Pathology - Research and Practice 225 (2021) 153559

Contents lists available at ScienceDirect

Pathology - Research and Practice

journal homepage: www.elsevier.com/locate/prp

Neonatal streptozotocin treatment rapidly causes different subtype of hepatocellular carcinoma without persistent hyperglycemia in 4CS mice fed on a normal diet

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ARTICLE INFO

Keywords:
Animal model
Hyperglycemia
Hepatocellular carcinoma
HCC subclasses
Streptozotocin

ABSTRACT

Although diabetes mellitus (DM) is a well-known risk factor for hepatocellular carcinoma (HCC), the underlying mechanisms have not yet to be defined. We previously reported that DIAR mice fed with standard murine diet developed type 1 diabetes and HCC at age of 16 weeks old with a neonatal streptozotocin treatment (n-STZ). Because DIAR mice did not manifest obesity nor develop steatohepatitis, hyperglycemia with streptozotocin trigger or streptozotocin alone might turn on the hepato-carcinogenesis. An insulin-recruitment to DIAR-nSTZ mice showed an increased frequency of HCC during the first 12 weeks of age, although the diabetic indications notably improved. To elucidate the role of hyperglycemia in hepato-carcinogenesis, we performed a head-to-head comparative study by using 4CS mice and DIAR mice with n-STZ treatment. Newborn 4CS mice and DIAR mice were divided into STZ treated group and control group. The blood glucose levels of DIAR-nSTZ mice increased at age of eight weeks, while that of 4CS-nSTZ mice were maintained in the normal range. At eight weeks old, three out of five DIAR-nSTZ mice (60%) and one out of ten 4CS-nSTZ mice (10%) developed multiple liver tumors. At age of 12 weeks old, all eight of DIAR-nSTZ mice (100%) and two of 10 4CS-nSTZ mice (20%) developed multiple liver tumors. At 16 weeks old, all animals of DIAR-nSTZ and 4CS-nSTZ mice occurred liver tumors. DIAR-nSTZ showed hyperglycemia and HCC, and 4CS-nSTZ developed HCC without hyperglycemia. These results were interpreted that the onset of HCC maybe not related to the presence or absence of hyperglycemia but nSTZ treatment. On the other hand, since the carcinogenesis of 4CS-nSTZ is delayed compared to DIAR-nSTZ, hyperglycemia may play a role in the progression of carcinogenesis. Histologically, the liver tumor appeared irregularly trabecular arrangements of hepatocytes with various degrees of nuclear atypia. By immunohistochemical analyses, all liver tumors showed positive staining of glutamine synthetase (GS), an established human HCC marker. The expression pattern of GS was divided into a strong diffuse pattern and weak patchy pattern, respectively. The liver tumor showing the weak GS-patchy pattern expressed biliary/stem markers, EpCAM, and SALL4, partially. Because 4CS-nSTZ mice did not show any metabolic complications such as gaining

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https://doi.org/10.1016/j.prp.2021.153559
Received 8 March 2021; Received in revised form 13 July 2021; Accepted 17 July 2021
Available online 21 July 2021

body weight or high blood glucose level, it is a unique animal model with a simple condition to investigate hepatic carcinogenesis by excluding other factors.

1. Introduction

Hepatocellular carcinoma (HCC) has become one of the most frequent causes of cancer death globally, only second to lung cancer [1,2]. It is well known that HCC is associated with either hepatitis B or C viral infections or other causes such as alcohol overuse, metabolic disturbance, and toxic agents, among others [3,4]. Because of the establishment of blood screening and the recent dramatic advances of therapeutic drugs against the hepatitis virus, the number of viral-induced HCC decreases. In contrast, the number of metabolic diseases including diabetes mellitus (DM) is rapidly increasing in the developed countries. It has been reported that DM and insulin resistance are epidemiologically established risk factors for HCC [5–7]. However, the underlying biological mechanism linked DM and HCC has not been addressed.

The chemical-induced HCC model is frequently used as a simple liver carcinogenesis model [8–10]. Although aflatoxin is clinically known as a liver carcinogen [11], the most frequently used experimental model is the diethylnitrosamine (DEN)-induced HCC model [9,10,12]. However, DEN-induced HCC model was a time-consuming required for six months or an even longer time, and the success rate was low. Using carbon tetrachloride (CCL_4) and high-fat diets (HFD) combined with DEN not only accelerated HCC development but also increased the frequency of HCC [12]. On the other hand, there has been an increasing reports of carcinogenic models characterized with steatohepatitis [13–15]. We have reported that galectin-3 KO mice [16], MSG mice [17,18], and TSOD mice [19] developed HCC at 15 months age. Ejima et al. reported similar findings that HCC occurred in the diet-induced nonalcoholic steatohepatitis (NASH) model [20]. The STAM mice, which are widely used as a NASH-developed carcinogenic model against the background of type 1 diabetes, were created by combining streptozotocin (STZ) administration with HFD in the neonatal period [21,22]. In the current study, we administered a single dose of STZ in neonatal DIAR (ddY, Institute for Animal Reproduction) mice (DIAR-nSTZ mice). DIAR-nSTZ mice on a standard murine diet developed HCC in a short time period [23]. The HCC developed in DIAR-nSTZ mice were histologically mimicked human HCC by expressing Glutamine synthetase (GS). We observed that pancreatic islets were struck down with STZ treatment and the DIAR-nSTZ mice exhibited hyperglycemia complications. Based on these results, we hypothesized that hyperglycemia might urge the onset or/and progression of HCC in DIAR-nSTZ mice. We then designed a series of experiments to detect the effect of insulin-recruitment in DIAR-nSTZ mice. Unexpectedly, the frequency of HCC was not decreased in the DIAR-nSTZ mice with insulin-recruitment during the first 12 weeks of age, despite the diabetic indications notably improved [24]. These results implied that hyperglycemia might not play a synergetic role with STZ in carcinogenesis. In another preliminary experiment, 4CS mice with neonatal STZ treatment exhibited a rapid normalization of blood glucose level without overexpressing insulin. To elucidate the role of hyperglycemia in hepato-carcinogenesis, we performed a head-to-head comparative study using 4CS mice and DIAR mice with n-STZ treatment.

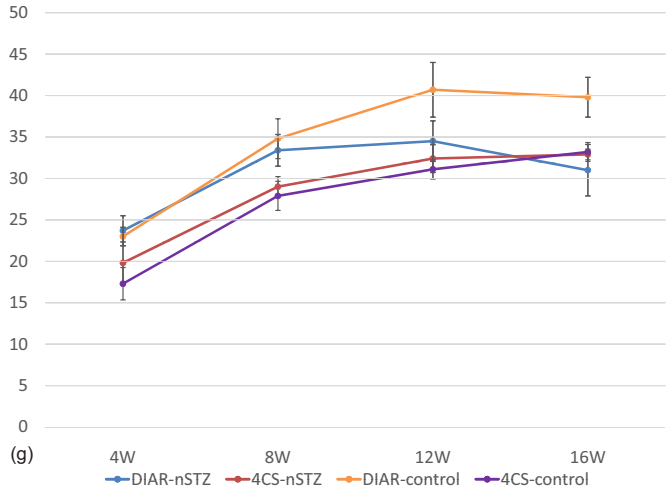

Fig. 1. The transition of the body weight (g) DIAR-nSTZ mice showed a marked decrease in their body weight as compared to the DIAR-control mice. In contrast, 4CS-nSTZ mice exhibited similar body weight transitions as compared to the 4CS-controls The number of mice examined: DIAR-nSTZ mice: 4 weeks: 26, 8 weeks: 21, 12 weeks: 16 and 16 weeks: 8, 4CS-nSTZ mice: 4 weeks: 34, 8 weeks: 28, 12 weeks: 18 and 16 weeks: 8, DIAR-control mice: 4 weeks: 20, 8 weeks: 15, 12 weeks: 10 and 16 weeks: 5, 4CS-control mice: 4 weeks: 20, 8 weeks: 15, 12 weeks: 10 and 16 weeks: 5.

2. Methods

Newborn male 4CS mice (4 days of a reproductive cycle) were accommodated at the Institute of Animal Reproduction (Ibaraki, Japan). These mice were randomly divided into two groups with or without STZ treatment. Thirty-four and 20 mice were engaged in 4CS-nSTZ and 4CS-control groups, respectively. At 36 h after birth, STZ was subcutaneously injected (60 mg/kg) into the treatment group (4CS-nSTZ mice), whereas the same volume of 0.9% NaCl physiological solution was injected into the control group (4CS-control mice). All mice were maintained on a standard murine diet. Mice in each group were assessed physiologically and histopathologically at 4, 8, 12, and 16 weeks. Likewise, 26 and 20 newborn male DIAR mice were engaged in DIAR-nSTZ and DIAR-control groups, respectively. All DIAR mice were parallelly examined as well at the same age of 4CS mice using the same procedure. Body weights of all mice were measured once a week. Blood was taken once from all mice every four weeks for the measurement of glucose level. The data were analyzed parallelly between the DIAR-nSTZ and DIAR-control groups.

All institutional and national guidelines for the care and use of laboratory animals were abided by the researchers involved in this study. The animal experiment guidelines specified in the Institute for Animal Reproduction (Ibaraki, JAPAN), (Permission number: IarAW No. 27–11) which were well aligned with the rules of guidance on animal research ethics from the International Association of Veterinary Editors' Consensus Author Guidelines on Animal Ethics and Welfare, were followed in all the animal experiments.

This article does not involve in any studies with human subjects.

Histological evaluation.

Morphological assessment of the liver:

After fixing the liver with 10% neutral buffered formalin, whole livers were cut through at 2 mm intervals and was examined grossly for tumor appearance. All sections with grossly visualized tumors were embedded in paraffin and thin-cut to 4 μm for microscopy with morphological and immunohistochemical assessment. Deparaffinized sections were stained with hematoxylin-eosin, dehydrated in 100% ethanol, cleared by xylene, mounted with NEW M-X (Matsunami Glass Industries, Osaka, Japan), and then examined by microscopy.

For immunohistochemistry, primary antibodies against glutamine synthetase (GS, Abcam, Cambridge, UK), sal-like protein 4(SALL4, Abnova, Taipei, TW), and epithelial cell adhesion molecule (EpCAM, Abcam, Cambridge, UK) were applied. After deparaffinization, antigen retrieval was carried out on the specimens, followed by endogenous

Table 1
Blood glucose level (mg/dL).

	4 W	5 W	8 W	12 W	16 W
DIAR-nSTZ	520 ± 63.4 (n = 26)	578 ± 42.8 (n = 21)	> 600 (n = 21)	> 600 (n = 16)	> 600(n = 8)
4CS-nSTZ	231 ± 75.9 (n = 34)	321 ± 99.3 (n = 28)	178 ± 52.3 (n = 28)	142 ± 25.1 (n = 18)	161 ± 13.1 (n = 8)
DIAR-control	146 ± 35.7 (n = 20)	N.D.	217 ± 56.0 (n = 15)	205 ± 40.3 (n = 10)	210 ± 34.2 (n = 5)
4CS-control	126 ± 19.6 (n = 20)	N.D.	152 ± 53.3 (n = 15)	113 ± 6.6 (n = 10)	121 ± 8.9 (n = 5)

600 mg / dL is the detection limit.
N. D.: Not tested

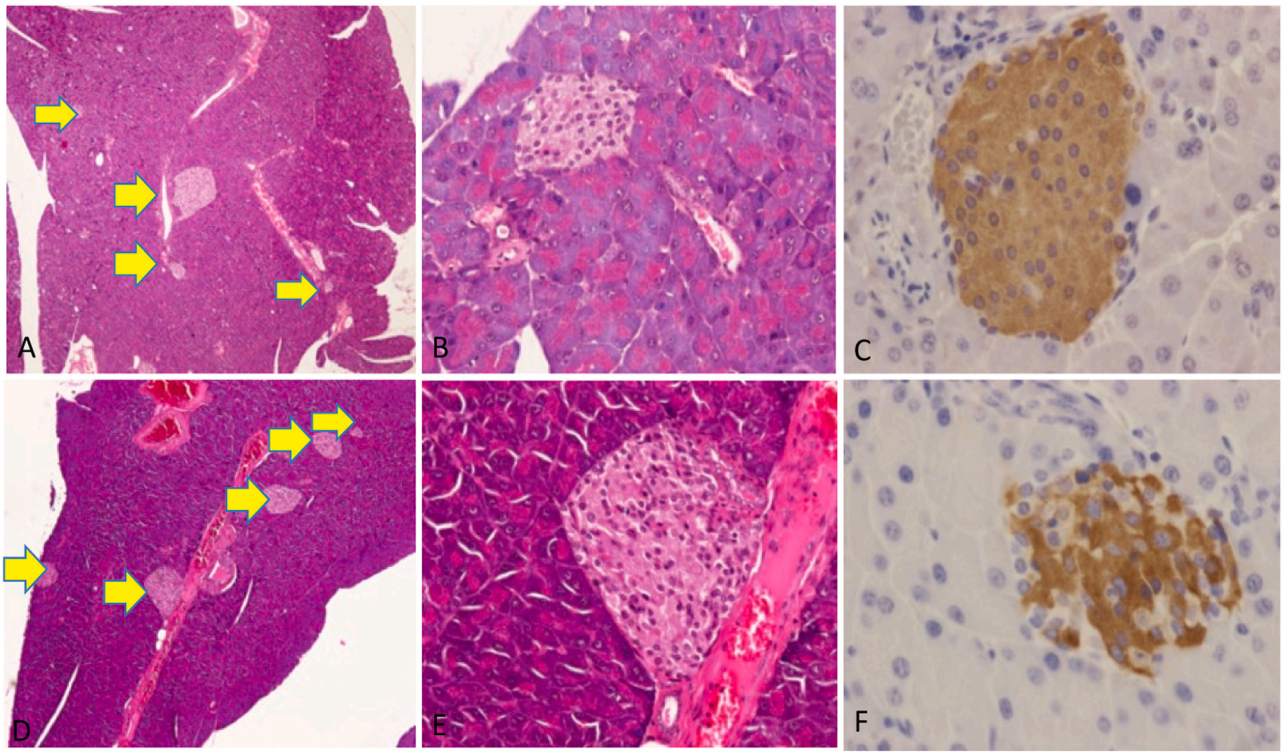

Fig. 2. Pathological change of pancreatic islet cells of 4CS at eight weeks of age. A-C:4CS-control mice. D-F: 4CS-nSTZ mice A, D: Distribution of pancreatic islets (Arrows). (x40 magnification) B, E: Morphology of representative pancreatic islet cells. (x200 magnification) C, F: Immunostaining of insulin (x400 magnification).

peroxidase blocking using 5% H_2O_2 in methanol for 5 min at room temperature (RT). Following incubation with 5% bovine serum albumin (BSA, Sigma-Aldrich Japan K. K., Tokyo, Japan) to block nonspecific binding, the specimens were incubated overnight at 4 °C with prediluted primary antibodies. EnVision Polymer –horseradish peroxidase (K4001; Dako Denmark A/S, Glostrup, Denmark) were then applied and incubated 1 h at 4 °C. The immunoreaction was visualized by using 3,3′-diaminobenzidine (DAB, SK4100; Vector Laboratories Inc., Burlingame, CA, USA), and was lightly counterstained with hematoxylin.

Morphological assessment of the pancreas:

After fixation in 10% neutral buffered formalin, the maximum cut surface of the pancreas was embedded in paraffin and then cut at 4 μm thickness for morphology assessment by HE stain.

One or two field of view at a magnification of 100 times was randomly photographed from each individual for assessment, and the areas of pancreatic tissue and islets of Langerhans were quantified by image analysis software (CellSens Standard, Olympus, Japan).

3. Results

3.1. Bodyweight

DIAR-nSTZ mice showed a remarkable decreased body weight compared to DIAR-control mice. In contrast, 4CS-nSTZ mice exhibited similar body weight transitions as that of 4CS-controls (Fig. 1).

3.2. Blood glucose level

Blood glucose levels of the DIAR-nSTZ mice increased at the age of 4 weeks, and then maintained the higher levels compared to DIAR-controls after eight weeks. In contrast, the 4CS-nSTZ mice exhibited a slight slope of increasing blood glucose level from four weeks old to 5 weeks old and bent back to normal range after eight weeks age (Table 1).

3.3. Histopathology

Pancreas: Pancreatic islets almost completely disappeared in DIAR-nSTZ as similar as previously reported [18]. At eight weeks old, 21 normal-sized islets were recognized in the 12 fields of 100 times magnification from DIAR-control mice, while 11 size-reduced islets were recognized in the 12 fields of 100 times magnification from DIAR-nSTZ mice. The ratio of the islet areas per examined pancreatic area of the DIAR-nSTZ mice (0.22 ± 0.14%) was robustly reduced from that observed in the DIAR-control mice (2.02 ± 1.2%). In contrast, most islets remained at the normal range in the 4CS-nSTZ mice at four weeks of age as well as that in the 4CS-control mice. Immunohistochemically, the population of insulin-positive beta cells in islets of 4CS-nSTZ mice was similar as those of 4CS-control mice (Fig. 2). At eight weeks old, 42 islets

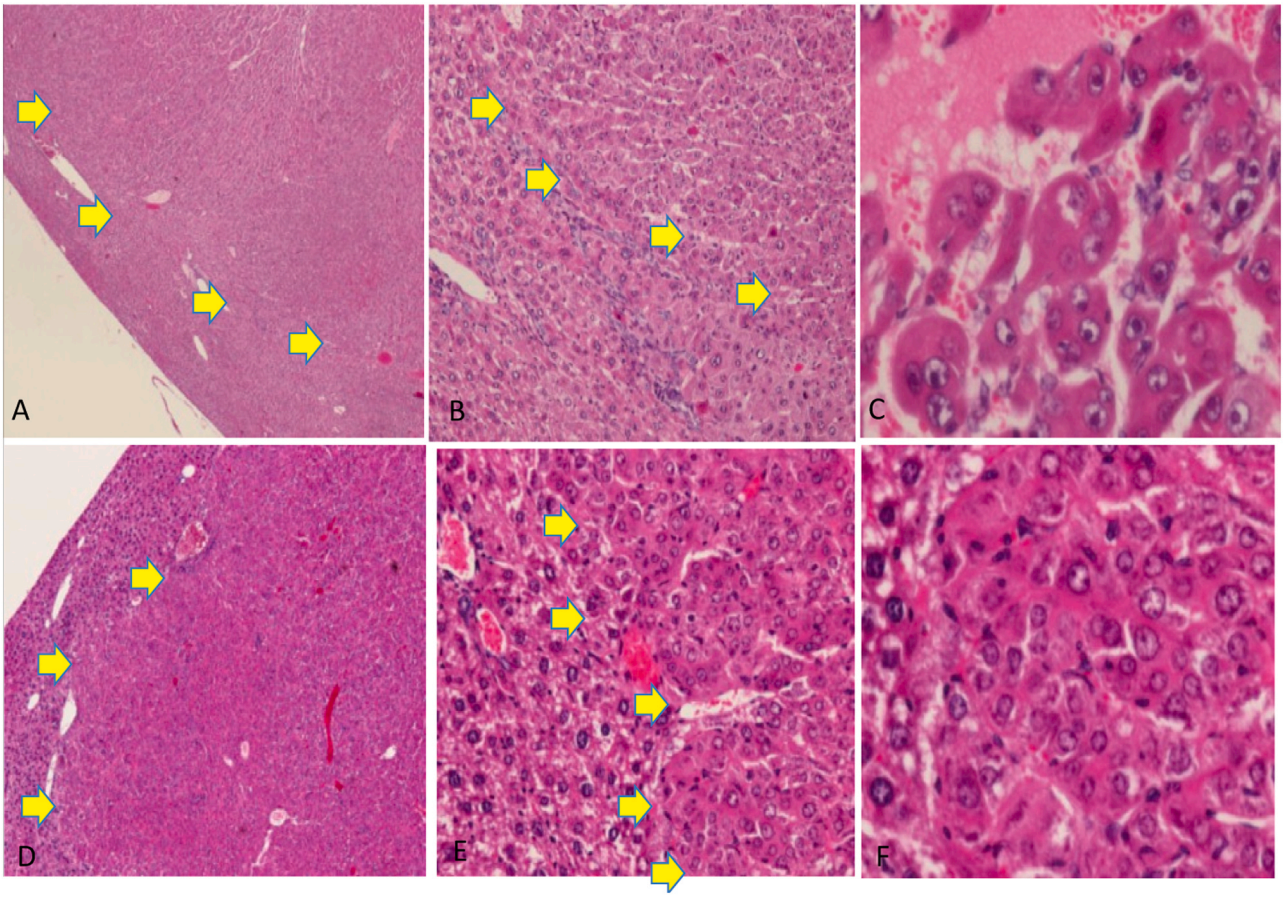

Fig. 3. Representative liver tumors of DIAR-nSTZ (A-C) and 4CS-nSTZ. (D-F) Arrows indicate tumor areas composed of atypical hepatocytes. (A, B, D, E) There are no fibrous capsules. Multinucleated atypical hepatocytes showed irregular thick trabecular arrangements. (B, C, E, F). (A, D: x40 magnification, B,E: x200 magnification, C,F: x400 magnification).

Table 2
Frequency of Liver tumor.

	4 W	8 W	12 W	16 W
DIAR-nSTZ	0/5 (0%)	3/5(60%)	8/8(100%)	8/8(100%)
4CS-nSTZ	0/6(0%)	1/10(10%)	2/10(20%)	8/8(100%)
DIAR-control	0/5(0%)	0/5(0%)	0/5(0%)	0/5(0%)
4CS-control	0/5(0%)	0/5(0%)	0/5(0%)	0/5(0%)

were recognized in the 12 fields of 100 times magnification from 4CS-control mice, while 29 islets were recognized in the 12 fields of 100 times magnification from 4CS-nSTZ mice. The ratio of the islet areas per examined pancreatic area of the 4CS-nSTZ mice (1.11 ± 0.84%) was no significant difference from that observed in the 4CS-control mice (1.57 ± 1.2%).

Liver: At age of four weeks old, no tumor was found in all examined mice. At eight weeks old, three out of five (60%) DIAR-nSTZ mice and one out of 10 (10%) 4CS-nSTZ mice exhibited a liver tumor about 2 mm in diameter. Histologically, these tumors appeared mild structural and nuclear atypia that mimicked the human dysplastic nodule. At 12 weeks age, all eight (100%) DIAR-nSTZ mice and two out of ten (20%) 4CS-nSTZ mice have developed liver tumors. The maximum size of liver tumor either in DIAR-nSTZ or 4CS-nSTZ mice was 2.5 mm in diameter. Some of these tumors showed moderate to severe nuclear and structural atypia without invasive growth those patterns mimicked human high-grade dysplastic nodules. At age of 16 weeks old, all DIAR-nSTZ mice and 4CS-nSTZ mice developed single or multiple liver tumors. The maximum size of these liver tumors either in DIAR-nSTZ mice or in 4CS-nSTZ mice was 8 mm in diameter. Histologically, these liver tumors appeared severe nuclear and structural atypia with a portal invasion that mimicked human HCC (Fig. 3). Frequency of the liver tumor of each group mice over the time was shown in Table 2. All liver tumors in DIAR-nSTZ mice and 4CS-nSTZ mice showed a positive reaction with GS, an established marker of human HCC (Fig. 4) with two characterized patterns, strong diffuse pattern (GS-diffuse) and weak patchy pattern (GS-patchy). Interestingly, tumors with weak GS-patchy pattern in nSTZ-treated mice also diffusely expressed EpCAM. Moreover, a perinuclear expression of SALL4 was also observed in partial GS- patchy tumors (Fig. 5). No liver tumor was recognized in DIAR-control or 4CS-control mice.

4. Discussion

We previously reported that DIAR-nSTZ mice on a regular diet developed type 1 diabetes and HCC without manifestation of steatohepatitis [25]. Since hyperglycemia was considered to enhance liver carcinogenesis [26], we hypothesized that DIAR-nSTZ mice on a regular mice diet could be a diabetes-induced HCC model. To examine this hypothesis, we treated DIAR-nSTZ mice with an excess of insulin administration. Although this insulin recruitment led to the mitigation of diabetes, HCC occurrence was observed as the same as that in DIAR-nSTZ mice without insulin recruitment [24]. To elucidate the role of hyperglycemia in hepato-carcinogenesis, we performed a head-to-head comparative study using both DIAR and 4CS mice and found that 4CS rapidly normalized the blood glucose levels after STZ treatment in contrast to high blood glucose levels maintained in DIAR-nSTZ mice.

Based on the results of present study, the two possibilities can be

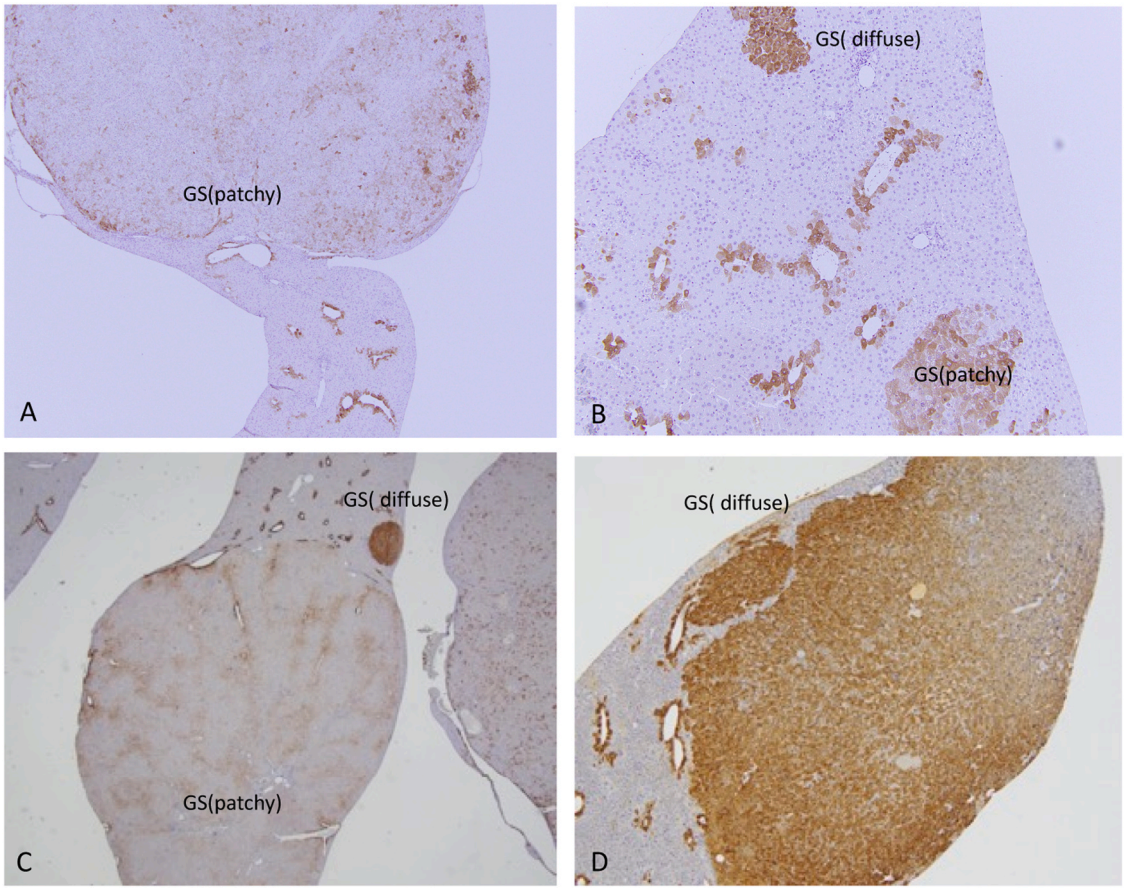

Fig. 4. Representative immunostaining of Glutamine synthetase (GS) in liver tumors of DIAR-nSTZ mice (A, B) and 4CS-nSTZ mice (C,D). All the liver tumors showed a positive reaction against glutamine synthetase (GS), a useful marker of dysplastic nodules and HCC. GS expression pattern was divided into strong diffuse pattern (GS-diffuse) and weak patchy pattern (GS-patchy). (A, C: x40 magnification, B, D: x100 magnification).

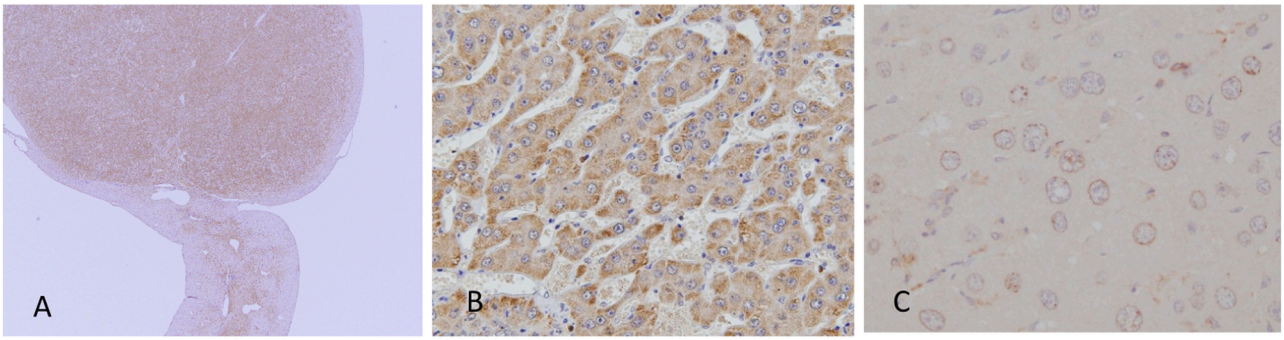

Fig. 5. Representative immunostaining of EpCAM and SALL4 in GS-patchy tumor of nSTZ-treated mice. A, B: GS-patchy tumor showed diffuse cytoplasmic expression of EpCAM. C: Part of tumor cells expressed perinuclear expression of SALL4. (A: x40 magnification, B, C: x400 magnification)).

speculated. First, since HCC was caused by nSTZ regardless of the presence or absence of hyperglycemia in 4CS mice, this model could be regarded as a nSTZ-induced carcinogenic model. Because STZ has a destructive effect on the islets of Langerhans, it was commonly used to induce type 1 diabetes in animal models [27,28]. Several studies have reported that the administration of STZ can be used for inducing carcinoma via DNA alkylation in situ [29,30], however there has not yet reported STZ-induced HCC animal model. We hypothesized that exposure to STZ during the neonatal period might be a potential cause of accelerating the development of HCC. In most of the previous reports, STZ was injected at around six weeks age. In this study, we administrated a single dose of STZ at 36 h after birth. At this time point, the blood-brain barrier is still immature, therefore injected STZ might leak into the brain. An encompassing analysis of the brain just before and after STZ injections will be taken in a further study to confirm if there is STZ leakage in the brain at neonatal period.

The other speculation is that hyperglycemia was involved in promoting but not initiating hepato-carcinogenesis. In our previous insulin supplementation experiment in DIAR-nSTZ mice, it was speculated that hyperinsulinemia might occur and trigger carcinogenesis. In this study, unfortunately, blood insulin levels were unmeasurable over the time due to the limited volume of collectible serum. Moreover, insulin supplementation was not applied and the population of insulin-positive cells in the pancreas of 4CS mice was not increased based on immunochemistry

analysis compared to their controls. Therefore, it was unlikely that hyperinsulinemia happened by STZ treatment either in 4CS -nSTZ mice and DIAR-nSTZ mice. Since neither 4CS-nSTZ mice nor DIAR-nSTZ mice developed obesity and steatohepatitis, hyperglycemia exhibited only in DIAR-nSTZ mice might play a key role in accelerating carcinogenesis.

Last but not the least, we would like to emphasize the uniqueness of 4CS-nSTZ mice as a chemical-induced HCC model compared to other well-known HCC animal models. In the DEN-induced model, HCC occurred at an average age of 50 weeks old [31,32], while the Solt-Farber method required surgical intervention to induce HCC at 4–8 weeks age [33,34]. In comparison, 4CS-nSTZ mice developed HCC in a short period of 16 weeks. Morphological and immunohistological characterizations of liver tumors in nSTZ-treated mice mimicked that of human HCC. Recently, based on immunohistochemical analysis HCC was divided into three subtypes: biliary/stem cell subtype, Wnt/β-catenin signaling subtype, and all-negative subtype. The Biliary/stem cell subtype tended to recurrence in the shortest time among the three subtypes [35]. For classifying the tumors occurred in our study into above three subtypes, immunohistochemical analysis of biliary/stem cell markers (SALL 4 and EPCAM) and Wnt/β-catenin signaling-related molecules (GS) were performed. In the liver tumors of 4CS-nSTZ mice and DIAR-nSTZ mice, both GS-diffuse pattern and GS-patchy pattern were observed. Interestingly, the tumors with GS- patchy pattern in DIAR-nSTZ mice also diffusely expressed EpCAM, and some of them expressed SALL4 as well. We classified tumors with GS-diffuse pattern into human-HCC-mimic Wnt/β-catenin signaling subgroup, but tumors with GS-patchy pattern into human-HCC-mimic biliary/stem cell group [36]. Genetic characterization of each tumor subgroup of these mice models will be investigated in further studies to further prove 4CS-nSTZ as a human-HCC-mimic animal model with rapid and thrifty tumor development on the regular murine diet.

5. Conclusions

Because 4CS-nSTZ mice developed liver tumors at very early age without any metabolic complications such as gaining body weight and high blood glucose level, it could be a unique and simple HCC animal model to investigate the carcinogenic process of the liver without other factors influence.

CRediT authorship contribution statement

Tomoko Kobayashi: Writing – original draft preparation, Methodology, Formal analysis, **Mayuko Ichimura-Shimizu:** Conceptualization, Writing – original draft preparation, Methodology, Formal analysis, **Takeshi Oya:** Writing – review & editing, Supervison, **Hirohisa Ogawa**: Data curation, Software, **Minoru Matsumoto and Yuki Morimoto and Satoshi Sumida and Takumi Kakimoto and Michiko Yamashita**: Software, Validation, Formal analysis: **Mitsuko Sutoh and Shunji toyohara and Ryoji Hokao:** Methodology, animal analysis **Chunmei Cheng:** Writing – review & editing, **Koichi Tsuneyama:** Project administration, Funding acquisition, Supervison.

Acknowledgments

We would like to thank Professors Yoshimi Bando and Hisanori Uehara in Tokushima University Hospital for their kind and thoughtful scientific advice. We also would like to thank Megumi Kume and Hitomi Umemoto for their diligent and exquisite technique assistance during the histological experiments.

Disclosure statement

Tomoko Kobayashi, Mayuko Ichimura-Shimizu, Takeshi Oya, Hirohisa Ogawa, Minoru Matsumoto, Yuki Morimoto, Mitsuko Sutoh, Syunji Toyohara, Ryoji Hokao, Koichi Tsuneyama declare that they have no conflict of interest.

Compliance with Ethical Requirements

Informed Consent in Studies with Human Subjects: This article does not contain any studies with human subjects. Animal Studies: All institutional and national guidelines for the care and use of laboratory animals were abided by the researchers involved in this study. The animal experiment guidelines specified in the Institute for Animal Reproduction (Ibaraki, JAPAN), (Permission number: IarAW No. 27-11) which were well aligned with the rules of guidance on animal research ethics from the International Association of Veterinary Editors' Consensus Author Guidelines on Animal Ethics and Welfare, were followed in all the animal experiments.

Financial support

This work was supported by a The Japan Society for the Promotion of Science (JSPS) KAKENHI Grant-in-Aid for Scientific Research (A) Number 17H00881 and (C) Number 18K07069.

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