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# Atf6α-null mice are glucose intolerant due to pancreatic β-cell failure on a high-fat diet but partially resistant to diet-induced insulin resistance

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ABSTRACT

Activating transcription factor 6α (ATF6α) is essential for the endoplasmic reticulum (ER) stress response. Since recent studies suggested that ER stress is involved in the pathogenesis of type 2 diabetes mellitus, we have analyzed *Atf6α*-null (*Atf6α*$^{-/-}$) mice challenged with metabolic overload or genetic manipulations. *Atf6α*$^{-/-}$ mice were fed a high-fat diet to create diet-induced obese (DO) mice, and were subjected to examination of glucose homeostasis with biochemical and morphological analysis of the pancreatic β-cell and liver tissues. *Atf6α*-null mice were also crossed with genetic models of diabetes caused either by insulin resistance (Agouti obese mice) or by impaired insulin secretion (*Ins2*$^{WT/C96Y}$ mice). *Atf6α*$^{-/-}$ DO mice were less glucose tolerant with blunted insulin secretion compared to littermates on a high-fat diet. Pancreatic insulin content was lower in *Atf6α*$^{-/-}$ DO mice with the swollen β-cell ER, a typical feature of cells with ER stress. In the liver of *Atf6α*$^{-/-}$ DO mice, XBP-1 splicing was increased, suggesting that higher ER stress was present. ATF6-deficient mice showed increased mRNA expressions of glucose-6-phosphatase and SREBP1c associated with a tendency for a higher degree of steatosis in the liver. However, *Atf6α*$^{-/-}$ DO mice exhibited higher insulin sensitivity with lower serum triglyceride levels. Similar phenotypes were observed in ATF6α-deficient Agouti mice. In addition, ATF6α-deficiency accelerated reduction in pancreatic insulin content in *Ins2*$^{WT/C96Y}$ mice. These data suggested that ATF6α contributes to both prevention and promotion of diabetes; it

*Abbreviations:* ATF6α, activating transcription factor 6α; BW, body weight; cDNA, complementary DNA; DO, diet-induced obese; eIF2α, eukaryotic initiation factor 2; ER, endoplasmic reticulum; G6Pase, glucose-6-phosphatase; GRP78, 78 kDa glucose-regulated protein; HFD, high-fat diet; IPGTT, intraperitoneal glucose tolerance test; ITT, intraperitoneal insulin tolerance test; IRE1, inositol requiring enzyme 1; PERK, PKR (double-stranded-RNA-dependent protein kinase)-like ER kinase; TG, triglyceride; UPR, unfolded protein response; VLDL, very low-density lipoprotein; WT, wild-type; XBP, X-box binding protein.

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protects β-cells from ER stress and suppresses hepatosteatosis, but plays a role in the development of hyperlipidemia and insulin resistance.



## 1. Introduction

Glucose homeostasis is maintained through fine regulation of insulin secretion from pancreatic β-cells and glucose production from the liver as well as glucose disposal into muscle and adipose tissues [1]. Recent studies have shown decreased pancreatic β-cell mass to be a common feature of subjects with type 2 diabetes mellitus (T2DM) [2-4]. Stress-mediated apoptosis is considered as one of the causes of β-cell loss. Pancreatic β-cells are especially vulnerable to endoplasmic reticulum (ER) stress [5,6] because of continuous and abundant production of insulin. In addition, ER stress and the activation of related stress signaling pathways are suggested to be an important mechanism underlying insulin resistance [7,8]. Recent studies showed that ER stress is present in adipose and liver tissues in obese humans [9-11].

ER stress is triggered when the amount of unfolded and misfolded proteins exceeds the folding capacity of the ER. This protein folding stress is recognized by three ER-resident transmembrane proteins, PERK (PKR (double-stranded-RNA-dependent protein kinase)-like ER kinase), IRE1 (inositol requiring enzyme 1) and ATF6α (activating transcription factor 6α). These ER stress sensor proteins initiate a series of signaling cascades known as the unfolded protein response (UPR) [12,13]. The UPR reduces ER stress in two ways, one of which is by reduction in ER protein load. This reduction is mediated by activation of PERK, which phosphorylates α subunit of eukaryotic translation initiation factor eIF2 (eIF2α), resulting in inhibition of global protein synthesis [14]. Another means to cope with ER stress is to increase protein folding capacity and to enhance activity of protein degradation. PERK-mediated inhibition of global protein synthesis paradoxically increases expression of ATF4. Activation of IRE1 leads to generation of an active form of transcription factor XBP-1 (X-box binding protein-1). These transcription factors together with ATF6α orchestrate transcriptional induction of specific genes which facilitate folding or degradation of misfolded proteins. Essential roles of PERK and IRE1α in cell survival have been established mainly through studies using *Perk*-null [15] and *Ire1α*-null [16] cells and mice. Furthermore, mutations of the *EIF2AK3* gene encoding PERK in humans cause Wolcott–Rallison syndrome with diabetes mellitus in early infancy [17], demonstrating an essential importance of PERK signaling in β-cells.

In contrast to these sub-pathways of the UPR, studies of the ATF6α-mediated pathway in the cell or organ physiology have recently been commenced. ATF6α is a type II transmembrane glycoprotein located on the ER membrane. In response to ER stress, ATF6α is cleaved and the N-terminus 50 kDa protein moves to the nucleus. Once in the nucleus ATF6α binds to the ER stress response elements in ER stress genes, which include molecular chaperones GRP78 and GRP94, augmenting ER capacity to assist folding of secretory and membrane proteins [13,18]. Recently, *Atf6α* knockout mice were generated [19,20]. Using cells from these mice, ATF6α was revealed to function as a critical regulator of ER quality control proteins.

In humans, expression of GRP78, a downstream effecter of ATF6α, was reportedly increased in T2DM β-cells [21]. Expressions of molecular chaperones downstream of ATF6α were also shown to be augmented in human adipose tissue and liver with obesity [10,11]. Furthermore, the ATF6α-Met67Val substitution with increased intrinsic activity was found to cause elevation in plasma cholesterol levels [22]. Several single nucleotide polymorphisms in the *ATF6α* gene were found to associate with T2DM [23-25], although a lack of association between genetic polymorphisms in the locus and glucose metabolism was also recently reported [26]. These data suggest an important role of ATF6α in development of obesity and T2DM. However, the causal relationship is not clear nor has the role of ATF6α been established in whole body glucose homeostasis. Therefore, in this study, we have analyzed glucose homeostasis in *Atf6α*-null mice challenged with metabolic overload or genetic manipulations.

## 2. Materials and methods

### 2.1. Animals

The Tohoku University Institutional Animal Care and Use Committee approved all animal experiments. Generation of *Atf6α*-null mice was described previously [20]. Heterozygous *Atf6α* knockout mice ($Atf6\alpha^{+/-}$) were backcrossed to female wild-type C57BL/6J mice eight times. Homozygous knockout mice were produced by intercrossing male and female heterozygotes. Genotyping for *Atf6α* knockout allele was performed using the forward primer 5′-CTTCTGAGGCGGAAAGAACCAGCTG-3′ and the reverse primer 5′-TTTGCAAGTC CAATGGGCC TCTC-3′. Reverse transcription PCR for detecting expression of *Atf6α* mRNA was conducted using primers 5′-CCAACAGAAAGCCGCATT-3′ and 5′-TGGACAGCCAT-CAGCTGA GA-3′. $Ins2^{WT/C96Y}$ (Akita) mice (C57BL/6J background, [27]) were purchased from Charles River. Male $Ins2^{WT/C96Y}$ mice were mated with female $Atf6\alpha^{-/-}$ mice to generate $Atf6\alpha^{+/-}Ins2^{WT/C96Y}$ mice which were further mated with female $Atf6\alpha^{-/-}$ to produce $Atf6\alpha^{-/-}Ins2^{WT/C96Y}$ mice. Genotyping for $Ins2^{WT/C96Y}$ mice was as described previously [27]. C57BL/6J Ham Slc-$A^{y/+}$($A^y$) mice were obtained from Japan SLC. Male $A^y$ mice were mated with female $Atf6\alpha^{-/-}$ mice to generate $Atf6\alpha^{+/-}A^y$ mice which were further mated with female $Atf6\alpha^{-/-}$ to produce $Atf6\alpha^{-/-}A^y$ mice. Offspring positive for the $A^y$ gene were recognized by agouti coat color. All agouti experiments were performed on the F1 generation. The mice were kept under standard, specific pathogen-free conditions with a constant dark/light cycle and free access to standard

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mouse chow (MF; Oriental Yeast, Tokyo, Japan) and water. The high-fat diet (rodent diet with 60% energy from fat; D12492) (HFD) was purchased from Research Diets (New Brunswick, NJ, USA) and was freely accessible.

### 2.2. Physiological studies

Body weights (BW) were measured once every other week. Blood samples were collected from the tail vein. Intraperitoneal glucose tolerance test (IPGTT: 2 g glucose/kg BW) was started by injecting 20% glucose solution. Intraperitoneal insulin tolerance test (ITT) was performed using the regular insulin (0.75 U/kg BW for mice on normal chow and 1.5 U/kg BW for mice on an HFD). Blood glucose levels were measured by the glucose oxidase method using a Glutest glucose sensor device (Sanwa Kagaku Kenkyusho, Nagoya, Japan). Serum insulin levels were determined using an insulin ELISA kit (Morinaga Institute of Biological Science, Tokyo, Japan).

### 2.3. Pancreatic insulin content and hepatic triglyceride content

Mice were killed by cervical dislocation. Pancreases were removed and homogenized in acid/ethanol (0.7 mol/l HCl/ethanol 25:75) and left at −20 °C for 48 h, with sonication every 24 h. Homogenates were then centrifuged (8000*g* for 10 min) and the insulin content of the acid/ethanol supernatant fraction was measured using insulin ELISA. Frozen livers were homogenized and triglycerides were extracted with CHCl3:CH3OH (2:1, v:v), dried and resuspended in 2-propanol [28]. Triglyceride content was measured using Lipidos liquid kit (TOYOBO, Osaka, Japan).

### 2.4. Immunohistochemistry

Pancreases were removed and fixed in 4% formalin. Formalin-fixed paraffin-embedded sections of pancreas were deparaffinized and re-hydrated. The sections were then incubated with a guinea pig anti-insulin IgG (DAKO Japan, Kyoto, Japan) diluted 1:1000 for 1 h at room temperature.

### 2.5. Quantitative RT-PCR

Total RNA was isolated from 0.05 g liver with Isogen (Wako Pure Chemical, Osaka, Japan), and cDNA was synthesized with a Transcriptor First Strand cDNA Synthesis Kit (Roche Diagnostics, Mannheim, Germany) using 1 μg total RNA. Complimentary DNA synthesized from total RNA was evaluated using the real-time quantitative PCR system (Light Cycler Quick System 350S; Roche Diagnostics). The relative amounts of mRNA were calculated with β-actin mRNA as the invariant control. The primers used are described in the Supplementary Table.

### 2.6. Ultrastructural analysis

We performed ultrastructural analyses of pancreatic β-cells from 25-week-old mice using electron microscopy. The samples of cells were fixed with 2% glutaraldehyde plus 2% paraformaldehyde in 0.1 M phosphate buffer (PB, pH 7.4) at 4 °C, and subsequently post-fixed with 2% osmium tetroxide in 0.1 M PB at 4 °C for 2 h. Then, the specimens were dehydrated in a graded ethanol, replaced in propylene oxide, and embedded in the epoxy resin. Ultrathin sections were obtained by ultramicrotomy technique. Ultrathin sections stained with uranyl acetate at 60 °C for 20 min and modified Sato's lead solution for 5 min were submitted to transmission electron microscope observation (JEM-1200EX, JEOL). The β-cells were distinguished from α- and δ-cells by the appearance of their secretory granules. The β-cell granules had a white halo, not evident in α- and δ-cell granules.

### 2.7. Statistical analysis

Data are presented as means ± S.E., unless otherwise indicated. Differences between groups were assessed by Student's t test.

## 3. Results

### 3.1. Glucose homeostasis in ATF6α-deficient mice on normal chow

Founder *Atf6α* heterozygous knockout mice [20] were backcrossed to the C57BL/6J mice for at least 8 generations and heterozygous knockout male and female mice were mated to generate whole body *Atf6α* homo knockout mice (Fig. 1A). In the present study, we analyzed male mice, since preliminary studies showed similar phenotypes in female *Atf6α*$^{-/-}$ mice. When *Atf6α*-null mice were fed with standard chow, their body weights (BW) tended to be lower than those of wild-type (WT) mice (Fig. 1B). Blood glucose levels measured in non-fasted states were comparable between two strains (data not shown). Neither blood glucose excursions (Fig. 1C) nor insulin secretory responses (Fig. 1D) differed significantly between WT and *Atf6α*$^{-/-}$ mice when mice were subjected to an intraperitoneal glucose tolerance test (IPGTT). Insulin sensitivity, estimated by an intraperitoneal insulin (0.75 U/kg BW) tolerance test (ITT), showed that *Atf6α*$^{-/-}$ mice were somewhat more insulin sensitive, although the difference was not statistically significant (Fig. 1E). However, when ITT was performed using 2.0 U/kg BW of insulin, 5 out of 6 *Atf6α*$^{-/-}$ mice showed severe hypoglycemia of less than the lower detection limit (1.1 mM) of the glucose monitoring device at 60, and 90 min, while blood glucose levels of all WT mice were higher than 2.0 mM at 90 min (Fig. 1F), suggesting that insulin sensitivity were higher in *Atf6α*$^{-/-}$ than in WT littermates.

### 3.2. Glucose homeostasis in ATF6α-deficient mice fed with a high fat diet

Impaired glucose homeostasis is often associated with obesity. To further investigate roles of ATF6α in glucose homeostasis, we fed *Atf6α*$^{-/-}$ mice with a 60% high fat diet (HFD) to generate diet-induced obese (DO) animals. ATF6α-

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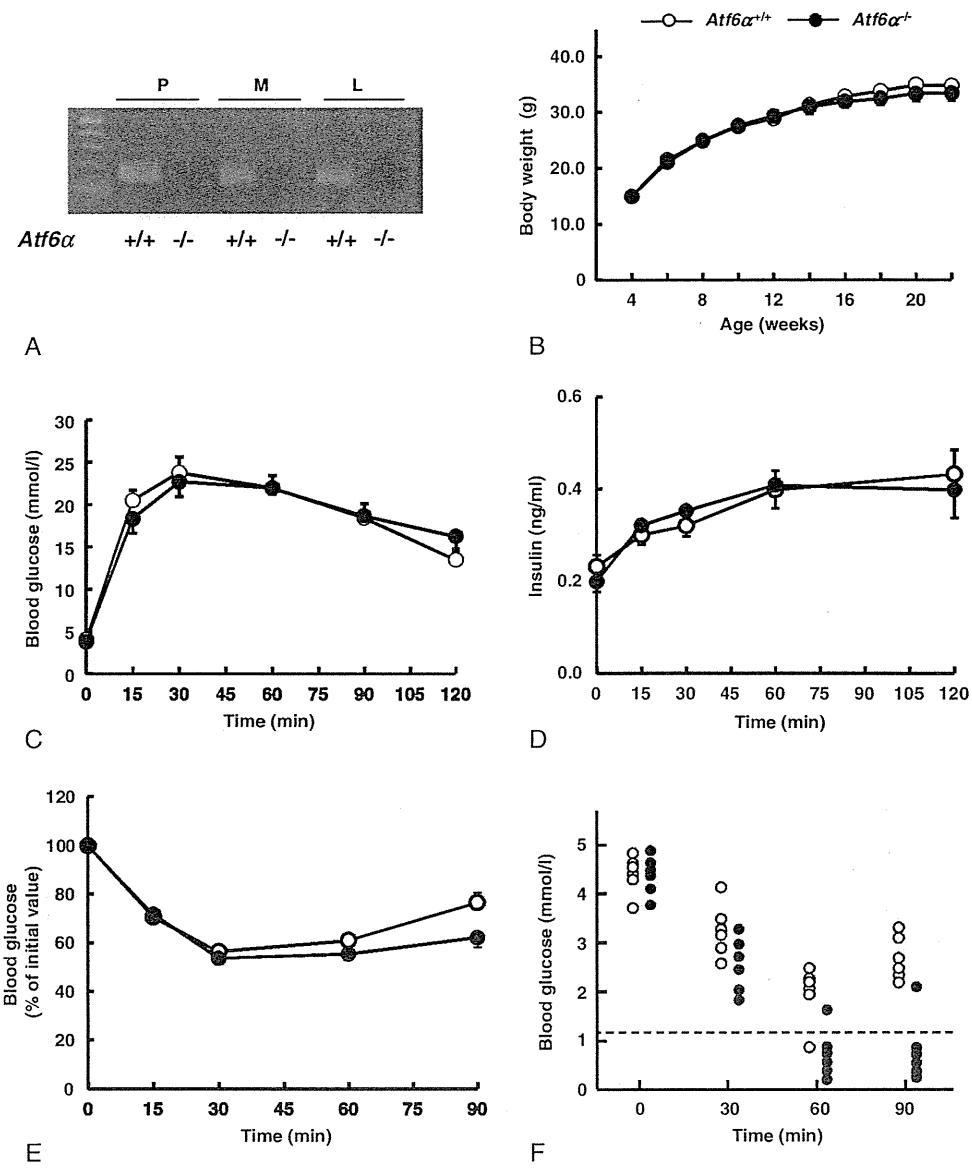


**Fig. 1 – Glucose homeostasis in ATF6α-deficient mice fed with normal chow. A, Reverse transcription-PCR analysis of *Atf6α* mRNA expression in the pancreas (P), skeletal muscle (M) and liver (L) from *Atf6α*$^{+/+}$ and *Atf6α*$^{-/-}$ mice. B, Body weight of *Atf6α*$^{+/+}$ (white circles, *n* = 9) and *Atf6α*$^{-/-}$ (black circles, *n* = 13) mice. Data from three cohorts are combined. C and D, Intraperitoneal glucose tolerance tests (2 g/kg body weight) were performed at 17–19 weeks of age in *Atf6α*$^{+/+}$ (white circles, *n* = 9) and *Atf6α*$^{-/-}$ (black circles, *n* = 13) mice. Blood glucose (C) and plasma immunoreactive insulin levels (D) were measured. E, Insulin tolerance test (0.75 U/kg body weight) was performed at 21 weeks of age in *Atf6α*$^{+/+}$ (white circles, *n* = 7) and *Atf6α*$^{-/-}$ (black circles, *n* = 9) mice. F, Insulin tolerance test (2.0 U/kg body weight) was performed at 23 weeks of age in *Atf6α*$^{+/+}$ (white circles, *n* = 6) and *Atf6α*$^{-/-}$ (black circles, *n* = 6) mice. In one *Atf6α*$^{+/+}$ mouse at 60 min and 5 *Atf6α*$^{-/-}$ mice at 60 and 90 min, blood glucose levels were below the detection limit (1.1 mM, dashed line).**

deficient DO mice tended to gain less weight (Fig. 2A) and showed similar blood glucose levels to those of WT-DO mice at non-fasted states (Fig. 2B). An IPGTT exhibited that ATF6α-deficient DO mice were less glucose tolerant (Fig. 2C) with reduced insulin secretion (Fig. 2D). On an HFD, it was evident that ATF6α-deficient mice were more insulin sensitive (Fig. 2E). We also measured serum lipid content in these mice. As shown in Fig. 2F, an HFD-induced increase in the serum triglyceride (TG) concentration was partially suppressed in *Atf6α*$^{-/-}$DO mice.

### 3.3. *Pancreatic β-cells suffered from ER stress in ATF6α-deficient mice fed with a high fat diet*

Although serum insulin levels in *Atf6α*$^{-/-}$DO mice (Fig. 2D) were lower than those in WT-DO mice, they were 3 times

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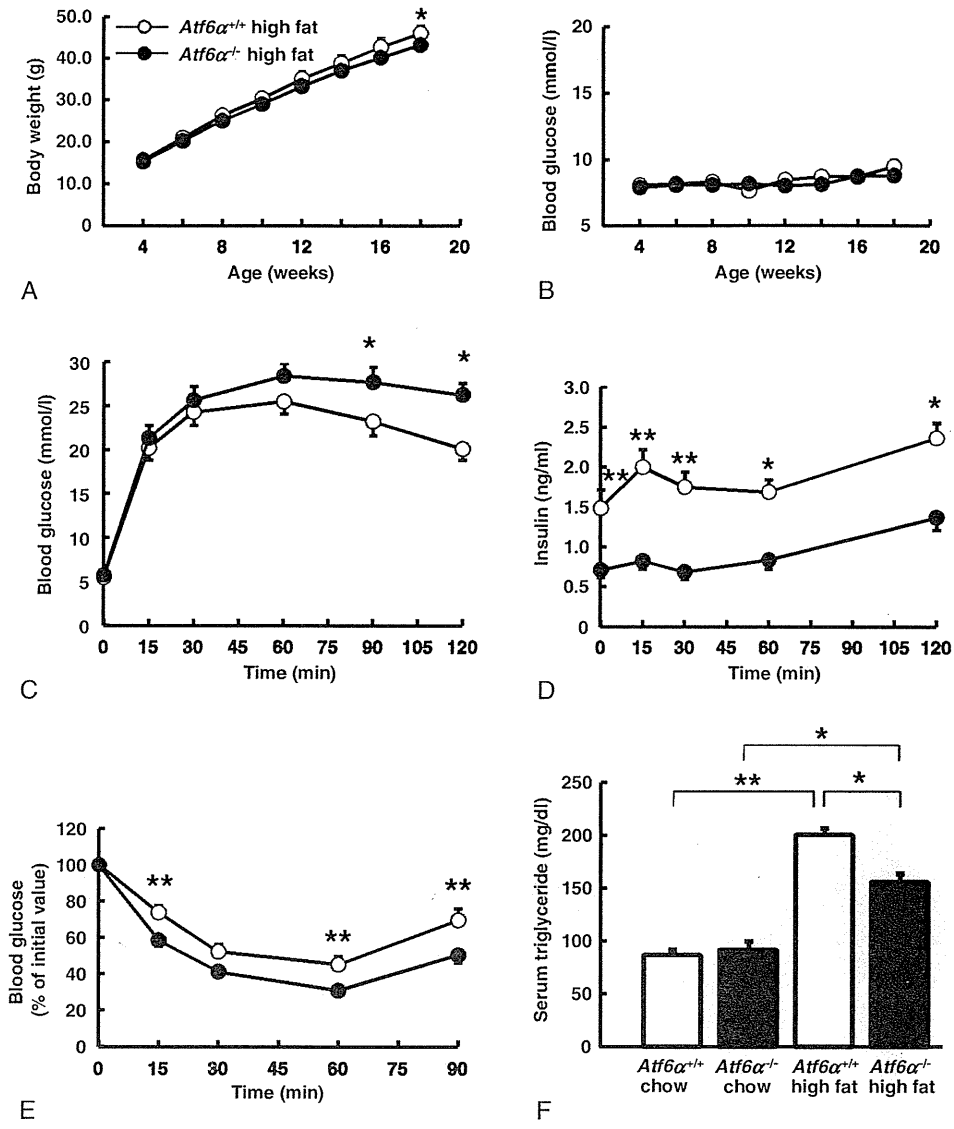


**Fig. 2 – Metabolic homeostasis in ATF6α-deficient mice fed with an HFD. A and B, Body weight (A) and non-fasted glucose (B) were measured in *Atf6α*$^{+/+}$ (white circles, $n = 12$) and *Atf6α*$^{-/-}$ (black circles, $n = 17$) mice fed with an HFD. *$P < .05$. C and D, Intraperitoneal glucose tolerance test (1.5 g/kg body weight) was performed at 17–19 weeks of age in *Atf6α*$^{+/+}$ (white circles, $n = 12$) and *Atf6α*$^{-/-}$ (black circles, $n = 17$) mice on an HFD. Blood glucose (C) and plasma immunoreactive insulin levels (D) were measured. *$P < .05$, **$P < .01$. E, Insulin tolerance test (1.5 U/kg) was performed at 20–22 weeks of age in *Atf6α*$^{+/+}$ (white circles, $n = 12$) and *Atf6α*$^{-/-}$ (black circles, $n = 17$) mice on an HFD. **$P < .01$. F, Serum triglyceride levels were measured at the 17–19 weeks of age in *Atf6α*$^{+/+}$ ($n = 8$) and *Atf6α*$^{-/-}$ ($n = 7$) mice on normal chow as well as *Atf6α*$^{+/+}$ ($n = 11$) and *Atf6α*$^{-/-}$ ($n = 13$) mice on an HFD. *$P < .05$, **$P < .01$.**

higher than those in ATF6α-deficient mice fed with normal chow (Fig. 1D). The homeostasis model assessment of insulin resistance index was 2.20, 1.66, 9.81 and 4.58, for WT on normal chow, *Atf6α*$^{-/-}$ on normal chow, WT on an HFD and *Atf6α*$^{-/-}$ on an HFD, respectively. These results indicated that *Atf6α*$^{-/-}$DO mice developed insulin resistance, although milder than WT mice. Therefore, it was reasonable to speculate that lower insulin secretion in *Atf6α*$^{-/-}$DO mice could result from β-cell failure in adaptation to increased insulin resistance. In order to gain insight into β-cell homeostasis in *Atf6α*$^{-/-}$DO mice, pancreatic insulin content was measured. While pancreatic insulin content increased upon HFD feeding in WT mice, *Atf6α*$^{-/-}$ mice did not respond to the HFD by increasing insulin production (Fig. 3A). We then analyzed ultrastructure of β-cells in *Atf6α*$^{-/-}$ mice fed with an HFD. As indicated in Fig. 3B, while the ER in β-cells showed compact reticulate structure in WT-DO mice, in *Atf6α*$^{-/-}$DO mouse β-cells, frequently observed was swollen ER, a typical phenotype of ER stressed cells [29]. These data indicate that ATF6α-deficient β-cells suffered from ER stress due to increased demands of insulin production, and suggest that lower insulin secretion was, at least partly, caused by ER

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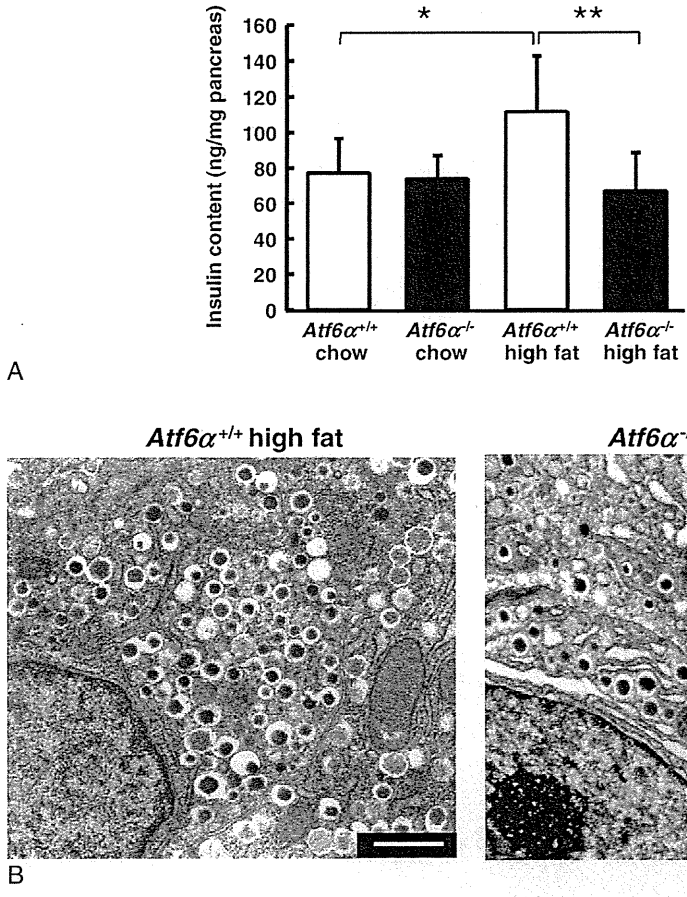


**Fig. 3 – Pancreatic β-cell homeostasis in *Atf6α*$^{-/-}$ mice on an HFD. A, Pancreatic insulin content was measured in *Atf6α*$^{+/+}$ ($n$ = 6) and *Atf6α*$^{-/-}$ ($n$ = 5) mice on normal chow as well as *Atf6α*$^{+/+}$ ($n$ = 10) and *Atf6α*$^{-/-}$ ($n$ = 13) mice on an HFD at 25 weeks of age. *$P$ < .05, **$P$ < .01. B, Ultrastructural analysis was performed on islets of *Atf6α*$^{+/+}$ ($n$ = 3) and *Atf6α*$^{-/-}$ ($n$ = 3) mice on HFD at 25 weeks of age. Representative micrographs of β-cells are shown. Bars, 1 μm.**

stress-induced failure in maintaining insulin content in ATF6α-deficient pancreas.

### 3.4. *Impact of ATF6 deficiency on liver homeostasis in mice fed with a high fat diet*

It is known that ER stress is increased in the liver of HFD-fed mice [7]. Thus, we analyzed effects of ATF6α deficiency on UPR gene expressions in the liver. XBP-1 mRNA splicing was augmented (Fig. 4A) and ATF4 mRNA levels was increased (Fig. 4B) in livers of *Atf6α*$^{-/-}$DO mice, indicating that higher ER stress existed when the ATF6α-deficient liver was metabolically overloaded. Since recent studies have indicated the involvement of UPR mediators in hepatic lipogenesis or glycolysis [30,31], we measured expressions of lipogenic or glycolytic enzymes. We found that mRNA expressions of a gluconeogenic enzyme glucose-6-phosphatase (G6P) and those of PPAR-γ and SREBP1c, master regulators of lipogenic enzymes, were increased in ATF6α-deficient DO mice (Fig. 4B). These findings have prompted us to measure liver TG content and to conduct morphological analysis of the liver in these animals. Liver TG content tended to increase in *Atf6α*$^{-/-}$DO mice although the difference failed to reach statistical significance ($P$ = .069, Fig. 4C). Liver sections showed increased lipid droplets in *Atf6α*$^{-/-}$DO mice (Fig. 4D), suggesting that steatosis was enhanced with ATF6α deficiency.

### 3.5. *ATF6α deficiency in Agouti yellow obese mice, a genetic diabetes model with increased insulin resistance*

To confirm effects of ATF6α deficiency on insulin resistance, ATF6α-deficient mice were crossed with Agouti yellow ($A^y$) mice. $A^y$ mice develop obesity and insulin resistance because of blockade of hypothalamic melanocortin-4 receptors due to ectopic expression of agouti peptide and thus are resistant to the satiety [32,33]. The phenotypes of *Atf6α*$^{-/-}$$A^y$ mice were similar to those of *Atf6α*$^{-/-}$DO mice. Body weights of *Atf6α*$^{-/-}$$A^y$ mice were significantly lower than those of $A^y$ mice (Fig. 5A). Although blood glucose levels were similar between the two strains (Fig. 5B), glucose excursion after an intraperitoneal glucose challenge was greater with reduced insulin secretion in *Atf6α*$^{-/-}$$A^y$ mice (Figs. 5C and 5D). Insulin sensitivity estimated by ITT was also higher in ATF6α-deficient $A^y$ mice (Fig. 5E). Pancreatic insulin content was approximately 50% lower in *Atf6α*$^{-/-}$$A^y$ mice than in WT mice at 25 weeks of age (Fig. 5F).

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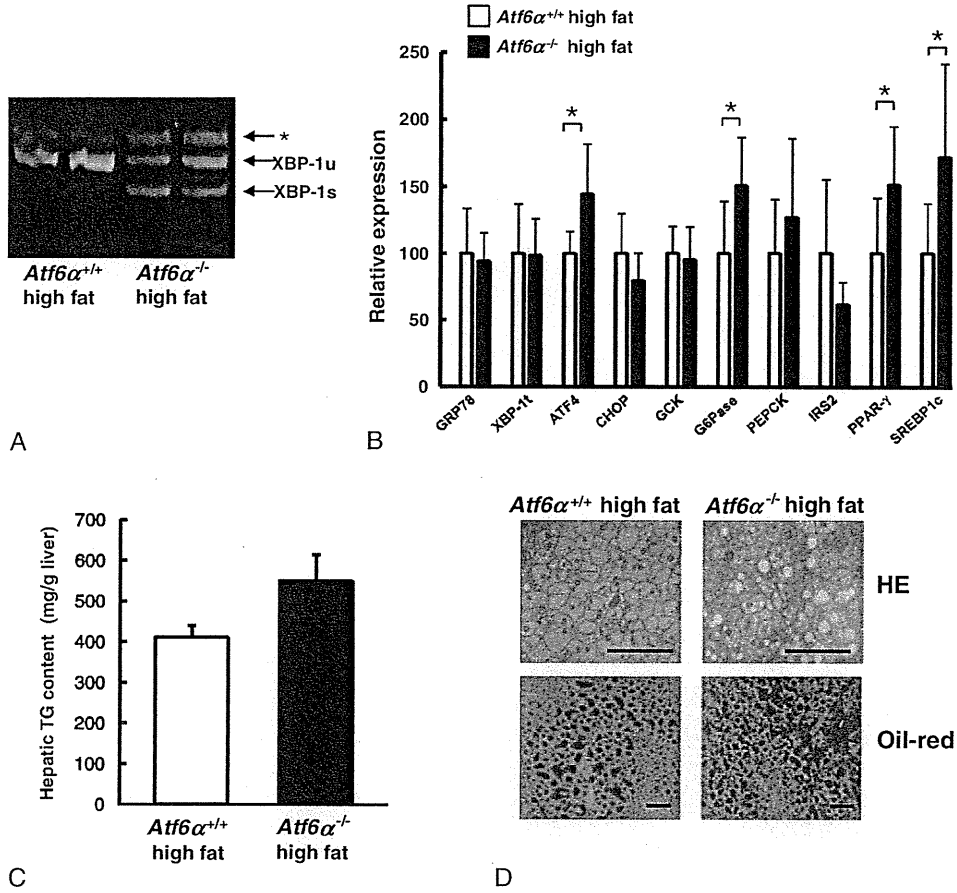


**Fig. 4 – Impact of ATF6 deficiency on liver homeostasis in mice on an HFD. A, Spliced (XBP-1s) and unspliced (XBP-1u) forms of XBP-1 mRNA were analyzed in liver of WT and *Atf6α*$^{-/-}$ mice on an HFD. The data are representative of 5 experiments. *Non-specific band. B, Quantitative RT-PCR was conducted using total RNA extracted from *Atf6α*$^{+/+}$ ($n$ = 6) and *Atf6α*$^{-/-}$ ($n$ = 11) mouse liver on an HFD at 25 weeks of age. XBP-1t: total XBP-1 mRNA. Data are presented as β-actin corrected value ± SD. *$P < .05$. C, TG content of liver was measured using liver homogenates from *Atf6α*$^{+/+}$ (white bars, $n$ = 7) and *Atf6α*$^{-/-}$ (black bars, $n$ = 9) mice on an HFD at 21 weeks of age. D, *Atf6α*$^{+/+}$ and *Atf6α*$^{-/-}$ mouse liver sections were stained with hematoxylin–eosin and oil-red O at 25 weeks of age. Bars, 100 μm.**

### 3.6. *ATF6α deficiency in Ins2*$^{WT/C96Y}$ *mice, a genetic diabetes model with ER-stress induced β-cell failure*

Studies on pancreas of *Atf6α*$^{-/-}$DO mice showed that ATF6-deficient β-cells suffered from ER stress. Thus, roles of ATF6α in β-cells under ER stress were also studied in *Atf6α*$^{-/-}$ mice bred with *Ins2*$^{WT/C96Y}$ (Akita) mice. In the latter mutant mice, insulin molecule with a Cys96Tyr mutation cannot fold correctly and causes ER stress in β-cells, leading to β-cell death and diabetes [27]. Reduction in BW and exaggerated hyperglycemia were evident in *Atf6α*$^{-/-}$*Ins2*$^{WT/C96Y}$ mice compared with *Atf6α*$^{+/+}$*Ins2*$^{WT/C96Y}$ mice (Figs. 6A and 6B). Pancreas insulin content from *Atf6α*$^{-/-}$*Ins2*$^{WT/C96Y}$ mice was 50% of that from *Atf6α*$^{+/+}$ *Ins2*$^{WT/C96Y}$ mice at 4–5 weeks of age, which was already reduced to one-third that from WT pancreas (Fig. 6C), with the majority of islets being smaller than those in *Ins2*$^{WT/C96Y}$ mice (Fig. 6D). These data showed that ATF6α contributes to the protection of β-cells against ER stress caused by mutated insulin Cys96Tyr in vivo.

## 4. Discussion

ATF6α, an ER stress sensor molecule, plays a critical role in initiation and development of the UPR. Recent studies indicated that ER stress itself and/or the UPR signaling are implicated in several components of the diabetes pathophysiology, including reduced β-cell mass, increased insulin resistance, ectopic lipid accumulation and hyperlipidemia. Thus, it has been speculated that ATF6α deficiency would have an impact on metabolic homeostasis. Our analysis of ATF6α-deficient mice revealed that ATF6α contributes to both prevention and promotion of the metabolic disorder in vivo.

In the absence of environmental challenges, *Atf6α*$^{-/-}$ mice maintained normal glucose homeostasis with normal insulin secretory responses, indicating that β-cells without ATF6α have the capacity to deal with the large amount of proinsulin under the physiological condition. However, when metabolic overload further demanded to produce and secrete greater

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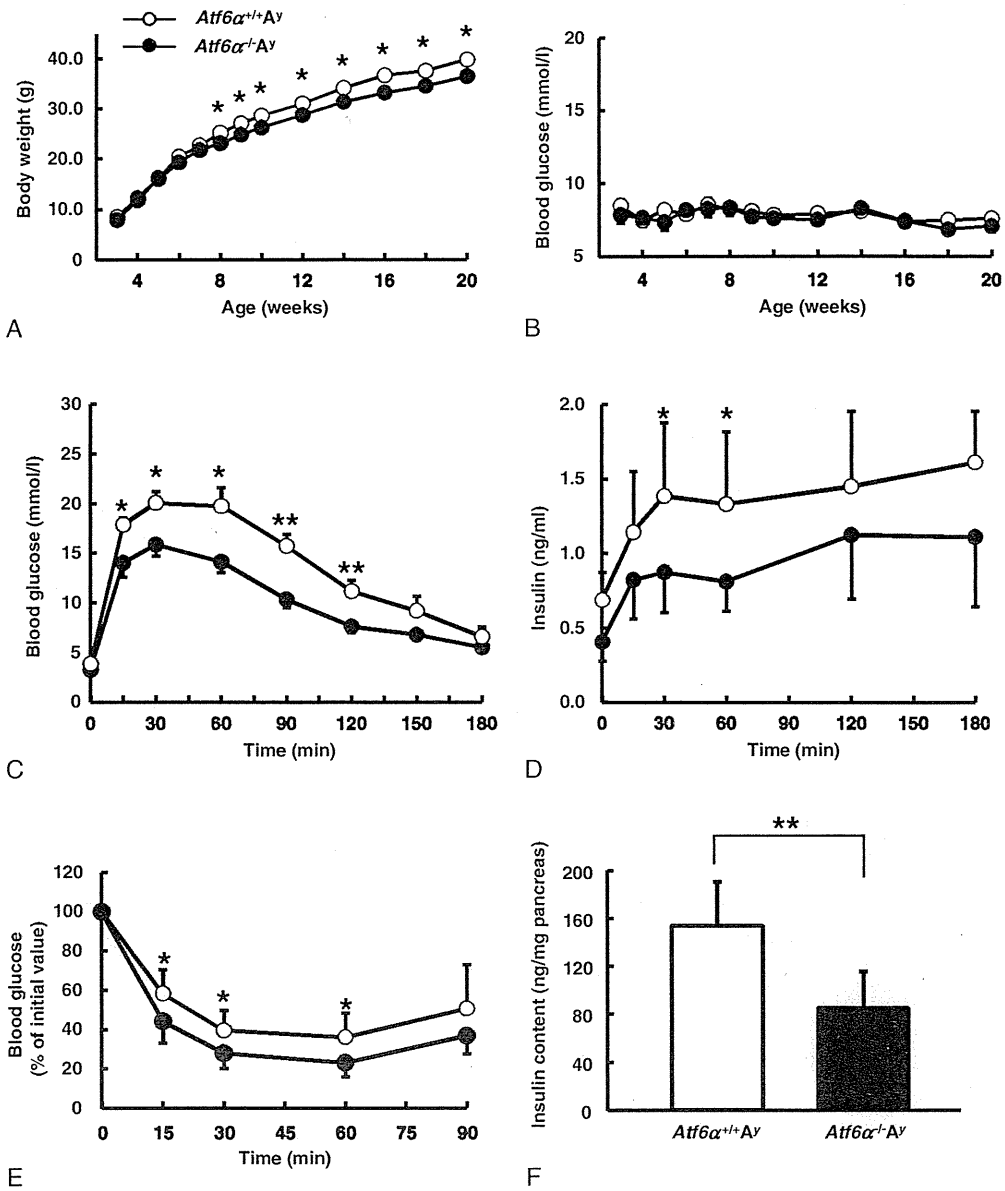


**Fig. 5 – Glucose homeostasis in ATF6α-deficient $A^y$ mice. A and B, Body weight (A) and glucose levels (B) on fed conditions were measured in $Atf6\alpha^{+/+}A^y$ (white circles, $n = 10$) and $Atf6\alpha^{-/-}A^y$ (black circles, $n = 13$) mice. Data from four cohorts are combined. *$P < .05$. C and D, Intraperitoneal glucose tolerance tests (1.5 g/kg body weight) were performed at 17–19 weeks of age in $Atf6\alpha^{+/+}A^y$ (white circles, $n = 7$) and $Atf6\alpha^{-/-}A^y$ (black circles, $n = 8$) mice. Blood glucose (C) and plasma immunoreactive insulin levels (D) were measured. *$P < .05$, **$P < .01$. E, Insulin tolerance tests (1.5 U/kg) were performed (E) at 20–22 weeks of age in $Atf6\alpha^{+/+}A^y$ (white circles, $n = 7$) and $Atf6\alpha^{-/-}A^y$ (black circles, $n = 8$) mice. *$P < .05$. F: Pancreatic insulin content was measured in $Atf6\alpha^{+/+}A^y$ ($n = 6$) and $Atf6\alpha^{-/-}A^y$ ($n = 8$) mice at 25 weeks of age. **$P < .01$.**

amount of insulin, the ATF6α-deficient β-cell failed to cope with the stress. Thus, we observed swollen ER, a typical phenotype of the stressed ER [29], in the β-cell of $Atf6\alpha^{-/-}$DO mice. The β-cell failure under the HFD seemed to be also caused by increased circulating lipid levels, as lipotoxicity is also a cause of ER stress [34]. We did not examine β-cell mass in $Atf6\alpha^{-/-}$DO mice. Therefore, it was not clear that lower pancreatic insulin content in $Atf6\alpha^{-/-}$DO mice resulted either from reduced insulin production in individual β-cells or from reduction in β-cell mass. Nonetheless, we prefer the latter possibility, since islets became smaller when the *Atf6α* gene was deleted in β-cells with misfolded insulin molecules in Akita $Ins2^{WT/C96Y}$ mice. These data suggest that ATF6α plays an important role in β-cell survival under ER stress conditions.

It has been known that ER stress plays a role in metabolic regulation in the liver. ATF6α reportedly suppresses gluconeogenesis by inhibiting transcription of gluconeogenic enzymes [35,36]. In accordance with this notion, we observed increased G6P expression, possibly contributing to impaired glucose homeostasis in ATF6α-deficient DO mice.

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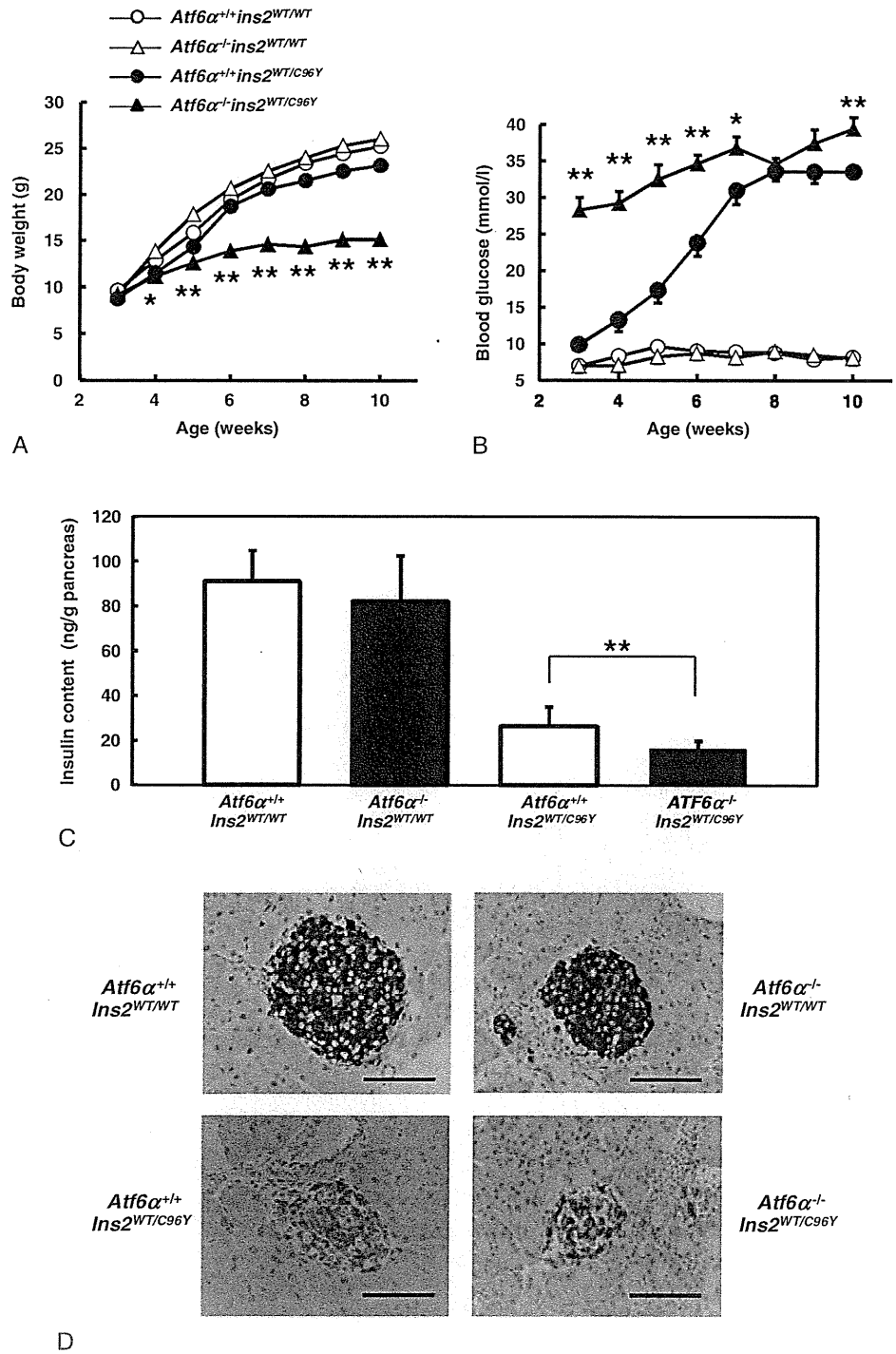


**Fig. 6 – ATF6α deficiency exaggerated hyperglycemia in $Ins2^{WT/C96Y}$ mice. A and B, Body weight (A) and glucose levels (B) on fed conditions were measured in $Atf6\alpha^{+/+}$ (white circles, $n$ = 12), $Atf6\alpha^{+/+}Ins2^{WT/C96Y}$ (black circles, $n$ = 16), $Atf6\alpha^{-/-}$ (white triangles, $n$ = 13) and $Atf6\alpha^{-/-}Ins2^{WT/C96Y}$ (black triangles, $n$ = 15) mice. *P < .05, **P < .01 compared with the $Atf6\alpha^{+/+}Ins2^{WT/C96Y}$ mice. C, Pancreatic insulin content of $Atf6\alpha^{+/+}$ ($n$ = 8), $Atf6\alpha^{-/-}$($n$ = 9), $Atf6\alpha^{+/+}Ins2^{WT/C96Y}$ ($n$ = 11) and $Atf6\alpha^{-/-}Ins2^{WT/C96Y}$ ($n$ = 11) mice at 4–5 weeks of age. **P < .01. D, Pancreas section of mice with indicated genotypes at 4–5 weeks of age was immunostained for insulin. Bars, 100 μm.**

Recent studies have also revealed a link between ER stress and hepatic steatosis. $Atf6\alpha^{-/-}$ mice [19,37], mice with liver-specific deletion of functional eIF2α [19], and liver-specific IRE1α knockout mice [19], all developed hepatic steatosis when a pharmacological ER stress inducer, tunicamycin (a protein glycosylation inhibitor) was injected. Here, we

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demonstrated that greater levels of ER stress were present with increased tendency to develop a higher degree of hepatosteatosis in liver of ATF6α-deficient DO mice. Thus, our data indicate that not only acute pharmacological ER stress but also chronic ER stress promoted the development of hepatosteatosis. It has been postulated that ER stress-mediated activation of metabolic master regulator proteins, such as PPAR-γ and SREBP1c, contributes to liver steatosis [19,30]. A direct role of ATF6α in regulation of lipid homeostasis has been also postulated: ATF6α attenuates lipogenesis in the liver by suppressing SERBP2 [38]. Interestingly, we observed that despite increased lipid accumulation in the liver, circulating triglyceride levels were lower in *Atf6α*$^{-/-}$DO mice. These data suggest defects in impaired very low-density lipoprotein (VLDL) formation or triglyceride transfer to the circulation. A recent article indicated that ER stress-mediated reduction in stability of apolipoprotein B-100, a major protein component of VLDL, caused lipid accumulation in the liver of *Atf6α*$^{-/-}$ mice injected with tunicamycin [37]. A similar mechanism might be involved in the liver of *Atf6α*$^{-/-}$DO mice under chronic ER stress, which leads to liver steatosis.

An interesting observation made in this study was that ATF6α deficiency resulted in improved insulin sensitivity. *Atf6α*-null mice were partially resistant to development of insulin resistance resulting from metabolic overload induced by two different manners: placing on a high fat diet or introducing the agouti mutation causing hyperphagia. The mechanism by which ATF6α deficiency made mice partially resistant to the development of insulin resistance is currently unknown. It has been shown that the serum triglyceride level is a factor of insulin resistance [39,40]. Therefore, partial suppression in the development of hypertriglyceridemia might cause improved insulin sensitivity in ATF6α-deficient DO mice.

The present data indicate that ATF6α protects pancreatic β-cells from ER stress-induced cell damage. It also seems to protect liver tissue from steatosis under the HFD, but contributes to development of hyperlipidemia and insulin resistance in mice. Thus, ATF6α affects whole body glucose homeostasis through tissue specific actions. A limitation of the present study, in which whole body knockout mice were analyzed, is that some phenotypes of one tissue could be influenced by those of other tissues with ATF6α deficiency. Future studies using tissue specific ATF6α knockout mice would clarify these issues.

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REVIEW

Yasunobu Matsuda · Takafumi Ichida · Manabu Fukumoto

# Hepatocellular carcinoma and liver transplantation: clinical perspective on molecular targeted strategies

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**Abstract** Hepatocellular carcinoma (HCC) has an aggressive clinical course with frequent recurrence and metastasis. Orthotopic liver transplantation has been the only curative tool for unresectable HCC; therefore, recent advances in molecular targeted therapy may improve the prognosis of HCC. The multiple kinase inhibitor sorafenib and the macrolide antibiotic rapamycin are currently the most promising agents for treating unresectable HCC. A large population-based clinical trial revealed that sorafenib significantly prolonged the overall survival of HCC patients. However, subsequent clinical studies showed that sorafenib rarely reduced tumor volume and inadequately prolonged survival of patients with severe liver damage. To improve its therapeutic effect, the development of a predictive biomarker and a sorafenib-based combination is awaited. Another molecular targeting agent, rapamycin, has now been considered as a putative agent for preventing tumor recurrence in post-liver transplantation HCC patients, because it not only has immunosuppressive activity but also exerts an anti-tumor effect. In the near future, a combination of molecular targeting agents, such as sorafenib and rapamycin, may become a standard protocol for treating unresectable HCC. For specifying cases with more effective and less harmful modalities, further investigation in clinical and basic research to identify unexpected effects are needed.

**Key words** Hepatocellular carcinoma · Liver transplantation · Molecular targeted therapy · Rapamycin · Sorafenib

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## Introduction

Recent advances in molecular biology have revealed a number of molecules involved in the development and progress of human diseases. In the field of cancer research, several therapeutic agents have been developed for targeting the molecules specifically expressed in cancer cells, and clinical trials have now revealed their favorable effects in cancer therapy (Fig. 1). For example, imatinib mesylate, a selective inhibitor of Bcr-Abl, has been found to significantly improve the survival of Philadelphia chromosome-positive chronic myeloid leukemia. Bevacizumab, a humanized monoclonal antibody toward vascular endothelial growth factor A (VEGF-A), has proven efficacy in metastatic colon cancer and non-small cell lung cancer. Bortezomib, a proteasome inhibitor, has been reported to be effective in relapsing multiple myeloma. However, with the use of molecular targeting agents, unexpected findings have also emerged: imatinib treatment frequently results in short-lived remission in blast crisis patients,[1] many tumors are found to acquire drug resistance to bevacizumab,[2] and the type of myeloma with plasmacytotic differentiation is found to be refractory to bortezomib.[3] Therefore, to ensure the successful achievement of molecular targeted therapy, or to avoid unexpected interactions, a thorough investigation of the functional mechanism of the molecular targeting agent is inevitable.

Hepatocellular carcinoma (HCC) is one of the most common human cancers, and its incidence is still increasing worldwide. The etiology of HCC is diverse. HCC arises in patients with hemochromatosis[4] or chronic liver disease induced by hepatitis virus (HBV, HCV), alcohol abuse, and radioactive agents such as thorotrast.[5] In developed countries, the incidence of HCC is now increasing in individuals with nonalcoholic fatty liver diseases.[6] It should be noted that the majority of HCC patients have severe hepatic dysfunction, irrespective of the etiological background. Moreover, because many patients are diagnosed at a late stage, curative therapies such as surgical resection or liver transplantation (LT) are precluded.[7] Locoregional therapies

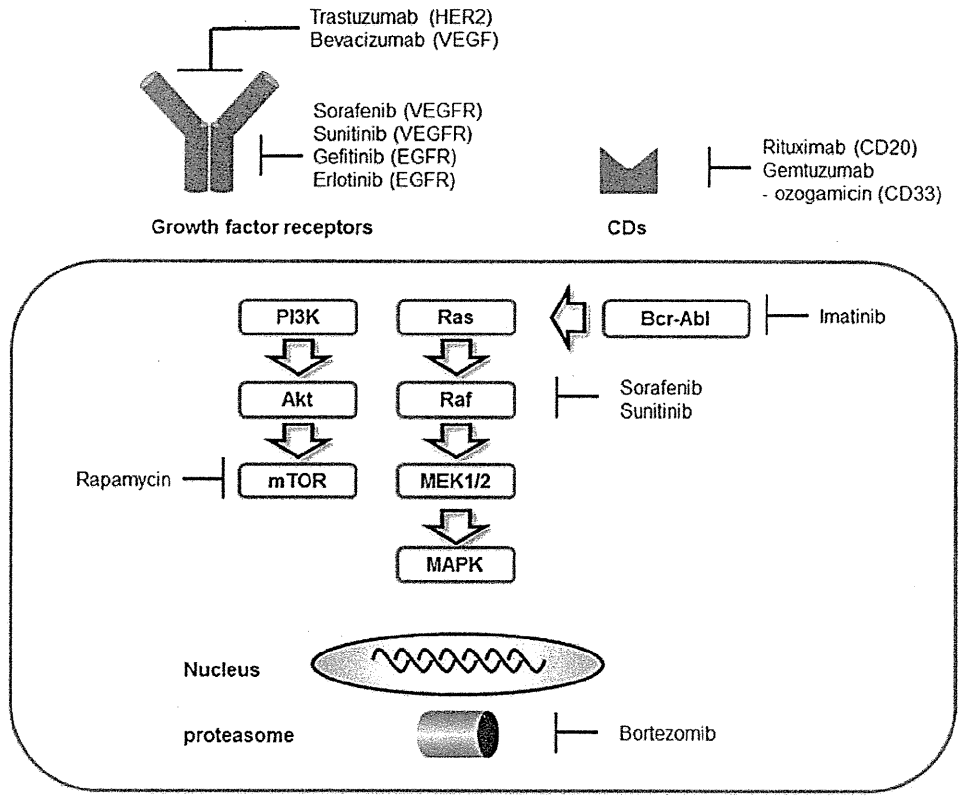


**Fig. 1.** Molecular target drugs against cancer cells. *HER2*, human epidermal growth factor receptor-2; *VEGF*, vascular endothelial growth factor; *VEGFR*, vascular endothelial growth factor receptor; *EGFR*, epidermal growth factor receptor; *PI3K*, phosphoinositide 3-kinase; *mTOR*, mammalian target of rapamycin; *MEK*, mitogen-activated protein kinase kinase; *MAPK*, mitogen-activated protein kinase; *Bcr-Abl*, oncogene fusion protein consisting of BCR and ABL associated with Philadelphia chromosome

[radiofrequency ablation and transarterial chemoembolization (TACE)] are widely used as substitute therapeutic tools, but the prognosis remains poor.[8] A multicenter survey in Japan showed that the best outcome in unresectable HCC had a 5-year survival of less than 30% in patients who underwent TACE.[9] Because HCC is a well-characterized tumor with frequent recurrence and metastasis, the poor response to either conventional chemotherapy or radiotherapy is a major problem to be solved.[10,11]

To date, orthotopic LT has been the only curative option for patients with unresectable HCC, although the high incidence of post-transplant recurrence enhanced by immunosuppressive agents is a major problem.[12] Up to now, there has been no successful systemic chemotherapy for patients with advanced HCC,[13] leading to the view that advanced HCC should be recommended for molecular targeted therapy. In this review, we focus on the clinical perspectives of two molecular targeting agents used for unresectable HCC, the multiple kinase inhibitor sorafenib and the macrolide antibiotic rapamycin, because accumulating evidence suggests that these agents may signify a step forward in molecular targeted therapy for HCC.

## Raf signaling and liver diseases

In various types of cancer cells, the Ras oncogene plays a central role in cell transformation. Ras activates c-Raf (Raf-1), a mitogen-activated protein kinase (MAPK) kinase, which phosphorylates and activates the serine/threonine-specific extracellular signal-regulated protein kinases ERK1 and ERK2 and the cascade of MAPK/ERK kinases MEK1 and MEK2.[14] Intriguingly, Raf-1 has been found to be deeply involved in hepatocarcinogenesis. For example, human hepatitis B virus X protein (HBx), a presumed strong promoter of HCC development, translocates Raf-1 to the mitochondria in oxidative stress.[15] HCV core protein increases the kinase activity of Raf-1, suggesting that Raf-1 may contribute to hepatocyte growth regulation in the HCV-infected liver.[16] Raf-1 also binds to the C-terminal of HCV-encoded nonstructural protein 5A (NS5A), suggesting that Raf-1 is crucial for HCV replication.[17] Moreover, activated Raf-1 is overexpressed in about 90% of liver cirrhosis cases and in 100% of HCC cases.[18] All these lines of evidence indicate that manipulating Raf-1 activity may be of value in treating HCC.

## Clinical trials of sorafenib

Sorafenib (Nexavar, BAY43-9006; Bayer/Onyx Pharmaceuticals) is a multitargeting small molecule that not only inhibits the receptor tyrosine kinases VEGF receptor-2 (VEGFR-2), VEGFR-3, Flt-3, platelet-derived growth factor (PDGF) receptor, and fibroblast growth factor receptor (FGFR)-1 but also blocks the activity of Raf serine-threonine kinase.[19] In 2008, a promising prospect for sorafenib monotherapy was provided by a multicenter

double-blind Phase III trial (the Sorafenib HCC Assessment Randomized Protocol, SHARP), which was conducted in Europe, North America, South America, and Australasia.[20] In the trial, 602 patients with advanced HCC without previous systemic treatment were randomly divided into either a sorafenib group (sorafenib 400 mg twice daily) or a placebo group. Overall survival in the sorafenib group was significantly longer than in the placebo group (10.7 vs. 7.9 months), indicating a 44% increase in overall survival [hazard ratio (HR), 0.69; $P < 0.0001$]. The median time to radiologic progression was 5.5 months and 2.8 months in the sorafenib-treated group and placebo group, respectively (HR, 0.58; $P < 0.0001$). The disease control rate (a composite of complete response, partial response, and stable disease) was significantly higher in the sorafenib group (43% vs. 32%; $P = 0.002$).

In the following year, 2009, a subsequent Asia-Pacific study was reported.[21] In this trial, 226 HCC patients from China, South Korea, and Taiwan were randomly assigned to sorafenib or placebo. Median overall survival was 6.5 months and 4.2 months in the sorafenib group and placebo group, respectively (HR, 0.68; $P = 0.014$). The disease control rate was 53% and 12% in the sorafenib group and placebo group, respectively. The efficacy of sorafenib seems convincing because the SHARP and Asia-Pacific studies were based on individuals from different geographic and ethnic backgrounds.

## Unresolved problems of sorafenib efficacy

As already described, both SHARP and Asia-Pacific trials clearly indicated the efficacy of sorafenib, showing promise in the treatment of unresectable HCC. However, the results also raised new concerns about this agent. First, in both studies, most patients had Child-Pugh class A cirrhosis and an Eastern Cooperative Oncology Group performance status (ECOG PS) score of 0–1, and information about patients with severe liver dysfunction was lacking. In this regard, an Austrian group recently reported a trial of sorafenib on HCC patients of Child-Pugh class A, B (medium degree of liver dysfunction), and C (severe degree of liver dysfunction).[22] They showed that Child-Pugh class C patients had a limited life expectancy after the treatment (median overall survival, 1.5 months), suggesting that only supportive care remained for such patients. Similarly, a German group reported that the median survival of patients treated with sorafenib was better in Child-Pugh class A than Child-Pugh class B cirrhosis.[23] The reason for the association between degree of liver function and efficacy of sorafenib is unknown. To clarify which individuals may benefit from sorafenib treatment, further clinical trials are awaited.

Second, although the SHARP and Asia-Pacific trials showed that the disease control rate was improved by sorafenib, tumor shrinkage was rare and no complete response was observed. A partial tumor response was seen in only 2% of the SHARP participants and in only 3.3% of the Asia-Pacific participants, which seems far removed from the results of preclinical studies of sorafenib in cultured cells or in animal models. The significant discrepancy of the anti-tumor effect of sorafenib between laboratory and bedside indicates that some physiological factors may influence the functional mechanism of this agent.

Third, a reliable biomarker for predicting the effect of sorafenib has not been established. There have been only a few reports of complete remission of HCC with sorafenib monotherapy.[24–28] In these reports, the patients' age ranged from 54 to 78 years old, the tumor marker α-fetoprotein level ranged from 3.1 to 13,599 ng/ml, and the etiology was hemochromatosis in one, HBV in one, and HCV in two. Because the number of cases was small and their clinical parameters showed no similarities, predicting those cases likely to benefit from sorafenib is difficult. In Japan, 15 of 3,700 HCC patients treated with sorafenib were reported to have complete remission with sorafenib,[29] and a difference in ethnic background may be one of the possible reasons for differing sorafenib efficacies.

## Sorafenib-based combination therapy

To date, there have been a few reports of patients who had complete remission of HCC with a combination of sorafenib and other therapeutic modalities, such as radioembolization[30] or anti-retroviral drugs.[31] However, the number of the patients is limited, and the clinical interpretation is not clear. Recently, clinical trials of sorafenib-based combination therapy have been reported in larger numbers of patients. An Italian group reported a Phase II multicenter study of a combination of sorafenib and long-acting octreotide.[32] In this study, the patients with advanced HCC (Child-Pugh A or B) received sorafenib (800 mg/day for 28 days) with a following week of rest and long-acting octreotide (40 mg) for 28 days. Median time to progression of the patients receiving the combination therapy was 7.0 months [95% confidence interval (CI), 3.0–10.9 months], and median overall survival was 12 months (95% CI, 6.3–17.4 months). The disease control rate was 76%, which was much higher than sorafenib monotherapy (43% in the SHARP study and 53% in the Asia-Pacific study). Octreotide is an analogue of somatostatin, whose receptor is expressed in HCC. Many studies reported that octreotide monotherapy was less effective in advanced HCC.[33] Therefore, octreotide may be effective only when combined with another molecular targeted agent.

Very recently, a double-blind phase II multinational study of sorafenib and the chemotherapeutic drug doxorubicin was conducted. In this trial, the patients received 60 mg/m$^2$ doxorubicin intravenously every 21 days plus either 400 mg sorafenib or a placebo orally twice a day.[34] After the treatment, median time to progression was 6.4 months in the sorafenib-doxorubicin group (95% CI, 4.8–9.2 months), and 2.8 months (95% CI, 1.6–5.0) in the doxorubicin monotherapy group ($P = 0.02$). Progression-free survival was 6.0 months (95% CI, 4.6–8.6) and 2.7 months

(95% CI, 1.4–2.8) in these groups, respectively ($P = 0.006$). These results indicate that the treatment with sorafenib plus doxorubicin could achieve a favorable survival compared with doxorubicin alone, although the significance of the synergism of sorafenib remains to be defined. To determine the best protocol for sorafenib-based combination therapy, further clinical trials are awaited as well as basic research.

## mTOR pathway and hepatocarcinogenesis

The mammalian target of rapamycin (mTOR) pathway is a fundamental in the signaling of protein synthesis.[35–38] When insulin stimulates the cells, a downstream target of serine/threonine kinase Akt phosphorylates and inactivates tuberous sclerosis complex 1 or 2. This signaling leads to the activation of the small G-protein Ras homologue enriched in brain (Rheb) and the mTOR complex 1 (mTORC1), which consists of mTOR, a regulatory associated protein of mTOR (RAPTOR), and mammalian LST8/ G-protein β-subunit-like protein. mTORC1 phosphorylates the translation control proteins p70 ribosomal S6 kinase 1 (S6K1) and eukaryotic initiation factor 4E binding protein 1 (4EBP1). Phosphorylated S6K1 plays an important role in the regulation of cell growth, cell-cycle progression, and cell proliferation by enhancing the translation of components required for protein synthesis.[35,39] Hyperphosphorylated 4EBP1 disrupts binding to the eIF4E translation initiation factor, leading to the activation of cap-dependent translation.[40] mTOR also forms the complex mTORC2, consisting of mTOR, mLST8 and rapamycin-insensitive companion of mTOR (Rictor), leading to full activation of Akt.[41]

Several studies have reported that the mTOR pathway is activated in about half the cases of human HCC. Sieghart et al.[42] immunohistochemically investigated the mTOR pathway (PTEN, Akt, p70S6K1, and 4EBP) in patients with HCC who underwent LT and detected an activated mTOR pathway in 40% of the patients. However, they found no relationship between mTOR signaling and either disease-free or overall survival. Sahin et al.[43] examined the active state of mTOR and S6K in HCC patients by immunohistochemistry, reporting that S6K overexpression was positively correlated with tumor nuclear grade, inversely correlated with tumor size, but not associated with the proliferation index. In contrast, Baba et al.[44] investigated the relationship between active p70S6K and prognosis in patients with HCC who underwent LT or liver resection, finding that phosphorylated p70S6K was detected in 24.5% and correlated with overall survival in patients with clear margin-resected HCC. Villanueva et al.[45] examined the status of ribosomal protein S6 (RPS6), a downstream effector of mTOR, in HCC patients. They showed that RPS6 was activated in half the tumors and correlated well with recurrence of HCC. Interestingly, active mTOR was observed to be predominantly localized in the plasma membrane in nonmalignant liver cirrhosis but was typically detected in the cytoplasm in HCC cells. Zhou et al.[46] examined PTEN, Akt, p27, and S6RP expression in the cases with HCC by immunohistochemical analysis, finding that phosphorylated forms of Akt, PTEN, and S6RP were independent prognostic factors for HCC. Taken together, S6RP might be a preferable prognostic factor for HCC rather than p70S6K, and the active status of the downstream effector of the mTOR pathway may be correlated with the prognosis of HCC.

## Rapamycin as a preventive agent for HCC recurrence

During the past few decades, LT has become an efficacious therapy for patients with severe liver damage. LT seems to be a logically ideal therapy for HCC, because it not only removes occult tumors in the residual liver but also improves liver function. In fact, LT provides excellent outcomes for HCC patients when the tumors conform to the Milan criteria (single nodule ≤5 cm, or two or three nodules ≤3 cm), with 5-year survival rates of 70% and low recurrence rates.[47] Nevertheless, the possibility of HCC recurrence in post-transplant recipients cannot be excluded because they require immunosuppressive medication. It should be noted that calcineurin inhibitors (cyclosporine and tacrolimus), which have been widely used for immunosuppressive therapy after LT, have been reported to induce tumor growth and metastasis.[48–50]

Rapamycin is a macrolide antibiotic and antifungal drug that was originally identified from *Streptomyces hygroscopicus*. After rapamycin was found to have both immunosuppressive and antiproliferative effects, this agent has been regarded as a useful therapeutic adjuvant in cancer.[51–53] Because rapamycin specifically interacts with its partner FKBP12 and directly inhibits the mTOR pathway, it leads to a reduction in synthesis of components of the translation process and cell-cycle arrest at the $G_1$ phase.[54,55] Rapamycin is now becoming an attractive therapeutic tool for LT patients in the hope that it may both inhibit rejection and prevent the recurrence of HCC.[56–61]

To date, there have been several reports of a large population of HCC patients receiving rapamycin for post-transplantation maintenance. Zhou et al.[62] retrospectively examined the patients who underwent LT for HCC that exceeded the Milan criteria and found that the recipients receiving sirolimus (rapamycin) had a better overall survival compared with FK506 (another type of macrolide immunosuppressant). The mean disease-free survival period was 519 ± 43 days in the rapamycin group and 477 ± 48 days in the FK506 group ($P = 0.234$), and multivariate analysis showed that the immunosuppressive protocol ($P = 0.015$) was a significant factor affecting overall survival. Very recently, a larger LT population (2,491 adults with HCC and 12,167 with non-HCC) was analyzed, using data from the Scientific Registry of Transplant Recipients, which includes all donors, waiting-list candidates, and transplant recipients in the United States.[63] Multivariate analysis showed that only anti-CD25 antibody induction and sirolimus-based maintenance therapy were associated with improved survival of the HCC patients after LT (HR, 0.64; 95% CI, 0.45–0.9; $P \leq 0.01$; HR, 0.53; 95% CI, 0.31–0.92, $P \leq 0.05$,

respectively). The other drugs, including calcineurin inhibitors, did not have a significant effect on survival. Notably, in non-HCC recipients, sirolimus maintenance showed a trend of lower survival, indicating that the therapeutic benefit of rapamycin on post-LT HCC patients may be based on its anticancer effect. At present, a prospective randomized clinical trial, the SiLVER Study (the use of sirolimus in LT patients with HCC; http://www.clinicaltrials.gov) is now being undertaken by a group in the University Hospital Regensburg endorsed by the European Liver and Intestine Transplant Association (13 countries within Europe, Canada, and Australia).[64]

## Combination therapy of sorafenib and rapamycin

The therapeutic efficacy of sorafenib in advanced HCC and preventive effect of rapamycin in post-LT patients may easily lead to the idea that a combination of these agents may be more effective for treating HCC. However, the question remains unanswered whether the combination therapy of sorafenib and rapamycin would be only applicable for HCC patients who underwent liver transplantation. We surmise that this protocol may bring about a significant breakthrough in the treatment of HCC patients, irrespective of whether liver transplantation was performed. Basic research suggests that this is likely to be the case. Using a highly metastatic human HCC xenograft mouse model, Wang at al.[65] reported that a combination of the drugs significantly enhanced the inhibitory effect on primary tumor growth compared with either rapamycin alone ($P < 0.001$) or sorafenib alone ($P < 0.001$). Huynh et al.[66] investigated the in vivo anticancer activity of sorafenib on four patient-derived HCC xenografts and found that the activity of the mTOR pathway (p70S6K, S6RP, 4EBP1) was induced in xenografts less sensitive to sorafenib. In such a tumor, treatment with sorafenib plus rapamycin blocked the sorafenib-induced activation of the mTOR target and inhibited tumor growth. Similarly, Newell et al.[67] reported that a combination of sorafenib and rapamycin enhanced tumor necrosis and ulceration of HCC xenografts, supporting the rationale for a combination of these agents in clinical studies.

There have been no reports of large-scale trials of a combination of these molecular targeted agents in advanced HCC. Recently, a few HCC cases with a favorable response to this combination have been reported.[68,69] At present, a Phase I trial of sorafenib and temsirolimus (a recently developed rapamycin analogue) in combination for advanced HCC has just started.[70] These molecular targeted agents may well open a new era for the treatment of HCC.

## Conclusion

Recent advances in molecular biology have revealed many altered molecular signaling pathways in human HCC, e.g., the p53 pathway, the p16/p27/RB1 pathway, the transforming growth factor-β pathway, the Wnt/β-catenin/adenomatous polyposis coli pathway, the E-cadherin/integrin pathway,

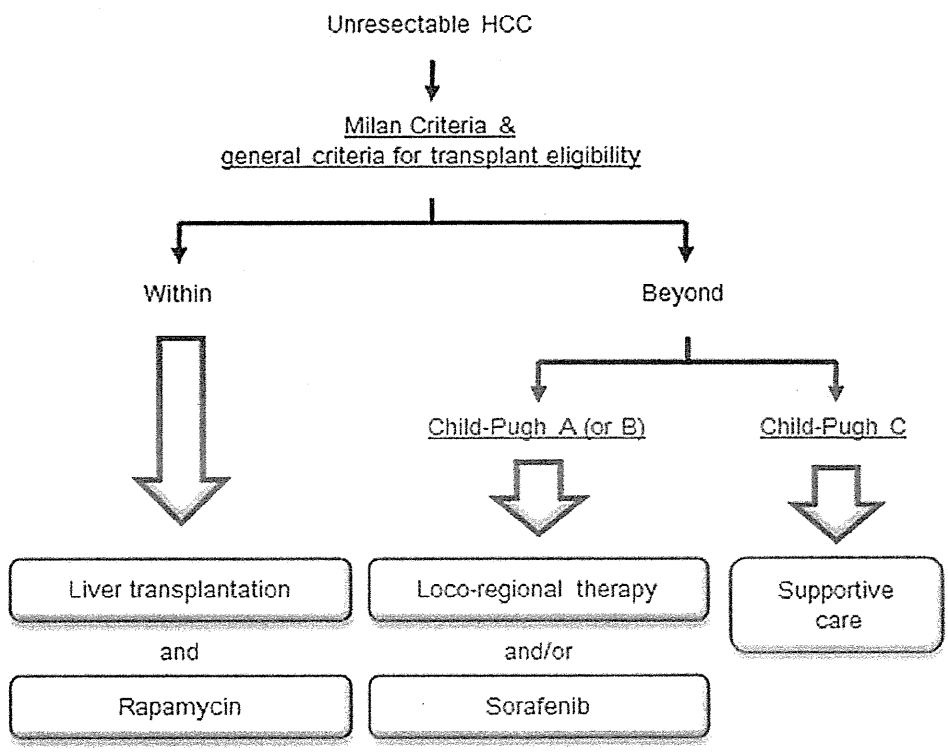


**Fig. 2.** Proposed treatment algorithm for unresectable hepatocellular carcinoma (*HCC*). *Milan Criteria*: a basis for selecting patients with hepatocellular carcinoma for liver transplantation (unifocal tumor mass ≤5 cm in diameter or multifocal tumors < 4 in number, each ≤3 cm in diameter with no evidence of extrahepatic manifestations and vascular invasion). *Child-Pugh*: the scoring system assessing prognosis of liver cirrhosis, consisting of five clinical parameters (total bilirubin, serum albumin, prothrombin time, ascites, hepatic encephalopathy). Grade A, mild; Grade B, medium; Grade C, severe. Locoregional therapy: radiofrequency ablation and transarterial chemoembolization, etc.

and MEK/ERK signaling.[71-73] However, because HCC shows diverse genetic alterations in the multidrug resistance phenotype,[74] it is not easy to form a consensus on molecular targeted therapy. Attempts to treat HCC by molecular targeted therapy have only recently started, and development of a standard protocol is underway.

At present, sorafenib and rapamycin are the only molecular targeting agents proven to have a significant effect on the survival of patients with advanced HCC in large-scale clinical trials. Intriguingly, accumulating evidence suggests that each agent can be used appropriately as the situation demands, i.e., sorafenib for treatment of patients with unresectable advanced HCC and rapamycin for preventing HCC recurrence in post-transplant recipients. More interestingly, as described in the present review, a combination of sorafenib and rapamycin may be promising in the treatment of tumors refractory to sorafenib monotherapy (Fig. 2). It is too early to be confident, however, because basic research studies have reported that the functional mechanisms of both sorafenib and rapamycin are more complicated than anticipated. Further studies of the functional mechanism of the molecular targeting agents are required to provide greater improvements in HCC therapy.

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