Biochemical and Biophysical Research Communications 417 (2012) 601–606

Contents lists available at SciVerse ScienceDirect

Biochemical and Biophysical Research Communications

journal homepage: www.elsevier.com/locate/ybbrc

Liver tumor formation by a mutant retinoblastoma protein in the transgenic mice is caused by an upregulation of c-Myc target genes

Bo Wang [a], Keisuke Hikosaka [a], Nishat Sultana [a], Mohammad Tofael Kabir Sharkar [a], Hidenao Noritake [a,b], Wataru Kimura [a], Yi-Xin Wu [a], Yoshimasa Kobayashi [b], Tadayoshi Uezato [a], Naoyuki Miura [a,*]

[a] Department of Biochemistry, Hamamatsu University School of Medicine, 1-20-1 Handa-yama, Higashi-ku, Hamamatsu 431-3192, Japan
[b] Department of Internal Medicine, Hamamatsu University School of Medicine, 1-20-1 Handa-yama, Higashi-ku, Hamamatsu 431-3192, Japan

ARTICLE INFO

Article history:
Received 25 November 2011
Available online 11 December 2011

Keywords:
Liver tumor
Myc
Foxm1
Skp2
Bmi1
AP-1

ABSTRACT

The retinoblastoma (Rb) tumor suppressor encodes a nuclear phosphoprotein that regulates cellular proliferation, apoptosis and differentiation. In order to adapt itself to these biological functions, Rb is subjected to modification cycle, phosphorylation and dephosphorylation. To directly determine the effect of phosphorylation-resistant Rb on liver development and function, we generated transgenic mice expressing phosphorylation-resistant human mutant Rb (mt-Rb) under the control of the rat hepatocyte nuclear factor-1 gene promoter/enhancer. Expression of mt-Rb in the liver resulted in macroscopic neoplastic nodules (adenomas) with ~50% incidence within 15 months old. Interestingly, quantitative reverse transcriptase-PCR analysis showed that *c-Myc* was up-regulated in the liver of mt-Rb transgenic mice irrespective of having tumor tissues or no tumor. In tumor tissues, several c-Myc target genes, *Foxm1, c-Jun, c-Fos, Bmi1* and *Skp2*, were also up-regulated dramatically. We determined whether mt-Rb activated the *Myc* promoter in the HTP9 cells and demonstrated that mt-Rb acted as an inhibitor of wild-type Rb-induced repression on the *Myc* promoter. Our results suggest that continued upregulation of c-Myc target genes promotes the liver tumor formation after about 1 year of age.

1. Introduction

Human hepatocellular carcinoma (HCC) is one of the most common types of malignant cancer in Asia and Africa. HCC normally develops as a consequence of underlying liver disease and is most often associated with cirrhosis. Surgical resection and liver transplantation are current best curative options to treat liver cancer. However, recurrence or metastasis is quite common in patients who have had a resection and survival rate is 30% to 40% at 5 years postoperatively [1–4]. Although the major viral and environmental risk factors for HCC development have been unraveled, the molecular mechanism involved in hepatic carcinogenesis is still poorly understood [5].

The retinoblastoma gene is the first-identified human tumor suppressor gene and is considered as a core element governing cell cycle progression, cell proliferation, differentiation, apoptosis, and senescence through its ability to bind the transcription factor E2F-1 that activate transcription of genes required for S phase [6]. Molecular genetic studies have identified abnormalities of this tumor suppressor gene in a large proportion of human cancers including HCC [7]. Germ line mutations in the *Rb* gene predispose individuals to bilateral retinoblastoma as well as osteosarcoma [8,9]. Somatic *Rb* inactivation contributes to the development of these tumors as well as to other tumors in different organs like bladder, lung, breast and prostate [10–13].

Although a vast amount of data has been accumulated on the role of Rb in cancer development for several cancer entities, only limited insight is available on the role of Rb in HCC development [14]. Most of the data show that deregulation, loss or inactivation of Rb is an obligatory step in tumor formation. Intriguingly, studies in human HCCs showed not only absent but also a high incidence of Rb protein over-expression (~50%) [15]. This finding suggests that active Rb protein may have some interesting biological consequences that contribute to liver tumorigenesis. Now, there are two ways to investigate the function of a gene *in vivo*. One is to create knockout mice lacking a gene function and the other is to create transgenic mice in which the gene is expressed ectopically or over-expressed [16]. The liver-specific Rb loss in mice shows ectopic cell cycle entry and aberrant ploidy whereas the loss of Rb does not lead to any detectable hyperplasia or tumorigenesis in the liver of adult mice [17]. We found that hepatocytes in the WT-Rb transgenic mice show

Abbreviations: HCC, hepatocellular carcinoma; HNF-1, hepatocyte nuclear factor-1; WT, wild type; mt-Rb, mutant retinoblastoma; PBS, phosphate-buffer saline; HE, hematoxylin and eosin; PAGE, polyacrylamide gel electrophoresis; dpc, days postcoitus.

* Corresponding author. Fax: +81 534352369.
E-mail address: nmiura@hama-med.ac.jp (N. Miura).

0006-291X/$ - see front matter © 2011 Elsevier Inc. All rights reserved.
doi:10.1016/j.bbrc.2011.12.014

resistance to fulminant hepatitis and carcinogenesis whereas its liver develops normally [16]. The over-expressed WT-Rb protein in hepatocytes can be phosphorylated, so the liver of transgenic mice does not show any chronic damages. And liver is a special organ that exhibits an exquisitely controlled cell cycle, wherein hepatocytes are maintained in quiescence until stimulated to proliferation. Previous studies showed that the phosphorylation-resistant Rb exhibits greater activity than the WT protein [18]. On these bases, we generated transgenic mice expressing phosphorylation-resistant human Rb (mt-Rb) under the control of the hepatocyte nuclear factor-1 (HNF-1) gene promoter/enhancer to predict the expression of mt-Rb protein only in the liver, not in other organ. We found that mt-Rb resulted in the *c-Myc* up-regulation in the liver of mt-Rb transgenic mice and then c-Myc activated several target genes in the tumor. These results indicate that up-regulation of *Foxm1*, *Bmi1* and several target genes promotes the liver tumor formation.

2. Materials and methods

2.1. Construction of the mutant Rb transgenic mice

The animal procedures were approved by the experimental animal ethics committee at Hamamatsu University School of Medicine.

The BamHI fragment of wild-type human Rb cDNA was subcloned into pKF18 k vector (Takara, Kyoto, Japan). Site-directed mutagenesis was performed using Mutan-Express km Enzyme/Oligo Set (Takara #6090, Kyoto). Mutagenic oligonucleotides were 5′-ATGCCGCCCAAAGCCCCCCGAAAAAC-3′ (T5A), 5′-GGTTCACCTCGAGCCCC CAGGCGAGGT-3′ (T252A), 5′-TTTGAAACACAGAGAGCCCCACGAAAAAGTAA C-3′ (T356A), 5′-ATTCCTCCACACGCTCCAGTTAGGAC-3′ (T373A), 5′-GCAGATATGTATCTTGCTCCTGTAAGAGCTCCAAAGAAAAAAGG-3′ (S608A, S612A), 5′-CACATTCCTCGAGCCCCTTACAAGTTT-3′ (S788A), 5′-CCTTACAAGTTTCCTAGTGCCCCCTTACGGATTCC-3′ (S795A), 5′-GGGAACATCTATATTGCCCCCCTGAAGGCTCCATATAAAATTTCAGAA-3′ (S807A, S811A), 5′-GAAGGTCTGCCAGCCCCAACAAAAATGACT-3′ (T821A) and 5′-ATACCCATTAATGGTGCACCTCGAGCCC-3′ (S249A). All mutations were confirmed by sequencing. The mutant Rb (mt-Rb) cDNA was cut with BamHI, filled-in with Klenow enzyme and ligated with a ClaI linker. The ClaI fragment of mt-Rb cDNA was replaced into the Rb cDNA position of HNF1-Rb to make HNF1-mtRb. HNF1-NZ was described previously [16]. Equal amounts of HNF1-mtRb and HNF1-NZ were mixed and injected into fertilized eggs.

2.2. Western blot analysis of mt-Rb protein in HTB9 cells and liver cells

HTB9 cells were transfected with 2 μg mt-Rb expression vector. Two days after transfection, cells were collected and resuspended. Lysates were collected by centrifugation and protein concentration was determined using BCA protein assay kit (Peirce). Twenty μg of the total protein was resolved by 10% SDS–PAGE and transferred onto PVDF membranes.

Liver cells were isolated from mt-Rb transgenic and WT mice and then nuclear extracts were prepared as described previously [19]. Then lysates were recovered by centrifugation and protein concentration was determined by the same method as in culture cells. Monoclonal anti-Rb antibody (Santa Cruz, CA) was used for Western blotting analysis.

2.3. Analysis of Rb binding activity to E2F-1 by co-immunoprecipitation

HTB9 cells were co-transfected with 2 μg Rb and 4 μg E2F-1 expression vector DNAs. Two days after transfection, cells were collected and lysed by binding buffer (10 mM Tris–HCl, pH 7.4, 150 mM NaCl, 1% Triton X-100, 1 mM EDTA, 0.3 mM PMSF, 0.5% Nonidet P-40). The lysates were collected by centrifugation and then incubated with anti-E2F-1 antibody (Santa Cruz). The immune complexes were collected on protein G Sepharose, solubilized with SDS sample buffer for immunoblotting by standard methods.

2.4. Animals, gross and histological analyses

Experimental animal protocols and animal procedures are followed with the international criteria of animal experimentation and were approved by the Hamamatsu Medical University Animal Care Center. Body weights were measured before sacrifice. Livers were obtained by autopsy, weighed and examined for the presence of tumor. For routine microscopy, Livers were fixed in 4% paraformaldehyde at 4 °C overnight for frozen section preparation and then dehydrated in 20% sucrose in PBS overnight. The specimen were frozen and sectioned at 8 μm. But for paraffin section, liver tissues were embedded in paraffin after fixation with paraformaldehyde and the sections were cut at 4 μm. Both were stained with hematoxylin and eosin according to standard methods.

2.5. Isolation of total liver mRNA and quantitative reverse transcription (qRT)-PCR assay

Total mRNA was extracted from the liver of wild type and mt-Rb transgenic mice at 11 months old using CsCl method. The quantity and quality of RNA samples were measured by absorbance at 260 and 280 nm. pRT-PCR was done as described previously [20] using the Step One Plus Real-Time PCR System (Applied Biosystems, Bedford, MA) using 24 sets of primers (Supplementary Table S1).

2.6. Transfection analysis of transcriptional activities of WT-Rb and mt-Rb on c-Myc promoter

PCR was performed against mouse genomic DNA with 5′-GGGATTGGTGGCTCTTGGTG-3′ and 5′-CTCCCTCTGTCTCTCGCTGG as primers. The PCR product (about 2.0 kbp) was subcloned into the EcoRV site of pGL4.10 (luc2, Promega, WI) to make the c-Myc-luc2 plasmid. The fixed amount of c-Myc-luc2 plasmid and the various amounts of WT-Rb and/or mt-Rb expression vectors were cotransfected into HTB9 cells with Fugene HD (Roche). In 36 h, the cell extracts were prepared and the luciefrase activity was measured using dual luciferase system (Promega). Supplementary methods are available on line.

3. Results

3.1. Rb alanine substitution mutations and generation of mt-Rb transgenic mice

Rb contains 16 potential phosphorylation sites that are distributed among the protein [21,22] (Fig. 1A). Seven of these sites are located within C domain which is sufficient for c-Abl binding and is required for association with many non-LXCXE containing Rb-binding proteins like E2F-1 [23,24]. Two sites are located in the spacer region between A and B domains which bind the LXCXE motif and one other site is in A domain. The remaining sites are in the N-terminal region of which function is still not clear since the A, B, C and spacer domains are sufficient for activity of Rb function in most assays [25].

We mutagenized the important 12 sites out of 16 consensus sites in mt-Rb where 8 serines and 4 threonines were substituted into alanines (Fig. 1A). For *in vitro* analysis, the WT-Rb and mt-Rb

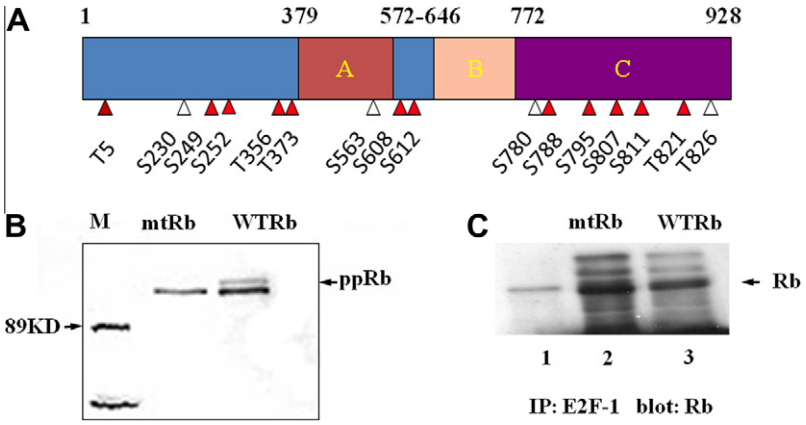

Fig. 1. Generation of phosphorylation-resistant Rb protein. (A) Schematic presentation of mutant human Rb protein. The pocket domains A, B and C were shown. Out of 16 cyclin-dependent kinase phosphorylation sites (red and white triangles), 12 sites were mutated (red triangles). (B) Phosphorylation status of WT-Rb and mt-Rb by Western blotting analysis. Phosphorylated Rb (ppRb) was detected only in cells transfected with WT-Rb expression vector (right), but not in those with mt-Rb expression vector (middle). (C) Co-immunoprecipitation of E2F1 and Rb. Cell extracts from mt-Rb expression vector-transfected cells (lane 2) and WT-Rb expression vector-transfected cells (lane 3) were incubated with anti-E2F1 antibody and the immunoprecipitates were subjected to 10% SDS–polyacrylamide gel electrophoresis. The transferred nylon membrane was incubated with anti-Rb antibody and visualized with ECL. Lane 1 shows positive control of Rb protein.

proteins were expressed in HTB9 (Rb protein-negative) cells using the cytomegalovirus enhancer/actin promoter-driven vector (CX-N2) [26]. Western blot analysis showed WT-Rb migrated as a doublet; the upper band was phosphorylated Rb protein (ppRb) and the lower band was unphosphorylated Rb protein (Fig. 1B, right lane). As compared with the wild type, the mt-Rb protein migrating in only one unphosphorylated form suggested that the mt-Rb was resistant to phosphorylation in cultured cells (Fig. 1 B, middle lane).

To confirm the E2F-1 binding activity of the mt-Rb protein, mt-Rb or WT-Rb and E2F-1 expression vectors were cotransfected into HTB9 cells. The proteins were subjected to co-immunoprecipitation and the result showed that mt-Rb bound to E2F-1 as similarly as WT-Rb did (Fig. 1C, lanes 2 and 3). This result showed that the phosphorylation site mutations did not disrupt the binding activity of mt-Rb to E2F-1.

3.2. Targeted expression of mt-Rb protein in the liver

To directly determine the effects of phosphorylation-resistant Rb on liver development and cancer progression, we generated transgenic mice in which the human mt-Rb cDNA was controlled by the rat HNF-1 gene enhancer/promotor and obtained 2 mouse lines, TGX and TGZ. We measured the expression of Rb protein in the liver of transgenic and wild-type adult mice by Western blotting (Supplementary Fig. S1A). Large amount of mt-Rb protein was detected in the liver of TGX mice (lanes 5 and 6) and only a small amount of Rb in TGZ mice (lanes 3 and 4). In contrast, a small amount of the mouse endogenous Rb was also detected in wild-type mice (lanes 1 and 2). Next, we investigated the developmental expression pattern of β-galactosidase as an indicator of expression of mt-Rb protein by the X-gal staining. TGX embryos showed strong staining in the liver at 11.5 dpc (Supplementary Fig. S1B) while wild-type and TGZ embryos showed no staining (Supplementary Fig. S1C and data not shown). The rat endogenous HNF-1 gene is expressed in the liver, kidney, and intestine. But in our previous and current studies, 9 kbp of HNF1 promoter/enhancer directed the LacZ expression only in the liver (Fig. S1B and Ref. [16]). Furthermore, we isolated total RNA from the liver of wild-type, TGX and TGZ mice and determined whether mouse Rb (endogenous) and human Rb (exogenous) mRNAs were expressed in each liver by the RT-PCR method (Supplementary Fig. S2). The result showed that only mouse Rb mRNA was expressed in the wild-type and TGZ mice while human Rb and mouse Rb mRNAs were

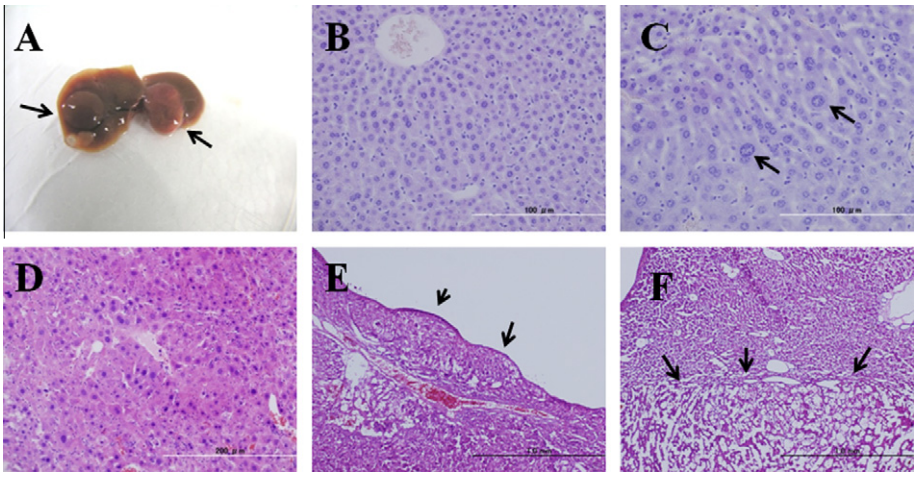

Fig. 2. Development of tumors in the liver of mt-Rb transgenic mice. (A) Macroscopic liver specimen from the mt-Rb transgenic mice at 1 year old. Tumors (adenoma) protruding from the surface of liver lobe were identified macroscopically (arrows). (B) Liver of wild-type mouse at 11 months old, 400×. (C) Liver of mt-Rb transgenic mice at 8 months. Liver cell dysplasia (large cell) was observed (arrowes), 400×. (D) Liver tumor tissue of mt-Rb transgenic mice at 11 months old. Dysplastic hepatocytes and disorganized lobular architecture were observed, 200×. (E) Multiple neoplastic nodules were observed (arrows) in the liver tumor tissue of mt-Rb mice at 11 months old, 40×. (F) A big nodule (lower part) was observed in the liver tumor tissue of mt-Rb mice at 15 months old. Arrows show a boundary between normal and tumor tissues, 40×.

expressed in TGX mice. We interpreted that the LacZ and mt-Rb genes were not expressed due to an unknown reason in the TGZ transgenic mice and investigated only the TGX transgenic mice thereafter.

3.3. Tumor development in mt-Rb transgenic mice

The liver size and architecture in the TGX transgenic mice was normal in newborns (data not shown). There were no significant differences between the mt-Rb mice and wild-type mice until 6 months old. Then the TGX mice developed hepatocyte dysplasia and foci followed by an appearance of adenomas. The incidence of liver tumors in the TGX mice that was identified macroscopically (Fig. 2A) are summarized in Supplementary Table S2. About 50% of female and male TGX mice developed adenomas with no differences in numbers and sizes. In contrast, the age-matched B6 mice developed no adenomas.

The cell dysplasia was first noted after 8 months and the histological examination showed that some hepatocytes and nuclei were larger than normal. They had a nearly normal nuclear-cytoplasmic ratio and a pale cytoplasm (compare Fig. 2C to Fig. 2B). A prominent feature of liver tumor in the mt-Rb transgenic mice was the early development of focal lesions which was coincident with the development of the dysplastic changes. Neoplastic nodules were first

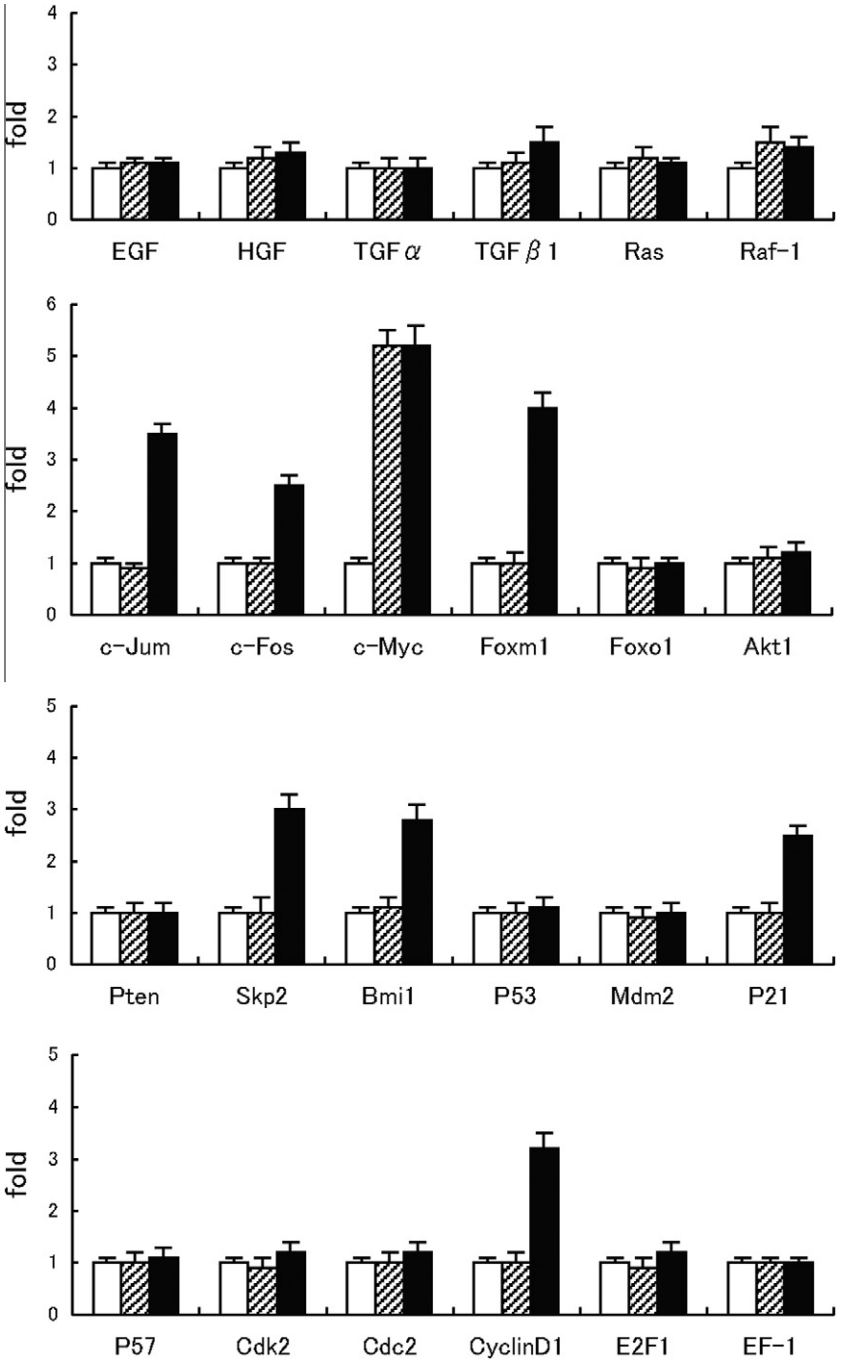

Fig. 3. qRT-PCR analysis of gene expression in the liver of transgenic mice. Total RNA was isolated from the liver of wild-type (open bars) and the mt-Rb transgenic mice without macroscopic tumors (shadowed bars) and those with macroscopic tumors (filled bars) at the age of 11 months and amplified quantitatively with a set of primers for each mRNA ($n = 5$).

noted in the liver of mt-Rb transgenic mice after 11 months. Microscopic examination showed the neoplastic nodules were composed of trabecular hepatocytes, arranged in sheets and cords which resemble normal hepatocytes but have some deviations in cell and nuclear size (larger cell), and were lacking of lobular architectures (Fig. 2D). Lower magnification view of the liver tissue from 11 months old (Fig. 2E) and 15 months old (Fig. 2F) transgenic mice showed the interface between the tumor which contained multiple nodules and septa, and the hepatic parenchyma which has no fibrosis and cirrhosis. Macroscopically, the livers contained solitary nodules of relatively small size bulging from the surface of the liver (Fig. 2A). Over all, both these foci and visible neoplasms had the characteristics of hepatocellular adenoma.

3.4. Gene expression in the transgenic mouse liver

To know the molecular mechanism of the tumor formation in mt-Rb transgenic mice, we determined whether the gene expression pattern was altered or not. qRT-PCR analyses of whole liver revealed that c-Myc mRNA was dramatically up-regulated in both non-tumorous and tumorous livers of mt-Rb transgenic mice compared with WT mice (Fig. 3). Only in tumorous liver, *Foxm1*, *Bmi1*, *c-Jun*, *c-Fos*, and *Skp2* were also up-regulated. These results suggested that the constituted expression of mt-Rb can up-regulate the expression of *c-Myc*. Therefore, the tumorigenesis was significantly induced when the other genes mentioned above were up-regulated.

In the tumorous liver of mt-Rb transgenic mice, P21 mRNA was up-regulated. This result suggested that apoptosis might be enhanced due to an increase of P21 during liver tumorigenesis in the transgenic mice. Furthermore, several cell cycle-related genes, such as CyclinD1, was up-regulated in the tumors. In contrast, EGF, HGF, TGFα, TGFβ, Ras, Raf-1, Foxo1, Akt1, Pten, p53, Mdm2, p57, Cdk2, and E2F-1 mRNAs had no significant changes (Fig. 3).

3.5. Mutant Rb protein reversed the repression of the wild-type Rb protein onto Myc gene

All mt-Rb transgenic mice showed up-regulation of c-Myc mRNA in the liver. So we investigated whether the mt-Rb protein activated the *c-Myc* gene. We connected the promoter region of *c-Myc* gene to the luciferase gene to make the c-Myc-luc2 construct.

Various amounts of WT-Rb and mt-Rb expression vectors were cotransfected into HTB9 cells and the luciferase activities were measured. The WT-Rb proteins repressed the *c-Myc* promoter dramatically while the mt-Rb protein repressed very weakly (Fig. 4A). Next, we transfected various amounts of mt-Rb expression vector with a fixed amount of WT-Rb expression vector into HTB9 cells. Interestingly, the mt-Rb protein inhibited the repression of WT-Rb protein (Fig. 4B). This result indicates that mt-Rb acts as an inhibitor to WT-Rb.

4. Discussion

Here we report that targeted expression of mt-Rb in the mouse liver exhibited macroscopic neoplastic nodules with about 50% incidence within 15 months old. These tumorous tissues had the characteristics of hepatocellular adenoma. *Rb* is the first-identified human tumor suppressor gene, and many lines of evidence have shown that loss or inactivation of Rb is an obligatory step in tumor formation. Paradoxically, Rb also has an apoptosis inhibition function. Since a defect in apoptosis is one of well-known oncogenic mechanisms, this functional property predicts that deregulated Rb expression may be oncogenic [27]. This concept is supported by the observations made in mice that express phosphorylation-resistant Rb in the mammary gland [28], and that express caspase-resistant Rb in p53-null knockout mice [29]. In addition to the inhibition of apoptosis, it is conceivable that constitutively active Rb may result in the accumulation of damaged DNA and the acquisition of transforming mutations [28]. These and our results suggest that the constitutively active Rb protein might have an oncogenic character.

Intriguingly, we found that the level of c-Myc mRNA was dramatically increased in both tumorous and non-tumorous livers of 11 months old transgenic mice compared with WT mice. Recently, it has been shown that a complex cross-regulation between Myc and Rb is mediated through the control of E2F in MYC-induced T cell lymphomagenesis [30]. Another study showed that down-regulation of MYC can activate Rb pathway through CDK4 and $p15^{INK4b}$ in STAT5A-induced senescence [31]. Consistent with these reports, our results may raise the possibility of derepressive activity of mt-Rb on WT-Rb on the *Myc* promoter (Fig. 4B). Up-regulation of c-Myc mRNA in non-tumorous liver suggests that the c-Myc up-regulation by itself does not cause a tumor formation.

It has been reported that c-Myc binds to an E-box of *Foxm1* promoter and up-regulates the expression of *Foxm1* in the liver [32,33]. Furthermore, c-Myc regulates multiple target genes including *c-Jun*, *Bmi1*, *CyclinD1 and TGFβ1* [32]. Another important finding of our study is the dramatic up-regulation of Foxm1 mRNA in the tumor in the mt-Rb transgenic liver. Foxm1 works as a cell cycle regulator, especially at S and G2/M phases [34]. Foxm1

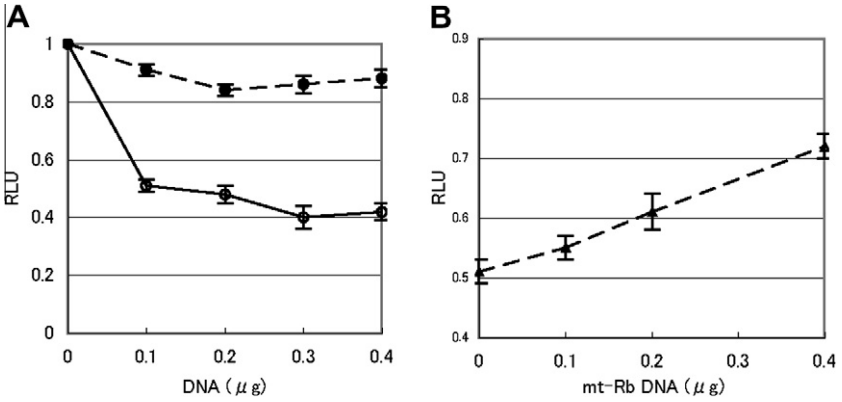

Fig. 4. mt-Rb reversed the repression to *c-Myc* promoter by WT-Rb. (A) The fixed amount (0.2 μg) of Myc-luc2 plasmid and various amounts of WT-Rb (solid line) or mt-Rb (broken line) expression vector were cotransfected into HTB9 cells. In 36 h, the cell extracts were prepared and the luciferase activities were measured and expressed as Mean ± SD (n = 3). (B) The Myc-luc2 plasmid (0.2 μg), WT-Rb expression vector (0.2 μg) and various amounts of mt-Rb expression vectors were cotransfected into HTB9 cells. The luciferase activity of cell extracts was measured and expressed as Mean ± SD (n = 3).

increases expression of numerous mitotic genes, such as *Cyclin A2*, *Cdc 25B phosphatase*, *CyclinB1* and *Skp2*, and down-regulates *P21* and *P27* [34]. However, *Foxm1* is not the only c-Myc target gene, and many of the cell cycle genes downstream of *Foxm1* have also been shown to be direct targets of c-Myc [33,35,36]. Our result showed that up-regulation of *c-Myc* target genes, *Foxm1*, *Bmi1*, *Skp2*, *AP-1*(*c-Jun/c-Fos*) and *CyclinD1*, was observed only in the tumors of mt-Rb liver. Bmi1, Skp2, AP-1 and CyclinD1 are known to be involved in oncogenesis [37–40]. In summary, the over-expression of phosphorylation-resistant Rb resulted in liver tumor formation. Our results suggest that the continued high expression of several c-Myc target genes promotes the liver tumor formation.

5. Conflict of interest statement

None.

Acknowledgment

This work was supported by Grants-in-Aids from Ministry of Education, Culture, Science, Sports and Technology of Japan.

Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.bbrc.2011.12.014.

References

[1] D. Motola-Kuba, D. Zamora-Valdes, M. Uribe, N. Mendez-Sanchez, Hepatocellular carcinoma. An overview, An. Hepatol. 5 (2006) 16–24.
[2] H. Blum, Hepatocellular carcinoma: therapy and prevention, World J. Gastroenterol. 11 (2005) 7391–7400.
[3] E.K. Teo, K.M. Fock, Hepatocellular carcinoma: an Asian perspective, Dig. Dis. 19 (2001) 263–268.
[4] R.N. Aravalli, C.J. Steer, E.N. Cressman, Molecular mechanisms of hepatocellular carcinoma, Hepatology 48 (2008) 2047–2063.
[5] P.E. Zondervan, J. Wink, J.C. Alers, et al., Molecular cytogenetic evaluation of virus-associated and non-viral hepatocellular carcinoma: analysis of 26 carcinomas and 12 concurrent dysplasias, J. Pathol. 192 (2000) 207–215.
[6] W. Du, J. Pogoriler, Retinoblastoma family genes, Oncogene 25 (2006) 5190–5200.
[7] G. Scambia, S. Lovergine, V. Masciullo, RB family members as predictive and prognostic factors in human cancer, Oncogene 25 (2006) 5302–5308.
[8] D.H. Abramson, R.M. Ellsworth, F.D. Kitchin, G. Tung, Second nonocular tumors in retinoblastoma survivors. Are they radiation-induced?, Ophthalmology 91 (1984) 1351–1355
[9] M.F. Hansen, A. Koufos, B.L. Gallie, et al., Osteosarcoma and retinoblastoma: a shared chromosomal mechanism revealing recessive predisposition, Proc. Natl. Acad.Sci. USA 82 (1985) 6216–6220.
[10] J.W. Harbour, S.L. Lai, J. Whang-Peng, A.F. Gazdar, J.D. Minna, F.J. Kaye, Abnormalities in structure and expression of the human retinoblastoma gene in SCLC, Science 241 (1988) 353–357.
[11] Y. Kubota, K. Fujinami, H. Uemura, et al., Retinoblastoma gene mutations in primary human prostate cancer, Prostate 27 (1995) 314–320.
[12] E.Y. Lee, H. To, J.Y. Shew, R. Bookstein, P. Scully, W.H. Lee, Inactivation of the retinoblastoma susceptibility gene in human breast cancers, Science 241 (1988) 218–221.
[13] H. Miyamoto, T. Shuin, S. Torigoe, Y. Iwasaki, Y. Kubota, Retinoblastoma gene mutations in primary human bladder cancer, Br. J. Cancer 71 (1995) 831–835.
[14] A. Teufel, F. Staib, S. Kanzler, A. Weinmann, H. Schulze-Bergkamen, P.R. Galle, Genetics of hepatocellular carcinoma, World J. Gastroenterol. 13 (2007) 2271–2282.
[15] A.M. Hui, X. Li, M. Makuuchi, T. Takayama, K. Kubota, Over-expression and lack of retinoblastoma protein are associated with tumor progression and metastasis in hepatocellular carcinoma, Int. J. Cancer 84 (1999) 604–608.
[16] T. Ichihara, Y. Komagata, X.L. Yang, et al., Resistance to fulminant hepatitis and carcinogenesis conferred by overexpression of retinoblastoma protein in mouse liver, Hepatology 33 (2001) 948–955.
[17] C.N. Mayhew, E.E. Bosco, S.R. Fox, et al., Liver-specific pRB loss results in ectopic cell cycle entry and aberrant ploidy, Cancer Res. 65 (2005) 4568–4577.
[18] S. Barrientes, C. Cooke, D.W. Goodrich, Glutamic acid mutagenesis of retinoblastoma protein phosphorylation sites has diverse effects on function, Oncogene 19 (2000) 562–570.
[19] H. Yamada, S. Hirai, S. Ikegami, Y. Kawarada, E. Okuhara, H. Nagano, The fate of DNA originally existing in the zygote nucleus during achromosomal cleavage of fertilized echinoderm eggs in the presence of aphidicolin: microscopic studies with anti-DNA antibody, J. Cell Physiol. 124 (1985) 9–12.
[20] R. Pascale, M.M. Simile, D.F. Calvisi, et al., Role of HSP90, CDC37, and CRM1 as modulators of $p16^{INK4A}$ activity in rat liver carcinogenesis and human liver cancer, Hepatology 42 (2005) 1310–1319.
[21] A.S. Lundberg, R.A. Weinberg, Functional inactivation of the retinoblastoma protein requires sequential modification by at least two distinct cyclin-cdk complexes, Mol. Cell Biol. 18 (1998) 753–761.
[22] S. Mittnacht, Control of pRB phosphorylation, Curr. Opin. Genet. Dev. 8 (1998) 21–27.
[23] X.Q. Qin, T. Chittenden, D.M. Livingston, W.G. Kaelin Jr., Identification of a growth suppression domain within the retinoblastoma gene product, Genes. Dev. 6 (1992) 953–964.
[24] S. Huang, E. Shin, K.A. Sheppard, et al., The retinoblastoma protein region required for interaction with the E2F transcription factor includes the T/E1A binding and carboxy-terminal sequences, DNA Cell Biol. 11 (1992) 539–548.
[25] S. Huang, N.P. Wang, B.Y. Tseng, W.H. Lee, E.H. Lee, Two distinct and frequently mutated regions of retinoblastoma protein are required for binding to SV40 T antigen, EMBO J. 9 (1990) 1815–1822.
[26] H. Niwa, K. Yamamura, J. Miyazaki, Efficient selection for high-expression transfectants with a novel eukaryotic vector, Gene 108 (1991) 193–199.
[27] D.W. Goodrich, The retinoblastoma tumor-suppressor gene, the exception that proves the rule, Oncogene 25 (2006) 5233–5243.
[28] Z. Jiang, E. Zacksenhaus, Activation of retinoblastoma protein in mammary gland leads to ductal growth suppression, precocious differentiation, and adenocarcinoma, J. Cell Biol. 156 (2002) 185–198.
[29] H.L. Borges, J. Bird, K. Wasson, et al., Tumor promotion by caspase-resistant retinoblastoma protein, Proc. Natl. Acad. Sci. USA 102 (2005) 15587–15592.
[30] R. Opavsky, S.Y. Tsai, M. Guimond, et al., Specific tumor suppressor function for E2F2 in Myc-induced T cell lymphomagenesis, Proc. Natl. Acad. Sci. USA 104 (2007) 15400–15405.
[31] F.A. Mallette, M.F. Gaumont-Leclerc, G. Huot, G. Ferbeyre, Myc down-regulation as a mechanism to activate the Rb pathway in STAT5A-induced senescence, J. Biol. Chem. 282 (2007) 34938–34944.
[32] P.C. Fernandez, S.R. Frank, L. Wang, et al., Genomic targets of the human c-Myc protein, Genes Dev. 17 (2003) 1115–1129.
[33] W.E. Blanco-Bose, M.J. Murphy, A. Ehninger, et al., C-Myc and its target FoxM1 are critical downstream effectors of constitutive androstane receptor (CAR) mediated direct liver hyperplasia, Hepatology 48 (2008) 1302–1311.
[34] J. Laoukili, M. Stahl, R.H. Medema, FoxM1: at the crossroads of aging and cancer, Biochim. Biophys. Acta 1775 (2007) 92–102.
[35] Q.M. Guo, R.L. Malek, S. Kim, et al., Identification of c-Myc responsive genes using rat cDNA microarray, Cancer Res. 60 (2000) 5922–5928.
[36] Z. Li, S. VanCalcar, C. Qu, W.K. Cavenee, M.Q. Zhang, B. Ren, A global transcriptional regulatory role for c-Myc in Burkitt's lymphoma cells, Proc. Natl. Acad. Sci. USA 100 (2003) 8164–8169.
[37] J.S. Dovey, S.J. Zacharek, C.F. Kim, J.A. Lees, Bmi1 is critical for lung tumorigenesis and bronchioalveolar stem cell expansion, Proc. Natl. Acad. Sci. USA 105 (2008) 11857–11862.
[38] M. Gstaiger, R. Jordan, M. Lim, C. Catzavelos, J. Mestan, J. Slingerland, Skp2 is oncogenic and overexpressed in human cancers, Proc. Natl. Acad. Sci. USA 98 (2001) 5043–5048.
[39] R. Eferl, E.F. Wagner, AP-1: a double-edged sword in tumorigenesis, Nat. Rev. Cancer 3 (2003) 859–868.
[40] H. Yamamoto, T. Ochiya, F. Takeshita, et al., Enhanced skin carcinogenesis in cyclin D1-conditional transgenic mice: cyclin D1 alters keratinocyte response to calcium-induced terminal differentiation, Cancer Res. 62 (2002) 1641–1647.