Hepatocarcinogenesis in Mice with β-Catenin and Ha-Ras Gene Mutations

Naomoto Harada,1 Hiroko Oshima,1,2 Masahiro Katoh,1 Yositaka Tamai,1 Masanobu Oshima,1,2 and Makoto M. Taketo2

1Banyu Tsukuba Research Institute (Merck), Tsukuba, and 2Department of Pharmacology, Graduate School of Medicine, Kyoto University, Kyoto, Japan

ABSTRACT
We have established previously a mouse strain containing a mutant β-catenin allele of which exon 3 was sandwiched by loxP sequences [Catnblox(ex3)]. In this mouse strain, a Wnt-activating β-catenin mutation alone is insufficient for hepatocarcinogenesis, but additional mutations or epigenetic changes may be required. Here we report that hepatocellular carcinoma develops at the 100% incidence in mice with simultaneous mutations in the β-catenin and H-ras genes that are introduced by adenovirus-mediated Cre expression. Although H-ras mutation alone rapidly causes large cell dysplasia in the hepatocytes, these cells show no autonomous growth within 1 week after infection of the Cre-adenovirus. However, simultaneous induction of an additional mutation in the β-catenin gene causes a clonal expansion of such dysplastic cells, followed by nodular formation and development of hepatocellular carcinoma. These results indicate that β-catenin mutations play a critical role in hepatocarcinogenesis in cooperation with another oncogene and that these mice provide a convenient model to investigate early steps of hepatocarcinogenesis.

INTRODUCTION
Hepatocellular carcinoma (HCC) is one of the most common human malignant tumors (1). Although the molecular mechanisms of hepatocarcinogenesis are not fully elucidated, many mutations have been found that inactivate tumor suppressor genes and stimulate expression of oncogenes or growth factors (2). Various transgenic or knockout mouse lines have been established as HCC models with long expression of oncogenes or growth factors (2). Various transgenic or knockout mouse lines have been established as HCC models with long latency periods of hepatocarcinogenesis (3, 4), which suggests a requirement of multiple and successive genetic alterations in these mice.

β-Catenin is one of the key downstream effectors in the Wnt signaling pathway that plays an important role in various human cancers (5, 6). Mutations at the serine and/or threonine residues near the NH2 terminus in the β-catenin gene prevent their phosphorylation by the APC-axin-glycogen synthase kinase 3β complex and subsequent degradation through the ubiquitin-proteasome pathway (7–12). Stabilized β-catenin associates with Tcf/Lef transcription factors, translocates into the nucleus, and activates transcription of a new set of genes (13, 14). We established previously a mouse strain containing a mutant β-catenin allele of which exon 3 was sandwiched by loxP sequences [Catnblox(ex3)] (15). Although the Cre-mediated deletion of the APC-axin-glycogen synthase kinase 3β-phosphorylation sites from β-catenin causes numerous adenomatous polyps in the intestines (15), the same deletion by itself does not form any neoplastic foci in the liver (16).

The ras family genes encode small GTPase proteins that transduce signals from the transmembrane receptors to the nucleus (17). Mutations at the hot-spots (codons 12, 13, and 61) in Ras proteins lead to defects in the GTPase activity and constitutive activation of the downstream signals (17). Such mutations were found in ~30% of human tumors. Although Ras mutations are rare in human liver tumors (18), elevated expression of transforming growth factor α or insulin-like growth factor II have been reported in some human HCC, which causes activation of the Ras signaling pathways (19). In addition, Harvey-ras (H-ras) mutation is detected frequently in the spontaneous and carcinogen-induced mouse liver tumors (20, 21). Accordingly, we have constructed a new transgenic mouse strain [Tglox(ex3)/H-ras*] in which an activated H-ras (H-rasG12V) protein is expressed on Cre-mediated recombination and investigated whether stabilized β-catenin can cause HCC in cooperation with activated H-ras.

MATERIALS AND METHODS

Transgenic Mice. Tglox(ex3)/H-ras* transgenic mice were generated as follows. The 1.4 kb lox-polyA-lox cassette from the plasmid pBS302 (Invitrogen, Groningen, the Netherlands) was subcloned into the Nol site downstream of cytomegalovirus (CMV) promoter of the plasmid pcDNA3.1 Hygro(+) (Invitrogen), 2.6 kb PvuII fragment of which was replaced with a Nol linker fragment. The 4.8 kb Smal-BamHI fragment from human activated Ha-ras gene (22) was then inserted into the XhoI site of the resulting plasmid (pCMV-loxP-hpA). The 7.3-kb whole-insert fragment was excised by Nol and injected into FVB/N fertilized eggs. Offspring mice were genotyped by PCR with the tail DNA using primers H-ras-F1 (5′-GCTCCATGTCGAACGTGGGC-3′) and bpA-R (5′-GGTGIACCTCTCCAGGGTCA-3′) at 94°C for 30 s, 60°C for 1 min, and 72°C for 1 min, for 35 cycles. Nine independent transgenic mouse lines were obtained, two of which were used to generate compound heterozygous mutants. Construction of Catnblox(ex3) knockout mice have been described previously (15), and they were backcrossed to the C57BL/6N strain for five generations.

Cre-Mediated Recombination in Liver. Construction of the cre-expressing recombinant adenovirus (AdCMV-cre) has been described previously (16). The diluted aliquots of AdCMV-cre (106 plaque-forming units/100 μl) were injected into the tail vein of 7-week-old mice.

Western Immunoblot Analysis. Tissue lysates were prepared by sonication in lysis buffer [10 mM HEPES (pH 7.8), 10 mM KCl, 0.1 mM EDTA, and 0.1% NP40] containing protease inhibitors (phenylmethylsulfonyl fluoride, aprotinin, pepstatin, leupeptin, and antipain.) Aliquots of 50 μg total protein were resolved by SDS-PAGE, transferred to a membrane, and detected by antibodies against β-catenin (Sigma Chemical Company, St, Louis, MO) or H-ras (Nihonkayaku, Tokyo, Japan) coupled with the ECL-detection system (Amersham-Pharma Biotech Inc., Piscataway, NJ).

Bromodeoxyuridine (BrDUr) Labeling. BrDUr labeling and detection kit (Roche Diagnostics, Mannheim, Germany) was used. Mice were injected i.p. with 250 μl of BrDUr solution 4 h before they were killed. Tissue samples were fixed with 10% formalin in PBS at 4°C overnight, dehydrated, and embedded in paraffin wax. Deparaffinized sections were microwaved in 10 mM citrate buffer (pH 6.0) for 10 min, denatured in 2 N HCl at 37°C for 2 min and neutralized in 0.1 M boric acid/NaOH (pH 8.5) at 37°C for 2 min before immunostaining.

Histology and Immunohistochemical Analyses. Sections were prepared and stained with H&E as described previously (15). For immunohistochemistry, sections were incubated with antibodies against β-catenin, H-ras, cyclin D1 (Oncogene Research Products, Boston, MA), or BrDUr (Becton Dickinson Immunocytometry Systems, San Jose, CA), respectively. The primary antibodies were detected using the Elite ABC rabbit kit (Vector Laboratories, Burlingame, CA) as described (15).
RESULTS

High Incidence of HCC in the Catnblox(ex3):Tglox(pA)H-ras* Mice Infected with AdCMV-cre. We constructed a new transgenic mouse strain \([T_g^{lox/pA}H-ras^*}\) in which a human activated H-ras (H-ras\(^{G12V}\)) protein is expressed on Cre-mediated recombination (Fig. 1A). Nine independent transgenic lines were established, carrying 4–17 copies of the transgene as determined by Southern hybridization analysis (Fig. 1B). Two independent transgenic lines, HR3 and HR7, were crossed with Catnblox(ex3):Tglox(pA)H-ras* compound mutant mice to introduce both \(\beta\)-catenin and H-ras mutations into the hepatocytes by Cre-mediated recombination, AdCMV-cre (16) was injected into the compound mutant mice through the tail vein at 10\(^8\) plaque-forming units/mouse (Fig. 1C). As shown in Fig. 1D, large liver tumors were found in the infected Catnblox(ex3):Tglox(pA)H-ras* (HR-3) mice, whereas livers from the infected single mutant or wild-type littermates appeared normal. Many HCC lesions were also found in the other compound mutant strain (HR-7) infected with AdCMV-cre (data not shown). The incidence of liver tumors for each genotype is summarized in Table 1 (Exp. 1). It should be noted that Catnblox(ex3):Tglox(pA)H-ras* (HR-7) showed 100% incidence of HCC and started to die with swollen abdomen 3–4 months after infection (Fig. 1E). The incidence of HCC in the HR-3 line was 78% at the same age, which could be explained by possible inactivation of the transgene, because the HR-3 locus was mapped on X-chromosome, whereas HR-7 on an autosome (data not shown). We have not detected any gross morphological or histological changes in other organs.

Table 1 Incidence of liver tumors in the mouse strains

| Genotype | Exp. 1 | Exp. 2 |
|----------|-------|-------|
|          | 6 months P.I. | 1–4 weeks P.I. | 5–14 weeks P.I. |
|          | HCC (%) | Dysplastic cell (%) | Nodules/HCC (%) |
| Δex3:ras*3 | 7/9 (78) | N.D. | N.D. |
| +:ras*3 | 0/0 (0) | N.D. | N.D. |
| Δex3:ras*7 | 7/7 (100) | 5/5 (100) | 8/8 (100) |
| +:ras*7 | 0/0 (0) | 4/4 (100) | 8/7 (0) |
| Δex3:+ | 0/14 (0) | 0/6 (0) | 0/7 (0) |
| +:+ | 0/8 (0) | 0/3 (0) | 0/7 (0) |

* P.I., postinfection; N.D., not determined; HCC, hepatocellular carcinoma.
To confirm expression of the mutant H-ras and β-catenin proteins in HCC, we prepared cell lysates from the tumor and adjacent normal liver tissues, and analyzed by Western analysis. As shown in Fig. 1F, expression of the mutant β-catenin and overexpression of H-ras was detected only in the tumor tissues, but not in the normal liver or other tissues (data not shown). Weak expression of H-ras in the normal liver is likely derived from the endogenous mouse H-ras protein, cross-reacting with the antibody for human H-ras.

Upon histological analysis of the HCC developed in the AdCMV-cre-infected Catnblox(ex3:Tglox(pA)H-ras* mice, most HCC tissues were well-differentiated with a compact, and occasionally trabecular pattern. The lobular architecture was lost in these tumors. Representative sections of the HCC with “compact” and “trabecular” types are shown in Fig. 2, A and B, respectively. The trabecules consisted of multiple cell layers in which large dysplastic hepatocytes were found with a high nucleocytoplasmatic ratio, numerous mitotic figures, and necrosis. Tumor cells often compressed the adjacent normal liver tissue (Fig. 2, C and D), and occasionally showed intrahepatic invasions (Fig. 2A). These histological features are very similar to those commonly found in human HCC.

Upon immunohistochemical analysis, overexpression of β-catenin (Fig. 2E) and H-ras (Fig. 2F) were detected only in the tumor cells, but not in the normal hepatocytes. These results are consistent with those of the Western analysis. Cyclin D1, one of the target genes of the Tcf/Leif/β-catenin transcription factor (23), was also overexpressed in the tumor cells (Fig. 2G). Consistent with the result, the labeling index with BrdUrd-uptake was increased in the tumor, with a high proliferation rate of the tumor cells (Fig. 2H). These data indicate that simultaneous mutations in the β-catenin and H-ras genes are sufficient to induce HCC in the mouse liver.

**Early Processes of Hepatocarcinogenesis in the Catnblox(ex3):Tglox(pA)H-ras* Mice.** Because Catnblox(ex3):Tglox(pA)H-ras* (HR-7) mice infected with the AdCMV-cre showed a very high incidence of HCC (100% at 6 months postinfection), we then investigated earlier processes of hepatocarcinogenesis in these mice. We infected another group of the compound mutant mice and controls (Table 1, Exp. 2), and euthanized them at various time points from 1 to 14 weeks after infection. At 4 weeks, some small foci (<1 mm ø) were visible under a dissection microscope on the surface of the liver only in the compound mutant mice (Fig. 3A). At 2 months, many large nodules (2–3 mm ø) were found macroscopically (Fig. 3B), whereas livers from the control mice all appeared normal (data not shown). The incidence of HCC in the Catnblox(ex3):Tglox(pA)H-ras* (HR-7) mice was again 100% between 5 and 14 weeks after infection.

As early as 1 week after infection, dysplastic hepatocytes with large nuclei and high nucleocytoplasmic ratio were detected in the liver sections of the compound mutant mice, although no gross morphological abnormalities were observed (Fig. 3E). Such cells were observed in all of the compound mutant mice at 1–4 weeks after infection. Interestingly, these dysplastic hepatocytes were surrounded by inflammatory cells (Fig. 3, D and E, insets) including lymphocytes, neutrophils, and macrophages (Kupffer cells), which were not reported in other HCC models (24–26). Hardly any inflammatory cells were observed in the AdCMV-cre-infected β-catenin mutant mice (Fig. 3C) or wild-type mice (data not shown), ruling out the possibility that the inflammation was caused by the adenoviral infection itself (see “Discussion”). As the acute inflammation subsided by the fourth week after infection (Fig. 3, G and H), the dysplastic hepatocytes persisted and started to grow, and formed multifocal dysplastic nodules by the fifth week (Fig. 3K), followed by formation of HCC (Fig. 3N).

**Proliferative Activation of the Dysplastic Hepatocytes by β-Catenin Mutation.** Interestingly, these large dysplastic hepatocytes were detected not only in livers from the compound mutant mice but also in the Tglox(pA)H-ras* mice (i.e., without β-catenin mutation; Fig. 3D), whereas they were not detected in the Catnblox(ex3) (Fig. 3C) or wild-type mice (data not shown). These data suggest that newly expressed activated human H-ras should be sufficient to cause a morphological change of hepatocytes in the mouse liver. Importantly, however, dysplastic cells in the Tglox(pA)H-ras* mice did not show any proliferative changes (Fig. 3, G, J, and M), and neither dysplastic nodules nor HCC developed in the Tglox(pA)H-ras* transgenic mouse livers even at 17 months postinfection of AdCMV-cre, although we cannot exclude the possibility that HCC might develop much later like in some HCC models (27).

To additionally characterize the early changes in the compound mutant mouse liver, serial sections of the dysplastic hepatocytes were analyzed immunohistochemically. Upon staining with an anti-H-ras antibody, a strong signal was detected in the membrane of the dysplastic hepatocytes in both Tglox(pA)H-ras* transgenic mice (data not shown) and compound mutant mice (Fig. 4C). On the other hand, cellular β-catenin level and BrdUrd incorporation in the dysplastic hepatocytes increased only in the compound mutant mice (Fig. 4, B and D), but not in the Tglox(pA)H-ras* transgenic mice (data not shown). As we reported previously (16), hepatocytes with the β-catenin mutation alone were nontumorigenic and quiescent despite the nuclear accumulation of β-catenin (data not shown). Taken together, these data demonstrate that combination of the H-ras and β-catenin mutations are sufficient to activate proliferation of the dysplastic hepatocytes, which is schematically summarized in Fig. 4E.

**DISCUSSION**

In the present study, we developed a new model for mouse HCC by simultaneously introducing β-catenin and H-ras mutations in the liver of compound transgenic mice [Catnblox(ex3):Tglox(pA)H-ras*]. Although cooperation of two oncogenes, such as H-ras/c-myc, SV40 T-antigen/ H-ras, and transforming growth factor α/c-myc have been reported to accelerate hepatocarcinogenesis (24, 28), the present model is distinct from others in several features. In most other transgenic models, sequential development of HCC is observed, i.e., hepatic dysplasia, degeneration, nodular regeneration of hepatocytes, hepatocellular adenoma, and subsequent HCC with a “nodule-in-nodule” pattern (3). In contrast, neither regenerative nor adenomatous lesions are observed in our model, but malignant neoplastic lesions appear rapidly after the clonal expansion of the dysplastic hepatocytes. Because transgenes are expressed ubiquitously in the liver in other models, it is still unclear whether large dysplastic hepatocytes are the direct precursor lesion of HCC or they simply induce degeneration of hepatocytes, followed by a secondary regeneration and hyperplastic growth of the surrounding cells. In our model, stabilized β-catenin and activated H-ras are expressed simultaneously in the same hepatocytes infected with adenovirus, leading to generation of dysplastic cells. Accordingly, the dysplastic hepatocytes in the present model are the direct precursor lesion of HCC. Although it takes long latency periods before hepatocarcinogenesis in other transgenic models (4), our model develops HCC at a 100% incidence within 2 months after the infection.

Although single transgenic mice, i.e., Catnblox(ex3) or Tglox(pA)H-ras* did not develop any HCC on infection with AdCMV-cre, there was an interesting difference between the two lines. As we reported previously, β-catenin mutation alone did not cause any morphological changes in the liver (16). In contrast, activated H-ras caused dysplastic changes in hepatocytes immediately after the infection of AdCMV-cre. It is worth noting that mild hepatocyte dysplasia is found in H-ras.
transgenic mice in which the transgene product is expressed consti-
tutively as one of the self-proteins (24). Appearance of large dysplas-
tic hepatocytes is one of the earliest changes also in other mouse HCC
models expressing SV40 T-antigen (24), c-myc (25), transforming
growth factor α (26), and so forth, although molecular mechanisms
are not clear. In the hepatocyte transformation, it is conceivably that
some common signals in the pathways of these oncoproteins are
activated by the H-ras mutation as well. Despite the rapid appearance

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**Fig. 2.** Histological analysis of hepatocellular carcinoma (HCC) developed in $\text{Catnb}^{\text{lox(ex3)}};\text{TgloxpA}^\text{H-ras*}$ (HR-3) mice. A–D, representative H&E-stained sections of HCC in the $\text{Catnb}^{\text{lox(ex3)}};\text{TgloxpA}^\text{H-ras*}$ (HR-3) mice inoculated with $10^8$ plaque-forming units/mouse Ad-CMV-cre. Arrows in A show intrahepatic invasion. E–H, immunohistochemical staining of the sections serial to that in C and D for β-catenin (E), H-ras (F), cyclin D1 (G), and bromodeoxyuridine (H), respectively. Bars, 100 µm for A and B; 500 µm for C; and 100 µm for D–H.
of large dysplastic hepatocytes, no HCC developed from H-ras transgenic mice at least for 1 year after infection, which is different from other H-ras transgenic lines reported previously (24, 27). The number of hepatocytes expressing activated H-ras is much less in the present model than those in other ubiquitous expression mice. The expression level of H-ras in our transgenic mice may also be lower than in other models because of the CMV promoter. Additionally, the genetic background may also affect the incidence of HCC (29). It remains to be seen whether HCC develops in our model after >1 year post-infection.

Activated H-ras alone is insufficient for clonal expansion of the dysplastic hepatocytes, but an additional mutation in the β-catenin gene can cause HCC, suggesting that one of the roles of the stabilized β-catenin in hepatocarcinogenesis may be to stimulate proliferation of the dysplastic cells generated by activated H-ras. This interpretation is supported further by the fact that cyclin D1,
one of the target genes of Wnt signaling, is overexpressed in HCC in the compound mutant mice (Fig. 2G). Although cooperation of H-ras and c-myc, another target of Wnt signaling, in hepatocarcinogenesis was reported, we could not detect any overexpression of c-myc in HCC (data not shown). As mutations in the β-catenin gene are often found in HCC developed in transgenic mouse lines.

Fig. 4. Immunohistochemical analysis of the dysplastic hepatocytes. A–D, serial liver sections containing the atypical hepatocytes at 11 weeks after infection stained with H&E (A), anti-β-catenin (B), anti-H-ras (C), and anti-bromodeoxyuridine (D) antibodies. E, schematic diagram showing the effect of β-catenin or H-ras mutation alone and compound mutations on the hepatocarcinogenesis. Bars, 100 μm.
with c-myc as well as H-ras transgenes (30), stabilized β-catenin may cooperate not only with H-ras but also with various oncogenes in hepatocarcinogenesis. Although no β-catenin mutations were found in HCC of SV40 T-antigen transgenic mice (31), mutations in or activation of other Wnt signaling genes may be contributing to the hepatocarcinogenesis (32).

We have observed inflammatory responses against dysplastic hepatocytes expressing activated H-ras, consistent with a previous report that overexpression of the H-ras gene enhanced inflammation in the liver (33). Regeneration of hepatocytes caused by this acute inflammation may help accumulate additional genetic mutations in other oncogenes, although it remains to be determined what other mutation(s) are involved in the β-catenin and H-ras genes. Our investigations can be extended further with helper-dependent adenovirus (34) or naked DNA (35) that should express Cre without causing much inflammation.

In conclusion, we have established a new mouse HCC model with β-catenin and H-ras mutations simultaneously introduced by an adenovirus-mediated liver-specific Cre-expression system. We have demonstrated that neither H-ras nor β-catenin mutation alone is sufficient for hepatocarcinogenesis, but both are when combined. Both β-catenin mutations and activation of H-ras signaling pathways, such as overexpression of transforming growth factor α or insulin-like growth factor II, are often found in human HCC. In the present mouse model, it takes only a short latency period of several weeks with 100% incidence. Accordingly, this HCC model provides an ideal system to investigate early steps of hepatocarcinogenesis, as well as to evaluate anticancer drug candidates.

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