2-Acetylaminofluorene Up-regulates Rat mdr1b Expression through Generating Reactive Oxygen Species That Activate NF-κB Pathway

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Overexpression of multidrug resistance genes and their encoded P-glycoproteins is a major mechanism for the development of multidrug resistance in cancer cells. The hepatocarcinogen 2-acetylaminofluorene (2-AAF) efficiently activates rat mdr1b expression. However, the underlying mechanisms are largely unknown. In this study, we demonstrated that a NF-κB site on the mdr1b promoter was required for this induction. Overexpression of antisense p65 and IκBα partially abolished the induction. We then delineated the pathway through which 2-AAF activates NF-κB. 2-AAF treatment led to the increase of intracellular reactive oxygen species (ROS) which causes activation of IKK kinases, degradation of IκBα (but not IκBβ), and increase in NF-κB DNA binding activity. Consistent with the idea that ROS may participate in mdr1b regulation, antioxidant N-acetylcysteine inhibited the induction of mdr1b by 2-AAF. Overproduction of a physiological antioxidant glutathione (GSH) blocked the activation of IKK kinase complex and NF-κB DNA binding. Based on these results, we conclude that 2-AAF up-regulates mdr1b through the generation of ROS, activation of IKK kinase, degradation of IκBβ, and subsequent activation of NF-κB. This is the first report that reveals the specific cis-elements and signaling pathway responsible for the induction of mdr1b by the chemical carcinogen 2-AAF.

A major problem in cancer chemotherapy is the development of resistance to a wide range of structurally and functionally unrelated anti-cancer drugs. One major mechanism in the development of multidrug resistance (MDR) is by overexpressing MDR1 gene and its encoded P-glycoprotein. It is generally believed that the overexpressed P-glycoprotein facilitates the efflux of anticancer drugs from the cytoplasm, thereby reducing the intracellular drug content to sublethal level. In humans, there are two classes of MDR genes: MDR1, which is involved in multidrug resistance, whereas MDR2, in the lipid transport. There are three mdr gene homologues in rodents, but only mdr1a and mdr1b confer multidrug resistance; while mdr2 functions as a lipid transporter.

Levels of MDR1 expression is frequently elevated in human hepatocellular carcinoma (HCC) (1, 2). Elevated expression of mdr gene transcripts and their encoded P-glycoprotein is also seen in rodent HCC (3, 4). However, mechanisms of the elevation of MDR expression in HCC are largely unknown. In the present study, we investigated the mechanisms of elevated hepatic mdr1b expression in rats induced by hepatocarcinogen 2-acetylaminofluorene (2-AAF). 2-AAF is a hepatocarcinogen that has been frequently used in the development of HCC in experimental animals. AAF is a genotoxic agent. Reaction of electrophilic 2-AAF derivatives with nucleophilic DNA results in the formation of DNA adducts (5). In the treated animals, DNA adducts are proportional to dose in both target tissues, liver and bladder; whereas tumor formation increases linearly with response to dose only in the liver (6). It is believed that 2-AAF also induces liver cancers through non-genotoxic effects, such as the promotion of cell proliferation (7). We (4) and others (8, 9) have previously demonstrated that rat HCC induced by 2-AAF exhibited elevated expression of mdr1b. Using a rat hepatoma cell line H4-II-E, we demonstrate here that the induction of mdr1b expression by 2-AAF is mediated by the activated NF-κB, which recognizes a cis-acting element located upstream of mdr1b promoter. We also demonstrated that the activation is mediated through oxidative stress induced by 2-AAF, as evidenced by the observations that induction of mdr1b expression can be regulated by the redox modulators.

MATERIALS AND METHODS

Reagents—Reagents were purchased from the following companies: Radioactive isotopes [32P]dCTP, [32P]UTP, and [14C]chloroamphenicol from ICN Biomedicals (Costa Mesa, CA); poly(dI-dC)(dl-dC) and acetylcoenzyme A from Amersham Pharmacia Biotech; oligonucleotides from SigmaGenosys Inc. (Houston, TX); anti-IκBα, anti-IκBβ, anti-IKKα, and anti-β-actin antibodies from Santa Cruz Biotechnology Inc. (Santa Cruz, CA); N-acetoxy-2-acetylaminofluorene (2-AAAF) was purchased from Sigma Chemical Co. (St. Louis, MO). MDR1 expression was determined by the reverse transcription polyamide chain reaction product between −245 and +123 bp of mdr1b gene into the KpnI and HindIII sites of pGL3-luciferase vector (Promega, Madison, WI). pNFrβ-Luc and pFR-Luc were obtained from Stratagene (La Jolla, CA). pcDNA1 was purchased from Invitrogen (Carlsbad, CA). Antisense p65 (sp65) was a gift from Tom Maniatis (13). Flag-tagged expression vectors pCMV4-F-IκBα and pCMV4-F-IκBβ were generously provided by S. Ghosh (14).

RNase Protection Assay—The rat mdr1b and 18 S rRNA probes were synthesized by in vitro transcription as described previously (4). Either 20 or 1 μg of total RNA was used for mdr1b or 18 S rRNA, respectively. The protected RNA products were analyzed on a 7% denaturing PAGE gel and quantified using a densitometer (Molecular Dynamics, Sunnyvale, CA).

Cell Culture, 2-AAF Treatment, and Transfection—The rat hepatoma cell line H-4-II-E (ATCC 1548) and human embryonic kidney cell line

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NF-κB Kinase Activity Assay—

IkK Kinase Activity Assay—Cells were treated with various concentrations of 2-AAF for 24 h (a) or with 10 μM 2-AAF for different times as indicated (b). RNA was extracted and subjected to RNase protection assay as described under “Materials and Methods.” The autoradiograph is representative of results from three independent experiments.

RESULTS

2-AAF and 2-AAAF Up-regulate Rat mdr1b mRNA Expression in H4-II-E Cells—We (4) and other (8, 9) have previously demonstrated that expression of mdr1b in hepatoma cells and in liver cancers can be induced by 2-AAF. To confirm that induction of rat mdr1b mRNA could be seen in a cultured cell system, we measured mdr1b mRNA expression by RNase protection assay in rat hepatoma cell line H4-II-E, following 2-AAF treatment. Fig. 1 shows a concentration- and time-dependent increase of mdr1b mRNA levels. Densitometric analyses revealed that maximal levels (7.5-fold) of induction were at 100 μM (panel A). Induction of mdr1b mRNA was seen 3 h after treatment with 100 μM 2-AAF, reached maximum at 5 h, and declined thereafter. Treatment of H4-II-E cells with 10 μM 2-AAAF, a major active metabolite of 2-AAF, also induces mdr1b expression to similar levels, indicating that 2-AAAF is a much more potent inducer than 2-AAF (data not shown).

The NF-κB-binding Site Is Required for 2-AAF Induction of mdr1b—To determine the DNA sequences responsible for the induction, a set of progressive deletion constructs −243RMI-CAT, −241RMI-CAT, and −163RMI-CAT (11, 12) were stably transfected into H4-II-E cells and their responses to 2-AAF treatment were measured by CAT assay. Mass cultures each consisted of more than 20 positive clones were used. The reason for using stably transfected cells rather than the transient transfection approach was because mdr1b gene expression is sensitive to cellular stress and the transfection per se is a stress inducing procedure. Moreover, the transfection efficiency in H4-II-E cells is generally low. As shown in Fig. 2, −243RMI-CAT and −214RMI-CAT constructs were responsive to 2-AAF treatment, but −163RMI-CAT completely lost its responsiveness. Thus DNA sequences critical for the 2-AAF induction are located between −214 and −163 bp, a region containing previously identified binding sites of p53 and NF-κB (11, 12). To determine whether the NF-κB site in this region is involved in the 2-AAF induction, we made a stably transfected cell line containing a reporter construct with mutation at this site. As shown in Fig. 2, mutation of the NF-κB site abolished the induction of mdr1b expression by 2-AAF. These results thus identified that the NF-κB site located at −167 to −158 bp of the rat mdr1b promoter are responsible for the induction of mdr1b expression by 2-AAF. Silverman and Hill (9) reported that the DNA sequence between −214 and −178 bp was important for the basal and carcinogen inducible promoter activity. However, these investigators failed to dissect the critical sequences that are involved within this region.

Down-regulation of NF-κB Abolishes the Induction of mdr1b−Luc by 2-AAF—NF-κB usually consists of p50 and p65 subunits. In most unstimulated cells, NF-κB is tightly controlled by a class of ankyrin containing inhibitors IκBs, which bind to NF-κB subunits and sequester them in the cytoplasm. To con-
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2-AAF induction of mdr1b promoter requires intact NF-κB motif. H4-II-E cells were stably transfected with wild type or mutant reporter constructs as indicated and CAT activity was measured in the absence or presence of 100 μM 2-AAF for 24 h. Values shown are average of three independent experiments in which each group was tested in duplicate. Error bars represent S.D. values. In the schematic diagram, the position of NF-κB site relative to the deletion end points is indicated. Mutation of NF-κB site is indicated by "X".

firm the involvement of NF-κB in 2-AAF induction of mdr1b, we established a stable cell line from 293 cells by transfecting −245 mdr1b-Luc. We then transiently transfected recombinant plasmids encoding NF-κB inhibitors IκBα and antisense p65, respectively, into this cell line. As indicated in Fig. 3, transfecting both antisense p65 and IκBα expressing plasmids abolished the induction by 2-AAF. As a control, no effect on 2-AAF induction was observed when the empty vector was used. These results further demonstrated that NF-κB is involved in the 2-AAF induction of mdr1b expression.

2-AAF Activates NF-κB DNA Binding Activity and NF-κB-mediated Transcription—We then examined whether induction of mdr1b expression by 2-AAF is mediated by an increase of NF-κB DNA binding activity. H-4-II-E cells were exposed to 2-AAF, nuclear extracts were prepared. As shown in Fig. 4A, DNA binding activity of NF-κB became detectable at 2 h, continued to increase until it reached a maximum at 5 h post-treatment and declined thereafter. The binding was verified by virtue that the complex could be competed by an excess of unlabeled wild type mdr1b-κB oligonucleotide fragment, but not by a mutant mdr1b-κB oligonucleotide. The supershift by anti-p50 antibody and partial reduction of binding using anti-p65 antibody also support the binding specificity (Fig. 4B). The rise and fall of NF-κB binding activity is generally consistent with the levels of mdr1b mRNA as detected by RNase protection assay (Fig. 1B).

We also examined the effect of 2-AAF on the transactivation activity of NF-κB using an artificial luciferase reporter pNFκB-Luc, which contains five copies of NF-κB consensus sequence. pFR-Luc, containing five copies of Gal4 consensus sequence, was used as a control. These two reporters were stably transfected into H4-II-E cells and exposed to 20–100 μM 2-AAF for 24 h. As shown in Fig. 4C, 2-AAF treatment led to a dose-dependent induction of pNFκB-Luc, but not pFR-Luc. Taken together, these results suggest that 2-AAF is able to activate both DNA binding and transactivation activities of NF-κB in H4-II-E cells.

2-AAF Induces Degradation of IκBβ, but Not IκBα—TNF-α and interleukin-1β, the two best characterized NF-κB activators, activate NF-κB through phosphorylation-dependent degradation of IκBs, resulting in the release and subsequent nuclear translocation of NF-κB (16, 17). To investigate how 2-AAF activates NF-κB, we performed Western blotting analysis with IκB antibodies to examine whether 2-AAF causes degradation of IκB proteins (Fig. 5A). Strikingly, no degradation of IκBα was observed. In the same experiment, exposure of H4-II-E cells to 1 nM TNF-α for 20 min led to nearly complete degradation of IκBα, indicating that the phosphorylation and proteolysis mechanisms were functional in these cells. The same membrane was stripped and re-probed with IκBβ antibody. As shown in Fig. 5A, after exposure to 2-AAF, IκBβ became partially degraded at 2 h and still remained so after 5 h, following a time course compatible with that for the activation of NF-κB DNA binding activity. In several independent experiments, we were able to obtain reproducible results.

To further strengthen the observation that IκBβ but not IκBα is degraded in response to 2-AAF treatment, we transfected Flag-tagged IκBα and IκBβ expressing plasmids into 293 cells and examined the effect of 2-AAF on their degradation, using anti-Flag M2 antibody. The reason for using 293 cells for transient transfection was because the transfection efficiency using anti-Flag M2 antibody. As indicated in Fig. 5B, 2-AAF treatment had no effect on the stability of F-IκBα, but caused degradation of F-IκBβ. The time course of the induced F-IκBβ degradation was earlier than that found in Fig. 5A. This is probably due to the fact that different cell lines (H4-II-E versus 293), different carcinogens (2-AAF versus 2-AAAF), and different IκBs (endogenous versus exogenously transfected) were used. These experiments suggest that the signal initiated by 2-AAF causes preferential degradation of IκBβ.

2-AAF Induces mdr1b through Generating Intracellular ROS—NF-κB has long been regarded as an important sensor of oxidative stress (18, 19). Expression of NF-κB can be induced by a variety of extracellular influences, including growth factors, UV irradiation, heat shock, and anti-tumor drugs. Many of
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To determine whether oxidative stress plays an important role in up-regulation of mdr1b expression, we first applied a strong oxidant H₂O₂ to H4-II-E cells stably transfected with mdr1b-Luc, pNFκB-Luc, and pFR-Luc recombinants, respectively. Six hours after H₂O₂ exposure, both mdr1b-Luc and pNFκB-Luc were highly activated in a dose-dependent manner, but H₂O₂ had no effect on pFR-Luc (Fig. 6A). These results suggest that the mdr1b promoter, which contains a NF-κB binding site, is sensitive to oxidative stress.

To further demonstrate the role of oxidative stress in the regulation of NF-κB-mediated mdr1b expression, we next investigated whether antioxidants would down-regulate AAF-induced mdr1b expression using stably transfected cells containing mdr1b-Luc, pNFκB-Luc, and pFR-Luc. N-Acetylcysteine (NAC) is a thiol reducing agent, which is commonly used as an anti-oxidant to block the ROS-induced stress. The transfected cells were treated with 100 μM 2-AAF in the absence or presence of increasing concentrations of NAC for 24 h and luciferase activity was determined. As shown in Fig. 6B, NAC at concentrations of 1–15 mM was able to partially abolish the 2-AAF induced expression of mdr1b-Luc and pNFκB-Luc, but 2-AAF and NAC had no effect on pFR-Luc. These results support the role of ROS in the regulation of mdr1b expression.

GSH is an important physiological antioxidant in mammalian cells and high level of GSH could be achieved by over-expressing the heavy subunit of γ-glutamylcysteine synthetase, a rate-limiting enzyme of GSH synthesis. A γ-glutamylcysteine synthetase overexpressing cell line H9 was established from H4-II-E cells and was previously shown to have elevated levels of intracellular GSH (15, 24). We treated H9 and H4-II-E cells in parallel with different doses of 2-AAAF and compared their steady-state mdr1b mRNA levels by RNase protection assay. As indicated in Fig. 6C, the induction levels of mdr1b mRNA by 2-AAAF were significantly lower at each concentration in H9 cells than that in H4-II-E cells. In H4-II-E cells, 2-AAAF treatment induced mdr1b mRNA by up to 7.3-fold, however, in H9 cells the maximal induction was only 2-fold. Taken together, these results indicated that 2-AAF (or 2-AAAF) up-regulates mdr1b expression through generation of ROS and inhibition of intracellular ROS was able to block the induction of mdr1b mRNA by 2-AAF.

2-AAF and 2-AAAF Increase Intracellular ROS and Overexpression of γ-Glutamylcysteine Synthetase Reduces ROS—To
substantiate that 2-AAF or 2-AAAF generate ROS in H4-II-E cells and to investigate whether overproduction of GSH suppresses the generation of ROS, we performed flow cytometric assay to measure intracellular ROS levels. Both H4-II-E and H9 cells were treated with either 100 μM 2-AAF or 10 μM 2-AAAF for time intervals ranging from 20 min to 6 h and ROS levels were measured using a non-fluorescent dye dichlorofluorescein diacetate, which becomes highly fluorescent upon oxidation by intracellular ROS. A representative set of histograms from untreated controls and 100 μM 2-AAF 1 h treatments was shown in Fig. 7A. The average values of the mean fluorescence intensity from triplicate treatments were plotted in Fig. 7B. In H4-II-E cells, the ROS level was rapidly increased and reached a peak at 1 h after treatment. Levels of ROS were then decreased, but reached a second peak at 4 h. This two-wave profile was reproducible. The underlying mechanism of this ROS wave is unclear at present. In contrast, the increase of ROS in H9 cells was much less dramatic, with a maximum only 50% of that of H4-II-E cells. When 2-AAAF was used, we also obtained a similar result (data not shown), indicating that in terms of both mdr1b induction and ROS generation, 2-AAF and 2-AAAF are nondistinguishable.

2-AAF Up-regulates mdr1b through NF-κB

Since mdr1b is a NF-κB-regulated gene and NF-κB is an important oxidative stress responder, we then compared 2-AAF induced NF-κB DNA binding activity in H4-II-E (Fig. 8A) and GSH overproducing H9 cells (Fig. 8B). Nuclear extracts were obtained from both cell lines treated with 100 μM 2-AAF for different times as indicated. This figure is representative of at least four independent experiments and each point is the average of the mean fluorescence intensity of triplicate treatments.
2-AAF activated NF-κB DNA binding in both cell lines, but the maximal induction level in H9 cells were about 40% lower than that in H4-II-E cells. This result indicates that suppression of ROS was correlated with suppression of the 2-AAF induced activation of NF-κB signaling.

2-AAF Activates IKK through Generating ROS—Phosphorylation and ubiquitination dependent degradation of IkB proteins is one of the most common mechanisms through which various extracellular stimuli activate NF-κB (25–27). IKK kinase complex has been shown to phosphorylate IkBs in cells treated with these inducers. To address the questions whether IKK activation is involved in 2-AAF induction of mdr1b and whether suppression of ROS also down-regulates IKK kinase activity, we performed in vitro kinase assay to measure IKK activity in H4-II-E and H9 cells treated with 2-AAF. As shown in Fig. 9, treatment of H4-II-E cells with 2-AAF increased IKK activity 1.3-1.8-fold with a kinetics and induction level compatible with the NF-κB DNA binding activity. In a parallel experiment, IKK activity remained unchanged after 2-AAF treatment in H9 cells. The specificity of the phosphorylation of recombinant GST-Iκα substrate was confirmed by the absence of phosphorylation of GST (panel C in Fig. 9). In addition, the induction of IKK activity in H4-II-E cells was not due to elevation of IκKα protein synthesis, since Western blotting analysis with IκKα antibody failed to reveal significant increase of IκKα protein. Thus, we conclude that induction of mdr1b expression by 2-AAF is mediated through the generation of ROS that activate IKK kinase activity and the downstream NF-κB signaling.

DISCUSSION

Activation of NF-κB Signaling and Induced mdr Gene Expression by 2-AAF in Rat Hepatoma—Previous studies have demonstrated that mdr1b expression is frequently up-regulated in HCC developed by various hepatocarcinogenic programs. However, the mechanisms of this activation are largely unknown. In this study, we studied mechanisms of mdr1b up-regulation in rat hepatoma cells induced by 2-AAF. We identified that the NF-κB-binding site on the mdr1b promoter is necessary for the induction by 2-AAF. We further demonstrated that treatment of rat hepatoma cells with 2-AAF increases DNA binding activity of NF-κB through activation of IKK, which degrades IkBβ but not IkBα. Our present study reveals a sequence of events involved in the activation of mdr1b by 2-AAF as shown in Fig. 10. This is the first demonstration that activation of mdr1b expression by a hepatocarcinogen is mediated by the NF-κB signaling.

In most cases, NF-κB activation involves phosphorylation and degradation of IkBα, which in turn releases NF-κB and leads to the nuclear translocation of NF-κB. However, we were not able to observe IkBα degradation after 2-AAF treatment, but instead IkBβ was degraded. These observations were also supported using recombinant DNA containing Flag-tagged IκBα and IκBβ in transfection assays followed by 2-AAF treatments. To the best of our knowledge, this is the first observation showing that activation of NF-κB pathway is mediated by IκBβ but not IκBα degradation.

Unlike IκBα, IκBβ itself is not regulated by NF-κB and thus it is not rapidly resynthesized after degradation. For this reason, IκBβ degradation is thought to associate with prolonged activation of NF-κB (14, 28). In agreement with this notion, our data showed that 2-AAF caused a relatively slow and prolonged degradation of IκBβ and activation of NF-κB DNA binding, in comparison with that caused by cytokines, e.g. TNF-α. The activation of IKK in the 2-AAF-treated cells could be demonstrated by the in vitro kinase assay experiments, suggesting that the mechanisms of preferential degradation of IκBβ may lie downstream from the IKK activation. At present it is unclear why IκBβ is preferentially degraded, but possibilities can be offered: One possibility is that there may exist a 2-AAF-inducible inhibitor which preferentially shields IκBα from phosphorylation by IKK. Alternatively, an IκBβ-specific kinase which preferentially recognizes and phosphorylates IκBβ but not IκBα may be induced by 2-AAF. Recent studies have identified an IκBβ-specific interacting protein (29). Moreover, activation of NF-κB by multiple distinct IκB kinase complexes has been demonstrated (30). There results suggest that there may be additional IκB-interacting proteins and/or kinases involved in the activation of NF-κB by 2-AAF. Another possibility is that both IκBα and IκBβ are phosphorylated, but their subsequent proteolytic degradation is differentially regulated. Further studies on the kinetics and extent of IκBα and IκBβ phosphorylation as well as their degradation will be helpful to differentiate these possibilities.

Recent studies have demonstrated that multiple pathways are responsible for activation of NF-κB. In addition to the mechanism reported here which involves the degradation of IκBβ, other alternative mechanisms may also play a role in the NF-κB activation in the absence of IκBα degradation. Beraud et
al. (31) found that hypoxia, reoxygenation, and the perivascular-induced IκBα phosphorylation at tyrosine 42 and this led to NF-κB activation independent of IκBα proteolysis. They observed that the regulatory subunit of phosphatidylinositol 3-kinase could recognize tyrosine-phosphorylated IκBα and sequester it from binding to NF-κB. Alternatively, several reports have shown that NF-κB subunits p65 and p50 are phosphorylated and phosphorylation increases NF-κB DNA binding activity or transactivation potential (32, 33). Moreover, the degradation of other NF-κB inhibitors including IκBα, IκBε, and p105 may also contribute to NF-κB activation by 2-AAF without degradation of IκBα. To define these possibilities, more detailed studies are warranted.

**Redox Regulation of NF-κB Signaling and mdr Gene Expression**—We also presented evidence showing that ROS levels are elevated in H-4-II-E cells treated with 2-AAF. Induction of ROS is much reduced in the GSH overproducing H9 cells. These results are consistent with our previous report demonstrating that overproduced antioxidant GSH in cultured cells suppressed oxidative stress induced by cytotoxic agents, including TNF-α, phorbol ester, and okadaic acid (24). Concomitantly, we found that induction of IKK activity, NF-κB DNA binding activity, and mdr1b expression is retarded in the H9 cells as compared with the parental H-4-II-E cells. These results strongly suggest that, like MRPI (15), expression of mdr1b can be regulated by redox conditions. The identification of IKK as an upstream redox sensor of NF-κB activating signal by 2-AAF is consistent with those reported by Chen et al. (34) demonstrating that the activity of IκB kinase β (IKKβ) was significantly elevated in cells exposed to prooxidant vanadate. However, our results may differ from those reported by Li and Karin (35). These investigators reported that induction of IKK activity by TNF-α (and therefore IκBα phosphorylation/degradation) was not affected in cells treated with NAC (35).

Aside from the involvement of IKK as a target of redox regulation, oxidative stress may cause protein conformational changes. Particularly, oxidation of methionyl residues may render proteins susceptible to proteasomal degradation (36). In fact, oxidized methionine residues are commonly found in many human hematopoietic and solid tumors (42, 43). III. NF-κB signaling can be activated by a wide variety of stimuli, including genotoxic and nongenotoxic factors. As liver is the major detoxification reservoir of xenobiotics, many of these excellular stimuli are known to induce liver cancers in experimental animals. (iv) Evidence has accumulated that ROS plays an important role in hepatocarcinogenesis in animal models and suppression of ROS retards liver cancer progression in these models (37, 44). Greater than 10-fold increases of mdr1b expression in Fisher rats can be induced by 2-AAF (4). On the other hand, liver neoplastic lesions can be chronically induced in these animals by this hepatocarcinogen. The results described in this article thus provide a molecular basis for further investigation on the roles of NF-κB signaling in hepatocarcinogenesis and the induction of mdr1b gene expression in the process. These experiments are currently being investigated in this laboratory. These studies may eventually lead to a better understanding on the mechanisms of liver cancer development and the evolution of drug resistance in this devastating disease.

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