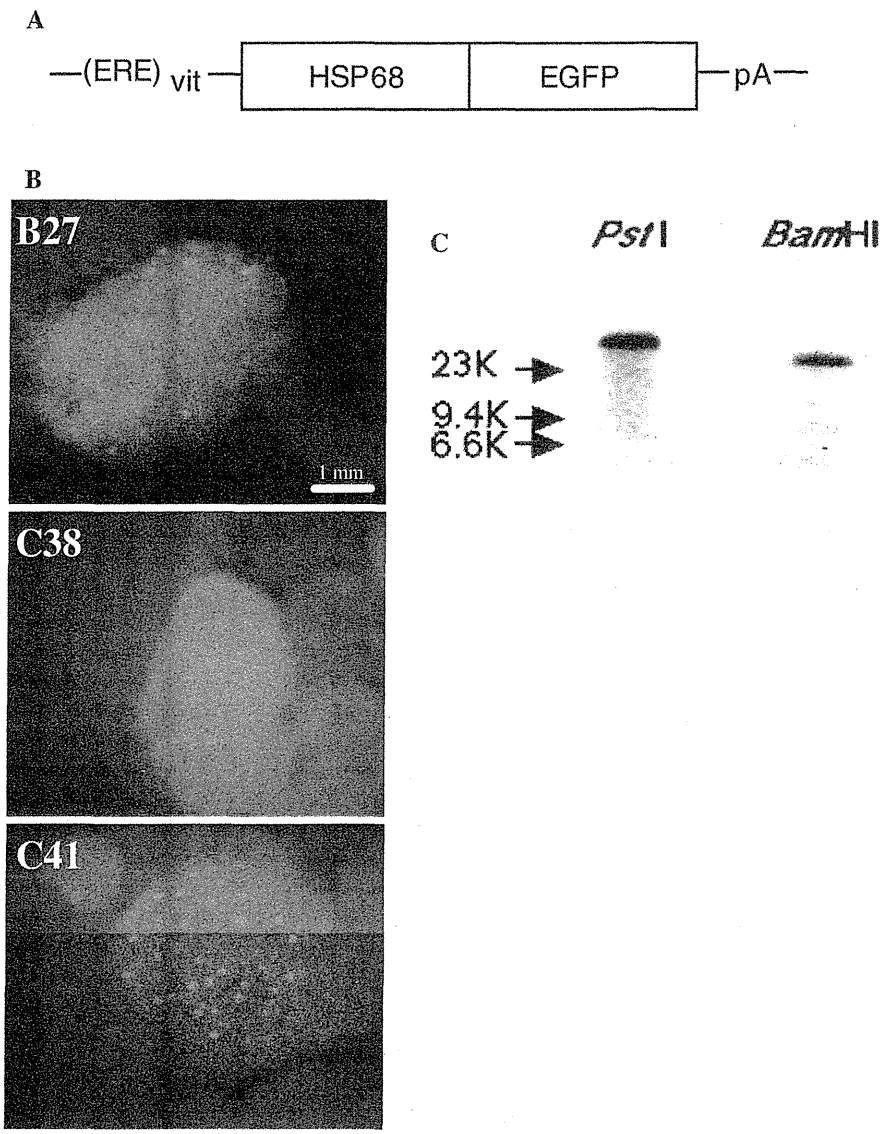

Fig. 1 Schematic view of the transgene, ovarian expression of the transgene and Southern blot analysis of the genomic DNA of the generated transgenic mice. **a** The transgene (2 kbp) consists of the estrogen-responsive element of the Xenopus leavis A2 vitellogenin gene and a minimal promoter of the heat shock protein 68 gene (HSP68) linked to the EGFP cDNA (EGFP) and SV40 polyadenylation sequence (pA). **b** Assessment of EGFP expression in the ovary of three founder lines in vivo. Imaging of EGFP fluorescence was performed with a fluorescence stereomicroscope for the unfixed ovary. *Scale bar* is 1 mm. **c** Total genomic Southern blot analysis on mouse of line C41. Genomic DNA from $Ar^{+/+}$ mice with the EGFP gene was digested completely with either *Pst*I or *Bam*HI. A radiolabeled fragment of cDNA coding for EGFP was used as a probe

Visualization and recording of EGFP fluorescence

Imaging of EGFP fluorescence was carried out using a fluorescence stereomicroscope (MZ FLIII; Leica, Deerfield, NJ) with a filter set, composed of a 480/440-nm excitation filter and a 510-nm barrier filter. The images were recorded using a cooled digital color charge-coupled device camera (C4742-95, Hamamatsu Photonics, Hamamatsu, Japan) mounted on the stereomicroscope.

To obtain images of EGFP fluorescence in sections, ovary and testis were fixed in a solution of 4 % (v/v)

paraformaldehyde at 4 °C for 1 h and then immersed in phosphate-buffered saline containing 10 % (w/w) sucrose at 4 °C for 15 h. The tissues were embedded in optimal cutting temperature compound (Sakura, Tokyo Japan) and cut into 15-μm-thick sections. After DNA was stained with 4,6-diamidino-2-phenylindole·2HCl (DAPI), sections were observed under a fluorescence microscope (Olympus BX50, Olympus, Tokyo Japan).

Quantification of EGFP fluorescence

To prepare tissue extracts containing EGFP, tissues (~0.2 g) from five transgenic mice for each genotype at 2-month-old were homogenized in 2 ml of 10 mM Tris–HCl (pH 7.2) containing 1 mM EDTA and 0.2 % (w/w) sodium dodecyl sulfate using a Polytron PT1200 homogenizer (KINEMATICA, Lucerne, Switzerland). After centrifugation at 2,000×*g* for 10 min at 4 °C, the supernatants containing EGFP were recovered for fluorometry using a spectrofluorometer (RF-5300PC, Shimadzu Corp., Kyoto, Japan). The filters used were 480 nm for excitation and 510 nm for emission. Purified recombinant EGFP (BD Clontech, Palo Alto, CA, USA) was employed as a standard to calibrate the amounts of EGFP expressed in the tissues of the transgenic mice (Toda et al. 2004). The amounts of EGFP in the extracts are expressed as picograms of EGFP per microgram of total protein of the tissue extracts examined.

Induction of ovulation

Powder (15 mg) of 17β-estradiol (E2) (Sigma, minimum 96 % purity) was first dissolved in 0.8 ml of ethanol, then diluted to 8 ml with sesame oil (Nacalai Tesque, Kyoto, Japan). Pregnant mare serum gonadotropin (PMSG) (Serotropin®, ASKA Pharmaceutical Co., Ltd., Tokyo, Japan) and human chorionic gonadotropin (hCG) (Wako Pure Chemical Industries, Osaka, Japan) were dissolved in 0.9 % NaCl solution at 500 and 2,500 international units (IU)/ml, respectively. Ovulatory induction was conducted according to a protocol described previously (Toda et al. 2012) with slight modifications. In Group A, $Ar^{-/-}$ mice at 4 weeks of age were supplemented with E2 on days 1, 4 and 5 (18 mg/kg body weight, subcutaneous injection). In Groups B, C, and D, E2 supplementation on day 1, day 4, and day 5 was, respectively, omitted (Table 2). The mice were injected with PMSG (25 IU/mouse, intraperitoneal injection) on day 4. At 48 h after PMSG injection (on day 6), hCG (25 IU/mouse, intraperitoneal injection) was administered. $Ar^{+/+}$ mice were stimulated with 5 IU of PMSG and hCG on day 4 and day 6, respectively. Ovulatory response was observed under a fluorescence stereomicroscope at 15 h after the hCG injection.

Statistical analysis

Data are expressed as the mean ± SEM. The significance of differences was analyzed by unpaired *t* test using InStat software (GraphPad Software, Inc., San Diego, CA, USA). *P* values less than 0.05 were considered significant.

Results

Generation and selection of transgenic mice expressing EGFP protein

Twelve (7 males and 5 females) of the 59 founder lines expressed functional EGFP. Fluorescence stereomicroscopic analysis revealed that three of the 12 founders expressed the EGFP gene in oocytes (Fig. 1b). As line C41 showed the strongest fluorescence in the ovary among these three lines under a fluorescence stereomicroscope. We characterized line C41 more extensively. We noticed no impairment in fertility in line C41, producing on average 6.5 ± 1.7 offspring per litter (male, 3.2 ± 1.3; female, 3.7 ± 1.7) (n = 30 litters). Total genomic Southern blot analysis by digestion of the murine DNA with *Pst*I or *Bam*HI, of which the recognition sequence does not exist within the EGFP gene, gave a single band hybridized with the EGFP probe, suggesting that the transgene was incorporated at a single locus in the genome (Fig. 1c). Approximately 6 copies of the EGFP transgene were estimated to be incorporated into the genome when analyzed by PCR amplification.

Quantitative analysis of EGFP expression using 2-month-old mice of line C41 revealed high expression in heart and adrenal gland in both female and male mice (Fig. 2). The analysis also showed that a relatively high level of EGFP expression (more than 20 pg EGFP/μg protein) was detected in the lung and uterus in $Ar^{+/+}$ females and in the skeletal muscle,

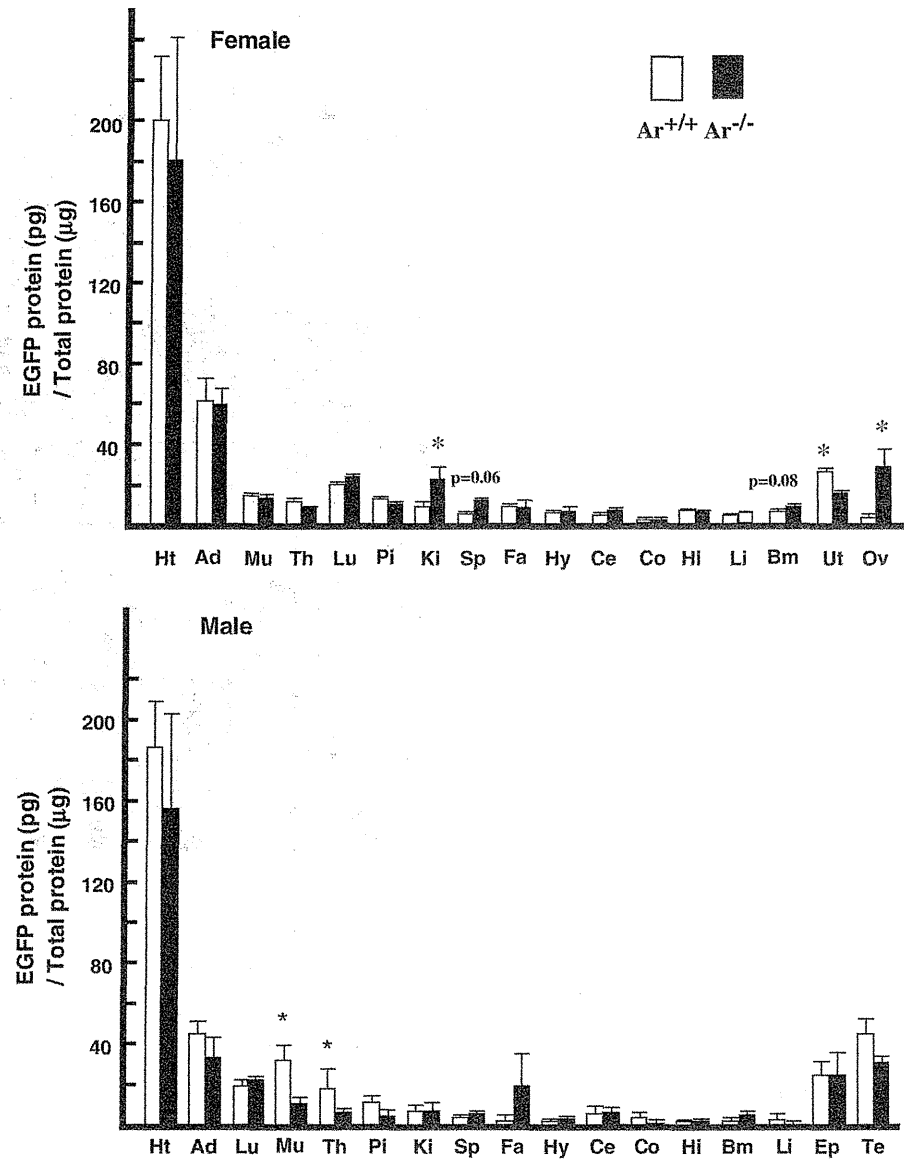

Fig. 2 Tissue distribution of EGFP in mouse of line C41. Quantitative analysis of EGFP expression using 2-month-old females (*upper panel*) and males (*lower panel*). The amounts of EGFP were measured by fluorescent spectrophotometry in various tissues including heart (*Ht*), adrenal gland (*Ad*), skeletal muscle (*Mu*), thymus (*Th*), lung (*Lu*), pituitary gland (*Pi*), kidney (*Ki*), spleen (*Sp*), gonadal fat pad (*Fa*), hypothalamus (*Hy*), cerebellum (*Ce*), cortex (*Co*), hippocampus (*Hi*), liver (*Li*), bone marrow (*Bm*), uterus (*Ut*), ovary (*Ov*), epididymis (*Ep*) and testis (*Te*) The amounts are expressed as pg EGFP per μg total protein for each tissue examined. The analysis was carried out on five animals per group. Open and closed bars indicate $Ar^{+/+}$ and $Ar^{-/-}$ mice, respectively. $^*P < 0.05$ relative to $Ar^{-/-}$ mice fed a control chow diet. The *error bars* represent the SEM

epididymis and testis in $Ar^{+/+}$ males. Low levels of the EGFP expression (<20 pg/μg protein) were detected in the other several tissue sites examined (Fig. 2). Quantification of EGFP expression in the females of lines B27 and C38 showed high levels of expression in heart and adrenal gland as observed in line C41 (Table 1). We also detected high levels of EGFP expression in the kidney, fat pad, liver and ovary in females of line B27, but the expression level in the lung and uterus was not marked as observed in line C41. In contrast to lines C41 and B27, EGFP expression was generally high in all tissue examined,

Table 1 Tissue distribution of EGFP in female $Ar^{+/+}$ mice of lines B27 and C38

	Line B27	Line C38
Heart	36.1 ± 3.2	41.8 ± 9.7
Adrenal gland	43.6 ± 7.5	54.9 ± 2.6
Skeletal muscle	4.4 ± 0.5	16.1 ± 2.8
Thymus	9.8 ± 2.7	13.0 ± 3.5
Lung	12.9 ± 0.6	30.9 ± 9.0
Pituitary gland	9.9 ± 1.1	70.0 ± 15.3
Kidney	36.9 ± 4.9	64.9 ± 14.2
Spleen	9.1 ± 0.4	9.6 ± 1.0
Fat pad	28.4 ± 4.9	34.1 ± 4.2
Hypothalamus	15.5 ± 2.5	46.3 ± 4.9
Cerebellum	18.5 ± 0.9	35.1 ± 4.1
Cortex	14.4 ± 1.8	34.9 ± 5.5
Hippocampus	12.9 ± 1.5	35.2 ± 4.5
Liver	22.5 ± 2.6	18.9 ± 3.7
Bone marrow	9.1 ± 0.6	ND
Uterus	11.0 ± 1.9	34.6 ± 8.4
Ovary	20.2 ± 5.0	29.0 ± 2.9

The amounts were expressed as EGFP (pg) per total protein (μg) for each tissue

ND not determined

except for skeletal muscle, thymus, spleen and liver in females of line C38.

Expression of the EGFP gene in $Ar^{-/-}$ mice of line C41

The expression levels of EGFP in mice of line C41 lacking *Cyp19a* ($Ar^{-/-}$ mice) were compared to those in the $Ar^{+/+}$ mice to assess the estrogen dependence of transgene expression. High levels of expression were detected in heart, adrenal gland, lung and uterus, as observed in $Ar^{+/+}$ females. In addition, the kidney and ovary expressed the EGFP gene at high levels in $Ar^{-/-}$ females. Of these tissues, the kidney, uterus and ovary showed a significant difference compared with the expression levels in $Ar^{+/+}$ females (Fig. 2). Although not statistically significant, expression levels in the spleen and bone marrow were higher in $Ar^{-/-}$ mice than in $Ar^{+/+}$ females. In $Ar^{-/-}$ male mice, the expression was reduced in the muscle and thymus, and increased in the gonadal fat pad compared with those in the $Ar^{+/+}$ males. Whereas uterine expression of the transgene was higher in $Ar^{+/+}$ mice than in $Ar^{-/-}$ mice, the differences in the expression levels in the other tissue sites seemed to be marginal between $Ar^{+/+}$ and $Ar^{-/-}$ mice.

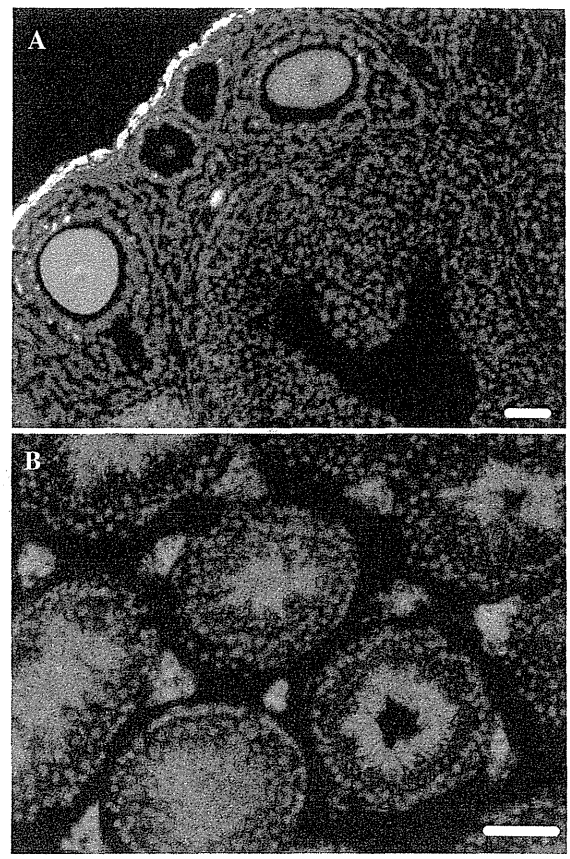

Fig. 3 Expression of the EGFP gene in the gonadal tissues of mouse of line C41. Imaging of EGFP fluorescence expressed in the $Ar^{+/+}$ ovary (**a**) and testis (**b**) was performed with a fluorescence microscope for sections of the fixed tissues. Leydig cells in the interstitial space of testis (**b**) showed non-specific fluorescence, as testicular sections of mice without the EGFP gene revealed similar fluorescent images under our experimental conditions. *Scale bar* is 100 μm

Expression of EGFP in germ cells

Next, we examined the gonadal expression of EGFP more closely in mice of line C41. The results clearly demonstrated that the expression was restricted to oocytes and was found in neither granulosa nor theca cells in the ovary. Furthermore, oocytes before preantral stages did not express the EGFP gene (Fig. 3a). Examination using testicular sections revealed that the spermatids were the cells expressing the EGFP gene.

We detected EGFP expression neither in spermatogonia, spermatocytes nor mature spermatozoa (Fig. 3b).

Ovulatory induction of mice expressing the EGFP gene

We employed the transgenic mouse line C41 to evaluate the ovulatory response of the $Ar^{-/-}$ mouse. When ovulation was induced in the $Ar^{+/+}$ mice by gonadotropin injections at standard doses (5 IU of PMSG and hCG), the fluorescent oocytes were observed in the oviducts at 15 h after hCG injection (Fig. 4a, b). As reported previously (Toda et al. 2001), when $Ar^{-/-}$ females were stimulated with the same dose of gonadotropins, no EGFP-positive oocytes were detectable in the oviducts (data not shown). In contrast, upon treatment of $Ar^{-/-}$ mice with the alternative super-ovulatory protocol (Toda et al. 2012), EGFP-positive oocytes were detected in the oviducts (Fig. 4c). These results demonstrated that the current transgenic mouse line expressing EGFP in oocytes allows us to assess ovulatory responses accurately and conclusively.

Next, we examined which E2 supplementations, given at three points within the protocol: days 1, 4 and 5, are essential for the ovulatory induction in $Ar^{-/-}$ mice. Ovulatory response could be detected in five out of twelve $Ar^{-/-}$ mice examined after the treatment without the E2 supplementation on day 1 (Table 2). In contrast, when the supplementation on day 4 ($n = 11$) or on day 5 ($n = 14$) was omitted, no $Ar^{-/-}$ mice showed ovulation, indicating that E2 supplementation after gonadotropin stimulation is essential for ovulatory induction in $Ar^{-/-}$mice.

Discussion

The EGFP transgenic mouse line C41 was fortuitously generated. Southern blot analysis using total genomic DNA of the present transgenic mouse suggested that the transgene is integrated at a single unique site in the genome. Comparison of the abundance of EGFP fluorescence between $Ar^{+/+}$ and $Ar^{-/-}$mice of line C41 revealed that the uterine expression was higher in the former mice than in the latter, indicating that the expression seems to be estrogen-dependent. However, we detected higher expression of the transgene in the

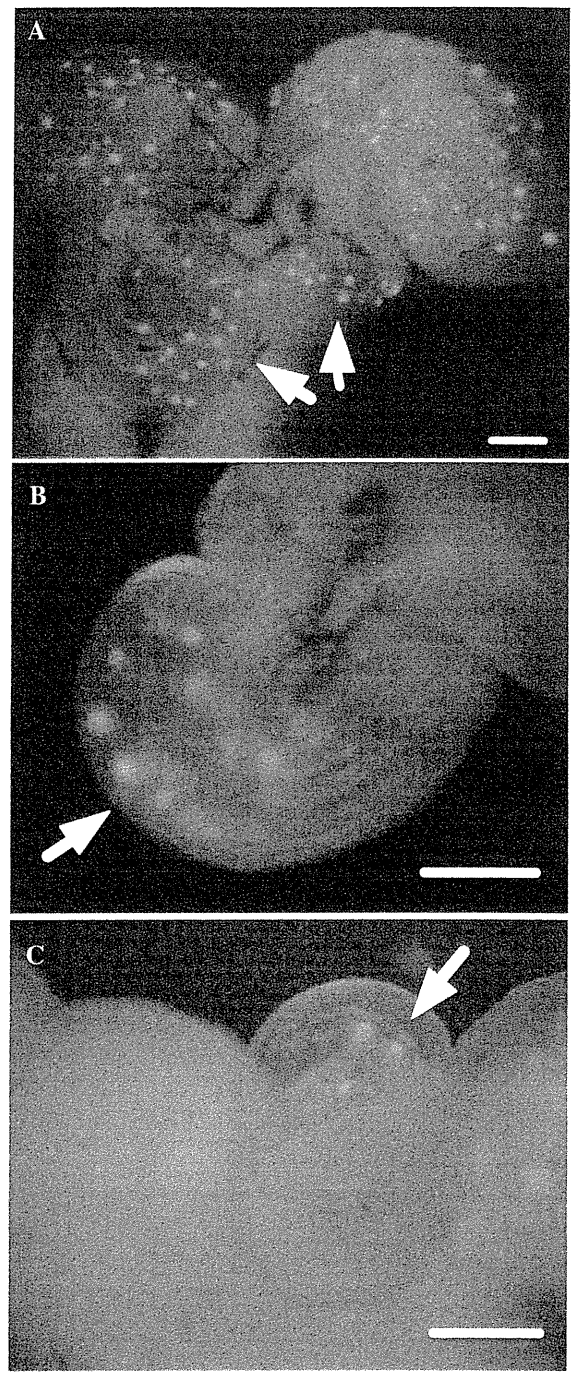

Fig. 4 Ovulated oocytes in the oviducts observed with a fluorescence stereomicroscope. In $Ar^{+/+}$ (**a**, **b**) and $Ar^{-/-}$ females (**c**) of line C41 at 4 weeks of age, ovulation was induced. Ovulatory responses were evaluated under a fluorescent stereomicroscope at 15 h after the hCG injection. *Arrows* indicate ovulated oocytes expressing EGFP. *Scale bar* is 0.5 mm

Table 2 Ovulatory response of $Ar^{-/-}$ mice

	Day 1	Day 4		Day 5	Day 6	Number of mice examined	Number of mice ovulated	Number of oocytes ovulated	Reference
	E2 (18 mg/kg)	E2 (18 mg/kg)	PMSG (25 IU)	E2 (18 mg/kg)	hCG (25 IU)				
Group A	+	+	+	+	+	60	42	6.9 ± 0.9	Toda et al. (2012)
Group B	−	+	+	+	+	12	5	4.4 ± 1.1	This study
Group C	+	−	+	+	+	11	0	−	This study
Group D	+	+	+	−	+	14	0	−	This study

ovary in the $Ar^{-/-}$ mice than that in $Ar^{+/+}$ mice. Furthermore little differences in the expression levels in the pituitary gland, which is highly sensitive to estrogenic chemicals (Toda et al. 2005), were observed between 2-month-old $Ar^{+/+}$ and $Ar^{-/-}$mice. We thus concluded that the EGFP transgene in line C41 was expressed in an estrogen-independent manner in vivo. Shedding of the estrogen-dependency might be caused by silencing the function of the regulatory element due to genomic integration of the transgene (Ristevski 2005). It is also tempting to speculate that some regulatory networks of gene expression have become functional in heart, adrenal gland and germ cells due to the influence of regulatory elements neighboring the integration site of the transgene. Nevertheless, quantitative analysis of EGFP expression showed high levels of its expression in heart and adrenal gland in three different founder lines, indicating that the transcriptional controlling units including the DNA fragment containing the ERE of the Xenopus leavis A2 vitellogenin gene and the transcription driving sequence from the mouse HSP68 gene might preferentially promote the expression of the EGFP gene in the tissue sites.

Histological analysis of gonadal tissues of line C41 revealed the expression of EGFP in spermatids in the testis and oocytes in ovarian follicles beyond preantral stages. We did not observe the expression in spermatogonia nor in oocytes in primordial follicles. Thus the expression of the EGFP transgene in line C41 might depend on factor(s) that might get to be functional after the germ cells progress to a certain stage of development. We also observed no EGFP expression in spermatozoa present in the lumen of seminiferous tubules or epididymis. The negative expression in spermatozoa seems to be due to removal of cytoplasm during a process of development of compact and slender spermatozoa from spermatids.

It was reported that the expression of EGFP in vivo negatively correlates with the developmental competence of preimplantation mouse embryos (Devgan et al. 2004) and also affects metabolic activities (Li et al. 2013). Current transgenic mouse line C41 did not reveal a detectable sign of reproductive difficulty. Nevertheless the litter size was smaller than that of our previous EGFP reporter, line KT2 (Toda et al. 2004), showing on average 8.9 ± 2.2 offspring per litter (n = 19 litters). This might reflect some detrimental effects of EGFP expression in oocytes on reproductive activity. However the differences in the litter size might be attributable to differences in the genetic background between the two lines.

Genetically modified murine models such as mice lacking the SULT1E1 gene (Gershon et al. 2007) or LRH-1 gene (Duggavathi et al. 2008) demonstrated that ovarian estrogens at high levels were detrimental to ovulatory induction. In order to elucidate the minimum requirements of E2 supplementations for ovulatory induction in aromatase-deficient mice, we employed $Ar^{-/-}$ mice of line C41, in which fluorescent oocytes are readily detectable within the oviduct, allowing us to evaluate accurately the ovulatory responses after stimulation with various combinations of stimulants including E2. The results demonstrated an absolute requirement of E2 supplementation at the time when gonadotropins were administered for ovulatory induction in $Ar^{-/-}$ mice. It is well established that luteinizing hormone receptor (LH/CG-R) expression was induced by synergic actions of E2 and FSH (Richards et al. 1979; Knecht et al. 1985). We confirmed the synergism in aromatase-deficient mice as well (Toda et al. 2012). It is thus interesting to examine whether or not different protocols for E2 supplementation can alter the synergic actions of E2 with FSH for LH/CG-R expression in $Ar^{-/-}$ mice, which might explain the observed differences in the

ovulatory responses in $Ar^{-/-}$ mice. Furthermore, recent studies employing microarray analysis identified a number of estrogen-dependent genes in the murine ovary (Liew et al. 2011; Binder et al. 2013). Expression analysis on those ovarian genes under the present experimental conditions using $Ar^{-/-}$ mice might be able to delineate a novel set of ovulation-related genes induced by synergic action of E2 and FSH.

In summary, various studies characterized mouse lines that harbor a reporter transgene in germ cells (Zambrowicz et al. 1993; MacGregor et al. 1995; Yeom et al. 1996; Lewandoski et al. 1997; Vidal et al. 1998; Yoshimizu et al. 1999; de Vries et al. 2000; Han et al. 2004; Lan et al. 2004; Nayernia et al. 2004; Tanaka et al. 2004; Cronkhite et al. 2005; Payer et al. 2006; Gallardo et al. 2007; West et al. 2009; Nicholas et al. 2009). Of these lines, some reporters have limitations in that they are sex-specific (Zambrowicz et al. 1993; Vidal et al. 1998; Nayernia et al. 2004) and/or may be limited in expression to the early stages of germ cell differentiation (MacGregor et al. 1995; Tanaka et al. 2004). The current transgenic mice expressed EGFP in germ cells at later stages of differentiation. We exploited this advantage to investigate the ovulation of $Ar^{-/-}$ mice, which are totally anovulatory (Fisher et al. 1998; Toda et al. 2001), demonstrating that our current EGFP transgenic mouse provides an alternative tool to study germ cell biology, including oogenesis, ovulation and senescence.

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ORIGINAL ARTICLE—LIVER, PANCREAS, AND BILIARY TRACT

Type 2 diabetes mellitus is associated with the fibrosis severity in patients with nonalcoholic fatty liver disease in a large retrospective cohort of Japanese patients

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Abstract
Background The prevalence of nonalcoholic fatty liver disease (NAFLD) and metabolic syndrome have been increasing worldwide. The associations between metabolic factors and the histologic severity of NAFLD have not yet been clarified. Therefore, we studied the relationships between relevant metabolic factors and the histological severity of NAFLD.
Methods In a cross-sectional multicenter study conducted in Japan, we examined 1,365 biopsy-proven NAFLD patients. The frequencies of underlying lifestyle-related diseases and their relationships to the NAFLD histology were investigated.
Results The hepatic fibrosis stages (Stage 0/1/2/3/4) were 22.6/34.1/26.7/14.5/2.1 (%) in the male patients, and 16.2/31.7/23.9/21.6/6.6 (%) in the female patients. Dyslipidemia was present in 65.7 % (hypertriglyceridemia, 45.3 %; increased low-density lipoprotein cholesterol, 37.5 %; decreased high density lipoprotein cholesterol, 19.5 %) of patients. Hypertension was present in 30.2 %, and diabetes mellitus (DM) in 47.3 %. The fibrosis stage increased with age, especially in postmenopausal females. The body mass index was positively correlated with the fibrosis stage. Deterioration of glucose control was positively correlated

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with the fibrosis stage, this correlation being more prominent in females. Multivariate analysis identified age and DM as significant risk factors for advanced fibrosis. No significant correlation of the fibrosis stage was observed with hypertension. There was a negative correlation between the serum triglyceride levels and the fibrosis stage. *Conclusions* DM appeared to be a significant risk factor for advanced fibrosis in patients with NAFLD, and would therefore need to be properly managed to prevent the progression of NAFLD.

Keywords NAFLD · Histology · Diabetes mellitus · Retrospective study

Abbreviations

NAFLD	Nonalcoholic fatty liver disease
NASH	Nonalcoholic steatohepatitis
IR	Insulin resistance
DM	Diabetes mellitus
NAFL	Nonalcoholic fatty liver
BMI	Body mass index
CT	Computed tomography
AST	Aspartate aminotransferase
ALT	Alanine aminotransferase
GGT	Gamma glutamyl transpeptidase
ChE	Cholinesterase
HDL	High density lipoprotein
LDL	Low-density lipoprotein
FPG	Fasting plasma glucose
HbA1c	Hemoglobin A1c
FFA	Free fatty acid
CRP	C-reactive protein
IRI	Immunoreactive insulin
HOMA-IR	Homeostasis model assessment-insulin resistance
SD	Standard deviation
IGT	Impaired glucose tolerance
NGT	Normal glucose tolerance

Introduction

Nonalcoholic fatty liver disease (NAFLD) is one of the most commonly encountered chronic liver disease in the world. According to Japanese annual health check reports, 9–30 % of Japanese adults suffer from NAFLD [1–3]. Since it is now known that almost 10–20 % of individuals with NAFLD have nonalcoholic steatohepatitis (NASH), the prevalence of NASH is estimated to be 1–3 % in the adult Japanese population, similar to the prevalence reported from Western countries.

Nonalcoholic fatty liver disease includes a wide spectrum of liver diseases, ranging from nonalcoholic fatty liver (NAFL), a benign and non-progressive condition, to NASH, which can progress to liver cirrhosis and hepatocellular carcinoma even in the absence of a history of significant alcohol consumption [4–7]. Furthermore, NASH is considered to be the hepatic manifestation of metabolic syndrome, and has been shown to be associated with obesity, insulin resistance (IR) and abnormalities of glucose and lipid metabolism [8–16]. Importantly, the rates of nonalcoholic fatty liver (NAFL) and NASH are expected to continue to grow with the developing pandemic of obesity and diabetes mellitus, to become global public health concerns.

Owing to the difficulties in diagnosing NAFLD (NAFL and/or NASH) and referral bias, it has been difficult to determine the prognostic factors in patients with NAFLD. NAFLD is a complex disease with multiple etiopathogenetic factors, including obesity, type 2 DM, dyslipidemia, hypertension, and other diseases associated with metabolic dysregulations. Recent reports have suggested that DM is an independent risk factor for NAFLD [17–19]. Despite the high prevalence and potentially serious nature of this disease, relatively little is known about the metabolic factors that might be associated with the histological severity of NAFLD.

The purpose of this study was to conduct a retrospective investigation of the association between metabolic factors and the histologic severity of NAFLD in a large cohort of Japanese patients with NAFLD.

Patients and methods

Patient population

A total of 1,365 biopsy-proven NAFLD patients seen between 2001 and 2012 were enrolled from institutes affiliated with the Japan Study Group of NAFLD (JSG-NAFLD), represented by the following nine hepatology centers in Japan: Hiroshima University, Kyoto Prefectural University of Medicine, Yokohama City University, Kochi Medical School, Saga Medical School, Osaka City University, Nara City Hospital, Kurume University, and Saiseikai Suita Hospital. A portion of the patients (76.8 %; 1,048 out of 1,365) had also been involved in the previous JSG-NAFLD study [20, 21]. Informed consent was obtained from each patient, and the study was conducted in conformity with the ethical guidelines of the 7th revision of the Declaration of Helsinki (in October 2008) [22] and the approval of the ethics and research committees of the hospitals. In all patients, the current and past daily alcohol intake was less than 20 g per day; details regarding alcohol consumption were obtained independently by at least two physicians and confirmed by close family members. None

of the patients were receiving any medications that could cause NASH. Among the patients, those with the following disorders were excluded: secondary causes of steatohepatitis, drug-induced liver disease, alcoholic liver disease, viral hepatitis, autoimmune hepatitis, primary biliary cirrhosis, α1-antitrypsin deficiency, hemochromatosis, Wilson's disease, and biliary obstruction. [23].

Study design

A complete physical examination was performed on each patient within 1 month prior to the liver biopsy, as reported previously [24]. The body mass index (BMI) was calculated as the weight (kg) divided by height (m)-squared. Obesity was defined as a BMI of greater than 25, according to the criteria of the Japan Society for the Study of Obesity [25]. Computed tomography (CT) was used to determine the visceral fat area at the level of the umbilicus [26], as previously reported [24]. Dyslipidemia was diagnosed based on serum cholesterol levels higher than 220 mg/dl and/or high-density lipoprotein cholesterol levels lower than 40 mg/dl and/or triglyceride levels over 150 mg/dl. Hypertension was diagnosed if the patient was on antihypertensive medication and/or had a resting recumbent blood pressure of $\geq$ 130/85 mmHg on at least two occasions. Hyperuricemia was diagnosed based on serum uric acid levels higher than 7.0 mg/dl. DM was diagnosed according to the 2006 World Health Organization (WHO) criteria [27].

Venous blood samples were taken in the morning following overnight fasting for 12 h. The laboratory evaluation in all patients included a blood cell count, hemoglobin, platelet count; and the serum levels of aspartate aminotransferase (AST), alanine aminotransferase (ALT), AST/ALT ratio, lactate dehydrogenase (LDH), alkaline phosphatase (ALP), gamma glutamyl transpeptidase (GGT), cholinesterase (ChE), total bilirubin, direct bilirubin, albumin, total cholesterol, triglycerides, high density lipoprotein (HDL) cholesterol, low-density lipoprotein (LDL) cholesterol, fasting plasma glucose (FPG), hemoglobin A1c (HbA1c), immunoreactive insulin (IRI), ferritin, uric acid, free fatty acid (FFA), and hyaluronic acids, were measured periodically during the treatment using the standard techniques of clinical chemistry laboratories.

Insulin resistance was calculated by the homeostasis model assessment-insulin resistance (HOMA-IR) using the following formula: HOMA-IR = fasting insulin (μU/ml) × plasma glucose (mg/dl)/405 [28].

Pathology

Patients enrolled in this study underwent percutaneous liver biopsy under ultrasonic guidance after obtaining informed consent. Formalin-fixed, paraffin-embedded liver sections were stained routinely with hematoxylin-eosin, silver reticulin, and Masson trichrome. All the specimens were examined by an experienced pathologist who was unaware of the clinical and biochemical data of the patients. Histological diagnosis for NAFLD was performed according to the methods of Matteoni et al. [6]. Grading and staging was classified according to Brunt et al. [29] and Kleiner et al. [30], as previously reported. In brief, steatosis was graded as follows: grade 1 (5–33 % of hepatocytes affected), grade 2 (34–66 % of hepatocytes affected), or grade 3 (> 66 % of hepatocytes affected). Necroinflammation was graded from grade 0 (absent) to 3 (1, occatonal ballooned hepatocytes and no or very mild inflammation; 2, ballooning of hepatocytes and mild-to-moderate portal inflammation; 3, intra-acinar inflammation and portal inflammation). Fibrosis was staged from grade 0 (absent) to 4 (1, perisinusoidal/pericellular fibrosis; 2, periportal fibrosis; 3, bridging fibrosis; 4, cirrhosis).

Statistical analyses

The data were statistically analyzed using R software, version 3.0.0. Continuous variables were expressed as mean ± standard deviation (SD). Qualitative data are expressed as numbers, with percentages shown in parentheses.

Statistically significant differences in the quantitative data were determined using the *t* test or Mann–Whitney *U* test. Multivariate analysis was carried out by logistic regression. Differences were considered to be statistically significant at *P* values of less than 0.05.

Results

Patient characteristics

A total of 1,365 biopsy-proven patients with NAFLD were enrolled in this study. The demographic and clinical characteristics of the male and female NAFLD patients are shown in Supplemental Table 1. Of the total, 709 were males. The mean age of the patients was 51.0 ± 14.9 years (45.7 ± 15.1 and 56.8 ± 12.4 years for males and females, respectively). Whereas no significant differences were observed in the BMI, blood pressure, waist circumference, and visceral fat area between the male and female patients, the subcutaneous fat area and L/S ratio were significantly higher in the female patients. Statistically significant differences were observed in the white blood cell count, hemoglobin, and serum levels of transaminases, AST to ALT ratio, LDH, ALP, GGT, ChE, total and direct bilirubin, albumin, triglycerides, HDL cholesterol, fasting

Table 1 Prevalences of metabolic abnormalities in NAFLD patients

Variable	Percentage
BMI ≥ 25	73.0
Hypertension	39.9
Dyslipidemia	65.7
Hypertriglyceridemia	45.3
Hyper-LDL cholesterolemia	37.5
Hypo-HDL cholesterolemia	19.5
DM	47.3
Hyperuricemia	30.2

glucose, HbA1c, ferritin, uric acid, and hyaluronic acid between the male and female patients, as shown in Supplemental Table 1.

The frequencies of the metabolic abnormalities in the NAFLD patients are shown in Table 1. Obesity, as defined by the criteria of the Japan Society for the Study of Obesity, was seen in 73.0 % of the NAFLD patients, hypertension was found in 39.9 %, dyslipidemia in 65.7 % (hypertriglyceridemia, 45.3 %; hyper-LDL cholesterolemia, 37.5 %; hypo-HDL cholesterolemia, 19.5 %), type 2 diabetes in 47.3 %, and hyperuricemia in 30.2 % of the patients.

Distribution of the metabolic factors by the histological findings

The fibrosis stages (Stage 0/1/2/3/4) were 22.6/34.1/26.7/14.5/2.1 (%) in males, and 16.2/31.7/23.9/21.6/6.6 (%) in females, respectively. The distribution of the fibrosis stage in the different age groups in both genders is shown in Supplementary Fig. 1. Whereas the percentage of patients with advanced fibrosis (Stage 3 and 4) increased gradually with age in both genders, significant increase was seen after the age of 60 years in the females.

The prevalences of obesity (BMI ≥ 25) for each fibrosis stage are shown in Supplementary Fig. 2. The percentages of patients with obesity for each fibrosis stage (Stage 0/1/2/3/4) were 61.3/73.3/79.9/86.4/80.0 (%) in males, and 57.1/72.9/74.4/75.9/74.4 (%) in females, respectively. The prevalence of obesity showed a linear increase with progression of the fibrosis stage in the male NAFLD patients. However, no such increase was observed in the female NAFLD patients between Stage 1 and Stage 4.

The prevalences of dyslipidemia for each fibrosis stage are shown in Figs. 1 and 2. The percentages of patients with hypertriglyceridemia for each fibrosis stage (Stage 0/1/2/3/4) were 56.3/57.7/54.8/51.0/26.7 (%) in males, and 34.0/39.5/39.1/30.2/12.2 (%) in females, respectively. The percentages of patients with hyper-LDL cholesterolemia for each fibrosis stage (Stage 0/1/2/3/4) were 38.6/36.2/41.3/30.4/18.2 (%) in males, and 43.4/38.0/43.6/36.8/11.1 (%) in females, respectively. The prevalence rates of dyslipidemia (hypertriglyceridemia and hyper-LDL cholesterolemia) decreased with progression of the fibrosis stage, especially in Stage 4.

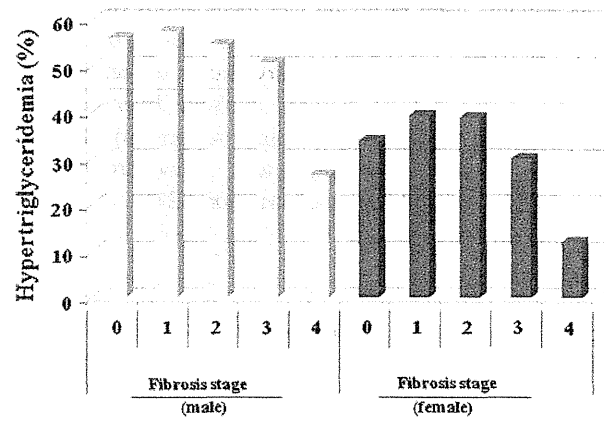

Fig. 1 Prevalence of hypertriglyceridemia for each stage of fibrosis. The *horizontal axis* shows the fibrosis stage and the *longitudinal axis* shows the percentage of patients with hypertriglyceridemia

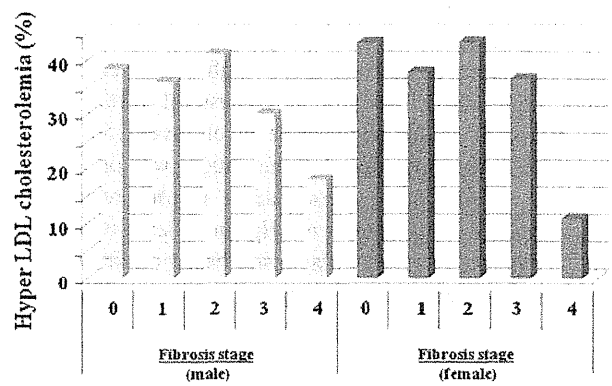

Fig. 2 Prevalence of hyper-LDL cholesterolemia for each stage of fibrosis. The *horizontal axis* shows the fibrosis stage and the *longitudinal axis* shows the percentage of patients with hyper-LDL cholesterolemia

The prevalence of hypertension for each fibrosis stage was shown in Fig. 3. The percentages of patients with hypertension for each fibrosis stage (Stage 0/1/2/3/4) were 17.9/34.0/40.3/51.4/42.9/35.3 (%) in males, and 35.3/50.0/47.7/50.0/23.9 (%) in females respectively.

The prevalences of impaired glucose tolerance, including DM, for each fibrosis stage are shown in Fig. 4. The percentages of patients with DM for each fibrosis stage (Stage 0/1/2/3/4) were 23.7/32.8/53.7/65.8 (%) in males, and 34.7/45.2/60.9/64.7 (%) in females, respectively. The percentages of patients with impaired glucose tolerance (IGT) in each fibrosis stage (Stage 0/1/2/3/4) were 6.6/18.5/17.6/16.2 (%) in males, and 15.3/10.6/14.1/14.1 (%) in females, respectively. The percentages of patients with

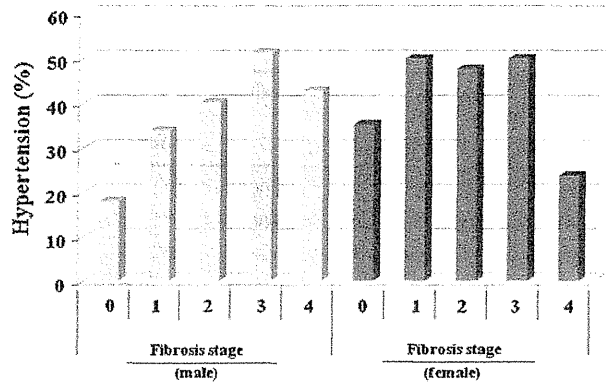

Fig. 3 Prevalence of hypertension for each stage of fibrosis. The *horizontal axis* shows the fibrosis stage and the *longitudinal axis* shows the percentage of patients with hypertension

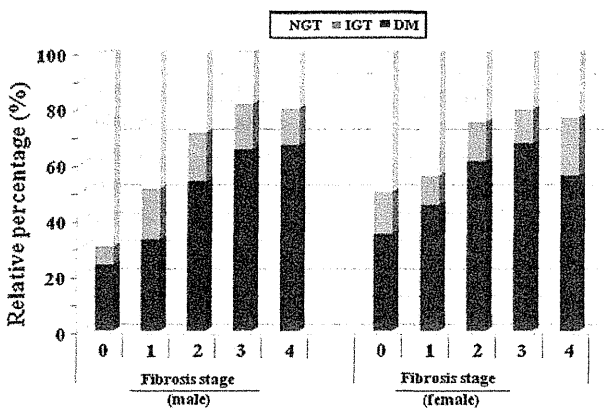

Fig. 4 The glucose tolerance pattern for each fibrosis stage in male and female NAFLD patients. The *horizontal axis* shows the fibrosis stage and the *longitudinal axis* shows the normal glucose tolerance, impaired glucose tolerance, or DM

normal glucose tolerance (NGT) were 69.7/48.7/28.7/17.9 (%) in males, and 50.0/44.2/25.0/21.2 (%) in females, respectively. The percentage of patients with DM increased with progression of the fibrosis stage in both male and female NAFLD patients.

Factors associated with advanced fibrosis

Factors associated with advanced fibrosis were examined (Table 2). NAFLD patients with advanced fibrosis were older, more likely to be female, and obese. The BMI, visceral fat area, and liver/spleen (L/S) ratio were significantly higher in NAFLD patients with advanced fibrosis. Furthermore, significant increases of the serum level of AST, AST/ALT ratio, ALP, GGT, total and direct bilirubin, fasting glucose, HbA1c, IRI, HOMA-IR, ferritin, FFA, and hyaluronic acid, and decreases of hemoglobin, platelet count, ChE, albumin, total cholesterol, triglycerides, LDL cholesterol, and uric acid were observed in the patients with advanced fibrosis. In cases with high fasting plasma glucose levels, HOMA-IR does not reflect insulin resistance exactly, and was assumed to be a reference level. To investigate the factors that might be related to the progression to advanced fibrosis, univariate analysis was performed between NAFLD patients with advanced fibrosis and those with no or mild fibrosis, as shown in Table 3. The results of the analysis revealed obesity (BMI $\geq$ 25), hypertension, hypotriglyceridemia, hyper-LDL cholesterolemia, DM, and hyperuricemia as risk factors for advanced fibrosis. Multivariate analysis identified older age, low serum triglyceride and DM as risk factors for advanced fibrosis.

Discussion

Many factors have been reported to be implicated in the pathogenesis of NAFLD, including obesity, DM, dyslipidemia and hypertension. However, it is still unclear how the metabolic factors might affect the pathogenesis and progression of NAFLD [11, 20, 31–34]. Therefore, identifying the risk factors for the deterioration of NAFLD would be useful for designing therapeutic strategies not only for the liver itself, but also for these metabolic diseases. Whereas a large number of papers have reported the differences in the clinical features between NAFL and NASH, comparisons of the clinical features by the histological severity are scarce. In this study, we retrospectively investigated the associations between metabolic factors and the histologic severity of NAFLD in a large cohort of 1,365 biopsy-proven NAFLD patients, considered as one of the largest-scale studies in the world to date.

The first important finding of our study was that the severity of fibrosis advanced gradually with age in the male patients with NAFLD, while it increased only in those women over 60 years of age. This gender difference may be attributable to menopause in females [35, 36].

The second important finding of our study was the association between obesity and fibrosis severity in NAFLD patients. We compared the prevalence of obesity and the histological severity of NAFLD. As shown in Supplementary Fig. 2, whereas the prevalence of obesity increased with the progression of fibrosis in males, the prevalence remained at approximately 70 % in all age groups of females.

It has been reported that 42–72 % of patients with NAFLD, including NASH, have dyslipidemia [37, 38]. Consist with these reports, dyslipidemia was present in 65.7 % of patients in our study, including hypertriglyceridemia in 45.3 %, increased serum low-density lipoprotein cholesterol in 37.5 %, and decreased serum high-density

Table 2 Comparison for the demographic and clinical characteristics between patients with mild (Stage 0–2) and advanced (Stage 3, 4) fibrosis with NAFLD

Variable	All cases (n = 1,365)	Stage 0–2 (n = 1,062)	Stage 3, 4 (n = 303)	P value	P value (after adjustment for age/sex)
Age	51 ± 14.9	49 ± 15.0	57 ± 12.8	<0.0001	
Gender (male/female)	709/656	591/471	118/185	<0.0001	
Clinical and anthropometric measure					
Body mass index (kg/m^2)	27.9 ± 4.8	27.7 ± 4.8	28.6 ± 4.7	0.0006	<0.0001
BMI ≥ 25 (%)	73.0	71.2	79.5	0.0054	<0.0001
Waist circumference (cm^2)	96.7 ± 13.5	96.1 ± 12.4	98.1 ± 15.4	0.2372	0.0239
Subcutaneous fat area (cm^2)	220.7 ± 103.9	221.1 ± 110.7	219.7 ± 87.1	0.4631	0.4865
Visceral fat area (cm^2)	151.9 ± 65.9	144.4 ± 56.8	168.5 ± 80.3	0.0025	0.0007
L/S ratio	0.75 ± 0.30	0.73 ± 0.29	0.81 ± 0.32	0.0013	0.3528
Blood pressure sys. (mmHg)	127 ± 16.9	127 ± 15.7	124 ± 21.3	0.5343	0.0867
Blood pressure dia. (mmHg)	77 ± 11.1	77 ± 11.0	76 ± 11.5	0.6701	0.6802
Laboratory studies					
White blood cells (/μl)	6,330 ± 1,616.9	6,348 ± 1,583.4	6,272 ± 1,717.7	0.7037	0.6377
Hemoglobin (g/dl)	14.5 ± 1.6	14.6 ± 1.6	14.2 ± 1.6	<0.0001	0.6617
Platelet count (×10^4/μl)	22.4 ± 10.0	23.7 ± 10.4	18.0 ± 6.7	<0.0001	<0.0001
AST (IU/l)	57 ± 38.9	52 ± 36.0	72 ± 44.6	<0.0001	<0.0001
ALT (IU/l)	88 ± 60.3	87 ± 60.2	92 ± 60.4	0.1319	0.0003
AST/ALT	0.72 ± 0.3	0.67 ± 0.3	0.89 ± 0.4	<0.0001	<0.0001
LDH (IU/l)	210 ± 55.1	209 ± 56.7	213 ± 49.9	0.1446	0.5835
ALP (IU/l)	258 ± 111.0	250 ± 103.5	284 ± 130.1	<0.0001	0.0003
GGT (IU/l)	91 ± 103.4	88 ± 103.2	101 ± 103.4	<0.0001	0.0023
ChE (IU/l)	374 ± 106.5	383 ± 104.7	345 ± 107.3	<0.0001	0.0004
Bilirubin, total (mg/dl)	0.89 ± 0.39	0.86 ± 0.36	0.97 ± 0.45	0.0024	0.7731
Bilirubin, direct (mg/dl)	0.21 ± 0.16	0.19 ± 0.13	0.26 ± 0.22	<0.0001	0.2974
Albumin (g/dl)	4.46 ± 0.43	4.50 ± 0.39	4.29 ± 0.50	<0.0001	<0.0001
Total cholesterol (mg/dl)	209 ± 41.9	212 ± 41.5	200 ± 41.6	<0.0001	<0.0001
Triglyceride (mg/dl)	164 ± 102.6	170 ± 107.7	145 ± 79.7	<0.0001	0.0226
HDL cholesterol (mg/dl)	51 ± 15.7	51 ± 16.2	51 ± 13.9	0.3545	0.0297
LDL cholesterol (mg/dl)	130 ± 37.9	133 ± 37.2	123 ± 38.8	<0.0001	0.0009
Fasting plasma glucose (mg/dl)	114 ± 37.8	111 ± 36.1	123 ± 41.4	<0.0001	0.0001
HbA1c (NGSP) (%)	6.32 ± 1.2	6.26 ± 1.2	6.67 ± 1.4	<0.0001	0.0003
IRI (μIU/ml)	15.2 ± 18.5	13.7 ± 11.7	20.1 ± 31.4	<0.0001	<0.0001
HOMA-IR	4.89 ± 10.0	3.98 ± 5.6	6.84 ± 15.5	<0.0001	0.0012
Ferritin (ng/ml)	260.2 ± 475.8	255.8 ± 522.4	275.4 ± 254.5	0.5642	0.5421
Uric acid (mg/dl)	5.9 ± 1.5	6.0 ± 1.5	5.7 ± 1.3	0.0297	0.8563
Free fatty acid (μEq/l)	0.41 ± 0.3	0.36 ± 0.3	0.56 ± 0.3	<0.0001	<0.0001
Hyaluronic acid (ng/ml)	64.3 ± 168.9	42.2 ± 66.9	145.8 ± 329.9	<0.0001	<0.0001

Comparison between patients with mild (Stage 0–2) and advanced (Stage 3, 4) fibrosis using the Chi-square test for binary variables and logistic regression of group indicator on continuous variables

lipoprotein cholesterol in 19.5 % of patients. However, as the third important finding of our study, dyslipidemia tended to decrease in prevalence as the fibrosis stage progressed. Multivariate analysis revealed a negative correlation between the serum triglyceride levels and the fibrosis stage (OR = 0.5687, 95 % CI 0.394–0.821). This result may reflect a deterioration of lipid metabolism with the progression of liver fibrosis towards liver cirrhosis.

The fourth finding of our study was the recognition of a relationship between hypertension and the fibrosis severity in NAFLD patients. In our NAFLD population, hypertension was present in 30.2 %. Whereas no obvious trends in

Table 3 Multiple regression analysis to identify predictive factors for the advanced fibrosis

Variable	All cases	Stage 0–2	Stage 3, 4	Univariate odds ratio (95 % CI)	*P* value	Multivariate odds ratio (95 % CI)	*P* value
Age (mean)	51.0	49.2	57.5	1.042 (1.032–1.053)	<0.0001	1.036 (1.021–1.051)	<0.0001
Female (%)	48.1	44.4	61.1	1.967 (1.516–2.553)	<0.0001	1.180 (0.787–1.768)	0.423
BMI ≥ 25 (%)	73.0	71.2	79.5	1.566 (1.149–2.133)	0.0045	1.568 (0.991–2.481)	0.0545
Hypertension (%)	39.9	38.0	47.3	1.468 (1.063–2.027)	0.0198	0.943 (0.641–1.387)	0.7640
Hypertriglyceridemia (%)	45.3	82.7	34.7	0.566 (0.432–0.739)	<0.0001	0.663 (0.453–0.970)	0.0343
Hyper-LDL cholesterolemia (%)	37.5	39.6	30.7	0.676 (0.496–0.920)	0.0129	0.885 (0.596–1.313)	0.5444
Hypo-HDL cholesterolemia (%)	19.5	19.7	18.9	0.836 (0.671–1.343)	0.7680		–
DM (%)	47.3	42.1	64.9	2.544 (1.948–3.320)	<0.0001	2.387 (1.603–3.553)	<0.0001
Hyperuricemia (%)	30.2	32.1	24.4	0.684 (0.485–0.965)	0.0308	1.058 (0.693–1.617)	0.793

the prevalence of hypertension were observed in females, comparison of the relationship between the prevalence of hypertension and the stage of fibrosis, except for Stage 4, revealed a tendency towards increase in the prevalence of hypertension with progression of the fibrosis stage. In general, blood pressure is considered to have an effect on the rate of progression of NAFLD. Systolic and diastolic blood pressures have been reported to be correlated with the liver fat content, and patients with systolic hypertension were reported to be correlated with the liver fat contents, and patients with systolic hypertension were reported to show a two-fold higher risk of development of NAFLD [39]. As shown in Fig. 3, the decrease in the rate of hypertension in NAFLD patients with Stage 4 liver fibrosis might be, at least in part, attributable to the hyperdynamic circulation, characterized by peripheral vasodilation and increased portal resistance, observed in patients with liver cirrhosis [40, 41].

Impaired glucose tolerance is well known to accompany NAFLD. While it appears clear that abnormal glucose tolerance, including DM, is a risk factor for NAFLD and vice versa, the relationship between abnormal glucose tolerance and the histological severity of NAFLD is still unknown. The fifth finding in our study was that the prevalence of DM increased with progression of the fibrosis stage (Fig. 4). Multivariate analysis identified DM as an independent risk factor for advanced fibrosis (OR = 2.8573, 95 % CI 1.941–4.207). In vitro, high glucose and high insulin concentrations, which are often observed in patients with NAFLD, were shown to stimulate connective tissue growth factor expression, which is known as one of the important mechanisms involved in the progression of hepatic fibrosis [42]. Furthermore, the cirrhotic condition is suspected to facilitate the development of hyperinsulinemia and hyperglycemia via the deteriorated liver function [43, 44]. Taken together, it would be reasonable to consider DM as both a cause and result of NAFLD [45].

In conclusion, we have reported the prevalences of lifestyle-related diseases, such as obesity, dyslipidemia, hypertension, and DM, in NAFLD patients according to the stage of fibrosis. Multivariate analysis identified DM as a significant risk factor for advanced fibrosis. Accordingly, impaired glucose tolerance, including DM, should be properly evaluated and managed for preventing the progression of NAFLD, even in the early stages of NASH.

Conflict of interest The authors declare that they have no conflict of interest.

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特集／肝炎から肝硬変・肝癌まで

非アルコール性脂肪肝炎(NASH)の診療

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はじめに

職場検診における血圧測定の励行が，高血圧を引き起こす最大の要因である塩分摂取量の制限につながり，脳出血症例が激減したのは記憶に新しい。そして，今日，メタボリックシンドローム検診の名の下に，高血圧や脂質異常症，あるいは糖尿病などの検診と同時に腹囲の測定が広く行われるようになり，今後，脳・心血管イベントの減少に繋がると期待されている。

このように検診で疾病を発見することが予後の改善に繋がると期待されるようになった背景には，高血圧，脂質異常，糖尿病領域における治療薬の進歩がある。優れた薬効を有する薬剤を医師が病状に応じて提供することで，動脈硬化巣の退縮など原因となる因子を改善することにより生活習慣病の治療が可能となった臨床的な意義は大きい。では，職場検診で最も高頻度に見つかる異常は高血圧，それとも脂質異常。私たちの身の回りで一番多く服用されている治療薬は，この2種類の疾患に対する薬剤である。にもかかわらず，検診で最も高頻度に異常を指摘されるのは肝機能である（図1）[1]。

本稿では検診で高頻度に異常を指摘されるにもかかわらず，治療に結びつくことの少ない慢性肝疾患の診療について紹介する。

I．日常診療で高頻度に出会う慢性肝疾患

検診で異常を指摘されてご紹介をいただく慢性肝疾患の中で，最も頻度の高い疾患が非アルコール性脂肪性肝疾患（NAFLD）である。ついで，アルコール性肝疾患，ウイルス性肝疾患と続く。このため，少なくともこの3疾患の鑑別を行う必要がある（図2）。

慢性肝炎を呈するウイルス性肝疾患で頻度の高いものは，B型慢性肝炎とC型慢性肝炎である。スクリーニングはHBs抗原とHCV抗体で行う。HBV-DNAあるいはHCV-RNAを検出すれば確定診断を下すことができる。他方，アルコール性肝疾患の診断はエタノールで60g/日以上の飲酒量があれば可能であるが，それ以下の場合でも女性を中心にアルコール性肝障害を認めることがある。病理学的には，アルコール性脂肪肝から肝線維症，肝硬変，そして肝細胞癌を合併することがある。

自宅で毎日飲酒を行っていると申告した脂肪肝を伴わない症例における肝機能検査の分布をみると，γ-GT＞100 IU/Lの異常値を示す症例は以外に少なく，ALT値の上昇も軽度で，異常値（ALT＞30 IU/L）を示す頻度は低い（図3）。

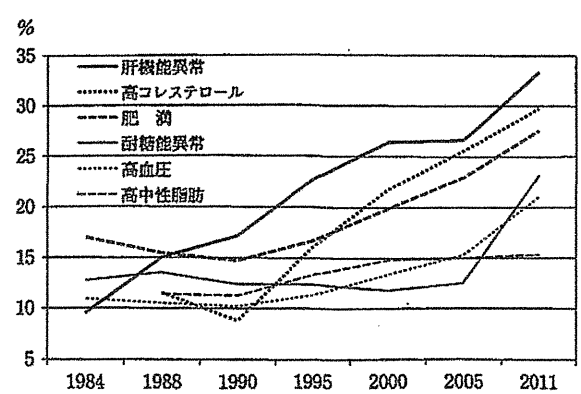

図1　人間ドック学会による検診6項目異常の推移

高知大学消化器内科

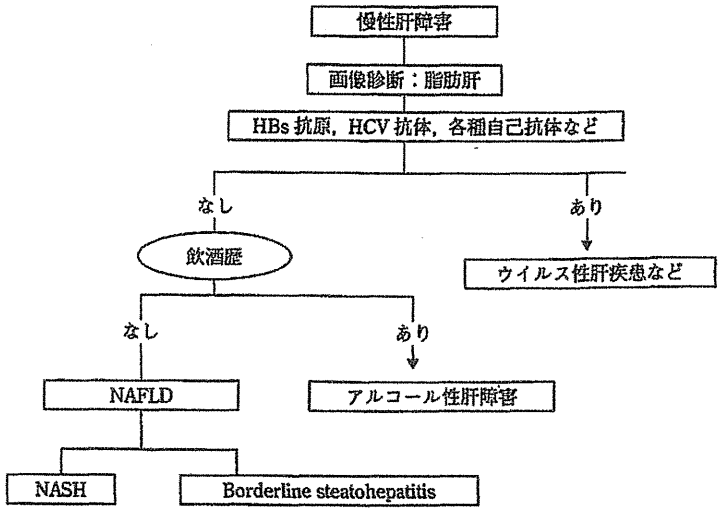

図　2　慢性肝疾患の鑑別チャート

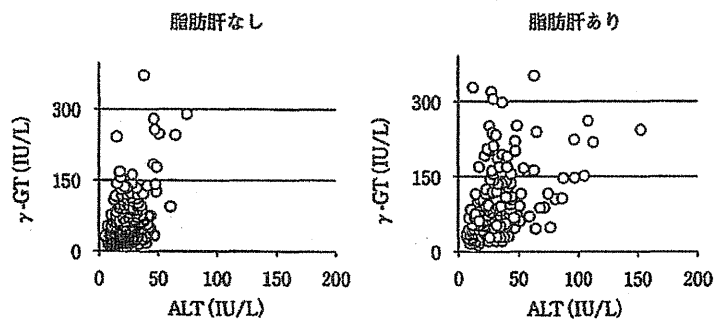

図　3　検診受診飲酒者における肝障害と脂肪肝

これに比して，脂肪肝を伴う症例ではγ-GT，ALT共に高値を示す症例の多いことが分かる。このことから，脂肪肝はアルコール性肝障害の増悪因子とみなすことも可能である。このようにゆっくりと進行する病態の他に，別項で詳述される急性増悪像としてのアルコール性肝炎があるが，アルコール性肝炎には重篤感があるので両者の鑑別は容易である。また，ウイルス性肝疾患に対しては，インターフェロンや核酸アナログ，アルコール性肝障害では嫌酒薬や断酒会などのように極めて有用な治療法が確立されてきた。有力な治療薬や治療をサポートする環境整備面で取り残された慢性肝疾患がNAFLDである。

Ⅱ．非アルコール性脂肪性肝疾患

ウイルス性慢性肝疾患対策が進み，検診を通じてアルコール性肝障害者に対するケアが浸透するにつれ，日常診療の場でも肥満を背景因子とする肝障害者の比率が徐々に高まってきた。検診で肝障害を指摘される頻度は1980年代から漸増し，2011年にはとうとう検診受診者の3割を超えた（図1）。肥満人口の増加に伴う脂肪肝の蔓延に加えて，飲酒習慣を有する男性成人の割合が徐々に低下して3人に1人となり非飲酒者が増加したことが大きな役割を果たしたと考えられる[2)3)]。飲酒歴のない肥満者ではALT高値を示す症例が多く，非アルコール性脂肪性肝疾患（NAFLD）と呼ばれる。このような症例では一般にAST<ALTで，γ-GTは正常あるいは軽度高値であることが多く，肝臓の線維化の進展と共にAST/ALT比が増加する（図4）。

肝臓はアルコール性肝障害と類似した脂肪肝を背景とする病変を呈し，脂肪肝，脂肪肝に炎症性細胞浸潤を伴う症例，風船様肝細胞や肝線維化を伴う症例，肝硬変，そして肝細胞癌を合併することがあるのはアルコール性肝障害と同

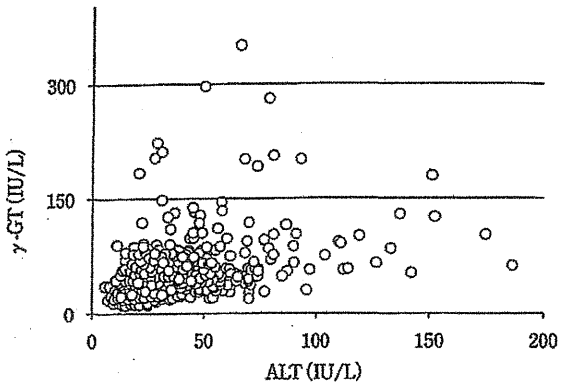

図 4　検診受診非飲酒脂肪肝症例における肝機能異常

様である[4]。非アルコール性脂肪性肝疾患の内，肝硬変を含む肝疾患関連死に至る症例の肝病変に共通する所見は肝臓の線維化や風船様肝細胞にしばしば観察されるマロリー小体の存在である。このため，風船様肝細胞や肝線維化を伴う非アルコール性脂肪性肝疾患は特に非アルコール性脂肪肝炎（NASH）と呼ばれる。

肥満を背景とする脂肪肝症例の多くは自覚症状に乏しいため，検診で脂肪肝を指摘されても放置されることが多い。また，NASH をきたす背景疾患も多様であり，高血圧や脂質異常症のように画一的な治療ですべての症例の肝病変が改善するわけではないため，NASH を適応疾患とする薬剤は未だ存在しない。しかし，成人の2％が罹患する疾患である NASH が肝疾患関連死をもたらす疾患であることを考慮すると，その背景疾患である NAFLD に対する積極的な介入が日常診療で必要であることは明らかである。

Ⅲ．非アルコール性脂肪性肝疾患の新分類

NASH は各種慢性肝疾患を除外した後，肝疾患関連死を惹起する因子を念頭に，肝臓の病理学的所見に基づいて診断される疾患である。このため，上述のように多様な病因を背景に発症していると考えられる。そこで最近，すべての NAFLD を母集団として，肝臓の病理学的所見を中心に NASH の診断を見直す提案がなされた（図5）[5]。

肝臓の最少構造単位は中心静脈を中心とした肝小葉であり，肥満に伴う脂肪肝は通常，中心静脈を中心とする zone 3 と呼ばれる領域に存在する肝細胞への大滴性脂肪滴の沈着を特徴とする。そこで，脂肪滴の沈着がこの領域を中心とする脂肪肝で，風船様肝細胞や肝線維化を伴う非アルコール性脂肪性肝疾患を新たに“definite” NASH と定義することにより，NASH の背景病変をできるだけ均質にしようという提案である。この提案は病理組織学的に病因が明らかに異なると想定される症例を NASH 関連疾患と位置づけることによって，典型的でより均質な NASH 症例を集積して，有効性の高い治療法を確立しようとの意図に基づいている。

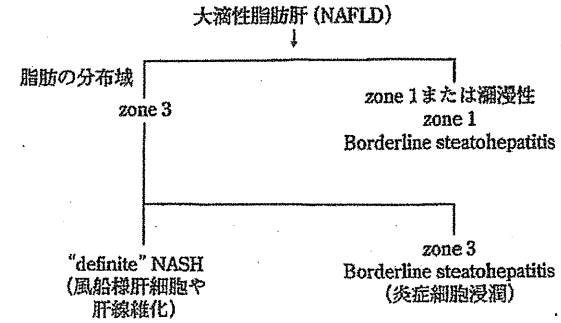

図 5　“definite” NASH と Borderline steatohepatitis
線維化を伴わない場合

脂肪滴の沈着が小葉構造に依存せず瀰漫性に分布する症例や門脈域を中心に分布する症例では，中心静脈領域を中心に脂肪滴の沈着が生じる典型的な NASH 症例とは異なる可能性が高く，治療反応性が異なるかも知れない。このような症例を新たに Borderline 1 の境界病変として捉えれば，NASH 発症の背景疾患をより均質なものにできるであろう。また，従来，NASH を臨床的に疑って肝生検を行っても，風船様肝細胞や肝線維化が認められない症例は単純性脂肪肝に分類せざるを得ず，NASH の診断を担当する病理医には重い負担となっていた。肝生検サンプル上に典型的な所見を見出すことができずに NASH と診断できない症例を Borderline 3 に分類して，NASH 疑診例として扱うことが可能となれば，病理医の負担は大きく軽減される。

Ⅳ．NASH の非観血的診断

上述のように NASH の診断には病理学的所見が用いられるが，NASH を疑った症例全てに肝生検を施行することは困難である。また，NASH の特徴的な病理所見が得られなかったか

らといって，有酸素運動による筋肉におけるインスリン感受性の回復に加えて，肥満の軽減を図るというNASH治療における基本方針が大きく変わるわけではない[6]。従って，日常診療ではNAFICスコア[7]のような指標を用いて，肝病変の程度を推測し，NASHを疑った症例を中心としてNAFLDの診断の基に積極的な治療を開始することが重要である。

V. 肥満を念頭においたNASH治療

多様な病因を背景に発症するNASHすべてを適応症とする薬剤は存在しない[5]。本稿では肥満を背景とするNASHを念頭に治療の方向性を示したい。この場合，まず行わなければならないのは過食と運動不足を主要な誘因とするNASHか，それ以外の誘因に基づくNASHであるかの判別である。今日のNAFLD増加の最大の誘因は肥満である。BMIが25を超えて肥満と呼ばれるようになると3割の症例で脂肪肝が観察される。NAFLDは脂肪肝を背景に発症する病態であり，NAFLDの一部として位置づけされるNASHにおいて肥満を解消できれば有効な治療法となると考えることは妥当であろう。

欧米のNAFLDでは心血管イベントによる死亡率が高いため，肥満の改善が強く求められている。本邦でもNASH・NAFLDでは内臓肥満に加えて，メタボリックシンドロームを構成する高血圧や脂質異常，耐糖能異常を合併する頻度が高いため，肥満の改善を通じて肝病変のみならず，心血管イベントの改善も可能と考えられる。NASHを適応症として，肝疾患関連死を改善する治療薬がない現状では，総死亡率の軽減を目標としてメタボリックシンドロームの構成要素の正常化を目指すこともなすべき一つの方策である。このため，NASH・NAFLDでは治療の第一段階として，内臓肥満の改善を目指した有酸素運動とカロリー制限による減量が推奨されている[6]。

しかしながら，有酸素運動とカロリー制限だけでは高血圧や脂質異常，耐糖能異常の早急な改善を行うことは容易ではないので，必然的に薬物療法を併用してその速やかな改善を目指すことになる。近時，このような薬物療法を通じてNASHの予後改善効果が模索されている。たとえば，高血圧治療薬であるアンギオテンシンII受容体阻害剤にはNADPH oxidaseの阻害作用があるため肝線維化抑制効果，ω-3系脂肪酸では脂肪酸のβ酸化亢進作用による脂質異常や脂肪肝の改善，チアゾリジン系薬剤ではインスリン感受性の改善を介して糖尿病を伴わないNASH症例の脂肪肝改善や肝線維化の改善効果など，その薬剤が本来目的とする病態の改善に加えてNASH・NAFLDの肝病変に対する改善効果も期待されている[4]。"definite" NASHの提案はこのような期待に対する回答を促し，新たな治療薬開発の促進に重要な役割を果たすことが期待される。

VI. NASHの長期予後

NAFLDの長期予後は必ずしも良好ではない。その原因は上述したようにメタボリックシンドロームの合併による脳・心血管イベントの好発である。これに対して，肝疾患としての長期予後は比較的良好である。なぜならば，肝硬変に進展するリスクを持つのはNASHであり，NAFLDの1割を占めるに過ぎないからである。従って，NAFLD症例では合併する生活習慣病の治療が優先される。

しかし，成人人口の2%程度をしめるNASHで，その2～3割が肝疾患関連死に直面する現状を過小評価してはならない。男性では中学生から肝臓の線維化が進展したNASH症例が出現し，30歳代よりNASHの罹患者が増加するのに対して，女性では閉経期以降にNASHに移行する症例が多く，肝臓の線維化は急速に進展する傾向が強い。女性の肝細胞癌の出現は70歳前後の肝硬変症例に集中するが，男性では肝硬変に至らない若年症例でも肝細胞癌が出現するので，留意が必要である。肝線維化を認めるNASHでは年率1～2%程度の肝硬変への移行，年率1%程度の肝疾患関連死が生じている[8][9]。

日常診療では有酸素運動とカロリー制限による改善を目的とした指導に留まらず，肝線維化の進展が疑われる症例に対してはNAFICスコアなどを併用して，インスリン高値例には運動と食事療法の併用によるインスリン感受性の回復，フェリチン高値例には瀉血，線維化進展例には肝病変の改善が期待される薬剤を選択する