Interferon (IFN) treatment in combination with ribavirin administration, which is now the first choice for HCV mono-infected patients,[6] is also a standard treatment for chronic hepatitis in HIV–HCV co-infected patients. Eradication of HCV is assumed to improve liver function, and normalization of serum aminotransferase (ALT) levels by IFN treatment may retard the progression of liver disease in HIV–HCV co-infected patients, even if they are on HAART. However, in general, the response rate to IFN treatment is lower in HIV–HCV co-infected patients than in HCV mono-infected patients.[7] The effects of IFN treatment on liver function and prognosis in HIV–HCV co-infected patients in Japan are yet undefined.

In 2004, we conducted a nationwide survey to determine the prevalence of HCV infection in HIV-infected patients by distributing a questionnaire to the hospitals in the HIV/AIDS Network of Japan, which revealed that 935 (19.2%) of 4877 HIV-positive patients were also positive for anti-HCV antibody.[8] In this study, we analyzed the progression of liver diseases and the impact of IFN treatment on the parameters of liver function in HIV–HCV co-infected patients in a multicenter retrospective study.

METHODS

Registry of patients with HIV–HCV co-infection

THE QUESTIONNAIRE REGARDING the current state of HIV–HCV co-infection was sent to the 366 hospitals in the HIV/AIDS Network of Japan in 2004, sponsored by the Japanese Ministry of Health, Labour and Welfare. One hundred seventy-six hospitals (48.1%) responded. The results, already published,[8] showed that HIV–HCV co-infected patients are concentrated in particular hospitals in big cities around Japan. Among these hospitals, we chose three hospitals in the Tokyo metropolitan area, and one each in the Hokkaido, Chubu, Osaka, Chugoku and Kyushu areas. These eight hospitals belong to the HIV/AIDS Network and had more HIV–HCV co-infected patients than other hospitals.

In the study, the following information was obtained from the hospitals regarding each HIV–HCV co-infected patient who visited the hospitals at least once between January and December in 2004: (1) age and sex of HIV-positive patients with anti-HCV; (2) possible transmission routes of HIV; (3) history of habitual alcohol intake; (4) date of the first and last visits; (5) counts of white blood cells, CD4-positive lymphocytes and platelets at the first and last visits; (6) levels of serum albumin and bilirubin at the first and last visits; (7) levels of HIV-RNA and HCV-RNA at the first and last visits; (8) history of IFN treatment with or without ribavirin; (9) history of HAART; and (10) history of jaundice, ascites, hepatic encephalopathy and hepatocellular carcinoma (HCC). The study sheets were completed by the physicians in charge and sent to the Department of Internal Medicine, University of Tokyo.

Ethical issues

The protocol of the current survey was approved by the ethical committee of each institution, and written informed consent was obtained from each patient.

Statistical analysis

The collected data were analyzed using Mann–Whitney's *U*-test whenever appropriate. *P*-values less than 0.05 were regarded as statistically significant.

RESULTS

Clinical backgrounds of registered patients

FROM THE EIGHT hospitals, 297 patients were registered. The number, age, sex, estimated transmission routes and history of habitual alcohol intake are shown in Table 1. Two hundred and ninety (97.6%) were male patients. The mean age of the patients was 37.9 ± 10.3.

HCV genotype was determined in 212 patients. One hundred seventeen (55.2%) patients were infected by genotype 1 HCV. Infection by genotypes 2, 3 or 4 HCV was found in 29 (13.7%), 40 (18.9%) and 2 (0.9%) patients, respectively. Twenty-four (11.3%) patients were infected by HCV of mixed genotypes. In the remaining 85 patients, the genotype was indeterminable or undetermined. The mean ages of patients infected by different HCV genotypes were similar (Table 1).

In 259 (87.2%) of 297 registered patients, HIV was most probably transmitted through the administration of blood products. Other transmission routes were sexual contacts among men who have sex with men (MSM) (4.0%), heterosexual contacts (3.0%) and intravenous drug use (IDU) (0.3%). Habitual alcohol consumption was noted in only one patient with genotype 1 HCV (0.6%).

Outcomes of IFN treatment in HIV–HCV co-infected patients

Serum HCV-RNA levels were available both at the first visit and registry to the study (i.e. the end of observa-

Table 1 Demography, transmission route and HCV genotypes in HIV–HCV co-infected patients

HCV genotype	Number (%)	HCV sub-genotypes	Viral load† (High : Low)	Age	Sex (Male : Female)	Transmission route				
						Transfusion	MSM	Hetero-sexual	IDU	Others
1	117 (55 2)	1a 31, 1b 43, 1a+1b 31, undetermined 12	31:11	38 3 ± 10 4	114:3	102	7	1	0	7
2	29 (13 7)	2a 16, 2b 11, undetermined 2	5:5	39 8 ± 9 5	29:0	24	1	1	0	3
3	40 (18 9)	3a 40	12:2	36 1 ± 8 9	40:0	38	0	0	0	2
4	2 (0 9)	4a 2	2:0	38 5 ± 2 1	2:0	2	0	0	0	0
Mixed	24 (11 3)	2a+3a 6, 1b+3a 3, others 15	11:0	38 7 ± 8 7	24:0	24	0	0	0	0
Others	85	Undetermined 85	6:1	36 2 ± 11 5	81:4	69	4	7	1	4
Total	297		67:19	37 9 ± 10 3	290:7	259 (87 2%)	12 (4 0%)	9 (3 0%)	1 (0 3%)	16 (5 5%)

†Viral loads are available in only a subset of patients High viral load: more than 1 Meq/mL by branched DNA-probe assay or more than 100 KIU/mL by Amplicor monitor assay

HCV, hepatitis C virus; HIV, human immunodeficiency virus; IDU, intravenous drug users; MSM, men who have sex with men

tion) in 158 patients. Of these 158, 60 patients (38.0%) received IFN treatment for HCV, and 35 of these 60 patients did it in combination with ribavirin. Those who did not complete the scheduled treatment were excluded from the current analysis.

As shown in Table 2, 26 (43.3%), 11 (18.4%) and 23 (38.3%) of the treated patients achieved sustained virological response (SVR), end-of-treatment virological response (ETR) and no virological response (NR), respectively. The SVR rate in patients with each genotype is shown in Table 2. The SVR rate in the patients who underwent IFN treatment in combination with ribavirin was 31.4% in total. The SVR rate in patients with each genotype who underwent IFN/ribavirin combination therapy is shown in Table 2.

All of the 26 patients who achieved SVR remained negative for serum HCV-RNA in the further follow-up periods. In contrast, none of the patients with ETR or NR became negative for serum HCV-RNA in the follow-up periods. In five patients who did not receive IFN treatment, HCV-RNA was negative at the end of the observation period, although it was positive at least twice before the registry. The profiles of the five patients are shown in Table 3.

Changes in liver function and associated complications (Table 4)

As mentioned above, the data on liver function and serum HCV-RNA positivity were available both at the first visit and registry (end of observation) in 158 of the 297 registered patients. The mean observation period was 9.5 ± 5.0 and 8.2 ± 8.2 years in the IFN-treated and IFN-untreated patients, respectively. Unfortunately, few, if any, patients underwent liver biopsy, because most HIV–HCV co-infected patients had coagulation disorders.

The annual change in the serum albumin concentration was +0.05 ± 0.42 g/dL in the IFN-treated patients, and −0.80 ± 0.82 g/dL in the non-IFN-treated patients. The annual change in the serum bilirubin concentration was +0.08 ± 0.38 mg/dL in the IFN-treated patients, while it was +0.15 ± 0.15 mg/dL in the non-IFN-treated patients. Among the IFN-treated patients, the serum bilirubin concentration decreased by 0.02 ± 0.08 mg/dL in the patients who achieved SVR, which was significantly larger than that in the non-IFN-treated patients at the end of the observation ($P < 0.05$). The annual changes in platelet counts were +0.06 ± 1.13 ($\times 10^4/\mu l$) in the IFN-treated patients and −0.94 ± 0.95 ($\times 10^4/\mu l$) in the non-IFN-treated patients. The change in platelet

Table 2 Virological response to interferon treatment in HIV–HCV co-infected patients

Genotype	Viral load (High Low)†	Response			Total
		SVR	ETR	NR	
(a) Response to interferon treatment in total (with or without ribavirin)					
1	9:6	7 (33.3%)	1	13	21
2	5:3	4 (40.0%)	2	4	10
3	5:1	5 (62.5%)	1	2	8
4	1:0	0	1	0	1
Mixed	5:1	2 (33.3%)	3	1	6
Others	6:2	8 (57.1%)	3	3	14
Total	31.13	26 (43.4%)	11	23	60
(b) Response to ribavirin/interferon combination therapy including peginterferon					
1	8:2	2 (15.3%)	0	11	13
2	1:2	1 (25.0%)	0	3	4
3	4:1	4 (66.7%)	1	1	6
4	1:0	0	1	0	1
Mixed	4:1	1 (20.0%)	3	1	5
Others	3:0	3 (50.0%)	1	2	6
Total	21:6	11 (31.4%)	6	18	35

†Viral loads are available in only a subset of patients. High viral load: more than 1 Meq/mL by Branched DNA-probe assay or more than 100 KIU/mL by Amplicor monitor assay.
ETR, end of treatment virological response; NR, no virological response; SVR, sustained virological response.

counts in the patients who achieved SVR was significantly larger than that in the non-IFN-treated patients ($P < 0.05$, Table 4).

No symptoms of hepatic failure (ascites or hepatic encephalopathy) were observed in the 60 IFN-treated patients while they were observed in six of the 98 non-IFN-treated patients. HCC was found in one IFN-treated patient after SVR, while it was found in two non-IFN-treated patients (Table 4).

Impact of HAART on liver function and associated complications (Table 5)

Information on HAART was available in 292 patients. The mean observation periods were 8.4 ± 4.2 years in 234 patients on HAART, and 9.8 ± 6.0 years in 58 patients not on HAART. Changes in the levels of albumin, bilirubin or platelet were similar between the two groups (statistically not significant). The morbidities of hepatic decompensation symptoms (ascites and hepatic encephalopathy) and HCC were not significantly different between the two groups. In total, nine patients had hepatic decompensation and seven had HCC, and the average age of such patients was 41.1 ± 14.0 years, which was much younger than that of HCV mono-infected patients with the same complications.[9]

DISCUSSION

IN THE CURRENT study, the features of liver disease in HIV–HCV co-infected patients in Japan were analyzed. The determination of HCV genotypes revealed that genotype 3 or 4, which is rarely seen in HCV mono-infected patients in Japan,[10] was found in a substantial fraction of HIV-infected patients. In addition, some of these patients were infected with HCV of mixed genotypes. These results are compatible with the fact that HCV is transmitted through imported blood products that were contaminated by HCV, as is the case with HIV infection.[11] Infection by HCV of mixed genotypes may reflect frequent administrations of blood products of different lots.

We evaluated the response rate to IFN treatment in HIV–HCV co-infected patients in Japan. Because the IFN treatment protocol varied between facilities, it was not easy to evaluate the effects of the treatments including IFN in this cohort. However, the regimen of ribavirin/IFN combination therapy was similar between the hospitals: the treatment period was 24 weeks in patients with HCV genotypes 2 and 3, and 48 weeks in those with HCV of other genotypes when either pegylated or standard IFN in combination with ribavirin was used.[12] Therefore, it may be possible to estimate the effect

Table 3 Clinical backgrounds of patients who spontaneously cleared HCV in HIV-infected patients

Patient no	Age	Sex	Transmission route	Observation period (years)	HCV-RNA (KIU/mL)	HCV genotype	HIV-RNA ($\times 10^2$/mL)	WBC (/μL)	CD4+ T cells (/μL)	Platelets ($\times 10^4$/mL)	ALT (U/l)	HAART
1	33	M	Transfusion	8 8	290	ND	200 000	4500	5	26 3	21	Yes
2	31	M	MSM	2 3	Positive†	ND	13 000	5760	931	22 7	29	Yes
3	27	M	Transfusion	9 3	>850	3a	180 000	4000	51	10 1	84	Yes
4	53	M	Transfusion	4 5	Positive†	1a	20 000	4800	296	35 4	24	No
5	22	M	Transfusion	7 8	220	ND	990	5500	125	33 1	44	Yes

†Positive: HCV-RNA was positive by qualitative PCR, but was not quantitatively determined
ALT, aminotransferase; HAART, highly active anti-retroviral therapy; HCV, hepatitis C virus; HIV, human immunodeficiency virus; MSM, men who have sex with men; ND, not determined; WBC, white blood cells

Table 4 Changes in clinical parameters and IFN treatment in HIV–HCV co-infected patients

	Outcome of IFN treatment	Number	Observation period (years)	⊿Albumin†	⊿Bilirubin‡	⊿Platelet§	Ascites/ encephalopathy	HCC
IFN-treated patients		60	9 5 ± 5 0	0 05 ± 0 42	0 08 ± 0 38*	0 06 ± 1 13	0	1
	SVR	26	9 1 ± 4 4	0 13 ± 0 59	(−) 0 02 ± 0 08*	0 14 ± 0 76*	0	1
	ETR	11	14 6 ± 7 0	(−) 0 07 ± 0 14	0 51 ± 1 04	0 07 ± 1 50	0	0
	NR	23	7 4 ± 2 0	0 01 ± 0 30	0 09 ± 0 30	(−) 0 18 ± 0 32	0	0
Non-IFN-treated patients		98	8 2 ± 8 2	(−) 0 80 ± 0 82	0 15 ± 0 15	(−) 0 94 ± 0 95	6	2
All		158	8 7 ± 4 7	(−) 0 45 ± 2 93	0 13 ± 0 52	(−) 0 59 ± 3 78	6	3

*$P < 0\ 05$ versus patients without IFN treatment
†⊿Albumin: changes in albumin concentration (g/dL)/observation period (years)
‡⊿Bilirubin: changes in bilirubin concentration (mg/dL)/observation period (years)
§⊿Platelet: changes in platelet count ($\times 10^4$/μL)/observation period (years)
ETR, end of treatment virological response; HCC, hepatocellular carcinoma; HCV, hepatitis C virus; HIV, human immunodeficiency virus; IFN, interferon; NR, no virological response; SVR, sustained virological response

Table 5 Changes in clinical parameters and HAART in HIV–HCV co-infected patients

	Number	Age	Sex (M : F)	Observation period (years)	⊿Albumin†	⊿Bilirubin‡	⊿Platelet§	IFN	Ascites/ encephalopathy	HCC
HAART (+)	234	37 8 ± 10 4	227:7	8 4 ± 4 2	(−) 0 002 ± 0 18	0 13 ± 0 53	(−) 0.40 ± 3 71	143 (61 1%)	6	5
HAART (−)	58	38 1 ± 10 5	58:0	9 8 ± 6 0	(−) 0 14 ± 0 18	0 03 ± 0 25	(−) 1 40 ± 3 30	30 (51 7%)	3	2

†⊿Albumin: changes in albumin concentration (g/dL)/observation period (years)
‡⊿Bilirubin: changes in bilirubin concentration (mg/dL)/observation period (years)
§⊿Platelet: changes in platelet count ($\times 10^4$/μL)/observation period (years).
HAART, highly active anti-retroviral therapy; HCC, hepatocellular carcinoma; HCV, hepatitis C virus; HIV, human immunodeficiency virus

of ribavirin/IFN combination therapy in HIV–HCV co-infected patients in this study.

The response rate to ribavirin/IFN combination therapy was 31.4% in total, and 15.3% in patients with HCV genotype 1, which are comparable rates to those achieved in previous studies on HIV–HCV co-infected patients in Western countries.[7] The low response rate in HIV–HCV co-infected patients compared with HCV mono-infected patients[12] may be attributed to several factors: impaired immune response, high HCV loads and viral quasi-species caused by frequent chances of transmission. Of these, high viral loads may be essential, because Table 2 shows that patients with genotype 1 HCV achieved SVR even by IFN monotherapy if their viral loads were low. In the era of IFN monotherapy, patients with favorable conditions were treated first of all: pretreatment viral loads in patients who received IFN monotherapy were lower than those who received PEG-IFN–ribavirin combination therapy. This may be the reason why the efficacy of PEG-IFN–ribavirin combination therapy was lower than that with IFN monotherapy in this study.

The serum bilirubin concentrations and platelet counts were improved in the patients who achieved SVR by IFN treatment. Although the response rate to IFN treatment is lower in HIV–HCV co-infected patients than in HCV mono-infected patients, the overall benefit of IFN treatment on liver function may be similarly expected in the patients who achieved SVR. HAART showed no impact on the liver function in HIV–HCV co-infected patients. Improvement of liver function can be expected only in IFN-treated patients, although there is a possibility that only patients with preserved liver function were able to receive IFN treatment. Given that liver disease is the major life-threatening factor in HIV-infected patients, IFN treatment should be considered in the early stage of HIV–HCV co-infection.

It should be noted that nine patients had hepatic decompensation and seven had HCC, and the average age of such patients was much younger than that of HCV mono-infected patients with the same complications.[9] This finding is compatible with reports from Western countries showing a faster progression of fibrosis[13] and earlier development of HCC.[14] A possibly interesting finding is that five patients (approximately 3% of patients whose serum HCV-RNA level was serially determined) cleared HCV-RNA from the serum without IFN treatment. Previous reports showed that some HIV-infected patients could spontaneously clear HCV-RNA.[15–17] The clearance of HCV among patients with chronic HCV infection is rare, although it has been

reported in Japan.[18] Three of the five patients had high HCV loads and low $CD4^+$ T-lymphocyte counts, which are generally thought to be unfavorable for spontaneous HCV clearance. A difference in immune status of HIV-infected patients from HCV mono-infected patients may be involved in such an observation, although further studies are awaited.

In summary, our study demonstrated that approximately 20% of HIV-infected patients are co-infected with HCV Some of the HIV–HCV co-infected patients had advanced liver disease such as ascites, encephalopathy or HCC at a younger age than HCV mono-infected patients, suggesting that the progression of liver disease may be more rapid in HIV–HCV co-infected patients than in HCV-mono-infected ones. Treatments with regimens including IFN, which may improve liver function and decrease liver-related death, should be considered in HIV–HCV co-infected patients.

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Association between Changes in Obesity Parameters and Incidence of Chronic Kidney Disease in Japanese Individuals

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Key Words
Chronic kidney disease · Body mass index · Waist circumference · Health screening

Abstract
Obesity increases the risk for chronic kidney disease (CKD). By analyzing data on individuals who underwent general health screening in two consecutive years, we investigated whether changes in body mass index (BMI) or waist circumference (WC) were associated with the appearance or disappearance of the CKD components; micro-/macroalbuminuria (≥30 mg urinary albumin per gram creatinine) and a low estimated glomerular filtration rate (eGFR; <60 ml/min/1.73 m^2). Logistic regression analysis showed that in men with micro-/macroalbuminuria at the first visit, a BMI reduction of ≥0.42 or a WC reduction of ≥3.0 cm over the 1-year period resulted in a significantly reduced incident of micro-/macroalbuminuria at the second visit. On the other hand, a BMI gain of ≥0.33 over 1 year in men without micro-/macroalbuminuria and a low eGFR at the fist visit significantly increased the incident of micro-/macroalbuminuria and a low eGFR, respectively, at the second visit. These findings indicate that lowering the obesity indexes in men with micro-/macroalbuminuria reduced the incidence of this condition at the 1-year follow-up and that, on the contrary, an increase in BMI in men without micro-/macroalbuminuria and a low eGFR at the first examination increased the risk of these conditions during the 1-year follow-up period.

Introduction

Chronic kidney disease (CKD), now recognized as a potential risk factor for cardiovascular disease as well as for end-stage renal disease [1], is a worldwide public health problem [2]. Several cross-sectional and longitudinal epidemiological studies showed that obesity may increase the prevalence and incidence of CKD [3–8] and end-stage renal disease [9], although there might be differences according to gender and ethnicity [9–11]. However, fewer studies have investigated whether changes in obesity indexes, such as body weight, body mass index (BMI), and waist circumference (WC), are associated with changes in CKD status [12–14]. In the current study, we retrospectively analyzed data on individuals who underwent general health screening at our institute for 2 consecutive years and investigated whether changes in obesity indexes were associated with changes in CKD status in these Japanese individuals.

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Subjects and Methods

Study Population

The study was approved by The Ethical Committee of Mitsui Memorial Hospital. At our institution, 3,312 (1,203 women, 2,109 men) individuals underwent general health screening including that on urinary albumin excretion between October 2005 and October 2006 (first visit) and also in the subsequent year (second visit). Among the 3,312 individuals, data on 2,861 (1,114 women, 1,747 men) who reported not taking anti-hypertensive drugs at both visits were used for the present study. The mean ± SD of the interval between the two visits of the enrolled individuals by the study subjects was 355 ± 52 days. Individuals who were taking antihypertensive medications were excluded from the analysis because certain depressor drugs may affect renal function and the extent of proteinuria [15, 16] and because the database did not include information on the class of drugs used. At the time of the health examination, recommendations may have given to overweight or obese subjects to reduce body weight. However, in analyzing data for this study, there was no intention to examine which strategies for weight control, if any, would have an impact on the status of CKD during the follow-up.

In Japan, regular health check-ups for employees are legally mandated; thus, the majority of these subjects did not have serious health problems. In addition, all or most of the costs of the screening are usually paid by the company to which they belong or by each subject. In addition, there are several courses in the heath screening program; however, which to choose is up to each individual, but not to physicians or company one belongs to. Therefore, the study population is not considered to be enriched for certain diseased condition.

Laboratory Analysis

Blood samples were taken from the subjects after an overnight fast. Serum levels of total cholesterol (TC), HDL-cholesterol (HDL-C), and triglycerides (TG) were determined enzymatically. Serum uric acid was measured by the uricase-peroxidase method, hemoglobin A_{1C} was determined using the latex agglutination immunoassay, and creatinine was determined by the enzymatic method. Plasma glucose was measured by the hexokinase method and serum insulin was measured by enzyme immunoassay.

Creatinine and urine albumin were measured by TBA-200FR (Toshiba Medical Systems, Tochigi, Japan) and by Accute (Toshiba Medical Systems), respectively, using commercially available kits, Accuras Auto CRE (Shino-test, Tokyo, Japan) and IATRO U-ALB by turbidimetric immunoassay (Mitsubishi Kagaku Iatron, Tokyo, Japan), respectively. Serum creatinine was calibrated using the following formula: serum creatinine (Jaffe method) = 0.2 + serum creatinine (enzyme method). Glomerular filtration rate (GFR) was estimated by equations of the simplified version of Modification of Diet in Renal Disease (MDRD) [17], where 0.881 is a coefficient for eGFR specific to the Japanese population [18]: estimated GFR (eGFR) for Japanese = 186.3 × (serum creatinine)$^{-1.154}$ × (age)$^{-0.203}$ × 0.881 × 0.742 (for females). eGFR values <60 ml/min/1.73 m^2 were classified as low [19]. For the diagnosis of micro-/macroalbuminuria, spot urine samples were collected and analyzed; micro-/macroalbuminuria was defined to be present when the urinary albumin excretion ratio (UAER), expressed as milligrams per gram creatinine, was ≥30 mg/g. Normoalbuminuria, microalbuminuria, and macroalbuminuria were defined as a UAER of <30, 30–299, and ≥300 mg/g, respectively. Micro-/macroalbuminuria and a low eGFR were considered to be the components of CKD [19]. The difference in BMI and WC between the two visits was designated as ΔBMI and ΔWC, respectively.

Statistical Analysis

Skewed variables (TG, UAER) are presented as median values (interquartile range). Other data are expressed as the mean ± SD unless stated otherwise. Analyses of variance, the Mann-Whitney U test, χ^2 tests, and logistic regression analysis were conducted as appropriate to assess the statistical significance of differences between groups using computer software, Dr. SPSS II (Chicago, Ill., USA). A value of $p < 0.05$ was taken to be statistically significant.

Results

Baseline Characteristics

The mean ± SD age of the individuals enrolled was 52.0 ± 10.1 years at the first visit (table 1). Of the 88 females and 149 males with micro-/macroalbuminuria, 83 and 134, respectively, had microalbuminuria and 5 and 15, respectively, had macroalbuminuria.

Changes in BMI and WC Values between the Two Visits

The mean BMI at the second visit was slightly lower than that at the first visit ($p < 0.001$, by paired t test) in men, but did not differ significantly in women. The mean WC at the second visit was slightly larger than that at the first visit ($p < 0.001$, by paired t test) in women and smaller ($p < 0.001$, by paired t test) in men. In this study, we calculated quartiles of WC or BMI by taking into the entire population. Ranges for each quartile for ΔBMI and ΔWC are shown in table 2. About half of the subjects had a decreased WC value at the second visit. The correlation coefficient between the first-visit BMI and ΔBMI was −0.09 ($p = 0.010$) in women and −0.09 ($p = 0.002$) in men, and that between the first-visit WC and ΔWC was −0.31 in women ($p < 0.001$) and −0.28 ($p < 0.001$) in men.

Changes in the Prevalence of Micro-/Macroalbuminuria and a Low eGFR between the Two Visits

Figure 1 shows the number of subjects with micro-/macroalbuminuria and a low eGFR at the first and second visits. Of those with micro-/macroalbuminuria at the first visit, 34% did not have micro-/macroalbuminuria at the second visit, but 4% of subjects who did not have micro-/macroalbuminuria at the first visit had de-

Table 1. Clinical characteristics and laboratory data

Variables	Women (n = 1,114)		Men (n = 1,747)	
	Visit 1	Visit 2	Visit 1	Visit 2
Age, years	51.3 ± 9.9	52.3 ± 9.9	52.5 ± 10.1	53.4 ± 10.1
Height, cm	157.1 ± 5.7	157.1 ± 7.8	169.7 ± 5.9	169.7 ± 5.9
Weight, kg	52.3 ± 7.7	52.3 ± 7.8	67.8 ± 9.2	67.6 ± 9.3
BMI	21.2 ± 2.9	21.2 ± 2.9	23.5 ± 2.7	23.5 ± 2.8
ΔBMI	–	0.0 ± 0.7	–	–0.1 ± 0.7
WC, cm	76.2 ± 8.6	76.9 ± 8.9	85.3 ± 7.5	85.0 ± 7.4
ΔWC, cm	–	0.7 ± 6.0	–	–0.3 ± 3.8
Systolic BP, mm Hg	116 ± 18	115 ± 18	124 ± 17	124 ± 18
Diastolic BP, mm Hg	72 ± 11	72 ± 11	79 ± 11	79 ± 11
Total cholesterol, mg/dl	217 ± 36	215 ± 34	210 ± 32	207 ± 31
LDL-cholesterol, mg/dl	130 ± 30	127 ± 30	128 ± 32	126 ± 31
HDL-cholesterol, mg/dl	69 ± 14	68 ± 14	56 ± 14	56 ± 13
TG, mg/dl	84 ± 46	84 ± 42	127 ± 80	126 ± 101
TG, median (interquartile range)	74 (55–99)	74 (54–101)	107 (77–152)	102 (74–144)
Uric acid, mg/dl	4.5 ± 0.9	4.5 ± 0.9	6.1 ± 1.2	6.0 ± 1.2
Fasting glucose, mg/dl	90 ± 17	91 ± 14	98 ± 21	99 ± 20
Hemoglobin A_{1C}, %	5.2 ± 0.6	5.2 ± 0.5	5.4 ± 0.8	5.5 ± 0.7
Antidiabetic medication, n (%)	5 (0.4)	9 (0.8)	46 (2.6)	58 (3.3)
Blood urea nitrogen, mg/dl	13.4 ± 3.2	13.6 ± 3.3	14.4 ± 3.5	14.5 ± 3.5
Serum creatinine, mg/dl	0.63 ± 0.09	0.62 ± 0.09	0.86 ± 0.28	0.84 ± 0.30
UAER, median (interquartile range)	7.5 (5.1–12.2)	7.9 (5.4–13.1)	5.2 (3.7–10.0)	5.6 (3.9–10.7)
UAER ≥30 mg/g Cr, n (%)	88 (7.9)	91 (8.2)	149 (8.5)	165 (9.4)
eGFR, ml/min/1.73 m^2	69.5 ± 9.3	70.1 ± 9.2	70.9 ± 10.0	71.8 ± 10.2
Low eGFR, n (%)	155 (13.9)	138 (12.4)	212 (13.1)	201 (11.5)
Current smoker	99 (8.9)	93 (8.3)	581 (33.3)	542 (31.0)

Data are means ± SD, median (interquartile range), n, or percentage. BMI = Body mass index; WC = waist circumference; BP = blood pressure; TG = triglycerides; UAER = urinary albumin excretion rate; eGFR = estimated glomerular filtration rate.

Table 2. Range for each quartile of ΔBMI and ΔWC

	Q1	Q2	Q3	Q4
ΔBMI	–5.33/–0.42 (–0.75)	–0.41/–0.04 (–0.21)	–0.04/0.32 (0.13)	0.33/3.67 (0.62)
ΔWC, cm	–21.0/–3.0 (–5.0)	–2.9/–0.1 (–1.5)	0.0/2.7 (1.0)	2.8/23.0 (5.0)

BMI = Body mass index; WC = waist circumference. Medians are given in parentheses.

veloped micro-/macroalbuminuria at the second visit (fig. 1a, b). Of individuals who had a low eGFR at the first visit, 28% did not have a low eGFR at the second visit, but alternatively, 3% of individuals who did not have a low eGFR at the first visit had a low eGFR at the second visit.

Association between Changes in BMI or WC and Albuminuric Status

Next, we investigated whether decreases in BMI and WC values were associated with changes in CKD status (fig. 2a, b). Logistic regression analysis adjusted for age, systolic blood pressure, HDL- and LDL-cholesterol, fast-

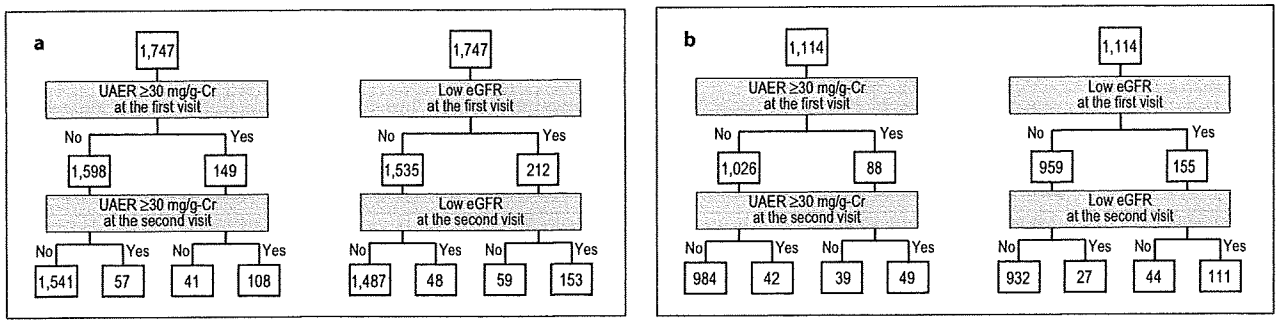

Fig. 1. Flow chart showing the number of men without micro-/macroalbuminuria or a low eGFR at the times of visit 1 and visit 2. **a** Men. **b** Women.

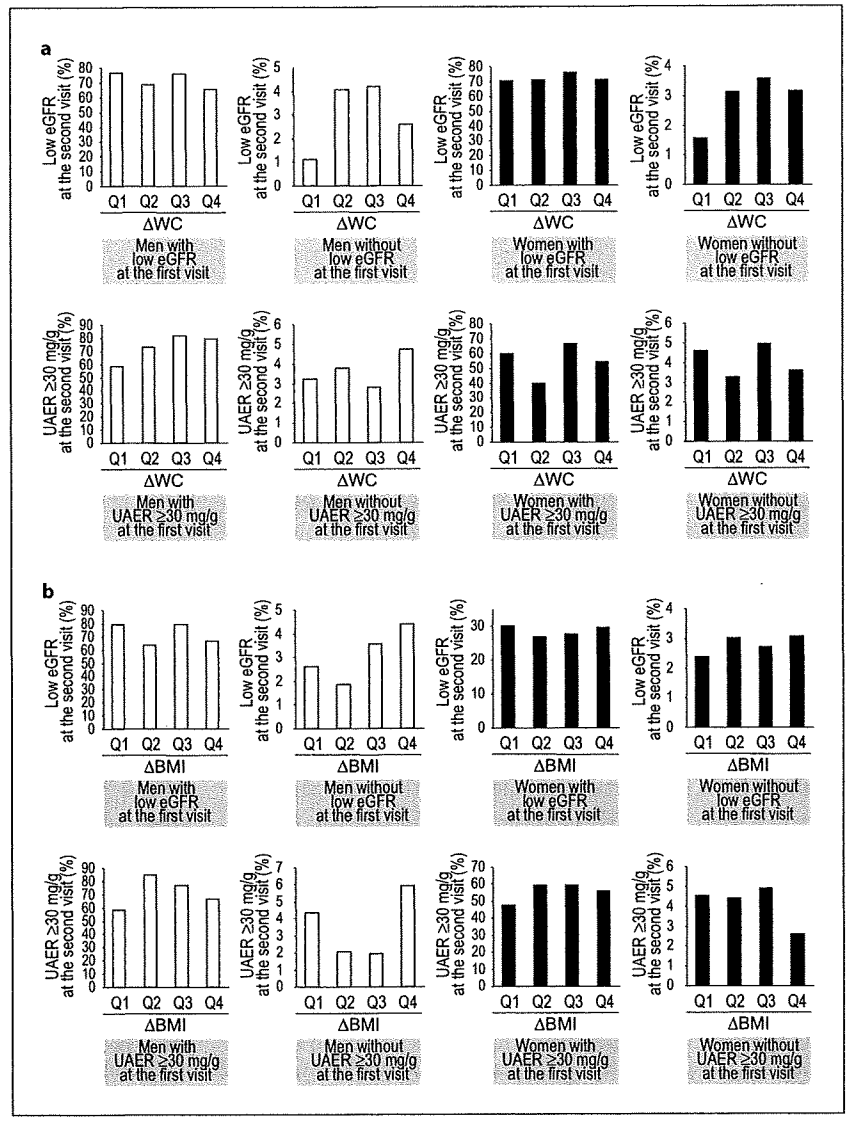

Fig. 2. Prevalence of a low eGFR and elevated levels of albuminuria at visit 2 in subjects with and without a low eGFR or micro-/macroalbuminuria at visit 1 according to quartiles of the difference in waist circumference between visit 1 and visit 2 (ΔWC) (**a**) and the difference in body mass index between the visit 1 and visit 2 (ΔBMI) (**b**).

Table 3. Logistic regression analysis with the lowest Δwaist circumference or Δbody mass index quartile as an independent variable and micro-/macroalbuminuria at the second visit as a dependent variable in individuals with micro-/macroalbuminuria at the first visit

Variables	Age adjusted		Multivariate adjusted*	
	OR (95% CI)	p value	OR (95% CI)	p value
Male (n = 149)				
ΔWC-Q2, Q3, Q4	1.00	-	1.00	-
ΔWC-Q1	0.36 (0.16–0.80)	0.012	0.31 (0.13–0.73)	0.007
Female (n = 88)				
ΔWC-Q2, Q3, Q4	1.00	-	1.00	-
ΔWC-Q1	1.20 (0.41–3.53)	0.735	1.01 (0.33–3.14)	0.987
Male (n = 149)				
ΔBMI-Q2, Q3, Q4	1.00	-	1.00	-
ΔBMI-Q1	0.37 (0.16–0.84)	0.018	0.36 (0.15–0.84)	0.018
Female (n = 88)				
ΔBMI-Q2, Q3, Q4	1.00	-	1.00	-
ΔBMI-Q1	0.51 (0.17–1.54)	0.232	0.52 (0.16–1.74)	0.289

BMI = Body mass index; WC = waist circumference.
* Multivariate adjusted: Adjusted for age, systolic blood pressure, HDL-cholesterol, LDL-cholesterol, fasting plasma glucose, and smoking status.

ing plasma glucose, and smoking status showed that, compared with the higher three ΔBMI quartiles, the lowest ΔBMI quartile (≥0.42 reduction) was associated with a significantly lower risk for micro-/macroalbuminuria at the second visit in men who had micro-/macroalbuminuria at the first visit (table 3). Similarly, compared with the higher three ΔWC quartiles, the lowest ΔWC quartile (≥3.0 cm reduction) was associated with significantly lower risk for micro-/macroalbuminuria at the second visit in men who had micro-/macroalbuminuria at the first visit. In contrast, in women, who had micro-/macroalbuminuria at the first visit, neither a ≥0.42 reduction in BMI nor a ≥3.0-cm reduction in WC significantly reduced the prevalence of micro-/macroalbuminuria at the second visit. Compared with the lower three ΔBMI quartiles, the highest ΔBMI quartile (≥0.33 gain) was associated with a significantly higher risk for micro-/macroalbuminuria at the second visit in men who did not have micro-/macroalbuminuria at the first visit (table 4).

Association between Changes in BMI or WC and a Low eGFR Status

Compared with the lower three quartiles, the highest ΔBMI quartile (≥0.33 gain) was associated with a significantly higher risk for a low eGFR at the second visit in men who did not have a low eGFR at the first visit (tables 5–6). The lowest quartile of either ΔBMI or ΔWC was not associated with reduced risk for a low eGFR at the second visit in those who had a low eGFR at the first visit in either gender.

Discussion

In the current study, we demonstrated that a WC reduction of ≥2.8 cm or a BMI reduction of ≥0.42 over a period of one year in men with micro-/macroalbuminuria at the first visit significantly reduced the risk for micro-/macroalbuminuria at the second visit (OR 0.31, 95% CI 0.13–0.73 and OR 0.36, 95% CI 0.15–0.84, respectively), after multivariate adjustment. On the other hand, a BMI gain of ≥0.33 over one year in men without micro-/macroalbuminuria or a low eGFR at the first visit significantly increased the risk at these conditions at the second visit (OR 2.50, 95% CI 1.44–4.37 and OR 1.94, 95% CI 1.04–3.61, respectively). Neither of these associations reached statistical significance in women. These data collectively suggest that the albuminuric status may be altered when men with micro-/macroalbuminuria have a substantial decrease in WC or BMI, and, in reverse, the

Table 4. Logistic regression analysis with the highest Δwaist circumference or Δbody mass index quartile as an independent variable and micro-/macroalbuminuria at the second visit as a dependent variable in individuals without micro-/macroalbuminuria at the first visit

Variables	Age adjusted		Multivariate adjusted*	
	OR (95% CI)	p value	OR (95% CI)	p value
Male (n = 1,598)				
ΔWC-Q1, Q2, Q3	1.00	–	1.00	–
ΔWC-Q4	1.52 (0.83–2.78)	0.177	1.62 (0.88–2.99)	0.120
Female (n = 1,026)				
ΔWC-Q1, Q2, Q3	1.00	–	1.00	–
ΔWC-Q4	0.87 (0.44–1.69)	0.674	0.87 (0.44–1.70)	0.677
Male (n = 1,598)				
ΔBMI-Q1, Q2, Q3	1.00	–	1.00	–
ΔBMI-Q4	2.41 (1.39–4.19)	0.002	2.50 (1.44–4.37)	0.001
Female (n = 1,026)				
ΔBMI-Q1, Q2, Q3	1.00	–	1.00	–
ΔBMI-Q4	0.57 (0.25–1.31)	0.185	0.60 (0.26–1.37)	0.221

BMI = Body mass index; WC = waist circumference.
* Multivariate adjusted: Adjusted for age, systolic blood pressure, HDL-cholesterol, LDL-cholesterol, fasting plasma glucose, and smoking status.

Table 5. Logistic regression analysis with the lowest Δwaist circumference or Δbody mass index quartile as an independent variable and a low eGFR at the second visit as a dependent variable in individuals with a low eGFR at the first visit

Variables	Age adjusted		Multivariate adjusted*	
	OR (95% CI)	p value	OR (95% CI)	p value
Male (n = 212)				
ΔWC-Q2, Q3, Q4	1.00	–	1.00	–
ΔWC-Q1	1.39 (0.68–2.88)	0.369	1.33 (0.63–2.80)	0.454
Female (n = 155)				
ΔWC-Q2, Q3, Q4	1.00	–	1.00	–
ΔWC-Q1	0.84 (0.38–1.85)	0.664	0.90 (0.39–2.08)	0.808
Male (n = 212)				
ΔBMI-Q2, Q3, Q4	1.00	–	1.00	–
ΔBMI-Q1	1.60 (0.80–3.23)	0.185	1.49 (0.73–3.04)	0.276
Female (n = 155)				
ΔBMI-Q2, Q3, Q4	1.00	–	1.00	–
ΔBMI-Q1	0.73 (0.30–1.81)	0.500	0.91 (0.34–2.38)	0.833

BMI = Body mass index; WC = waist circumference.
* Multivariate adjusted: Adjusted for age, systolic blood pressure, HDL-cholesterol, LDL-cholesterol, fasting plasma glucose, and smoking status.

Table 6. Logistic regression analysis with the highest Δwaist circumference or Δbody mass index quartile as an independent variable and a low eGFR at the second visit as a dependent variable in individuals without a low eGFR at the first visit

Variables	Age adjusted		Multivariate adjusted*	
	OR (95% CI)	p value	OR (95% CI)	p value
Male (n = 1,535)				
ΔWC-Q1, Q2, Q3	1.00	-	1.00	-
ΔWC-Q4	0.84 (0.39–1.83)	0.667	0.88 (0.40–1.91)	0.737
Female (n = 959)				
ΔWC-Q1, Q2, Q3	1.00	-	1.00	-
ΔWC-Q4	1.30 (0.60–2.86)	0.508	1.37 (0.62–3.03)	0.432
Male (n = 1,535)				
ΔBMI-Q1, Q2, Q3	1.00	-	1.00	-
ΔBMI-Q4	1.98 (1.07–3.66)	0.030	1.94 (1.04–3.61)	0.037
Female (n = 959)				
ΔBMI-Q1, Q2, Q3	1.00	-	1.00	-
ΔBMI-Q4	1.22 (0.53–2.83)	0.644	1.23 (0.53–2.89)	0.631

BMI = Body mass index; WC = waist circumference.
* Multivariate adjusted: Adjusted for age, systolic blood pressure, HDL-cholesterol, LDL-cholesterol, fasting plasma glucose, and smoking status.

status of albuminuria or a low eGFR may be altered when men without micro-/macroalbuminuria or a low eGFR, respectively, gain BMI substantially, although such a relationship was not apparent in female subjects. Future studies should be directed toward elucidating whether these observed gender differences were, in part, due to the greater prevalence of other risk factors, such as increased blood pressure, elevated fasting glucose levels, and reduced insulin sensitivity [20, 21], in men than in women.

Several studies have investigated the possible association between the obesity index and CKD. A high BMI has been reported to be associated with CKD [6, 10, 11]. Chou et al. [22] reported that in elder Taiwanese, the waist-hip ratio, body weight and WC, but not BMI, were predictors of a low eGFR, and that among these predictors, the waist-hip ratio may be the best anthropometric index for predicting a low eGFR. Foster et al. [23] showed that the association between obesity with an increased risk of developing stage 3 CKD was not independent, but was confounded by other cardiovascular disease risk factors. These findings suggest that the mode of association between certain obesity index and CKD might differ according to the study design and population studied.

Whether changes in obesity parameters would result in changes in CKD status has also been investigated in several previous studies. Changes in body weight were found to be associated with parallel changes in albuminuria in 6,894 participants of the Prevention of Renal and Vascular End-Stage Disease (PREVEND) study during a 4.2-year follow-up period [12]. In addition, moderate weight loss induced by a hypocaloric and normoproteinic diet in overweight patients with chronic proteinuria resulted in a significant decrease in proteinuria [13]. Furthermore, weight loss induced by an inhibitor of gastrointestinal lipase was associated with the reduction of urinary albumin excretion [14]. Therefore, most, if not all, studies showed that body weight reduction in overweight subjects resulted in a reduction of proteinuria, which was in agreement with the observation in the current study. Compared to the association between changes in obesity parameters and proteinuria, fewer numbers of studies have analyzed the relationship between change in body weight and change in eGFR. In the above-mentioned analysis in the PREVEND study, weight loss or gain did not significantly bring about a change in GFR [12]. Other studies showed that GFR was decreased after weight loss in extremely obese patients, presumably by the mechanisms of amelioration of obesity-associated hyperfiltration [24, 25]. In the current study, BMI gain of ≥ 0.33 was associated with a significantly higher risk for a low eGFR

at the second visit in men, but not in women, who were free from a low eGFR at the first visit. Taking all these results together, it is suggested that the relationship between weight loss and GFR change may also differ according to the target population. Interestingly, high BMI is known to be associated with better survival in dialysis patients [26] designated as a risk factor paradox [27].

The current study has several limitations. First, we retrospectively analyzed data on individuals who underwent general health screening at our institute in two consecutive years; therefore, individuals who did not visit our institute the following year for unknown reasons were not enrolled in the current study, which may cause some biases. Second, we excluded subjects those who were taking anti-hypertensive agents during either visit. This may have excluded from the study population some hypertensive subjects with proteinuria. Whether or not a body weight change results in a change in CKD status in such hypertensive individuals is nonetheless an important question. However, we do not have data on which class of anti-hypertensive agents had been used, which might affect the development, amelioration or elimination of CKD. Third, we used the MDRD equation for the estimation of GFR, which may result in a certain degree of inaccuracy. In addition, changes in weight will be affected not only by the changes in fat mass, but also those in muscle mass, and eGFR determined by MDRD formula is also highly dependent on muscle mass, as this formula takes only serum creatinine into account. We have to be careful in interpreting the results of the current study, as changes in muscle mass will lead to bias when assessing the association between obesity parameters and eGFR. Fourth, our findings may not be immediately applicable to non-Japanese populations, as the GFR estimated using serum creatinine is again more than slightly affected by muscle mass.

In conclusion, a BMI reduction of $\geq$0.42 or a WC reduction of $\geq$3.0 cm over a 1-year period in men with micro-/macroalbuminuria at the first visit significantly reduced the risk for micro-/macroalbuminuria at the second visit, and a BMI gain of $\geq$0.33 over a period of a year in men without micro-/macroalbuminuria or a low eGFR at the first visit significantly increased the risk for micro-/macroalbuminuria or a low eGFR during the 1-year follow-up. Such associations were not statistically significant in female subjects. Our data indicated that reducing body weight in overweight/obese men with micro-/macroalbuminuria and that maintaining an ideal body weight in non-overweight men without micro-/macroalbuminuria or a low eGFR are both important targets of lifestyle in terms of renoprotection.

Acknowledgements

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Announcement

The Verband Deutsche Nierenzentren e.V. (Association of German Nephrology Centers) Announces the Bernd Tersteegen Award 2009

Dr. Bernd Tersteegen, the founder of the Verband Deutsche Nierenzentren (DN) e.V., was dedicated to the improvement of outpatient treatment modalities in end-stage renal disease. Specifically, he focused on further technical development of hemodialysis. The Bernd Tersteegen award was established following Dr. Tersteegen's death in 1995. The prize is awarded internationally both for basic and particularly for clinical research related to chronic renal insufficiency and to advances in the treatment of end-stage renal disease.

The annual award of EUR 8,000 is provided by Roche Pharma AG (Grenzach, Germany). The award is usually given to a single applicant but may be shared under certain circumstances. Applicants should be physicians, researchers or engineers who are involved in research in the area of renal failure and renal replacement therapy. Only research papers that have been published in 2008 or 2009 or have not yet been published are suitable for submission. Papers should be written in German or English. Review articles, dissertations, university habilitation works and manuscripts already entered in other competitions may not be submitted.

Five copies of the work must be submitted by July 15, 2009, to the following address:

Verband Deutsche Nierenzentren (DN) e.V.
Priv. Doz. Dr. med. Werner Kleophas, President
Kleine Klotzbahn 23
DE–42105 Wuppertal (Germany)

The members of the prize committee are chosen by the executive board of the DN. The president of the DN serves as chairman of the committee.

In the case in which no work is found suitable for the award, the prize money is carried over to the following year. An appeal is not allowed.

The award will be conferred at the Annual Meeting of the Association of German Nephrology Centers of the DN in Mannheim, Germany, on November 21, 2009. The presence of the award winner at the award ceremony is required. The award winner will be informed in due time.

Hepatitis B Virus X Protein Shifts Human Hepatic Transforming Growth Factor (TGF)-β Signaling from Tumor Suppression to Oncogenesis in Early Chronic Hepatitis B

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Hepatitis B virus X (HBx) protein is suspected to participate in oncogenesis during chronic hepatitis B progression. Transforming growth factor β (TGF-β) signaling involves both tumor suppression and oncogenesis. TGF-β activates TGF-β type I receptor (TβRI) and c-Jun N-terminal kinase (JNK), which differentially phosphorylate the mediator Smad3 to become C-terminally phosphorylated Smad3 (pSmad3C) and linker-phosphorylated Smad3 (pSmad3L). Reversible shifting of Smad3-mediated signaling between tumor suppression and oncogenesis in HBx-expressing hepatocytes indicated that TβRI-dependent pSmad3C transmitted a tumor-suppressive TGF-β signal, while JNK-dependent pSmad3L promoted cell growth. We used immunostaining, immunoblotting, and *in vitro* kinase assay to compare pSmad3L- and pSmad3C-mediated signaling in biopsy specimens representing chronic hepatitis, cirrhosis, or hepatocellular carcinoma (HCC) from 90 patients chronically infected with hepatitis B virus (HBV) with signaling in liver specimens from HBx transgenic mice. In proportion to plasma HBV DNA levels, early chronic hepatitis B specimens showed prominence of pSmad3L in hepatocytic nuclei. HBx-activated JNK/pSmad3L/c-Myc oncogenic pathway was enhanced, while the TβRI/pSmad3C/p21^{WAF1} tumor-suppressive pathway was impaired as human and mouse HBx-associated hepatocarcinogenesis progressed. Of 28 patients with chronic hepatitis B who showed strong oncogenic pSmad3L signaling, six developed HCC within 12 years; only one of 32 patients showing little pSmad3L developed HCC. In contrast, seven of 30 patients with little Smad3C phosphorylation developed HCC, while no patient who retained hepatocytic tumor-suppressive pSmad3C developed HCC within 12 years. *Conclusion:* HBx shifts hepatocytic TGF-β signaling from the tumor-suppressive pSmad3C pathway to the oncogenic pSmad3L pathway in early carcinogenic process. Hepatocytic pSmad3L and pSmad3C assessment in HBV-infected liver specimens should prove clinically useful for predicting risk of HCC. (HEPATOLOGY 2009;49:1203-1217.)

Hepatocellular carcinoma (HCC) is the fifth most common cancer worldwide and one of the most deadly, causing approximately 600,000 deaths yearly.[1] The overall incidence of HCC continues to rise, especially in western Europe and the United States.[2] During the past 20 years, striking advances have enhanced our understanding of HCC. More than 85% of HCC cases are related to known hepatitis B virus (HBV) and hepatitis C virus (HCV).

Abbreviations: Ab, antibody; HBV, hepatitis B virus; HBx, hepatitis B virus X. HCC, hepatocellular carcinoma; HCV, hepatitis C virus; HSC, hepatic stellate cells; IgG, immunoglobulin G; JNK, c-Jun N-terminal kinase; PPM1A, protein phosphatase magnesium 1A; pSmad3C, C-terminally phosphorylated Smad3; pSmad3L, linker-phosphorylated Smad3; SCP1-3, small C-terminal domain phosphatase 1-3; TGF-β, transforming growth factor β; TβRI, TGF-β type I receptor; TβRII, TGF-β type II receptor.

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A strong correlation between chronic HBV infection and HCC occurrence has long been apparent according to epidemiologic evidence and the finding of integrated HBV DNA sequences in virtually all HBV-related HCC.[3] Hepatitis B virus X (HBx) oncoprotein has been implicated in HBV-mediated hepatocarcinogenesis,[4,5] and persistent high-level expression of HBx protein in transgenic mouse liver results in hyperplasia leading to HCC, with no preceding inflammation.[6] Although HBx does not bind DNA directly, HBx activates Ras/mitogen-activated protein kinase pathways including extracellular signal-regulated kinase and c-Jun N-terminal kinase (JNK),[7] resulting in tumor cell growth and survival.

Transforming growth factor β (TGF-β) can inhibit epithelial cell growth, acting as a tumor suppressor. During carcinogenesis, however, cancer cells gain advantage by selective reduction of the tumor-suppressive activity of TGF-β together with augmentation of its oncogenic activity.[8] This led us to hypothesize that alterations in the TGF-β signal transduction pathway could be involved in the development of HCC in long-standing HBV infection.

Smads are central mediators of signals from the receptors for TGF-β superfamily members to the nucleus.[9] Smads are modular proteins with conserved Mad-homology 1, intermediate linker, and Mad-homology 2 domains.[10] The catalytically active TGF-β type I receptor (TβRI) phosphorylates the C-terminal serine residues of receptor-activated Smads, which include Smad2 and the highly related protein Smad3. The linker domain can undergo regulatory phosphorylation by other kinases including mitogen-activated protein kinases and cyclin-dependent kinases.[11-14] In contrast to the clearly activating role of the C-terminal phosphorylation events, the regulation of Smad activity by phosphorylation of the linker region is complex. Linker phosphorylation of Smad2 during human colorectal carcinogenesis results in cytoplasmic retention of Smad2 and inhibition of tumor-suppressive TGF-β signaling.[11,15] However, Smad3 phosphorylated at the linker region (pSmad3L) is localized predominantly to cell nuclei in actively growing Ki-67–immunoreactive colon cancer with distant metastasis.[15] Reversible shifting of Smad-dependent signaling between tumor suppression and oncogenesis in hyperactive Ras-expressing cells indicates that Smad3 phosphorylated at the C-terminal region (pSmad3C) transmits a tumor-suppressive TGF-β signal, whereas oncogenic activities such as cell proliferation and invasion are promoted by the pSmad3L pathway.[16] In addition, Roberts' group[17] has recently reported that Smad3 is critical for Ras/JNK-mediated transformation. Taken together, these findings indicate that oncogenic TGF-β signaling results from the functional collaboration of Ras and Smad3 rather than from Ras-mediated inhibition of the Smad3 pathway. Linker phosphorylation of Smad3 indirectly inhibits C-terminal phosphorylation, minimizing tumor-suppressive pSmad3C signaling.[16] Notably, pSmad3L-mediated signaling in activated hepatic stellate cells (HSCs) promotes liver fibrosis by stimulating extracellular matrix deposition.[13,18]

The role of HBV and HCV in tumor formation appears to be complex and may involve both direct and indirect mechanisms.[19] Integration of HBV DNA into the host genome occurs at early steps of clonal tumor expansion. Alternatively, chronic liver inflammation and hepatic regeneration induced by host cellular immune responses can increase the risk of HCC development. During progression of HCV-related chronic liver disorders, hepatocytes affected by chronic inflammation undergo a transition from the tumor-suppressive pSmad3C pathway to the JNK/pSmad3L pathway.[20] Our present studies extend the previous observations to HBV-related hepatocarcinogenesis. We study Smad3 phosphorylation profiles in HBV-infected human liver and HBx transgenic mouse liver, concluding that HBx oncoprotein in early stages of chronic hepatitis B contributes directly to hepatocarcinogenesis by shifting hepatocytic Smad3-mediated signaling from tumor suppression to oncogenesis.

Patients and Methods

Patients, Follow-up, and Detection of HCC. Ninety patients with HBV-related chronic liver disease underwent liver biopsy at the Department of Gastroenterology and Hepatology of Kansai Medical University Hospital between 1992 and 1994. All patients were seropositive for hepatitis B surface antigen (Abbott Laboratories, North Chicago, IL) and were seronegative for anti-HCV antibody (Ortho Diagnostics, Tokyo, Japan). Patients included 70 with chronic hepatitis, 10 with cir-

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rhosis, and 10 with HCC. Sixty of the chronic hepatitis patients were enrolled in a program for early diagnosis of HCC; the other 10 were lost to follow-up. HBV DNA (Roche Diagnostics, Tokyo, Japan) and hepatitis B envelope antigen (Abbott Laboratories) were measured at the time of liver biopsy. During the surveillance period, patients were followed up with abdominal ultrasonography and plasma alpha-fetoprotein determinations every 3 to 6 months. We also made a random choice of 20 chronic hepatitis B specimens with little fibrosis (F1) and little inflammation (A1) from the liver biopsy specimens of the patients showing high plasma HBV DNA levels.

Necroinflammatory activity and fibrotic stage were graded histologically according to the classification of Desmet and colleagues.[21] We counted and scored pSmad3, HBx, and c-Myc positivity in hepatocytes as follows: 0, no positivity; 1, <25%; 2, 25% to 50%; 3, 50% to 75%; 4, >75%.[20] Written informed consent was obtained from each patient according to the Helsinki Declaration. We also obtained approval for this study from our institutional ethics committee.

Reverse-Transcription Polymerase Chain Reaction. Reverse-transcription polymerase chain reaction of TGF-β type II receptor (TβRII), Smad2, and Smad4 genes was performed as described.[15]

Domain-Specific Antibodies Against the Phosphorylated Smad3. Two polyclonal anti-phospho–Smad3 sera—α pSmad3L (Ser 208/213) and α pSmad3C (Ser 423/425)—were raised against the phosphorylated linker and C-terminal regions of Smad3 by immunization of rabbits with synthetic peptides. Relevant antisera were affinity-purified using phosphorylated peptides as described.[13]

Transgenic Animals. HBx transgenic mice were derived by microinjection of a 1151-bp HBV DNA fragment containing the HBx gene with its own regulatory elements and polyadenylation signal into fertilized eggs of CD-1 mice. An independent line (H9) was derived from founders.[6]

Immunohistochemical and Immunofluorescence Analyses. Immunohistochemical and immunofluorescence analyses were performed as described.[18] Primary antibodies (Abs) used in this study included mouse monoclonal anti-HBx Ab (2 μg/mL; Abcam, Cambridge, UK), mouse monoclonal anti-c-Myc Ab (10 μg/mL; Santa Cruz Biotechnology, Santa Cruz, CA), and mouse monoclonal anti-$p21^{WAF1}$ Ab (0.5 μg/mL; DAKO, Glostrup, Denmark), in addition to the affinity-purified rabbit polyclonal anti-pSmad3L (2 μg/mL) and anti-pSmad3C (0.5 μg/mL) as described above. Anti-pSmad3C Ab cross-reacted weakly with C-terminally phosphorylated Smad2: to block binding of anti-pSmad3C Ab to phosphorylated domains in Smad2, anti-pSmad3C Ab was adsorbed with 1 μg/mL C-terminally phosphorylated Smad2 peptide.

For immunohistochemical analyses, sections exposed to primary Abs were then incubated with peroxidase-labeled polymer conjugated to goat anti-mouse or anti-rabbit immunoglobulin G (IgG) (DAKO). Finally, sections were developed with 3,3'-diaminobenzidine tetrahydrochloride (DAB; Vector Laboratories, Burlingame, CA), counterstained with Mayer's hematoxylin (Merck, Darmstadt, Germany), and mounted under coverslips.

For double-labeling immunofluorescence analyses, sections exposed to a pair of primary Abs (rabbit plus mouse) were then incubated in a 1:500 dilution of goat anti-rabbit IgG conjugated with a red fluorophore (Alexa Fluor 594; Molecular Probes, Eugene, OR) and goat anti-mouse IgG conjugated with a green fluorophore (Alexa Fluor 488; Molecular Probes). Images were obtained with a fluorescence microscope (Carl Zeiss Microimaging, Oberkochen, Germany).

Immunoprecipitation and Immunoblotting. pSmad3L and pSmad3C immunoblots on Smad3 immunoprecipitates of cell extracts from frozen tissues representing either HCC or underlying liver diseases were performed as described.[20]

In Vitro *Kinase Assay.* *In vitro* kinase assay was performed as described.[12]

Statistical Analyses. The Kaplan-Meier method was used to determine the cumulative probability of appearance of HCC during the 12-year follow-up period. HCC occurrence curves were compared between patients with abundant (scores 3 to 4) and those with sparse (scores 0 to 2) Smad3L/C phosphorylation, by means of the log-rank test. For continuous variables, the optimal cutoff threshold for defining groups was established using receiver operating characteristics curves. All parameters with P values less than 0.10 in the univariate analysis were selected for multivariate analysis, which was performed using the Cox proportional hazards model.[22] P values less than 0.05 were considered significant. The Mann-Whitney U test was used to identify significant differences in hepatocytic pSmad3L and pSmad3C positivity among fibrotic stages.

Results

Two Distinct Hepatocytic Smad3 Signaling Pathways in Human Chronic Hepatitis B: pSmad3L- and pSmad3C-Dominant Types. We initially analyzed mutations of TβRII, Smad2, and Smad4 genes in 10 HCC and six cirrhotic liver samples, finding no mutations in

Table 1. Clinicopathologic Features, Smad3L/C Phosphorylation, and HBx and c-Myc Positivities in Specimens from Patients with HBV-Related Chronic Liver Disease

		Fibrotic Stage*				
	Nomal	F1	F2	F3	F4	HCC
Patients, n	2	20	27	23	10	10
Sex (male/female), n	2/0	13/7	19/8	17/6	5/5	10/0
Age (years), mean ± SD	57.0 ± 9.9	35.5 ± 14.3	34.3 ± 13.9	43.1 ± 13.7	59.6 ± 7.6	54.0 ± 15.1
pSmad3L staining, n†						
0	2	0	0	0	0	0
1	0	8	6	2	1	0
2	0	6	6	7	1	0
3	0	3	11	11	2	5
4	0	3	4	3	6	5
pSmad3C staining, n†						
0	0	0	0	0	0	0
1	0	0	4	4	2	4
2	0	4	9	12	7	3
3	2	11	7	5	1	3
4	0	5	7	2	0	0
Activity, n*						
A0	2	1	0	0	0	0
A1	0	17	6	1	0	1
A2	0	2	19	11	3	7
A3	0	0	2	11	7	2
HBx staining, n†						
0	2	0	0	0	1	1
1	0	6	5	3	2	3
2	0	7	11	8	3	3
3	0	3	7	6	2	2
4	0	4	4	6	2	1
c-Myc staining, n†						
0	2	0	0	0	0	0
1	0	2	4	1	1	0
2	0	9	10	8	3	1
3	0	6	8	8	3	3
4	0	3	5	6	3	6
Histology of HCC (well/moderate)‡						6/4
TNM stage (I/II/III/IV)ʃ						4/4/2/0
Size of tumor (cm), mean ± SD						2.2 ± 0.3
AST (IU/L), mean ± SD	22.5 ± 3.5	68.6 ± 56.1	92.8 ± 65.8	79.7 ± 51.8	82.0 ± 53.1	71.0 ± 36.4
ALT (IU/L), mean ± SD	24.0 ± 2.8	104 ± 83.5	141 ± 97.5	84.5 ± 83.1	68.2 ± 52.3	59.1 ± 32.2
Platelet count (× 10^9/L), mean ± SD	25.0 ± 4.2	17.1 ± 3.6	15.8 ± 4.9	14.1 ± 7.1	9.7 ± 6.7	9.0 ± 3.7
AFP (ng/mL), mean ± SD	2.1 ± 1.3	6.8 ± 4.6	14.8 ± 12.2	66.2 ± 138	132 ± 208	164 ± 184

Abbreviations: AFP, alpha-fetoprotein; ALT, alanine aminotransferase; AST, aspartate aminotransferase; pSmad3L, linker-phosphorylated Smad3; pSmad3C, C-terminally phosphorylated Smad3; SD, standard deviation; TNM, tumor-node-metastasis.

*Necroinflammatory activity and fibrotic stage are determined histologically according to Desmet's classification.

†Hepatocytic Smad3 phosphorylation is scored as follows:. 0. no phosphorylation; 1. <25% Smad3 phosphorylation; 2, 25% to 50% Smad3 phosphorylation; 3, 50% to 75% Smad3 phosphorylation; 4, >75% Smad3 phosphorylation. Extent of HBx and c-Myc expression is indicated as that of pSmad3L positivity.

‡Histological grading of HCC is classified according to the criteria of the International Working Party.

ʃTNM is classified by the International Union Against Cancer and American joint Committee on Cancer.

any sample. This confirms the low probability of mutations in HCC tissues, which has been reported recently.[23]

To investigate domain-specific phosphorylation mediating Smad3 signaling *in vivo*, we generated two Abs specific to each phosphorylation site, and determined the distribution of pSmad3L and pSmad3C in chronic hepatitis B and C specimens. Table 1 shows clinical background and positivity for pSmad3L and pSmad3C in 90 patients with HBV-related chronic liver diseases. We also studied HCC occurrence over 12 years in 60 patients with chronic hepatitis B who were enrolled in a program for early diagnosis of HCC (Table 2). We recently reported that Smad3 was phosphorylated at the linker region, particularly in groups of hepatocytes adjoining collagen fibers in portal tracts in chronic hepatitis C.[20] In contrast, the distribution of pSmad3L and pSmad3C in chronic

Table 2. Clinicopathologic Features, Smad3L/C Phosphorylation, and HCC Incidence in Specimens from Patients with HBV-Related Chronic Hepatitis

Patient No.	Sex	Age	Incidence of HCC	pSmad3L Staining*	pSmad3C Staining*	Fibrotic Stage†	Inflammatory Activity†	HBV DNA (log copies/mL)	HBeAg
1	M	62	○	4	2	3	3	5.4	+
2	F	44	○	4	2	2	2	5.5	−
3	M	22	○	4	2	2	2	5.2	−
4	F	20		4	4	3	3	3.0	−
5	M	43		4	4	2	2	4.5	−
6	M	30		4	2	2	3	4.0	−
7	M	30		4	2	2	3	5.6	−
8	M	65	○	3	2	3	3	4.0	+
9	F	56	○	3	2	3	2	3.7	+
10	M	52	○	3	1	1	1	6.4	−
11	F	40		3	1	1	1	6.9	−
12	M	44		3	1	3	3	5.1	−
13	M	45		3	1	3	3	3.8	−
14	M	28		3	2	3	1	3.0	−
15	M	60		3	2	3	2	2.8	−
16	M	44		3	2	3	3	5.2	+
17	M	44		3	2	3	3	3.2	+
18	M	44		3	2	3	3	4.4	−
19	F	26		3	2	2	1	4.9	−
20	M	20		3	2	2	1	2.9	+
21	M	59		3	2	2	2	4.4	−
22	M	43		3	3	2	2	3.2	+
23	M	29		3	3	3	2	6.2	−
24	M	29		3	3	3	2	3.0	−
25	M	25		3	4	1	1	3.5	−
26	F	33		3	4	2	2	4.6	+
27	M	19		3	3	3	2	5.6	−
28	M	63		3	4	2	2	5.1	−
29	M	52	○	2	1	3	2	3.7	−
30	M	44		2	2	3	3	5.2	−
31	M	29		2	2	3	2	3.9	+
32	F	46		2	4	3	3	3.2	−
33	M	25		2	2	1	2	5.0	−
34	F	23		2	3	1	1	2.1	−
35	F	31		2	3	2	2	3.9	+
36	F	26		2	3	1	1	2.4	−
37	M	35		2	3	1	1	5.6	−
38	M	20		2	3	2	1	3.2	+
39	F	56		2	3	3	2	5.1	+
40	F	36		2	3	3	2	2.6	−
41	F	25		2	3	2	1	5.1	−
42	F	23		2	4	2	1	3.5	−
43	F	41		2	4	1	1	2.0	+
44	M	29		2	4	2	2	4.5	−
45	M	31		2	4	1	1	5.9	+
46	M	42		2	1	2	2	3.7	−
47	M	24		1	1	2	2	3.9	+
48	M	28		1	2	3	2	3.8	−
49	F	11		1	2	2	2	3.0	+
50	M	40		1	1	2	2	3.2	−
51	M	37		1	2	2	2	3.0	−
52	F	10		1	2	2	2	2.3	−
53	M	16		1	3	1	1	5.1	−
54	M	41		1	3	1	1	4.3	−
55	M	40		1	3	1	1	2.2	−
56	M	53		1	3	1	1	2.7	−
57	M	27		1	3	1	1	4.6	−
58	M	53		1	4	1	1	3.3	−
59	M	30		1	4	2	2	2.1	−
60	F	22		1	4	1	0	3.7	−

Abbreviations: F, female; HBeAg, hepatitis B e antigen; HBV, hepatitis B virus; HCC, hepatocellular carcinoma; M, male; pSmad3C, C-terminally phosphorylated Smad3; pSmad3L, linker-phosphorylated Smad3.

*Hepatocytic Smad3 phosphorylation is scored as follows: 0, no phosphorylation; 1, <25% Smad3 phosphorylation; 2, 25% to 50% Smad3 phosphorylation; 3, 50% to 75% Smad3 phosphorylation; 4, >75% Smad3 phosphorylation.

†Necroinflammatory activity and fibrotic stage are determined histologically according to Desmet's classification.