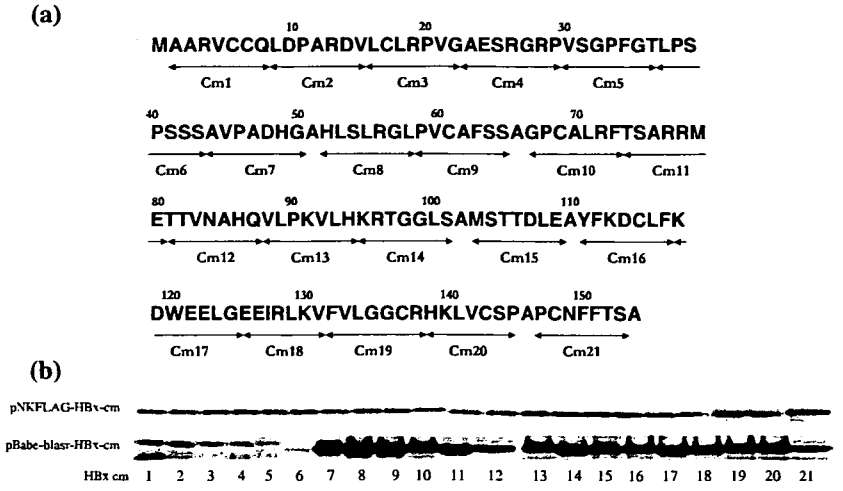

Fig. 5. Expression of hepatitis B virus X protein (HBx) library of clustered alanine substitution mutants in BJ-human telomerase reverse transcriptase (hTERT) cells. (a) Schematic representations of a series of clustered alanine substitution mutants (cm1 to cm21) of HBx. The amino acid locations of the clustered mutations are shown. (b) Detection of the mutated HBx proteins. Total cell lysates prepared from BJ-hTERT cells transfected with the mutant HBx expression vectors were fractionated by sodium dodecylsulfate–polyacrylamide gel electrophoresis and subjected to western blot analysis with anti-FLAG M2 antibody.

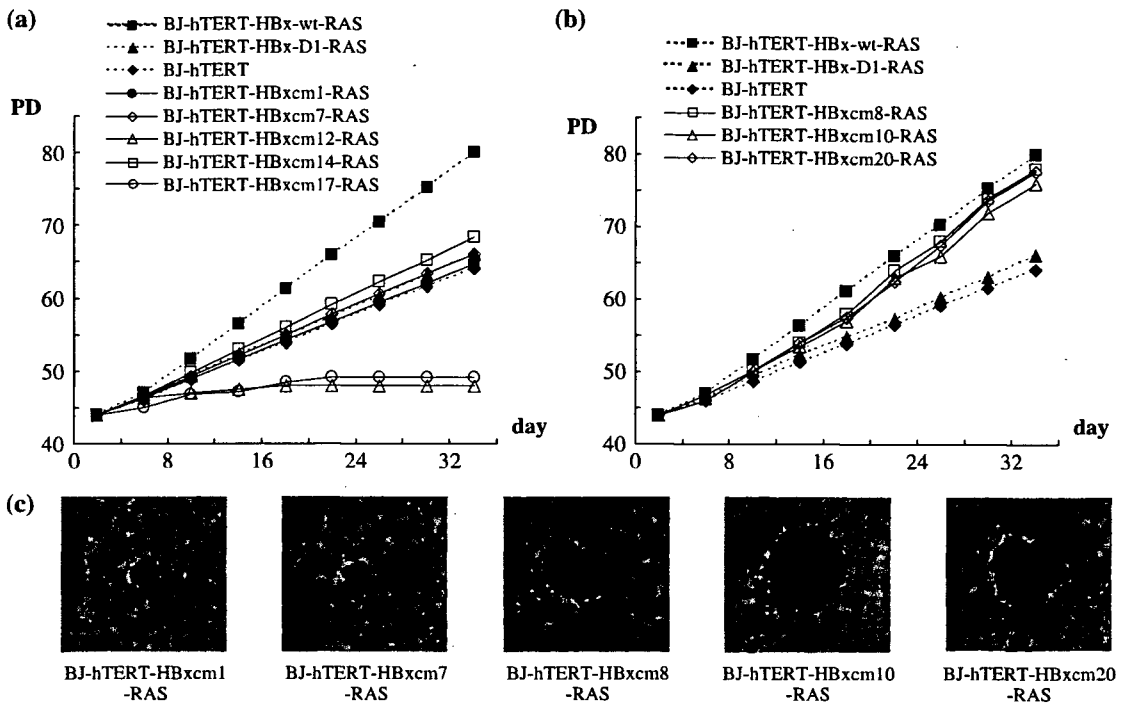

Fig. 6. Critical regions of hepatitis B virus X protein (HBx)-wt for tumorigenic function. (a) Effect of HBx-cm1–7 and HBx-cm11–18 failed to overcome H-RAS^{V12}-induced cellular senescence. Cell proliferation curves of several HBx-cm clones introduced with BJ-human telomerase reverse transcriptase (hTERT)-H-RAS$^{V(12)}$ in addition to those of BJ-hTERT cells (filled diamonds), H-RAS^{V12}-introduced BJ-hTERT-HBx-wt cells (filled squares) and BJ-hTERT-HBx-D1 cells (filled triangles) are shown. HBx-cm1, -cm7, -cm12, -cm14 and -cm17 were selected. HBx-cm1 (closed circles) and HBx-cm7 (open diamonds) represent HBx-cm1–7-introduced BJ-hTERT-H-RAS^{V12} cells. HBx-cm12 (open triangles) represents HBx-cm11–13-introduced BJ-hTERT-H-RAS^{V12} cells. HBx-cm14 (open squares) represents HBx-cm14–16-introduced BJ-hTERT-H-RAS^{V12} cells. HBx-cm17 (open circles) represents HBx-cm17 and HBx-cm18-introduced BJ-hTERT-H-RAS^{V12} cells. pBabe-puro-RAS-infected cells were selected with 1 μg/mL puromycin. After 10 days of drug selection at population doubling (PD) 44, triplicate samples of 1×10^5 cells were plated and grown under normal conditions. (b) Effect of HBx-cm8–10 and HBx-cm19–21 overcomes H-RAS^{V12}-induced cellular senescence. Cell proliferation curves of several HBx-cm clones introduced into BJ-hTERT-H-RAS^{V12} in addition to those of BJ-hTERT cells (filled diamonds), H-RAS^{V12}-introduced BJ-hTERT-HBx-wt cells (filled square) and BJ-hTERT-HBx-D1 cells (filled triangles) are shown. HBx-cm8 (open squares) and HBx-cm10 (open triangles) represent HBx-cm8–10-introduced BJ-hTERT-H-RAS^{V12} cells. HBx-cm20 (open diamonds) represents HBx-cm19–21-introduced BJ-hTERT-H-RAS^{V12} cells.

and HBx-cm18 were found to be critical for overcoming active RAS-induced senescence as the BJ-hTERT-RAS clones expressing these HBx-cm mutants failed to proliferate, meaning that these had no ability to overcome active RAS-induced cellular senescence at all (Fig. 6) (Table 1). Among the BJ-hTERT-HBx-cm cells, expression levels of HBx-cm1 to HBx-cm6 were very weak, like that of HBx-D1. Furthermore, the protein bands of HBx-cm1 to HBx-cm5 migrated slightly slower than those of HBx-cm6 and the other HBx-cm mutants in the coactivation domain in SDS-PAGE analysis (see Discussion).

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Table 1. Degree of proliferation of H-RAS^{V12}-introduced BJ-hTERT-HBx-cm cells

Cell type	Degree of proliferation
HBx-cm1[†]	+[‡]
HBx-cm2	+
HBx-cm3	+
HBx-cm4	+
HBx-cm5	+
HBx-cm6	+
HBx-cm7	+
HBx-cm8	++[§]
HBx-cm9	++
HBx-cm10	++
HBx-cm11	–
HBx-cm12	–[¶]
HBx-cm13	–
HBx-cm14	+
HBx-cm15	+
HBx-cm16	+
HBx-cm17	–
HBx-cm18	–
HBx-cm19	++
HBx-cm20	++
HBx-cm21	++

[†]HBx-cm1–21 in this table represent HBx-cm1–21-introduced BJ-hTERT-H-RAS^{V12} cells. [‡]Same as BJ-hTERT-HBx-D1-H-RAS^{V12} cells. [§]Same as BJ-hTERT-HBx-wt-H-RAS^{V12} cells. [¶]Senescence.

Discussion

Hepatitis B virus X protein has long been suspected to be positively involved in HBV-associated HCC, but its molecular role in hepatocarcinogenesis remains unclear. Although HBx is involved directly in the transformation of immortal rodent cells *in vitro* and in tumor formation in the livers of nude mice, the oncogenic activity of HBx itself remains to be elicited as the reproducibility of these experiments has been seriously controversial.[5] Furthermore, the positive role of HBx has not been addressed with human primary cells or human immortal cells. To our knowledge, our report is the first to show that HBx retains the ability to overcome RAS-induced senescence of immortalized human cells, although it is not sufficient for immortalizing human primary cells or transforming human immortal cells. hTERT-immortalized human cells stably expressing HBx-wt and RAS can form colonies in soft agar and tumors in nude mice in a cell-number-dependent manner. HBx can overcome RAS-induced senescence of BJ cells, but HBx-wt and active RAS could not immortalize the human fibroblasts. Although our findings are different to a report showing that HBx itself retains the transforming ability in NIH3T3 cells,[9] they are similar to results in rodent immortal embryonic fibroblast cells.[10]

To determine the region of HBx responsible for the ability to overcome RAS-induced senescence, we used two truncation mutants: HBx-D1 (aa 51–154), which exhibits transcriptional coactivation function and augments HBV transcription and replication,[8] and HBx-D5 (aa 1–50), which harbors the negative regulatory domain of transcriptional modulation.[6] When HBx-D1 and H-RAS^{V12} were introduced into BJ-hTERT cells, HBx-D1 was similar to wild-type HBx in overcoming RAS-induced senescence in the PD analysis and in SA-β-gal staining. Therefore, HBx-D1 alone seems to be sufficient for overcoming active RAS-induced senescence and for anchorage-independent growth, but it is not sufficient for BJ-hTERT + H-RAS^{V12} + HBx-D1 cells to form visible colonies in soft agar and tumors in nude mice. HBx alone may be sufficient for overcoming RAS-induced senescence, but hTERT is required for immortal proliferation of the transformed cells with H-RAS^{V12} and HBx. As HBx-D1 exhibits a similar ability to HBx-wt in overcoming RAS-induced senescence and anchorage-independent growth, but not in immortalizing human fibroblasts, HBx-D1 may harbor all of the critical abilities of HBx. However, HBx-D1 is different from HBx-wt in the ability to form visible colonies in soft agar and to form tumors in nude mice.

The coactivation function was recently mapped by scanning a HBx library of clustered alanine substitution mutants (HBx-cm library), and two separate sequences in HBx-D1 were found to be critical.[8] Using the same HBx-cm library, we attempted to map the sequences critical for overcoming RAS-induced senescence. We have identified three different phenotypes among the HBx-cm mutants: those phenotypes are like HBx-wt, HBx-D1 and HBx-D5 (Fig. 6). HBx-cm mutations within the D5 region, cm1 to cm7, have the ability to partially overcome OIS, whereas those within the D1 region (cm8–10, cm14–16 and cm 19–21) fail to exhibit the overcoming ability. The HBx-D5 phenotype is even found among the HBx-cm mutants (cm13, cm17 and cm18) that are defective in the coactivation function.[8] These results indicate that the ability to fully overcome OIS requires two putative functions carried by the D1 and D5 regions of the HBx protein. Because HBx-D5 does not have a positive or negative effect on RAS-induced senescence (Figs 2,3,4c), the negative regulatory domain may be active only in full-length HBx. The very low expression of HBx-D1 in human primary cells and hTERT-immortalized cells may be due to the selection result of clones, reflecting that a high level of HBx-D1 protein was eliminated due to a toxic effect of the coactivation domain,[5] or due to deletion of the N-terminal domain that has some critical role in stabilizing HBx in the expression system. Both of these may actually occur. The former is supported by the enrichment of cells expressing HBx-D1 during the early stages of drug selection. The latter is highly possible as expression levels of HBx-cm1 to HBx-cm6 covering most of the N-terminal domain were very low, as for HBx-D1. Pang *et al.* recently reported a stabilization mechanism of HBx through direct interaction with Pin1,[35] which binds phosphorylated serine and the next proline. The target serine residue is within the N-terminal domain or within the region covered by HBx-cm6. Interestingly, the HBx-cm1 to HBx-cm5 bands migrated more slowly than the HBx-cm6 band (Fig. 5b), supporting the possibility that the N-terminal domain may be critical for Pin1 binding to stabilize HBx. One interesting possibility that remains to be tested is that activation of the degradation pathway of HBx causes the toxic effect on cell proliferation. This possibility may explain the low expression of HBx-D1 and the cm mutants in the N-terminal domain. In this context, it remains unclear at present the reason for the rather stable expression of two bands of HBx-cm7 that seem to confer the same phenotype as HBx-D1 in the characterization of the cells.

The region of D1 that is responsible for overcoming RAS-induced senescence should be defined. Because some HBx-cm mutants defective in coactivation function still exhibit the ability to overcome OIS, it seems that the coactivation function is dispensable for the role. More than a dozen host factors have been reported to interact directly with the HBx-D1 region, including p53,[36,37] Smad4,[38] DDB1,[39,40] and two core subunits of the proteasome.[5] It is especially important to determine whether the binding of HBx to p53 is responsible for the ability to overcome RAS-induced senescence, as the direct binding of p53 to HBx was found to suppress p53-dependent gene activation.[5,37]

Although we have shown here that the D5 region of HBx has an indispensable biological role in anchorage-independent cell growth, the critical role of the D5 region in overcoming OIS remains obscure. The ability of the D5 region in full-length HBx

to support anchorage-independent growth will provide a good experimental system for revealing the function of the negative regulatory domain of HBx, as no host factor has been reported to interact specifically with the D5 region.

Our results clearly indicate that HBx retains the ability to overcome RAS-induced senescence in human cells immortalized by hTERT, although HBx alone could neither immortalize nor transform human cells. The ability of HBx to collaborate with active RAS in cell transformation may explain its role in hepatocellular carcinogenesis. Our findings, however, were obtained using an experimental model with immortalized cells derived from human fibroblasts. Our results may not reflect the role of HBx in HBV-infected liver, as overcoming the processes of OIS seems to vary with tissue and tumor type.[41] The role of HBx should therefore be addressed using human hepatocytes and immortalized human hepatocytes. The former, however, are quite difficult to obtain whereas the latter are available at present. It had been immortalized by introducing the other viral oncogene SV LT.[42,43]

Acknowledgments

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Gene expression profiles in peripheral blood mononuclear cells reflect the pathophysiology of type 2 diabetes

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Abstract

We hypothesized that systemically circulating peripheral blood mononuclear cells (PBMCs) reflect the pathophysiology of type 2 diabetes. PBMCs were obtained from 18 patients with type 2 diabetes and 16 non-diabetic subjects. The expression of genes in the PBMCs was analyzed by using a DNA chip followed by statistical analysis for specific gene sets for biological categories. The only gene set coordinately up-regulated by the existence of diabetes and down-regulated by glycemic control consisted of 48 genes involved in the c-Jun N-terminal kinase (JNK) pathway. In contrast, the only gene set coordinately down-regulated by the existence of diabetes, but not altered by glycemic control consisted of 92 genes involved in the mitochondrial oxidative phosphorylation (OXPHOS) pathway. Our findings suggest that genes involved in the JNK and OXPHOS pathways of PBMCs may be surrogate transcriptional markers for hyperglycemia-induced oxidative stress and morbidity of type 2 diabetes, respectively.

Keywords: c-Jun N-terminal kinase; Diabetes; DNA chip; Gene expression; Glycemic control; Oxidative stress; Mitochondria; Oxidative phosphorylation; Peripheral blood mononuclear cell

Diabetes is caused by absolute and/or relative deficiency of insulin action due to genetic disposition and environmental factors. Therefore, the diagnosis of diabetes requires a comprehensive understanding of hereditary aspects as well as habit and environmental effects. A long-term duration of diabetes causes chronic vascular complications. The underlying mechanism causing diabetic pathophysiology involves hyperglycemia itself and protein glycation. Additionally, bioactive mediators such as plasminogen activator-1, vascular endothelial growth factor, fatty acids, and adipocytokines secreted from the liver and adipose tissue can cause oxidative stress and thereby promote insulin resistance [1] and vascular complications [2]. We revealed one of the systemic manifestations of diabetes in our previous work, which showed that the hepatic gene expression profile of patients with type 2 diabetes is altered from that of patients without diabetes [3,4]. The livers of patients with type 2 diabetes had gene expression profiles indicative of increased angiogenesis, a reduced stress-defence system [3], and altered mitochondrial oxidative phosphorylation (OXPHOS) [4].

Owing to the multiple and complicated causes of the onset of diabetes, the search for conventional biomarkers that reflect diabetic pathophysiology and predict prognosis is an important issue. Glycated proteins such as haemoglobin A_{1C} HbA_{1C} and glucoalbumin are used as surrogate markers for long-term glycemic control [5,6]. Albuminuria

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is predictive of not only future diabetic nephropathy but also cardiovascular events [7,8]. High-sensitivity C-reactive protein (hs-CRP) has been found to be an independent indicator of coronary heart disease [9]. However, the use and clinical significance of these markers are limited.

Systemically circulating peripheral blood mononuclear cells (PBMCs) are considered to be a unique tissue affected by the host condition and may reflect oxidative stress caused by high levels of glucose, insulin, free fatty acids, and tissue-derived circulating bioactive mediators. To verify the hypothesis that the gene expression of PBMCs changes in response to diabetic circumstances, we comprehensively compared global gene expression profiles of PBMCs between patients with and without type 2 diabetes and between patients with type 2 diabetes before and after glycemic control, by using DNA microarray technology. We extracted the metabolic pathways coordinately altered in the PBMCs of patients with type 2 diabetes and identified the c-Jun N-terminal kinase (JNK) and OXPHOS pathways as surrogate transcriptional markers for hyperglycemia-induced oxidative stress and morbidity of type 2 diabetes, respectively. This finding may lead to the novel and powerful application of gene expression profile analysis of PBMCs for exploring the pathophysiology of diabetes.

Materials and methods

Patients. Eighteen patients with type 2 diabetes admitted to Kanazawa University Hospital between 2002 and 2004 and sixteen non-diabetic subjects were enrolled in this study. The clinical characteristics of the study subjects are shown in Table 1. No subjects had chronic inflammatory diseases such as collagen diseases and infectious diseases, all tested negative for the hepatitis B and C viruses, and all reported drinking less than 20 g/day of ethanol. The patients were diagnosed based on criteria established by an expert committee on the diagnosis and classification of diabetes mellitus [10]. The patients with diabetes were treated with diet therapy alone, oral hypoglycemic agents, or insulin as described in Table 1. Some patients were prescribed with agents for hypertension and dyslipidemia such as statins, angiotensin-converting enzyme inhibitors, or angiotensin II receptor type 1 blockers (Table 1). Overweight subjects were defined as those with a body mass index (BMI) $\geqslant 25\ kg/m^2$, which is the Japanese criteria of obesity [11].

All patients were treated for hyperglycemia at our outpatient clinic for 328 ± 235 days, and their blood samples were analyzed before and after glycemic control (Table 1).

All patients provided written informed consent for this study. The experimental protocol was approved by the relevant ethics committee of our institution and was carried out in accordance with the Declaration of Helsinki.

Laboratory studies. After an overnight fast, venous blood samples were withdrawn from each patient. Serum samples were assayed for plasma glucose, HbA_{1C}, total cholesterol, triglycerides, HDL cholesterol, insulin, alanine aminotransferase, aspartate aminotransferase (AST), hs-CRP, free fatty acids, and adipocytokines such as adiponectin, leptin, and tumor necrosis factor-α (TNF-α).

Isolation of RNA from PBMCs and amplification of antisense RNA. Heparinized blood samples were withdrawn from the peripheral vessels of subjects, and mononuclear cells were isolated by the Ficoll density-gradient method as previously described [12]. Total RNA was isolated from PBMC samples by using a Micro RNA isolation kit (Stratagene, La Jolla, CA) and RNeasy mini column (QIAGEN, Chatsworth, CA). Antisense RNA was synthesized and amplified using 2 μg of the isolated RNA with an Amino Allyl MessageAmp aRNA kit (Ambion, Austin, TX).

Preparation of fluorescently labelled cDNA and microarray hybridization. To label the probes, approximately 5 μg of amplified aRNA was chemically coupled to Cy3 or CY5 mono-reactive dye (Amersham) in accordance with the manufacturer's protocol. As a reference for each hybridization, we used aRNA samples prepared from the PBMCs of a 29-year-old healthy man. Reference aRNAs were labelled with Cy3, and test sample aRNAs were labelled with Cy5. Hybridization experiments were as described (http://www.dna-chip.co.jp/thesis/AceGeneProtocol.pdf). Briefly, the labelled probes were purified on Microcon 30 columns (Millipore, Bedford, MA); each mixture was concentrated to 31 μL. After fragmentation, 25 μL of 5× standard saline citrate (SSC), 5 μL of 10% sodium dodecyl sulphate, 8 μL of 50× Denhardt's solution, 1 μL of salmon sperm DNA (10 μg/μL), 20 μL of 5 M tetramethyl ammonium chloride, and 10 μL of formamide were added. Each 100-μL aliquot was used as a hybridization probe for the oligo-DNA chip (AceGene®Human Oligo Chip 30 K, HitachiSoft, Japan). The slides were covered with glass coverslips, fixed in a hybridization cassette (TeleChem, Sunnyvale, CA), and hybridized at 65 °C for 16 h. The slides were washed in 2× SSC and 0.03% sodium dodecyl sulphate for 5 min, 1× SSC for 5 min, and 0.2× SSC for 5 min.

Image analysis. The fluorescence intensity of each spot on the hybridized oligo-DNA microarray plate was obtained with a DNA microarray scan array G (Perkin-Elmer, Wellesley, MA). The images were quantified by DNASIS array v. 2.6 software (Hitachi Software Engineering Co., Ltd., Yokohama, Japan). The signal intensity of each spot was calibrated by subtracting adjacent background signals. To normalize the data, we averaged the intensities of all spots obtained with Cy3 and Cy5 in each of the 16 rectangles and adjusted the intensity of each corrected DNA spot by the average intensity ratio Cy5/Cy3 = 1.0. This global normalization of intensity provided a smaller variance of the Cy5/Cy3 ratio and almost equivalent results as normalization using housekeeping genes.

Hierarchical clustering of the gene expression in the patients was assessed by calculating Pearson's product-moment correlation coefficient using BRB-Array Tools software (NCBI, NIH, USA) [13]. The data were log transformed, normalized, mean centered, and applied to the average linkage clustering. The resulting dendrogram indicated the order in which patients were grouped based on the similarities of their gene expression patterns. The gene cluster data are presented graphically, and the analyzed genes are arranged as ordered by the clustering algorithm, so that genes with the most similar expression patterns are placed adjacent to each other.

Statistical analysis. All data are expressed as means ± SEM. To test the significance of expression ratios of individual genes or pathways, we used supervised analyses with a permutation-based method by using the BRB-Array Tools software [13]. This is the Class Comparison Tool based on univariate *F*-tests to find genes differentially expressed between predefined clinical groups. The permutation distribution of the *F*-statistic, based on 2000 random permutations, was also used to confirm statistical significance. We screened a total of 535 human gene sets, which included 285 BioCarta pathways, 101 KEGG pathways, and 149 gene sets previously described [14].

We normalized the expression levels of the 48 genes involved in the JNK pathway and 92 genes involved in the OXPHOS pathway to a mean of 0 and a variance of 1 across all samples. The mean centroid is the mean of the normalized gene expression levels [4,14]. A P value of <0.005 was considered significant.

Results

Patients characteristics

Clinical characteristics of the study subjects and the patients with diabetes before and after glycemic control

Table 1
Clinical characteristics of the patients with type 2 diabetes (T2DM) before (pre) and after (post) glycemic control

	Non-DM	T2DM	
		Pre	Post
No. (M:F)	16 (14:2)	18 (10:8)	18 (10:8)
Age (years)	26 ± 2	55 ± 17*	(after 328 ± 235 days)
BMI (kg/m^2)	21.1 ± 1.9	26.0 ± 5.4*	27 ± 5.1
Fasting plasma glucose (mmol/l)	4.6 ± 0.7	16.2 ± 15.4*	7.28 ± 2.11**
HbA_{1C} (%)	ND	11.0 ± 2.7	6.8 ± 1.5**
Total cholesterol (mmol/l)	4.62 ± 0.72	5.07 ± 0.85	5.04 ± 1.13
Triglyceride (mmol/l)	1.17 ± 0.60	1.80 ± 2.09	1.17 ± 0.65
HDL-cholesterol (mmol/l)	1.55 ± 0.31	1.34 ± 0.31	1.37 ± 0.31
Alanine aminotransferase (IU/l)	14 ± 8	35 ± 23 *	28 ± 15
hs-CRP (μg/dl)	0.070 ± 0.093	0.097 ± 0.088	0.13 ± 0.15
Tumor necrosis factor-α (pg/ml)	1.1 ± 0.65	1.3 ± 0.31	1.6 ± 0.53
Adiponectin (μg/ml)	8.5 ± 2.2	8.0 ± 4.7	11 ± 9
Leptin (ng/ml)	0.65 ± 0.31	12.6 ± 13.1 *	8.5 ± 2.2
Free fatty acids (mEq/L)	0.65 ± 0.31	1.0 ± 0.38*	0.94 ± 0.38
Treatments for diabetes			
Diet therapy alone		7	2
Metformin		2	1
Pioglitazone		2	2
Sulphonylureas		6	3
Insulin		5	11
Treatments for hypertension and hyperlipidaemia			
ACE-I or ARB		2	3
Statins		6	5

ACE-I, Angiotensin converting enzyme inhibitor; ARB, Angiotensin receptor type I blocker; Non-DM, Non-diabetic subjects; T2DM, Patients with type 2 diabetes. Data are expressed as means ± SD.
* $P < 0.05$ vs. Non-DM.
** $P < 0.05$ vs. Pre.

are shown in Table 1. Age, BMI, and levels of fasting plasma glucose, HbA_{1c}, alanine aminotransferase, leptin and free fatty acids were significantly increased in patients with type 2 diabetes. There were no significant differences in levels of adiponectin and inflammatory markers such as hs-CRP and TNF-α between diabetic and non-diabetic subjects (Table 1). The patients with diabetes were treated mainly with insulin and improved in glycemic control as described in Table 1. The use of drugs that may affect the gene expression in the PBMCs such as metformin, pioglitazone, angiotensin converting enzyme inhibitor, angiotensin receptor type I blocker or statins were not changed in most patients between before and after glycemic control. There were no significant differences in levels of inflammatory markers and adipocytokines between before and after glycemic control (Table 1).

Differential gene expression in PBMCs obtained from patients with type 2 diabetes mellitus

To explore whether the gene expression in PBMCs obtained from patients with type 2 diabetes differs from that of non-diabetic subjects, we applied supervised and non-supervised learning methods to classify the gene expression profiling. With a hierarchical clustering analysis, a non-supervised learning method, using 29,597 non-filtered genes, the patients were roughly clustered into three groups: patients with diabetes before glycemic control, patients with diabetes after glycemic control, and non-diabetic subjects.

Supervised learning methods based on the compound covariate predictor revealed that, among the various clinical parameters, only two clinical parameters, glycemic control and the presence of diabetes, significantly classified these patients (Supplementary Table 1). In contrast, age, gender, and BMI were not clinical determinants of gene expression profiling (data not shown).

Pathways that determine diabetes and the glycemic level

To examine which signalling pathways were evoked in PBMCs in type 2 diabetes, we compared the gene expression profiles obtained from type 2 diabetes patients and non-diabetic subjects. Moreover, we compared the gene expression in PBMCs obtained from pre-treated and post-treated patients with type 2 diabetes to explore which signalling pathway could be rescued by the treatment of type 2 diabetes. We screened a total of 535 human pathways determined by BioCarta, the KEGG pathway, and Affymetrix (http://www.affymetrix.com) and extracted the metabolic pathways that were significantly altered in the PBMCs of the subject groups (Table 2, Supplementary Table 2). Various pathways such as the OXPHOS (VOXPHOS_h), MAPK (ST_JNK_MAPK_Pathway_h), and electron transport chain pathways were significantly altered between subjects with and without diabetes (Table

2, Supplementary Table 2). On the other hand, pathways involved in the stress response, such as the MAPK, TNF signalling, apoptosis, and mTOR signalling pathways, were significantly altered after glycemic control (Table 2, Supplementary Table 2). These pathways were not significantly altered by age, gender, or BMI in the PBMCs of the patients with diabetes (data not shown).

The JNK pathway reflects hyperglycemia

The only pathway genes coordinately altered commonly by the existence of diabetes (pre-treated diabetic patients vs. non-diabetic subjects) and by glycemic control (pre-treated vs. post-treated diabetic patients), but not altered between post-treated diabetic patients and non-diabetic subjects, were the 48 genes involved in the JNK pathway (Table 2, Supplementary Table 3). With respect to individual genes, 10 of 48 genes involved in the JNK pathway were significantly up-regulated ($P < 0.05$), and 14 of 48 genes were significantly down-regulated after glycemic control ($P < 0.05$).

To further address the significance of the JNK pathway in the pathophysiology of type 2 diabetes, we computed the mean centroid of the JNK genes as previously described [4,14]. The JNK mean centroid was significantly higher in patients with diabetes compared with non-diabetic subjects and was significantly decreased after glycemic control (Fig. 1A).

We next evaluated the correlation of the expression level of JNK genes in the PBMCs and clinical or biochemical parameters of individuals with type 2 diabetes (Table 3). The JNK mean centroid in the PBMCs was significantly correlated with levels of fasting plasma glucose and A1C. Thus, the up-regulation of the JNK genes in the PBMCs may be associated with hyperglycemia.

OXPHOS pathway reflects morbidity of type 2 diabetes

The only pathway genes coordinately altered by the existence of diabetes (pre-treated diabetic patients vs. non-diabetic subjects and post-treated diabetic patients vs. non-diabetic subjects), but not altered by glycemic control (pre-treated vs. post-treated diabetic patients), were the 92 genes involved in the mitochondrial OXPHOS pathway and 96 genes involved in the electron transport chain pathway, which share most of the same genes (Table 2, Supplementary Table 4). With respect to individual genes, 30 of 92 genes involved in the OXPHOS pathway were significantly down-regulated, and no genes were significantly up-regulated in diabetes ($P < 0.05$).

The OXPHOS mean centroid was significantly down-regulated in patients with diabetes compared with non-diabetic subjects, whereas it was not significantly altered after glycemic control (Fig. 1B).

As shown in Table 3, the OXPHOS mean centroid in the PBMCs did not significantly correlate with the fasting levels of plasma glucose and HbA_{1C}. Thus, the down-regulation of OXPHOS genes may be determined genetically and may reflect the morbidity of type 2 diabetes.

Discussion

In the present study, we demonstrated the possibility that gene expression profiles in PBMCs reflect the pathophysiology of type 2 diabetes. As type 2 diabetes is a multifactorial disorder [15], a comprehensive approach

Table 2
Significantly altered pathways in diabetic PBMC

	Pathway description	No. of genes	T2DM-Pre vs. Non-DM		T2DM-Post vs. T2DM-Pre	
			LS permutation *p*	KS permutation *p*	LS permutation *p*	KS permutation *p*
1	Electron_Transport_Chain_h	96	0.0000905	0.0087215	0.3297651	0.1435376
2	VOXPHOS_h	92	0.0009636	0.0269323	0.2323794	0.0899932
3	HOXA9_DOWN_h	42	0.011004	0.0045108	0.0643224	0.2061987
4	4fcer1 Pathway_h	44	0.01321	0.0186952	0.4412977	0.4359556
5	pparaPathway_h	62	0.0165551	0.1204174	0.6542218	0.7514925
6	MAP00620_Pyruvate_metabolism_h	40	0.0169308	0.0955992	0.6148643	0.6883807
7	p38mapkPathway_h	55	0.0278384	0.0945286	0.0759194	0.184746
8	CR_IMMUNE_FUNCTION_h	57	0.0298111	0.0008373	0.5589565	0.8259213
9	keratinocytePathway_h	53	0.0298347	0.0827755	0.2315696	0.316895
10	ST_Fas_Signaling_Pathway_h	81	0.0309935	0.1369283	0.1961165	0.053945
11	metPathway_h	47	0.0314289	0.2487016	0.2889148	0.1813162
12	CBF_LEUKEMIA_DOWNING_AML_h	81	0.0356794	0.3177211	0.0464059	0.0042596
13	ST_B_Cell_Antigen_Receptor_h	48	0.0370735	0.2309135	0.1514077	0.2013602
14	ST_JNK_MAPK_Pathway_h	48	0.0463635	0.0181573	0.0082456	0.0048617
15	erkPathway_h	40	0.0473747	0.0488313	0.1155434	0.0388996
16	INSULIN_2F_DOWN_h	40	0.0529183	0.0283123	0.0496545	0.0783795
17	MAP00071_Fatty_acid_metabolism_h	46	0.0548243	0.0433901	0.9451761	0.9641461
18	bcrPathway_h	42	0.066755	0.3680323	0.5129712	0.678769
19	MAP00561_Glycerolipid_metabolism_h	54	0.0672738	0.0125806	0.9263149	0.9473428
20	integrinPathway_h	45	0.0748778	0.4564322	0.0871219	0.1522387

Non-DM, Non-diabetic subjects; Post, After glycemic control; Pre, Before glycemic control; T2DM, Patients with type 2 diabetes.

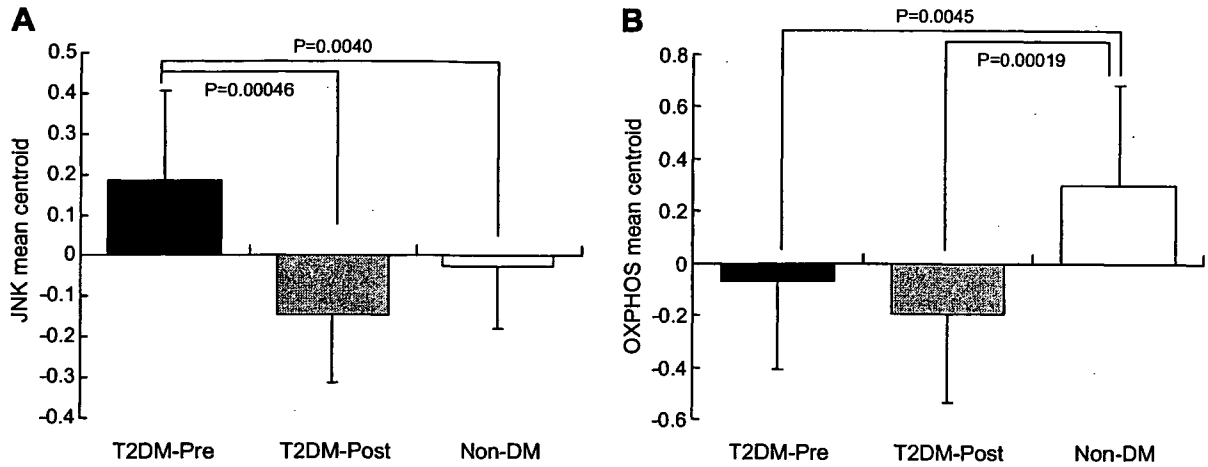

Fig. 1. JNK mean centroid (A) and OXPHOS mean centroid (B) in patients with type 2 diabetes (T2DM) before (Pre) and after (Post) glycemic control and in non-diabetic subjects (Non-DM). The mean centroid of the OXPHOS genes was computed as described in Methods. The values are means ± SD.

Table 3
Correlation of mean centroid of the genes involved in pathways for OXPHOS and JNK with the clinical parameters in patients with type 2 diabetes

	JNK mean centroid		OXPHOS mean centroid	
	Pearson's r	P	Pearson's r	P
BMI	0.018	0.920	−0.252	0.143
Fasting plasma glucose	0.468	0.018	0.097	0.644
HbA_{1C}	0.393	0.022	0.169	0.338
Fasting insulin	0.528	0.360	0.739	0.153
Alanine aminotransferase	0.232	0.208	0.127	0.496
Total cholesterol	0.154	0.417	−0.024	0.900
Triglyceride	0.098	0.606	0.093	0.624
HDL-cholesterol	0.030	0.874	0.120	0.527
hs-CRP	−0.068	0.789	−0.333	0.177
TNF-α	−0.355	0.125	−0.287	0.219
Adiponectin	−0.184	0.411	0.281	0.205
Leptin	−0.215	0.336	−0.166	0.459
Free fatty acids	0.255	0.291	0.302	0.209

identifying biological pathways or co-regulated gene sets associated with the diseases should be required to understand the molecular signature of type 2 diabetes [4]. Thus, we screened known human pathways and extracted the metabolic pathways that were significantly altered in the PBMCs of the subject groups.

We found that the distinct pathophysiology of patients with type 2 diabetes was reflected by coordinate alterations in the gene expression levels of the JNK and mitochondrial OXPHOS pathways in PBMCs; the former reflected hyperglycemia-associated oxidative stress, and the latter reflected an intrinsic alteration in patients with type 2 diabetes.

It has been recognized that, in endothelial cells, hyperglycemia causes mitochondrial superoxide production that leads to oxidative stress via glucose-induced activation of protein kinase C, increased formation of glucose-derived advanced glycation end-products, and increased glucose flux through the aldose reductase pathway [2]. Such diabetes- or hyperglycemia-induced oxidative stress may cause endoplasmic reticulum stress leading to activation of the JNK pathway in pancreatic beta-cells and hepatocytes [16]. The activation of JNK suppresses insulin biosynthesis and interferes with insulin action. Indeed, the suppression of JNK in diabetic mice was found to improve insulin resistance and ameliorate glucose tolerance [16]. Thus, the JNK pathway plays a central role in the pathogenesis of type 2 diabetes and could be a potential target for diabetes therapy. This glucose-induced oxidative stress can occur systemically, especially in PBMCs that uptake glucose in an insulin-independent manner. In the present study, the genes involved in the JNK pathway of the PBMCs were coordinately up-regulated in diabetes and significantly down-regulated after glycemic control, whereas the inflammatory markers (hs-CRP and TNF-α) and adipocytokines (adiponectin and leptin) were not altered. Thus, it might be possible that we can estimate the glucose-induced oxidative stress in pancreatic beta cells, hepatocytes, and endothelial cells simply by analyzing the gene expression profile in the PBMCs of patients with type 2 diabetes.

On the other hand, the OXPHOS pathway may predict the existence of diabetes because it was coordinately down-regulated in the PBMCs of patients with type 2 diabetes,

but was not altered by glycemic control. Emerging evidence supports the potentially unifying hypothesis that insulin secretory failure and insulin resistance, both of which are prominent features of type 2 diabetes, are caused by mitochondrial dysfunction [17]. Indeed, type 2 diabetes is associated with the coordinate down-regulation of genes involved in OXPHOS in skeletal muscle [14] and adipose tissue [18]. This alteration might occur genetically and systemically in patients with type 2 diabetes, except in the liver in which excess metabolites of glucose and fatty acids may up-regulate the genes involved in OXPHOS [4]. Thus, it might be possible to predict the predisposition or onset of type 2 diabetes simply by analyzing the gene expression profile of PBMCs.

Although future studies are necessary to clarify the effects of age, gender, type of diabetes, complications, treatment regimens for diabetes, other pharmacological treatments for hypertension and hyperlipidemia, etc., the present study suggests the diagnostic potential of gene expression analysis of PBMCs in patients with type 2 diabetes.

Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.bbrc.2007.07.006.

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Impact of Diabetes on Recurrence of Hepatocellular Carcinoma after Surgical Treatment in Patients With Viral Hepatitis

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OBJECTIVES: Consensus has been reached that diabetes is a risk factor for development of HCC, but the impact on postoperative recurrence is still controversial. To clarify this point, we analyzed the relationship of postoperative recurrence rate of HCC and coexistence of diabetes in the patients with viral hepatitis.

METHODS: A total of 90 patients who had undergone curative resection for HCC were analyzed. They were divided into two groups with and without diabetes, and the recurrence-free survival rates after surgical treatment and the factors contributing to recurrence were examined.

RESULTS: Kaplan-Meier survival analysis showed the recurrence-free survival rates in the diabetic group were significantly lower than those in the nondiabetic group ($P = 0.005$) and overall survival rates in the diabetic group were significantly lower than those in the nondiabetic group ($P = 0.005$). These results were emphasized in the analysis of patients infected with hepatitis C virus. Univariate and multivariate analyses showed diabetes was a significant factor contributing to HCC recurrence after treatment. Furthermore, multivariate analysis in HCC patients with diabetes showed Child-Pugh classification B ($P = 0.001$) and insulin therapy ($P = 0.049$) were significant factors contributing to HCC recurrence after treatment.

CONCLUSIONS: The results of the present study suggest that diabetes is a risk factor for the recurrence of HCV-related HCC and decreases the overall survival rates after surgical treatment. HCV-related HCC patients with diabetes should be closely followed for postoperative recurrence.

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INTRODUCTION

Hepatocellular carcinoma (HCC) is the fifth most frequent malignant neoplasm in the world (1), and its prevalence is particularly high in Asia and Africa. The recent increase in its prevalence has attracted the attention of researchers (2, 3). Surgical therapy provides complete cure for HCC, but the indication is limited to a relatively small number of patients. Recent remarkable advances in diagnostic imaging techniques and systematic hepatectomy have been improving the prognosis of patients with HCC, but these techniques have not provided satisfactory results (4, 5), because of a high post-treatment recurrence rate characterizing HCC. Previous studies have noted that factors contributing to recurrence include gender, alcohol consumption, hepatitis C virus (HCV) infection, hepatic reserve, liver fibrosis degree, tumor size, tumor differentiation degree, vascular factor, and alpha-fetoprotein (AFP) level (6–9).

On the other hand, recent studies have reported that coexistence of diabetes is a risk factor for the progression of liver fibrosis and the development of HCC in chronic hepatitis C (10, 11). These reports suggest that coexistence of diabetes is also involved in the high postoperative recurrence rate of HCC. However, it is controversial whether diabetes is an independent risk factor for the post-treatment recurrence of HCC. Ikeda *et al.* (12) reported that diabetes was a risk factor for the recurrence of HCC after surgical treatment, but Poon *et al.* (13) and Toyoda *et al.* (14) reported that this was not the case. The discrepancy among these reports is probably due in part to the difference in the etiology of liver disease in the patients studied.

In this study, to clarify the controversial point about diabetes and HCC recurrence, we examined the impact of diabetes on the postoperative recurrence of HCC in 90 patients who had undergone curative resection for HCC. In addition, we classified the HCC patients into groups of hepatitis B

virus (HBV)- and HCV-related HCC patients, and performed a close analysis of the impact of diabetes on the postoperative recurrence of HCC in each group.

PATIENTS AND METHODS

Patients

A total of 150 patients were diagnosed with primary HCC and underwent surgical treatment in Kanazawa University Hospital between June 1987 and May 2004. Of these patients, 90 were analyzed who had HBV or HCV infection and underwent curative resection.

HCCs were detected by imaging modalities such as ultrasound scan, dynamic CT scan, MR imaging, and abdominal arteriography. The diagnosis of HCC was made by typical hypervascular tumor staining on angiography in addition to using typical findings, which showed hyperattenuation areas in the early phase and hypoattenuation in the late phase on dynamic CT (15).

All resected tumors were examined pathologically for the degree of differentiation of HCC, vascular invasion, and persistence of tumor in the surgical stump. Pathological degree of differentiation of HCC was assessed according to the general rules for the clinical and pathologic study of primary liver cancer (16).

Treatment and Follow-Up

In selecting surgery as a treatment option for HCC, we considered the following criteria: (a) good general condition of the patient whose Karnofsky performance status was over 80, (b) primary HCC, (c) Child-Pugh classification A or B, (d) the number of HCC was solitary and no CT, MRI, or angiographic evidence of vascular invasion or distant metastasis. Curative resection was defined as complete excision of the tumor with tumor-free surgical margins and no local recurrence at the surgical margin within 6 months after surgery.

Patients were followed postoperatively on an outpatient basis by abdominal ultrasound, dynamic CT, or MRI at 3-month intervals for at least 60 months. Recurrence was diagnosed by dynamic CT or MRI, and the date of recurrence was defined as the date of examination when the recurrence of HCC was noted. In patients with recurrent HCC, the recurrence-free period was defined as the time between the date of surgery and the date of recurrence. We confirmed the date of the patient's last visit to our hospital and checked the status of HCC using each patient's medical record.

Laboratory and Virologic Testing

Blood samples were tested for hepatitis B surface antigen (HBs-Ag) and hepatitis C virus antibody (HCV-Ab) by commercial immunoassays (Fuji Rebio, Tokyo, Japan). Serum AFP level was measured by enzyme immunoassay (AxSYM AFP, Abbott Japan, Tokyo, Japan). Diabetes was diagnosed according to the American Diabetes Association criteria for type II diabetes (17) and the severity of liver disease (stage of fibrosis) was evaluated according to the criteria of Desmet *et al.* (18).

Statistical Analysis

Between-group differences were assessed by univariate analysis with Student's t-test for numerical data and the $\chi 2$ test with Yates' correction (or Fisher's exact test where appropriate) for nominal data. Overall survival and recurrence-free survival was examined using the method of Kaplan-Meier, and differences were assessed by the log-rank test. Impact factors for the recurrence of HCC after hepatic resection were analyzed by univariate and multivariate analysis using Cox proportional hazards model. Seventeen variables were analyzed, consisting of age, gender, etiology, body mass index (BMI), prevalence of alcohol abuse, diabetes, hemoglobin A1c (HbA1c), liver fibrosis degree, Child-Pugh classification, platelet count, alanine aminotranseferase (ALT), T-bil, Alb, AFP, tumor size, tumor differentiation degree, and the presence of vascular invasion. $P < 0.05$ was considered statistically significant.

RESULTS

Comparison of Baseline Characteristics

Of the 90 patients (75 men and 15 women, with a mean age of 61.0 yr) who were followed and analyzed, 30 were diagnosed as having coexistence of diabetes, and 60 had no diabetes. The characteristics of the patients in both groups

Table 1. Characteristics of Patients

Characteristic	Patients With Diabetes (N = 30)	Patients Without Diabetes (N = 60)	*P* Value
Median age (yr)	62.0	60.6	0.453
Gender (male/female)	24/6	51/9	0.560
Etiology (HBV/HCV/HBV + HCV)	8/22/0	17/40/3	0.438
Body mass index (kg/m^2)	23.5	22.73	0.316
Alcohol abuse (+/−)	13/17	27/33	0.881
HbA1c (%)	6.4	4.8	<0.001
HOMA-IR	4.1	3.3	0.399
Platelet count ($\times 10^4/\mu$L)	12.2	13.5	0.284
ALT (IU/L)	69.8	56.4	0.318
Total bilirubin (mg/dL)	0.9	0.8	0.510
Albumin (g/dL)	4.1	4.2	0.341
AFP (ng/mL)	417	395	0.931
Fibrosis (F1/F2/F3/F4)	0/4/4/22	4/5/5/46	0.732
Inflammatory grading (A1/A2/A3)	11/17/2	24/33/3	0.385
Child-Pugh grade (A/B/C)	24/6/0	53/7/0	0.294
Tumor size (mm)	34.3	29.8	0.359
Diff. degree (wel/mod/por)*	11/12/7	20/19/21	0.264
Vascular invasion (+/−)	11/19	20/40	0.757
Date of operation (1987–1995/1995–2000/2001–2004)	8/14/8	13/30/17	0.538

*Histological degree of HCC: wel = well differentiated; mod = moderately differentiated; por = poorly differentiated.

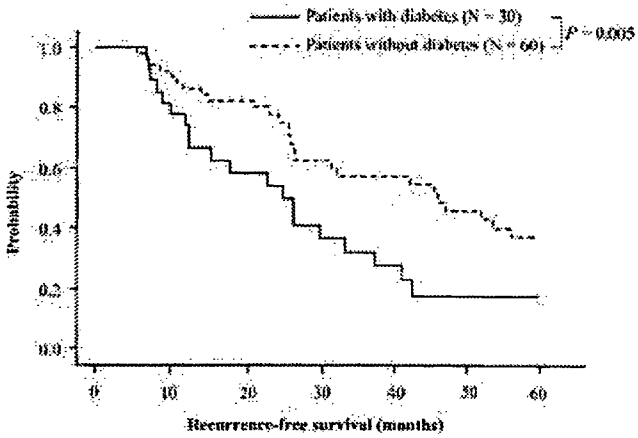

Figure 1. Kaplan-Meier curves for recurrence-free survival in the groups of patients with and without diabetes.

are shown in Table 1. No significant differences were noted between the two groups in age, gender, HBV or HCV infection rate, BMI, or prevalence of alcohol abuse. HbA1c was significantly higher, at 6.4%, in the diabetic group than in the nondiabetic group with an HbA1c of 4.8% ($P < 0.001$). There were no significant differences in platelet count, ALT, T-bil, Alb, AFP, or Child-Pugh classification between the two groups. In addition, no significant differences were observed in the liver fibrosis degree, or in the size, degree of differentiation, and presence of microscopic vascular invasion of resected HCC. Homeostasis model assessment-insulin resistance (HOMA-IR) of the patients with diabetes, which was high compared with that of Japanese healthy subjects (19), was higher than that of the patients without diabetes, although it was not statistically significant.

Impact of Diabetes on Recurrence After Surgical Treatment of HCC

Next, the diabetic and nondiabetic groups were compared for the rate of HCC recurrence after surgical treatment. HCC recurred after surgical treatment in 49 patients, consisting of 22 diabetic patients (73.3%) and 27 nondiabetic patients (45.0%). The mean time to recurrence was 32.8 months (range 8–60 months) and the median time to recurrence was 29.4 months.

Figure 1 shows the Kaplan-Meier curves for recurrence-free survival of the patients with and without diabetes. The recurrence-free survival rates 1, 2, 3, 4, and 5 yr after surgical treatment were 77.8%, 55.6%, 36.0%, 16.7%, and 16.7%, respectively, in the diabetic patient group, and 89.5%, 80.4%, 56.8%, 45.0%, and 36.6%, respectively, in the nondiabetic patient group; all rates except 1 yr were significantly lower in the diabetic patient group ($P = 0.155$, $P = 0.010$, $P = 0.009$, $P = 0.002$, and $P = 0.005$).

To further examine the degree of contribution of diabetes to the postoperative recurrence of HCC, we performed univariate and multivariate analysis. Univariate analysis identified the following variables as factors significantly contributing to HCC recurrence after surgical treatment: presence of diabetes ($P = 0.003$), high HbA1c level ($P < 0.001$), and Child-Pugh classification B against A ($P < 0.001$) (Table 2). When we conducted multivariate analysis, we chose variables that had already pointed out a risk factor for HCC recurrence and the P value was lower than 0.1 in univariate analysis. As a result, multivariate analysis of these variables showed that the presence of diabetes (risk 2.9, 95% CI 1.5–5.4, $P < 0.001$) and Child-Pugh classification B against A (risk 3.6, 95% CI 1.7–7.7, $P = 0.001$) were significant factors contributing to HCC recurrence after surgical treatment (Table 3).

Table 2. Univariate Proportional Hazard Model for Recurrence of HCC After Surgical Treatment

Variable	Hazard Ratio	95% CI	P Value
Age (yr)	1.0	1.0–1.1	0.258
Gender (male)	0.5	0.3–1.1	0.104
Etiology (HCV)	1.0	0.5–1.8	0.965
Body mass index (>25 kg/m^2)	1.2	0.6–2.3	0.554
Alcohol abuse (+)	1.1	0.7–2.0	0.644
Diabetes (+)	2.4	1.3–4.2	0.003
HbA1c (%)	1.5	1.2–1.9	<0.001
Fibrosis (F4)	1.4	0.7–3.1	0.349
Child-Pugh grade (B)	3.1	1.6–6.0	<0.001
Platelet count ($\times 10^4/\mu$L)	1.0	0.9–1.0	0.465
ALT (IU/L)	1.0	1.0–1.1	0.717
Total bilirubin (mg/dL)	1.4	0.6–3.6	0.451
Albumin (g/dL)	1.4	0.8–2.4	0.225
AFP (>200 ng/mL)	1.0	0.5–1.8	0.906
Tumor size (>50 mm)	1.5	0.7–1.2	0.213
Diff. degree (P)	1.1	0.6–2.1	0.675
Vascular invasion (+)	1.2	0.4–1.7	0.490

Impact of Diabetes on Prognosis After Surgical Treatment of HCC

To examine the impact of diabetes on prognosis of HCC patients, we analyzed the overall survival rates after surgical treatment. Figure 2 shows the Kaplan-Meier curves for overall survival of the patients with and without diabetes after surgical treatment of HCC. The overall survival rates 1, 2, 3, 4, and 5 yr after surgical treatment were 100%, 88.9%, 75.0%, 63.6%, and 45.5%, respectively, in the diabetic patient group, and 100%, 98.1%, 88.9%, 85.7%, and 76.3%, respectively, in the nondiabetic patient group; the rates of more than 3 yr were significantly lower in the diabetic patient group ($P = 1.000$, $P = 0.073$, $P = 0.028$, $P = 0.039$, and $P = 0.005$).

Table 3. Multivariate Proportional Hazard Model for Recurrence of HCC After Surgical Treatment

Variable	Hazard Ratio	95% CI	P Value
Diabetes	2.9	1.5–5.5	<0.001
Fibrosis (F4)	1.9	0.8–4.5	0.148
Child-Pugh grade (B)	3.6	1.7–7.7	0.001
AFP(>200 ng/mL)	0.7	0.3–1.5	0.390
Diff. degree (P)	0.9	0.5–1.8	0.776
Vascular invasion (+)	2.0	1.0–4.0	0.061

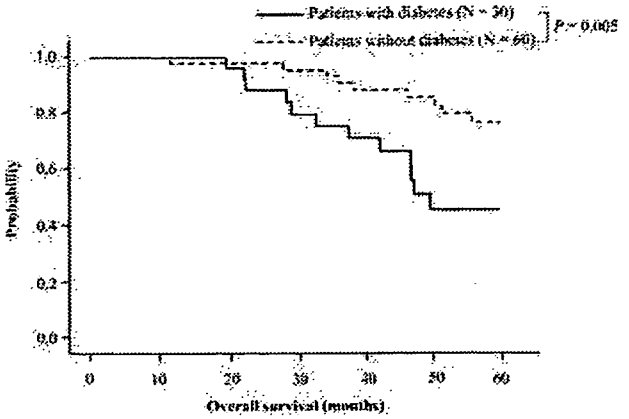

Figure 2. Kaplan-Meier curves for overall survival in the groups of patients with and without diabetes.

These curves indicated that overall survival rates were significantly lower in the diabetic patient group ($P = 0.005$).

Differential Impact of Diabetes on Prognosis After Surgical Treatment Between HBV- and HCV-Infected Patients

Next, we classified the HCC patients into HBV- and HCV-related HCC patients, and examined the impact of diabetes on recurrence-free and overall survival rates after surgical treatment. We divided all patients into 25 HBs-Ag (+), HCV-Ab (−) patients (with HBV-related HCC) and 62 HBs-Ag (−), HCV-Ab (+) patients (with HCV-related HCC), and further divided these two groups of patients into four groups according to the presence or absence of diabetes. In 62 patients who were HCV-Ab positive, 53 patients were also positive for HCVRNA. The other 9 patients were not examined for HCVRNA. The clinical profiles of these four groups of patients are shown in Table 4. Three HBs-Ag (+), HCV-Ab (+) patients, who were not complicated by diabetes, were excluded from the analysis. There were no significant differences between the groups of HBV-related HCC patients with and without diabetes in age, gender, BMI, prevalence of alcohol abuse, platelet count, ALT, total bilirubin, Child-Pugh classification, liver fibrosis degree, tumor size, tumor differentiation degree, or the presence of vascular invasion, except for Alb and AFP. Similarly, there were no significant differences between the groups of HCV-related HCC patients with and without diabetes. The HbA1c levels were higher in the groups of diabetic patients with HBV- or HCV-related HCC.

The Kaplan-Meier curves for recurrence-free survival in the groups of HBV-related HCC patients with and without diabetes are shown in Figure 3. The recurrence-free survival rates 1, 3, and 5 yr after surgical treatment were 85.7%, 57.1%, and 42.9%, respectively, in the diabetic patient group, and 76.5%, 46.7%, and 40.0%, respectively, in the nondiabetic patient group, showing no significant differences between the two groups ($P = 0.596$, $P = 0.670$, and $P = 0.827$). In the analysis of overall survival in the groups of HBV-related HCC patients with and without diabetes by the method of Kaplan-Meier, it indicated that there was no difference between the two groups ($P = 0.505$) (Fig. 4).

Figure 5 shows the Kaplan-Meier curves for recurrence-free survival in the groups of HCV-related HCC patients with and without diabetes. The recurrence-free survival rates 1, 2, 3, 4, and 5 yr after surgical treatment were 75.0%, 38.9%, 22.2%, 11.1%, and 11.1%, respectively, in the diabetic patient group, and 94.6%, 83.9%, 62.1%, 35.0%, and 29.2%, respectively, in the nondiabetic patient group, indicating that the recurrence-free survival rates were significantly lower in the diabetic patient group than in the nondiabetic patient group ($P = 0.030$, $P < 0.001$, $P < 0.001$, $P < 0.001$, and $P < 0.001$).

In the analysis of overall survival in the groups of HCV-related HCC patients with and without diabetes, the overall survival rates 1, 2, 3, 4, and 5 yr after surgical treatment were 100%, 88.9%, 76.5%, 64.7%, and 43.8%, respectively, in the diabetic patient group, and 100%, 100%, 96.7%, 92.5%, and

Table 4. Characteristics of Patients With HBV- or HCV-Related HCC

	Patients With HBV-Related HCC			Patients With HCV-Related HCC		
Characteristic	With Diabetes (N = 8)	Without Diabetes (N = 17)	*P* Value	With Diabetes (N = 22)	Without Diabetes (N = 40)	*P* Value
Median age (yr)	61.6	57.3	0.3	62.2	62.9	0.737
Gender (male/female)	7/1	13/4	0.5	17/5	35/5	0.302
Body mass index (kg/m^2)	23.4	23.2	0.9	23.5	22.7	0.348
Alcohol abuse (+/−)	2/6	6/11	0.6	11/11	21/19	0.853
HbA1c (%)	5.9	4.6	0.07	6.6	4.9	<0.001
Fibrosis (F1/F2/F3/F4)	0/0/2/6	3/2/0/12	0.8	0/4/2/16	1/3/5/31	0.680
Child-Pugh grade (A/B)	6/2/0	15/2	0.4	18/4	35/5	0.551
Platelet count (× 10^4/μL)	11.5	13.9	0.3	12.4	13.5	0.520
ALT (IU/L)	31.8	42.3	0.6	84.2	63.5	0.233
Total bilirubin (mg/dL)	0.9	0.9	1.0	0.9	0.8	0.303
Albumin (g/dL)	3.8	4.2	0.02	4.2	4.2	0.983
AFP (ng/mL)	1,056	121	0.001	162	328	0.460
Tumor size (mm)	28.9	31.2	0.8	36.0	29.6	0.295
Diff.degree (W/M/P)	2/2/4	5/7/5	0.3	9/10/3	14/11/15	0.058
Vascular invasion (+/−)	1/7	4/13	0.5	12/10	15/25	0.548

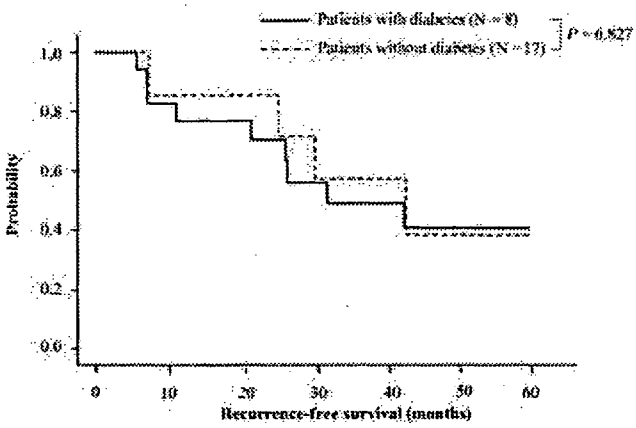

Figure 3. Kaplan-Meier curves for recurrence-free survival in HBV patients with diabetes and HBV patients without diabetes.

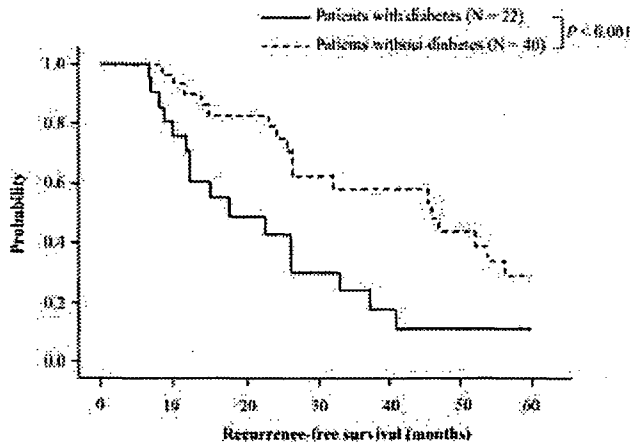

Figure 5. Kaplan-Meier curves for recurrence-free survival in HCV patients with diabetes and HCV patients without diabetes.

82.6%, respectively, in the nondiabetic patient group, indicating that the overall survival rates of more than 3 yr were significantly lower than in the nondiabetic patient group ($P = 1.000$, $P = 1.000$, $P = 0.035$, $P = 0.015$, $P = 0.004$) (Fig. 6).

Factors Associated With Recurrence-Free Survival After Surgical Treatment for HCC in Patients With Diabetes

Finally, we performed univariate and multivariate analyses to determine the variables that might affect the postoperative recurrence of HCC in the 30 HCC patients with diabetes, consisting of 17, 4, and 9 patients receiving insulin therapy, oral hypoglycemic drugs, and no treatment, respectively. Univariate analysis identified Child-Pugh classification B as a factor significantly contributing to the postoperative recurrence of HCC ($P < 0.001$) (Table 5). When we conducted multivariate analysis, we chose variables that had been already pointed out as a risk factor for HCC recurrence and whose P value was lower than 0.1 in univariate analysis. Multivariate analysis identified Child-Pugh classification B (risk 40.0, 95% CI 4.4–362.1, $P = 0.001$) and the presence of insulin therapy (risk 3.9, 95% CI 1.0–15.3, $P = 0.049$) as factors significantly contributing to the postoperative recurrence of HCC (Table 6). In multivariate analysis, both factors showed significant P value. Based on the results, we considered that both factors contribute to recurrence of HCC independently.

DISCUSSION

In the present study, univariate and multivariate analyses identified the presence of diabetes as a factor significantly contributing to the recurrence of HCC after surgical treatment. The results are consistent with the findings of Ikeda *et al.* (12). They analyzed a population of 64 HBV-related HCC patients and a larger population of 144 HCV-related HCC patients, but did not compare the postoperative recurrence rate between the two populations. In our study, 25 and 62 patients with HBV- and HCV-related HCC, respectively, were included, similar to the proportion of such patients in the study by Ikeda *et al.* (12), presumably leading to similar results. On the other hand, none of the variables that have been reported to contribute to the postoperative recurrence of HCC, such as liver fibrosis degree, Alb level, AFP level, tumor differentiation degree,

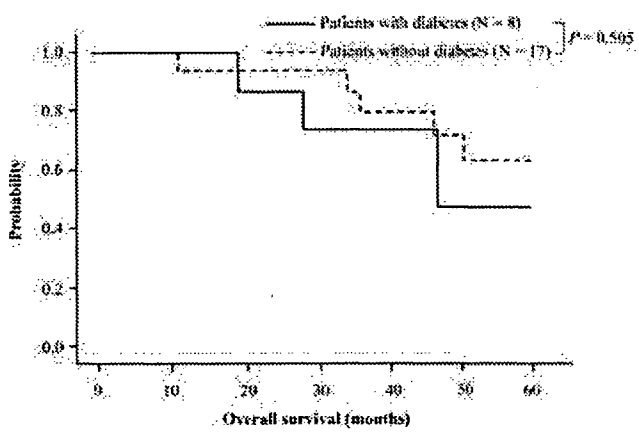

Figure 4. Kaplan-Meier curves for overall survival in HBV patients with diabetes and HBV patients without diabetes.

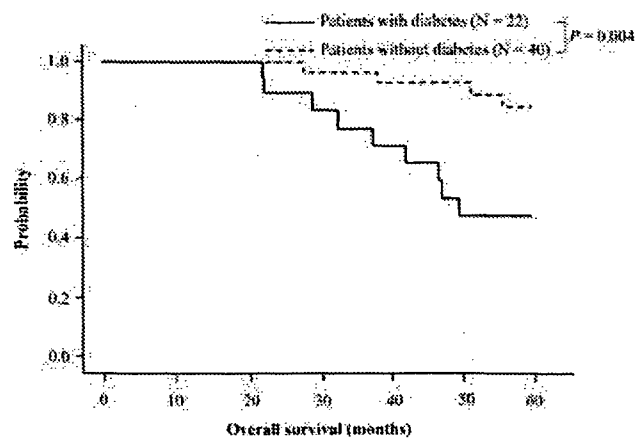

Figure 6. Kaplan-Meier curves for overall survival in HCV patients with diabetes and HCV patients without diabetes.

Table 5. Univariate Proportional Hazard Model for Recurrence of HCC After Surgical Treatment in Diabetic Patients

Variable	Hazard Ratio	95% CI	*P* Value
Age (yr)	1.0	1.0–1.1	0.534
Gender (male)	0.8	0.3–2.2	0.681
Etiology (HCV)	0.5	0.2–1.4	0.183
Body mass index (>25 kg/m^2)	1.0	0.4–2.6	0.938
Alcohol abuse (+)	1.0	0.4–2.3	0.972
HbA1c (%)	1.4	1.0–1.9	0.059
Fibrosis (F4)	1.2	0.5–3.3	0.651
Child-Pugh grade (A/B)	11.8	3.2–43.9	<0.001
Platelet count ($\times 10^4/\mu$L)	1.0	0.9-1.1	0.548
ALT(IU/L)	1.0	1.0–1.0	0.699
Total bilirubin (mg/dL)	0.6	0.2–2.5	0.506
Albumin (g/dL)	1.2	0.5–2.9	0.689
AFP (>200 ng/dL)	0.9	0.4–2.3	0.899
Tumor size (>50 mm)	0.8	0.7-1.2	0.668
Diff degree (W/M/P)	0.9	0.4–2.0	0.723
Vascular invasion	0.9	0.4–2.2	0.843
Insulin therapy	2.5	1.0–6.6	0.058

and the presence of vascular invasion, were identified as significant factors. This is probably because of our criteria for surgical treatment and that patients who recurred within 6 months after surgery were excluded from the study, resulting in the inclusion of only a population with little variation in these variables.

Next, groups of patients with HBV- and HCV-related HCC were separately examined for the impact of diabetes on the recurrence of HCC after surgical treatment. No significant differences in the recurrence-free survival rates determined by the Kaplan-Meier curve were noted between the HBV-related HCC patient groups with and without diabetes, which was similar to the results reported by Poon *et al.* (13), Toyoda *et al.* (14), and Huo *et al.* (20). In contrast, the recurrence-free survival rate was significantly lower in the group of HCV-related HCC patients with diabetes than in the group of HCV-related HCC patients without diabetes. From the above findings, we concluded that the coexistence of diabetes was a factor contributing to the recurrence of HCC after surgical treatment in HCV-related HCC patients, and that the results of analysis of all HCC patients reflected those in the HCV-related HCC patients. In addition, the results of the analysis for the prognosis of HCV-related HCC patients after surgical treatment showed that the overall survival rate was significantly lower in the diabetic patient group than in the nondiabetic group. These results suggest that more frequent recurrence may contribute to shorter survival in HCV-related HCC patients with diabetes.

Table 6. Multivariate Proportional Hazard Model for Recurrence of HCC After Surgical Treatment in Diabetic Patients

Variable	Hazard Ratio	95% CI	*P* Value
Insulin therapy (+)	3.9	1.0–15.3	0.049
Fibrosis (F4)	2.2	0.5–9.8	0.306
Child-Pugh grade (B)	40.0	4.4–362.1	0.001
AFP (>200 ng/mL)	2.1	0.5–8.8	0.289
Diff degree (P)	0.6	0.1–2.8	0.542
Vascular invasion (+)	1.7	0.4–7.6	0.513
Etiology (HCV)	2.0	0.3–12.2	0.460
HbA1c (%)	1.1	0.8–1.6	0.629

To our knowledge, only one study has examined the impact of diabetes on the recurrence of HCC after surgical treatment separately in HBV- and HCV-related HCC patients. Contrary to the results of this study, Huo *et al.* (20) have reported that diabetes is not a risk factor for the recurrence of HCV-related HCC. The clinical characteristics of HCC patients, such as the number of tumors, tumor diameter, and background liver histology, differed between their study and ours, and the presence or absence of vascular invasion and hepatic reserve indicated by Child-Pugh classification were unknown in their study, which makes direct comparison difficult, but partially accounts for the different results. Although, to date, no studies have reported that, as shown in this study, there is a possibility that diabetes differently affects the postoperative recurrence of HCC in the groups of patients with HBV- or HCV-related HCC.

This may be because of different mechanisms of carcinogenesis in the two groups (21). It appears that neither HBV nor HCV damages liver cells, but these viruses induce chronic inflammation in the liver, and facilitate mutations in liver cells, leading to their malignant transformation (22, 23). Our previous study using the microarray technique showed that the genes expressed in the liver differed markedly between HBV- and HCV-related liver disease patients (24). This genetic heterogeneity is considered to be associated with different modes of pathogenesis of HBV- and HCV-related HCC (25–28). Previous studies have shown that, in HCV-related HCC, chronic inflammation and oxidative stress are closely associated with hepatocellular death and regeneration (29–33). Highly insulin-resistant diabetics show increased peripheral lipolysis and hepatic accumulation of free fatty acids (34, 35). The β-oxidation of fatty acids in mitochondria is decreased in these patients, and they are under high oxidative stress. We also previously reported that the gene expression profile in the liver of diabetic patients shows increasing fibrogenic, angiogenic, tumorigenic, and stress responsive factors (36). Taken together, these observations suggest that the coexistence of diabetes promotes the progression of liver fibrosis and development of HCC in HCV-related liver disease (37). In contrast, in HBV-related liver disease, integration of the virus genome into the host DNA appears to induce HCC (38–40). Therefore, in such a mechanism of carcinogenesis, the coexistence of diabetes may have little synergistic effect.

Finally, we examined variables that might contribute to the recurrence of HCC after surgical treatment in HCC patients with diabetes. Since insulin therapy is often administered to diabetic patients who have difficulty in controlling blood sugar, or who have advanced liver disease, this factor may be involved in the recurrence of HCC in those under insulin therapy. Therefore, we included HbA1c, liver fibrosis degree,

and Child-Pugh classification together with insulin therapy in multivariate analysis. As a result, multivariate analysis identified Child-Pugh classification B and insulin therapy as significant factors contributing to the postoperative recurrence of HCC. These findings suggest that insulin therapy and Child-Pugh classification B are independent risk factors for postoperative recurrence.

The mechanism by which insulin promotes HCC recurrence is unknown. However, the results of this study are consistent with the report that insulin acts as a tumor growth factor *in vitro* (41). In animal models, insulin has been shown to be a promoter of colonic carcinogenesis (42). Although there has been much debate about the use of insulin and the risk of cancer development, no consensus has been reached (43–45). A recent study has indicated that insulin therapy is a risk factor for the postoperative recurrence of colorectal cancer (46). These findings show the possibility that insulin therapy promotes HCC recurrence after surgical treatment. It should be discussed how to use insulin therapy in HCC patients with diabetes in the future.

There is a limitation to our study, because our study is retrospective and on a not so large population. However, the results of the present study suggest that diabetes is a risk factor for the recurrence of HCV-related HCC and decreases the overall survival rates after surgical treatment. HCV-related HCC patients with diabetes should be closely followed for post-treatment recurrence, and blood sugar control may also be important to reduce the rate of recurrence. However, since the use of insulin to treat diabetes in HCC patients may promote tumor recurrence, treatment methods for blood sugar control require further evaluation.

STUDY HIGHLIGHTS

What Is Current Knowledge

- Diabetes accumulates liver fibrosis for chronic hepatitis C.
- Diabetes is a risk factor for hepatocellular carcinoma (HCC).
- HCC has high recurrence rate after curative surgery.

What Is New Here

- Hepatitis C virus (HCV) related patients with diabetes have a higher possibility of HCC recurrence.
- HCV-related patients with diabetes have poorer prognosis.
- Controlling of blood sugar may reduce HCC recurrence.
- Insulin therapy may accumulate HCC recurrence.

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CONFLICT OF INTEREST

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Infection of human hepatocyte chimeric mouse with genetically engineered hepatitis C virus and its susceptibility to interferon

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Abstract **We developed a reverse genetics system of hepatitis C virus (HCV) genotypes 1a and 2a using infectious clones and human hepatocyte chimeric mice. We inoculated cell culture-produced genotype 2a (JFH-1) HCV intravenously. We also injected genotype 1a CV-H77C clone RNA intrahepatically. Mice inoculated with HCV by both procedures developed measurable and transmissible viremia. Interferon (IFN) alpha treatment resulted in greater reduction of genotype 2a HCV levels than genotype 1a, as seen in clinical practice. Genetically engineered HCV infection system should be useful for analysis of the mechanisms of resistance of HCV to IFN and other drugs.**

Keywords: Human hepatocyte chimeric mouse; Human serum albumin; HCV RNA; Interferon

1. Introduction

The hepatitis C virus (HCV) infects an estimated 170 million people worldwide [1]. HCV causes persistent infection in adults leading to chronic hepatitis, liver cirrhosis, and hepatocellular carcinoma [2,3]. The most effective therapy for viral clearance is a 48-week combination therapy of pegylated interferon (IFN)-alpha and ribavirin. However, the success rate of this combination therapy is only about 50% [4]. Development of new anti-HCV drug had been severely restricted by the absence of a cell culture system that supports the efficient replication of HCV, as well as the lack of a small animal model. A cell culture system has been developed recently using a unique genotype 2a HCV gnome (JFH-1), which does not require adaptive mutations for efficient replication [5–7]. Chimpanzee was the only useful animal for the study of HCV until recently, although the availability of this model is severely restricted [8]. Recently, HCV-infected mice have been developed by inoculating HCV-infected human serum into chimeric urokinase-type plasminogen activator (uPA)-severe combined immunodeficiency (SCID) mice with engrafted human hepatocytes [9]. This HCV-infected mouse model has been reported to be useful for evaluating anti-HCV drugs such as IFN-alpha and anti-NS3 protease [10]. We have generated a human hepatocyte chimeric mouse where mouse hepatocytes were extensively replaced by human hepatocytes [11], and established a genetically engineered hepatitis B virus (HBV) system [12]. Using this mouse, we show in this paper the development of reverse genetics system of genotypes 1a and 2a after intrahepatic injection of transcribed RNA and intravenous injection of cell culture-produced virus, respectively. We also show here that HCV in these mice can be transmitted to naïve mice. Interferon treatment of these mice resulted in a greater reduction of HCV titer in genotype 2a clone infected mice than in genotype 1a infected mice. As these results are consistent with our clinical experience, we consider this model suitable for the study of resistance of HCV against IFN and other drugs.

2. Materials and methods

2.1. Generation of human hepatocyte chimeric mice and quantification of human serum albumin

Generation of the uPA$^{+/+}$/SCID$^{+/+}$ mice and transplantation of human hepatocytes were performed as described recently by our group [11,12]. All mice used in this study were transplanted with frozen

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Abbreviations: HBV, hepatitis B virus; HCV, hepatitis C virus; HSA, human serum albumin; IFN, interferon; SCID, severe combined immunodeficiency; uPA, urokinase-type plasminogen activator

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human hepatocytes obtained from one donor. Infection, extraction of serum samples, and sacrifice were performed under ether anesthesia. Mouse serum concentrations of human serum albumin (HSA) correlate with the repopulation index [11], and were measured as described previously [12]. The experimental protocol was approved by the Ethics Review Committee for Animal Experimentation of Graduate School of Biomedical Sciences, Hiroshima University.

2.2. HCV RNA transcription and inoculation into chimeric mice

A plasmid containing the full-length genotype 1a HCV cDNA clone, pCV-H77C, was kindly provided by Dr. Robert H. Purcell (National Institutes of Health). Ten micrograms of plasmid DNA, linearized by *Xba*I (Promega, Madison, WI) digestion, was transcribed in a 100-μl reaction volume with T7 RNA polymerase (Promega) at 37 °C for 2 h [13], and analyzed by agarose gel electrophoresis. Each transcription mixture was diluted with 400 μl of phosphate-buffered saline (PBS) and injected into the liver of chimeric mice. Transcripts of plasmid pJFH-1 containing the full-length HCV genotype 2a were transfected into Huh7 cells as described previously [6]. Seventy-two hours after transfection, 200 μl of the culture medium was injected intravenously into the chimeric mice. IFN-treatment was also performed by intramuscular injection of diluted IFN solutions. IFN-alpha was a kind gift from Hayashibara Biochemical Labs, Inc. (Okayama, Japan). Serum samples collected every 2 weeks after inoculation were frozen at −80 °C until further analysis.

2.3. Human serum samples

For control infection experiments, human serum containing a high titer of genotype 1b HCV (2.2×10^6 copies/ml) was obtained from a patient with chronic hepatitis after obtaining a written informed consent. The individual serum samples were divided into small aliquots and separately stored in liquid nitrogen until use.

2.4. RNA extraction and amplification

RNA was extracted from serum samples by Sepa Gene RV-R (Sankojunyaku, Tokyo), dissolved in 8.8 μl RNase-free H_2O, and reverse transcribed by using a random primer (Takara Bio, Inc., Shiga, Japan) and M-MLV reverse transcriptase (ReverTra Ace, TOYOBO Co., Osaka, Japan) in a 20 μl reaction mixture according to the instructions provided by the manufacturer. One microliter of cDNA solution was amplified by Light Cycler (Roche Diagnostic, Japan, Tokyo) for quantitation of HCV. The primers used for amplification were 5′-TTTATCCAAGAAAGGACCC-3′ and 5′-TTCACGCAGAAAGCGTCTAGC-3′. The amplification conditions included initial denaturation at 95 °C for 10 min, followed by 45 cycles of denaturation at 95 °C for 15 s, annealing at 55 °C for 5 s, and extension at 72 °C for 6 s. The lower detection limit of this assay is 10^3 copies/ml. Nested PCR was used with the outer primers NC1 (5′-CAACACTACTCGGCTAGCAGT-3′) and NC2 (5′-CCTGTGAGGAACTACTGTC-3′) and inner primers cc6 (5′-TTTATCCAAGAAAGGACCC-3′) and cc7 (5′-TTCACGCAGAAAGCGTCTAGCttc-3′). The amplification condition included 35 cycles of 94 °C for 30 s, 58 °C for 1 min 30 s, and 72 °C for 1 min after 5 min of initial denaturation at 94 °C followed by 7 min of final extension using Gene Taq (Wako Pure Chemicals, Tokyo) with anti-Taq high according to the instructions provided by the manufacturer (TOYOBO).

2.5. Histochemical analysis of mouse liver

Histopathological analysis and immunohistochemical staining using an antibody against HSA (Bethyl Laboratories Inc.) were performed as described previously [12].

3. Results

3.1. High serum HCV RNA titer in human hepatocyte chimeric mice after inoculation of serum samples obtained from HCV-infected patient

We inoculated 50 μl of genotype 1b serum samples into five chimeric mice intravenously to test their susceptibility to HCV infection. All mice became positive for HCV RNA by nested PCR at 2 weeks after inoculation (Fig. 1). The viremia reached a plateau level at 6–8 weeks after infection, and persisted for more than 12 weeks.

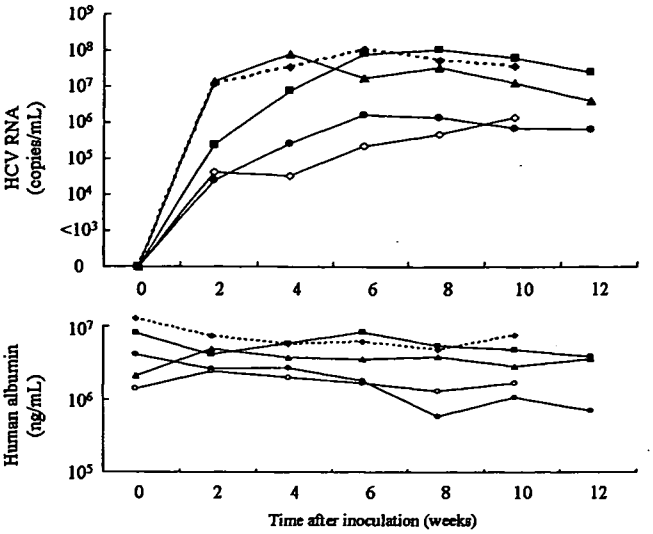

Fig. 1. Serial changes in HCV RNA and human serum albumin in sera of mice inoculated with human serum samples positive for genotype 1b HCV. Fifty microliter serum samples were injected intravenously into each mouse. Mice serum samples were obtained every 2 weeks after injection, and HCV RNA titer was analyzed.

3.2. Infection with in vitro-transcribed genotype 1a HCV RNA and cell culture generated genotype 2a HCV

In the next step, we tried to establish infection of cloned HCV using infectious genotype 1a and genotype 2a clones. In these experiments, we used two different strategies to establish infection using these two clones because genotype 1a has not been confirmed to replicate in cell culture system. We used genotype 1a HCV RNA (CV-H77C), which has been reported to be infectious to chimpanzee [13]. In vitro-transcribed HCV RNA was directly injected intrahepatically in three chimeric mice. We also infected three chimeric mice by intravenous injection of Huh7 cell-produced genotype 2a HCV after transfection of in vitro transcribed RNA from an infectious clone JFH-1. This clone has been shown to be infectious to a chimpanzee [6] and a chimeric mouse [7]. All mice developed measurable viremia 2 weeks after inoculation. At 6 weeks after inoculation, HCV RNA titer was 2.4×10^7 copies/ml (range: 8.8×10^6–2.9×10^7 copies/ml) in genotype 1a HCV-infected mice, and 2.5×10^5 copies/ml (range: 1.4×10^5–3.7×10^5 copies/ml) in genotype 2a HCV-infected mice (Fig. 2).

3.3. Passage experiment of HCV to naïve chimeric mice

We then performed passage experiments using naïve mice. Each of three mice was inoculated intravenously with 10 μl serum samples obtained from the above genotype 1a and genotype 2a HCV-infected mice at week 6. Two weeks after injection, all mice developed measurable viremia, and the titer was 8.5×10^6 copies/ml (range: 1.4×10^6–2.4×10^7 copies/ml) in genotype 1a, and 1.7×10^5 copies/ml (range: 1.5×10^5–2.5×10^5 copies/ml) in genotype 2a HCV-infected mice (Fig. 3).

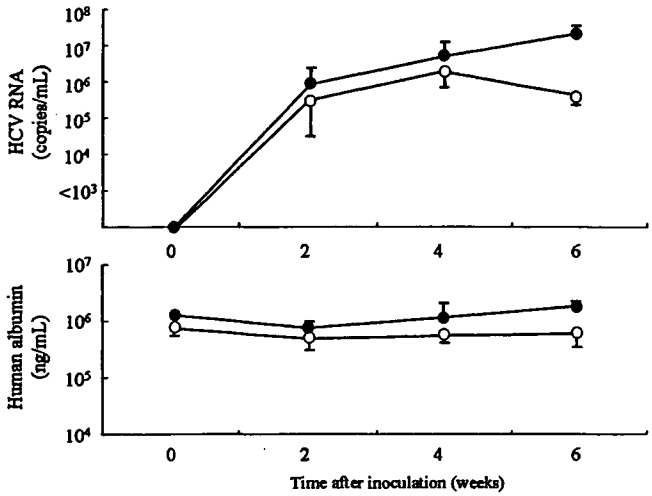

Fig. 2. Changes in HCV RNA and human albumin concentrations in serum of mice infected with clonal HCV. Each of three mice were inoculated intrahepatically with in vitro transcribed genotype 1a HCV RNA (closed circles) or intravenously with a culture medium collected from Huh7 cells transfected with JFH-1 genome intravenously (open circles). Data are mean ± S.D.

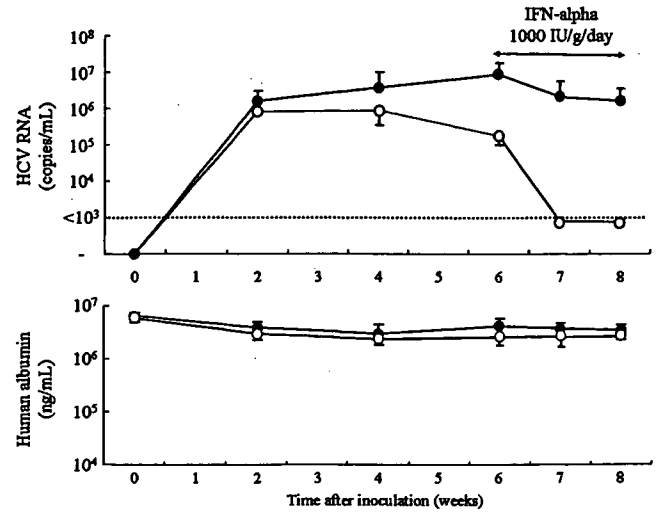

Fig. 3. Passage experiment and response to IFN-alpha therapy in mice infected with HCV genotypes 1a and 2a clones. Serum samples (10 μl) obtained from genotype 1a and 2a clonal HCV-infected mice sera (see Fig. 2) were inoculated intravenously into each of three naïve chimeric mice. Six weeks after infection, all six mice were injected intramuscularly with 1000 IU/g/day of IFN-alpha daily for 2 weeks. Closed circles: genotype 1a HCV-infected mice, open circles: genotype 2a HCV-infected mice. Data are mean ± S.D.

3.4. Variable susceptibility of HCV clones to IFN therapy

We treated each of the three mice infected with genotype 1a and 2a clones by passage experiments with 1000 IU/g of IFN-alpha daily for 2 weeks. Such treatment induced only a slight decrease in HCV in genotype 1a-infected mice; the viral load decreased only 0.6 and 0.7 log after 1 and 2 weeks of treatment, respectively (Fig. 3). In contrast, the same treatment reduced HCV genotype 2a RNA to undetectable levels after 1 and 2 weeks of IFN therapy. During IFN-treatment, serum HSA levels did not decrease in mice infected with genotype 1a or 2a HCV. Histopathological examination showed no morphological changes or apoptotic hepatocytes in replaced

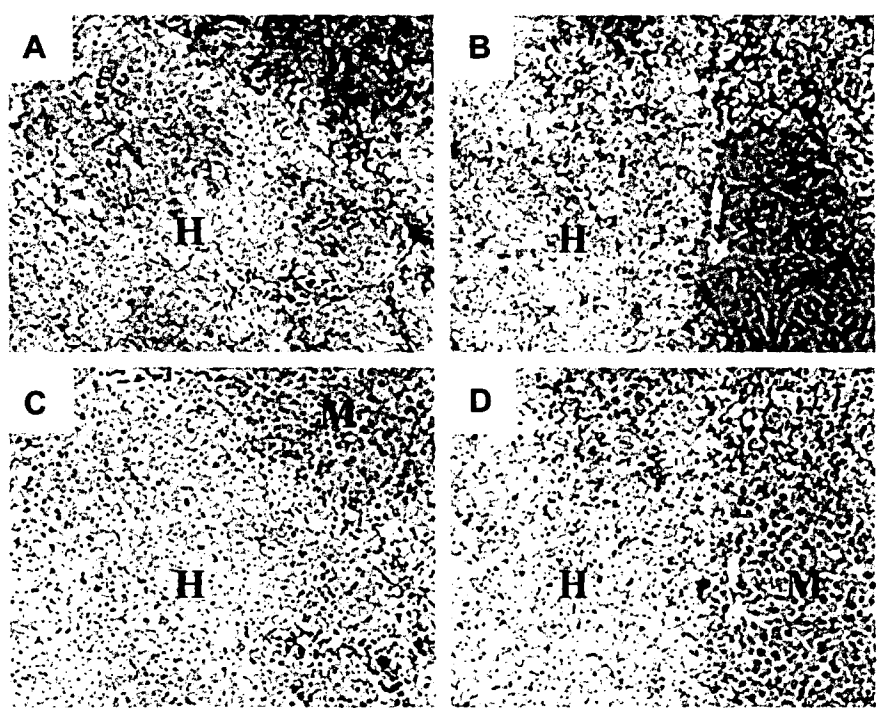

Fig. 4. Histochemical analysis of the tissues of infected chimeric mice. Liver samples obtained from mice infected with genotype 1a (A, C) and genotype 2a (B, D) stained with hematoxylin–eosin staining (A, B) or by immunohistochemical staining with anti-human serum albumin antibody (C, D). Regions are shown as human (H) and mouse (M) hepatocytes, respectively. (Original magnification, ×100.)