The phosphorylated retinoid X receptor-α promotes diethylnitrosamine-induced hepatocarcinogenesis in mice through the activation of β-catenin signaling pathway

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Abstract

Previous studies have shown that phosphorylation of the retinoid X receptor-α (p-RXRα) is associated with the development of hepatocellular carcinoma (HCC). However, these findings were revealed using HCC cell lines that express phosphorylated-RXRα (p-RXRα) proteins; therefore, it remains unclear whether p-RXRα affects hepatocarcinogenesis in vivo. Therefore, to investigate the biological function of p-RXRα in vivo, we developed a doxycycline-inducible ES cell line and transgenic mouse, both of which overexpress the phosphomimetic mutant form of RXRα, T82D/S260D, in a doxycycline-dependent manner. We found that the development of liver tumors, especially high-grade adenoma and HCC, was enhanced in diethylnitrosamine (DEN)-treated T82D/S260D-inducible mice. Moreover, the increased incidence of liver tumors in the transgenic mice was attributable to the promotion of cell cycle progression. Interestingly, the expression of β-catenin protein and its target gene cyclin D1 was elevated in the liver tumors of DEN-treated T82D/S260D-inducible mice, concurrent with increased cytoplasmic and nuclear β-catenin protein expression, indicating its stabilization and transcriptional activation. These results indicate that p-RXRα promotes DEN-induced hepatocarcinogenesis in mice through the activation of the β-catenin signaling pathway, suggesting that p-RXRα may serve as a possible therapeutic target for HCC.

Abbreviations: APC, adenomatous polyposis coli; DEN, diethylnitrosamine; DOX, doxycycline; HCC, hepatocellular carcinoma; LEF, lymphoid enhancer factor; PCNA, proliferating cell nuclear antigen; qRT-PCR, quantitative real-time reverse transcription-polymerase chain reaction; RARs, retinoic acid receptors; RXRα, retinoid X receptor-α; TCF, T-cell factor; TUNEL, terminal deoxynucleotidyltransferase-mediated dUTP-biotin nick end labeling.

Original Article

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in HBV- or HCV-related HCC. Moreover, altered expression of RARs has been reported to be associated with malignant transformation of animal tissues or cultured cells (12,13). Our previous studies have reported that a malfunction of RXRα due to its aberrant phosphorylation plays a role in the development of HCC (14,15). In HCC cell lines, RXRα is constitutively phosphorylated at both threonine 82 and serine 260 owing to the activation of Ras/MAPK signaling (14), and it accumulates by avoiding ubiquitination and proteasome-mediated degradation (15). The accumulation of phosphorylated-RXRα (p-RXRα) protein interferes with the functioning of the remaining normal RXRα in a dominantly-negative manner, thereby promoting the development of HCC cells (14). In contrast, acyclic retinoid, a synthetic retinoid that can inhibit the phosphorylation of RXRα and restore its receptor function (16), has been reported to inhibit the growth of human hepatoma cells (16–19). Thus, our previous in vitro studies have revealed the important role of p-RXRα in hepatocarcinogenesis.

Although our previous studies showed that p-RXRα is associated with the development of HCC cell lines, the effects of p-RXRα expression on hepatocarcinogenesis in vivo remain unknown. Therefore, in the current study, we established a phosphomimetic mutant form of human RXRα, T82D/S260D, transgenic mice using a doxycycline (DOX)-dependent expression system to investigate the role of T82D/S260D expression in hepatocarcinogenesis in mice.

Materials and methods

Molecular cloning and gene targeting in ES cells
A phosphomimetic mutant form of human RXRα cDNA, T82D/S260D, in which threonine 82 and serine 260 were mutated to aspartate, respectively, was generated as described previously (14), and then cloned into pcr2.1-TOPO. Sequence-verified T82D/S260D cDNA was subcloned into a unique EcoR1 site of the pBS31 prime vector (20,21). KH2 ES cells obtained from Open Biosystems, Huntsville, AL were used to insert a single copy of T82D/S260D by flippase recombination into the ColIa1 locus under the control of a minimal CMV tetracycline-inducible promoter using a previously described method (20), and ES cells were selected for hygromycin resistance.

Mouse generation
Fertilized zygotes were isolated from the oviducts of day-0.5 pregnant B6D2F1 females and allowed to develop to the blastocyst stage in culture. Subsequently, 7-12 ES cells were injected into the blastocysts, which were then transferred into day-2.5 pseudo-pregnant females.

Doxycycline treatment
Mice were administered 2 mg/ml (wt/vol) DOX (D9891; Sigma–Aldrich, St. Louis, MO) in their drinking water, which was supplemented with 10 mg/ml sucrose. For culture cells, DOX was used at a concentration of 2 μg/ml (w/v).

RNA preparation and quantitative real-time reverse transcription-polymerase chain reaction
Total RNA was extracted using the RNaqueous-4PCR kit (Ambion, Carlsbad, CA) according to the manufacturer’s instructions. Thereafter, cDNA was synthesized using the SuperScript III First-Strand Synthesis System (Invitrogen Life Technologies, Carlsbad, CA). Quantitative real-time reverse transcription-polymerase chain reaction (qRT-PCR) analysis using the fluorescent SYBR green method was performed according to previously described protocols (22). The expression of each gene was normalized to that of β-actin using the standard curve method. Primer sequences are shown in Supplementary Table S1, available at Carcinogenesis Online.

Western blotting analysis
Western blotting analysis was performed as described previously (22). The following primary antibodies were used: anti-RXRα (rabbit IgG, 1:500 dilution, sc-553; Santa Cruz Biotechnology, Inc., Santa Cruz, CA), anti-β-catenin (mouse IgG, 1:8000 dilution, #610154; BD Transduction, San Jose, CA), anti-Rb (mouse IgG, 1:500 dilution, #554136; BD Pharmingen, San Jose, CA), anti-cyclin D1 (rabbit IgG, 1:1000 dilution, sc-753; Santa Cruz Biotechnology), antiproliferating cell nuclear antigen (PCNA) (mouse IgG, 1:2000 dilution, #2586; Cell Signaling Technology, Danvers, MA) and anti-GAPDH (rabbit IgG, 1:2000 dilution, #2118; Cell Signaling Technology).

Animals and experimental procedures
Heterozygous Rosa26::M2rtTA mice with heterozygous tetO-T82D/S260D allele (Rosa+/Rxr+) were generated by breeding homozygous Rosa26::M2rtTA male mice with homozygous tetO-T82D/S260D allele (Rosa/Rosa; Rxr/Rxr) to C3H/HeN females without the Rosa26::M2rtTA allele or tetO-T82D/S260D allele (SLC Japan, Shizuoka, Japan). In contrast, heterozygous Rosa26::M2rtTA mice (Rosa+/+), which served as a control for Rosa+/Rxr+ mice, were generated by breeding homozygous Rosa26::M2rtTA (Rosa/Rosa) males to C3H/HeN females without Rosa26::M2rtTA allele or tetO-T82D/S260D allele (SLC Japan) (Supplementary Figure S1A, available at Carcinogenesis Online). First, to investigate the spontaneous development of liver tumors in vivo, T82D/S260D-inducible male mice (Rosa+; Rxr+/+) and control male mice (Rosa+) were administered tap water containing 2 mg/ml (wt/vol) DOX (D9891; Sigma–Aldrich) starting at 4 weeks of age and killed at either 6 (Supplementary Figure S1B, available at Carcinogenesis Online) or 8 (Supplementary Figure S1C, available at Carcinogenesis Online) months of age for both macroscopic inspection and histological analysis. Next, to induce liver tumors, the liver carcinogen diethylnitrosamine (DEN) (442687; Sigma–Aldrich) dissolved in 0.9% saline was administered intraperitoneally (25 mg/kg of body weight) to 15-day-old male pups, which were obtained from each breeding pair described above. The DEN-treated T82D/S260D-inducible male mice (Rosa+; Rxr+/+) and DEN-treated control male mice (Rosa+) were given tap water containing 2 mg/ml (wt/vol) DOX from 4 weeks to 6 months of age. At 6 months of age, both genotypes of mice were killed for both macroscopic inspection and histological analysis (Supplementary Figure S1D, available at Carcinogenesis Online). All mice received humane care and were housed at the Gifu University Life Science Research Center in accordance with the Institutional Animal Care Guidelines. All animal experiments were approved by the Institutional Committee on Animal Experiments of Gifu University.
Macroscopic inspection, histopathologic analysis, immunohistochemical analysis and terminal deoxynucleotidyltransferase-mediated dUTP-biotin nick end labeling assay

After killing the mice, the livers were immediately removed and weighed, and the number of hepatic tumors on the surface of the left lobe with diameter ≥1 mm was macroscopically counted. Maximum sagittal sections of three sublobes (left lateral, medial and right medial lobes) were histopathologically examined. Four-micrometer-thick sections of 10% buffered formalin-fixed, paraffin-embedded liver sections were stained with hematoxylin and eosin for conventional histopathology. The numbers and the histological grades of liver tumors were evaluated macroscopically according to previously described criteria by which liver tumors were classified as Grade 1, 2 or 3 adenomas and HCC depending on the atypical degree (23). Per these criteria (23), liver tumors were defined as Grade 1: the lesion showed a focal trabecular pattern with single cell plates; Grade 2: the lesion showed a prominent trabecular pattern with plates of two cell layers, slight cellular pleomorphism and increased cell size; Grade 3: the lesion was composed of prominent abnormal trabeculation with more than two cell layers, increased N/C ratio, high mitotic rates and marked cellular pleomorphism; and HCC: the lesion showed features similar to that of Grade 3, but necrosis was present with no visible remnant of adenoma (Supplementary Figure S2A, available at Carcinogenesis Online). In addition, foci of cellular alteration (FCA), which are hepatic preneoplastic lesions with a basophilic cytoplasm and hyperchromatic nuclei (24), were also evaluated (Supplementary Figure S2B, available at Carcinogenesis Online). Immunohistochemistry was performed using an avidin–biotin immunoperoxidase assay, according to a previously described protocol (22). The following primary antibodies were used: anti-PCNA (rabbit IgG, 1:100 dilution, ab2426; Abcam, Cambridge, MA), anti-cleaved caspase 3 (rabbit IgG, 1:100 dilution, #9661; Cell Signaling Technology) and anti-β-catenin (mouse IgG, 1:500 dilution, #610154; BD Transduction). Apoptotic cells in the liver were evaluated using the ApopTag peroxidase in situ apoptosis detection kit (#57100; Millipore, Billerica, MA), which labels DNA strand breaks by the terminal deoxynucleotidyltransferase-mediated dUTP-biotin nick end labeling (TUNEL) method, according to the manufacturer’s instructions.

Statistical analysis

Statistical analysis was performed using GraphPad Prism 4 software (GraphPad Software, Inc., San Diego, CA). The mean ± standard deviation (SD) was calculated for all parameters determined. Statistical significance was evaluated using either Student’s t-test or Welch’s t-test for paired samples. Statistical significance was set at P < 0.05.

Results

Inducible expression of a phosphomimetic mutant form of the human RXRα (T82D/S260D) in ES cells and mice

We generated DOX-inducible T82D/S260D ES cells, in which a phosphomimetic mutant form of the human RXRα gene, T82D/S260D, can be induced under the control of a tetracycline-responsive regulatory element (Figure 1A). Upon treatment of these ES cells with DOX, the expression of T82D/S260D mRNA was significantly increased compared with that in ES cells without DOX treatment (Figure 1B). Increased expression of T82D/S260D protein, which was evaluated using an anti-RXRα antibody, was also confirmed in DOX-treated ES cells (Figure 1C). Next, we administered DOX to T82D/S260D-inducible mice (Rosa+/; Rxr+/) to confirm the expression of the phosphomimetic mutant form of human RXRα, T82D/S260D, in the liver. As shown in Figure 1D and E, the administration of DOX induced a time-dependent increase in T82D/S260D mRNA and protein expression in the liver, indicating that the phosphomimetic mutant form of human RXRα was effectively induced in these transgenic mice by the administration of DOX.

T82D/S260D-inducible mice are highly susceptible to the development of DEN-induced liver tumors

We investigated the effects of a phosphomimetic mutant form of human RXRα, T82D/S260D, on the spontaneous development of liver tumors in vivo. Initially, both T82D/S260D-inducible male mice (Rosa+/; Rxr+/) and control male mice (Rosa+) were administered DOX starting at 4 weeks of age and were killed at 6 months of age (Supplementary Figure S1B, available at Carcinogenesis Online). Macroscopic inspection and microscopic analysis revealed no development of liver tumors in both genotypes of mice, and histological findings of the liver were comparable between both genotypes (data not shown). We then extended DOX treatment up to 8 months of age (Supplementary Figure S1C, available at Carcinogenesis Online); however, the development of liver tumors was not observed in either group (data not shown). To induce liver tumors, we administered the liver carcinogen DEN (25 mg/kg of body weight) intraperitoneally to 15-day-old male pups of each group and started DOX treatment at 4 weeks of age, and killed the mice at 6 months of age (Supplementary Figure S1D, available at Carcinogenesis Online). As shown in Table 1 and Figure 2, the number and maximum size of macroscopic liver tumors were significantly increased in DEN-treated T82D/S260D-inducible mice (Rosa+/; Rxr+/) compared with those in DEN-treated control mice (Rosa+). In addition, microscopic analysis revealed that the number of high-grade liver tumors, including grade 3 adenoma and HCC, was significantly increased in DEN-treated T82D/S260D-inducible mice (Rosa+/; Rxr+/) (Table 2). No signs of liver cirrhosis, such as pseudolobule formation and liver fibrosis, were observed in either mouse genotype (data not shown). Thus, these results indicate that a phosphomimetic mutant form of human RXRα, T82D/S260D, by itself, does not initiate liver tumorigenesis, but promotes hepatocarcinogenesis following the administration of DEN.

Development of liver tumors in DEN-treated T82D/S260D-inducible mice is primarily attributed to the promotion of cell proliferation

The retinoid receptor RXRα plays a role in maintaining homeostasis by regulating fundamental cell activities, including cell proliferation and apoptosis (7,25). Previous studies have shown that RXRα loses its receptor function by undergoing phospho-modifications, which are associated with hepatocarcinogenesis in vitro (14,17–19,26,27). We then investigated the levels of cell proliferation and apoptosis in liver tumors developed in either DEN-treated
T82D/S260D-inducible mice (Rosa+/; Rxr+/) or DEN-treated control mice (Rosa+). Immunohistochemistry for PCNA showed that the percentage of PCNA-positive cells per liver tumor was significantly increased in DEN-treated T82D/S260D-inducible mice (Rosa+/; Rxr+) compared with that in DEN-treated control mice (Rosa+) (Figure 3A). In contrast, the levels of apoptosis, as ascertained by immunohistochemical staining of tumor sections with TUNEL and cleaved caspase 3, were identical in both genotypes of mice (Figure 3B). The expression of the anti-apoptotic Bcl-2 and Bcl-xl genes tended to increase in the liver tumors of DEN-treated T82D/S260D-inducible mice (Rosa+/; Rxr+), but no difference was observed in the gene expression levels of pro-apoptotic Bax and Bad genes between the two genotypes (Figure 3C). Thus, our findings suggest that the increased liver tumors observed in DEN-treated T82D/S260D-inducible mice are primarily attributed to the promotion of cell proliferation.
The β-catenin signaling pathway is activated in the liver tumors of DEN-treated T82D/S260D-inducible mice

We previously reported that an impaired receptor function of RXRα due to its phosphorylation is associated with the growth of HCC cells, which was attributed to either a decrease in RARβ and p27 or an increase in cyclin D1 (17–19). Therefore, we examined the effect of T82D/S260D expression on the mRNA levels of these molecules. As shown in Figure 4A, the expression levels of RARβ and p27 mRNA in liver tumors and non-tumorous liver tissues were comparable between DEN-treated T82D/S260D-inducible mice (Rosa/+; Rxr+/+) and DEN-treated control mice (Rosa/+). In contrast, the expression levels of cyclin D1 mRNA were significantly increased in the liver tumors of DEN-treated T82D/S260D-inducible mice (Rosa/+; Rxr+/+). Cyclin D1 is known as a downstream target of β-catenin/Tcf transcription (28), and the activation of the Wnt/β-catenin pathway promotes the development of several types of cancer, including HCC (29–33). As shown in Figure 4B, the levels of β-catenin and cyclin D1 proteins in the liver tumors of DEN-treated T82D/S260D-inducible mice (Rosa/+; Rxr+/+) were higher than in the liver tumors of DEN-treated control mice (Rosa/+). In addition, the level of phosphorylated Rb protein, which induces G1-S checkpoint transition of the cell cycle under the control of cyclin D1 (34,35), was also increased in the liver tumors of DEN-treated T82D/S260D-inducible mice (Rosa/+; Rxr+/+).

To histologically evaluate the activation of the β-catenin signaling pathway, it is important to determine the subcellular and nuclear localization of β-catenin, because signal transduction via this protein involves its post-transcriptional stabilization and translocation into the nucleus (37). Therefore, we compared the localization of β-catenin protein in precancerous Grade 3 adenomas, defined according to previous criteria (23), between DEN-treated T82D/S260D-inducible mice (Rosa/+; Rxr+/+) and DEN-treated control mice (Rosa/+). As shown in Figure 4C, the expression of β-catenin protein in DEN-treated control mice (Rosa+/+) was primarily localized in the membrane of tumor cells. In contrast, relatively strong cytoplasmic expression of β-catenin protein was observed in DEN-treated T82D/S260D-inducible mice (Rosa/+; Rxr+/+), which was localized in the nucleus in some tumor cells of DEN-treated control mice (Rosa/+). Overall, these findings suggest that in DEN-treated T82D/S260D-inducible mice (Rosa/+; Rxr+/+), β-catenin signaling pathway is activated even in precancerous lesions of HCC and may play a role in promoting hepatocarcinogenesis.

Reduced expression of Pleckstrin homology domain-containing family B member 1 (Plekhb1) mRNA may be associated with DEN-induced liver tumorigenesis in T82D/S260D-inducible mice

We also investigated other molecular targets that can be regulated by a phosphomimetic mutant form of human
Figure 3. Effects of T82D/S260D expression on cellular proliferation and apoptosis in DEN-induced liver tumors. (A) Liver sections from DEN-treated control mice (Rosa/+) and DEN-treated T82D/S260D-inducible mice (Rosa++; Rxr+/+) were stained with anti-PCNA antibody. Representative images from each group are shown in the left panels. PCNA-positive cells were counted and expressed as a percentage of the total number of cells per liver tumor. The positive cell indices are shown in the right panels. The dotted lines indicate the margin of liver tumors. Scale bar, 200 μm. (B) TUNEL and cleaved caspase 3-positive cells in liver tumors were evaluated using an apoptosis detection kit and immunohistochemical analysis, respectively. Representative images from each group are shown in the left panels. Sections of rat thymus were used as the positive control in each experiment. The dotted lines indicate the margin of liver tumors. Scale bar, 200 μm. (C) The mRNA expression levels of Bax, Bad, Bcl-xL and Bcl-2 in liver tumors were detected by qRT-PCR using specific primers. Transcript levels were normalized to that of β-actin. Data are presented as mean ± SD (n = 3). *P < 0.05, Student’s t-test.
RXRα. Previously, Plekb1 was shown to be downregulated in human HCC (38). In addition, the Plekb1 gene was identified to have a canonical retinoic acid response element (RARE) in the promoter regions, and the levels of Plekb1 expression in germ cells were downregulated in RARα conditional knockout mice (39). Given that the overexpression of T82D/S260D inhibited transactivation through RARE in normal human hepatocytes (14), we estimated the association between the expression of T82D/S260D and Plekb1 mRNA. We then focused on validating gene expression in the liver of DOX-treated T82D/S260D-inducible mice (Rosa/+; Rxr/+) using qRT-PCR. In contrast with the significant increase in T82D/S260D mRNA levels after DOX treatment, the mRNA expression of Plekb1 was significantly reduced, suggesting a negative correlation between the expression of each gene (Supplementary Figure S3A, available at Carcinogenesis Online). Notably, compared with DEN-treated control mice (Rosa/+), the mRNA level of Plekb1 in the liver tumors of DEN-treated T82D/S260D-inducible mice (Rosa/+; Rxr+/+) was significantly reduced (Supplementary Figure S3C, available at Carcinogenesis Online). In contrast, increased T82D/S260D mRNA expression was observed in liver tumors of DEN-treated T82D/S260D-inducible mice (Rosa/+; Rxr+/+) (Supplementary Figure S3C, available at Carcinogenesis Online). Collectively, these results suggest that the expression of Plekb1 mRNA may be negatively regulated by T82D/S260D expression, and the reduced expression of the Plekb1 gene may be associated with DEN-induced liver tumorigenesis in T82D/S260D-inducible mice (Rosa/+; Rxr+/+) (Figure 4D).

**Discussion**

In the present study, our findings demonstrate that the in vivo expression of T82D/S260D promotes DEN-induced
hepatocarcinogenesis in mice through the activation of the \( \beta \)-catenin signaling pathway.

\( \beta \)-Catenin is a structural protein in the cadherin-mediated cell–cell adhesive system which plays a role in the differentiation and repair of normal tissues (40). It is also known to act as a mediator in the canonical Wnt signaling pathway; inappropriate activation of this pathway has been implicated in the development of several types of malignancies, including HCC (30–33). A previous study showed that overexpression of \( \beta \)-catenin accelerates liver tumorigenesis and progression to HCC following DEN exposure (41). In this study, a high prevalence of high-grade liver tumors and increased expression of \( \beta \)-catenin protein in tumor cells was observed in DEN-treated T82D/S260D-inducible mice (Table 2, Figure 4B and C), suggesting the involvement of the \( \beta \)-catenin signaling pathway in hepatocarcinogenesis in this experimental model.

Nuclear and cytoplasmic \( \beta \)-catenin expression has been shown to play an important role in tumor progression (30,42). The stabilized cytoplasmic \( \beta \)-catenin enters the nucleus by binding to the T-cell factor (TCF) and lymphoid enhancer factor (LEF) family of proteins, and induces the transcription of target genes, including cyclin D1. Cyclin D1 promotes the transition between the G1-S checkpoint of the cell cycle by influencing the activity of Rb protein and induces cell proliferation in the cell cycle (34,35). Indeed, cytoplasmic and nuclear accumulation of \( \beta \)-catenin in HCC tissues has been reported in previous clinical and experimental studies (13,29–31,43,44), and the amplification of cyclin D1 genes and its overexpression have been shown to be associated with aggressive forms of liver tumors, including HCC (45,46). In this study, \( \beta \)-catenin protein was localized in the cytoplasm and the nucleus of liver tumor cells in DEN-treated T82D/S260D-inducible mice, and the downstream proliferative signals, such as cyclin D1 and phosphorylated Rb proteins, were elevated in these liver tumors (Figure 4B and C), indicating the activation of the \( \beta \)-catenin signaling pathway in these mouse models. Our findings suggest that the acceleration of hepatocarcinogenesis observed in DEN-treated T82D/S260D-inducible mice is attributable to the activation of the \( \beta \)-catenin signaling pathway. In addition, as previously indicated (45,46), increased cyclin D1 expression may be associated with the high prevalence of high-grade liver tumors observed in this mouse model.

Previous studies have reported that retinoid receptors, including RAR\( \alpha \) and RXR\( \alpha \), directly interact with \( \beta \)-catenin and regulate the Wnt/\( \beta \)-catenin signaling pathway. Han et al. revealed that RXR\( \alpha \) overexpression directly inhibited both \( \beta \)-catenin/TCF/LEF transcriptional activity and \( \beta \)-catenin protein levels in colorectal cancer cells, whereas downregulation of RXR\( \alpha \) by small interfering RNA abolished these inhibitory effects and elevated both \( \beta \)-catenin protein levels and \( \beta \)-catenin/TCF/LEF transcriptional activity (47). In addition, a previous study using transgenic mice expressing the RAR\( \alpha \)-dominant negative form in hepatocytes showed that the reduction of the RAR\( \alpha \)/\( \beta \)-catenin complex caused an increase in the \( \beta \)-catenin/TCF complex, thus inducing the expression of cyclin D1 and leading to hepatocarcinogenesis (13). Thus, RXR\( \alpha \) and RAR\( \alpha \) regulate free \( \beta \)-catenin protein levels by directly forming a complex with \( \beta \)-catenin, thereby inhibiting the Wnt/\( \beta \)-catenin/TCF function. Notably, the direct regulation of \( \beta \)-catenin by these retinoid receptors is perturbed by not only the reduced expression of retinoid receptors, but also their malfunction (13,29,43,47). T82D/S260D, the phosphomimetic mutant form of human RXR\( \alpha \) used in this study, has been reported to interfere with the function of the remaining normal RXR\( \alpha \) in a dominant-negative manner (14). Previous studies combined with our present findings may lead to the hypothesis that the in vivo expression of T82D/S260D may inhibit complex formation between normal RXR\( \alpha \) and \( \beta \)-catenin, thereby causing an increase in the free levels of \( \beta \)-catenin, activating the Wnt/\( \beta \)-catenin signaling pathway, inducing overexpression of cyclin D1 and thereby contributing to hepatocarcinogenesis in DEN-treated T82D/S260D-inducible mice (Figure 4D).

The levels of free \( \beta \)-catenin are regulated by two adenomatous polyposis coli (APC)-dependent pro teaseosomal degradation pathways: glycogen synthesis kinase-3\( \beta \)-regulated pathway involving the APC/Axin complex and a p53-inducible pathway involving Siah-1 (48,49). Mutations in either the key components of the above two pathways, such as APC, Axin and p53, or \( \beta \)-catenin itself, have been reported to lead to dysregulation of \( \beta \)-catenin turnover in several malignancies, including HCC, subsequently resulting in its cytoplasmic and nuclear accumulation and abnormal activation of TCF/LEF-regulated genes that are involved in oncogenesis (43,50). To date, there have been no reports of an association between retinoid receptor malfunction and the above-mentioned mutations. However, given that the \( \beta \)-catenin signaling pathway was activated in the liver tumors of DEN-treated T82D/S260D-inducible mice, the possibility that the in vivo expression of T82D/S260D may be associated with these mutations cannot be ruled out. Further experiments are required to address this issue.

The levels of the Plekhh1 gene were significantly reduced in liver tumors of DEN-treated T82D/S260D-inducible mice compared with those of DEN-treated control mice (Supplementary Figure S3C, available at Carcinogenesis Online). Given that the gene expression has been revealed to be downregulated in human HCC (38), it has been suggested that the reduced expression of Plekhh1 gene may be associated with DEN-induced liver tumorigenesis in T82D/S260D-inducible mice (Figure 4D). Further experiments are necessary to elucidate the underlying molecular mechanisms.

In conclusion, our findings demonstrate that p-RXR\( \alpha \) plays a role in chemically induced hepatocarcinogenesis in mice. The fact that the abnormal phosphorylation of RXR\( \alpha \) is involved in liver carcinogenesis in vivo suggests that p-RXR\( \alpha \) may serve as a possible therapeutic target for HCC.

Supplementary material
Supplementary data are available at Carcinogenesis online.

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Conflict of Interest Statement

None declared.

Authors’ contributions

H.S., Y.Y. and M.S. collaborated with the conception and design of the experiment. H.S., M.K. and Y.S. performed the experiments and acquired the data and images. H.S., Y.Y., K.I. and H.T. analyzed the data. H.S. wrote the main manuscript text and prepared all the figures and tables. A.H., Y.Y. and M.S. supervised the manuscript preparation. All authors reviewed the manuscript.

References

1. El-Serag, H.B. (2011) Hepatocellular carcinoma. N. Engl. J. Med., 365, 1118–1127.
2. European Association for the Study of the Liver. (2018) EASL clinical practice guidelines: management of hepatocellular carcinoma. J. Hepatol., 69, 182–236.
3. Njei, B. et al. (2015) Emerging trends in hepatocellular carcinoma incidence and mortality. Hepatology, 61, 191–199.
4. Llovet, J.M. et al.; SHARP Investigators Study Group. 2008) Sorafenib in advanced hepatocellular carcinoma., 339, 378–390.
5. Brux, J. et al.; RESORCE Investigators. 2017) Regorafenib for patients with hepatocellular carcinoma who progressed on sorafenib treatment (RESORCE): a randomised, double-blind, placebo-controlled, phase 3 trial. Lancet, 389, 56–66.
6. Kudo, M. et al. (2018) Lenvatinib versus sorafenib in first-line treatment of patients with unresectable hepatocellular carcinoma: a randomised phase 3 non-inferiority trial. Lancet, 391, 1163–1173.
7. Chambon, P. (1996) A decade of molecular biology of retinoic acid receptors. FASEB J., 10, 940–954.
8. Sun, S.Y. et al. (2002) Retinoids and their receptors in cancer development and chemoprevention. Crit. Rev. Oncol. Hematol., 41, 41–55.
9. Huan, B. et al. (19929063) Retinoid X receptor RXR alpha binds to and trans-activates the hepatitis B virus enhancer. Proc. Natl. Acad. Sci. U.S.A., 89, 9059.
10. Benbrook, D. et al. (1988) A new retinoic acid receptor identified from a hepatocellular carcinoma. Nature, 333, 669–672.
11. Zhang, R. et al. (2021) Genetic variant of RXR involved in the vitamin D metabolic pathway was linked to HCV infection outcomes among a high-risk Chinese population. Infect. Genet. Evol., 87, 104641.
12. Bushue, N. et al. (2010) Retinoid pathway and cancer therapeutics. Adv. Drug Deliv. Rev., 62, 1285–1298.
13. Yanagitani, A. et al. (2004) Retinoic acid receptor alpha dominant negative form causes steatohepatitis and liver tumors in transgenic mice. Hepatology, 40, 366–375.
14. Matsushima-Nishiwaki, R. et al. (2001) Phosphorylation of retinoid X receptor alpha at serine 260 impairs its metabolism and function in human hepatocellular carcinoma. Cancer Res., 61, 7675–7682.
15. Adachi, S. et al. (2002) Phosphorylation of retinoid X receptor suppresses its ubiquitination in human hepatocellular carcinoma. Hepatology, 35, 332–340.
16. Matsushima-Nishiwaki, R. et al. (2003) Molecular mechanism for growth suppression of human hepatocellular carcinoma cells by acyclic retinoid. Carcinogenesis, 24, 1335–1339.
17. Shimizu, M. et al. (2004) Synergistic effects of acyclic retinoid and OSI-461 on growth inhibition and gene expression in human hepatoma cells. Clin. Cancer Res., 10, 6710–6721.
18. Suzuki, M. et al. (2002) Growth inhibition of human hepatoma cells by acyclic retinoid is associated with induction of p21(CIP1) and inhibition of expression of cyclin D1. Cancer Res., 62, 3997–4006.
19. Suzuki, M. et al. (2004) Acyclic retinoid activates retinoic acid receptor beta and induces transcriptional activation of p21(CIP1) in HepG2 human hepatoma cells. Mol. Cancer Ther., 3, 309–316.
20. Beard, C. et al. (2006) Efficient method to generate single-copy transgenic mice by site-specific integration in embryonic stem cells. Genesis, 44, 23–28.
21. Hochdelinger, K. et al. (2005) Ectopic expression of Oct-4 blocks progenitor-cell differentiation and causes dysplasia in epithelial tissues. Cell, 121, 465–477.
22. Sakai, H. et al. (2010) Genetic ablation of Tnalpha demonstrates no detectable suppressive effect on inflammation-related mouse colon tumorigenesis. Chem. Biol. Interact., 184, 423–430.
23. Jang, J.J. et al. (1992) Progressive atypia in spontaneous and N-nitrosodimethylamine-induced hepatocellular adenomas of C3H/HeNcr mice. Carcinogenesis, 13, 1541–1547.
24. Uehara, T. et al. (2021) The DEN and CCl4-induced mouse model of fibrosis and inflammation-associated hepatocellular carcinoma. Carc. Protoc., 1, e211.
25. Germain, P. et al. (2006) International Union of Pharmacology. LXIII. Retinoid X receptors. Pharmacol. Rev., 58, 760–772.
26. Shimizu, M. et al. (2009) Strategy and mechanism for the prevention of hepatocellular carcinoma: phosphorylated retinoid X receptor alpha is a critical target for hepatocellular carcinoma chemoprevention. Cancer Sci., 100, 369–374.
27. Yoshimura, K. et al. (2007) Phosphorylated retinoid X receptor alpha loses its heterodimeric activity with retinoic acid receptor beta. Cancer Sci., 98, 1868–1874.
28. Tetsu, O. et al. (1999) Beta-catenin regulates expression of cyclin D1 in colon carcinoma cells. Nature, 398, 422–426.
29. Easswaran, V. et al. (1999) Cross-regulation of beta-catenin-LEF/TCF and retinoid signaling pathways. Curr. Biol., 9, 1415–1418.
30. Lustig, B. et al. (2003) The Wnt signaling pathway and its role in tumor development. J. Cancer Res. Clin. Oncol., 129, 199–221.
31. Asaoka, Y. et al. (2020) Clinical implications of WNT/beta-catenin signaling for hepatocellular carcinoma. Glob. Health Med, 2, 269–272.
32. Chen, J. et al. (2016) The microtubule-associated protein PRC1 promotes early recurrence of hepatocellular carcinoma in association with the Wnt/beta-catenin signalling pathway. Gut, 65, 1522–1534.
33. Liu, L.J. et al. (2016) Aberrant regulation of Wnt signaling in hepatocellular carcinoma. World J. Gastroenterol., 22, 7486–7499.
34. Edamoto, Y. et al. (2003) Alterations of RB1, p53 and Wnt pathways in hepatocellular carcinomas associated with hepatitis C, hepatitis B and alcoholic liver cirrhosis. Int. J. Cancer, 106, 334–341.
35. Levy, L. et al. (2002) Genetic alterations and oncogenic pathways in hepatocellular carcinoma. Ann. N. Y. Acad. Sci., 963, 21–36.
36. Kelman, Z. et al. (1995) Structural and functional similarities of prokaryotic and eukaryotic DNA polymerase sliding clamps. Nucleic Acids Res., 23, 3613–3620.
37. Robinfeld, B. et al. (1996) Binding of GSK3beta to the APC-beta-catenin complex and regulation of complex assembly. Science, 272, 1023–1026.
38. Ho, D.W. et al. (2015) TCGA whole-transcriptome sequencing data reveals significantly dysregulated genes and signaling pathways in hepatocellular carcinoma. Front. Med., 9, 322–330.
39. Law, S.M. (2013) Retinoic Acid Receptor Alpha in Germ Cells Is Important for Mitosis of Spermatogonia, Spermatogonial Differentiation and Meiosis. School of Molecular Biosciences. ProQuest LLC: Washington State University, p. 291.
40. Wei, Y. et al. (2002) Altered expression of E-cadherin in hepatocellular carcinoma: correlations with genetic alterations, beta-catenin expression, and clinical features. Hepatology, 36, 692–701.
41. Nejak-Bowen, K.N. et al. (2010) Accelerated liver regeneration and hepatocarcinogenesis in mice overexpressing serine-45 mutant beta-catenin. Hepatology, 51, 1603–1613.
42. Tien, L.T. et al. (2005) Expression of beta-catenin in hepatocellular carcinoma. World J. Gastroenterol., 11, 2398–2401.
43. Xiao, J.H. et al. (2003) Adenomatous polyposis coli (APC)-independent regulation of beta-catenin degradation via a retinoid X receptor-mediated pathway. *J. Biol. Chem.*, 278, 29954–29962.
44. Ando, N. et al. (2007) Expression of retinoid X receptor alpha is decreased in 3'-methyl-4-dimethylaminoazobenzene-induced hepatocellular carcinoma in rats. *Oncol. Rep.*, 18, 879–884.
45. Nishida, N. et al. (1994) Amplification and overexpression of the cyclin D1 gene in aggressive human hepatocellular carcinoma. *Cancer Res.*, 54, 3107–3110.
46. Ito, Y. et al. (1999) Expression and prognostic roles of the G1-S modulators in hepatocellular carcinoma: p27 independently predicts the recurrence. *Hepatology*, 30, 90–99.
47. Han, A. et al. (2008) A direct protein-protein interaction is involved in the suppression of beta-catenin transcription by retinoid X receptor alpha in colorectal cancer cells. *Cancer Biol. Ther.*, 7, 454–459.
48. Ha, N.C. et al. (2004) Mechanism of phosphorylation-dependent binding of APC to beta-catenin and its role in beta-catenin degradation. *Mol. Cell.*, 15, 511–521.
49. Liu, J. et al. (2001) Siah-1 mediates a novel beta-catenin degradation pathway linking p53 to the adenomatous polyposis coli protein. *Mol. Cell.*, 7, 927–936.
50. Polakis, P. (2000) Wnt signaling and cancer. *Genes Dev.*, 14, 1837–1851.