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Nrf2, but not β-catenin, mutation represents an early event in rat hepatocarcinogenesis

Patrizia Zavattari[1,†,*], Andrea Perra[1,†,*],Silvia Menegon[2], Marta Anna Kowalik[1],Annalisa Petrelli[2], Maria Maddalena Angioni[1], Antonia Follenzi[3], Luca Quagliata[4], Giovanna Maria Ledda-Columbano[1], Luigi Terracciano[4], Silvia Giordano[2] and Amedeo Columbano[1]

Author Information

[1]Department of Biomedical Sciences, University of Cagliari, Cagliari, Italy
[2]University of Torino School of Medicine, Candiolo Cancer Institute-FPO, Torino, Italy
[3]Department of Health Sciences, University of Piemonte Orientale, Novara, Italy
[4]Institute of Pathology, University Hospital, Basel, Switzerland
[†]These authors equally contributed to this work.

[*]Address reprint requests to: Patrizia Zavattari, Ph.D., Department of Biomedical Sciences, Unit of Biology and Genetics, University of Cagliari, Cittadella Universitaria di Monserrato, SP 8, Km 0.700-09042, Monserrato, Cagliari, Italy. E-mail: pzavattari@unica.it; tel: +39-070-6754101; fax: +39-070-6754100. Or to: Andrea Perra, M.D., Ph.D., Department of Biomedical Sciences, Unit of Oncology and Molecular Pathology, University of Cagliari, Via Porcell 4, 09124 Cagliari, Italy. E-mail:andreaperra@omeca.it; tel: +39-070-6758346; fax: +39-070-666062.

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Abstract

Hepatocellular carcinoma (HCC) develops through a multistage process, but the nature of the molecular changes associated with the different steps, the very early ones in particular, is largely unknown. Recently, dysregulation of the NRF2/KEAP1 pathway and mutations of these genes have been observed in experimental and human tumors, suggesting their possible role in cancer development. To assess whether *Nrf2/Keap1* mutations are early or late events in HCC development, we investigated their frequency in the rat Resistant Hepatocyte model, consisting of the administration of diethylnitrosamine followed by a brief exposure to 2-acetylaminofluorene. This model enables the dissection of all stages of hepatocarcinogenesis. We found that *Nrf2/Keap1* mutations were present in 71% of early preneoplastic lesions and in 78.6% and 59.3% of early and advanced HCCs, respectively. Mutations of*Nrf2* were more frequent, missense, and located in the Nrf2-Keap1 binding region. Mutations of *Keap1* occurred at a much lower frequency in both preneoplastic lesions and HCCs and were mutually exclusive with those of *Nrf2*. Functional *in vitro* and *in vivo*studies showed that *Nrf2* silencing inhibited the ability of tumorigenic rat cells to grow in soft agar and to form tumors. Unlike *Nrf2*mutations, those of *Ctnnb1*, which are frequent in human HCC, were a later event as they appeared only in fully advanced HCCs (18.5%). *Conclusion*: In the Resistant Hepatocyte model of hepatocarcinogenesis the onset of *Nrf2* mutations is a very early event, likely essential for the clonal expansion of preneoplastic hepatocytes to HCC, while *Ctnnb1* mutations occur only at very late stages. Moreover, functional experiments demonstrate that *Nrf2* is an oncogene critical for HCC progression and development. (HEPATOLOGY2015;62:851-862)

Abbreviations

aHCC: advanced HCC

DENA: diethylnitrosamine

eHCC: early HCC

GS: glutamine synthase

HCC: hepatocellular carcinoma

qRT-PCR: quantitative reverse-transcriptase polymerase chain reaction

R-H: resistant-hepatocyte

Hepatocellular carcinoma (HCC) is the third most frequent cause of cancer-related deaths worldwide.[1] Unfortunately, our knowledge of the genomic alterations implicated in HCC initiation and progression is still fragmentary. Moreover, different from other solid tumors, no driving genetic alteration to which tumor cells are addicted and that can be effectively targeted has ever been identified. In this scenario, HCC is probably one of the tumor types where a more complete understanding of the underlying genetic alterations could have a major impact on the development of new treatment strategies. Also known as *NFE2L2*, *NRF2* is of particular interest as conflicting results have been reported concerning the role of the NRF2/KEAP1 pathway in cancer development.[2] It is a master transcriptional activator of genes encoding enzymes that protect cells from oxidative stress and xenobiotics by induction of transcriptional regulation of several antioxidant enzymatic pathways and various drug efflux pumps and members of the multidrug resistance protein family.[3] It is negatively regulated and targeted to proteosomal degradation by KEAP1.[4] It was reported in several studies that either loss of NRF2-KEAP1 interaction or point mutations in the *KEAP1* or *NRF2* gene are often associated with primary tumors.[3] As for human HCC, two recent studies using whole-exome sequencing have revealed mutations of either *NRF2* (6.4%) or *KEAP1* (8%),[5, 6] suggesting that dysregulation of this pathway may play a relevant role in a subset of human HCCs. Dysregulation of the Nrf2/Keap1 pathway, monitored through analysis of the expression profile of HCC, has also been described in two mouse models where tumors developed in the liver of transforming growth factor-α–c-myc double knockout or in the liver of mice subjected to treatment with diethylnitrosamine plus phenobarbital.[7, 8] In both these studies, as well as in humans, however, gene mutation and/or dysregulation of the Nrf2/Keap1 pathway were investigated only at the final biological endpoint, namely, HCC. This makes it difficult to understand whether alteration of this pathway is critical for HCC development or if it represents one of the several molecular changes featured in HCC. The same problem applies also to mutations in the *Ctnnb1* gene; in fact, while mutations of this gene are frequent in human HCC,[9] whether they are involved in HCC initiation[10] or take place only during HCC progression[11] is still a matter of debate.

Recently, we have shown that the Nrf2/Keap1 pathway is dysregulated in the very early steps of the carcinogenic process in rat liver.[12]Therefore, this study investigated whether, similar to the human situation, mutations of *Nrf2* and/or *Keap1* could be responsible for the alteration of this pathway. Using the same animal model, we also investigated the occurrence of *Ctnnb1* mutations in the different stages of hepatocarcinogenesis. The results demonstrate that (1) mutations of *Nrf2* are extremely frequent in rat HCC, (2) they represent a very early event in the tumorigenic process occurring in early preneoplastic stages, and (3) mutations of *Ctnnb1,* a gene frequently mutated in human HCC, occur only during the progression from early to advanced HCC.

Materials and Methods

Resistant-Hepatocyte Model

Guidelines for the Care and Use of Laboratory Animals were followed during the investigation. All animal procedures were approved by the Ethical Committee of the University of Cagliari and the Italian Ministry of Health. Male Fischer F-344 rats (100-125 g) were purchased from Charles River (Milan, Italy). Animals were injected intraperitoneally with diethylnitrosamine (DENA; Sigma, St. Louis, MO) at a dose of 150 mg/kg body weight. After a 2-week recovery period, rats were fed a diet containing 0.02% 2-acetylaminofluorene (Sigma) for 1 week, followed by a two-thirds partial

hepatectomy and by an additional week of the 2-acetylaminofluorene diet. Animals were then returned to the basal diet and killed at 10 weeks (preneoplastic nodules), 10 months (early HCCs [eHCCs]), or 14 months (advanced HCCs [aHCCs]) after DENA (for a schematic representation of the experimental protocol, see Supporting Fig. S1A). Histologic classification of preneoplastic nodules as eHCCs or aHCCs was performed as described[13] and as detailed in the Supporting Information. Lung metastases were macroscopically identified in aHCC-bearing rats and further analyzed by hematoxylin and eosin staining.

Immunohistochemistry

Frozen liver sections were collected and fixed in −20°C acetone for 20 minutes at room temperature. Paraffin-embedded sections were incubated overnight with the following antibodies: anti–placental glutathione *S*-transferase (MBL, Nagoya, Japan), anti–glutamine synthase (GS; Sigma-Aldrich), anti-NQO1 (Abcam, Cambridge, MA), and anti-NRF2 (Santa Cruz Biotechnology, Dallas, TX) and detected by antirabbit HRP antibody and 3,3'-diaminobenzidine (Dako Envision). Sections were counterstained with Harris hematoxylin.

Laser-Capture Microdissection

We microdissected 38 early preneoplastic nodules, 14 eHCCs, and 27 aHCCs. Serial frozen sections of rat livers (16 μm thick) were attached to 2-μm RNase free polyethylene naphthalate membrane slides (Leica, Wetzlar, Germany). Microdissection (Leica, LMD6000) was followed by hematoxylin and eosin staining.

Quantitative Reverse-Transcriptase Polymerase Chain Reaction Analysis

We isolated RNA from microdissected preneoplastic and neoplastic lesions using MirVana (Ambion, Austin, TX), as reported.[12]Retrotranscription was performed starting from 0.5 μg RNA/sample using the High Capacity Kit (Applied Biosystems, Carlsbad, CA). Gene expression was assessed by quantitative reverse-transcriptase polymerase chain reaction (qRT-PCR) using specific TaqMan probes (Nqo1, Gclc, Gsta4, Gs, Nrf2, Gapdh, and β-actin; Applied Biosystems) or Express Sybr Green (Invitrogen, Paisley, UK) for Keap1 and β-actin. The relative levels were determined using the ΔΔCT method.

Sequencing

Genomic DNA was extracted from the same samples used for qRT-PCR analysis with minor modifications (see Supporting Information). To identify *Nrf2* mutations, we analyzed 38 rat preneoplastic lesions, 14 eHCCs, and 27 aHCCs obtained from five, three, and 14 rats, respectively (Supporting Fig. S1B). Nine fragments corresponding to the five exons of rat *Nrf2* gene (annealing 66°C-60°C) were amplified using a Touch-down PCR protocol. For details of the amplification protocols, please see the Supporting Information. 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 fluorescence-based Sanger's direct sequencing in an ABI 3130 DNA capillary sequencer. The mutation nomenclature described in this study is given according to the numbering of the amino acids in the human protein sequence (Ensembl).

Transient Transfection of HEK293T Cells

In a 5% CO_2 atmosphere HEK293T cells (ATCC, Manassas, VA) were cultured in Dulbecco's modified Eagle's medium with 10% fetal bovine serum (Lonza, Basel, Switzerland). For transient transfection, an NRF2 lentiviral construct (217EX-T3128-Lv157; GeneCopoeia, Rockville, MD) and a KEAP1 encoding plasmid (pCDNA3-T7-Keap1; AddGene, Cambridge, MA) were used. The mutant forms of NRF2 (V32E and E82G) were obtained using the QuikChange II XL Site-Directed Mutagenesis Kit (Agilent Technologies). Using the calcium phosphate technique, HEK293T cells were transfected with 10 μg of NRF2 and KEAP1 constructs. Extraction of RNA was performed upon 48 hours using the miRNeasy Mini Kit (Qiagen). Expression of NQO1 was evaluated by qRT-PCR as described above. Western blot analysis was performed as described.[12]

Stable Transduction With Nrf2 Targeting Short Hairpin RNA

Cells isolated from an HCC-bearing rat (resistant-hepatocyte [R-H] cells),[12] cultured in Roswell Park Memorial Institute 10% fetal bovine serum medium, were stably transduced with the 217CS-SH213J-LVRH1P lentiviral vector containing a specific custom-designed *Nrf2*targeting short hairpin RNA (GeneCopoeia). Transduced cells were selected by treatment with puromycin (1 μg/mL for 7 days; Invitrogen). Anchorage-independent growth in soft agar was performed, embedding 3000 cells/well in 0.5% agar (SeaPlaque) in a 24-well plate. At the end of the experiment grown colonies were stained with crystal violet and quantified by counting all visible colonies.

Wild-type and stably *Nrf2* silenced R-H cells (1,000,000/rat) were injected subcutaneously in the flank of F-344 syngeneic rats (n = 6 rats/group). Tumor size was measured twice a week by caliper. Subcutaneous tumor volume was calculated using the formula 4/3π (*D*/2) (*d*/2) 2, where *d* is the minor tumor axis and *D* is the major tumor axis. Rats were sacrificed 28 days after injection, and tumors were excised.

Statistical Analysis

Data are expressed as mean ± standard deviation or mean ± standard error. Analysis of significance was done by the Student *t* test and by one-way analysis of variance using GraphPad software (La Jolla, CA). *P* values were considered significant at $P < 0.05$.

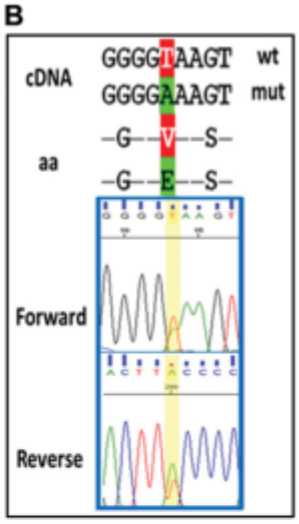
Results

Onset of Nrf2 and Keap1 Mutations Is an Early Event in Hepatocarcinogenesis

The R-H model allows us to dissect the several stages of the carcinogenic process (Supporting Fig. S1A).[14] To investigate whether*Nrf2/Keap1* mutations take place early in the carcinogenic process and are therefore fundamental for HCC progression, we microdissected preneoplastic nodules arising 10 weeks after DENA initiation; these lesions were identified by their positivity to the placental form of glutathione *S*-transferase.[15] As published works indicate that most *Nrf2* mutations occur in exon 2,[16] we sequenced this exon in 38 preneoplastic lesions and in eight age-matched control livers, using Sanger fluorescence-based sequence analysis. Mutational analysis showed that 25/38 (65.8%) preneoplastic lesions did exhibit *Nrf2* mutations. All of them were missense, the most frequent being at codons 32 (8/25, V32E) and 77 (4/25, D77G) (Fig. 1A,B). Twenty-four of 25 *Nrf2* mutations were located in the *Nrf2* regions coding for either the LxxQDxDLG motif (spanning amino acids 17-32) or the DxETGE motif (amino acids 77-82) (Fig. 1C).[16] Both these motifs, evolutionarily conserved, bind to the Kelch domain in Keap1. Analysis of the predicted effects of these changes (MUpro software, athttp://www.ics.uci.edu/~baldig/mutation.html, PolyPhen-2, at http://genetics.bwh.harvard.edu/pph2/) showed that all the mutations profoundly impair the binding between Keap1 and Nrf2 and should be considered as activating mutations (data not shown). Given the extremely high frequency of *Nrf2* mutations in early lesions, we sequenced the remaining *Nrf2* exons (exons 1, 3, 4, 5); but no further mutations were detected. Because Keap1 mutations disrupting the interaction with Nrf2 have already been described in human HCCs,[4]we also sequenced *Keap1* exons coding for the Kelch domain, involved in the binding to Nrf2.[17] Notably, *Keap1* mutations were found in two samples lacking *Nrf2* mutations (Fig. 1A). Sequencing of *Keap1* exons not involved in Nrf2 binding was also performed, but no further mutations were found. Complementary DNA sequencing of *Nrf2* full-length transcripts confirmed the presence of the same mutations (data not shown). Analysis of age-matched control livers revealed no *Nrf2/Keap1* mutation. Thus, *Nrf2/Keap1* mutations represent very early events in the hepatocarcinogenic process.

A

Aminoacid substitution	Nucleotide substitution	Number of mutated preneoplastic lesions
Nrf2_V32E	GTA > GAA	8
Nrf2_D77G	GAT > GGT	4
Nrf2_L30H	CTT > CAT	2
Nrf2_E79G	GAG > GGG	2
Nrf2_T80A	ACC > GCC	2
Nrf2_E82G	GAA > GGA	2
Nrf2_W24R	TGG > AGG	1
Nrf2_D27G	GAC > GGC	1
Nrf2_D29G	GAT > GGT	1
Nrf2_F37S	TTT > TCT	1
Nrf2_E82K	GAA > AAA	1
Keap1_R413S	CGC > AGC	1*
Keap1_I566S	ATC > AGC	1*
Keap1_Y584X	TAT > TAA	1



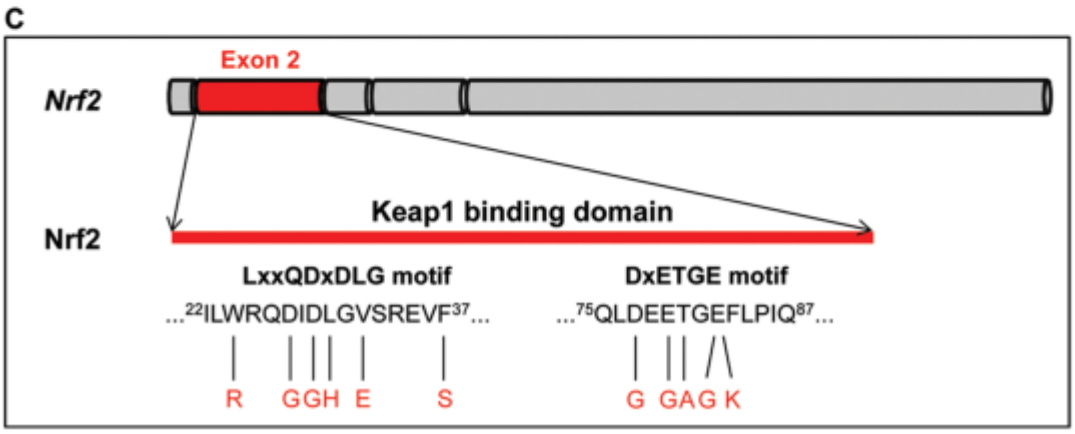


Figure 1. Mutations in *Nrf2/Keap1* in preneoplastic lesions. (A) Mutations in*Nrf2* and *Keap1* identified in 38 preneoplastic lesions. All PCR products were purified and sequenced by fluorescence-based Sanger's direct sequencing in an ABI 3130 DNA capillary sequencer. *These two mutations were identified in the same lesion. (B) Electropherogram showing the most frequent *Nrf2*mutation. (C) Scheme illustrating the position of *Nrf2* mutations. All mutations are located in the LxxDQxDLG and DxETGE motifs responsible for *Keap1*binding. Amino acid changes are shown in red.

Nrf2/Keap1 Mutations Lead To Pathway Activation

To directly establish whether *Nrf2* and *Keap1* mutations result in increased Nrf2 transcriptional activity, we analyzed the expression of three established target genes.[18] The results shown in Fig. 2A demonstrate that all three examined genes (*Nqo1*, *Gclc*, *Gsta4*) were strongly up-regulated in mutated preneoplastic lesions compared to control liver or to nonmutated preneoplastic nodules, suggesting that these are, actually, activating mutations.

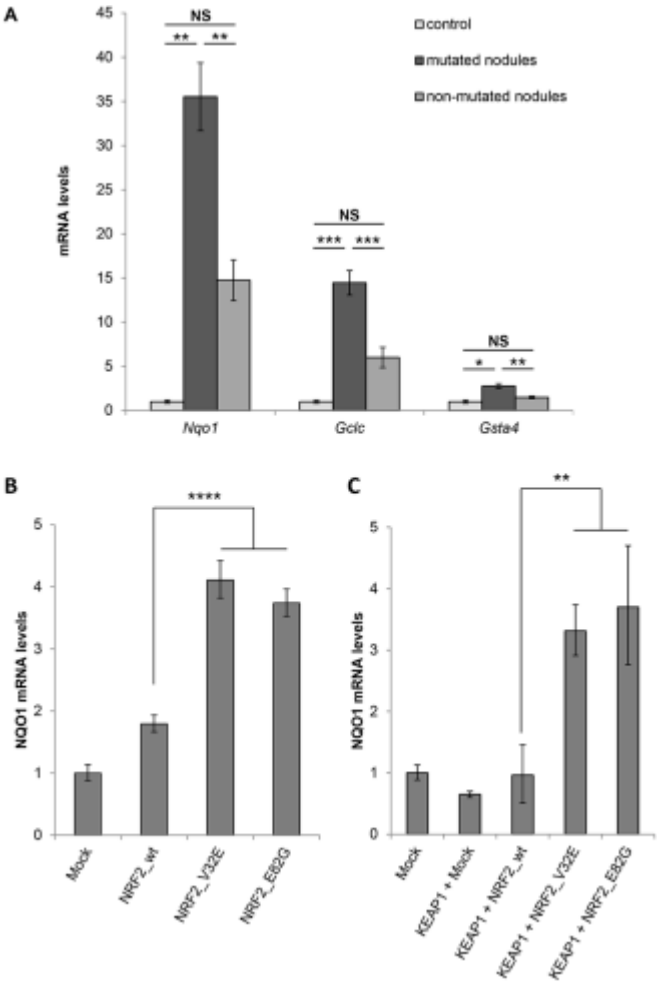


Figure 2. Mutations in *Nrf2* lead to pathway activation. (A) Expression levels of three Nrf2-target genes in *Nrf2/Keap1* mutated or nonmutated preneoplastic lesions. Messenger RNA levels of *Nqo1*, *Gclc*, and *Gsta4* were evaluated by qRT-PCR and normalized to age-matched control livers. Glyceraldehyde 3-phosphate dehydrogenase was used as endogenous control. Error bars represent standard deviation. ***$P < 0.001$, **$P < 0.01$, *$P < 0.05$. (B,C) Lentiviral vectors containing wild-type or mutated Nrf2 complementary DNAs were transfected alone (B) or in combination with a Keap1 expression vector (C) in HEK293T cells with the calcium-phosphate technique. Expression of *Nqo1* was evaluated by qRT-PCR 48 hours after transfection and normalized to mock transfected cells. Beta-actin was used as endogenous control. Error bars represent standard deviation. ****$P < 0.0001$, **$P < 0.01$. NRF2_V32E and NRF2_E82G are NRF2 mutated forms; "Mock" indicates empty vector. Abbreviation: mRNA, messenger RNA; NS, not significant.

To further assess if the mutations observed in preneoplastic lesions are indeed activating mutations, transient transfection of HEK293T cells with lentiviral vectors containing either wild-type or mutated *NRF2* complementary DNA (V32E and E82G) was performed (Supporting Fig. S2A). The results showed a significantly stronger activation of the NRF2 pathway (evaluated as *NQO1* transcription) in cells expressing the two analyzed mutated forms (Fig. 2B). Moreover, while cells transduced with both wild-type *NRF2* and *KEAP1* expression vectors did not show any increase of *NQO1* expression, a significant increase of this gene was observed in cells expressing the two *NRF2* mutants and*KEAP1* (Fig. 2C and Supporting Fig. S2B). These data provide evidence that the NRF2 mutated forms are indeed unable to interact with KEAP1 and thus sustain the increased pathway activation.

Nrf2 Mutations Occur at High Frequency in HCCs

Next, we investigated *Nrf2* mutations in eHCCs developed 10 months after DENA treatment. The lesions were composed of well-differentiated cells with minimal atypia, frequent fatty change, and increased nuclear/cytoplasmic ratio; they were not encapsulated and blended imperceptibly into adjacent nontumorous parenchyma. Normal lobular architecture was replaced by a compact pattern of thin trabeculae, with occasional small pseudoglandular or acinar

structures (Supporting Fig. S3A). No lung metastases were found in animals bearing eHCC. Advanced HCCs developed 4 months later and were composed of poorly differentiated cells, not resembling hepatocytes, arranged in thick trabeculae or pseudoglandular patterns. Pleomorphism, including bizarre giant cells, was frequently seen (Supporting Fig. S3B). Lung metastases (Supporting Fig. S3C) were found in 50% of the animals.

Eight of the 14 eHCCs (57.1%) showed missense mutation of *Nrf2*; three were located at codon 32 (V32E) and one each at codons 23 (L23P), 27 (D27G), 30 (L30R), 77 (D77G), and 82 (E82G) (Fig. 3A). Mutations of *Keap1* were also observed in three eHCCs. Interestingly,*Keap1* mutations, similar to what was observed in early lesions, were mutually exclusive with *Nrf2* mutations (Fig. 3A).

A

Aminoacid substitution	Nucleotide substitution	N° of mutated/N° of analyzed	
		11/14 eHCCs	16/27 aHCCs
Nrf2_V32E	GTA > GAA	3	7
Nrf2_L23P	CTT > CCT	1	
Nrf2_D27G	GAC > GGC	1	
Nrf2_L30R	CTT > CGT	1	
Nrf2_D77G	GAT > GGT	1	1
Nrf2_E82G	GAA > GGA	1	1
Nrf2_L30H	CTT > CAT		2
Nrf2_D29G	GAT > GGT		1
Nrf2_L30P	CTT > CCT		1
Nrf2_E79G	GAG > GGG		1
Nrf2_T80A	ACC > GCC		1
Keap1_L374P	CTG > CCG	1*	
Keap1_S390P	TCC > CCC	1*	
Keap1_L457X	TTG > TAG	1	
Keap1_V581E	GTG > GAG	1	1

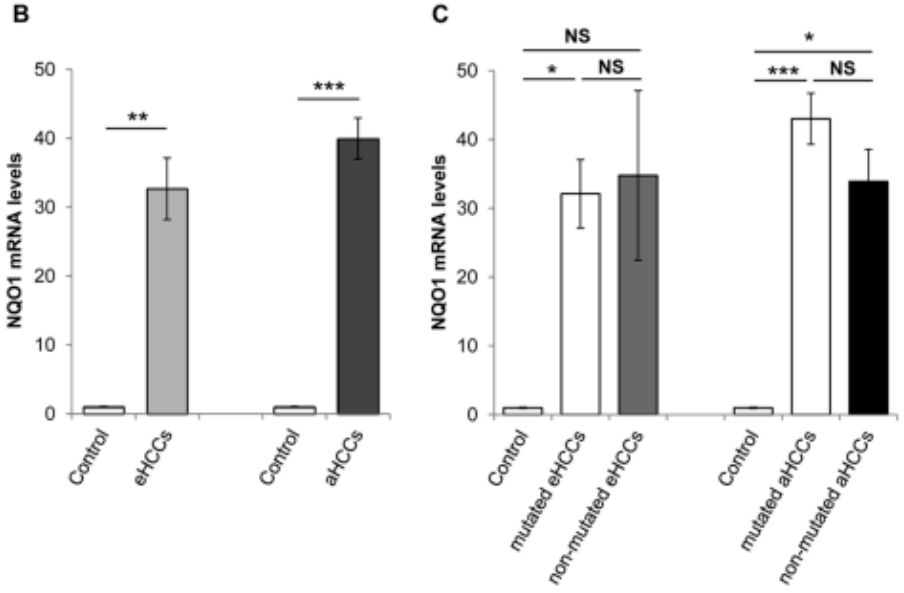


Figure 3. Mutations in *Nrf2/Keap1* and pathway activation in eHCCs and aHCCs. (A) Mutations in *Nrf2* and *Keap1* in 14 eHCCs and 27 aHCCs. All PCR products were purified and sequenced by fluorescence-based Sanger's direct sequencing in an ABI 3130 DNA capillary sequencer. *These two mutations were identified in the same lesion. (B) Expression levels of the Nrf2-target gene *Nqo1*, evaluated in all the eHCCs and aHCCs. (C) Expression levels of *Nqo1*, evaluated in Nrf2/Keap1 mutated or nonmutated eHCCs and aHCCs. Messenger RNA levels in *Nqo1* were evaluated by qRT-PCR and normalized to age-matched control livers. Glyceraldehyde 3-phosphate dehydrogenase was used as endogenous control. Error bars represent standard deviation. ***$P < 0.001$, **$P < 0.01$, *$P < 0.05$. Abbreviation: mRNA, messenger RNA; NS, not significant.

Mutations in *Nrf2* were present in 15/27 aHCCs (55.6%) developed at 14 months after DENA. Eleven mutations were located in the region coding for the LxxQDxDLG motif, while four were in the DxETGE motif. In particular, seven mutations were located at codon 32 (V32E), three at codon 30 (two L30H, one L30P) and one each at codons 29 (D29G), 77 (D77G), 79 (E79G), 80 (T80A), and 82 (E82G) (Fig. 3A). Only one mutation (V581E) of *Keap1* was detected in an aHCC devoid of *Nrf2* mutations. Therefore, mutation of *Nrf2/Keap1* accounted for 59.3% of the examined aHCCs. Age-matched control livers showed no mutations of *Nrf2* or *Keap1*.

When we investigated the activation of the Nrf2/Keap1 pathway, we found a strong up-regulation of *Nqo1* in eHCCs and aHCCs compared to normal liver (Fig. 3B). Interestingly, unlike preneoplastic lesions, no significant difference in *Nqo1* expression was found in mutated versus nonmutated HCCs (Fig. 3C), suggesting that at late stages the Nrf2/Keap1 pathway no longer depends on the presence of activating mutations only but can be activated by other mechanisms as well.[12]

The Nrf2/Keap1 Pathway Is Also Activated in Lung Metastases

Finally, we also examined the Nrf2/Keap1 pathway in lung metastases of rats sacrificed 14 months after DENA administration. The results showed the presence of multiple metastases in 50% of HCC-bearing rats (Supporting Fig. S3C). Lung metastases examined in five animals displayed strong and uniform cytoplasmic positivity for Nqo1 (Fig. 4A,B). Accordingly, the same metastases exhibited both cytoplasmic and nuclear positivity for Nrf2 (Fig. 4C). These data show that activation of the Nrf2 pathway persists up to the final stage of the carcinogenic process.

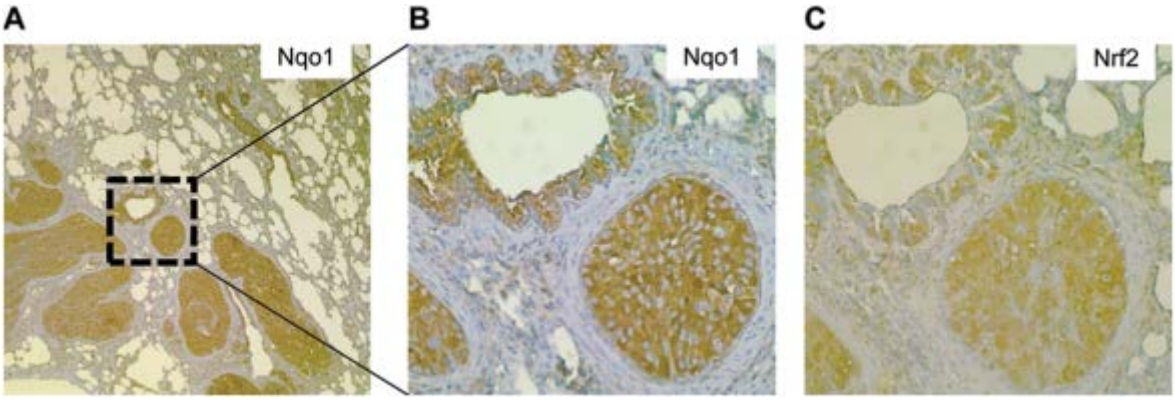


Figure 4. The Nrf2/Keap1 pathway is activated in lung metastases. (A) Representative photomicrograph of a lung displaying several HCC metastases, immunostained for Nqo1 (×10). (B) Higher magnification of the inset shown in (A). Ciliary cells and metastatic hepatocytes are intensely positive for Nqo1 staining (×20). (C) Serial section of the same area as in (B), immunostained for Nrf2 (×20).

Targeting Nrf2 Mutations Impairs Anchorage-Independent Growth and Tumorigenic Ability of HCC Cells

Because our results suggest a critical role of *Nrf2* in early onset and progression of HCC, to directly establish its oncogenic role, we moved to *in vitro* studies. Neoplastic hepatocytes were isolated by collagenase perfusion from the liver of a rat with multiple HCCs generated by the R-H model (R-H cells).[12] In agreement with our *in vivo* data, R-H cells bear a mutation of *Nrf2* (D38G). To establish the biological effect of the modulation of Nrf2 levels in HCC cells, we performed soft agar assays on *Nrf2*-silenced cells (Supporting Fig. S4). As shown in Fig. 5A, while R-H cells were able to grow in soft agar, *Nrf2* silencing caused a significant inhibition of their anchorage-independent growth ability. We then tested if *Nrf2* inhibition could impact their tumorigenic ability as well. Parental R-H cells were able to form tumors in all animals (six out of six) within 28 days when subcutaneously grafted into syngeneic F-344 rats; on the contrary, the tumorigenic ability of*Nrf2*-silenced cells was completely inhibited (Fig. 5B,C). No tumors, in fact, were observed up to 3 months after cell grafting (data not shown). These experiments suggest that *Nrf2* targeting can effectively interfere with HCC growth.

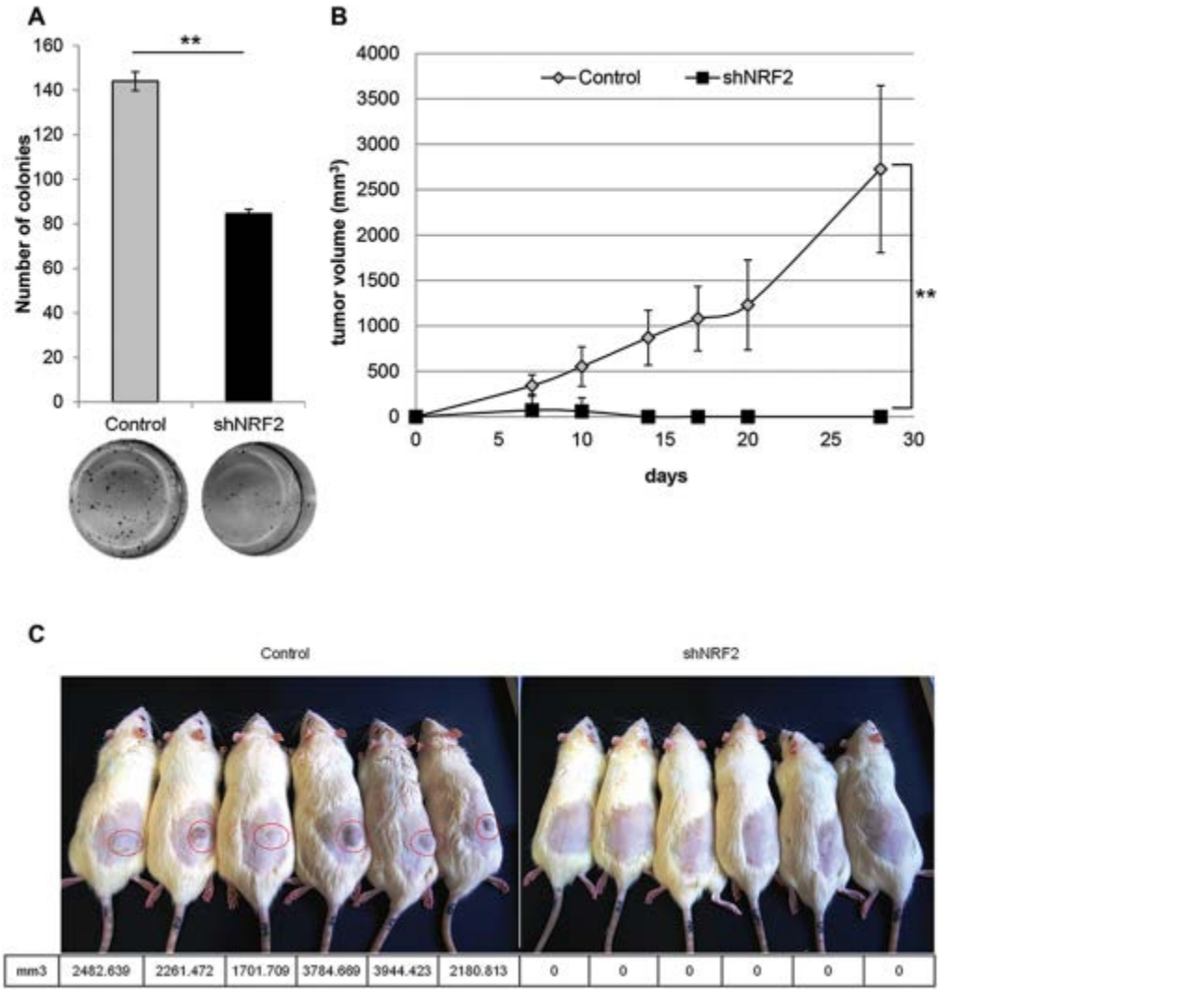


Figure 5. Silencing of *Nrf2* impairs anchorage-independent growth and tumorigenic ability of HCC cells. Resistant-hepatocyte cells were isolated by collagenase perfusion from the liver of a rat with multiple HCCs.[12] (A) The R-H cells were stably transduced with an Nrf2 short hairpin RNA lentiviral vector and puromycin-selected. Anchorage-independent growth was performed, embedding 3000 cells/well in soft agar. Ten days later grown colonies were stained with crystal violet (bottom panel) and quantified by counting all visible colonies (upper panel). (B) Lentivirus-transduced R-H cells (1×10^6) were subcutaneously injected into the right posterior flanks of syngeneic male Fischer F-344 rats (n = 12). The graph shows tumor size, evaluated twice weekly by caliper (triplicate measurements); approximate volume of the mass was calculated as indicated in Materials and Methods. Error bars represent standard deviation. $^{**}P < 0.01$. (C) Rats injected with Nrf2 silenced or not silenced R-H cells, 28 days postinjection. Abbreviation: sh, short hairpin.

Mutations of Ctnnb1 Are a Very Late Event in the R-H Model

Dysregulation of the Wnt/β-catenin pathway and occurrence of activating mutations in *CTNNB1* are among the most frequent alterations in human HCC (15%-33%).[5, 6, 9] However, whether *CTNNB1* mutations occur at early stages of the carcinogenic process is unclear. Therefore, we investigated *Ctnnb1* mutations in the same samples (38 preneoplastic lesions, 14 eHCCs, and 27 aHCCs) analyzed for Nrf2/Keap1. Interestingly, while five of 27 aHCCs (18.5%) exhibited missense mutations in hot spots of exon 3 (codons 32, 33, and 37, corresponding to human codons 31, 32, and 36), none of the 38 preneoplastic lesions, nor the eHCCs, harbored *Ctnnb1* mutations (Fig.6A). This indicates that mutations of this gene, unlike *Nrf2*, are a very late event in the process of hepatocarcinogenesis induced in this rat model. Evidence that either mutation of *Ctnnb1* or activation of the Wnt/β-catenin pathway is not involved in the early stages of hepatocarcinogenesis in this rat model is also supported by the following: (1) GS, a β-catenin target

gene[19] analyzed by both qRT-PCR and immunohistochemistry, was not expressed in preneoplastic nodules (Fig. 6B,C) and (2) GS expression was significantly increased only in advanced HCCs carrying *Ctnnb1* mutations (Fig. 6D). Interestingly, only those mutations resulting in TGT and TAT nucleotide substitutions, i.e., cysteine and tyrosine, were associated with enhanced expression of GS (Fig. 6D). The same mutations were found to be activating mutations in previous works.[20]

A

Aminoacid substitution	Nucleotide substitution	N° of mutated/N° of analyzed	
		0/14 eHCCs	5/27 aHCCs
Ctnnb1_D31H	GAT > CAT		1
Ctnnb1_D31Y	GAT > TAT		1
Ctnnb1_S32C	TCT > TGT		1
Ctnnb1_S36C	TCT > TGT		1
Ctnnb1_S36F	TCT > TTT		1
Aminoacid substitution	**Nucleotide substitution**	**N° of mutated/N° of analyzed**	
		1/14 eHCCs	1/27 aHCCs
Kras_G12V	GGT > GTT	1	
Kras_G12R	GGT > CGT		1

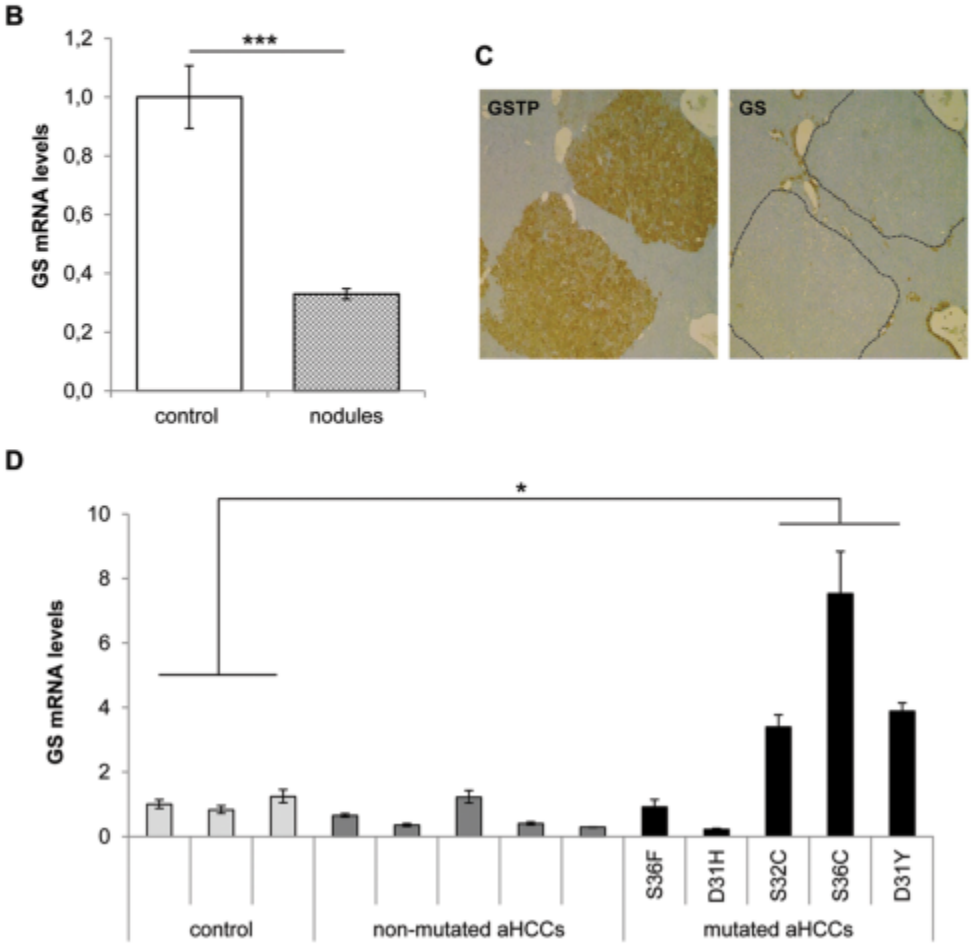


Figure 6. β-Catenin mutations are a late event in hepatocarcinogenesis. (A) Mutations in *Ctnnb1* and *Kras* found in 14 eHCCs and 27 aHCCs. All mutations identified in the *Ctnnb1* gene are located in exon 3, whereas *Kras*mutations are located in exon 1 and are missense mutations. (B) Expression of GS in 38 preneoplastic nodules was assessed by qRT-PCR and compared to age-matched control rat livers. The levels were calculated as fold change compared to control liver. Glyceraldehyde 3-phosphate dehydrogenase was used as endogenous control. Error bars represent the standard deviation of technical triplicates. ***$P < 0.001$. (C) Serial sections of preneoplastic nodules stained for the placental form of glutathione *S*-transferase (GSTP) and GS (4×). (D) Quantitative RT-PCR analysis of GS expression in 14-month HCCs with or without *Ctnnb1* mutations. The levels were calculated as fold change compared to age-matched control livers. Glyceraldehyde 3-phosphate dehydrogenase was used as endogenous control. *$P < 0.05$.

The *KRAS* gene is one of the most frequently mutated in human cancers.[21] However, an extremely low frequency of mutation of this gene has been reported in human HCC.[5, 6] In agreement with these findings, no mutations of *Kras* could be observed in 30 preneoplastic nodules. *Kras* mutations were found only in 1/14 eHCCs and 1/27 aHCCs. Both these mutations were in codon 12 (Fig. 6A).

Discussion

Hepatocellular carcinoma is among the most aggressive and prevalent cancers worldwide.[1, 22] Despite an enormous number of studies aimed at elucidating the exact nature of the molecular events fundamental for the development and progression of HCC, there is only a rudimentary understanding of the genetic/epigenetic events associated with the development of HCC. Moreover, due to the multistage nature of HCC, the molecular pathogenesis of this cancer cannot be properly understood without more information on the molecular alterations characterizing its early development. Unfortunately, knowledge about molecular events in early-stage HCC development is hampered by the clinical difficulty in the histomorphologic distinction between nonmalignant lesions and early HCC. Therefore, experimental models can help to identify genetic changes occurring at initial stages of the hepatocarcinogenic process. However, only few studies have investigated the presence of mutations in early lesions in nontransgenic mouse models,[11, 23] and no studies have been done in rats. In the present study we used a rat model characterized by a cirrhosis-like reaction limited to the very early stages of the carcinogenic process, while cirrhosis/fibrosis, usually associated with human HCCs, is no longer present at later stages of the process. Nevertheless, the molecular signature of rat HCCs strikingly overlaps with that of human HCCs characterized by poor prognosis.[12, 13]

We identified *Nrf2* mutations at an extremely high frequency and established that they occur at very early stages of the carcinogenic process. The NRF2/KEAP1 axis is an integrated redox-sensitive signaling system that regulates 1%-10% of human genes.[3] Notably, an oncogenic role of *NRF2* activation has recently been proposed, based on the finding that its overexpression and/or mutation takes place in many human cancers, including HCC,[5, 24, 25] and that cancers with high NRF2 levels are associated with poor prognosis.[26, 27]However, as pointed out by Sporn and Liby, whether *NRF2* plays a pro- or an antitumorigenic role in premalignant lesions or early malignancy is still unclear.[2] Indeed, while, on the one hand, *NRF2* can protect normal cells from oxidative stress and DNA-damaging electrophiles, on the other hand, it can confer cytoprotection against high endogenous levels of reactive oxygen species, thus increasing survival and resistance to chemotherapy of cancer cells.[27] The results stemming from our *in vivo* and *in vitro* studies prove that *Nrf2* plays a tumorigenic role and that inhibition of *Nrf2* is sufficient to impair colony-forming ability and *in vivo* growth of hepatocarcinoma cells. The finding that activating mutations of *Nrf2* occur at very early stages of the carcinogenic process suggests that activation of the *Nrf2/Keap1*pathway is mandatory for HCC progression and that its inhibition deeply affects the tumorigenic capacity of HCC cells.

Several studies have shown that many oncogenic pathways are involved in the modulation of metabolism.[28, 29] In this context, it is interesting to note that *Nrf2*, other than maintaining redox homeostasis in quiescent cells, is able to redirect glucose metabolism into anabolic pathways[30]; indeed, while increased *Nrf2* expression shifts glucose distribution toward purine nucleotide synthesis (through activation of the pentose phosphate pathway), *Nrf2* knockdown inhibits this process.[30] Even if the impact of Nrf2/Keap1 activation on the hepatocyte pentose phosphate pathway is unknown, it is conceivable that *Nrf2*-induced expression of this pathway may account (1) for the aggressive proliferation of *Nrf2*-overexpressing cells, by providing purines required for nucleic acid synthesis, and (2) for increased survival due to its ability to generate antioxidant species.

Most of the identified *Nrf2* mutations were located in the regions coding for either the LxxQDxDLG motif or the DxETGE motif (Fig. 1C). Both these motifs, evolutionarily conserved, bind to the Kelch domain in Keap1.[31, 32] The highest affinity is located in the ETGE motif[4, 16]; however, the weaker Keap1-binding DLG region has been shown to be more vulnerable to structural changes and fits into a role of the “latch” for the Nrf2-repression gate in the stress response.[16] On this basis, it seems that although the DLG motif has a lower affinity than the ETGE motif, mutations in the former motif may dramatically alter the Nrf2 transcriptional activity.

Interestingly, we observed that while Nrf2/Keap1 mutations result in a much higher increase of Nrf2-target genes in mutated preneoplastic lesions compared to nonmutated ones (Fig. 2A), the Nrf2/Keap1 pathway is activated to a similar extent in mutated and nonmutated HCCs. These results suggest that while gene mutations confer an advantage to preneoplastic hepatocytes, other events may be responsible for the activation of this pathway. Notably, up-regulation of the Keap1-targeting miR-200a was previously found in HCCs generated by the R-H model.[12]

Our results in rat liver provide evidence that the Nrf2/Keap1 pathway plays a tumorigenic role not only in the progression but also in the onset of HCC. Although in humans NRF2/KEAP1 mutations have been identified in HCCs,[5, 6] they were not found in dysplastic cirrhotic macronodules, where TERT mutation was the earliest identified event.[33] However, regulation of telomerase activity is different in humans and rodents. In fact, while telomerase activity is usually undetectable in adult human tissues, several organs of normal adult rodents display substantial amounts of telomerase activity; in particular, in the liver telomerase is expressed at a nearly constant level throughout life and decreases during liver regeneration.[34, 35] Indeed, we did not observe an increase of telomerase expression in any of the lesions we examined (data not shown). This remarkable difference between animal species may reflect the different regulation mechanisms of telomerase expression. As for the Nrf2/Keap1 pathway, it is important to underline that it can be activated also through mechanisms other than mutations, such as dysregulation of microRNAs targeting Keap1.[12] We can speculate that in a tumor not induced by chemicals, epigenetic alterations controlling the Nrf2/Keap1 pathway are more likely involved rather than mutations.

Because the R-H model is based on initial exposure to a genotoxic agent, it is conceivable that DENA might have induced Nrf2/Keap1 mutations in hepatocytes and that these mutations might provide an advantage for their clonal expansion; however, it is also possible that the promoting procedure based on the cytostatic effect of 2-acetylaminofluorene on regenerating liver might select, among the hepatocytes carrying different DENA-induced lesions, those displaying Nrf2/Keap1 mutations. Many studies, in fact, have recently highlighted the interplay between initiated/tumor cells and the microenvironment that can select the genetic alterations conferring the best clonogenic ability in a specific environment.

In the present study, *Cnntb1* mutations, among the most frequent in human HCC, were found only at very late stages (aHCC) of the tumorigenic process but not in preneoplastic nodules or early HCCs. Evidence that *Cnntb1* mutations are late events in the tumorigenic process, and thus β-catenin is unlikely a driving force in early HCC development, also came from a mouse model of hepatocarcinogenesis[11]; moreover, our data showing that *Ctnnb1* mutations are a late event are in agreement with other works that identified β-catenin mutation only in tumors (adenomas and HCCs) and not in cirrhotic nodules.[23, 33, 36] Taken together, all these findings indicate that β-catenin activating mutations are not involved in the onset of HCC but that they occur in already transformed cells and are probably implicated in the final steps of cancer progression. Because the R-H model reflects a histological and molecular signature similar to that of KRT-19-positive human HCCs characterized by poor prognosis,[13] results stemming from this model are potentially relevant for human hepatic tumorigenesis. In turn, they also suggest that studies aimed at analyzing not only genetic/epigenetic changes in advanced tumors but the chronological order of molecular changes starting from the very early steps of the process are highly relevant for the identification of new fundamental alterations.

In summary, the present study identifies *Nrf2* as the most frequently mutated gene in a rat model of chemically induced hepatocarcinogenesis and provides evidence for its mandatory activation in HCC development. In view of the recent finding of *NRF2*mutations in a subset of human HCC,[5] the possibility that *Nrf2* might represent a new targetable gene should be actively pursued. This study also provides evidence that *Cnntb1* mutations are a very late event as they are absent in preneoplastic hepatocytes and in eHCCs.

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