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Induction of rat liver tumor using the Sleeping Beauty transposon and electroporation

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ABSTRACT

The Sleeping Beauty (SB) transposon system has been receiving much attention as a gene transfer method of choice since it allows permanent gene expression after insertion into the host chromosome. However, low transposition frequency in higher eukaryotes limits its use in commonly-used mammalian species. Researchers have therefore attempted to modify gene delivery and expression to overcome this limitation. In mouse liver, tumor induction using SB introduced by the hydrodynamic method has been successfully accomplished. Liver tumor in rat models using SB could also be of great use; however, dose of DNA, injection volume, rate of injection and achieving back pressure limit the use of the hydrodynamics-based gene delivery. In the present study, we combined the electroporation, a relatively simple and easy gene delivery method, with the SB transposon system and as a result successfully induced tumor in rat liver by directly injecting the *c-Myc*, *HRAS* and *shp53* genes. The tumor phenotype was determined as a sarcomatoid carcinoma. To our knowledge, this is the first demonstration of induction of tumor in the rat liver using the electroporation-enhanced SB transposon system.

1. Introduction

Genetically-modified laboratory animals are of great importance in biomedical research. The Sleeping Beauty (SB) transposon system, an insertional mutagenesis system consisting of a transposon and SB transposase, represents an important tool for genetic studies [1]. A transposon is a DNA mobile element, flanked by inverted repeat sequences, that encodes transposase, which catalyzes mobilization and reintegration of the transposon into genomic DNA [2,3]. Integration of a transposon modified to encode a transgene provides prolonged expression of the transgene, thus allowing long term *in vivo* expression. This makes the SB transposon system a particularly attractive candidate for the development of molecularly-defined tumorigenesis models [4]. As rats are larger than mice in size, they are useful for some biomedical research in terms of surgical procedures, larger volume of blood collection and frequent parentral injection. Although hydrodynamics-based procedures are effective methods, which has been adapted to rats [5], several factors such as dose of DNA, injection volume, rate of injection and achieving back pressure limit their use when applying the hydrodynamic delivery method to larger animal species [6–9]. Combination of direct injection of SB transposon gene and electroporation enhanced transfection could provide sustained gene expression.

The present study demonstrated the delivery into rats of *c-Myc*, *HRAS* and *shp53* genes via the SB transposon system combined with electroporation. After introducing the oncogenes and shRNA against *p53*, we observed the development of tumors in the liver, which was then verified by phenotype analysis. To our knowledge, this is the first demonstration of the SB-based tumorigenesis in rat liver other than hydrodynamic method, which was enabled by the use of electroporation.

2. Materials and methods

2.1. Animals

Ten Sprague–Dawley rats (five males, five females; aged 7 weeks) were obtained from Orient Bio (Yongin, Korea). The rats

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were housed at the laboratory animal facility in the Asan Institute for Life Sciences under specific pathogen-free conditions in accordance with the guidelines of the Animal Care and Use Committee of Asan Institute for Life Sciences.

2.2. Plasmid construction

Plasmids encoding SB transposase (pPGK/SB13) and transposon vectors (PT2/BH) containing multiple cloning sites between two indirect repeat sequences (IR/DRs) were generous gifts from Drs. David Largaespada and Perry Hackett (University of Minnesota). cDNA encoding *c-Myc* or *HRAS* was inserted into the pCXEGFP, which was kindly provided by Dr. Masaru Okabe (Osaka University, Japan). The whole transcriptional cassettes were then cloned into PT2/BH. The PT2/shp53/GFP4 transposon plasmid encoding a short hairpin RNA against the tumor suppressor *p53* was a gift from Dr. John Ohlfest (University of Minnesota). DNA used for injection was prepared using EndoFree Plasmid Maxi kit (Qiagen).

2.3. Injection of plasmid DNA

Each animal was given a mixture of three types of transposon and the plasmid encoding the transposase, as described above. The molar ratio of transposase-encoding plasmids to transposon plasmids was 1:2. At first, three types of transposon were mixed in equal amounts, making up a total of 50 μg. Then, 50 μl of phosphate-buffered saline containing the transposase-encoding plasmids were added to the mixture. The mixture was loaded into an insulin syringe (31 G) and injected directly into the liver. The approximate lengths of the transposon and the transposase gene were 7000 kb and 5000 kb, respectively.

2.4. In vivo electroporation

Electroporation was conducted on the rats following the injection of DNA plasmid. The skin was shaved to expose a flat plain. A 3 cm incision was made on the ventral surface just above the liver. The injection site was electroporated using Cellectra (VGX International Inc. [Seoul, Korea]/Inovio [Blue Bell, PA, USA]) containing three needle probes at 0.2 A for 4 s (three pulses, pulse duration: 52 ms/pulse, interval separating pulses: 1 s) in accordance with the manufacturer's guidelines. The incision was closed with a subcuticular suture.

2.5. Animal PET Imaging

Decay-corrected radiochemical yields ranged from 60% to 70%, and after HPLC purification, the radiochemical purity was 98 ± 1.2% (mean ± SD). The specific activity of the [^{18}F]Flu-deoxyglucose (FDG) obtained was greater than 100 TBq/mmol. PET scans were performed using a microPET Focus 120 system (microPET Concorde Microsystems, Inc.) with resolutions of 1.18 mm (radial), 1.13 mm (tangential) and 1.44 mm (axial) to the center of the field of view. Each rat was injected with 7.4 MBq (0.2 mCi) and 37 MBq (1 mCi) [^{18}F]FDG into the tail vein, and 10 min static PET scans were obtained. Each rat was under isoflurane anesthesia during the uptake and scanning periods. Heating tools were used to maintain body temperature at approximately 37 °C. PET images were reconstructed by OSEM2D using a cut-off frequency of 0.5 cycles per pixel. No attenuation correction was applied.

2.6. Necropsy, histology and immunohistochemistry

The rats were humanely euthanized and subjected to necropsy 35 days after injection. Tumors were excised from surrounding circumferential tissue for histopathological examination. After macroscopic examination, the removed tissues were fixed in 10% neutral buffered formalin. The specimens were then embedded in paraffin blocks and cut into 3 μm thick sections. Staining was done with haematoxylin and eosin (H&E). Immunohistochemical markers (Table 1) used to subtype the tumor were as follows: CD45 (1:2,000), CD163 (1:2000) and CD68 (1:4000) for histiocytic sarcoma; desmin (1:1000) and myogenin (1:100) for pleomorphic rhabdomyosarcoma; HMB45 (1:1000) and S100 (1:400) for malignant melanoma; α-smooth muscle actin (α-SMA; 1:400) for leiomyosarcoma; pan-cytokeratin (1:3000), cytokeratin 20 (1:200) and cytokeratin 7 (1:4000) for undifferentiated carcinoma; and cyclin-dependent kinase 4 (CDK4; 1:1000) and murine double minute 2 (MDM2; 1:1000) for pleomorphic liposarcoma.

2.7. Detection of gene expression using quantitative real-time RT-PCR (RT-qPCR)

Expression of the transferred oncogenes (*c-Myc*, *HRAS*) and *p53* were analyzed by RT-qPCR. Samples were obtained from the liver tumor tissue and normal liver tissue. The Paradise Whole Transcript RT Reagent System (Arcturus, CA, USA) was used for RNA isolation and reverse transcription of the samples. All PCR reactions were performed in a Lightcycler 2.0 (Roche Applied Science) according to standard procedures [10]. The primers (all from Roche Diagnostics, Mannheim, Germany) used for identification of the

Table 1
Immunohistochemical profile of tumor developed in the liver.

Tissue marker	IHC antibody	Reactivity	Dilution	Type
Epithelial tissue marker	Pan-cytokeratin	+	1:1000	M
	Cytokeratin 7	–	1:1000	P
	Cytokeratin 20	–	1:1000	M
Muscular tissue marker	Myogenin	–	1:1000	M
	Desmin	–	1:4000	M
	α-smooth muscle actin	–	1:1000	M
Hematopoietic cell marker	CD45	–	1:2000	M
	CD163	–	1:2000	M
	CD68	–	1:2000	M
Melanoma marker	Melanoma	–	1:100	M
	S100	–	1:400	M
Adipose tissue marker	CDK4	–	1:1000	M
	MDM2	–	1:1000	M

M, monoclonal; P, polyclonal; + = positive; – = negative.
All antibodies were purchased from Abcam (Cambridge, England); see text for more information.

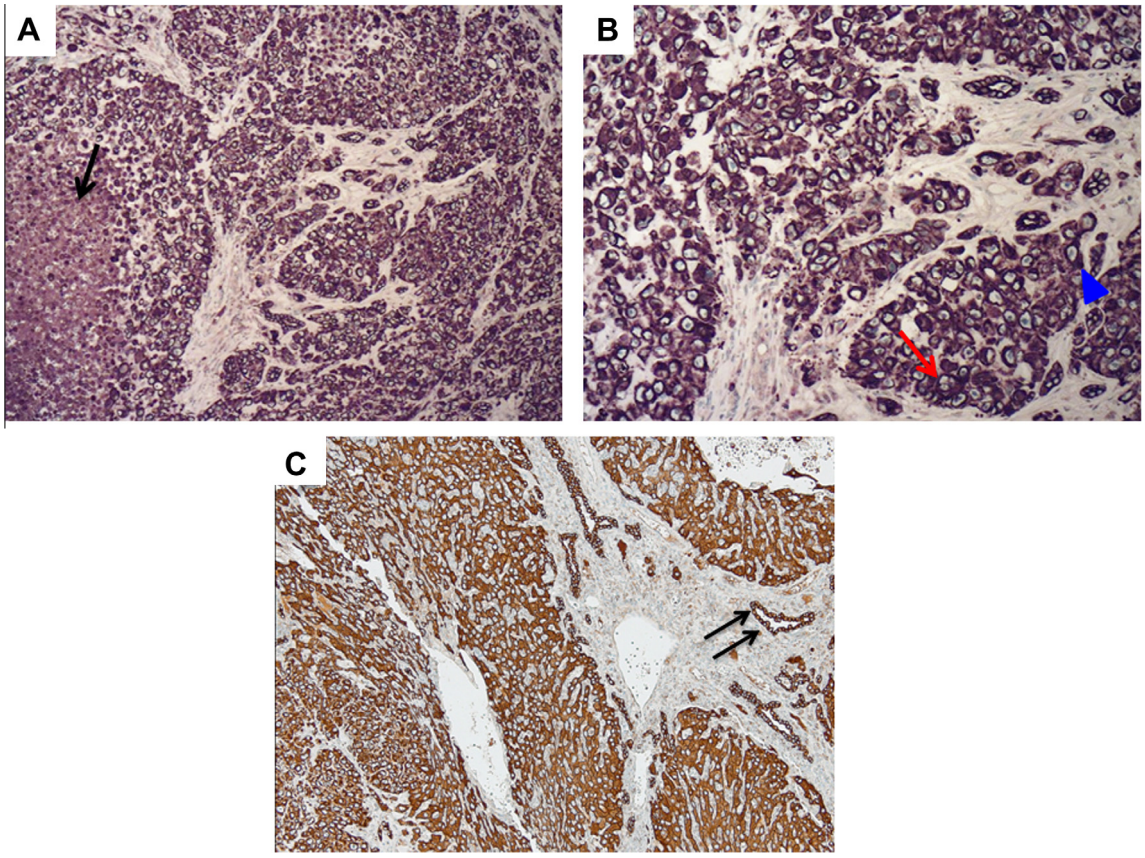

Fig. 1. Microscopic image of liver tumor tissue (A) Induced liver tumor (×100). Necrotic areas were observed in the central part of the tumor (arrow). (B) Tumors were consisted of highly pleomorphic and undifferentiated cells. Some of the tumor cells resembled epithelial cells whereas others appeared to be of mesenchymal cell origin. The poorly differentiated cells had slightly basophilic cytoplasm (blue arrowhead). Multinucleated giant cells, round to oval in shape, were occasionally visible. Bizarre cells with large and pale cytoplasm and with prominent nuclei were also observed (red arrow) (×200). (C) The only positive immunoreactivity the tumors exhibited was pan-cytokeratin, suggesting the epithelial origin of the tumor (×100). See negatively stained normal hepatocytes in the middle and positively stained normal bile duct epithelium (arrows).

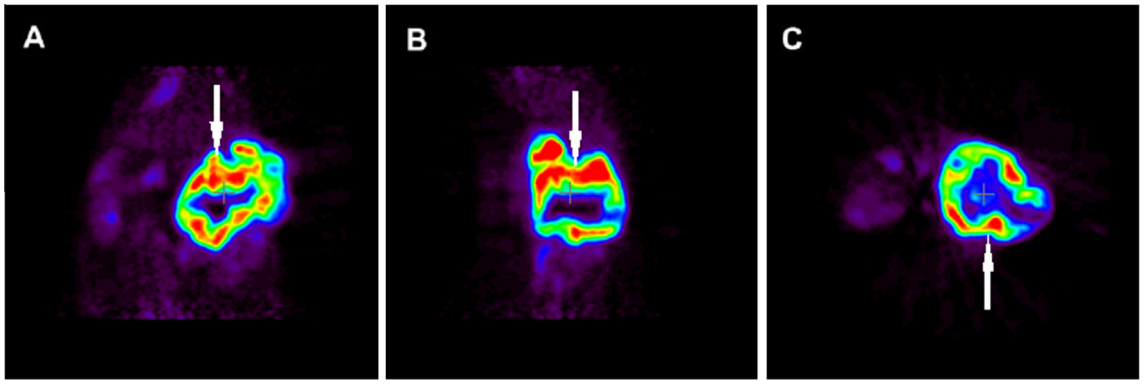

Fig. 2. PET imaging of induced tumors. Neoplasms were evident in coronal (A), sagital (B) and transverse (C) dimensions of the PET-CT images of the injection sites. Tumors are indicated by red-pinkish-color (arrows).

transcript levels were as follows; *c-Myc* (forward: TCC TGT ACC TCG TCC GAT TC, reverse: GGA GGA CAG CAG CGA GTC); *HRAS* (forward: GGA CGA ATA CGA CCC CAC TAT, reverse: TGT CCA ACA GGC ACG TCT C); *p53* (forward: TAA AGG ATG CCC GTG CTG, reverse: TCT TGG TCT TCG GGT AGC TG); the probes #12, #38 and #94 were individually from the Universal Probe Library (Roche Applied Science). Rat beta-actin (ACTB) gene (forward: CCC GCG AGT ACA ACC TTC T, reverse: CGT CAT CCA TGG CGA ACT) and Taqman probe (TIB MOLBIOL, Berlin, Germany) was used as a 'reference gene' to normalize gene expression. For reproducibility, experiments were repeated three times.

3. Results and discussion

3.1. Tumor observation

Plasmids containing SB transposase, a transposon vector, two oncogenes (*c-Myc* and *HRAS*) and shRNA against *p53* gene, were

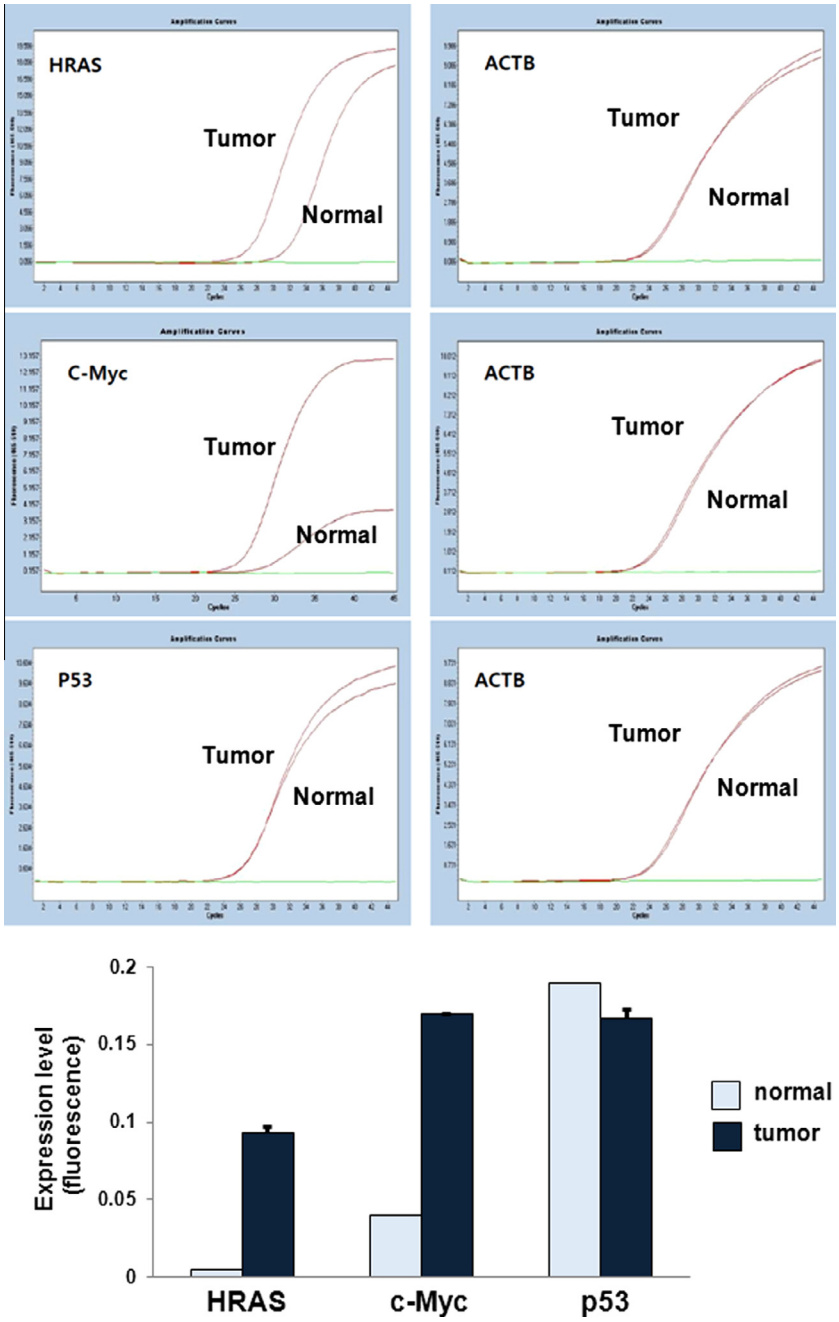

Fig. 3. Analysis of gene expression by RT-qPCR. Expression of the transferred genes, *c-Myc*, *HRAS*, and *p53*, were analyzed by RT-qPCR as described in Section 2. Upper panels show the representative RT-qPCR results (each gene plus beta-actin as a control) and lower panel shows the relative expression of the transferred genes in tumor and normal liver tissues (expressed as fluorescence level). Data shown in the bar graph represent average ± standard deviation (STDEV) obtained from three independent experiments.

directly injected into the liver of rats. Electroporation was then applied to the injection sites. Though the rats were non-transgenic, three of the five male recipients (3/5) and two of the five female recipients (2/5) subjected to the injection with transposons and the SB transposase followed by electroporation developed single or coupled and nodular neoplasms. The tumors were only positive to pan-cytokeratin and the phenotype was of a sarcomatoid carcinoma (Table 1).

3.2. Gross and microscopic findings

Microscopic observation revealed a well-marginated, ovoid nodule positioned in the liver where the DNA plasmids were injected. No metastatic foci were observed in non-injected sites. The tumor showed both epithelial and mesenchymal components with high cellularity, abundant mitosis, and apoptosis (Fig. 1A and B). Tumor cells were undifferentiated and showed pleomorphic features. The cells were round to oval, with pale basophilic cytoplasm, and hyperchromatic nuclei with prominent nucleoli (Fig. 1B). Multinucleated giant cells were occasionally visible. Necrotic areas were also seen in the central part of the tumor (Fig. 1A). All tumors had similar morphological features. In immunohistochemical analysis (Table 1 and Fig. 1C), only the pan-cytokeratin showed positivity and the other markers were totally negative, except in tumor associated stroma, suggesting the epithelial origin of the tumor. Consequently, we diagnosed this tumor

as sarcomatoid carcinoma. The present results provide a plausible method for the development of laboratory tumor models. The SB transposon system enables the production of tumor models with genetic complexity, involving a number of different mutations that can cause various histological subtypes, which is difficult in the present mouse model systems [11–15].

3.3. Animal PET imaging

Neoplasms were clearly observed in all three different dimensions – coronal, sagittal and transverse – on PET-CT images of the injection sites. The tumors indicated by red-pinkish color were apparently visible (Fig. 2).

3.4. Immunohistochemical findings

As shown in Table 1 and Fig. 1C, only pan-cytokeratin was positively expressed by the tumor cells and other markers were completely negative. These findings suggest that the tumor is of epithelial origin. We thus diagnosed the tumor as a sarcomatoid carcinoma.

3.5. Confirmation of gene expression using RT-qPCR

Real-time qPCR confirmed successful expression of the transferred genes, *c-Myc* and *HRAS* (Fig. 3). Expression of *HRAS* was increased nearly 19-fold in the tumor tissue compared to normal liver tissue and that of *c-Myc* was increased more than 4-fold in the tumor tissue. By contrast, expression level of *p53* was consistently reduced in the tumor tissues when compared to normal tissues.

The electroporation-enhanced SB transposon method can produce tumors at desired anatomical locations and in the desired species. Using this method, tumors can be produced in the lung, mammary gland, prostate and pancreas, among others; locations where cancers have not yet been generated by using the SB transposon system [16]. In addition, utilizing this method, it would be possible to cause tumorigenesis in other large-size laboratory animals, which can be of great advantage. An interesting characteristic of the tumors produced in this study was that no metastasis was found, even though highly malignant features were evident upon microscopic analysis. By contrast, other studies utilizing the SB transposon method experienced metastasis [3,17]. The electroporation-enhanced SB transposon method can produce diverse gene mutations embodied in a tumor, similar to human cancer, by multiple gene combinations. In conclusion, the present results implicate that the electroporation-enhanced SB transposon system can be useful for production of tumors in genes and sites of desired animals.

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