Characterization of Non-Alcoholic Steatohepatitis-derived Hepatocellular Carcinoma as a Human Stratification Model in Mice

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Abstract. The therapeutic strategy against hepatocellular carcinoma (HCC) is determined by tumor stage and liver function. Improvements of stratification contribute to extending the survival of patients. However, stratification has been attributed little attention in animal models largely due to the lack of suitable models. Herein we showed that the recently-reported, non-alcoholic steatohepatitis-derived HCC model (STAM model) is the first murine model in which the concept of human stratification is applicable by demonstrating the following features: (i) at least 4 detectable tumor nodules; (ii) average tumor growth rate of 150 % from 16 to 20 weeks of age; (iii) no visible metastasis; and (iv) relatively preserved liver function. These observations suggested that HCC in STAM mice is equivalent to stages B to C of the Barcelona Clinic Liver Cancer (BCLC) staging system for humans. Application of the stratification concept to experimental animals will create new avenues to establish pharmacological intervention against HCC.

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Hepatocellular carcinoma (HCC) is the most common type of primary liver cancer, the fifth most frequent form of cancer and the third most common cause of death (1). Approximately 500,000 new patients are diagnosed with HCC each year (2). Most cases of HCC have some background of liver damage, such as hepatitis and/or subsequent liver fibrosis (3). The therapeutic strategy against HCC is commonly determined by extra-hepatic manifestations, hepatic spare ability, as judged by the Child-Pugh grade, tumor number, tumor size and vascular infiltration, as judged by the Barcelona Clinic Liver Cancer (BCLC) staging system in actual clinical settings. Improvements of human stratification contribute to extending the survival of patients and to providing insights into the use of anti-HCC drugs, but there are still limited systematic therapeutic options for HCC treatment, thereby underlining the need for new molecular targets. Moreover, distinct from current clinical strategies, little attention has been paid to stratification in animal models largely due to the lack of suitable models. Given the importance of an experiment using animals for the development of anti-HCC therapeutics, stratification should also be considered in animal models. Xenograft models, which have only accommodated tumor size and carcinogen-induced HCC models and also have variable disease onset and poor reproducibility (4), are hard to establish stratification. Distinct clinical effects of sorafenib according to the staging of HCC have been revealed, thus it would be better to consider the staging of HCC in non-clinical studies for molecular-targeted drugs. These concepts have an impact on dose discovery trials and pharmacokinetic analyses of HCC drugs.

Fuji et al. compared STAM model mice, which represent patients who develop HCC among non-alcoholic fatty liver disease (NAFLD)/diabetic populations, to existing NAFLD/diabetes models, which do not show fibrosis/HCC.
(5). Because non-alcoholic steatohepatitis (NASH), which is the most extreme form of NAFLD, is recognized as an evolving major cause of non-viral HCC and may account for a large proportion of HCC in developed countries in recent years (6), investigation of NASH-derived HCC is important in addition to viral hepatitis-derived HCC. They reported the following results: (i) all male mice develop well-differentiated HCC according to histology without exception; (ii) HCC develops in the fibrotic liver but not in the intact liver; and (iii) dynamic computed tomography (CT) indicates that hypervascularity is present in the arterial phase and that washout occurs in the delayed phase. Thus, the NASH-derived HCC model (STAM model) fulfills the human criteria of clinical diagnosis. However, extra-hepatic manifestations, hepatic-spare ability, tumor number and staging system were not investigated in detail.

To investigate if the staging system can be applied to the animal model, we performed serological, histological and multiphase dynamic-enhanced CT evaluations in the present study. The results suggested that HCC in STAM mice is equivalent to stages C or, to a lesser extent, B of the BCLC staging system in humans. Engagement of animal experimentation from the perspective of clinical HCC will facilitate the development of new and improved therapeutics that are directly connected to human HCC, which may provide the opportunity for more successful chemoprevention against HCC.

Materials and Methods

Animal model. Pathogen-free 14-day pregnant C57BL/6j mice were purchased from CLEA Japan (CLEA, Tokyo, Japan). NASH-HCC was induced in male mice by a single subcutaneous injection of 200 μg of streptozotocin (STZ; Sigma, MO, USA) at 2 days after birth followed by feeding with high-fat diet, 32% fat (HFD32; CLEA, Tokyo, Japan) ad libitum after 4 weeks of age (5). All experimental procedures followed the Japanese Pharmacological Society Guidelines for Animal Use. The number of mice per group per time point for biochemical, histological and CT imaging analyses are indicated in the Table and Figure legends.

Biochemical analyses. Blood was collected from mice via cardiac puncture with or without anticoagulant (sodium citrate). For serum preparation, the blood samples were kept at room temperature for 30 min, followed by 4°C for 1 h and then centrifuged at 1,000 × g for 15 min at 4°C. The supernatant was collected and stored at −80°C. Plasma or serum albumin (Alb) and total bilirubin (T-bil) levels were measured by FUJI DRI-CHEm 7000 (Fujiﬁlm, Tokyo, Japan). Serum ammonia (NH3) levels were measured by NH3-PII (Fujifilm, Tokyo, Japan). Serum alpha fetoprotein (AFP) level was measured by mouse alpha-fetoprotein ELISA (APF) level was measured by mouse alpha-fetoprotein ELISA (Kamiya Biomedical, Seattle, WA, USA). Prothrombin time (PT) determination was performed according to the technique described by the Quick method (7) using ThromboCheck PT Plus (Sysmex Corp., Kobe, Japan). Briefly, blood samples were collected with sodium citrate as an anticoagulant, then citrated plasma samples (25 μl) were pipetted into cuvettes and finally 50 μl of ThromboCheck PT Plus was added to measure the clotting time.

Tissue sampling and histological analysis. For hematoxylin and eosin (HE) staining, left lateral livers were washed with chilled saline, fixed in 10% neutral-buffered formalin (Wako Pure Chemical Industries, Osaka, Japan) and embedded in paraffin. Paraffin sections were stained with HE.

For immunostaining, left lateral livers were washed with chilled saline, embedded into Tissue-Tek O.C.T. compound (Sakura Tissue Tek, Tokyo, Japan), frozen in liquid-nitrogen and stored at −80°C. Immunostaining was performed on O.C.T. or paraffin-embedded liver sections previously described (5). All sections were blocked with the BlockAce (DS Pharma Biomedical Co., Osaka, Japan) according to manufacturer’s instructions. Paraffin-embedded sections were subjected to antigen retrieval in an autoclave with a sodium citrate buffer and incubated overnight at 4°C with antibody specific for Glypican-3 (GPC3) (1:100; Cat# ab66596, Abcam, address). Staining for CD11c, type-IV collagen and BrdU were performed as described previously (5), all slides were subsequently processed with HRP-conjugated appropriate secondary antibodies and DAB solution (Nichirei Biosciences Inc., Tokyo, Japan).

Medical imaging (CT). Mice were scanned by CT using the Latheta CT system (LCT-200, Aloka, Tokyo, Japan) for the detection and characterization of HCC. The mice were mounted on a holder and placed in the CT system under pentobarbital sodium (Dainippon sumitomo pharma, Osaka, Japan) anesthesia. Contrast-enhanced images were obtained after the intravenous injection of 1.7 ml/kg iopamidol (Iopamiron, Bayer HealthCare AG, Berlin, Germany) via tail veins. Individual maximal tumor diameter was measured by CT images using the Osirix Imaging software (link or supplier). For calculating the tumor volume, the maximal (a) and minimal (b) diameters were measured in coronal and horizontal section and tumor volumes were calculated by the formula Volume (mm³)=1/2(a²×b). Tumor growth rates were calculated from tumor volumes at week 16 as 100%.

Statistical analyses. All statistical analyses were calculated using the GraphPad Prism™ (version 4.0; GraphPad Software, San Diego, USA). Data are expressed as the mean±standard deviation (SD). Student’s t-test was used for statistical analysis of the number of hepatocytes. p-Values <0.05 were considered to be statistically significant.

Results

Evaluation of tumor stage by a clinically equivalent procedure. To characterize the HCC by a clinically equivalent procedure, we investigated the kinetics of space-occupied lesions (SOLs) in the STAM mouse model using the dynamic CT system. The liver SOLs at week 20 were multiple and diagnosed as HCC because the SOLs showed HCC radiological hallmarks, arterial hypervascularity and late-phase washout (5). Since the number of tumors (single or 3 nodules) is important to consider curative treatments or others in BCLC, we first confirmed the number of SOLs. At least 4 tumor nodules were observed per individual (n=5), supporting previous reports. Because SOLs were detectable from 14 weeks of age, we investigated the mean tumor

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diameter (5 tumors per mouse) during weeks 14 through 20. The mean tumor diameter started to increase after week 16 and reached near 1 cm thereafter (Figure 1). Tumor growth rate was estimated using tumor diameter in accordance with a Response Evaluation Criteria in Solid Tumors (RECIST) fashion and the average growth rate of the tumors from week 16 to 20 was 150% (Table I). Tumor volume was also estimated separately and all SOLs showed increased tumor volume up to week 20 (Figure 2). In all STAM mice, there was no obvious spread to other organs including lung. Most STAM mice did not exhibit ascites, but some individuals showed ascites at week 20 (Figure 3b).

**Table I. Tumor growth rate.**

<table>
<thead>
<tr>
<th>Tumor</th>
<th>Weeks</th>
<th>16</th>
<th>17</th>
<th>18</th>
<th>19</th>
<th>20</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>100%</td>
<td>81%</td>
<td>116%</td>
<td>147%</td>
<td>177%</td>
<td></td>
</tr>
<tr>
<td>2</td>
<td>100%</td>
<td>120%</td>
<td>146%</td>
<td>150%</td>
<td>165%</td>
<td></td>
</tr>
<tr>
<td>3</td>
<td>100%</td>
<td>122%</td>
<td>128%</td>
<td>125%</td>
<td>166%</td>
<td></td>
</tr>
<tr>
<td>4</td>
<td>100%</td>
<td>139%</td>
<td>102%</td>
<td>120%</td>
<td>127%</td>
<td></td>
</tr>
<tr>
<td>5</td>
<td>100%</td>
<td>83%</td>
<td>81%</td>
<td>120%</td>
<td>123%</td>
<td></td>
</tr>
</tbody>
</table>

Tumor growth rate of HCC in STAM mouse from 16 weeks of age. The mean growth rate of the tumors from 16 weeks of age to 20 weeks of age was approximately 150%.

**Figure 1.** Mean tumor diameters of HCCs in STAM mice. Tumor diameters were measured in STAM mice (n=7).

**Table II. Laboratory data.**

<table>
<thead>
<tr>
<th></th>
<th>Normal (Week 20)</th>
<th>HCC (Week 20)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Alb (g/dl)</td>
<td>2.7±0.3</td>
<td>2.7±0.3</td>
</tr>
<tr>
<td>T-Bil (mg/dl)</td>
<td>0.73±0.31</td>
<td>0.43±0.06</td>
</tr>
<tr>
<td>NH3 (μg/dl)</td>
<td>249±77</td>
<td>274±59</td>
</tr>
<tr>
<td>PT</td>
<td>-</td>
<td>&lt;40%</td>
</tr>
<tr>
<td>AFP (pg/ml)</td>
<td>9.12±4.3</td>
<td>25.8±9.5</td>
</tr>
</tbody>
</table>


**Evaluation of liver function by serological parameters.** Fujii et al. reported that the STAM mice showed mild elevation of serum AST and ALT levels (5). To investigate hepatic spare ability related to Child-Pugh grade, we measured the serological parameters in the HCC-bearing mice at 20 weeks of age (Table II). Plasma Alb and T-bil levels revealed no significant change between HCC and normal mice. Serum NH3, as an index of hepatic encephalopathy, was also unchanged between HCC and age-matched control mice. Although the normal ranges of serum parameters are not always the same between human and mouse, it is considered that Alb, T-bil and NH3 were not elevated in HCC-bearing...
Figure 3. CT imaging of HCCs in STAM mice. CT images were captured using a micro-CT. Six slices per mouse were selected in order to chase the same tumors (A to G, indicated by white circles) between week 16 (a) and 20 (b). No visible lung metastasis and pleural effusion in both time periods.
STAM mice compared to normal mice. In contrast, HCC-bearing STAM mice showed marked prolongation of PT.

Evaluation of HCC-related parameters used in the clinical setting. We further investigated HCC-related parameters to obtain insights into the clinical relevance of HCC in this model. First, we observed the survival of STAM mice (n=55) to know cancer-related death. At week 12, which is corresponding to liver fibrosis stage in NASH (5), almost all mice survived (Figure 4). In parallel with SOL formation (week 14), mice started to die. Approximately 50% of mice survived for week 20.

Next, an important serological parameter, AFP, was elevated at week 20 (Table II), thereby supporting the notion that STAM mice developed HCC. We also investigated the protein expression of the human HCC marker GPC3 by immunohistochemical staining, as GPC3 mRNA was up-regulated in the HCC-laden liver. GPC3-positive tumor cells were detectable in HCC of STAM mice at week 20 (Figure 5a).

In addition in histology, tumor cell invasion into the portal tracts within the tumor was observed (Figure 5b). Finally, we observed that a number of CD11c+ dendritic cells (DCs) accumulated within HCC (Figure 5c). DCs were also detectable in lymphatics around tumor-associated portal tract (Figure 5d), suggesting that DCs can exit from the liver to hepatic lymph nodes.

Discussion

Because current animal models of HCC do not accommodate clinical stratification of humans, there has been a limit on the development to directly connect with clinical practice. Thus, we proposed the STAM model as the first animal model adopting clinical stratification of humans in the present study.

In view of liver function in HCC, the Child-Pugh grade is a gold standard and consists of hepatic encephalopathy, ascites, T-bil, Alb and PT activity. We examined hepatic encephalopathy by serum NH3 levels and found HCC-bearing mice did not show increased NH3 levels compared to normal mice, thus considered hepatic encephalopathy was not obvious in STAM mice at week 20. Similarly, the levels of T-Bil and Alb did not show significant differences between STAM and normal mice. Although the value between human and mouse is not always same, we considered that the levels of T-Bil and Alb were not out of normal ranges. Thus, we judged that the points of hepatic encephalopathy, T-Bil and Alb were 1. In contrast, marked prolongation of PT was of interest and judged as point 3. It was recently reported that HCC patients with diabetes showed increased levels of PT-INR (8), and this is consistent with our results. Because we demonstrated the NASH-pathology is a critical link between diabetes and HCC in STAM model, we consider that NASH-related dysregulation
of coagulation may have impacts on higher prolongation of PT in HCC. The exact mechanism remains as yet unclearly understood, thus further investigations are required. Ascites was estimated by CT. Although most STAM mice did not show ascites, minor population of STAM mice actually showed mild ascites. Thus, we judged the point of ascites was 1 to 2. In total, the point was 7 to 8. We therefore estimated that the hepatic spare ability of STAM mice was equivalent to Child-Pugh grade B in humans.

In view of tumor stage, we estimated HCC in STAM mice in accordance with the BCLC. The size/diameter of HCC between human and mouse cannot be compared directly. The maximum size of HCC in STAM mice reached over 1 cm, which is considered as >2 cm in human. The HCC in STAM mice exhibited a multi-nodular pattern, corresponding to BCLC stages B, C and D. As Child-Pugh grade is considered as B (that is, not C), BCLC stage D is excluded. In CT analysis in the present study, visible metastasis including macroscopic lymph node metastasis was not detected. However, we did not conclude the absence of lymph node metastasis because of the limitation of the number of mice examined for detailed histology. Invasion of HCC was observed within the portal area. Several vessels had disappeared and the structure of the portal area was often unclear. Considering together with the presence of ascites, we estimated that the tumor stage of STAM mice was equivalent to the BCLC stage C or, to a lesser extent, B.

The BCLC stage C would be a suitable stage to investigate the efficacy of chemotherapy or molecular-targeted drugs. To investigate the potential of STAM mice as a model used for drug development of anti-HCC drugs, we estimated HCC-related parameters that are used in the clinical setting. Overall survival is the most important end-point in clinical trial. Based on our survival observations (Figure 4), we considered overall survival could be estimated in STAM mice in pharmacological studies. Serum AFP would be used as a serological parameter like human HCC and GPC3 helps to distinguish between the cancerous and non-cancerous parts of hepatocellular lesions (9-11). Participation of DCs indicates that immune-modulators including vaccination could be investigated for their anti-HCC efficacy in this model.

There are some limitations to our study. First, the number of STAM mice evaluated in the present study was not sufficient for decisive evidence. Thus, further investigations including lymph node metastasis, immune responses and longer survival rates are needed. Second, the standard blood test values are different between mice and humans.
Therefore, the Child-Pugh grade is not directly applicable to mice, although we estimated the severity by comparison with normal mice. Third, the model cannot be applied directly to viral hepatitis-derived HCC. However, NASH has been revealed as a critical risk factor for HCC (12). In addition, recent investigations revealed that HCC that occurs even after achievement sustained virological response (SVR) in viral hepatitis-derived HCC, suggesting the importance of understanding the virus-independent HCC development pathway. NASH-derived HCC would contribute to this new intriguing research area.

To the best of our knowledge, this is the first study to bring a clinical stratification concept into animal experiments. We demonstrated that HCC in STAM mice was characterized into BCLC stage C or, to a lesser extent, B that was never shown in the previous animal models for HCC.

In conclusion, STAM model mice are consistent with clinical stratification of humans and are able to contribute to new therapeutic developments of HCC.

References


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