signaling molecules such as Akt. Furthermore, activation of tumor necrosis factor-alpha (TNF-α) and/or triglyceride accumulation-induced nuclear factor-κB (NF-κB) in the liver is shown to play a role in the insulin resistance of patients with HCV-related chronic liver disease as well. We, along with others, have recently found that branched-chain amino acids (BCAAs) and pigment epithelium-derived factor (PEDF) could improve HCV-associated insulin resistance *via* suppression of NF-κB and preservation of insulin signaling pathways. In this review, we discuss the mechanisms underlying the actions of BCAAs and PEDF, and their clinical implications in insulin resistance of patients with HCV infection. We also discuss here which chemical structures could contribute to insulin-sensitization in patients with HCV infection.

MOLECULAR PATHWAY OF INSULIN SIGNALING

Insulin is one of the anabolic hormones which regulate not only glucose metabolism, but also protein synthesis, lipid metabolism and cell proliferation through activation of various intracellular signaling molecules as shown in Fig. (**1**) [47-50]. Insulin binds to the extracellular α subunit of the insulin receptor and subsequently causes conformational changes of the insulin receptor, thus leading to the activation of tyrosine kinase domain in the β subunit of the insulin receptor [51]. Activation of the tyrosine kinase of the insulin receptor leads to a phosphorylation of tyrosine residues in the insulin receptor substrate (IRS), a central molecule of intracellular insulin signaling, and then to two major signaling pathways; the p85 regulatory subunit of phosphatidylinositol 3-kinase (PI3K) signaling pathway and mitogen-activated protein kinase (MAPK) signaling pathway [51].

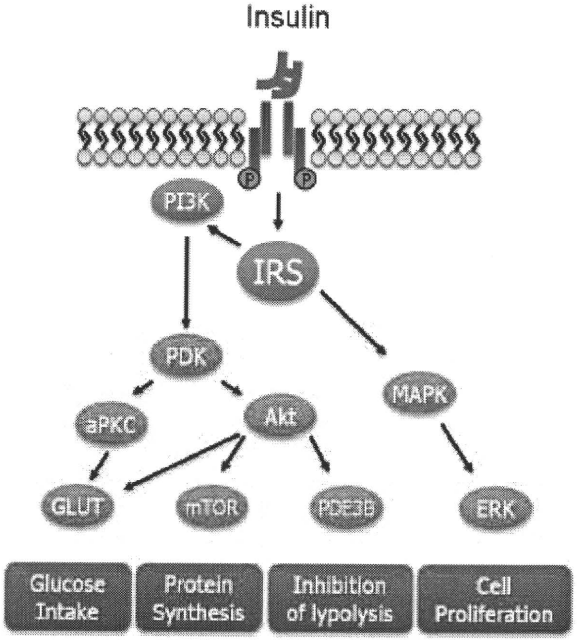

Fig. (1). Molecular pathways of insulin signaling. Abbreviations; aPKC, atypical protein kinase C; ERK, extracellular signal-regulated kinase; GLUT, glucose transporter; IRS, insulin receptor substrates; mTOR, mammalian target of rapamycin; MAPK, mitogen-activated protein kinase; PDE3B, phosphodiesterase 3B; PDK, phosphoinositide-dependent kinase; PI3K, phosphatidylinositol 3-kinase.

The PI3K cascade transmits insulin signaling. PI3K activates phosphoinositide-dependent kinase and initiates the activation of atypical protein kinase C (aPKC), resulting in glucose uptake through glucose transporter (GLUT) [52] and glycogen synthesis [53]. Phosphoinositide-dependent kinase (PDK) also activates Akt, leading to activation of its downstream effectors, GLUT 4, mammalian target of rapamycin (mTOR), and phosphodiesterase 3B (PED3B) and to subsequent glucose uptake, protein synthesis and inhibition of lipolysis, respectively [51, 54-56]. The MAPK kinase cascade is involved in cell proliferation through activation of extracellular signal-regulated kinase (ERK) [57].

MOLECULAR MECHANISMS OF HCV-ASSOCIATED INSULIN RESISTANCE

Insulin resistance is defined as an insensitivity of cells to the effects of insulin [54]. Under insulin resistance conditions, post-receptor insulin signaling cascades are disturbed [58]. Obesity is a well-known causative factor for insulin resistance, and inflamed adipose tissues release fatty acids and cytokines resulting in the impairment of intracellular insulin signals [59]. In addition, recent studies have shown that HCV itself could directly elicit insulin resistance [10, 16, 17, 20, 21, 23, 36, 60].

Down-Regulation of IRS1 and IRS2 through Up-Regulation of Suppressor of Cytokine Signaling (SOCS) Proteins

We have previously found that HCV core protein induces nuclear translocation of signal transducer and activation of transcription (STAT) 3 and subsequent up-regulation of SOCS proteins in various hepatoma cell lines [16]. The SOCS family of proteins has functional similarities and similar structural characteristics including a "SOCS box", a unique NH2-terminal domain of variable length, a central Src homology 2 domain, and a COOH-terminal [61-63]. The SOCS box acts as an adaptor to facilitate the ubiquitination of signaling proteins and their subsequent targeting to the proteasome by complexing with Elongins B and C [64, 65]. Since IRS1 and IRS2 are down-regulated in livers from HCV-core transgenic mice and in livers of patients with HCV infection [16, 20], HCV core-induced SOCS3 up-regulation may promote proteasomal degradation of IRS1 and IRS2 through ubiquitination, thus causing insulin resistance in patients with HCV infection as shown in Fig. (**2A**). The following observations further implicate of SOCS3 in HCV-elicited insulin resistance; 1) carbobenzoxy- L-leucyl-L-leucyl-L-leucinal, a potent proteasomal proteolysis inhibitor, inhibits HCV core-induced reductions in IRS1 and IRS2 of HepG2 cells [16], 2) ubiquitination of IRS1 and IRS2 is increased by transfection of HCV core [16], 3) HCV core does not cause down-regulation of IRS1 and IRS2 in SOCS3$^{-/-}$ mouse embryonic fibroblast cells [16], and 4) SOCS-3 immunoreactivity in HCV-infected liver was significantly increased in non-responders to interferon therapy compared with responders [66, 67].

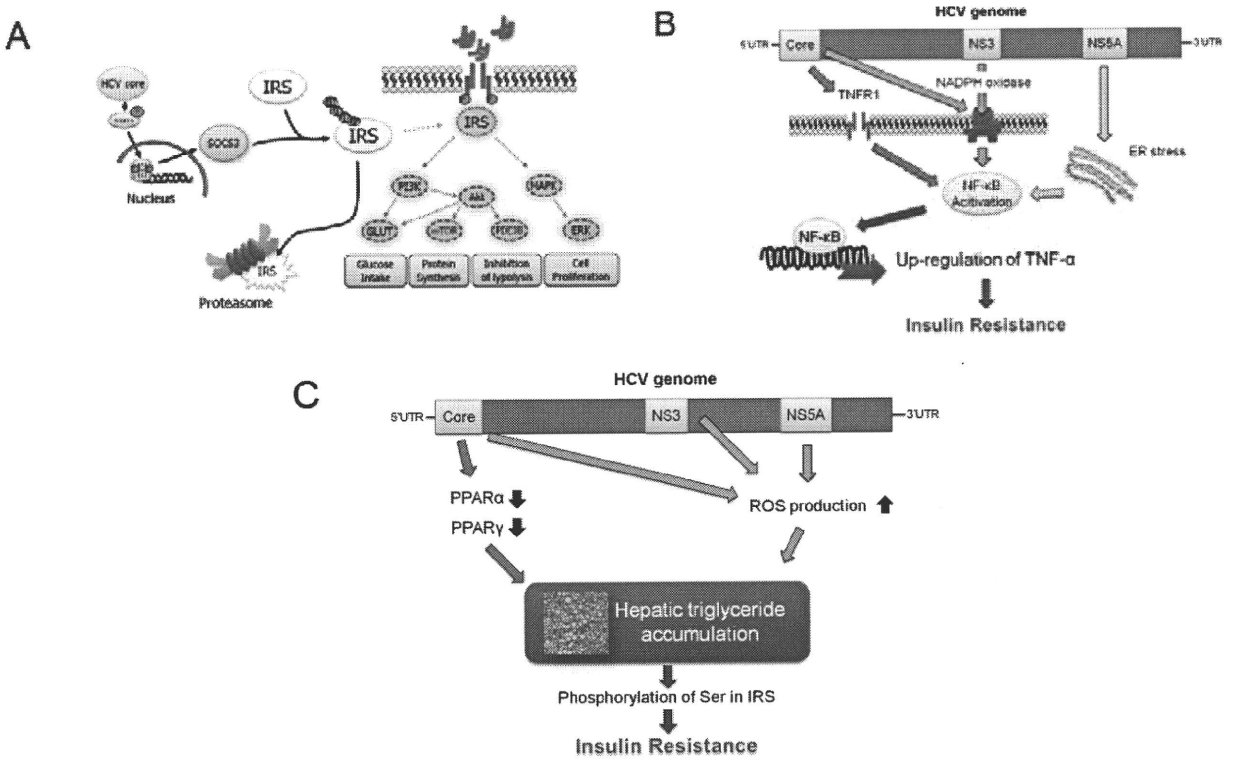

Fig. (2). Molecular mechanisms of HCV-associated insulin resistance. (**A**) Down-regulation of IRS1 and IRS2 through up-regulation of SOCSs. (**B**) Involvement of hepatic activation of NF-κB. (**C**) Participation of triglyceride accumulation in the liver. Abbreviations; ER, endoplasmic reticulum; ERK, extracellular signal-regulated kinase; GLUT, glucose transporter; HCV, hepatitis C virus; IRS, insulin receptor substrates; mTOR, mammalian target of rapamycin; MAPK, mitogen-activated protein kinase; NADPH, nicotinamide adenine dinucleotide phosphate; NF-κB, nuclear factor-kappaB; NS, nonstructural protein; PDE3B, phosphodiesterase 3B; PI3K, phosphatidylinositol 3-kinase; PPAR, peroxisome proliferator-activated receptor; ROS, reactive oxygen species; SOCS, suppressor of cytokine signaling; STAT, signal transducer and activator of transcription; TNF-α receptor 1; TNFR1, TNF-α receptor 1; Ub, ubiquitin, UTR; untranslated region.

Increased TNF-α Expression

Increased TNF-α expression is also involved in HCV-associated insulin resistance [68-71]. HCV core protein binds to the TNF-α receptor 1 (TNFR1), thus activating hepatic NF-κB as shown in Fig. (**2B**) [72, 73]. In addition, HCV core protein and HCV nonstructural protein (NS) 3 stimulate reactive oxygen species (ROS) production through activation of NADPH oxidase in HCV-infected cells [74-78]. ROS, in turn, activates NF-κB and subsequently up-regulates TNF-α expression [79-83], which could cause serine phosphorylation of IRSs and decrease the expression of the glucose transporter (GLUT) in peripheral tissues, thus leading to the development of insulin resistance as shown in Fig. (**2B**) [71]. HCV NS5A induces endoplasmic reticulum stress and also elicits NF-κB activation through Zeta-chain-associated protein kinase 70-mediated tyrosine phosphorylation of inhibitor of NF-κB-α [84].

Hepatic Triglyceride Accumulation

HCV core protein leads to hepatic triglyceride accumulation in transgenic mice, thus suggesting the direct steatogenic effect of HCV [85, 86]. Hepatic triglyceride accumulation is associated with resistance to antiviral treatments in patients with HCV chronic liver disease [87]. Furthermore, increased hepatic triglyceride activates various stress kinases, thus leading to phosphorylation of serine residues in the IRS1 and IRS2 proteins and subsequently causing insulin resistance [88]. Although the mechanism for HCV-associated triglyceride accumulation is not fully elucidated, HCV core protein and structural proteins cause hepatic triglyceride accumulation through increased ROS production as shown in Fig. (**2C**) [77, 89].

Peroxisome proliferator-activated receptors (PPARs) play a crucial role in lipid metabolism in the liver [88, 90-94]. Yamaguchi *et al.* found that HCV core decreased PPARα expression and down-regulated various lipid metabolism-associated gene expressions, including multidrug resistance protein 2, carnitine palmitoyl transferase, and acyl-CoA oxidase, thus leading to hepatic triglyceride accumulation as shown in Fig. (**2C**) [94]. PPARγ is also a key regulator of hepatic lipid metabolism [92]. Activation of PPARγ increases mRNA levels of representative genes of fatty acid oxidation such as acyl-CoA oxidation and reduces triglyceride accumulation in the liver [90]. Liver-specific disruption of PPARγ reduces hepatic triglyceride content in leptin-deficient A-ZIP/F-1 mice, an animal model of type 2 diabetes mellitus, and in wild type mice [93, 95]. De Gottardi *et al.* reported that PPARγ mRNA was decreased in the liver of

patients with HCV infection and was associated with the severity of hepatic steatosis [91]. Furthermore, HCV core protein reduces expression levels of PPARγ and increased triglyceride accumulation in a hepatoma cell line [91].

Thus, amelioration of insulin signaling by down-regulation of SOCS, suppression of TNF-α expression, and hepatic triglyceride accumulation is a novel therapeutic target for HCV-associated insulin resistance.

BCAAs

BCAAs include three amino acids with aliphatic side-chains and comprise the three essential amino acids that cannot be synthesized endogenously in humans; valine, leucine, and isoleucine, as shown in Fig. (**3**). BCAAs are constituents of protein like other amino acids and are required for protein synthesis. In addition, BCAAs are known to have some relevant pharmacologic properties in muscle-protein synthesis, immune system functioning, and ammonia metabolism.

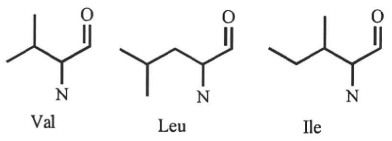

Fig. (3). Chemical structures of BCAAs.

PHARMACOLOGIC PROPERTIES OF BCAAs

Muscle-Protein Synthesis

BCAAs are mainly metabolized in skeletal muscle [96, 97] and serve as both a non-specific source of carbon for oxidation as fuel for the muscle [98] and as a precursor for the synthesis of other amino acids and proteins [99]. BCAAs have beneficial effects for decreasing exercise-induced muscle damage and promoting muscle-protein synthesis [100].

Immune System

Omission of valine, leucine, or isoleucine from the culture medium abolishes the proliferative activity of lymphocytes in response to phytohemagglutinin [101]. Administration of BCAAs improves maturation of monocyte-derived dendritic cells [102]. BCAAs also increase the number of liver-associated lymphocytes and augment lectin-dependent cellular cytototoxic activity in animals [103]. In human studies, treatment with BCAAs increases absolute lymphocyte count [104, 105] and improves phagocytic function of neutrophils, and natural killer activity of lymphocytes [106].

Ammonia Metabolism

Hyperammonemia is a common manifestation in patients with advanced liver cirrhosis and a causative factor for hepatic encephalopathy [107]. Hyperammonemia is caused by impaired hepatic ability to detoxify ammonia. BCAAs are source of glutamate, which detoxifies ammonia by glutamine synthesis in skeletal muscle and the brain [108, 109]. Therefore, BCAAs enhance detoxification ability of blood ammonia by incorporating ammonia in the process of glutamine production [110] and are currently used for the treatment for patients with hepatic encephalopathy.

EFFECTS OF BCAAs ON GLUCOSE METABOLISM

A couple of clinical studies have shown that protein-rich diets cause changes in plasma amino acid profile and improve insulin resistance, compared with normal diets having the same caloric content [111-114]. These findings suggest that amino acids have some pharmacologic effect on glucose metabolism. In contrast, plasma BCAAs levels are elevated in patients with obese or insulin resistance [115, 116]. Recently, Newgard *et al.* revealed the molecular mechanisms for BCAA-induced insulin resistance [116]. Thus, the oppose effects of BCAAs on glucose metabolism and insulin sensitization remain controversial. Herein, we summarize *in vitro*, *in vivo*, and clinical studies that examined the effects of BCAAs on glucose metabolism (Table **1**) and discuss the effects of BCAAs on glucose metabolism.

Effects of BCAAs on Insulin Secretion

Leucine is known to stimulate insulin secretion from pancreatic beta-cells [117, 118]. The mechanisms for leucine-induced insulin secretion are mediated by the metabolism of leucine *via* oxidative decarboxylation and allosteric activation of glutamate dehydrogenase in mitochondria by [119, 120]. Both acetyl-CoA and alpha-ketoglutarate are necessary as Kreb's cycle substrates to fully activate the beta-cell mitochondria, leading to insulin exocytosis induced by ATP [121]. In addition, some insulin signaling molecules such as AMP-activated protein kinase, pancreatic duodenal homeobox-1, and mTOR are involved in leucine-induced insulin secretion [121, 122]. Leucine-mediated activation of mTOR also contributes to enhance beta-cell function and the maintenance of beta-cell mass [121]. Thus, administration of leucine is a possible therapeutic strategy for diabetic patients with exhausted or decreased insulin secretion of beta-cells.

Effects of Intracellular Insulin Signaling Molecules

Among all amino acids, BCAAs have been demonstrated to modulate the insulin signaling cascade in muscle, adipose tissue, and liver (Table **1**). In high-fat diet mice, Zhang *et al.* showed that leucine supplementation improved glucose metabolism by reducing insulin resistance. Although leucine supplementation does not affect food intake, it increases uncoupling protein (UCP)-3 expression in skeletal muscle, brown adipose tissue, and white adipose tissue [123]. BCAAs, especially leucine, are reported to induce glucose uptake in primary rat muscle cells. Since the beneficial effect is inhibited by LY294002, a specific inhibitor of PI3K, leucine may stimulate insulin signaling pathways in skeletal muscle *via* preservation of PI3K activity [124, 125]. Similarly, in adipocytes from db/db mice, leucine augments the insulin-induced activation of the Akt/mTOR pathway [126]. Since insulin sensitivity is enhanced in p70 S6 kinase 1 (S6K1) $^{-/-}$ mice [127], mTOR/PI3K may be involved in the improvement in insulin resistance elicited by BCAAs.

The mTOR pathway is an important cascade for the regulation of insulin-stimulated glucose transport [128]. Nutrients including amino acids negatively affect insulin signaling through mTOR/S6K1 phosphorylation of IRS1 [129]. In a rat model of obesity-associated insulin resistance, BCAAs increase phospho-mTOR at Ser2448, phospho-S6K1 at

Table 1. Effects of BCAAs on Glucose Metabolism

	Cells/Animal models/Subjects	Amino acids	Administration term	Insulin resistance or glucose intolerance	Altered molecules or insulin effects	Reference
In vitro study	Primary rat muscle cells	Leucine	60 min	Improved	PI3K aPKC	[124]
	L6 skeletal muscle cells Primary rat muscle cells	BCAAs	180 min	Improved	PI3K aPKC	[125]
	Primary aciposytes from db/db mice	Leucine	80 min	Improved	Akt	[126]
In vivo study	High fat diet-male mice	Leucine	14-week	Improved	UCP-3 mTOR	[123]
	High fat diet-BCATm knockout mice	BCAAs	15-week	Improved	mTOR	[137]
	Exercise-trained rats	BCAAs	6-week	Improved	Hepatic and muscle glycogen contents	[213]
	Exercise-trained rats	BCAAs-enriched protein	4-week	Improved	Hepatic glucokinase	[131]
	CCl_4-treated rats	Leucine Isoleucine	60 min	Improved	GLUT1 GLUT4	[130]
	High fat diet-rats	BCAAs-enriched protein	6-week	Improved	N/A	[214]
	High fat diet-rats	BCAAs-enriched protein	6-week	Improved	N/A	[215]
	db/db mice	BCAAs	7-week	Improved	N/A	[216]
	Zucker fa/fa rats	Isoleucine	3-week	Improved	PI3K	[132]
	High fat diet-rats	BCAAs	16-week	Worsen	mTOR/S6K1, IRS1	[116]
Clinical study	Healthy male volunteers (n = 7)	Amino acids	120 min	Worsen	N/A	[142]
	Healthy elderly man (n = 30)	Leucine	12-week	No change	N/A	[143]
	Patients with type 2 diabetes mellitus (n =12)	High-protein diet	5-week	Improved	N/A	[217]
	Patients with chronic viral liver diseases (n = 12)	BCAAs	12-week	Improved	N/A	[33]
	Patients with HCV infection (n = 2)	BCAAs	6-week	Improved	N/A	[34]

Note. Abbreviation, aPKC, atypical protein kinase C; BCAAs, branched-chain amino acids; BCATm, mitochondrial branched chain amino acid aminotransferase; CCl_4, carbon tetrachloride; GLUT, glucose transporter; mTOR, mammalian target of rapamycin; N/A, not applicable; PI3K, phosphatidylinositol 3-kinase; UCP-3, uncoupling protein-3.

Thr389, and phospho-IRS1 at Ser302, leading to the development of insulin resistance [116]. However, in a rat model of liver cirrhosis, Nishitani *et al.* reported that administration of leucine and isoleucine decreases blood glucose levels by enhancing glucose uptake as a result of increased translocation of GLUT4 and GLUT1 to the plasma membrane of skeletal muscle [130]. Thus, effects of BCAAs on mTOR/S6K1 pathway are opposite and may be depend on the etiology of the insulin resistance.

Administration of BCAAs has also been reported to activate insulin signals in the liver. BCAAs-enriched protein activates hepatic glucokinase and results in increased hepatic glycogen contents [131]. Isoleucine also increases hepatic PI3K activity and improves insulin resistance in Zucker fa/fa rats, a model of severe insulin resistance [132]. Since insulin increases UCP-3 protein expression [133], translocation of GLUT4 and GLUT1 to the plasma membrane [134], glucokinase activity [135], and PI3K activity [136], BCAAs could improve insulin signals in various organs *via* various pathways.

Recently, She *et al.* clearly demonstrated an interaction between BCAAs and insulin resistance in mitochondrial

branched chain amino acid aminotransferase (BCATm) gene knockout mice. BCATm gene encodes the enzyme catalyzing the first step in peripheral BCAAs metabolism, and therefore, knockout of the BCATm gene leads to a significant elevation of plasma BCAAs levels. In $BCATm^{-/-}$ mice, fasting blood glucose and fasting serum insulin levels are decreased by 33% and 67%, respectively, and the homeostasis model assessment for insulin resistance (HOMA-IR) index, a marker of insulin resistance, is significantly lower compared to that of controls [137].

Taken together, BCAAs directly enhance insulin sensitivity by activating PI3K, Akt, and UCP-3. Since these pathways are down-stream of IRS, a target molecule for HCV-associated insulin resistance, BCAAs may be a candidate therapeutic agent for insulin resistance in patients with HCV infection.

Inhibition of Proteolysis

BCAAs inhibit proteolysis [96, 138, 139]. Indeed, BCAAs decrease expression levels of the genes involved in ATP-ubiquitin-dependent proteolysis [140, 141]. Since ubiquitin-proteasomal degradation of IRS1 and IRS2 is associated with HCV-associated insulin resistance, BCAAs may also contribute to improve insulin resistance by inhibiting degradation of IRS1 and IRS2. Inhibition of proteolysis is one of the possible mechanisms by which BCAAs combat insulin resistance.

Effects of BCAAs on Insulin Resistance

Clinical studies which evaluate effects of BCAA on glucose metabolism are summarized in Table **1**. No clinical studies show that BCAAs improve insulin resistance in healthy volunteers without apparent insulin resistance [142, 143]. However, we have recently demonstrated the beneficial effects of BCAAs administration on insulin sensitivity in chronic viral liver disease patients with insulin resistance [33]. Although body weight and plasma glucose concentration were unchanged, serum insulin levels and HOMA-IR index were significantly decreased after 60-days administration of BCAAs. Moreover, in two patients with HCV infection, BCAAs caused a decrease in both fasting insulin concentration and HOMA-IR index [34].

In contrast, elevated plasma concentration of BCAAs is reported in animal models of obesity and patients with insulin resistance [115, 116, 144]. Obesity-associated increase in BCAAs levels has been attributed to increased protein catabolism secondary to insulin resistance [145, 146] and impairment of BCAA metabolism [144]. Newgard *et al.* recently showed that BCAAs activate the mTOR/S6K1 pathway and phosphorylate multiple Ser residues of IRS, leading to the development of insulin resistance [116]. Although BCATm gene knockout mice showed increase in BCAA levels and reduction of insulin resistance [137], the absence of leucine-mediated suppression of proteolysis may contribute to the energy requirement for futile cycling of protein and subsequent reduction of insulin resistance [116]. Thus, the insulin-sensitizing effect of BCAAs remains controversial and may be only seen in patients with liver diseases, but not in obese patients with hyperinsulinemia or impairment of BCAA metabolism.

SAFETY OF BCAAs

Maple syrup urine disease is a genetic disorder impairing branched-chain alpha-keto acid dehydrogenase complex activity, resulting in the accumulation of BCAAs and branched-chain alpha-keto acids [147]. Patients with maple syrup urine disease often show severe neurological damage and mental retardation [148, 149], and therefore, tolerance limits for BCAAs have to be examined carefully.

Plasma BCAAs levels are decreased in patients with chronic liver diseases, especially liver cirrhosis [108, 150]. To detoxify ammonia, skeletal muscle uptakes and consumes BCAAs and subsequently increases clearance of BCAAs from plasma [108, 151]. Furthermore, BCAA catabolic enzymes are widely expressed throughout the body [152]. Thus, treatment with BCAAs is considered safe, as long as BCAA catabolism is at normal levels [153-155]. The prevalence of adverse effects of BCAAs is less than 15%, and major adverse effects are mild gastrointestinal symptoms such as abdominal distention, diarrhea, and constipation, but no neurological symptoms [156-162]. In general, adverse effects tend to disappear when treatment is discontinued.

PEDF

PEDF is a 50-kDa glycoprotein initially isolated from fetal human retinal pigment epithelial cells [163]. Sequence analysis of the 418 amino acids in human PEDF demonstrates a 27% identity to the serine protease inhibitor (serpin) prototype, α1-antitrypsin. PEDF has the typical serpin secondary and tertiary structure and belongs to the serpin superfamily [163-165]. Besides retinal pigment epithelial cells, PEDF is expressed in other parts of the eye (corneal epithelial cells and ciliary epithelium) [165], in other parts of the central nervous system (ependymal cells and motor neurons of the ventral horn) [166], and various cell types [166-169]. We have recently found that PEDF protein is expressed in human liver tissue, one of the target organs of insulin [170]. In addition, we have found that serum PEDF is elevated in patients with metabolic syndrome [167, 171]. Moreover, we have revealed an association between serum PEDF levels and HOMA-IR index in patients with HCV infection as shown in Fig. (**4**). These findings lead us to hypothesize that PEDF plays an important role in the development of HCV-associated insulin resistance. First, we review the structure-function relationship of PEDF and then, the molecular mechanisms by which PEDF improves insulin resistance.

STRUCTURE-FUNCTION RELATIONSHIP OF PEDF

Since HCV causes insulin resistance as well as the development of HCC, a hypervascular tumor, PEDF may have beneficial effects in HCV-related HCC patients *via* direct anti-tumor, anti-angiogenic and insulin-sensitizing properties. Here, we discuss which chemical structures could contribute to their multipotent functions.

Protein Structure Associated with Receptor-Binding

Receptor-binding activity of PEDF is preserved when the exposed loop is cleaved from its C-terminal end. PEDF pep-

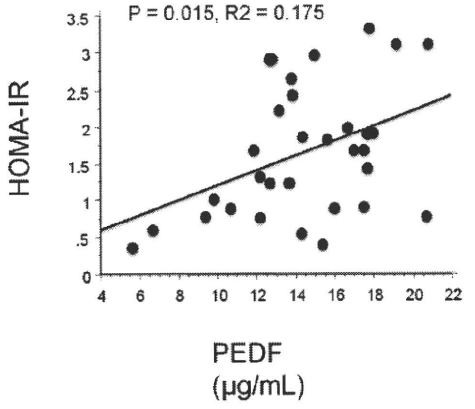

Fig. (4). An association between PEDF and HOMA-IR in patients with HCV infection. A positive correlation between serum PEDF levels and HOMA-IR index is seen in patients with HCV infection.

tides spanning residue position 32-380 and 44-121 have the ability to induce morphological differentiation and neurite outgrowth-inducing activity in human Y-79 retinoblastoma cells. Thus, N-terminal region of PEDF is a receptor-binding site [172] that has also been proven by the radioligand-binding assay and crystallization analysis [164, 173].

The heparin-binding site is in the loop region between sheet 2A and helix E [164, 174]. Site-directed mutagenesis examination revealed that Arg145, Lys146, and Arg148 are necessary for heparin binding [175]. Binding with heparin increases the proteolytic susceptibility of PEDF to trypsin and induces a conformational change in the vicinity of Lys178 [176]. Heparin facilitates the binding of PEDF to a receptor on the cell surface of retinoblastoma cells and structural change of the PEDF molecule is thought to be a mechanism for enhanced receptor binding [177]. These data indicate that a variety of PEDF functions are regulated by heparin-induced structural alteration as well as the N-terminal region.

Protein Structure Associated with Anti-Tumor Effect

PEDF contains putative hyaluronan (HA)-binding motifs. Becerra *et al.* examined the HA-binding region of PEDF by site-directed mutagenesis and identified $BXBX_2BX_2B$ and BX_3AB_2XB motifs as binding site HA (B, X, and A indicate basic amino acids, residues other than acidic amino acids, and negatively charged amino acids, respectively) [178]. These HA-binding proteins activate caspase-8, caspase-3, and poly (ADP-ribose) polymerase, which are triggers of apoptosis [179]. Moreover, the HA-binding proteins inhibit tumor growth on chorioallantoic membranes of chicken embryos and in nude mice xenograft models [179]. Thus, the HA-binding activity of PEDF may contribute to deposition in the extracellular matrix that subsequently has anti-tumor effects.

Protein Structure Associated with Anti-Angiogenic Effect

Collagen I-binding region is located opposite the heparin-binding region. In this region, the acidic amino acid residues Asp255, Asp257, and Asp299 are critical to collagen I-binding [175]. Mutation of the collagen I-binding region of PEDF is reported to cause tumor progression with neovascularization [180]. These data suggest that the collagen I-binding region of PEDF may play a crucial role in anti-angiogenesis.

MOLECULAR MECHANISMS UNDERLYING PEDF-MEDICATED IMPROVEMENT OF INSULIN RESISTANCE

PEDF is first characterized as a neurotrophic factor in 1989 [181]. Since then, a range of biological effects of PEDF has been disclosed. PEDF is now widely recognized as anti-tumor factor with an apoptotic and anti-angiogenic properties [163, 165, 182-186].

Inhibition of IkappaB Kinase (IKK) and NF-κB Activation

Under hyperglycemic, oxidative, and inflammatory conditions, advanced glycation end products (AGEs) progressively form and accumulate. Recently, we found that AGEs cause insulin resistance in Hep3B hepatoma cells where they activate Rac-1, and phosphorylate IRS1 at the Ser307 residue, and phosphorylate IKK [187, 188]. In insulin-exposed Hep3B cells, AGEs decrease tyrosine phosphorylation of IRS1 and inactivate PI3K [187, 188]. PEDF inhibits the harmful effects of AGEs on insulin sensitivity in Hep3B cells [187, 188]. Since serum levels of AGEs are higher in patients with HCV infection than in healthy subjects [189], PEDF could improve HCV-associated insulin resistance by inhibiting IKK as shown in Fig. (**5**).

As described above, activation of NF-κB and subsequent overproduction of inflammatory cytokines such as TNF-α play an important role in the development of HCV-associated insulin resistance [69, 72, 73, 84]. Zhang *et al* found that intravitreal injection of PEDF significantly reduced vascular hyper-permeability in rat models of diabetes and oxygen-induced retinopathy, correlating with the decreased levels of retinal inflammatory factors, including TNF-α [190]. In cultured retinal capillary endothelial cells, PEDF significantly decreases TNF-α expression under hypoxia [190]. Moreover, down-regulation of PEDF expression by siRNA results in significant increases of TNF-α secretion by retinal Müller cells [190]. Wang *et al.* found that PEDF inhibits high glucose-induced activation of NF-κB in cultured primary human renal mesangial cells. We also demonstrated that PEDF suppresses NF-κB activation in various types of cells including hepatoma cells [191-195]. These findings suggest that PEDF acts as an anti-inflammatory factor by blocking the NF-κB pathway and may combat HCV-associated insulin resistance as shown in Fig. (**5**).

Suppression of NADPH Oxidase Activity

HCV core protein and HCV NS3 stimulate ROS production through activation of NADPH oxidase in HCV transfected cells [74-78]. ROS activates NF-κB and up-regulates expression of TNF-α, which inactivates insulin-signaling cascade [79-83]. We have previously found that PEDF down-regulates mRNA levels of p22phox, Nox4, and gp91phox/Nox2, which are membrane components of

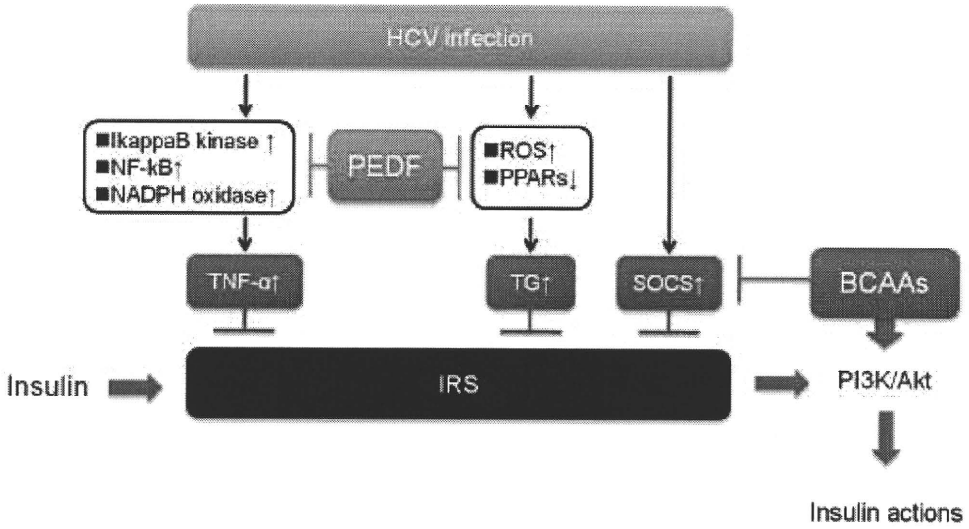

Fig. (5). Possible molecular mechanisms by which BCAAs and PEDF exert their insulin-sensitizing effects in patients with HCV infection. Abbreviations; BCAAs, branched-chain amino acids; HCV, hepatitis C virus; IRS, insulin receptor substrates; NADPH, nicotinamide adenine dinucleotide phosphate; NF-κB, nuclear factor-kappaB; PEDF, pigment epithelium-derived factor; PI3K, phosphatidylinositol 3-kinase; PPAR, peroxisome proliferator-activated receptor; ROS, reactive oxygen species; SOCS, suppressor of cytokine signaling; TG, triglyceride; TNF-α receptor 1.

NADPH oxidase, and decrease ROS derived from NADPH oxidase activity in endothelial cells [194]. We also found that PEDF inhibits ROS generation through suppression of NADPH oxidase activity *via* down-regulation of p22phox and gp91phox, and suppress the proliferation of smooth muscle cells induced by platelet-derived growth factor-BB [196]. Similarly, PEDF reduces ROS production *via* NADPH oxidase in T-cells and platelets [192, 194, 196-200]. Thus, PEDF may play a protective role against the development of HCV-associated insulin resistance *via* suppression of NADPH oxidase activity as shown in Fig. (**5**).

Reduction of Hepatic Triglyceride Accumulation *Via* Activation of PPARs

Hepatic triglyceride accumulation contributes to HCV-associated insulin resistance by decreasing PPARα and PPARγ expressions and subsequently various lipid metabolism-associated gene expressions [90, 92, 94]. Proteomic analysis revealed that PEDF is a potential regulator of lipid metabolism [201-203]. In fact, PEDF levels are correlated with severity of hepatic steatosis in both animals [204] and humans [171, 205]. In addition, hepatocytes isolated from PEDF null mice have about two-fold increase in triglyceride compared to hepatocytes from wild-type mice and hepatic triglyceride accumulation is an early event in livers from PEDF null mice [206]. Chung *et al.* found that PEDF is able to reduce the triglyceride content in a hepatoma cell line [207]. PEDF directly binds to PPARα and transfection of the PEDF gene in the presence of the PPARα/RXR heterodimer stimulates transcriptional activity of PPARα [207]. In addition, PEDF induces activation of PPARγ in various cell lines [207-212], PEDF may reduce hepatic triglyceride contents through induction of PPARγ not only in the liver, but also in other tissues thereby causing a redistribution of lipids. Thus, PEDF regulates hepatic lipid metabolism through modulation of PPARs activity and may ameliorate insulin resistance in patients with HCV infection as shown in Fig. (**5**).

CONCLUSION

We summarize the possible molecular mechanisms by which BCAAs and PEDF exert insulin-sensitizing properties in HCV-associated insulin resistance in Fig. (**5**). *In vitro-*, *in vivo-*, and human studies suggest that the following three pathways are responsible for HCV-associated insulin resistance in the liver; 1) proteasomal degradation of IRSs through up-regulation of SOCSs, 2) increased TNF-α expression, and 3) triglyceride accumulation. BCAAs improve insulin resistance by activation of insulin signaling molecules, which are down-stream of IRSs. In addition, BCAAs inhibit proteasomal degradation through down-regulation of ATP-ubiquitin-dependent proteolysis. Thus, BCAAs may improve insulin signaling pathways by two-different mechanisms. PEDF improves insulin resistance through down-regulation of TNF-α *via* suppression of IKK, NF-κB, and NADPH oxidase. PEDF also suppresses hepatic triglyceride accumulation by inhibition of ROS generation and activation of PPARs. Although direct evidence to show that BCAAs and PEDF improve insulin resistance in patients with HCV infection is still lacking, treatment with BCAAs and PEDF or pharmacological up-regulation of BCAAs and PEDF may be a promising therapeutic strategy for HCV-associated insulin resistance.

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ABBREVIATIONS

AGEs	=	advanced glycation end products
aPKC	=	atypical protein kinase C
BCAAs	=	branched-chain amino acids
BCATm	=	mitochondrial branched chain amino acid aminotransferase
CHOP	=	CCAAT/enhancer-binding protein homologous protein
ER	=	endoplasmic reticulum
ERK	=	extracellular signal-regulated kinase
GLUT	=	glucose transporter
HCC	=	hepatocellular carcinoma
HCV	=	hepatitis C virus
HNF-4	=	hepatocyte nuclear factor-4
HOMA-IR	=	the homeostasis model assessment for insulin resistance
IKK	=	inhibition of IkappaB kinase
IRS	=	insulin receptor substrates
MAPK	=	mitogen-activated protein kinase
mTOR	=	mammalian target of rapamycin
NADPH	=	nicotinamide adenine dinucleotide phosphate
NF-κB	=	nuclear factor-kappaB
NS	=	nonstructural protein
PDE3B	=	phosphodiesterase 3B
PDK	=	phosphoinositide-dependent kinase
PEDF	=	pigment epithelium-derived factor
PI3K	=	phosphatidylinositol 3-kinase
PPAR	=	peroxisome proliferator-activated receptor
ROS	=	reactive oxygen species
S6K1	=	S6 kinase 1
SOCS	=	suppressor of cytokine signaling
STAT	=	signal transducer and activator of transcription
TNF	=	tumor necrosis factor
TNFR1	=	TNF-α receptor 1
TG	=	triglyceride
Ub	=	ubiquitin
UCP-3	=	uncoupling protein-3
USF	=	upstream stimulatory factor
UTR	=	untranslated region

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Long-term trends of the incidence of hepatocellular carcinoma in the Nagasaki prefecture, Japan

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Abstract. The incidence of hepatocellular carcinoma (HCC) in Japan is still increasing. The aim of the present study was to analyze the epidemiological trend of HCC in the Western area of Japan, Nagasaki. A total of 1,807 patients with HCC diagnosed at our two hospitals between 1981 and 2005 were consecutively recruited for this study. Cohorts of patients with HCC were categorized into five-year intervals. The etiology of HCC was categorized into four groups: HCC-B: HBsAg positive, HCVAb negative, HCC-C: HCVAb positive, HBsAg negative, HCC-BC: both of HBsAg and HCVAb positive and HCC-nonBC: both of HBsAg and HCVAb negative. The number and proportion of HCC-B cases decreased from 1986 to 1990 and thereafter stabilized, whereas those of HCC-C reached the peak from 1995 to 2000 and thereafter decreased. On the other hand, the number and ratio of the HCC-nonBC cases continued to increase in the whole period. The male/female ratio of HCC-C patients decreased from 6.4 in the period 1981-1985 to 1.9 in 2001-2005, indicating clearly the increase of female patients. On the other hand, the male/female ratio of other types of HCC patients did not change during the period. HCC patients rapidly increased from 1981 to 2000 and this increase was originated from that of HCC-C. The increase of the median age and the number of female patients with HCC-C was also demonstrated. The increase in the number and the proportion of the HCC-nonBC patients was also significant.

Introduction

Primary liver cancer is the most common primary cancer of the liver accounting for ~6% of all human cancers. It is estimated that half a million cases occur worldwide annually, making primary liver cancer the fifth most common malignancy in men and the ninth in women (1-6). Hepatocellular carcinoma (HCC) accounts for 85 to 90% of primary liver cancers (7) and the age-adjusted HCC mortality rate has increased in recent decades in Japan (8). Similarly, a trend of increasing rates of HCC has been reported from several developed countries in North America, Europe and Asia (9,10). HCC often develops in patients with liver cirrhosis caused by hepatitis B virus (HBV), hepatitis C virus (HCV), excessive alcohol consumption, or nonalcoholic fatty liver disease. Of the hepatitis viruses which cause HCC, HCV is predominant in Japan (11-14).

Although the age-adjusted incidence of HCC has increased in Japan, sequential changes in background features of HCC patients are not fully understood (15). Yoshizawa reports that deaths due to HCC in Japan have continued to increase in males, particularly in those older than 60 years of age in the past 3 decades, although the reasons for this are unclear (16). To clarify factors affecting epidemiological changes in Japanese HCC patients, especially the change in age distribution and gender, we analyzed the underlying features of HCC patients in a two major liver center-based study.

Patients and methods

Patients. A total of 1,807 patients with HCC diagnosed between January 1981 and December 2005 in the Liver Disease Center, National Nagasaki Medical Center and in the outpatient clinic of The First Department of Internal Medicine, Nagasaki University Hospital, were consecutively recruited for this study. The diagnosis of HCC was based on AFP levels and imaging techniques including ultrasonography (USG), computerized tomography (CT), magnetic resonance imaging (MRI), hepatic angiography (HAG) and/or tumor biopsy. The diagnostic criteria for HCC were either a confirmative tumor biopsy or elevated AFP (≥20 ng/ml) and neovascularization in HAG and/or CT. Cohorts of patients with HCC were categorized into five-year intervals (1981-1985, 1986-1990, 1991-1995, 1996-2000 and 2001-2005).

Etiology of HCC. Sera were stored at -80°C until use. A diagnosis of chronic HCV infection was based on the presence of HCVAb (microparticle enzyme immunoassay; Abbott

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Key words: hepatitis C virus, hepatocellular carcinoma, aging, Japan

Table I. The characteristics of HCC patients, 1981-2005.

Period	1981-1985	1986-1990	1991-1995	1996-2000	2001-2005	Total
Number	240	316	369	419	463	1807
Gender						
Male	194	257	268	314	314	1347
Female	46	59	101	105	149	460
Ratio (male/female)	4.2	4.4	2.7	3.0	2.1	2.9
Age (y.o) (IQR)	57 (6.5)	61 (5.1)	63 (5.4)	66 (5.1)	68 (6.3)	64 (6.5)
Hepatitis virus						
HCC-B	95	70	80	67	100	412
HCC-C	111	213	240	292	278	1134
HCC-B+C	8	8	9	11	10	46
HCC-nonBC	26	25	40	49	75	215

Gender: 2000-2005 vs. 1981-1985 p=0.0003; 2000-2005 vs. 1986-1990 p≤0.0001; 2000-2005 vs. 1991-1995 p=0.1330; 2000-2005 vs. 1996-2000 p=0.0197. Age: 2000-2005 vs. 1981-1985 p≤0.0001; 2000-2005 vs. 1986-1990 p≤0.0001; 2000-2005 vs. 1991-1995 p≤0.0001 and 2000-2005 vs. 1996-2000 p=0.0292. IQR, interquartile range.

Laboratories) and HCV-RNA detected by polymerase chain reaction (PCR), whereas diagnosis of chronic HBV infection was based on the presence of hepatitis B surface antigen (HBsAg) (enzyme-linked immunosorbent assay; Abbott Laboratories).

Statistical analysis. The data were analyzed by the Mann-Whitney test for the continuous ordinal data between two qualitative variables. The standard deviation was calculated based on the binomial model for the response proportion. P<0.05 was considered statistically significant.

Results

Clinical features of the studied patients. A total of 1,807 patients with HCC were diagnosed at our hospital from 1981 to 2005. There were 1,347 male (75%) and 460 female (25%) patients, with a median age of 64 years. The proportion of patients diagnosed as HCC-B (HBV-associated: HBsAg positive, HCVAb negative) was 23% (412 of 1,807), whereas 63% (1,134 of 1,807) had HCC-C (HCV-associated: HCVAb positive, HBsAg negative) and an additional 3% (46 of 1,807) had HCC associated with both viruses. The remaining 215 patients (12%) showed both of the virus markers negative.

As shown in Table I and Fig. 1, the number and proportion of HCC-B cases decreased from 1986 to 1990 and thereafter stabilized, whereas those of HCC-C reached the peak in the period 1996-2000 and thereafter decreased. The number and proportion of the HCC-nonBC (HBsAg and HCVAb negative) cases continued to increase in the whole period.

Background features for patients with HCC. Fig. 2 shows the median age at diagnosis of HCC-B, HCC-C and HCC-nonBC in five-year intervals (1981-1985, 1986-1990, 1991-1995, 1996-2000 and 2001-2005). The median age of patients at diagnosis of HCC-C showed a steadily significant increase from 58 to 69 years of age during the period. The median age of patients with HCC-B and HCC-nonBC did not significantly change during the period.

Fig. 3 shows the age distribution of patients with HCC-B and HCC-C with the five 5-year intervals. There was no difference in the age distribution of patients with HCC-B during these periods. In contrast, HCC-C obviously had a trend to increase in the number of patients aged >65 years.

Table I shows that the male/female ratio of HCC patients decreased from 4.2 in the period 1981-1985 to 2.1 in 2001-2005, indicating clearly the increase of female patients. In analysis of patients in HCC-C, the male/female ratio in the periods 1981-1985, 1986-1990, 1991-1995, 1996-2000 and 2001-2005 were 6.4, 4.8, 2.5, 2.7 and 1.9, respectively (1981-1985 vs. 2001-2005, p≤0.0001) (Table II). The ratio became clearly smaller, indicates an increase in female patients with HCC-C. On the other hand, the male/female ratio of other types of HCC patients did not significantly change during the period.

Discussion

This was a two major liver center-based study designed to examine the sequential change in the background of HCC patients during the past 25 years, 1981-2005. More than 80% of our patients had chronic HBV or HCV infections. During the observation period, the number and proportion of HCC-B cases decreased in the period 1986-1990 and thereafter reached a plateau, whereas HCC-C reached a peak in the period 1995-2000 and thereafter slightly decreased. On the other hand, the number and the proportion of HCC-nonBC gradually increased in the periods of 1981-1985, 1986-1990, 1991-1995, 1996-2000 and 2001-2005 being 26 (11%), 25 (8%), 40 (11%), 49 (12%) and 75 (16%), respectively. Previous studies from Japan reported that the proportion of HCC-C had been increased and reached a plateau in the

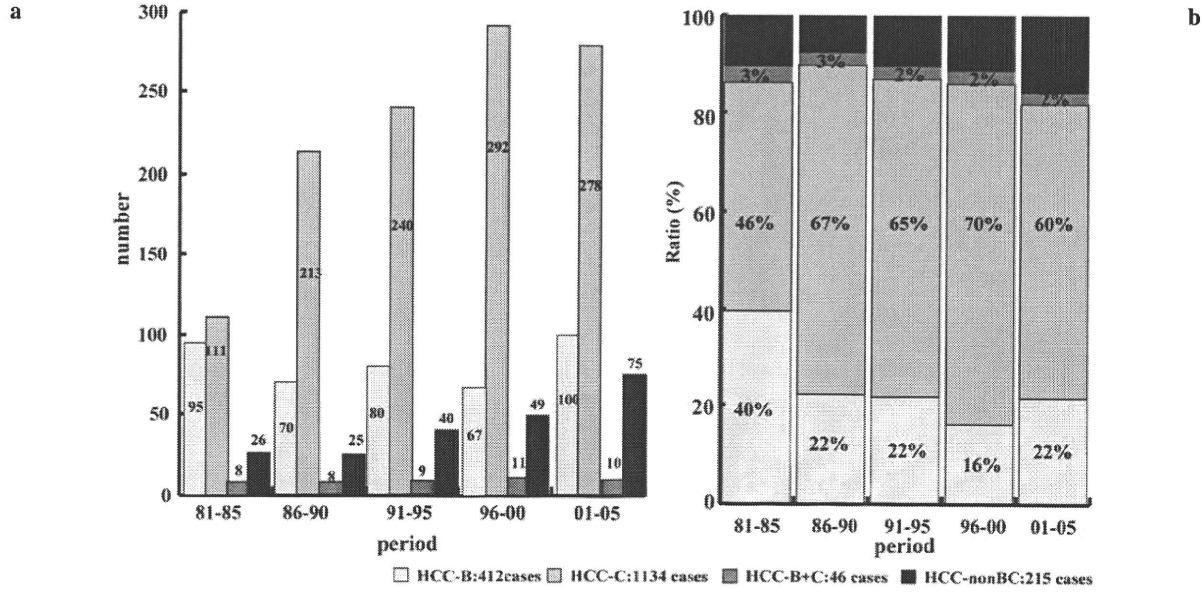

Figure 1. Sequential changes in the number (a) and ratio (b) of HCC patients categorized by etiology during the period 1981-2005 with 5-year intervals.

Table II. The number and male/female ratio of HCC patients during the period of 1981-2005.

Period	1981-1985	1986-1990	1991-1995	1996-2000	2001-2005	Total
Number	240	316	369	419	463	1807
Total						
Male	194	257	268	314	314	1347
Female	46	59	101	105	149	460
Ratio (male/female)	4.2	4.4	2.7	3.0	2.1	2.9
HCC-B						
Male	69	54	61	55	74	313
Female	26	16	19	12	26	99
Ratio (male/female)	2.7	3.4	3.2	4.6	2.9	3.2
HCC-C						
Male	96	176	172	212	182	838
Female	15	37	68	80	96	296
Ratio (male/female)	6.4	4.8	2.5	2.7	1.9	2.8
HCC-nonBC						
Male	21	20	29	40	51	1347
Female	5	5	11	9	24	460
Ratio (male/female)	4.2	4.0	2.6	4.4	2.1	2.9

HBV and nBnC: NS. HCV: 2000-2005 vs. 1981-1985 p≤0.0001; 2000-2005 vs. 1986-1990 p≤0.0001; 1996-2000 vs. 1981-1985 p=0.0033; 1996-2000 vs. 1986-1990 p=0.0084; 1991-1995 vs. 1981-1985 p=0.0024 and 1991-1995 vs. 1986-1990 p=0.0058.

period of 1981-2001 (8,15,17-19). However, in our study, the number and proportion of HCC-C cases decreased in the period 2001-2005. This may be due to interferon therapy associated with a decreased incidence of HCC (20-24). Iron depletion for chronic hepatitis C patients is a promising modality for lowering the risk of progression to HCC (25,26). Oral supplementation with oral branched-chain amino acids has been useful in the prevention HCC (27). Finally, the chronically HCV-infected population is aging in Japan. Yoshizawa reported that age-specific prevalence for the presence of HCVAb among ~300,000 voluntary blood donors from Hiroshima in 1999 clearly increased with the

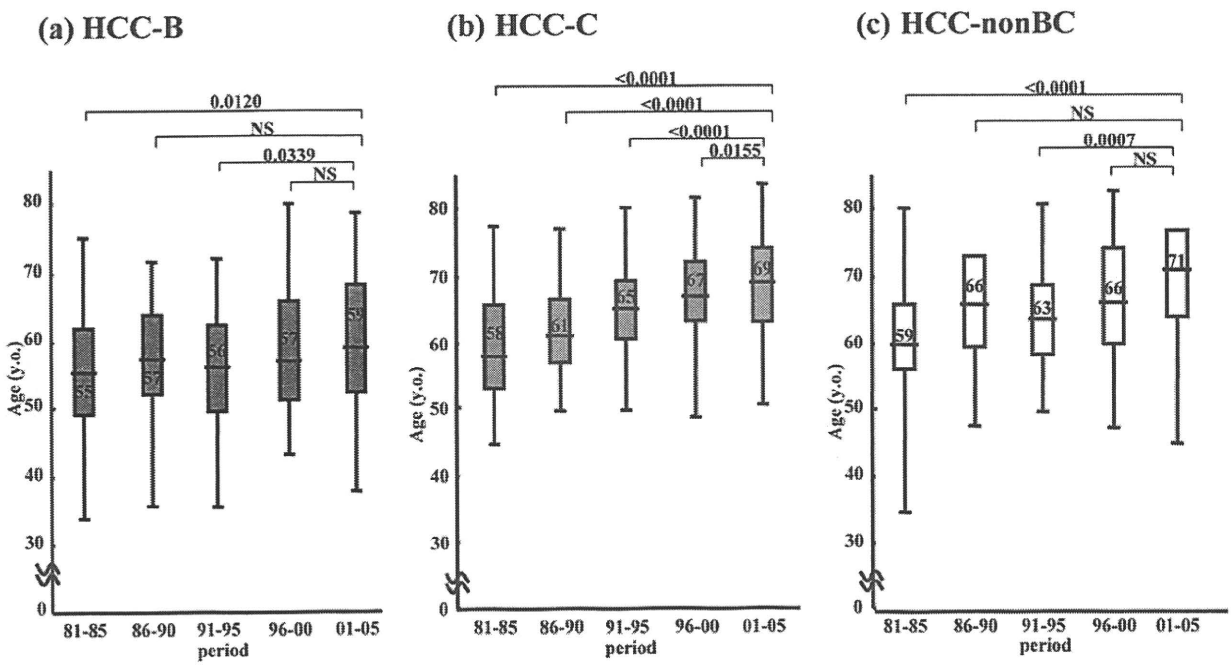

Figure 2. Sequential changes in the median age of HCC patients categorized by etiology during the period, 1981-2005 with 5-year intervals. (a) HCC-B, (b) HCC-C and (c) HCC-nonBC type $p<0.05$.

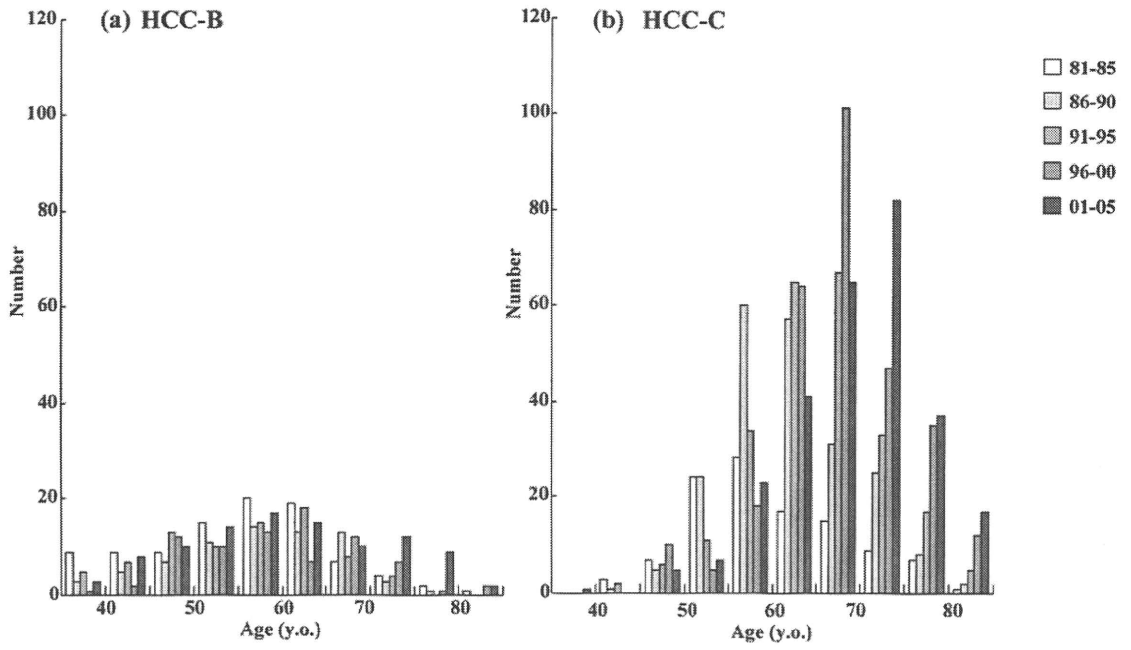

Figure 3. Changes in the age distribution of patients with HCC-B and HCC-C during the period, 1981-2005 with 5-year intervals.

age, reaching the highest proportion of 7% in individuals who were >70 years old (15,16). In this study, the median age of patients with HCC-C steadily increased from 58 to 69 years of age during the studied period. *i.e.* HCV infected people become older and they were regarded as a high risk for HCC.

In almost all populations, males have higher liver cancer proportions than females, with the male/female ratios usually averaging between 2:1 and 4:1 (7). However, the male/female ratio of HCC in Japan was 4.5 in the period 1983-1985 and 2.57 in 2000-2001 (17). In analysis of background features among HCC patients, HCC-B and HCC-nonBC cases revealed no significant change, whereas the male/female ratio of patients with HCC-C steadily decreased from 6.4 to 1.9 during the period. We suggest that the increase of female

patient with HCC-C was caused by the aging of HCV infected people. The increase of females among HCC patients was considered to increase because of HCC-C.

It is known that 2 to 4 decades of chronic HCV infection are required to develop cirrhosis and subsequent HCC (28-31). The number of HCC cases has increased in Japan, because individuals infected with HCV in the past have grown old and have reached the cancer-bearing age. The prevalence of HCV infection in young Japanese individuals is low and the incidence of HCVAb is very low because of preventative actions against HCV infection such as the screening of blood products for HCV and the use of sterile medical equipment (32). Additionally, we showed that the number and proportion of patients with HCC-C cases decreased together with an increase in the median age, whereas the number and ratio of HCC-nonBC steadily increased during the studied period. Based on these findings it may be expected that the incidence of HCC-nonBC in Japan may continue to increase even after the consequence of the HCV epidemic level off in the near future, although Japan is far advanced with regard to HCC-C.

In summary, HCC patients rapidly increased from 1981 to 2000 and this increase originated from HCC-C and the increase of the median age and the number of female patients with HCC-C. Increase in the number and proportion of the HCC-nonBC patients are also significant.

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Mini-review

Cancer stem cells in hepatocellular carcinoma: Recent progress and perspective

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ABSTRACT

Although the "cancer stem cell (CSC)" hypothesis was first proposed roughly 50 years ago, recent progress in stem cell biology and technologies has successfully achieved the identification of CSCs in a variety of cancers. CSCs are defined as a minor population which possesses a prominent ability to generate new tumors that faithfully reproduce the phenotype of original tumors in xenotransplant assays. Additionally, CSCs are able to self-renew and generate differentiated progenies to organize a hierarchical cell system in a similar fashion to normal stem cells. Although not all types of cancer follow the CSC theory, it provides an attractive cellular mechanism to account for the therapeutic resistance and recurrence of the disease. A minor population with CSC properties has been detected in a number of established hepatocellular carcinoma (HCC) cell lines and extensive analyses characterizing the CSC system in primary HCC samples are now ongoing. Considering that HCC has high rates of recurrence and mortality, novel therapeutic approaches are urgently required. Although the clinical relevance of CSCs remains elusive, deep understanding of the cellular organization of HCC may allow us to develop therapies targeting specific cell types such as CSCs.

1. Introduction

Cancer is usually unicellular in origin [1,2], although cancer cells constitute functional heterogeneity in a wide variety of cancers [3]. Classically, two general theories have been debated in terms of carcinogenesis [4]. The stochastic model indicates that a few cells which acquired proliferative potential via stochastic events are responsible for tumor formation. The alternative hypothesis, namely, the hierarchical model, postulates that a small subset of cells generates a hierarchical organization containing varied downstream descendants, proliferates extensively, and initiates tumors at high frequency.

Stem cells, generally defined by an ability to differentiate into multiple cell lineages and self-renew, contribute to not only organogenesis but also regeneration in response to the injury of tissues and organs [5]. Recent advancements in stem cell biology have allowed for the identification and characterization of stem cells in a variety of tissues and organs. On the other hand, it has been documented that solid tumors such as breast cancer and colon cancer contain a small subset of tumorigenic cells which can generate new tumors in xenograft transplantation [6,7]. This minor population of cells, termed cancer stem cells (CSCs) or tumor initiating cells (TICs), possesses stem cell-like properties and contributes to a hierarchical structure containing varied progenies in a similar fashion to normal stem cells. Successful detection of CSCs in a wide variety of cancers supports the hierarchical carcinogenesis theory.

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