Research Article

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Serum-based tracking of *de novo* initiated liver cancer progression reveals early immunoregulation and response to therapy

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Abstract

Background & Aims: Liver inflammatory diseases associated with cancer promoting somatic oncogene mutations are increasing in frequency. Preclinical cancer models that allow for the study of early tumor progression are often protracted, which limits the experimental study parameters due to time and expense. Here we report a robust inexpensive approach using *Sleeping Beauty* transposition (SBT) delivery of oncogenes along with Gaussia Luciferase expression vector GLuc, to assess *de novo* liver tumor progression, as well as the detection of innate immune responses or responses induced by therapeutic intervention.

Methods: Tracking *de novo* liver tumor progression with GLuc was demonstrated in models of hepatocellular carcinoma (HCC) or adenoma (HCA) initiated by hydrodynamic delivery of SBT oncogenes.

Results: Rising serum luciferase levels correlated directly with increasing liver tumor burden and eventual morbidity. Early detection of hepatocyte apoptosis from mice with MET+CAT transfected hepatocytes was associated with a transient delay in HCC growth mediated by a $CD8^+$ T-cell response against transformed hepatocytes. Furthermore, mice that lack B cells or macrophages had an increase in $TUNEL^+$ hepatocytes following liver MET transfection demonstrating that these cells provide protection from MET-induced hepatocyte apoptosis. Treatment with IL-18+IL-12 of mice displaying established HCC decreased tumor burden which was associated with decreased levels of serum luciferase.

Conclusions: Hydrodynamic delivery of the SBT vector GLuc to hepatocytes serves as a simple blood-based approach for real-time tracking of pathologically distinct types of liver cancer. This revealed tumor-induced immunologic responses and was beneficial in monitoring the efficacy of therapeutic interventions. Published by Elsevier B.V. on behalf of the European Association for the Study of the Liver.

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Abbreviations: SBT, *Sleeping Beauty* Transposition; GLuc, Gaussia Luciferase expression SBT vector; HCC, hepatocellular carcinoma; HCA, hepatocellular adenoma; $GLuc^+$ hepatocytes, GLuc vector integrated in hepatocytes; HSB2, *Sleeping Beauty* transposase; MET, human c-Met; PT3, empty SBT vector; CAT, Δ90Nβ-catenin; AKT, Myr-AKT; ConA, concanavalin A; WT, wild-type.

Introduction

Up to 70–90% of all liver cancers are detected in patients suffering from chronic inflammatory liver diseases due to obesity, chronic hepatitis C and liver fluke infections that increase the frequency of deleterious somatic mutations in oncogenes and tumor suppressor genes [1–9]. Because liver inflammatory diseases due to obesity [10] and hepatitis C infections [11] have a globally increasing frequency, it is important to understand which somatic mutations in oncogenes drive liver cancer development and/or progression and how inflammation facilitates this process.

The advent of *Sleeping Beauty* transposition (SBT) technology has provided experimental tools to investigate how mutations of oncogenes lead to the development of liver cancer [12,13]. This non-viral-based gene transfection system can be used to induce liver tumors by stably integrating oncogenes into hepatocytes. The induction of tumors using the SBT system allows the analysis of how inflammation and tumor immunity regulate the entire spectrum of tumor growth including the early stages of neoplasia, which cannot be addressed using transplantable tumors. Although carcinogens such as diethylnitrosamine (DEN) also allow us to study liver tumor formation, butl often induces a myriad of undefined mutations and off target events, while the SBT system can be best used to study how specific oncogenes collaborate in the formation of tumors.

Mouse cancer models using SBT, carcinogens and transgenic mice are amenable for the study of tumor formation at the early stages of neoplasia but are limited by the variable and often protracted time for development/progression of tumors, depending on the oncogene combinations used for initiation. *In vivo* imaging could be used to follow tumor progression but this approach can be prohibitively expensive, limited by the number of mice that

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can be scanned and requires dedicated staff to run complex instrumentation. Thus, a tumor-tracking method that could be used sequentially in individual mice over a long period of time would allow an assessment of tumor status in real-time. Such an approach may reveal therapeutic windows, be used to extrapolate tumor burden and survival times and potentially elucidate immunoregulatory events.

In this study, we investigated the use of the SBT GLuc vector for tracking nonlinear oncogene-induced liver tumor progression. Using two distinct *de novo* liver tumor models, we demonstrated that the level of serum luciferase released by GLuc vector integrated in hepatocytes (GLuc$^+$ hepatocytes) significantly correlated with the volume of liver tumors, thereby providing a minimally-invasive method to track in living mice the growth of *de novo*-generated liver tumors. Using this minimally-invasive method, parameters can be measured that would otherwise require euthanasia of mouse cohorts with an associated higher experimental cost. The evaluation of immunogenic *de novo* liver tumors using GLuc$^+$ hepatocytes also allowed us to identify inflection points that revealed CD8$^+$ T-cell mediated transient tumor inhibitory effects during tumor progression. Concordantly, we show for the first time that stable expression of luciferase by hepatocytes can also be used to efficiently track, in real-time, the outcome of therapies against oncogene-driven *de novo* liver tumors.

Materials and methods

Plasmid constructs

Plasmids encoding the *Sleeping Beauty* transposase (HSB2) and transposons containing human c-Met (MET) and empty vector control (PT3), were a generous gift from Dr. Xin Chen (UCSF, San Francisco, Ca.). Transposon constructs containing Δ90Nβ-catenin (CAT) and Myr-AKT (AKT) were previously reported [14]. The gene for Gaussia Luciferase was PCR-amplified and cloned into pENTR (Invitrogen) and subsequently Gateway-cloned into the PT3 destination vector to produce a Gaussia luciferase transposon (GLuc) vector.

Experimental model

C57/BL6J (wild-type [WT]), B6.129S2-Cd8a (CD8$^{-/-}$), B6.129S2-Cd4/J (CD4$^{-/-}$), B6.129S2-lgh-6 (Bcell$^{-/-}$) and CD1d$^{-/-}$ (NKT$^{-/-}$) mice were purchased from The Jackson Laboratory and were bred and maintained at the Frederick National Laboratory in a specific pathogen free facility in accordance with an approved Animal Care and Use Protocol. Oncogene constructs were delivered to hepatocytes using the hydrodynamic transfection technique. Briefly, constructs containing either MET+CAT or AKT+CAT along with HSB and GLuc vector were mixed in saline at a volume of 10% vol/mouse weight and injected via tail vein into aged and sex matched 8–12 week old C57/BL6J or B6.129S2-Cd8a mice over 5–7 seconds as previously described [12]. MET+CAT induces liver tumors with a pathology consistent with hepatocellular carcinoma (HCC) while AKT+CAT induces liver tumors histopathologically characterized as hepatocellular adenoma (HCA) [14].

Immunohistochemistry (IHC) staining and analysis

Liver tissues were fixed with 10% normal buffered formalin overnight and then transferred to 70% ethanol. Paraffin blocks were made from the fixed tissues. Slides cut from the blocks were analyzed for apoptosis using TUNEL staining, proliferation using BrdU at 1:50 (Invitrogen Life Technologies), β-catenin at 1:200 (Abcam) and N-ras (F155 Santa Cruz). For IHC slide analysis, five non-overlapping pictures were taken from at least two separate lobes and images were analyzed using CellProfiler (http://www.cellprofiler.org/) with modified Ki67 pipeline.

Immunotherapeutic regimen

Recombinant murine IL-12 and IL-18 was purchased from PeproTech Inc. (Rocky Hill, NJ). Stock aliquots of cytokines were diluted with HBSS and mice were injected intraperitoneally with vehicle control (VC) or IL-12 (0.3 μg) + IL-18 (0.3 μg) on days 20–24 and 28–31 post-initiation of liver tumors with MET+CAT oncogenes.

qPCR quantitation of oncogenes in the liver

Livers were harvested and snap frozen on a dry ice and 2-Methylbutane mixture for later manipulations. Frozen livers were mechanically shattered into small fragments and genomic DNA was isolated using a Gentra Puregene Tissue Kit (Qiagen) according to manufactures instructions. Oncogene copy number per 100 ng of liver DNA was determined using qPCR analysis with TaqMan probes MET (Hs 01564484_m1), CTNNB1 (CAT) (Hs 00355049_m1), and AKT (mm 01331626_m1) (ABI) and comparing it to a standard curve made using the oncogene plasmids.

Tumor assessment by magnetic resonance imaging (MRI)

Animal imaging was performed by Frederick National Laboratory Small Animal Imaging Program. MRI was performed with a 3.0T clinical scanner (Philips Intera Achieva, Philips Medical Systems, Einthoven, NL) to detect the onset of tumors, monitor their progression, and track the changes in the liver volume biweekly. Data was acquired using a 40-mm diameter solenoid receiver coil (Philips Research, Hamburg, Germany). The mice were anesthetized in an induction chamber with 3% isoflurane in O_2 at 1 L/min flow rate and then placed in a custom made mouse holder within the imaging coil. During an imaging session, the anesthesia level was modified between 1.5–2.0% isoflurane to maintain a 40 bpm pulmonary rate, and their physiology monitored (Biopac System Inc., Goleta, CA). Body temperature was maintained at 37 °C by supplying warm air (Small Animal Instruments, Inc., Stony Brook, NY) around the mouse holder. Multi slice T2 weighted turbo spin echo (T2w-TSE) sequence was applied in coronal view with respiratory triggering to minimize the motion artifacts. An imaging volume of 36 × 27 × 18 mm was chosen to cover the mouse abdominal cavity. The images were acquired with a repetition time (TR) 4500 ms, echo time (TE) 65 ms, with an in plane resolution of 0.190 × 0.190 mm, and slice thickness 0.5 mm. Serial images obtained in 8 biweekly consecutive imaging sessions were used to calculate the changes in tumor and liver volumes.

Gaussia Luciferase assay

Mouse blood was collected using retro orbital bleeds in a serum separator tube (Becton Dickinson co.) and then serum was recovered by centrifuging tubes for 3 minutes at 20,000 g and then 5 μl of serum was added to 100 μl of PBS and plated on a 96 well flat bottom white polystyrene assay plate (Costar 3362). Serum luciferase levels were determined using the BioLux Gaussia Luciferase Assay kit (New England BioLabs) according to manufacturer's direction and luminescence measurements were acquired using FLUOstar Omega microplate reader (BMG LABTECH) after controlled injection of 50 μl of substrate mixture into plates containing the serum PBS mixture.

Liver leukocyte isolation and flow cytometric analysis

Liver leukocytes were isolated as previously described [15]. Isolated leukocytes were counted and 1×10^6 cells were Fc-blocked with monoclonal 2.4G2 prior to staining with appropriately titered monoclonal antibodies (CD8a PerCP and PD-1 PE; eBioscience) used in conjunction with Live/Dead reagent; Invitrogen). Data was collected using a LSRII Special Order System equipped with solid state blue (488 nm), red (640 nm) and violet (405 nm) lasers and then analyzed using FloJo Vx0.6.

Transfection of mouse hepatoma Hepa1–6 cells

Hepa1–6 cells were maintained in DMEM containing 1.45 g of glucose/liter (Sigma), supplemented with 2 mM glutamine, 50 μg/ml gentamycin sulfate, and 10% (v/v) heat-inactivated fetal calf serum. Targefect-Hepatocyte reagent (Targeting Systems, CA) was used for PT3-GFP or PT3-MET plasmid transfection into Hepa1–6 cells according to the manufacturer's protocol. Cells were harvested