

2016 Hepatocellular Carcinoma: Global View

## Alpha-fetoprotein-targeted reporter gene expression imaging in hepatocellular carcinoma

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### Abstract

Hepatocellular carcinoma (HCC) is one of the most common cancers in Eastern Asia, and its incidence is increasing globally. Numerous experimental models have been developed to better our understanding of the pathogenic mechanism of HCC and to evaluate novel therapeutic approaches. Molecular imaging is a convenient and up-to-date biomedical tool that enables the visualization, characterization and quantification of biologic processes in a living subject. Molecular imaging based on reporter gene expression, in particular, can elucidate tumor-specific events or processes by acquiring images of a reporter gene's expression driven by tumor-specific enhancers/promoters. In this review, we discuss the advantages and disadvantages of various experimental HCC mouse models and we present *in vivo* images of tumor-specific reporter gene expression driven by an alpha-fetoprotein (AFP) enhancer/promoter system in a mouse model of HCC. The current mouse models of HCC development are established by xenograft, carcinogen induction and genetic engineering, representing the spectrum of tumor-inducing factors and tumor locations. The imaging analysis approach of reporter genes driven by AFP enhancer/promoter is presented for these different HCC mouse models. Such molecular imaging can provide longitudinal information about carcinogenesis and tumor progression. We expect that clinical application of AFP-targeted reporter gene expression imaging systems will be useful for the detection of AFP-expressing HCC tumors and screening of increased/decreased AFP levels due to disease or drug treatment.

**Key words:** Alpha-fetoprotein; Hepatocellular carcinoma; Molecular imaging; Reporter gene; Tumor-specific enhancer/promoter

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**Core tip:** It is essential to establish an appropriate animal model of hepatocellular carcinoma (HCC) for monitoring the disease progression and evaluating therapeutic interventions with anticancer drugs. Reporter gene-based molecular imaging can elucidate tumor-specific events or processes through acquisition of images of reporter gene expression driven by tumor-specific enhancers/promoters. In this paper, we describe the advantages and disadvantages of various animal models of HCC and present images of *in vivo* reporter gene expression controlled by alpha-fetoprotein enhancer/promoter in the various HCC animal models.

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## INTRODUCTION

Molecular imaging methods can provide novel insights into biological and physiological processes through visualization of cellular or molecular events in a living organism. The greatest significance of molecular imaging methods lies in their ability to non-invasively and repetitively obtain longitudinal and quantitative biological information in an *in vivo* setting<sup>[1]</sup>. Moreover, when compared to analytical methods involving biopsied tissues, the non-invasive nature of molecular imaging is less time-consuming and provides more reliable inferences to the organism.

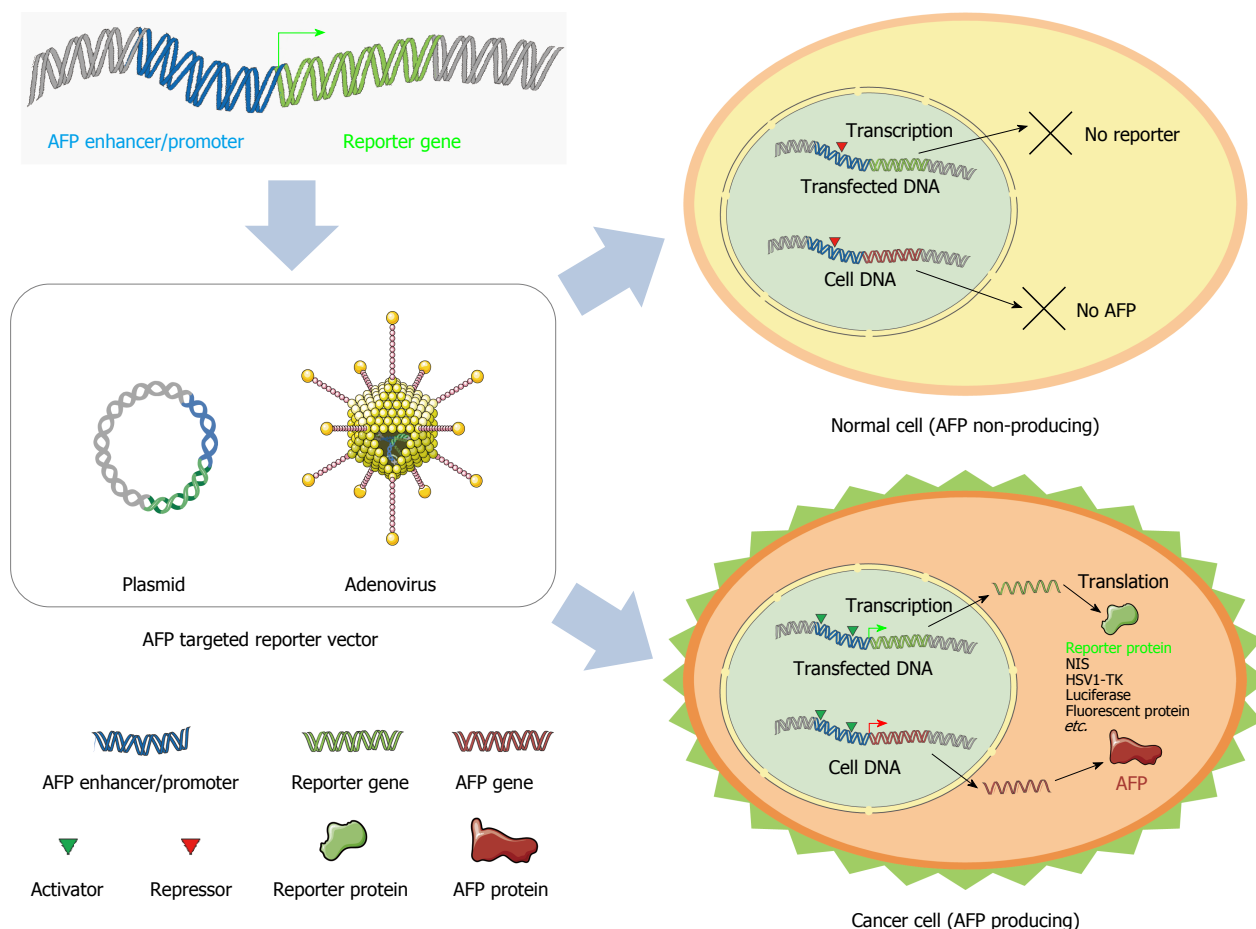
When applied to evaluate the efficacy and therapeutic mechanism of new drugs, molecular imaging can help to determine biochemical or metabolic changes *in vivo* by use of Tc-99m labeled annexin V (for apoptosis), Tc-99m labeled vascular endothelial growth factor or Cu-64/In-111/Tc-99m labeled arginylglycylaspartic acid peptide (for angiogenesis), Cu-64 labeled methylthiosemicarbazone or F-18 labeled fluorozomycin arabinoside (for hypoxia), or F-18 labeled 2-deoxy-D-glucose, F-18 labeled 3'-deoxy-3'-fluorothymidine or F-18 labeled fluoroethyltyrosine (for glucose, nucleotide or amino acid metabolism)<sup>[2]</sup>. The pharmacokinetic and pharmacodynamic properties of new drugs, including tissue biodistribution at designated time points and the binding affinity of ligands to their target receptors, can also be determined by nuclear

medicine imaging using radioisotope-labeled drugs<sup>[3]</sup>. At present, the applicability of molecular imaging methods to drug development is increasing in scale and scope, with the added benefit of providing further clarification of basic biological phenomena<sup>[4]</sup>.

The currently available molecular imaging methods are classified into the following categories according to their technical modalities: optical imaging (fluorescence and bioluminescence); magnetic resonance imaging (MRI); nuclear medicine imaging, including scintigraphy, positron emission tomography (PET) and single-photon emission computed tomography (commonly known as SPECT); and others, including ultrasound imaging and photoacoustic imaging. Optical imaging has high sensitivity, but its clinical utility is limited due to its low depth of penetration. MRI has high resolution and excellent soft-tissue contrast, but poor sensitivity and the expensive cost of the MRI equipment have proven prohibitive to its widespread application. Nuclear medicine imaging has high sensitivity and unlimited depth penetration, but again cost of the equipment is prohibitive and its limited spatial resolution is another limiting factor. Ultrasound imaging has high spatial/temporal resolution and a much more affordable (relatively low cost) profile, but is limited to vascular compartments and its reliability can be operator-dependent<sup>[5,6]</sup>. Therefore, several multimodality imaging instruments, such as PET/CT, PET/MR, and optical imaging/computed tomography (CT), have been developed to overcome the distinct disadvantages of each and now play an important role in basic and clinical research.

Molecular imaging with reporter gene expression (also known as molecular genetic imaging) is defined as an imaging method that makes use of reporter gene expression in a target cell or tissue. Various imaging reporter genes have been developed for optical and nuclear medicine imaging, with the most popular being those encoding firefly luciferase, a variety of fluorescent proteins, the herpes simplex virus type 1 thymidine kinase (HSV1-tk) and the sodium iodide symporter (NIS)<sup>[5,7]</sup>. To localize and track target cells *in vivo*, a reporter gene driven by a strong constitutive promoter, such as that of cytomegalovirus (commonly referred to as CMV), is first introduced into target cells. The reporter gene-expressing target cell is injected into a living organism and images are then acquired at designated time points following target cell injection. Because the *HSV1-tk* gene is applied for therapy as well as imaging, this gene expression can be monitored by visualization with an imaging technique<sup>[5]</sup>. Therefore, combining molecular imaging and gene therapy can allow for real-time evaluation of location and duration of expression of a therapeutic gene, and successful application of this method in clinical practice has been reported<sup>[8,9]</sup>.

Because tumor-specific enhancers/promoters, such as the alpha-fetoprotein (AFP) enhancer/promoter



**Figure 1** Cancer-specific reporter gene expression mediated by the alpha-fetoprotein enhancer/promoter. A reporter gene was delivered into cells using a vector system, such as a plasmid or adenovirus. In alpha-fetoprotein (AFP)-producing cancer cells, the level of the transcriptional activator of the *AFP* gene is high and thus the AFP enhancer/promoter is active. In contrast, in normal (non-cancer, non-AFP producing) cells, expression of the transcriptional repressor of the *AFP* gene is high and the *AFP* enhancer/promoter is inactive. The reporter protein is activated only in cancer cells with a high AFP level. AFP: Alpha-fetoprotein; HSV1-TK: Herpes simplex virus type 1 thymidine kinase; NIS: Sodium iodide symporter.

(Figure 1) and human telomerase reverse transcriptase, are highly active in cancer cells but not in non-cancer cells, they may serve as markers of cancer pathogenesis and progression that can be monitored by imaging of reporter gene expression. Indeed, several studies have already shown the utility of such reporter gene expression imaging using various tumor-specific enhancers/promoters, including survivin, mucin-1, carcinoembryonic antigen, prostate specific antigen and progression elevated gene-3; these studies are summarized in Table 1<sup>[10-20]</sup>.

Hepatocellular carcinoma (HCC) is one of the most common cancers worldwide, and is currently ranked fifth in incidence and second in mortality among males<sup>[21]</sup>. Fetal liver cells normally express AFP, but expression decreases rapidly after birth. In adults, however, AFP expression has been found to resume under abnormal conditions, including liver cancer and cirrhosis. Although the diagnostic accuracy of AFP in small HCC (< 3 cm) is limited, it has been a useful and convenient diagnostic tool for HCC since the 1970s<sup>[22]</sup>. Thus, many of the studies of HCC-specific imaging have used AFP-targeted reporter gene expression.

It is essential to establish an HCC animal model for monitoring of HCC progression and therapeutic interventions. In this review, we discuss methods for preparation of experimental HCC mouse models and the particular advantages and disadvantages of each. The AFP-specific reporter gene system, *in vivo* imaging applications for hepatocarcinogenesis and detection of AFP-positive HCC are also presented in the context of the mouse models.

## EXPERIMENTAL HCC MOUSE MODELS

Mouse models of HCC development are established by xenografting, carcinogen induction or genetic engineering, according to the tumor-inducing factor and tumor location of research interest. Table 2 provides an overview of the various HCC mouse modeling methods and their key advantages and disadvantages.

### Xenograft HCC models

Xenograft HCC models involve inoculation of cultured HCC cells into immune-deficient mice, such as the

**Table 1 Reporter gene expression imaging with tumor-specific enhancers/promoters**

Enhancer/promoter	Reporter gene (delivery method)	Imaging modality	Targeted tumor	Ref.
Survivin	<i>hNIS</i> (adenovirus)	Gamma camera	Ectopic xenograft PC-3 (prostate cancer) HepG2 (hepatoma) A375 (melanoma)	Huang <i>et al</i> <sup>[10]</sup>
	<i>fLuc</i> (adenovirus)	Bioluminescent imaging	Orthotopic xenograft McA-RH7777 (rat hepatoma)	Ahn <i>et al</i> <sup>[11]</sup>
Mucin-1	<i>fLuc</i> (adenovirus)	Bioluminescent imaging	Ectopic xenograft (metastasis) KPL-1 (breast cancer)	Huyn <i>et al</i> <sup>[12]</sup>
Hepatocarcinoma-intestine-pancreas (HIP)	<i>NIS</i> (adenovirus)	SPECT-CT	DEN-induced HCC (rat)	Hervé <i>et al</i> <sup>[13]</sup>
Prostate specific antigen (PSA)	<i>fLuc/HSV1-sr39tk</i> (adenovirus)	Bioluminescent imaging/ PET	Ectopic xenograft LNCaP (prostate cancer)	Iyer <i>et al</i> <sup>[14]</sup> Jiang <i>et al</i> <sup>[15]</sup>
Carcinoembryonic antigen (CEA)	<i>HSV1-tk</i> (adenovirus)	Gamma camera	Ectopic xenograft MOD (murine breast cancer)	Qiao <i>et al</i> <sup>[16]</sup>
	<i>hNIS</i> (adenovirus)	Gamma camera	Ectopic xenograft TT (medullary thyroid cancer)	Spitzweg <i>et al</i> <sup>[17]</sup>
Progression elevated gene (PEG)-3	<i>fLuc/HSV1-tk</i> (plasmid)	Bioluminescent imaging/ SPECT-CT	Ectopic xenograft (metastasis) MeWo (melanoma) MDA-MB-231 (breast cancer)	Bhang <i>et al</i> <sup>[18]</sup>
Telomerase reverse transcriptase (TERT)	<i>hNIS</i> (plasmid)	SPECT-CT	Ectopic xenograft Hep3B (hepatoma)	Kim <i>et al</i> <sup>[19]</sup>
	<i>GFP</i> (lentivirus)	Fluorescence imaging	Ectopic xenograft HepG2 (hepatoma) SGC-7901 (gastric cancer) SW480 (colon cancer)	Yu <i>et al</i> <sup>[20]</sup>

fLuc: Firefly luciferase; GFP: Green fluorescent protein; hNIS: Human sodium iodide symporter; HSV1-TK: Herpes simplex virus type 1-thymidine kinase; PET: Positron emission tomography; SPECT-CT: Single-photon emission computed tomography-computed tomography.

**Table 2 Experimental hepatocellular carcinoma animal models**

Category	Inducing factor	Latency	Advantages	Disadvantages
Xenograft Ectopic Orthotopic	Cancer cell line	Several weeks	Fast and easy modeling Easy detection of tumorigenesis	Less clinical relevancy Only reflect the characteristics of the selected cells
Carcinogen-induced Genetically engineered	DEN	5-10 mo	More clinical relevancy	Lengthy time and high cost to model
	HBV-derived	12-24 mo	Uncovering the molecular mechanisms of hepatocarcinogenesis	Difficult to detect tumorigenesis
	HCV-derived	12-24 mo	Closely mimic the pathophysiological features of human HCC	
	Oncogene-derived	Several weeks		

DEN: Diethylnitrosamine; HBV: Hepatitis B virus; HCC: Hepatocellular carcinoma; HCV: Hepatitis C virus.

athymic or severe combined immunodeficiency (SCID) strains. Xenograft models can be generated ectopically, by subcutaneous injection of HCC cells into extrahepatic locations such as the flank or thigh, or orthotopically, by direct injection into the liver; the latter approach more accurately reflects the *in vivo* tumor environment<sup>[23]</sup>. Xenograft models are considered an easy, rapid and efficient means by which to demonstrate proofs of concept when appropriate cell lines are selected<sup>[24]</sup>. However, this model has less clinical relevancy than autochthonous HCC models (which are beyond the scope of this review and not discussed herein).

### Carcinogen-induced HCC models

Carcinogens are subdivided into two classes, namely

the genotoxic and non-genotoxic (or epigenetic) types. Genotoxic carcinogens irreversibly damage DNA, leading to genetic alterations and interference with normal biological processes; these types of carcinogens comprise chemical or non-chemical agents, such as radiation (ultraviolet or ionizing) and viruses. Non-genotoxic carcinogens do not directly adduct DNA structure, but alter cellular metabolism and promote uncontrolled malignant division. In general, the effect of carcinogens is insidious because they may not be immediately toxic<sup>[23,25-27]</sup>. Carcinogen-induced HCC mouse models are principally used for evaluation of human hepatocarcinogenesis, and these studies must take into consideration that HCC in both human and mouse can be affected by additional contributing

factors - *e.g.*, sex, age and genetic background<sup>[28]</sup>.

Several chemical carcinogens can induce HCC formation to establish the mouse model system; the most commonly used are diethylnitrosamine (DEN)<sup>[29]</sup>, aflatoxin<sup>[30]</sup>, thioacetamide<sup>[31]</sup>, and carbon tetrachloride<sup>[32]</sup>. Among these, DEN is the preferred agent, since the other chemical carcinogens have human toxicity and yield low tumor incidence and delayed carcinogenesis<sup>[23]</sup>. The carcinogenic property of DEN arises from its alkylation of cellular DNA<sup>[33]</sup> and generation of reactive oxygen species<sup>[34]</sup>. DEN is generally administered to the mice as a single dose between postnatal days 12-15. The single-dose DEN administration to generate HCC must consider the dosage applied as well as the sex and age of the animal<sup>[35-37]</sup>. Research groups using the single-dose protocol that was originally described by Vesselinovich and Mihailovich have reported that intraperitoneal injection of DEN results in generation of HCC in 100% of B6C3F1 male mice after 44 wk<sup>[38-40]</sup>. In addition, the role of androgen receptor (AR) status has been demonstrated as important in DEN-induced murine hepatocarcinogenesis<sup>[41]</sup>.

### Genetically engineered HCC models

As a consequence of the known significant differences between mouse and human carcinogenesis, carcinogen-induced HCC mouse models are not appropriate for determining the molecular mechanisms of human hepatocarcinogenesis. In contrast, genetically engineered mouse (GEM) models closely mimic the pathophysiological and molecular features of human HCC<sup>[42]</sup>. GEM modeling, then, is more feasible for studies to understand the complexities of human diseases, such as HCC, and for assessment of the molecular mechanisms of tumor generation, progression and maintenance in particular<sup>[43]</sup>. GEM models have already been successfully used in investigations of specific genes and their interactions with other genes<sup>[28]</sup>. Numerous GEMs have been developed to study liver tumorigenesis in mice, with the most frequently used ones involving overexpression (transgenic mice) or deletion (knockout mice) of a specific gene<sup>[44]</sup>. Moreover, these GEM models have been developed by various techniques, including pronuclear injection (additive transgenesis), homologous recombination (targeted transgenesis using embryonic stem cell technology), and RNA interference (to generate knockdown mice)<sup>[45]</sup>.

More than 80% of HCCs in humans are attributable to infection with the hepatitis B virus (HBV) and/or the hepatitis C virus<sup>[46]</sup>. The genome of HBV is characterized by four overlapping open reading frames, which encode surface, core, polymerase and X (HBx) proteins. HBx transgenic mice are more sensitive to a single DEN-injection than their non-transgenic counterparts<sup>[47,48]</sup>. In general, mutation or overexpression of the *Myc* gene is associated with

tumorigenesis, including that of HCC. A mouse model with overexpression of the human transforming growth factor- $\alpha$  (TGF- $\alpha$ ) under control of the methallothionein (MT) 1 promoter develops HCC. Concordantly, it has been reported that *Myc*- and TGF- $\alpha$  over-expressing transgenic mice are genetically close to human HCC and that the overexpression profile is related with prognosis<sup>[49]</sup>. In transgenic mice with over-expression of epidermal growth factor (EGF) under the control of the albumin promoter, the overexpression of secreted EGF leads to generation of multiple highly malignant hepatic tumors<sup>[50]</sup>. In addition, other DNA viruses, such as simian vacuolating virus 40 (SV40), have the potential to cause tumors<sup>[51]</sup>.

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## AFP-TARGETED REPORTER GENE EXPRESSION IMAGING IN HCC MOUSE MODELS

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AFP is a biomarker of HCC, and much progress has been made in our understanding of the mechanistic underpinnings of AFP expression in HCC. Because the *AFP* gene becomes re-expressed in HCC, tumorigenesis can be monitored using reporter gene expression imaging. However, few AFP-targeted reporter gene-imaging studies have been published; those studies on reporter gene imaging driven by AFP enhancer/promoter in HCC mouse models are presented in Table 3<sup>[52-60]</sup>.

### Xenograft HCC models

Xenograft tumor models are preferred for use in cancer research because of their ease of tumor establishment. Several studies of AFP-targeted imaging or therapeutic effects in xenograft ectopic HCC mouse models established by the ectopic approach have been reported which carried out assessment via optical or nuclear medicine imaging modalities. A reporter gene under the control of the AFP enhancer/promoter can be delivered into cells using a plasmid vector (cell transfection) or adenoviral vector (systemic or intratumoral administration) system.

Jin *et al.*<sup>[52]</sup> and Willhauck *et al.*<sup>[53]</sup> used a plasmid system under the control of the AFP enhancer/promoter to achieve stable *NIS* gene expression in an HCC cell line. The AFP-targeted *NIS* gene expression system functioned well *in vitro* and *in vivo*, indicating the feasibility of HCC-specific reporter gene expression systems for diagnosis and therapy of AFP-positive HCC. However, this plasmid system proved to be limited in terms of its ability to deliver the reporter gene to the target region *in vivo* due to use of a plasmid-transfected HCC cell line. In other studies, an adenoviral vector system using the AFP enhancer/promoter was injected intratumorally in a xenograft HCC model established by the ectopic approach<sup>[54,55]</sup>. Those results indicated the potential of *in vivo* AFP-

**Table 3** Reporter gene expression imaging by alpha-fetoprotein enhancer/promoter system

Tumor model	Mouse strain	Induction of HCC	Reporter gene	Delivery system	Injection route	Ref.
Xenograft	NOD/SCID	HuH-7 cells	<i>hNIS</i>	Adenovirus <sup>1</sup>	IV	Kim <i>et al.</i> <sup>[56]</sup>
	BALB/c nude		<i>TK/fLuc</i>	Adenovirus <sup>1</sup>	IT	Park <i>et al.</i> <sup>[57]</sup>
	NOD/SCID		<i>fLuc</i>	Adenovirus <sup>1</sup>	IV	Kim <i>et al.</i> <sup>[58]</sup>
	BALB/c nude	HepG2 cells	<i>hNIS</i>	Plasmid <sup>2</sup>	-	Jin <i>et al.</i> <sup>[52]</sup>
	CD-1 nude		<i>hNIS</i>	Adenovirus <sup>1</sup>	IT	Klutzb <i>et al.</i> <sup>[54]</sup>
	BALB/c nude		<i>hNIS</i>	Adenovirus <sup>1</sup>	IT	Ma <i>et al.</i> <sup>[55]</sup>
	NMRI nude		<i>hNIS</i>	Plasmid <sup>2</sup>	-	Willhauck <i>et al.</i> <sup>[53]</sup>
Carcinogen-induced	C57BL/6	DEN	<i>fLuc</i>	Adenovirus <sup>1</sup>	IV	Kim <i>et al.</i> <sup>[58]</sup>
	FVB/N	DEN	<i>TK/fLuc</i>	Transgenesis <sup>3</sup>	-	Lu <i>et al.</i> <sup>[59]</sup>
	C57BL/6	DEN	<i>fLuc</i>	Transgenesis <sup>3</sup>	-	Park <i>et al.</i> <sup>[60]</sup>

<sup>1</sup>Adenovirus: *in vivo* delivery of reporter gene; <sup>2</sup>Plasmid: use of reporter gene transfected cells; <sup>3</sup>Transgenesis: use of transgenic reporter mice. DEN: Diethylnitrosamine; fLuc: Firefly luciferase; hNIS: Human sodium iodide symporter; IT: Intratumoral injection; IV: Intravenous injection; NOD/SCID: Non-obese diabetic/severe combined immunodeficiency; TK: Thymidine kinase; HCC: Hepatocellular carcinoma.

targeted imaging and a radiotherapeutic approach in HCC.

Kim *et al.*<sup>[56]</sup> and Park *et al.*<sup>[57]</sup> reported *in vivo* systemic delivery of an adenoviral vector system with a reporter gene (NIS, fLuc or HSV1-tk) driven by the AFP enhancer/promoter. Those studies showed that targeted imaging and therapy through systemic delivery of adenoviral vector was possible. Also, Kim *et al.*<sup>[58]</sup> introduced AFP-targeted bioluminescent imaging performed using adenovirus in xenograft and carcinogen-induced HCC tumor models. Thus, the adenovirus system is capable of facilitating AFP-targeted imaging and therapy in carcinogen-induced HCC as well as in xenograft tumor models.

### Carcinogen-induced HCC models

Generally, carcinogen-induced HCC animal models can be established upon exposure of genetically susceptible mice to a variety of chemical carcinogens, such as DEN. Induction of HCC using carcinogens is more difficult than by the xenograft approach because of the longer generation time and greater difficulty of verification of AFP expression.

Lu *et al.*<sup>[59]</sup> reported that a hepatocarcinogenesis reporter (HCR) transgenic mouse model enables monitoring of tumorigenesis by bioluminescent and nuclear medicine imaging. In their HCR mouse model, the HSV1-tk and fLuc genes were concurrently expressed under the control of the AFP enhancer/promoter. The bioluminescent signal was then detected during the early stage of DEN-induced HCC, prior to neoplastic transformation. Detection at later stages revealed high expression of fLuc and HSV1-tk. In another study, Park *et al.*<sup>[60]</sup> demonstrated non-invasive monitoring of AFP expression and DEN-induced hepatocarcinogenesis using bioluminescent imaging in transgenic mice with the fLuc gene under the control of the AFP enhancer/promoter. These two transgenic mouse models enabled *in vivo* monitoring of AFP expression throughout the entire disease course and lifetime of the afflicted animal. These studies suggested the usefulness of AFP-targeted reporter

gene expression imaging for non-invasive *in vivo* evaluation of hepatocarcinogenesis.

## CONCLUSION

We have presented here the various experimental HCC animal models, including xenograft, carcinogen-induced and genetically engineered HCC models, and discussed their characteristics. Non-invasive real-time *in vivo* molecular imaging of reporter gene expression under the control of tumor-specific enhancers/promoters can provide longitudinal information about carcinogenesis and tumor progression. We expect that AFP-targeted reporter gene expression imaging systems will be applied for the detection of AFP-expressing HCC tumors and screening of increased/decreased AFP levels due to disease or drug treatment.

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