on resident CCR9-expressing pDCs in the liver because emerging evidence has shown that immature pDCs function as anti-inflammatory cells.[13] We found a higher proportion of $CCR9^+$ pDCs cells in the liver, which preferentially express Siglec-H, B220, PDCA-1, and CD8α, but which show lower expression of major histocompatibility complex class II and CD80 and CD86 than macrophages/cDCs. Moreover, liver $CCR9^+$ pDCs showed a tolerogenic function; they suppressed the proliferation of naive CD4 T cells and instructed these cells to become $Foxp3^+$ regulatory T cells. It is likely that pDCs induce regulatory T cells in the inflamed liver rather than in the lymph nodes, because pDCs are generally recognized to be resident cells and do not migrate to the lymph nodes.[29]

These findings suggest that liver pDCs in the steady state are key players in maintaining liver tolerance. However, after administration of con A, the change in the actual number of pDCs in the liver and the phenotypic characteristics of $CCR9^+$ pDCs was low. Furthermore, con A-treated *CCR9*$^{-/-}$ mice did not develop hepatitis. To solve the discrepancy, we reevaluated CCR9 expression on other APCs in the con A-treated liver and noticed a dramatic change in CCR9 expression between normal and inflammatory livers; in con A-treated mice, pDCs down-regulate CCR9 expression, whereas macrophages/cDCs increase and up-regulate CCR9 expression. This was surprising in light of previous reports indicating that CCR9 molecules are expressed on lymphocytes and pDCs but not macrophages/cDCs[8,12,13]; however, one paper recently reported CCR9-expressing macrophages in patients with rheumatoid arthritis.[30] Therefore, we reconsidered that the up-regulation of CCR9 molecules in liver macrophages during the development of con A-induced hepatitis is closely associated with the gain of inflammatory responses.

Because *CCR9*$^{-/-}$ mice do not show spontaneous liver inflammation, it is possible that the induction of liver inflammation requires the action of specific inflammatory $CCR9^+$ macrophages rather than down-regulation of CCR9 expression in liver pDCs. In this regard, the finding that *CCR9*$^{-/-}$ mice injected with con A and transferred with $CCR9^+$ macrophages from con A-treated WT mice developed hepatitis, although this was not seen with $CCR9^+$ pDCs, $CCR9^-$ pDCs, or $CCR9^-$ macrophages from con A-treated WT mice, clearly indicates that the liver $CCR9^+$ macrophages are the key inflammatory cells responsible for the induction of con A-induced T-cell hepatitis. It is quite important to notice that $CCR9^+$ macrophages play a role not only in this con A model but also in the acute carbon tetrachloride-induced hepatitis model, indicating a broad pathologic involvement of the CCR9/CCL25 axis in acute liver inflammation.

It was still unclear which cell subsets were mainly responsible for conducting acute liver injury in this model. To clarify this issue, we first showed that the accumulation of $CCR9^+$ macrophages in the liver is not sufficient to establish hepatic injury in *RAG-2*$^{-/-}$ mice, in which T cells are genetically absent. Importantly, the absence of $CCR9^+$ macrophages resulted in milder hepatitis with less activation of CD4 T cells and NKT cells in *CCR9*$^{-/-}$ mice. In addition, the adoptive transfer of $CCR9^+$ macrophages resulted in recovery from hepatic injury at least partially by activation of CD4 T cells. These results suggest that the initial induction of $CCR9^+$ macrophages in the liver and subsequent interaction between $CCR9^+$ macrophages and T/NKT cells are essential in this model.

Collectively, our data show for the first time that the emergence of CCR9-expressing macrophages in the liver is centrally involved in inducing liver inflammation, resulting in the development of inflammatory liver diseases. In terms of the clinical relevance, not only the efficacy of anti-CCL25 mAb for the prevention of con A–induced hepatitis, but also the presence of human counterpart cells of murine $CCR9^+$ macrophages, $CCR9^+CD14^+$ $CD16^{high}$ cells, in human PB of AH clearly indicate that blockade of the CCR9-CCL25 pathway would provide a feasible strategy for treating human AH. Therefore, our proposal for treating acute liver inflammation, such as fulminant liver injury by hepatitis viruses or autoimmune hepatitis, is to target pathologic $CCR9^+$ macrophages/monocytes. The present results point to what we believe to be a novel treatment for these diseases in the near future; however, further assessments are warranted in terms of the specific sites in the liver (center for liver immunity) that produce CCL25 and the modulating factor to induce CCR9 expression in macrophages.

Supplementary Materials

Note: To access the supplementary material accompanying this article visit the online version of *Gastroenterology* at www.gastrojournal.org and at doi:10.1053/j.gastro.2011.10.039.

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Conflicts of interest

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ORIGINAL ARTICLE

Novel electric power-driven hydrodynamic injection system for gene delivery: safety and efficacy of human factor IX delivery in rats

T Yokoo[1,4], K Kamimura[1,4], T Suda[1], T Kanefuji[1], M Oda[2], G Zhang[3], D Liu[3] and Y Aoyagi[1]

The development of a safe and reproducible gene delivery system is an essential step toward the clinical application of the hydrodynamic gene delivery (HGD) method. For this purpose, we have developed a novel electric power-driven injection system called the HydroJector-EM, which can replicate various time–pressure curves preloaded into the computer program before injection. The assessment of the reproducibility and safety of gene delivery system *in vitro* and *in vivo* demonstrated the precise replication of intravascular time–pressure curves and the reproducibility of gene delivery efficiency. The highest level of luciferase expression (272 pg luciferase per mg of proteins) was achieved safely using the time–pressure curve, which reaches 30 mm Hg in 10 s among various curves tested. Using this curve, the sustained expression of a therapeutic level of human factor IX protein (>500 ng ml^{-1}) was maintained for 2 months after the HGD of the pBS-HCRHP-FIXIA plasmid. Other than a transient increase in liver enzymes that recovered in a few days, no adverse events were seen in rats. These results confirm the effectiveness of the HydroJector-EM for reproducible gene delivery and demonstrate that long-term therapeutic gene expression can be achieved by automatic computer-controlled hydrodynamic injection that can be performed by anyone.

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Keywords: gene delivery; hydrodynamic gene delivery; hemophilia; human factor IX

INTRODUCTION

Gene therapy has shown promising results in treating major disease, including cancer, cardiovascular diseases and hemophilia,[1] and the popularity of gene therapy for these diseases continues to grow. Among the various methods of gene transfer, hydrodynamic gene delivery (HGD) was first reported to be a simple and effective gene delivery method for the mouse liver in 1999.[2,3] Since then, HGD has been extensively studied and used in small animals for various types of gene delivery research, including gene expression, gene functional analysis, gene regulation, drug metabolism, establishment of animal models, drug discovery, viral vectors and nanoparticles (for recent reviews, see Suda and Liu,[4] Herweijer and Wolff,[5] Kamimura and Liu,[6] Kamimura *et al.*[7] and Bonamassa *et al.*[8]). The method of HGD consists of the rapid and pressurized injection of a large amount of isotonic DNA solution into a target organ *via* vessels.[9–12] However, traditional HGD technology has not been directly applied to humans owing to safety issues related to an unexpected large volume and high intravascular pressure during gene delivery, and difficulty in maintaining reproducibility due to the variability of responsiveness to the pressure in target organs.

In the past decade, our team and others have improved several aspects of the HGD procedure in addressing these issues. First, a catheter-based procedure was included to reduce injection volume and achieve site-specific DNA delivery.[13–18] Eastman *et al.*[13] reported successful isolated liver-targeted HGD in rabbits with a volume of 15 ml kg^{-1}; Yoshino *et al.*[14] reported single-lobe-specific gene delivery in pigs using catheterization and occlusion of the portal vein (PV); and Alino *et al.*[15] reported successful gene delivery of the human α-1 antitrypsin gene by catheterization. Fabre *et al.*[16] isolated the liver by double occlusions in the inferior vena cava (IVC) and performed whole liver-targeted HGD from the IVC.[16] A recent report from Alino's group[19] demonstrated the efficacy of this procedure in *ex vivo* human liver segments.[19] Second, we developed a CO_2-pressurized injector, called the 'HydroJector', for a sustained injection power for the maintenance of injection parameters.[20] This injector cleared the problem of a sustained flow rate injection by traditional manual hydrodynamic injection or simple pump injection associated with high variability in gene expression *in vivo* due to resistance in the area.[14] We recently showed that these two improvements yield a catheter-based regional liver lobe-specific HGD by inserting the catheter into each hepatic vein followed by the CO_2-pressurized injection of gene delivery. We called this development the 'Image-guided regional HGD', and it achieved 100- to 1000-fold improved gene expression compared with previous reports,[14,15] with a volume of 1% body weight (BW) that was 10-fold less than was used in those reports. Moreover, the sequential injection of multiple lobes can be safely performed with this procedure.[17] We also reported the applicability of this procedure to swine muscle.[18] While carefully studying the physiological data and gene delivery efficiency of 'Image-guided regional HGD', we determined that the level of

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gene expression in the liver lobe after HGD depends on the intravascular pressure upon injection, which is affected by various factors, including placement of the catheter, leakage to the IVC, intravenous anastomosis, balloon inflation and differences in histological structures such as fibrotic tissue.[17] For example, in swine liver, sustained injection power of 300 psi for 15 s resulted in luciferase activity of 10^7 relative light units (RLU) per mg of proteins in the right medial, left medial and left lateral lobes, and 10^4 level in the right lateral lobe. This 1000-fold difference was proven to be due to the anatomically larger number of leakage pathways to IVC of the solution from the right lateral lobe.[17] The time–pressure curve during these injections showed a sustained peak pressure of 75 mm Hg over the entire injection period in area achieved 10^7 level and an irregularly shaped curve reaching a peak pressure of 70 mm Hg in 5 s and declining slowly thereafter for 10 s was seen in 10^4 area. These results indicate that the time–pressure curve upon the injection is significantly related to gene delivery efficiency and reproducibility.[17,18]

Therefore, we realized that the remaining challenge for the use of the catheter-based 'Image-guided regional HGD' procedure in humans is to control the time–pressure curves for the precise reproducibility and controllability of gene delivery. For this purpose, we have developed a novel electric power-driven hydrodynamic injection system, called the HydroJector-EM, and evaluated its gene delivery efficiency, reproducibility and safety in this study. The significant advantage of this injector is its precise replication of time–pressure curves that have been preloaded into the program for various targets by flexibly controlling the injection power. This has never been achieved with a manual injection or a simple pump. Furthermore, the HydroJector-EM consists of clinically approved parts and disposable products, such as syringes and injection tubes, for future clinical applications. Here we report for the first time the precise reproducibility and controllability of safe and efficient HGD using our new system. Our long-term study demonstrated sustained therapeutic gene expression of human factor IX in rats. The results indicate that anyone can achieve safe, efficient and therapeutic HGD using this new system; thus, the clinical applications of HGD procedures can be realized.

RESULTS

A major obstacle in developing clinically applicable HGD technology is precise reproducibility of gene delivery and safety of the procedure. The HydroJector-EM developed in this study can precisely replicate time–pressure curves that have been preloaded into the program and reproduces safe and effective gene delivery.

Development of the new gene injector

A major advantage of the HydroJector-EM is the flexible and fine computer control of the injection power to replicate precisely the programmed time–pressure curves for safe, efficient and reproducible gene delivery, which is unable to be achieved by the manual or the simple pump. In this new system, firstly we preload time–pressure curves before injection (Figure 1a, Step 1). Then, the electric power-driven injector pushes a sterilized syringe and injects its solution into the target region (Figure 1a, Step 2). The actual pressure in the target region during injection is transmitted to the programmed computer every 0.05 s through a pressure sensor placed in the vasculature connected to the target organ, such as PV in the liver, and amplifier (Figure 1a, Step 3). The system employs a pulse-width modulation control according to the pressure, and a program stored in the computer moves the syringe set in the injector and produces a preloaded time–pressure curve (Figure 1a, Step 4). If the actual pressure in the region is lower than the preloaded pressure curve, it sends the signal for a larger output to increase the pressure inside by a faster injection, and *vice versa*. This is the major difference from the previously developed CO_2-pressurized injector that provides sustained injection power output. To assess the controllability of this injector *in vitro*, various pressure curves—exponential (Figure 1b), convex (Figure 1c), stepwise (Figure 1d) and frequent spike (Figure 1e) pressure patterns—were preloaded into the system (gray solid lines in Figure 1), and hydrodynamic injections were performed into a specially constructed pressure-resistant reservoir connected to the HydroJector-EM. The actual pressure (black solid lines in Figure 1), and electromotor output power during the injections (black dotted lines in Figure 1) showed precise control of pressure and motor power over a short period, and even complex pressure patterns were precisely reproduced, suggesting a high level of controllability of the HydroJector-EM. This controllability of pressure during injection is important for reproducibility of an injection, as well as for safety in future human gene therapy, because it avoids unexpected too high pressure.

Pressure controllability of the HydroJector-EM *in vivo*

To examine whether the HydroJector-EM could reproduce the injection controllability *in vivo*, HGD was performed in rat livers using a catheter-based procedure (Figure 2). An injection catheter was inserted into the IVC and another catheter was placed in the PV close to the liver and was connected to a pressure sensor, which sent actual intrahepatic pressures to the programmed computer controlling the HydroJector-EM (Figure 2a). Using the same HydroJector-EM settings and various preloaded exponential time–pressure curves optimized *in vitro* (see Identification of the ideal time–pressure curves under Materials and methods section), pCMV-Luc plasmid solution in saline (5 μg ml^{-1}) was hydrodynamically injected into the whole liver. The representative time–pressure curves of i–iv are shown in Figure 2b. They were set to exponentially reach peak pressures of 15 mm Hg in 10 s (i), 15 mm Hg in 20 s (ii), 30 mm Hg in 10 s (iii) and 30 mm Hg in 20 s (iv) (see Materials and methods section). The actual intravascular pressures closely followed the preloaded computerized curves throughout the entire injection period (Figure 2c). The peak pressure achieved showed $<5\%$ delineation from the preloaded curves (for pressure i, actual pressure achieved 0.7 mm Hg higher than the preloaded 15 mm Hg), and the differences in injection volume between each animal injected with the same time–pressure curve were $<0.47\%$ BW (Figure 2d). These data support the high controllability of the HydroJector-EM *in vivo* using catheter-based procedure, resulting in reproducible hydrodynamic injection by replicating the time–pressure curves.

Efficiency and reproducibility of the HydroJector-EM-mediated gene delivery

The above time–pressure curves, using a manual injection method as a control, were completed safely, and liver, heart, lung and kidney tissues were harvested 3 h after the injections. The injection volume and time period for manual injection was set to be the same as curve iii with sustained injection flow rate showing a simple linear proportional time–pressure curve. The highest level of luciferase expression of 272 pg luciferase per mg of extracted proteins was obtained with pressure curve iii. This was 2–20 times higher than the levels achieved with pressure curves i, ii and iv, and with manual injection ($P<0.05$ for 'manual vs iii', but $P<0.01$ for 'iv vs iii' and $P<0.001$ for 'ii vs iii' and 'iv vs iii'; Figure 3a). No luciferase activity was detected in the heart, lung and kidneys (data not shown). Immunohistochemical staining using an anti-luciferase antibody was performed on livers collected 3 h after injection with pressure curves (Figures 3b–g). The largest number of the positively stained cells, 12.6%, was obtained with pressure curve iii, and was significantly 2.6–10 times higher than the levels achieved with pressure curves i, ii and iv ($P<0.001$ for 'i vs iii', 'ii vs iii' and 'iv vs iii'; Figure 3h). The level was

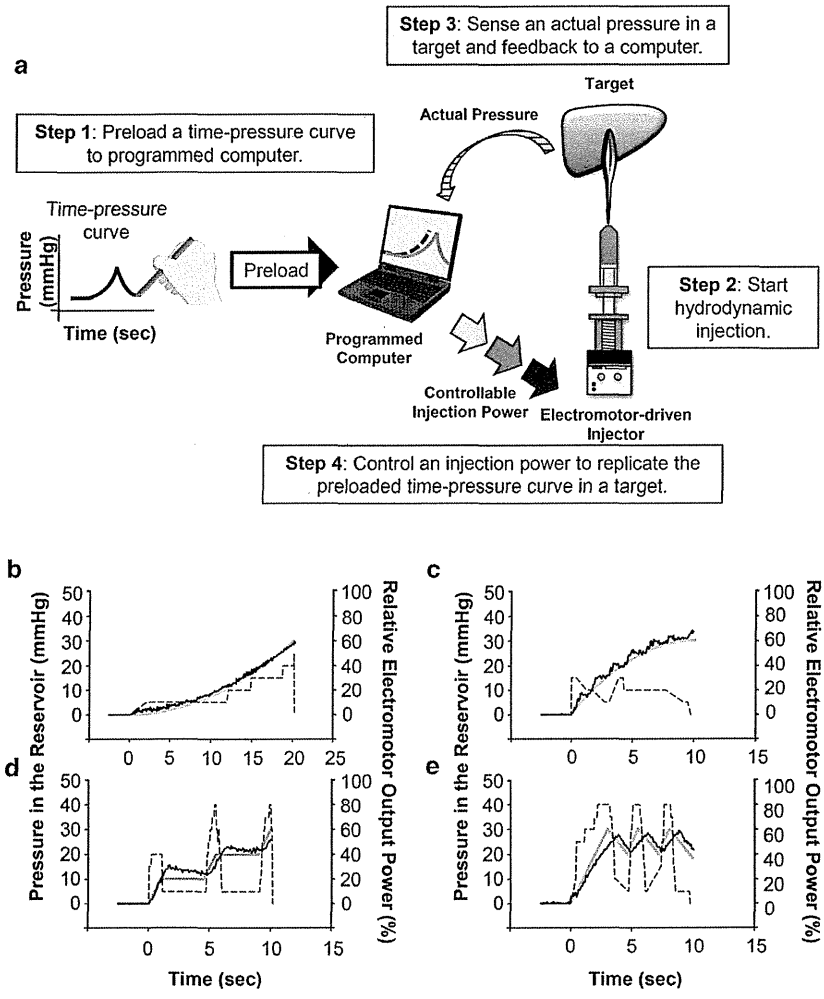

Figure 1. Development of the HydroJector-EM. Various time–pressure curves can be preloaded to the programmed computer. The electric power-driven injector injects a solution hydrodynamically by pushing the sterilized syringe. The actual pressure in the target during the injection is transmitted to the programmed computer every 0.05 s upon the injection through a sensor and amplifier. According to the actual pressure, the programmed computer controls the output electric-injection power moving to reproduce this preloaded pressure in the target region (**a**). Assessment of the controllability of the HydroJector-EM *in vitro* using a pressure-resistant reservoir connected to the HydroJector-EM (**b–e**). The exponential (**b**), convex (**c**), stepwise (**d**) and frequent spike (**e**) time–pressure patterns were preloaded to the system and tested for the reproducibility. Gray solid lines, black solid lines and black dotted lines represent time-dependent preloaded pressure, actual *in vitro* pressure and relative electromotor output power to its capacity, respectively.

not significantly different from the 10% positive cells obtained with manual injection (Figure 3h). These results suggest efficient and reproducible gene delivery by the HydroJector-EM and that pressure curve iii, which injects with a higher pressure over a shorter period, resulting in a more efficient gene delivery.

Physiological impacts

To examine the safety of our system, hematoxylin and eosin staining on the liver tissue and serum biochemical analysis were performed. The pressure curve iii injection, which achieved highest pressure over the shortest time among those tested, was compared with manual injection. Hematoxylin and eosin staining was performed on the liver tissues before (Figure 4a), immediately after (Figures 4b, c) and 3 h after (Figures 4d, e) the HydroJector-EM controlled injection with preloaded time–presure curve iii (Figures 4b, d) and manual injection (Figures 4c, e). There was a marked enlargement of the central veins and sinusoidal structure immediately following the injections (Figures 4b, c), and there were no marked differences between the injections. These enlargements recovered by 3 h post-injection, and no marked destruction of hepatocytes or necrotic tissues were seen 7 days after the injection (Figure 4f).

Blood samples were collected from the catheter placed in the IVC at the same time points as for the hematoxylin and eosin assays, and markers of liver status, including aspartate aminotransferase (AST), alanine aminotransferase (ALT) and lactate dehydrogenase (LDH) in the serum, were analyzed to assess tissue damage after injection with pressure curve iii and manual injection (Figures 4g, h). AST, ALT and LDH showed peak levels of 1418, 911 and 6671 IU ml^{-1}, respectively, 1 h after injection with pressure curve iii and 1228, 932 and 9065 IU ml^{-1}, respectively, 1 h after manual injection. These levels returned to normal 7 days after both injections, and no significant differences were seen. These results suggest that there are no significant differences in the physiological impact of computer-controlled and manual injections. These results also indicate that the HydroJector-EM can achieve efficient, reproducible and safe gene delivery *in vivo*.

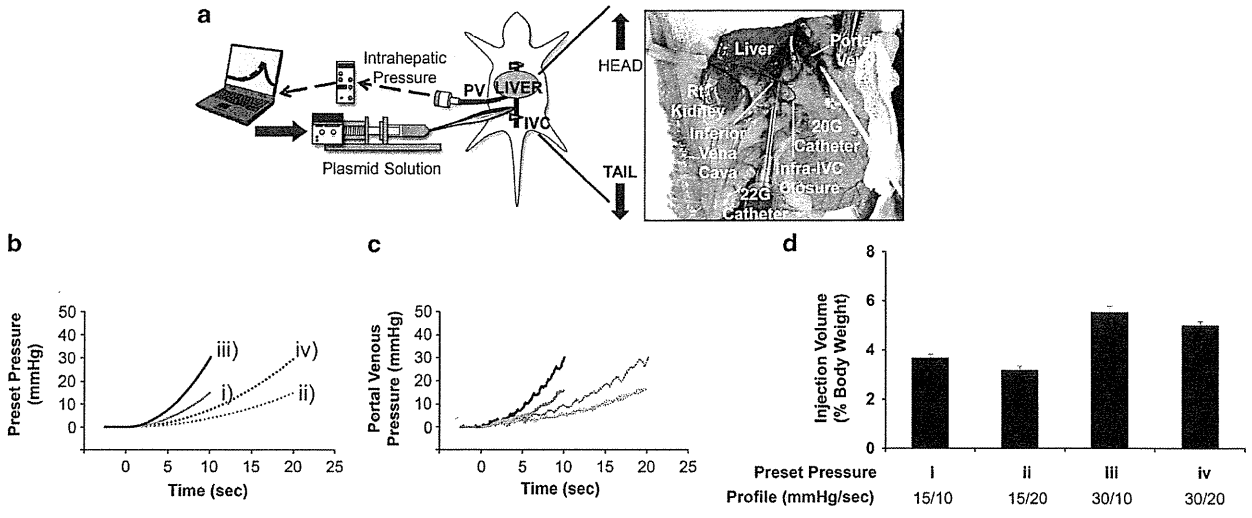

Figure 2. Pressure controllability of the HydroJector-EM in rat liver. (**a**) A pressure transducer was connected to a catheter (20 G) placed in the PV. Intravascular pressure was transmitted from the catheter to the computer controlling the electric power-driven gene injector. The injection catheter (22 G) was placed *via* the IVC of a rat (200 g) at the junction of the IVC and the hepatic veins followed by an injection of plasmid solution under temporal blood flow occlusions at the supra- and infrahepatic IVC. Various time–pressure curves were examined in rat liver to test whether intravascular pressure can be precisely controlled by the HydroJector-EM *in vivo*. (**b**) Time–pressure curves tested (peak pressure/injection period): i, gray solid line, 15 mm Hg/10 s; ii, gray dotted line, 15 mm Hg/20 s; iii, black solid line, 30 mm Hg/10 s; iv, black dotted line, 30 mm Hg/20 s. (**c**) Time-dependent actual portal venous pressures during the HydroJector-EM-controlled hydrodynamic injection. (**d**) The averages of injected volumes for pressure curves i–iv. The values represent mean ± s.d. ($n = 3$ for each curve).

Long-term gene expression study

Based on the above results, the pBS-HCRHP-FIXIA plasmid was hydrodynamically injected into rats using the HydroJector-EM, to examine the reproducibility of sustained gene expression. Pressure curve iii, which resulted in the highest gene delivery efficiency in the previous section, was utilized for the study, and the serum level of human factor IX was analyzed by enzyme-linked immunosorbent assay for 112 days. Additionally, injection using pressure curve i reaching 15 mm Hg and manual injection of 6% BW were tested for the same period. Human factor IX achieved its highest level of $5390 \pm 474\,ng\,ml^{-1}$ 7 days after injection with pressure curve iii. This was 17-fold higher than the level achieved with pressure curve i ($317 \pm 37\,ng\,ml^{-1}$; $P<0.001$) and 2.7-fold higher than the level achieved by manual injection ($2030 \pm 374\,ng\,ml^{-1}$; $P<0.01$). Human factor IX slowly decreased to the background level by 80 days after injection. Immunohistochemical staining using anti-human factor IX antibody was performed to confirm the results. Positively stained hepatocytes were detected 7 days after the injections (Figure 5, black arrows). Pressure curve iii injection resulted in 9.7% positively stained cells in four different sections (Figure 5d), whereas manual injection (Figure 5e) and injection with curve i (Figure 5c) resulted in 7.4% and 5.5% positive cells, respectively, while no stained cells were seen in saline-injected rats liver (Figure 5b). In addition, the level returned to this control level 112 days after the injection. Altogether, these data suggest that the HydroJector-EM gene transfer protocol can deliver plasmids safely and efficiently, and achieve a therapeutic level of human factor IX protein ($>500\,ng\,ml^{-1}$) sustained for 2 months.

DISCUSSION

The list of applications for gene therapy is growing rapidly, including cancer, heart disease and hemophilia.[1] Among the various gene delivery methods, HGD is relatively new,[7] having first been reported by Liu *et al.*[2] and Zhang *et al.*[3] in 1999 as a simple and efficient gene delivery method through the tail veins of small animals. The key aspects for successful HGD include injection power, intravascular pressure, volume, speed and time period.[9–12] Various modifications have been made to the original procedure to apply this method to human gene therapy. The catheter-based procedure contributed to the reduction of injection volume[13–19] and the CO_2-pressurized injection device, the HydroJector, maintained injection parameters, such as sustained injection power, volume, speed and period for examining the optimum parameters for gene delivery efficiency and safety in large animals.[20] We have proven that the combination of these developments have achieved liver lobe- and target muscle-specific gene delivery in large animal and called 'Image-guided regional HGD'.[17,18] To further extend the clinical applicability of our technology, we have focused on the gene injection time–intravascular pressure curves to ensure the reproducibility and controllability of gene delivery in this study and developed a new electric power-driven injector, the HydroJector-EM. We have demonstrated that: (1) it can precisely replicate the time–pressure curves preloaded into the system before HGD in animals and ensure reproducible gene delivery to various targets by flexibly changing the electric-injection power; (2) it consists of clinically approved parts and disposable products; and (3) it utilizes electric power as a physical force for HGD rather than the CO_2 gas used in previous studies, thereby reducing the risk of gas embolism (although this small amount of CO_2 gas is also used for phase contrast in angiography with patients who have allergies to contrast enhancements).

The HydroJector-EM showed efficient gene delivery with time–pressure curve iii reaching 30 mm Hg in 10 s, which was the highest pressure over the shortest period among tested. The level of luciferase expression was higher than the manual injection utilizing the same volume and period, probably due to the difference in pressure–time curves, 'exponential' with iii and simple 'linear proportional line' with fixed flow rate manual injection. However, the manual injection also achieved the therapeutic level of hFIX, although lower level than curve iii, the proximity of the efficacy of both procedures was revealed. The significant advantage of our injector and the difference from

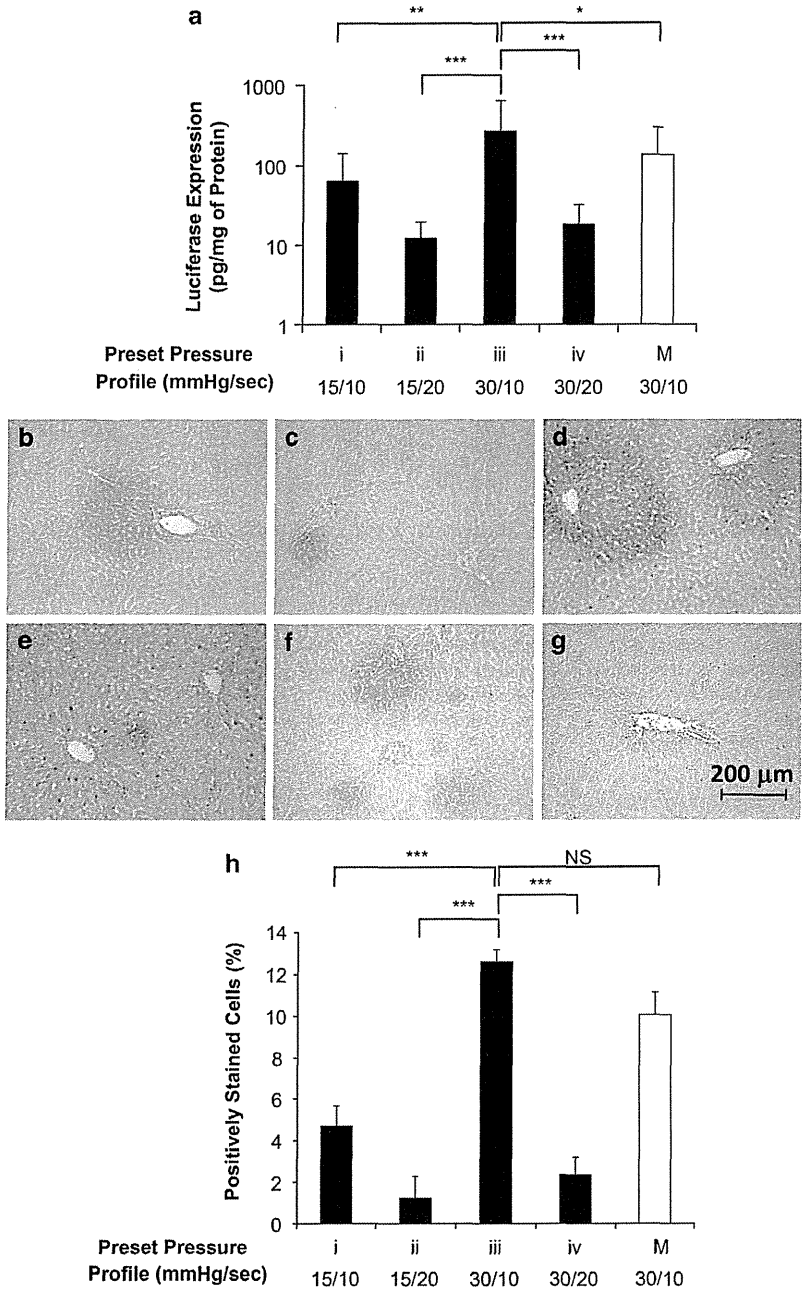

Figure 3. Gene delivery efficiency in rat liver. (**a**) A total of 12 rats received an injection of pCMV-Luc plasmid DNA using preloaded time–pressure curves shown in Figure 2b, and were euthanized 3 h later for tissue analysis of luciferase expression. Manual injection (M) with a volume of 6% BW in 10 s was performed as a control in three rats. The values represent mean ± s.d. (five samples from every liver lobe per rat from all three rats in each group, 15 samples for each curve and manual injection). Immunohistochemical (IHC) staining with anti-luciferase antibody was performed on liver tissues collected (**b**–**f**). Scale bar represents 200 µm (× 100). (**b**) Injected with curves of i; (**c**) ii; (**d**) iii; (**e**) iv; (**f**) manual injection; and (**g**) untreated animals. (**h**) Quantitative analysis of positively stained cells. Four different liver sections from three rats in each group and untreated rats were immunohistochemically stained with anti-luciferase antibody and a quantitative analysis was performed using the ImageJ software (version 1.6.0_20; National Institutes of Health). The values represent mean ± s.d. for $^{*}P<0.05$, $^{**}P<0.01$, $^{***}P<0.001$ and NS, no statistical significance. One-way analysis of variance (ANOVA) followed by Bonferroni's multiple comparison test.

the manual injection is that the reproducibility of our injection showing the less variability of gene expression in animals (Supplementary Figure S1). Since the gene delivery efficiency by the sustained flow rate injection by manual or single pump was affected by the possible leakage due to the intravenous anastomosis in large animals and the high intravascular pressure due to the species specific structure of target organ tissue, such as fibrotic tissue in pigs liver, this reproducibility achieved by the HydroJector-EM, that flexibly control the injection power, will exceed these matters and be efficient in the gene delivery in various large animals because the injection power changes according to the actual intravascular pressure upon the injection. The serum levels of AST, ALT and LDH reached peak in 1 h after the injection as reported previously,[12] with a traditional tail vein

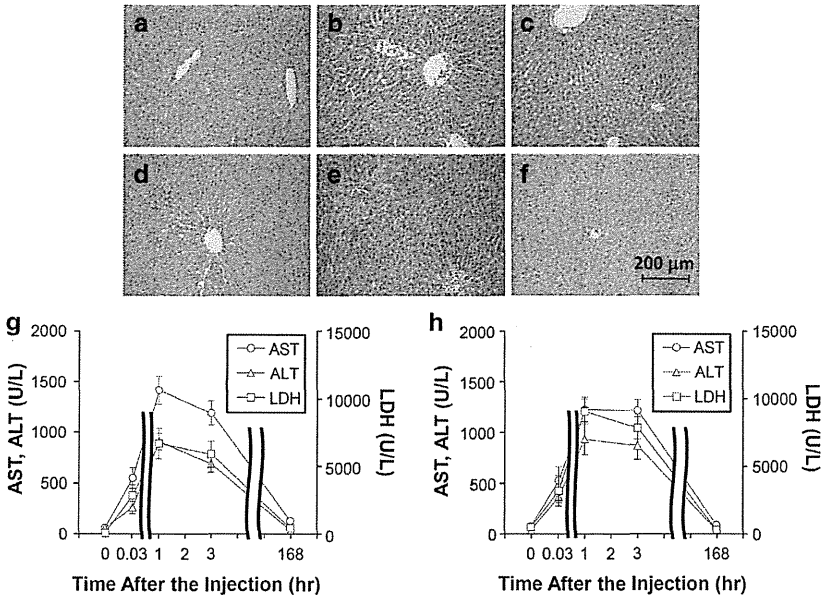

Figure 4. Physiological impacts in rats. Hematoxylin and eosin (HE) staining was performed on the liver tissue before (**a**), immediately after (**b**, **c**), 3 h (**d**, **e**) and 7 days after the HydroJector-EM controlled injection with time–pressure curve iii (**b**, **d**, **f**) and manual injection (**c**, **e**). Scale bar represents 200 μm (× 100). Blood biochemical analyses after the injections with pressure curve iii (**g**) and manual injection (**h**). Blood samples were collected from the catheter placed in the IVC before (time = 0), 2 min, 1 h, 3 h and 7 days after the injections. The values represent average of AST, ALT and LDH in the serum ($n = 3$ from each group). Open circular, triangular, square markers represent AST, ALT and LDH, respectively. The values represent mean ± s.d.

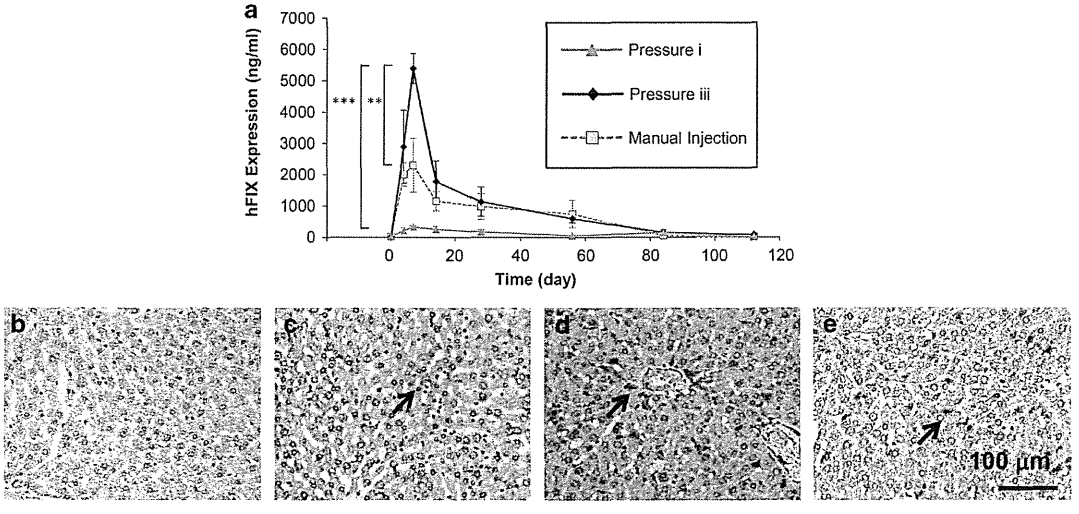

Figure 5. Long-term gene expression in rat liver. (**a**) Plasma concentrations of human factor IX. Blood samples were collected before, 4, 7, 14, 28, 56, 84 and 112 days after the hydrodynamic injection of human factor IX expressing plasmid DNA with pressure curves i and iii, and manual injection. Enzyme-linked immunosorbent assay was performed on those samples using a mouse anti-hFIX monoclonal antibody for plate coating, and the horse radish peroxidase-conjugated goat anti-hFIX polyclonal antibody. The values represent mean ± s.d. ($n = 3$ for each group). (**b**–**e**) Liver tissues were collected 7 days after the injection and IHC was performed with rabbit anti-hFIX polyclonal antibody (**b**, saline injected with curve of iii; **c**, plasmid injected with curve i; **d**, iii; **e**, manual). Scale bar represents 100 μm (× 200). Black arrows represent positively stained cells. $^{**}P < 0.01$, $^{***}P < 0.001$. Two-way factor repeated-measure analysis of variance (ANOVA) test followed by Bonferroni test. Gray solid line with gray triangular markers, black solid line with black diamond-shaped markers and gray dotted line with gray square markers represent hFIX concentration with pressure curve i, iii and manual injection.

injection of 7.5% BW volume. The level was 10-fold higher than that achieved by the tail vein injection,[12] probably due to the difference in injection site since our catheter-based procedure directly injects to the whole liver with temporal blood flow occlusion at IVC to reduce the injection volume.

Our long-term study showed a promising therapeutic level of hFIX expression that was sustained for more than 2 months. The level achieved was comparable to the results shown in previous studies by Miao's group[21–23] using mouse tail vein injection and ultrasound. For hemophilia gene therapy, a number of studies, especially those using an adeno-associated virus vector, have shown therapeutic and sustained hFIX expression in animals (for recent reviews, see Tuddenham[24] and Benveniste[25]). Interestingly, Brunetti-Pierri *et al.*[26] utilized a catheter-based procedure for

helper-dependent adenoviral vector transfer to the monkey liver and showed increased liver-specific transduction showing therapeutic level of gene expression with reduced systemic vector dissemination, suggesting the importance of a catheter-based procedure. While viral vectors are highly effective and have been used in quite a few clinical trials, the intrinsic carcinogenesis- and immune-response-stimulating properties of viral genomes and proteins remain the largest hurdle, and the injection of plasmid DNA is considered to be safer.[7] Therefore, the results obtained in this study suggest a promising option for hemophilia gene therapy, as the automatic-injector delivery of simple naked DNA achieved a therapeutic level that has never before been seen in rats. The slow decrease to the background level by 80 days after injection was probably due to factors, such as methylation of plasmid,[27] antibody to the human factor IX.[28]

In addition, the reproducible gene delivery efficiency based on the intravascular time–pressure curve may enable us to estimate the therapeutic effect upon gene delivery, and this new system suggests versatile applications, including combining with virus vector and protein delivery, among other possibilities.[9,20]

In summary, we have reported on the development and effectiveness of the HydroJector-EM. We have confirmed its reproducibility and controllability for gene delivery and long-term therapeutic gene expression. An assessment of its effectiveness in large animals, organs and disease models combined with a catheter-based procedure will be the next logical steps toward clinical applications. The fine-tuning of the procedure and plasmid engineering is also a necessary prerequisite for clinical trials of HGD.

MATERIALS AND METHODS

Materials

The pCMV-Luc plasmid containing firefly luciferase cDNA driven by a CMV promoter was purified by the method of CsCl-ethidium bromide gradient centrifugation and kept in a Tris-EDTA buffer. The pBS-HCRHP-FIXIA plasmid containing human factor IX gene driven by hAAT promoter was kindly provided by Dr Carl Miao's lab.[22] The purity of the plasmid preparation was checked by absorbency at 260 and 280 nm and 1% agarose gel electrophoresis. The luciferase assay kit was from Wako Pure Chemical Industries Ltd (Chuo-ku, Osaka, Japan). The electric power-driven motor for the injector was from Tsugawa EW Co. Ltd (Nishinari-ku, Osaka, Japan). The pressure transducer was from Kyowa Electronic Instruments Co. Ltd (Chofu, Tokyo, Japan). The charger amplifier unit AG3101 was from NEC Avio Infrated Technologies Co. Ltd (Shinagawa-ku, Tokyo, Japan). Wistar rats (female, 150–200 g) were purchased from Japan SLC Inc. (Hamamatsu, Shizuoka, Japan).

Hydrodynamic injection into rat liver

All animal experiments were conducted in full compliance with regulation and approved by the Institutional Animal Care and Use Committee at the Niigata University (Niigata, Japan). A midline skin incision was made under general anesthesia using isoflurane and 2,2,2-tribromoethanol (concentration, 0.016 g ml^{-1} in 0.9% saline; dose, 1.25 ml/100 g BW). With a small incision in the abdominal wall, an injection catheter (SURFLO 22 gauge; Terumo, Sibuya-ku, Tokyo, Japan) was inserted *via* the IVC and its tip placed at the junction of the IVC and the hepatic veins. A pressure transducer was connected to another catheter (SURFLO 20 gauge; Terumo) placed in the PV in hepatic hilar side, which reflected the intrahepatic sinusoidal pressure upon the injection.[29] Saline containing either pCMV-Luc or pBS-HCRHP-FIXIA plasmid DNA (5 μg ml^{-1}) was injected into the liver *via* the HydroJector-EM under the program with temporal blood flow occlusions at the supra- and infrahepatic IVC as used in small animals[20] and in non-human primates.[26,30] The incision made at the animals' abdomen was sutured after the procedure.

Identification of the ideal time–pressure curves

HGD was performed in rats with different settings of preloaded exponential time–pressure curves. Among the various pressure curves tested *in vitro* shown in Figure 1, exponential curves with a peak pressure of 15 and 30 mm Hg and injection period of 10 and 20 s were chosen for the gene delivery analysis since the other curves with higher pressure and longer period showed intolerableness in rats cardiovascular condition.

Manual injection procedure

To examine the safety and efficiency of the HydroJector-EM controlled injections, the steady manual injection of 6% BW, the same volume as used in pressure curve iii (5.5 ± 0.47%) in 10 s was performed.

Lucierase assay

HGD of pCMV-Luc plasmid DNA was performed with different settings of preloaded time–pressure curves. The rat was euthanized 3 h after the injection of pCMV-Luc plasmid DNA and tissue samples were collected from each lobe of the liver, kidney, lung and heart. Tissue samples for the luciferase assay were kept in −80 °C until use. A measure of 1 ml of lysis buffer (0.1 M Tris–HCl, 2 mM EDTA and 0.1% Triton X-100, pH 7.8) was added to each sample (~200 mg) and the samples were homogenized for 30 s with the tissue homogenizer (ULTRA-TURRAX T25 Digital; IKA, Staufen, Germany) at maximum speed. The tissue homogenates were centrifuged in a microcentrifuge for 10 min at 13 000 *g* at 4 °C. The protein concentration of the supernatant was determined by Protein Assay Kit (Bio-Rad, Hercules, CA, USA) based on Coomassie blue assay strategy. A measure of 10 μl of supernatant was mixed with 100 μl of luciferase assay reagent and the luciferase activity was measured in a luminometer (Luminescencer Octa AB-2270; Atto, Bunkyo-ku, Tokyo, Japan) for 10 s according to the previously established procedure.[2] Luciferase activity obtained as the relative light units was converted to luciferase mass using standard curve established using reagents and procedure from Analytic Luminescence Laboratory (San Diego, CA, USA).[2] The amount of luciferase protein in the tissue extract was calculated using the equation derived from the standard curve in which luciferase protein (pg) = 7.98 × 10^{-5} RLU + 0.093 (R^2 = 0.9999).[2]

Analysis of serum hFIX concentration

Animals were hydrodynamically injected with the pBS-HCRHP-FIXIA plasmid or saline. Blood samples were collected from their tail vein before, 4, 7, 14, 28, 56, 84 and 112 days after the gene delivery according to the preliminary data and plasma were collected for enzyme-linked immunosorbent assay. A mouse anti-hFIX monoclonal antibody (clone HIX-1, F2645l Sigma, St Louis, MO, USA) was used for plate coating, and the horse radish peroxidase-conjugated goat anti-hFIX polyclonal antibody (GAFIX-HRP; Affinity Biologicals, Ancaster, ON, Canada) was used as the second antibody for binding to hFIX that bound to the well.

Immunohistochemical stainings

Tissue samples for immunohistochemical stainings were collected 3 h after the injection for luciferase staining and 7 and 112 days after for hFIX staining. Four different liver sections from three rats in each group and untreated rats were immunohistochemically stained with anti-luciferase antibody or anti-hFIX antibody. Tissues were fixed in 10% formalin upon the collection and embedded in paraffin. Sections (10 μm) were made and standard immunohistochemistry was performed with a goat anti-luciferase polyclonal antibody (G7451, 1:50 dilution; Promega, Madison, WI, USA), Vecstatin Elite ABC Goat IgG Kit (PK-6105; Vector Laboratories Inc., Burlingame, CA, USA) and DAB chromogen tablet (Muto Pure Chemicals Co. Ltd, Bunkyo-ku, Tokyo, Japan) for luciferase staining. The rabbit anti-hFIX polyclonal antibody (HPA 000254, 1:100 dilution; Sigma), Vecstatin Elite ABC Rabbit IgG Kit (PK-6101; Vector Laboratories Inc.), and DAB chromogen tablet (Muto Pure Chemicals Co. Ltd) were used for hFIX staining.

Every three fields from each section (total of 36 fields for each curves and control) were captured and a quantitative analysis of positively stained cells was performed using the ImageJ software (version 1.6.0_20; National Institutes of Health, Bethesda, MD, USA) as previously reported method.[31]

Assessment of tissue damages

The blood samples were collected at the appropriate time points as in the previous study,[12] which were before (time = 0), 2 min, 1 h and 3 h after the hydrodynamic injection of pCMV-Luc from seven animals and additional data points before, 2 min and 7 days after the injection were collected from nine rats hydrodynamically injected with pBS-HCRHP-FIXIA. Automated concentration determination of ALT, AST and LDH was performed by BML

Inc. (Shibuya-ku, Tokyo, Japan). The liver tissue samples collected 3 h after the injection were analyzed by hematoxylin and eosin staining.

Statistical methods

The luciferase and enzyme-linked immunosorbent assay assays were statistically evaluated by analysis of variance followed by Bonferroni's multiple comparison test.

CONFLICT OF INTEREST

The authors declare no conflict of interest.

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Hepatology Research

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Original Article

Survey of non-B, non-C liver cirrhosis in Japan

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Aim: The aim of this survey was to reveal clinical features for each etiology of non-B, non-C liver cirrhosis (NBNC LC) in Japan.

Methods: In a nationwide survey of NBNC LC in Japan at the 15th General Meeting of the Japan Society of Hepatology, 6999 NBNC LC patients were registered at 48 medical institutions. Epidemiological and clinical factors were investigated.

Results: The percentage of NBNC LC among LC patients was 26%. NBNC LC patients were categorized into 11 types according to etiological agents: non-alcoholic steatohepatitis (NASH), 14.5%; alcoholic liver disease (ALD), 55.1%; fatty liver disease (FLD), except NASH, ALD, and other known etiology, 2.5%; primary biliary cirrhosis, 8.0%; other biliary cirrhosis, 0.8%; autoimmune hepatitis, 6.8%; metabolic disease, 0.6%; congestive disease, 0.8%; parasitic disease, 0.2%; other known etiology, 0.2%; and unknown etiology, 10.5%. Compared with previous surveys, the percentage of ALD remained unchanged, whereas that of NASH increased. The mean age and percentage of females were significantly higher in NASH patients than in ALD and FLD patients. Prevalence of diabetes mellitus was significantly higher in NASH and FLD patients than in ALD ones. Prevalence of hepatocellular carcinoma (HCC) in NBNC LC patients was 35.9%. Among NASH, ALD and FLD patients, 50.9%, 34.3% and 54.5% had HCC, respectively. Positivity of hepatitis B core antibody was significantly higher in HCC patients than in those without HCC (41.1% vs 24.8%).

Conclusion: This survey determined the etiology of NBNC LC in Japan. These results should contribute new ideas toward understanding NBNC LC and NBNC HCC.

Key words: alcoholic liver disease, hepatocellular carcinoma, non-alcoholic steatohepatitis, non-B, non-C liver cirrhosis

INTRODUCTION

A NATIONWIDE SURVEY of liver cirrhosis (LC) for each etiology has been conducted as the main theme on four occasions at the national academic conference in Japan. Therefore, many registered patients have been surveyed on uniform diagnostic criteria.[1] The 15th General Meeting of the Japan Society of Hepatology was held in October 2011. In a featured session in this meeting, we conducted a nationwide survey of non-B, non-C LC (NBNC LC) in patients at medical institutions in Japan. NBNC LC was the main theme of the featured session in this meeting for two reasons. First, the prevalence of non-alcoholic fatty liver disease (NAFLD) is increasing and has been recently reported to be approximately 20% in adults in Japan. Approximately 1% of adults in Japan are estimated to have non-alcoholic steatohepatitis (NASH).[2,3] Thus, NASH is the most common chronic liver disease not only in Western countries but also in Japan. NASH patients can develop LC and even hepatocellular carcinoma (HCC), although there have been few investigations concerning the incidence of LC associated with NASH (NASH LC)

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in Japan. Second, the number of NBNC HCC patients has been rapidly increasing, and it has been recently reported to account for approximately 15% of all HCC patients in Japan.[4] Most NBNC HCC patients seem to have LC with alcoholic liver disease (ALD LC); however, NASH LC has been noted as a high-risk group of NBNC HCC. Nevertheless, HCC complicated with NBNC LC of an unknown cause has been occasionally reported. Therefore, it is important to investigate the clinical features of NBNC LC, which will lead to the development NBNC HCC. Based on these backgrounds, we report the characteristics of NBNC LC in Japan. This was one of the programs of the 15th General Meeting of the Japan Society of Hepatology in 2011.

METHODS

Patient database

AT 48 MEDICAL institutions (all investigators are listed in Appendix I) (Table 1), 6999 subjects were diagnosed with NBNC LC based on the negative results for serum hepatitis B surface antigen (HBsAg), anti-hepatitis C antibody and hepatitis C virus (HCV) RNA. The patients registered in this study were clinically (laboratory examinations and imaging studies) and histologically diagnosed with LC based on the criteria proposed by a previous nationwide survey (the 44th Annual Meeting of the Japan Society of Hepatology in 2008).[1] The NBNC LC patients were categorized into 11 types according to etiology: (i) NASH; (ii) ALD; (iii) fatty liver disease (FLD); (iv) primary biliary cirrhosis (PBC); (v) other biliary cirrhosis (such as primary sclerosing cholangitis [PSC] and secondary biliary cirrhosis); (vi) autoimmune hepatitis (AIH) (including AIH-PBC overlap syndrome); (vii) metabolic disease (such as Wilson's disease, hemochromatosis and glycogen storage disease); (viii) congestive disease (including Budd–Chiari syndrome); (ix) parasitic disease (such as Japanese schistosomiasis); (x) other known etiology (such as sarcoidosis and drug-induced liver injury); and (xi) unknown etiology. The diagnosis of NASH was based on the following criteria: (i) absence of clinically significant alcohol consumption (intake of ≤20 g ethanol/day); (ii) appropriate exclusion of other liver diseases; (iii) complications with risk factors of steatosis such as obesity (in particular, visceral obesity), metabolic syndrome and diabetes mellitus; and (iv) the presence of steatosis on liver histology (histological

Table 1 Forty-eight medical institutions registered at the 15th General Meeting of the Japan Society of Hepatology on 2011

Akita University Graduate School of Medicine	Nara Medical University
Asahikawa-Kosei General Hospital	National Center for Global Health and Medicine
Asahikawa Medical University	Nihon University School of Medicine
(Division of Gastroenterology and Hematology/Oncology)	Niigata Prefectural Central Hospital
(Division of Metabolism and Biosystemic Science)	Niigata University Medical and Dental Hospital
Asahikawa Red Cross Hospital	Oji General Hospital
Chiba University	Osaka City University
Dokkyo Medical University	Osaka Police Hospital
Ehime Prefectural Central Hospital	Osaka Red Cross Hospital
Ehime University Graduate School of Medicine	Saiseikai Suita Hospital
Fukushima Medical University School of Medicine	Saitama Medical University
Gunma University Graduate School of Medicine	Sapporo City General Hospital
Hyogo College of Medicine	Sapporo-Kosei General Hospital
Iwate Medical University	Shinshu University School of Medicine
Jikei University School of Medicine, Katsushika Medical Center	Teikyo University School of Medicine
Juntendo University School of Medicine	Teine-Keijinkai Hospital
Kagawa University	Tokyo Medical and Dental University
Kanazawa Medical University	Tokyo Medical University Ibaraki Medical Center
Keio University School of Medicine	Tokyo Women's Medical University
Kumamoto University	Tottori University School of Medicine
Kurume University School of Medicine	University of Tokyo
Kyoto Second Red Cross Hospital	University of Yamanashi
Mie University Graduate School of Medicine	(First Department of Internal Medicine)
Musashino Red Cross Hospital	(First Department of Surgery)
Nagano Red Cross Hospital	Yamagata University Faculty of Medicine

diagnosis) or imaging studies (imaging diagnosis). The diagnosis of ALD was based on the proposed Diagnostic Criteria for Alcoholic Liver Disease by a Japanese study group for ALD (the Takada group).[5] The diagnosis of FLD was based on the following criteria: (i) alcohol consumption between that for NASH and ALD (i.e. intake of >20 g and <70 g ethanol/day); (ii) appropriate exclusion of other liver diseases; and (iii) the presence of steatosis on liver histology or imaging studies.

The following variables were used to investigate the clinical features of NBNC LC: age; sex; body mass index (BMI); prevalence of diabetes mellitus (DM), impaired glucose tolerance, hypertension and dyslipidemia; Child–Pugh classification; prevalence of gastroesophageal varices and HCC; and presence of hepatitis B core antibody (anti-HBc). In addition, the percentage of NBNC LC was investigated among all LC patients at each institution and was compared with previous reports. The ethics committees of the appropriate institutional review boards approved this study in accordance with the Declaration of Helsinki (2000).

Statistical analyses

Statistical tests were performed using the IBM SPSS Statistics ver. 21. The statistical significance of difference was determined using the χ^2-test, Mann–Whitney *U*-test and multivariate Cox's proportional hazard model as appropriate. $P < 0.05$ was considered statistically significant.

RESULTS

Percentage of NBNC LC among all LC patients

WE CALCULATED THE percentage of NBNC LC among all 25 020 LC patients at 37 registered institutions. The percentages of NBNC LC, hepatitis B virus (HBV)-related cirrhosis, HCV-related cirrhosis, and both HBV- and HCV-related cirrhosis were 26%, 12%, 60.9% and 1.1%, respectively. Compared with a previous nationwide survey (the 44th Annual Meeting of the Japan Society of Hepatology in 2008),[1] there was no significant difference between them (Table 2).

Frequency of each etiology among NBNC LC patients

We determined the frequency and percentage of each etiology among all 6999 NBNC LC patients at 48 registered institutions. The percentages of each etiology were as follows: NASH, 14.5%; ALD, 55.1%; FLD, 2.5%; PBC, 8.0%; other biliary cirrhosis, 0.8%; AIH, 6.8%; metabolic disease, 0.6%; congestive disease, 0.8%; parasitic disease, 0.2%; other known etiology, 0.2%; and unknown etiology, 10.5% (Table 3). Among 1015 NASH patients, 309 (30.4%) were diagnosed histologically, 402 (39.6%) were diagnosed by imaging studies and the method of diagnosis of 304 patients (30%) was not described in detail. Among 60 patients with other biliary cirrhosis, 71.7% had PSC and the rest had cholestatic diseases, except PBC and PSC (such as congenital biliary atresia and secondary biliary cirrhosis). Among 39 metabolic disease patients, 66.7% had Wilson's disease, 25.6% had hemochromatosis (glycogen storage disease, amyloidosis and citrullinemia in one patient each). All 12 cases of parasitic disease were Japanese schistosomiasis. Of 11 patients with other known etiology, two patients sarcoidosis, two post-liver transplantation, two post-hepatectomy, one drug-induced liver injury, one systemic lupus erythematosus-related liver injury and the diagnosis of the remaining patients was not described in detail.

Compared with the survey at the 44th Annual Meeting of the Japan Society of Hepatology in 2008,[1] the percentage of ALD among all NBNC LC patients did

Table 2 Percentage of NBNC LC among all patients with liver cirrhosis compared with the 44th Annual Meeting of the Japan Society of Hepatology on 2008[1]

	The 15th General Meeting of the Japan Society of Hepatology on 2011 ($n = 25\ 020$)	The 44th Annual Meeting of the Japan Society of Hepatology on 2008 ($n = 33\ 379$)	*P*-value
NBNC LC	26.0%	24.0%	N.S.
HBV-related cirrhosis	12.0%	13.9%	N.S.
HCV-related cirrhosis	60.9%	60.9%	N.S.
both HBV- and HCV-related cirrhosis	1.1%	1.2%	N.S.

P-values were analyzed by χ^2-test.
HBV, hepatitis B virus; HCV, hepatitis C virus; NBNC LC, non-B, non-C liver cirrhosis; N.S., not significant.

Table 3 Frequency of each etiology among patients with NBNC LC compared with the 44th Annual Meeting of the Japan Society of Hepatology on 2008[1]

	The 15th General Meeting of the Japan Society of Hepatology on 2011 ($n = 6999$)	The 44th Annual Meeting of the Japan Society of Hepatology on 2008 ($n = 8011$)	*P*-value
NASH	14.5%	8.7%	$P < 0.001$
ALD	55.1%	56.3%	N.S.
FLD	2.5%	-	-
PBC	8.0%	9.9%	$P < 0.001$
Other biliary cirrhosis	0.8%	1.2%	$P < 0.001$
AIH	6.8%	7.9%	$P = 0.018$
Metabolic disease	0.6%	1.2%	$P < 0.001$
Congestive disease	0.8%	1.2%	$P = 0.013$
Parasites	0.2%	0.4%	$P = 0.011$
Other known etiology	0.2%	0.8%	$P < 0.001$
Unknown etiology	10.5%	12.4%	$P < 0.001$

P-values were analyzed by χ^2-test.
AIH, autoimmune hepatitis; ALD, alcoholic liver disease; FLD, fatty liver disease; NASH, non-alcoholic steatohepatitis; NBNC LC, non-B, non-C liver cirrhosis; N.S., not significant; PBC, primary biliary cirrhosis.

not change (55.1% vs 56.3%), whereas that of NASH increased (14.5% vs 8.7%; $P < 0.001$) (Table 3).

Clinical features of NBNC LC patients

The male : female ratio for the NBNC LC patients was 1.93. The percentages of each etiology among 4608 male and 2391 female patients were as follows: NASH (9.5% and 24%), ALD (73.4% and 19.8%), FLD (3.4% and 0.9%), PBC (1.9% and 20%), other biliary cirrhosis (0.8% and 0.9%), AIH (1.5% and 17.1%), metabolic disease (0.5% and 0.8%), congestive disease (0.8% and 0.8%), parasitic disease (0.2% and 0.1%), other known etiology (0.1% and 0.2%) and unknown etiology (7.9% and 15.4%), respectively (Fig. 1). The male : female ratio for each etiology among the NBNC LC patients was as follows: NASH, 0.77; ALD, 7.12; FLD, 6.86; PBC, 0.18; other biliary cirrhosis, 1.73; AIH, 0.17; metabolic disease, 1.29; congestive disease, 2.17; parasitic disease, 5; other known etiology, 0.83; and unknown etiology, 0.99 (Table 4). Thus, the NASH patients were predominantly female as opposed to the ALD and FLD patients who were predominantly male.

The mean age at clinical diagnosis in the NBNC LC patients for NASH, ALD, FLD, PBC, other biliary cirrhosis, AIH, metabolic disease, congestive disease, parasitic disease, other known etiology and unknown etiology was 66.9, 60.3, 64.2, 63.6, 51.3, 64.5, 42.6, 52.7, 77.4, 56.1 and 68.8 years, respectively. In the patients with NASH, AIH, congestive disease and unknown etiology, the mean ages at clinical diagnosis of the male patients were lower than those of the female patients ($P < 0.001$). In contrast, in the ALD, FLD, PBC and metabolic disease patients, the mean ages at clinical diagnosis of the female patients were lower than those of the male patients ($P < 0.001$) (Table 5).

Regarding the risk factors of NASH, the following variables were investigated in the NASH, ALD and FLD patients: BMI and the prevalence of DM, impaired glucose tolerance (IGT), hypertension and dyslipidemia. BMI in the NASH, ALD and FLD patients was 27, 23.4 and 25 kg/m^2, respectively, and the differences among them were statistically significant. The prevalence of DM and IGT in the NASH and FLD patients (63% and 57%, respectively) was significantly higher compared with that in the ALD patients (31%) ($P < 0.001$). The prevalence of dyslipidemia in the NASH and FLD patients (25% and 29%, respectively) was significantly higher compared with that in the ALD patients (14%) ($P < 0.001$). The prevalence of hypertension in the NASH patients (52%) was significantly higher compared with that in the ALD and FLD patients (28% and 35%, respectively) ($P < 0.001$) (Table 6).

The levels of hepatic functional reserve based on the Child–Pugh classification for each etiology are summarized in Table 7. The percentages of moderate-to-low hepatic reserve (Child–Pugh class B and C) in the ALD and AIH patients (52.9% in both) were significantly higher compared with those in the NASH and FLD patients (35.8% and 27%, respectively) ($P < 0.001$).

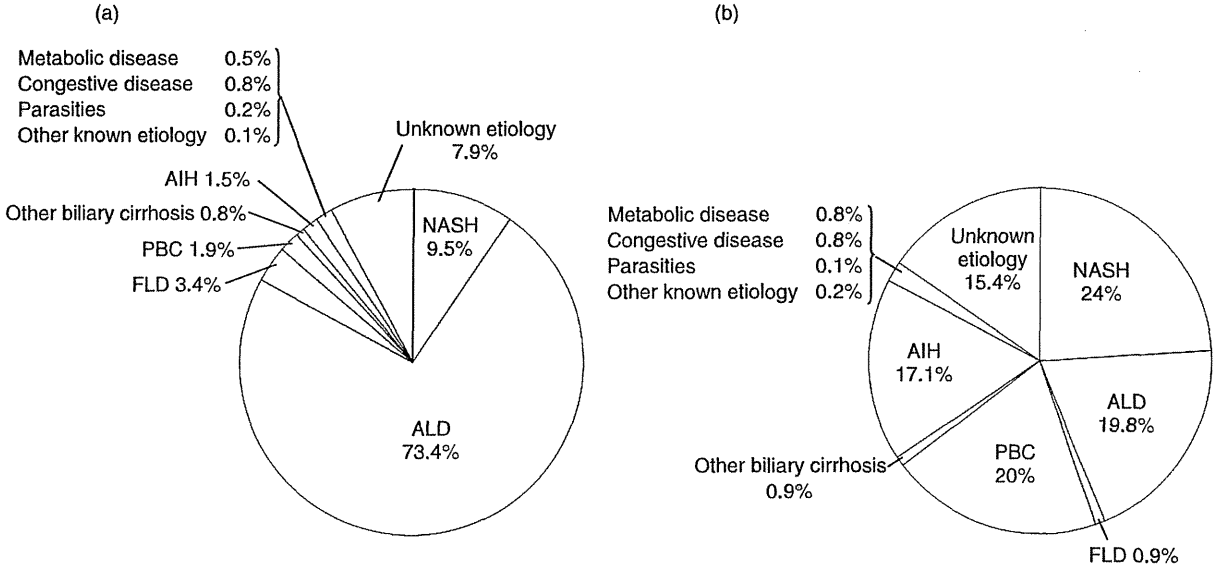

Figure 1 Frequency of each etiology among male or female patients with NBNC LC. (a) Male, (b) female. AIH, autoimmune hepatitis; ALD, alcoholic liver disease; FLD, fatty liver disease; NASH, non-alcoholic steatohepatitis; NBNC LC, non-B, non-C liver cirrhosis; PBC, primary biliary cirrhosis.

To determine the frequency of complicated portal hypertension patients, the prevalence of gastroesophageal varices was calculated. The prevalence in the ALD and PBC patients (54.5% and 61.9%, respectively) was significantly higher compared with that in the patients with NASH, FLD, AIH and unknown etiology (40.8%, 40.7%, 48.2% and 45.9%, respectively) ($P < 0.05$). Considering only patients with Child–Pugh class A, the prevalence of gastroesophageal varices in PBC patients was highest among all etiologies. ALD had significantly higher prevalence than NASH, the histology of which was very similar (Table 8).

Table 4 Male : female ratio of each etiology

	Male ($n = 4608$)	Female ($n = 2391$)	Male : female ratio
NASH	440	575	0.77
ALD	3381	475	7.12
FLD	151	22	6.86
PBC	87	477	0.18
Other biliary cirrhosis	38	22	1.73
AIH	69	409	0.17
Metabolic disease	22	19	1.29
Congestive disease	39	18	2.17
Parasites	10	2	5.00
Other known etiology	5	4	0.83

AIH, autoimmune hepatitis; ALD, alcoholic liver disease; FLD, fatty liver disease; NASH, non-alcoholic steatohepatitis; NBNC LC, non-B, non-C liver cirrhosis; N.S., not significant; PBC, primary biliary cirrhosis.

The prevalence of HCC in the NBNC LC patients was 35.9%. Among 2438 NBNC HCC patients, 51.9% were diagnosed with HCC simultaneously with the diagnosis of NBNC LC, 25.6% were diagnosed after, 1.4% were diagnosed before the diagnosis of NBNC LC and the diagnosis of the remaining patients was not described in detail. The male : female ratio for the NBNC HCC patients was 3.06. The percentage of each etiology among the HCC patients was as follows: NASH, 19.9%; ALD, 53.4%; FLD, 3.7%; PBC, 3.2%; other biliary cirrhosis, 0.2%; AIH, 4.9%; metabolic disease, 0.1%; congestive disease, 0.7%; parasitic disease, 0.1%; other known etiology, 0%; and unknown etiology, 13.8%. The percentage of NASH among the NBNC HCC patients was significantly higher than that among the NBNC LC patients (19.9% vs 14.5%, $P < 0.001$). The clinical diagnosis of HCC was made at a mean age of 67.2 years in all patients. The mean age of onset of HCC was 70.8, 64.8 and 68.4 years in the NASH, ALD and FLD patients, respectively, and the differences among them were significant ($P < 0.001$). The prevalence of

Table 5 The mean ages at clinical diagnosis in the patients with NBNC LC

	Total ($n = 6999$)	Male ($n = 4608$)	Female ($n = 2391$)	P-value (M vs F)
NASH	66.9 ± 11.6	64.8 ± 13.2	68.5 ± 9.8	$P < 0.001$
ALD	60.3 ± 11.0	60.9 ± 10.7	55.7 ± 12.1	$P < 0.001$
FLD	64.2 ± 11.8	64.7 ± 11.3	61.2 ± 15.0	$P < 0.001$
PBC	63.6 ± 12.1	66.0 ± 11.3	63.2 ± 12.0	$P < 0.001$
Other biliary cirrhosis	51.3 ± 20.7	52.0 ± 22.0	50.0 ± 19.0	$P < 0.001$
AIH	64.5 ± 12.2	63.3 ± 14.2	66.0 ± 11.7	$P < 0.001$
Metabolic disease	42.6 ± 18.2	44.0 ± 18.0	40.7 ± 19.0	$P < 0.001$
Congestive disease	52.7 ± 20.4	50.5 ± 20.7	57.4 ± 19.6	$P < 0.001$
Parasites	77.4 ± 5.9	76.5 ± 6.1	81.5 ± 2.1	$P < 0.001$
Other known etiology	56.1 ± 19.1	53.0 ± 18.7	58.7 ± 20.8	$P < 0.001$
Unknown etiology	68.8 ± 11.9	67.9 ± 13.0	69.8 ± 10.7	$P < 0.001$

All results are expressed as mean ± standard deviation. P-values were analyzed by Mann–Whitney U-test.
AIH, autoimmune hepatitis; ALD, alcoholic liver disease; FLD, fatty liver disease; NASH, non-alcoholic steatohepatitis; NBNC LC, non-B, non-C liver cirrhosis; PBC, primary biliary cirrhosis.

HCC in the patients with NASH, FLD and unknown etiology (50.9%, 54.5% and 47.5%, respectively) were significantly higher compared with that in the ALD, PBC and AIH patients (34.3%, 14.4% and 26.0%) ($P < 0.0001$). The percentage of moderate-to-low hepatic reserve (Child–Pugh class B and C) in HCC in AIH patients was significantly higher than those in the patients with NASH, FLD and unknown etiology

Table 6 Risk factors of NASH in the patients with NASH, ALD and FLD

Variable	NASH ($n = 1015$)	ALD ($n = 3856$)	FLD ($n = 173$)	P-value
Body mass index (kg/m^2)	27.0 ± 4.3	23.4 ± 6.4	25.0 ± 3.7	$P < 0.001$*,**
Diabetes mellitus or Impaired glucose tolerance	62.5%	37.5%	56.5%	$P < 0.001$*
Dyslipidemia	25.0%	13.5%	29.4%	$P < 0.001$* $P = 0.01$**
Hypertension	52.0%	28.2%	34.7%	$P < 0.001$*,**

ALD, alcoholic liver disease; FLD, fatty liver disease; NASH, non-alcoholic steatohepatitis.
Results of body mass index are expressed as mean ± standard deviation. P-values were analyzed by Mann–Whitney U-test and χ^2-test.
*NASH vs ALD, **NASH vs FLD.

Table 7 Levels of hepatic functional reserve based on the Child–Pugh classification

Child–Pugh classification	Class A	Class B	Class C	Percentages of both class B and C	P-value
NASH ($n = 783$)	503	222	58	35.8%	
ALD ($n = 2710$)	1276	867	567	52.9%	$P < 0.001$*
FLD ($n = 89$)	65	18	6	27.0%	
PBC ($n = 355$)	204	105	46	42.5%	
AIH ($n = 295$)	139	106	50	52.9%	$P < 0.001$** $P = 0.01$***
Unknown etiology ($n = 515$)	300	150	65	41.7%	

AIH, autoimmune hepatitis; ALD, alcoholic liver disease; FLD, fatty liver disease; NASH, non-alcoholic steatohepatitis; NBNC LC, non-B, non-C liver cirrhosis; PBC, primary biliary cirrhosis.
P-values were analyzed by χ^2-test.
*vs NASH, FLD, PBC and unknown etiology; **vs NASH and FLD; ***vs PBC and unknown etiology.

Table 8 Prevalence of patient with gastroesophageal varices

	Total	Child-Pugh classification		
		Class A	Class B	Class C
NASH ($n = 686$)	40.8% (280/686)*	31.8% (138/434)**	56.1% (111/198)	57.4% (31/54)
ALD ($n = 2365$)	54.5% (1289/2365)***	44.2% (486/1099)†	59.0% (447/757)	69.9% (356/509)
FLD ($n = 81$)	40.7% (33/81)	36.5% (23/63)	50.0% (7/14)	75.0% (3/4)
PBC ($n = 331$)	61.9% (205/331)††	53.5% (100/187)††	70.6% (72/102)	78.6% (33/42)
AIH ($n = 278$)	48.2% (134/278)	39.7% (52/131)	53.5% (53/99)	60.4% (29/48)
Unknown etiology ($n = 401$)	45.9% (184/401)	42.9% (94/219)	47.2% (60/127)	54.5% (30/55)

P-values were analyzed by Fisher's exact test or χ^2-test.
*$P < 0.05$, vs ALD, PBC and AIH; **$P < 0.01$, vs ALD, PBC and unknown etiology; ***$P < 0.05$, vs NASH, FLD and unknown etiology; †$P < 0.0001$ vs NASH; ††$P < 0.05$ vs NASH, ALD, FLD, AIH and unknown etiology.
AIH, autoimmune hepatitis; ALD, alcoholic liver disease; FLD, fatty liver disease; NASH, non-alcoholic steatohepatitis.

($P < 0.0001$). BMI in the NASH, ALD and FLD patients was 26.8, 24.0 and 25.8 kg/m^2, respectively, and the differences among them were statistically significant. (Table 9).

Table 10 shows the analysis of the risk factors associated with HCC in patients with ALD LC. Obesity and complication of DM were the risk factors of hepatic carcinogenesis in ALD LC patients as well as male sex and being older. Conversely, portal hypertension and anemia of ALD LC patients without HCC were worse than those with HCC. Accordingly, we investigated the comparison of the clinical features between the two ALD LC groups divided based on BMI (Table 11). Although the mean age was similar in these two groups, the prevalence of HCC in the ALD LC patients with obesity (BMI, ≥25 kg/m^2) was significantly higher compared with that in those without obesity (BMI, <25 kg/m^2) (48.3% vs 35.7%, $P < 0.001$) and similar to that in the NASH LC patients (48.3% vs 50.9%, not significant).

Of the NBNC LC patients, 31.3% were anti-HBc positive. Anti-HBc positivity was 30.7%, 30.8%, 34.7% and 43% in the patients with NASH, ALD, FLD and unknown etiology, respectively. The positivity was significantly higher in the patients with unknown etiology compared with the NASH, ALD and FLD patients ($P < 0.001$). Anti-HBc positivity was significantly higher in the HCC patients than in those without HCC (41.1% vs 24.8%, $P < 0.001$).

DISCUSSION

THIS NATIONWIDE SURVEY revealed the following clinical features in the NBNC LC patients:

1 Compared with the previous nationwide survey,[1] the percentage of ALD among the NBNC LC patients remained unchanged, whereas that of NASH increased.
2 The NASH LC patients were significantly older, predominantly female, heavier, hypertensive and more likely to have DM and HCC.
3 The ALD LC patients were significantly younger, predominantly male, had low hepatic reserve and were more likely to have portal hypertension than NASH LC.
4 The FLD LC patients were observed at an age between that of the NASH and ALD patients, were predominantly male (similar to the ALD patients) and were more likely to have DM and HCC similar to the NASH patients.
5 Approximately 10% of the NBNC LC patients still had an unknown etiology, and these patients were more likely to have HCC similar to both the NASH and FLD patients.
6 Anti-HBc positivity was significantly higher in the HCC patients than in those without HCC.

Although the natural history of NASH is not completely understood, Matteoni *et al.* reported that 23% of NASH patients progressed to cirrhosis within 10–15 years.[6] In addition, Starley *et al.* recently stated that approximately 26–37% of NASH patients demonstrate the progression of fibrosis over time periods up to 5.6 years, with up to 9% patients progressing to cirrhosis.[7] BMI and DM have been found to be independent risk factors associated with the progression of fibrosis in NASH patients.[8] Therefore, it is thought that the NASH LC patients in the present study had significantly more severe disease and were more likely to have DM. Conversely, the prevalence of NAFLD in Japan appears to be twice as high in males than in females;[9] however, the NASH LC patients in the present study were

Table 9 Clinical features of patients with HCC

	Percentage (%)	Prevalence of HCC (%)	Age of onset of HCC (years)	M : F ratio	Child–Pugh classification (A/B/C, %)	BMI	Platelet ($10^3/mm^3$)
Total ($n = 2438$)	100	35.9	67.2 ± 10.1	3.06	62.6/28.8/8.6	24.6 ± 4.0	127 ± 66
NASH ($n = 485$)	19.9	50.9*	70.8 ± 9.0**	1.06	66.0/28.9/5.1	26.8 ± 4.3‡‡	128 ± 61
ALD ($n = 1302$)	53.4	34.3	64.8 ± 9.4†	19.05††	60.3/29.8/9.9	24.0 ± 3.8	126 ± 66
FLD ($n = 91$)	3.7	54.5*	68.4 ± 8.8***	17.20††	82.5/15.9/1.6	25.8 ± 4.0‡‡‡	120 ± 61
PBC ($n = 79$)	3.2	14.4	68.0 ± 10.4***	0.32†††	53.2/35.9/10.9	22.3 ± 3.0	110 ± 54
Other biliary cirrhosis ($n = 4$)	0.2	6.8	–	–	–	–	–
AIH ($n = 119$)	4.9	26.0	68.8 ± 8.7**	0.23†††	42.5/42.5/15.0‡	24.3 ± 4.1	107 ± 60§
Metabolic disease ($n = 2$)	0.1	5.1	–	–	–	–	–
Congestive disease ($n = 16$)	0.7	32.0	52.0 ± 16.6	1.67	57.2/21.4/21.4	23.6 ± 3.2	127 ± 72
Parasites ($n = 3$)	0.1	30.0	–	–	–	–	–
Unknown etiology ($n = 337$)	13.8	47.5*	70.9 ± 10.9**	1.57	70.8/22.4/6.8	23.6 ± 3.7	143 ± 76

Results of age are expressed as mean ± standard deviation. *P*-values were analyzed by Mann–Whitney *U*-test and χ^2-test as appropriate.
*$P < 0.0001$, vs ALD, PBC and AIH; **$P < 0.0001$, vs ALD and congestive disease; ***$P < 0.001$, vs ALD and congestive disease; †$P < 0.001$, vs congestive disease; ††$P < 0.0001$, vs NASH, PBC, AIH and unknown etiology; †††$P < 0.0001$, vs NASH, ALD, FLD and unknown etiology; ‡$P < 0.0001$, vs NASH, FLD and unknown etiology; ‡‡$P < 0.0001$, vs ALD, PBC, AIH and unknown etiology; ‡‡‡$P < 0.0001$, vs ALD, PBC and unknown etiology; §$P < 0.001$, vs NASH and unknown etiology.
AIH, autoimmune hepatitis; ALD, alcoholic liver disease; BMI, body mass index; FLD, fatty liver disease; HCC, hepatocellular carcinoma; NASH, non-alcoholic steatohepatitis; NBNC LC, non-B, non-C liver cirrhosis; PBC, primary biliary cirrhosis.

predominantly female. Yasui *et al.* reported that NASH HCC patients were predominantly male, although the prevalence of cirrhosis among these patients was significantly lower in male patients compared with that in female patients.[10] These studies suggest that sex is implicated in the progression of fibrosis in NASH patients in Japan. In addition, the prevalence of HCC in the NASH LC patients in the present study was significantly higher compared with that in the previous nationwide survey (50.9% vs 31.5%, $P < 0.001$).[1] The incidence of NASH and NASH HCC has been gradually increasing in Japan, contrary to the decreased incidence of virus-related HCC.[4] Starley *et al.* found that as many as 4–27% of cases of NASH transform to HCC after the development of cirrhosis, and that the prevalence of HCC in NAFLD is 0–0.5%, whereas that of HCC in NASH is 0–2.8% over time periods of up to 19.5 years.[7] Yatsuji *et al.* reported the prospective evaluation of NASH LC and HCV-related LC (LC-C). They reported that NASH LC followed a course similar to that of LC-C, namely, complications of cirrhosis developed, including HCC (the 5-year cumulative rate of HCC development was 11.3% for NASH LC and 30.5% for LC-C).[11] Therefore, NASH LC patients need to be followed up carefully with respect to the occurrence of HCC, similar to LC-C patients.

Alcoholic liver disease remains the most prevalent cause of NBNC LC in Japan, accounting for approximately 55% of all NBNC LC cases. In the present study, the prevalence of HCC was significantly lower in the ALD LC patients than in the NASH LC patients, whereas the ALD LC patients were significantly younger and had a lower hepatic reserve. Regarding the comparison of outcomes with LC-C, Toshikuni *et al.* reported that the risk of HCC was lower in ALD LC than in LC-C, whereas the risk of hepatic decompensation and mortality was the same.[12] It is estimated that there are approximately 2.4 million heavy drinkers in Japan, and the number of ALD patients has been increasing because of increased alcohol consumption.[13] Therefore, ALD LC patients need to be followed up carefully with respect to the occurrence of hepatic decompensation, similar to LC-C patients. Obesity appears to be involved in the progression of ALD LC.[13] Accordingly, we investigated the risk factors associated with HCC and clarified that obesity and complication of DM could be the risk for hepatic carcinogenesis in ALD LC patients. The comparison of the clinical features between the two ALD LC groups divided based on BMI revealed that the prevalence of HCC in the ALD LC patients with obesity was significantly higher compared with that in those without obesity. Horie *et al.* also reported similar results.[14] Thus,

Table 10 Factors associated with HCC in patients with ALD

Factors	HCC (−), ($n = 2494$)	HCC (+), ($n = 1303$)	Univariate analysis, *P*-value	Multivariate analysis, *P*-value
Sex (M : F)	83.7%:16.3%	95.0%:5.0%	<0.0001	<0.0001
Age (years)	57.9 ± 11.0	64.8 ± 9.4	<0.0001	<0.0001
Body mass index (kg/m^2)	22.8 ± 3.8	24.0 ± 3.8	<0.0001	<0.0001
Hypertension (− : +)	77.4%:22.6%	61.9%:38.1%	<0.0001	0.068
Dyslipidemia (− : +)	87.0%:13.0%	81.6%:18.4%	<0.0001	0.482
Diabetes mellitus (− : +)	67.2%:32.8%	50.2%:49.8%	<0.0001	<0.0001
Child-Pugh classification (A : B + C)	38.5%:61.5%	60.3%:39.7%	<0.0001	0.188
Esophageal varices (− : +)	42.3%:57.7%	57.9%:42.1%	<0.0001	<0.0001
Ascites (− : +)	57.1%:42.9%	76.5%:23.5%	<0.0001	<0.0001
WBC (/mm^3)	6014 ± 3465	5532 ± 3484	0.001	0.547
Hemoglobin (g/dL)	11.3 ± 2.6	12.7 ± 2.2	<0.0001	<0.0001
Platelet (×10^3/mm^3)	114.6 ± 67.1	126.1 ± 65.5	<0.0001	0.104
AST (IU/L)	93 ± 209	65 ± 71	<0.0001	0.974
ALT (IU/L)	51 ± 118	45 ± 43	0.159	0.786
Bilirubin (mg/dL)	2.8 ± 3.9	1.6 ± 2.4	<0.0001	0.006
Albumin (g/dL)	3.3 ± 1.0	3.5 ± 0.7	<0.0001	0.281
PT%	69 ± 22	79 ± 19	<0.0001	0.628

Results of age are expressed as mean ± standard deviation. *P*-values were analyzed by Mann–Whitney *U*-test, χ^2-test and multivariate Cox's proportional hazard model as appropriate.
ALD, alcoholic liver disease; ALT, alanine aminotransferase; AST, aspartate aminotransferase; HCC, hepatocellular carcinoma; PT, prothrombin time; WBC, white blood cells.

obesity appears to be involved in the progression of HCC in ALD LC. Therefore, ALD LC patients with obesity need to be followed up carefully with respect to the occurrence of HCC, similar to NASH LC and LC-C patients. Not only abstinence from alcohol but also improvement in lifestyle is important to decrease the progression of ALD LC.

In the present study, we established a new clinical etiologic criterion: FLD. According to previous clinical etiologic criteria in Japan, mild drinkers (intake of >20 g and <70 g of ethanol/day) with steatohepatitis were not diagnosed with both NASH and ALD. The prevalence of minor homozygote or heterozygote type of the aldehyde dehydrogenase-2 gene (*ALDH2*), which oxidizes acetaldehyde to acetate and is a key enzyme in alcohol metabolism, is very high in Asian countries. The enzyme activity of a minor homozygote of *ALDH2* is completely defective. Moreover, the enzyme activity of a heterozygote is only 1/16th. Our survey is the first to reveal that these FLD LC patients were observed in 2.5% of NBNC LC patients. Considering the frequencies of mild drinkers and obese people in Japan, it is thought that the frequency of FLD LC is lower than that of LC with unknown etiology. This is because there were many patients whose amounts of daily alcohol intake were unknown; therefore, some were diagnosed as having an

Table 11 Clinical features of patients with ALD LC

	BMI <25 ($n = 1915$)	BMI ≥25 ($n = 749$)	*P*-value
Sex (M : F)	1644:317 (83.4%:16.6%)	692:57 (92.4%:7.6%)	$P < 0.001$
Age	60.2 ± 11.1	61.0 ± 10.2	N.S.
Diabetes mellitus	35.1%	43.9%	$P < 0.001$
HCC	35.7%	48.3%	$P < 0.001$

Results of age are expressed as mean ± standard deviation, *P*-values were analyzed by by Mann–Whitney *U*-test and χ^2-test as appropriate.
ALD, alcoholic liver disease; BMI, body mass index; HCC, hepatocellular carcinoma; N.S., not significant.

unknown etiology. Interestingly, the clinical features of the FLD LC patients overlapped with those of the NASH LC and ALD LC patients. Because the mean age of the FLD LC patients was between that of the NASH and ALD patients, the FLD LC patients were predominantly male, similar to the ALD LC patients, and they were more likely to have DM and HCC similar to the NASH LC patients. Horie *et al.* described a category such as FLD as overlap steatohepatitis.[13,14] The most important clinical feature in FLD LC patients was that the prevalence of HCC was high, similar to that in the NASH LC patients. This finding suggests that steatohepatitis per se is a potent risk factor of HCC, irrespective of alcohol consumption.

The LC patients with unknown etiology (or cryptogenic LC) were approximately 10% of the NBNC LC patients and were more likely to have HCC similar to the NASH and FLD patients. Some FLD LC patients whose daily alcohol intake was unknown may have been included in this group, and some "burnt-out" NASH LC patients whose liver showed complete disappearance of steatosis[15] may have also been included in this group. In addition, some patients who had been HBV carriers but had become HBsAg negative or those with occult HBV may have also been included in this group. Anti-HBc positivity was significantly higher in this group than in the NASH, ALD and FLD LC groups. Several studies have suggested a high prevalence of occult HBV among cryptogenic LC and NBNC HCC patients and also the participation of occult HBV in the progression to cirrhosis and occurrence of HCC.[16,17] In the present study, anti-HBc positivity was significantly higher in the NBNC LC patients with HCC than in those without HCC; however, the role of occult HBV in the progression to cirrhosis and carcinogenesis remains unclear. Occult HBV is defined as the presence of HBV DNA in the liver (with or without detectable HBV DNA in serum) for patients testing HBsAg negative.[18] Because of the lack of a HBV DNA assay in the present study, the impact of occult HBV on carcinogenesis could not be evaluated. Thus, a HBV DNA assay in the liver is needed for the evaluation of occult HBV on carcinogenesis. Although NBNC LC seemed to include varied etiology, occult HBV should be taken into account in the prediction of future HCC development in NBNC LC.

Our nationwide survey determined the etiology of NBNC LC in Japan. Future changes in etiology must be considered for the establishment of precise diagnostic strategies. We hope that these results contribute new ideas toward understanding NBNC LC and NBNC HCC.

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