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c-Myc-driven Hepatocarcinogenesis

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Abstract. *Background/Aim: Dysregulation of the c-Myc gene is frequently found in human hepatocellular carcinoma (HCC), often accompanied by genetic and epigenetic alterations in other cancer-related genes. Here, we investigated the tumorigenic potential of c-Myc in diverse genetic environments in which the Ras, Wnt/β-catenin, Sonic hedgehog, or P53 pathways were either activated or inactivated. Materials and Methods: Hydrodynamic tail vein injection was employed to administer expression transposons and generate transgenic livers expressing c-Myc together with a constitutively active form of RAS ($HRAS^{G12V}$), β-catenin ($\beta\text{-catenin}^{S33Y}$), Smo (SmoM2), or short hairpin RNA targeting P53 (shp53). Results: c-Myc was most tumorigenic when the RAS signaling pathway was activated, whereas no tumors were found in mice when either $\beta\text{-catenin}^{S33Y}$ or SmoM2 was co-expressed with c-Myc. Approximately 40% of mice had HCC when c-Myc was over-expressed under P53 inactivation. Furthermore, we investigated the effect of mutation in c-Myc on hepatocarcinogenesis. Conclusion: No significant differences in tumorigenic potential were found between wild type c-Myc and $c\text{-Myc}^{T58A}$, minimizing the role of the mutation in hepatocarcinogenesis.*

The *c-Myc* gene was discovered as the cellular homolog of the retroviral v-*myc* oncogene and subsequently found to be activated in a wide range of human cancers including Burkitt's lymphoma, colon cancer, melanoma, breast cancer, and myeloid leukemia (1). c-Myc plays critical roles in carcinogenesis by transcriptionally activating and repressing a plethora of genes involved in cell growth, proliferation, and differentiation (2-5). As tumors often harbor multiple genetic and epigenetic changes in cancer-related genes, activated proto-oncogenes and/or inactivated tumor suppressor genes are thought to collaborate during the initiation and progression of human carcinogenesis. Collaboration between c-Myc and various oncogenes has been suggested to promote carcinogenesis in a variety of tissues (6-8).

Hepatocellular carcinoma (HCC) is the most common type of primary liver cancer and one of the most lethal cancers. Liver cancers rank third in terms of mortality; they accounted for more than 800,000 deaths worldwide in 2020 (9). *c-Myc* is one of the first oncogenes that are found amplified in HCC (10). Over-expression of the *c-Myc* gene is frequently found in human HCC, which is typified by genetic heterogeneity displaying alterations in diverse cancer-related genes (11, 12). In HCC, the *TP53* and *CTTNB1* (encoding β-catenin) genes are found to be mutated at a frequency as high as 30% (13). The Sonic Hedgehog (SHH) signaling pathway promotes hepatic fibrosis and carcinogenesis and is activated in over 50% of human HCC (14-16). Here, to better understand the genetic mechanism underlying c-Myc-driven hepatocarcinogenesis, we investigated oncogenic synergism between c-Myc and activated oncogenes or inactivated tumor suppressor genes for hepatocarcinogenesis, employing a simple liver-specific transgenic approach (17, 18).

Deregulation of c-Myc in cancers is achieved through quantitative changes in the protein level such as increased or sustained expression of c-Myc. Mechanistically, over-expression of c-Myc is predominately caused by gene amplification of the c-*Myc* locus or, less commonly, by chromosomal translocation, which places c-*Myc* under a strong promoter or enhancer (19). In addition, point mutations in the c-*Myc* coding region are occasionally found in some cancers, notably in B-cell lymphoma. Although these mutations mostly increase protein stability, another mechanism of elevating c-Myc protein levels, studies have shown that the mutation at codon 58 leading to the substitution of alanine for threonine in c-Myc ($c\text{-Myc}^{T58A}$) is a gain-of-function

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mutation that endows the mutant c-Myc with an anti-apoptotic function (20, 21). In this study, we also investigated whether c-Myc harboring the T58A mutation has a higher oncogenic potential than wild-type c-Myc in the liver.

Materials and Methods

Plasmids. The plasmids, pT2/EGFP, pT2/HRAS^{G12V}, pT2/shp53, pT2/c-Myc, and pPGK-SB13 have been previously described (22, 23). The plasmid, pT2/c-Myc^{T58A} was constructed *via* site-directed mutagenesis replacing ACC (encoding threonine) at codon 58 of the c-*Myc* with GCC (encoding alanine). The ORFs encoding a constitutively active form of the murine β-catenin (β-catenin^{S33Y}) and Smo (SmoM2) were PCR amplified from pMXs-beta-catenin-S33Y (a gift from Shinya Yamanaka, Plasmid # 13371; Addgene, Watertown, MA, USA) and PD23 (a gift from Gregory Pazour, Plasmid # 164536; Addgene), respectively. The amplified PCR products were used to replace the ORF encoding EGFP in pT2/EGFP. The resulting plasmids are referred to as pT2/ β-catenin^{S33Y} and pT2/SmoM2, respectively.

Animal experiments. All experiments using live mice were approved by the Animal Policy and Welfare Committee of the Kyung Hee University. Wild-type C57BL/6 mice were purchased from the Orientbio (Seongnam, Republic of Korea). Hydrodynamic tail vein injection was previously described (24). Briefly, the total of 25 μg of transposons was mixed with 9 μg of pPGK-SB13, and then suspended in 2 ml of Lactated Ringer's solution. Each DNA solution was injected into the lateral tail veins of 6-week-old C57BL/6 mice (0.1 ml/g body weight) in less than 7 seconds.

Liver harvesting and histological analysis. Mice were deeply anesthetized by the inhalation of isoflurane. A midline laparotomy incision was performed, and their livers were removed and photographed. Excised livers were fixed overnight in freshly prepared 10% neutral-buffered formalin, embedded in paraffin and sectioned into 4-μm slices. Slices were stained with hematoxylin & eosin (H&E).

Immunohistochemistry (IHC). Paraffin sections were deparaffinized in xylene and rehydrated through a gradual decrease in ethanol concentration. The antigen epitopes were then unmasked using sodium citrate buffer (pH 6.0). Subsequently, the sections were incubated overnight at 4°C with the following primary antibodies: anti-c-Myc (ab32072; Abcam, Cambridge, UK), anti-Ki-67 (ab15580; Abcam), anti-cleaved caspase-3 (#9661, Cell Signaling Technology, Danvers, MA, USA), and anti-HNF4α antibodies (ab201460; Abcam). After primary antibody incubation, sections were incubated with biotinylated anti-rabbit IgG secondary antibodies (PK-7200; Vector Laboratories, Burlingame, CA, USA) followed by treatment with freshly prepared DAB substrates (PK-4100; Vector Laboratories). Sections were lightly counter-stained with hematoxylin and mounted. Slides were analyzed and photographed using a microscope (Eclipse Ti; Nikon, Tokyo, Japan) equipped with a digital camera.

Mouse survival and statistical analysis. Mice were monitored daily for illness symptoms. Kaplan–Meier survival data were analyzed using a log-rank test. Statistical analyses were conducted using an unpaired parametric Student's *t*-test. A value of $p<0.05$ was considered to indicate statistical significance.

Results

Oncogenic synergism of c-Myc with other cancer genes. Dysregulation of *c-Myc* and *TP53* is frequently found in human HCC (13, 25). In addition, studies have shown that RAS, Wnt/β-catenin, and SHH signaling are among the most frequently activated molecular signaling pathways during hepatocarcinogenesis (13, 26). To investigate the role of dysregulation of each gene or signaling pathway in the development of HCC, we developed transgenic mice in which livers expressed a constitutively active form of RAS (HRAS^{G12V}), β-catenin (β-catenin^{S33Y}), or Smo (SmoM2), utilizing hydrodynamic tail vein injection (HTVI) and the *Sleeping Beauty* transposon system (18). Likewise, transposons expressing c-Myc or those encoding a short hairpin RNA targeting TP53 (shp53) were delivered to murine livers *via* HTVI (Figure 1A). Mice were sacrificed and examined 50 weeks post HTVI. No mice in any of the groups developed a tumor in the liver (Table I). These data suggest that single oncogenes are not sufficient to induce liver tumors and, thus, synergism among cancer genes might be required to efficiently develop HCC.

Over-expression of the c-Myc gene is found in more than 50% of human HCC (10). HCC is also typified by clinical heterogeneity displaying different degrees of differentiation, mitotic activity, and vascular invasion of tumor cells, presumably due to its genetic heterogeneity. Based on the significance of c-Myc in human carcinogenesis and the fact that c-Myc alone is insufficient for hepatocarcinogenesis, we aimed to study oncogenic synergism between c-Myc and other cancer genes.

For this study, we developed double transgenic livers co-expressing HRAS^{G12V}, β-catenin^{S33Y}, or SmoM2 together with c-Myc (Figure 1B). Likewise, to study the role of TP53 inactivation together with c-Myc over-expression in hepatic carcinogenesis, transposons encoding a short hairpin RNA targeting TP53 (shp53) were co-delivered to the liver along with those expressing c-Myc *via* HTVI. Although mice co-expressing either β-catenin^{S33Y} or SmoM2 together with c-Myc did not develop liver tumors 50 weeks post HTVI, liver tumors were found in approximately 40% of mice expressing shp53 and c-Myc at the same time (Table I). Of note, mice hydrodynamically transfected with HRAS^{G12V} and c-Myc displayed signs of discomfort starting as early as 5 weeks post HTVI and liver tumors were observed in all mice in this group (Figure 2A and Table I). The data indicate that c-Myc favors dysregulation of particular cancer genes to promote carcinogenesis in the liver.

Differences between c-Myc plus HRAS^{G12V} and c-Myc plus shp53 HCC models. In addition to the difference in the timing of tumor onset and the frequency of tumor incidence, the c-Myc plus HRAS^{G12V} and c-Myc plus shp53 models revealed

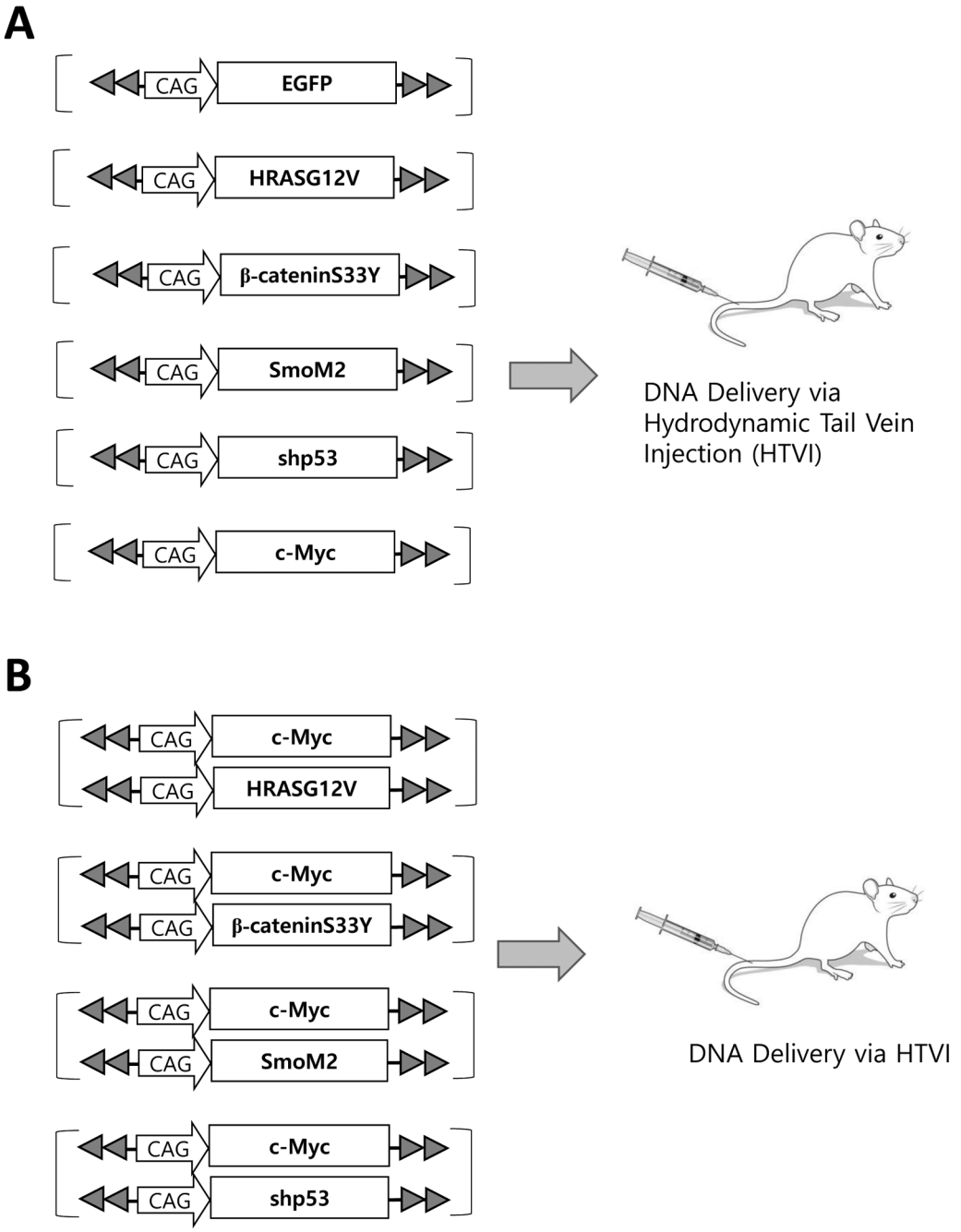

Figure 1. *Schematic illustration of the experimental procedures. (A) To generate single transgenic livers, hydrodynamic tail vein injection (HTVI) was performed to deliver each transposon to the liver. (B) To generate double transgenic livers, two kinds of transposons encoding the indicated oncogenes were mixed and delivered to the livers via HTVI.*

some different cellular and histological characteristics. Although both tumor models showed HCC phenotypes, histopathological examination revealed highly differentiated morphology of tumor cells in the c-Myc plus shp53 group, whereas tumors from the c-Myc plus $HRAS^{G12V}$ group consisted of cells with atypical hepatocyte morphology that were poorly differentiated (Figure 2B). High expression of the representative hepatocyte marker HNF4α was detected in neoplastic cells of the c-Myc plus shp53 tumors group, while HNF4α expression was undetectable in c-Myc plus $HRAS^{G12V}$

Table I. *Effects of expressed oncogenes on the induction of liver tumors.*

Oncogene(s) expressed	% of mice with liver tumors	Timepoint of liver harvest (weeks PHI)*
EGFP (control)	0 (0/10)	50
HRASG12V	0 (0/10)	50
β-cateninS33Y	0 (0/10)	50
SmoM2	0 (0/10)	50
shp53	0 (0/10)	50
c-Myc	0 (0/10)	50
c-MycT58A	0 (0/10)	50
c-Myc and HRASG12V	100 (25/25)	5-12
c-Myc and β-cateninS33Y	0 (0/25)	50
c-Myc and SmoM2	0 (0/25)	50
c-Myc and shp53	44 (11/25)	25-50

*Livers were harvested when mice displayed signs of discomfort. The timepoint of 50 weeks post hydrodynamic injection (PHI) was the experimental endpoint.

tumors (Figure 2B). In line with histological findings, expression analysis using a quantitative reverse-transcription polymerase chain reaction (qRT-PCR) revealed higher expression levels of hepatic stem cell markers such as EpCAM and CK19 in c-Myc plus $HRAS^{G12V}$ tumors than those in c-Myc plus shp53 tumors (Figure 2C). TGF-β signaling promotes epithelial-to-mesenchymal (EMT) transition and stem cell characteristics (27, 28). In addition, Notch signaling promotes development of cholangiocarcinoma, a primary liver cancer of bile duct origin (29, 30). Levels of TGF-β1, the ligand of TGF-β signaling, were higher in c-Myc plus $HRAS^{G12V}$ tumors than those in the control and c-Myc plus shp53 groups. The levels of the Notch1 ligand in the two tumor groups were comparable to that in the control group.

Tumorigenic potential of the wild-type c-Myc and c-Myc^{T58A} in livers. A mutation at codon 58 in c-Myc that substitutes alanine for threonine (*i.e.*, c-Myc^{T58A}) enhances the oncogenic potential of c-Myc in Burkitt's lymphoma and some other cancers (20, 31). Increased proliferation and decreased levels of apoptosis have been reported in cancer cells expressing c-Myc^{T58A} when compared to cancer cells expressing wild-type c-Myc (32, 33). To investigate whether such increased oncogenic potential of c-Myc^{T58A} is also observed in the liver, we generated transgenic livers expressing c-Myc^{T58A}. Similar to the results from livers expressing wild-type c-Myc, mice expressing c-Myc^{T58A} alone did not develop a tumor in their livers (Table I).

Next, we compared oncogenic potential between wild-type c-Myc and c-Myc^{T58A} under the genetic context of activated RAS signaling (Figure 3A). Comparison of animal survival following HTVI showed no significant differences between the c-Myc plus $HRAS^{G12V}$ and c-Myc^{T58A} plus $HRAS^{G12V}$ groups (Figure 3B). Furthermore, both groups developed HCC in the livers with a frequency of 100%, which displayed similar histopathological features (Figure 3C and D).

Cellular proliferation and apoptosis induced by c-Myc and c-Myc^{T58A}. To better characterize tumors induced by c-Myc plus $HRAS^{G12V}$ and c-Myc^{T58A} plus $HRAS^{G12V}$, the levels of cellular proliferation and apoptosis in the tumors were assessed (Figure 4A and B). No differences were found between tumors induced by c-Myc plus $HRAS^{G12V}$ and c-Myc^{T58A} plus $HRAS^{G12V}$. In line with these findings at the cellular level, molecular analysis also showed that expression levels of genes regulating cell cycle and apoptosis were not significantly different between the two groups (Figure 4C). Thus, all data indicated that c-Myc^{T58A} does not vary from wild-type c-Myc in promoting HCC synergistically with $HRAS^{G12V}$.

Oncogenicity of c-Myc and c-Myc^{T58A} under TP53 inactivation. We suspected that oncogenic RAS might have provided a unique genetic environment in which c-Myc^{T58A} and wild-type c-Myc displayed similar oncogenic functions. Thus, we tested the oncogenicity of wild-type and mutant c-Myc under the genetic context of *TP53* inactivation. Transposons encoding either wild-type c-Myc or c-Myc^{T58A} were delivered to murine livers together with those expressing shp53 (Figure 5A).

The HTVI experiment revealed that animal survival was not significantly different between c-Myc plus shp53 and c-Myc^{T58A} plus shp53 groups (Figure 5B). In addition, histopathological analysis showed similar morphological characteristics among tumors from the two groups (Figure 5C). Thus, in accordance with the findings under the genetic context of RAS activation, the wild-type c-Myc and c-Myc^{T58A} exerted similar tumorigenic effects in the liver under P53 inactivation.

Discussion

The c-*Myc* gene is one of the most frequently dysregulated genes in human cancers and is involved in multiple facets of tumorigenesis including tumor initiation, maintenance, and metastasis (3, 34, 35). In the present study we investigated the tumorigenic ability of c-Myc in the liver utilizing a liver-specific transgenic approach. Our study showed that although c-Myc alone is not sufficient for hepatocarcinogenesis, it can effectively synergize with activated RAS or TP53 inactivation to induce HCC. Tumors induced by c-Myc plus $HRAS^{G12V}$ were more malignant and had a lower degree of differentiation than those induced by c-Myc plus shp53. The representative hepatocyte marker HNF4α was strongly expressed in the nuclei of c-Myc plus shp53 tumor cells, whereas HNF4α expression was undetectable in c-Myc plus $HRAS^{G12V}$ tumors. Further, co-

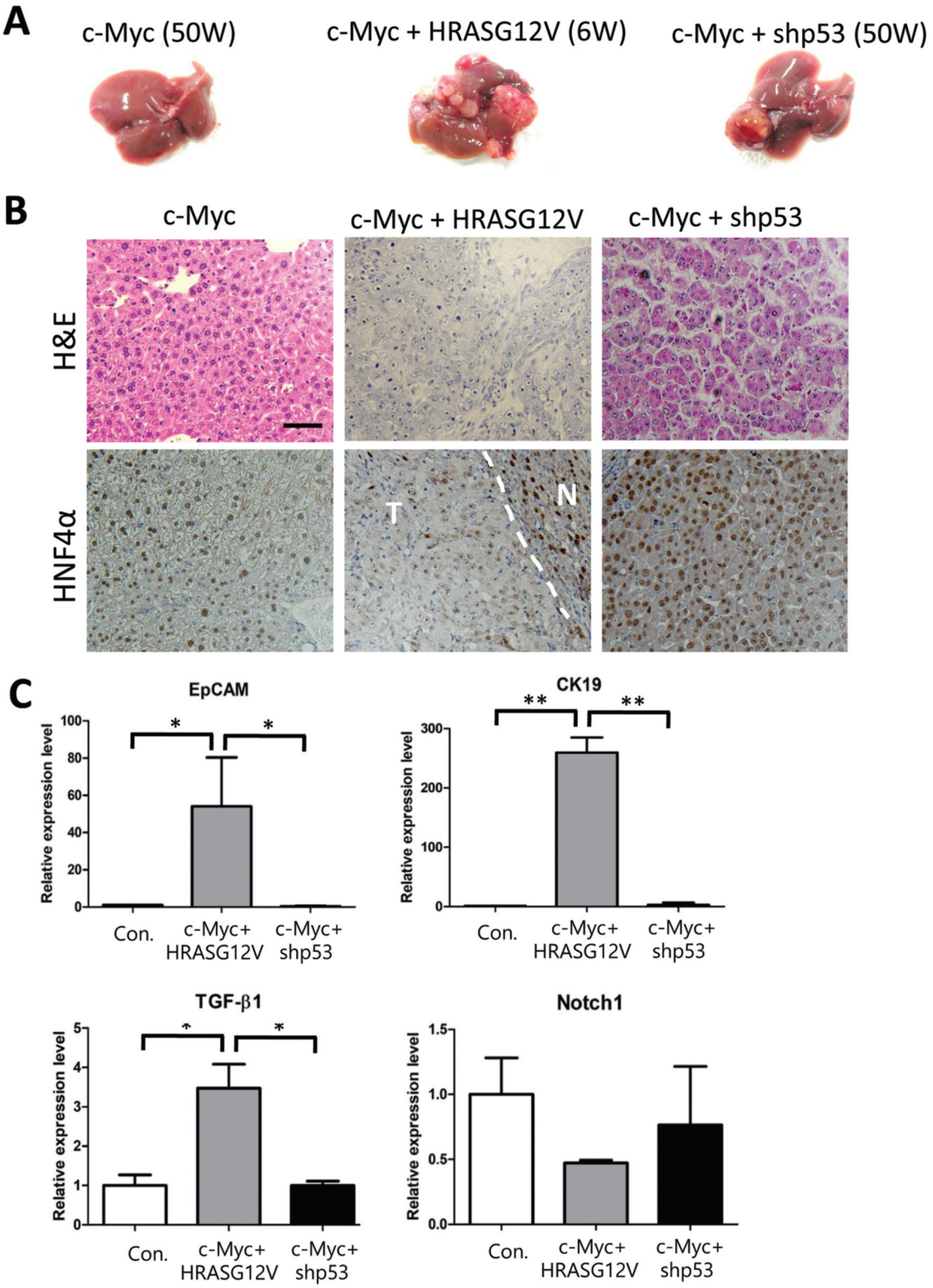

Figure 2. *Liver cancers induced by c-Myc plus HRAS^{G12V} and c-Myc plus shp53. (A) Gross morphology of representative livers harvested at the indicated time points following HTVI. (B) H&E staining and immunohistochemistry (IHC) images of paraffin sections from livers shown in A. In the HNF4α IHC image of c-Myc plus HRAS^{G12V}, "T" denotes tumor and "N" denotes non-tumorous liver parenchyma (boundary marked by white dashed line). Scale bar, 50 μm. (C) Expression levels of genes. Quantitative RT-PCR was performed to assess mRNA levels of the indicated genes in tumors induced by c-Myc plus HRAS^{G12V} and c-Myc plus shp53. Livers expressing EGFP were used as a control.*

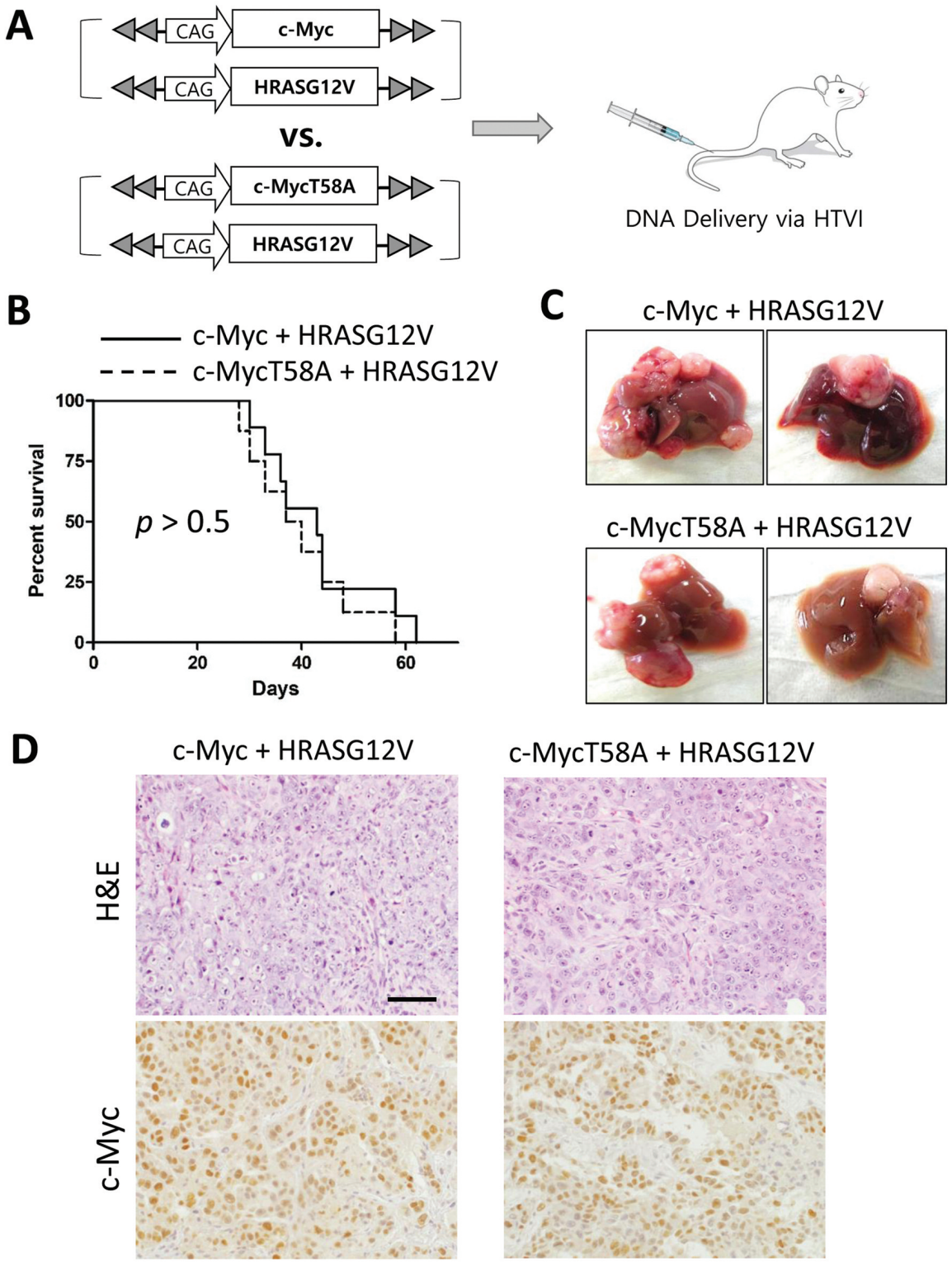

Figure 3. *Comparison of tumorigenic potentials between the c-Myc and c-Myc^{T58A}. (A) Schematic illustration of the experimental procedures. Transposons encoding the wild-type c-Myc and c-Myc^{T58A} were delivered to the liver together with those encoding HRAS^{G12V}. (B) Kaplan–Meier survival curves of mice expressing c-Myc plus HRAS^{G12V} versus those expressing c-Myc^{T58A} plus HRAS^{G12V} following HTVI. (C) Gross morphology of representative livers harvested at 6 weeks post HTVI. n=10. (D) Paraffin sections of c-Myc plus HRAS^{G12V} and c-Myc^{T58A} plus HRAS^{G12V} tumors were stained with H&E and anti-c-Myc antibodies. Scale bar, 50 μm.*

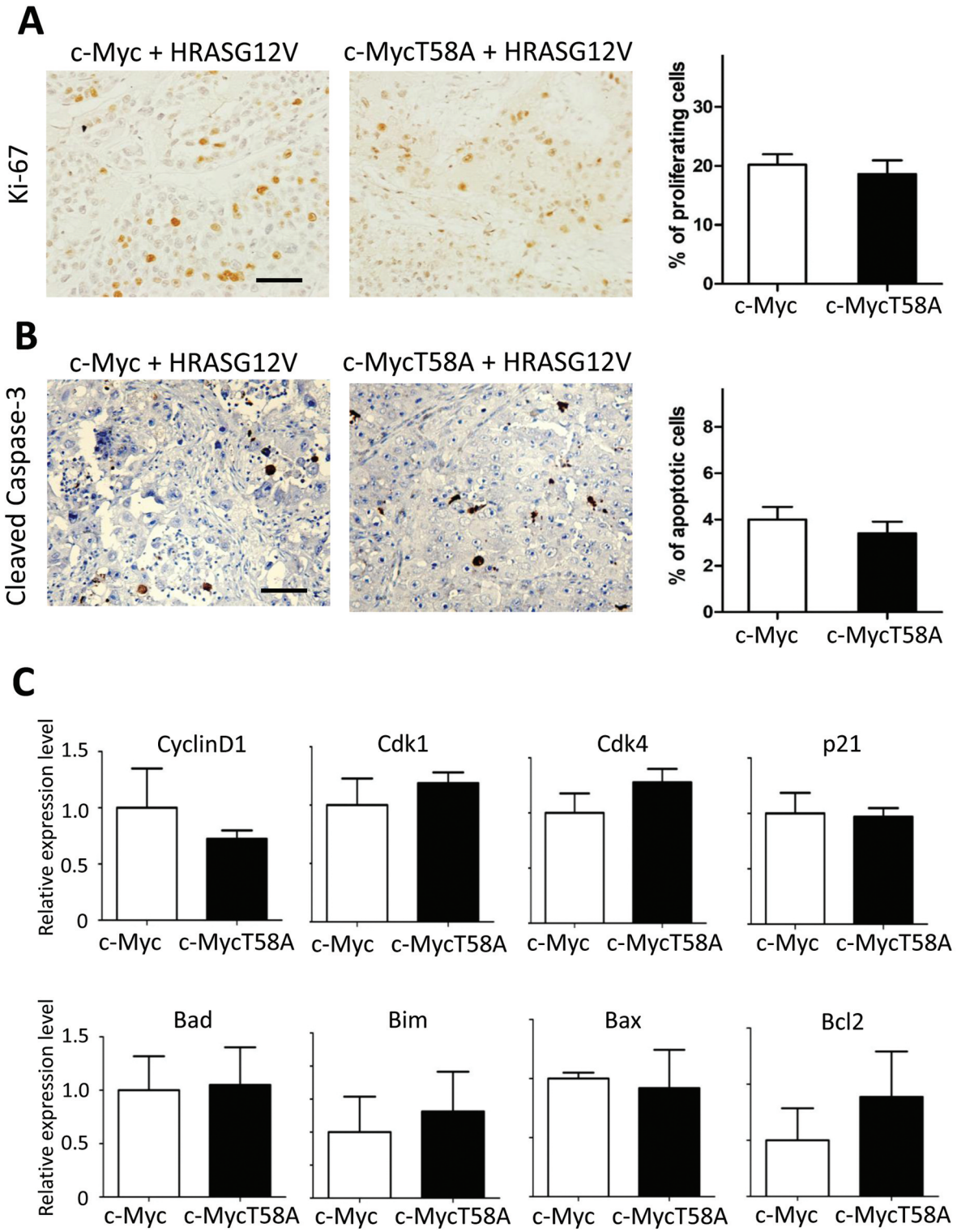

Figure 4. *Cell proliferation and apoptosis in tumors of c-Myc plus HRAS^{G12V} and c-Myc^{T58A} plus HRAS^{G12V} (A) IHC staining for Ki-67 in paraffin sections of indicated tumors, and percentages of proliferating cells in tumors based on the staining. Scale bar, 50 μm. (B) IHC staining for cleaved canspase-3 in tumor sections, and percentages of apoptotic cells in tumors based on the staining. Scale bar, 50 μm. (C) Expression levels of genes involved in cell cycle and apoptosis. Real-time RT-PCR was performed to quantitatively assess mRNA levels of the indicated genes in tumors induced by c-Myc plus HRAS^{G12V} and c-Myc^{T58A} plus HRAS^{G12V}.*

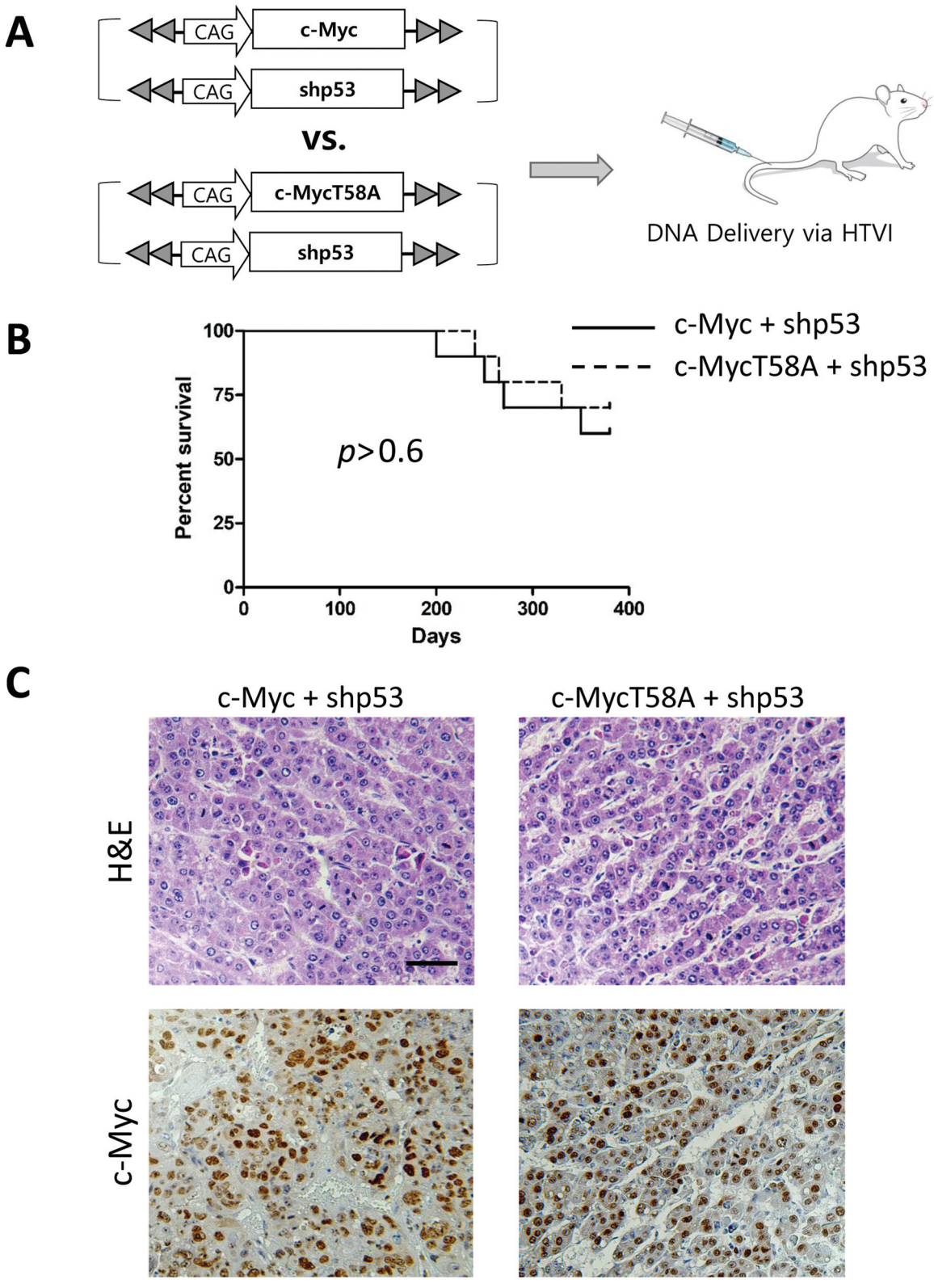

Figure 5. *Tumors expressing c-Myc plus shp53 and c-Myc^{T58A} plus shp53. (A) Schematic illustration of the experimental procedures. (B) Kaplan–Meier survival curves of mice expressing c-Myc plus shp53 versus those expressing c-Myc^{T58A} plus shp53 following HTVI. (C) Paraffin sections of c-Myc plus shp53 and c-Myc^{T58A} plus shp53 tumors were stained with H&E and anti-c-Myc antibodies. Scale bar, 50 μm.*

expression of c-Myc and $HRAS^{G12V}$ allowed HCC to develop as early as 5 weeks after HTVI, whereas tumors were not grossly observed in the c-Myc plus shp53 group up to 12 weeks post HTVI. The study shows that activated RAS synergized with c-Myc highly effectively to induce HCC. It is not clear from this study why β-$catenin^{S33Y}$ and SmoM2 failed to synergize with c-Myc to induce tumor in the liver. Although speculative, c-Myc-driven hepatocarcinogenesis might favor certain types of genetic aberrations to further enhance the tumorigenic potential of c-Myc. Further study is needed to address this issue.

Mutant $c\text{-}Myc^{T58A}$ has shown an increased transforming ability in certain types of cancers including B-cell lymphoma, mammary gland tumor, and squamous cell carcinoma (20, 31, 36). In addition to increased stability, $c\text{-}Myc^{T58A}$ acquires additional oncogenic functions such as suppression of apoptosis by down-regulating the pro-apoptotic BH3-only protein Bim, and promoting stem cell characteristics by activating stem cell genes encoding Lgr6, Sox2, and CD34 (20, 36). In our study, $c\text{-}Myc^{T58A}$ was not more tumorigenic than wild-type c-Myc in HCC. It should be pointed out that this comparison was made between the two under the genetic context of RAS activation and P53 inactivation; thus, it is possible that $c\text{-}Myc^{T58A}$ could be more hepatocarcinogenic than wild-type in other oncogenic environments.

In this study, we investigated hepatocarcinogenesis driven by c-Myc, employing a variety of transgenic mouse models developed by the HTVI method. The methodology allows transgenic livers with diverse genetic characteristics to be developed in less time and in a cost-effective manner. A simple liver-specific transgenic approach will broaden our understanding of cooperation between oncogenic signaling pathways in the liver.

Conflicts of Interest

The Authors declare that there are no conflicts of interest in relation to this study.

Authors' Contributions

HM, HP and SWR designed experiments, analyzed data, and wrote the manuscript. HM and HP performed experiments. SWR supervised the research. All Authors read and approved the final manuscript.

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