type *NRF2*, led to rapid HCC development and mortality.[28] Thus, it appears that the frequency of Nrf2 mutations greatly vary among HCCs occurring in different species (Figure 7).

Quite surprisingly, the results of the present work showed a complete lack of *Nrf2* gene mutations in liver mouse tumors generated by 4 different experimental protocols. This finding was particularly unexpected because DEN, the chemical used in the present study to induce HCC, is a genotoxic carcinogen that, in rats, induces Nrf2 gene mutation in 90% of preneoplastic lesions and in 25% of HCCs.[22] In this context, it should be noted that *NRF2* mutations in rats occur in HCCs generated by a regimen almost identical to the experimental protocol 3 of the present study, consisting of a single dose of DEN followed by a choline-deficient diet.[22] Furthermore, in light of the recent works showing cooperation between β-catenin and Nrf2 in promoting liver cancer,[27,28] it is even more puzzling to find a complete absence of *Nrf2* mutation and/or activation of this transcription factor in HCCs developed by DEN + TCPOBOP or TCPOBOP alone that displayed up to 80%–90% of mutations of *Ctnnb1*.[29]

The reason why DEN-induced *Nrf2* mutations confer a selective advantage to initiated hepatocytes in rat, but not mouse, liver is unknown. Metabolic activation of DEN to its carcinogenic electrophiles, generation of promutagenic lesions in DNA (ie, O^6-EtG), and their fixation into daughter cells, are similar in both species, as shown by the high incidence of HCC induced in both rats and mice.[38,39] Nevertheless, the type of mutation caused by DEN in mouse tumors greatly varies depending on genetic background, age, sex, and other factors. Indeed, although the majority of liver tumors generated by a single dose of DEN are mutated in either the *Ha-ras* or the *B-raf* gene,[40] the prevalence of *Ha-ras* mutated tumors was significantly higher in the susceptible C3H and B6C3F1 mouse strains (39%–50%) than in the comparatively resistant C57BL mouse (7%). In contrast, B-raf mutated tumors were more frequent in C57BL mice (68%) than in the other 2 strains (17%–45%).[41] It also is worth mentioning that *Ha-Ras* and *B-raf* mutations are rare in human HCCs, whereas more relevant similarities with human HCC are shown by liver tumors generated by mouse experimental models consisting of a single application of DEN followed by promoting procedures, such as TCPOBOP or phenobarbital; indeed, these tumors display 80%–90% of *Ctnnb1* mutations,[29,42] a condition resembling human HCC in which mutations of this

gene were among the most frequent (32.8%).[16] Our present study showing a complete lack of Nrf2 mutations in 52 HCCs generated with 4 different models of mouse hepatocarcinogenesis, 3 distinct strains, both sexes, different age of the animals, and different doses of DEN, suggests that Nrf2 mutations are a rare event in mouse HCC, possibly because they do not provide any selective growth advantage to chemically induced initiated mouse hepatocytes for their clonal expansion to HCC.

In this context, it should be noted that the results of experimental protocol 4 are apparently in contrast with those of Ngo et al,[35] who reported 100% of Nrf2 mutation in mouse HCCs induced by a single dose of DEN in the absence of promoting procedures. Although experimental protocol 4 closely resembles that used by Ngo et al,[35] differences nevertheless exist that may be responsible for the discrepancy concerning the frequency of Nrf2 mutation, as follows: (1) dose of DEN (15 vs 25 mg/kg); (2) route of administration: gavage vs intraperitoneal administration; (3) strain: B6C3F1 vs C57BL6/129SV; and (4) sex: females vs males. Although some of these differences (ie, dose or route of administration) were unlikely to account for the lack of Nrf2 mutation observed in our work, the different strain used in the present work may be the key to explain the discrepancy between the 2 studies. Indeed, although the incidence of *Ha-ras* gene mutations in spontaneously developed liver tumors was 50%–63% in mice of the same strain used in our study (B6C3), DEN-induced tumors showed a very low incidence of *Ha-ras* mutations (1 of 13 tumors; 7.7%).[43] On the other hand, the BrafV637E mutation, corresponding to the human BrafV600E mutation, was detected in 54 of 63 (85.7%) hepatic lesions induced by neonatal treatment with DEN in mice of the same strain used in our study (B6C3F1 mice).[44] Most important, the same work showed that whole-exome analysis performed in 4 tumors generated by neonatal treatment with DEN in B6C3F1 mice was able to identify 98 mutations, but none of them involved the *Nrf2* gene. Thus, the different strains used in our study and the study by Ngo et al[35] may well be responsible for the different results.

Independently of mutations, the present study did not show clear evidence of activation of the Keap1–Nrf2 pathway in the examined models. Indeed, the expression of Nrf2 target genes, such as *Nqo1*, *Hmox1*, *Gclc*, and *Gsta4* was not significantly changed in most of the tumors. The latter finding reasonably rules out the possibility that other mechanisms, such as increased nuclear translocation and transcriptional activity resulting from p62 accumulation, Nrf2 deglycation by fructosamine-3, Keap1 succination, and degradation by fumarate or other oncometabolite,[45] could be involved in mouse HCC development.

As mentioned previously, Nrf2 redirects glucose and glutamine into anabolic pathways suggestive of the metabolic reprogramming often associated with cancer cells.[33] According to the lack of Nrf2 mutations and its transcriptional activation, no significant and convincing evidence of metabolic reprogramming was found in the present study, as documented by the lack of increase of *Glut1*, *G6pdx*, and *Mct4* (representative of increased glucose consumption, PPP activation, and lactate extrusion), or *Gls*, responsible for glutaminolysis. Using a DEN + phenobarbital mouse experimental model, Unterberger et al[46] showed that both *Ha-ras* and *Ctnnb1* mutated tumors showed a reduction in the levels of glucose-6-phosphatase, a condition favoring tumor cells because this enzyme catches glucose as an energy source. However, although glucose-6-phosphatase may be used through the PPP, it resulted up-regulated in Ha-*ras*, Q27 but not in *Ctnnb1*, mutated tumors. It also was surprising that although transcriptional up-regulation of TCA cycle Q28 enzymes, such as isocitrate dehydrogenase and citrate synthase, was observed in *Ctnnb1* mutated tumors, increased lactate levels were observed in Ha-*ras* but not in *Ctnnb1* mutated tumors. These results were in line with those obtained by Yuneva et al,[47] who did not detect increased lactate levels in *MET*-induced mouse liver tumors characterized by activating β-catenin mutations, and suggested that glucose and glutamine metabolism in HCC varies with the nature of the activated oncogene.

In conclusion, our present study, unlike rat HCCs, shows the following: (1) no *Nrf2* mutation takes place in HCCs generated by distinct mouse models of chemically induced hepatocarcinogenesis (3 strains, both sexes, different ages of animals, and different doses of DEN); (2) no increased activation of the Keap1–Nrf2 pathway was observed in the same tumors; and (3) no change in the expression of genes involved in glycolysis, PPP, and glutamine pathway indicative of metabolic reprogramming was observed in mouse HCCs lacking Nrf2 activation but carrying *Ctnnb1* mutations. These results also suggest that for translational studies investigating the role of Nrf2 mutation/activation in chemically induced hepatocarcinogenesis, the mouse may not be the ideal model because it does not recapitulate the human landscape.

Materials and Methods

Female C3H mice (ages, 6–8 wks) were obtained from Charles River (Milan, Italy). Two-week-old C57BL/6 male mice were obtained from Charles River, and maintained at the University Amedeo Avogadro of East Piedmont (Novara, Italy). Seven-day-old B6C3F1 female mice (Charles River) were kept at the Istituto Nazionale Tumori (Milan, Italy). Guidelines for the Care and Use of Laboratory Animals were followed throughout the investigation. All animal procedures were approved by the Ethical Commission of the University of Cagliari, East Piedmont, and the Italian Ministry of Health.

Experimental Protocol 1

Mice were injected intraperitoneally with DEN at a dose of 90 mg/kg body weight. After a 1-week recovery period, mice were treated intragastrically with TCPOBOP (3 mg/kg body weight; Sigma-Aldrich) once weekly for 28 weeks. Q29 Another group of mice received TCPOBOP alone, once a week for 28 weeks. Untreated mice were used as a further control group.

Experimental Protocol 2

Mice were treated with intragastric injections of TCPOBOP (3 mg/kg body weight) once weekly for 42 weeks. Age-matched mice treated with dimethyl sulfoxide dissolved in corn oil were used as controls.

Experimental Protocol 3

A single intraperitoneal injection of DEN (25 mg/kg body weight) was given to 2-week-old male pups (C57BL/6 mice). At 6 weeks of age, animals were fed a CDAA obtained from Laboratorio Dottori Piccioni (Gessate, Italy) for the following 25 weeks.

Experimental Protocol 4

Seven-day-old B6C3F1 female mice received a single dose of DEN (15 mg/kg body weight in 0.9% NaCl solution) by gavage. Animals were killed 40 weeks later.

Laser-Capture Microdissection

Twenty HCCs from the livers of 8 mice treated with DEN + TCPOBOP and 11 HCCs from 5 animals given TCPOBOP alone (experimental protocols 1 and 2) were laser-microdissected as described previously.[22] Liver random areas of peritumoral tissue or control livers from age-matched untreated control mice also were microdissected. Macrodissection of 9 and 11 tumors was performed in experimental protocols 3 and 4, respectively.

DNA Sequencing

Total RNA was extracted from frozen liver samples using TRIzol Reagent (Thermo Fisher Scientific), according to the manufacturer's protocol. Total RNA was retrotranscribed using the High-Capacity cDNA Reverse Transcription Kit (Thermo Fisher Scientific). To identify *Nrf2* mutations, we amplified exon 2 of the mouse *Nrf2* gene, using a touch-down PCR protocol (annealing temperature, 66°C–60°C). To analyze mouse cDNA sequences corresponding to the second exon of *Nrf2*, we designed the following 2 primers: forward 5′-CCTCTGCTGCAAGTAGCCTC-3′ and reverse 5′-CAGGGCAAGCGACTCATGG-3′. All PCR products were amplified with High-Fidelity *Taq* polymerase (Platinum Taq DNA Polymerase High Fidelity; Invitrogen), purified (by exonuclease 1 and shrimp alkaline phosphatase), and sequenced by fluorescent-based Sanger direct sequencing in an ABI3130 DNA capillary sequencer.

Microarray Analysis

The microarray expression profiles of RNA in HCCs and adjacent noncancerous tissues described in our previous study[29] were re-analyzed from Gene Expression Omnibus (accession number: GSE113708). Analysis was performed in R studio. Quantile normalized data were downloaded using Geoquery and the limma package was applied to perform gene set testing and differential gene expression. Only genes whose expression differed by at least 1.5-fold were considered for further analysis.

Functional Analysis Using IPA

Rat standard gene symbols (RGD ids) were submitted to the Ingenuity IPA analysis pipeline. Analysis of the pathways was based on the number of genes significantly dysregulated (fold difference cut-off, ±1.5) with corresponding biological functions. The significance of each network and the connectivity was estimated in IPA.

qRT-PCR Analysis

The same cDNA used for gene sequencing also was used for qRT-PCR analysis. Total and microdissected RNA was retrotranscribed using the High-Capacity cDNA Reverse-Transcription Kit (Thermo Fisher Scientific). Analysis of *Nqo1*, *Hmox1*, *Gclc*, *Gsta4*, *Glut1*, *Mct4*, *G6pdx*, *Hk2*, *G6pc*, and *Gls* was performed using specific TaqMan probes (Thermo Fisher Scientific) and *Gapdh* as the reference gene.

Immunohistochemistry

Liver sections were fixed in 10% buffered formalin and processed for staining with H&E or immunohistochemistry. Paraffin-embedded tissue was cut into 4-μm sections, dewaxed, and hydrated. Endogenous peroxide was inactivated using hydrogen peroxide. Slides were microwaved in citrate buffer at pH 6.0 (ab93678; Abcam) or in EDTA buffer at pH 8.0 (ab64216), followed by overnight incubation with the primary antibodies Nqo1 (ab28947; Abcam), G6pd (ab87230; Abcam), and Gls (ab262716; Abcam). After washes, the sections were incubated with the appropriate polymer DAKO Envision secondary antibody at room temperature. Signal was detected using the VECTOR NovaRED Peroxidase (horseradish peroxidase) Substrate Kit (Vector Laboratories). Sections were counterstained with Harris hematoxylin solution (Sigma-Aldrich) and passed through the dehydration process and covered.

Statistical Analysis

Statistical significance was performed using the Student *t* test with Instat (GraphPad, San Diego, CA). The results of observations are presented as the means ± SD value. $P < .05$ was considered a significant difference between groups.

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

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