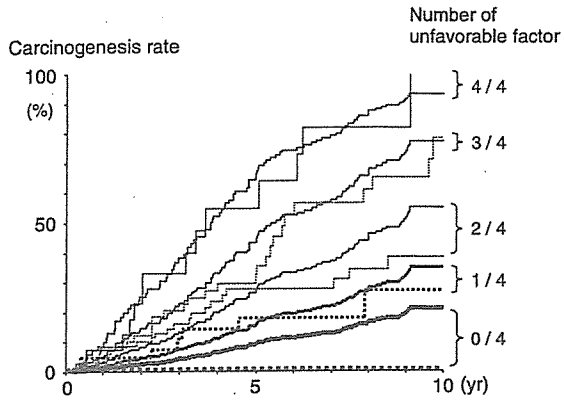

Fig. 3. Simulated HCC appearance curves with actual appearance rates of internal and external validation cohorts, according to the number of unfavorable risk factors. Five solid curves show simulated carcinogenesis rates drawn according to the number of unfavorable risk factors; none (the thickest line), one, two, three, and four (the thinnest line). Five dotted curves indicate actual HCC appearance curves of the validation cohort (Toranomon Hospital, 1991–2003).

development that combine several variables of patient data to indicate the probability of clinical outcome are powerful tools for assisting physicians in the decision-making process. Our model can be used for prediction of HCC in daily clinical practice by hepatologists, for education and information for individual patients, for selection of a candidate for a cancer prevention program, and for a proper stratification of cirrhotic patients in clinical trials for the purpose of cancer prevention. The consistency and reproducibility of the present model should also be confirmed by other institutions outside Japan.

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REVIEW ARTICLE

Ningen Dock 2006 ; **20** : 1－5

How and Why Do We Diagnose Metabolic Syndrome?

Kan Saito, Nobukazu Ishizaka, Ryozo Nagai

About a year has passed since the criteria for defining and diagnosing of metabolic syndrome (MetS) in the Japanese were established[1]. There are several differences between these criteria and other commonly used definitions of MetS, such as those of the World Health Organization (WHO) and the Adult Treatment Panel III of the National Cholesterol Education Program (NCEP-ATPIII). In some ways this is not surprising because the criteria might be expected to differ for different ethnic populations. On the other hand, it is important to understand why we should identify subjects with MetS. In this paper, we would like to discuss how and why it is necessary to diagnose MetS. (*Ningen Dock* 2006 ; **20** : 1－5)

Key Words : metabolic syndrome, insulin resistance, criteria, cardiovascular disease

Definition and Diagnosis of Metabolic Syndrome

Insulin resistance increases the risk of glucose intolerance, dyslipidemia and essential hypertension. Clustering of these cardiac risk factors, which enhances the risk for cardiovascular disease (CVD), has been variously called Reaven's syndrome, syndrome X[2], deadly quartet[3], and insulin-resistance syndrome. Recently, the World Health Organization (WHO) and the Adult Treatment Panel III of the National Cholesterol Education Program (NCEP-ATPIII) have proposed clinical criteria to diagnose the clinical entity 'metabolic syndrome (MetS)' in an effort to raise awareness of this problem.

There are several definitions of MetS, including those proposed by the WHO, the NCEP-ATPIII, the European Group for the Study of Insulin Resistance (EGIR), and the association of Clinical Endocrinologists (AACE), and the prevalence of MetS in the general population differs according to the definition of MetS that is used and to ethnicity. Although the WHO[4] and the NCEP-ATPIII[5] definitions of MetS are currently used the most frequently (**Table 1**), there is still no gold standard for diagnosis of MetS.

In Japan, criteria for defining and diagnosing MetS in the Japanese population were established in April 2005[1] (**Table 1**). Based on the understanding that intra-abdominal visceral fat accumulation plays an important role in the development of CVD risk, these criteria state that waist circumference is a mandatory requirement for the diagnosis of MetS.

The International Diabetes Federation (IDF) committee has also adopted waist circumference as a surrogate marker for abdominal adiposity and has recommended ethnicity-specific cutoff points for the waist circumference. This was because the central obesity is an early step in the etiological cascade leading to full MetS[6]. In the IDF definition, waist circumference is also a mandatory requirement and, unique to this definition, waist circumference thresholds are adjusted according to the ethnic groups (in Japan, WC≥90 cm in women or ≥85 cm in men, **Table 1**). Data suggest, however, that MetS diagnosed according to the IDF may not be a better diagnostic predictor for CVD than WHO-defined MetS (WHO-MetS) or NCEP-ATIII-defined MetS (NCEP-MetS) in Japanese diabetic patients[7].

In the IDF criteria and the definition of MetS in Japan, the cutoff for waist circumference is greater in women (90 cm) than in men (85 cm), which is quite unique and differs from the cutoff values not only for European and American populations but also for other Asian countries. These sex-specific cutoff values have been determined because the number of risk factors markedly increases when the area of visceral fat exceeds 100 cm^2, which corresponds to different cutoff values of waist circumference for males and females[8].

As summarized in the **Table 1**, the advocated diagnostic criteria for MetS differ substantially. Therefore, in comparing the prevalence of MetS and presumed hazard ratio for CVD in subjects with MetS in various populations, it is important to pay attention to the criteria that were used to diagnose MetS.

The existence of various diagnostic criteria for MetS seems to imply that the clinical entity of MetS remains somewhat fluid. This raises the question of whether we need to make a diagnosis of MetS for preventive medical care and clinical practice. For example, we already know that certain individuals with decreased levels of HDL cholesterol, a component of the MetS diagnostic criteria, may have a higher probability of future CVD events than those with normal levels. Nevertheless, we also have to understand why the concept of MetS is spotlighted worldwide.

MetS and CVD

The *raison d'etre* of MetS relies upon whether it helps to identify subjects at high risk for CVD and

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Table 1. Various criteria for the diagnosis of metabolic syndrome

WHO criteria[4]

Diabetes or IFG or IGT or insulin resistance (assessed by clamp studies), plus at least two of the following criteria :

1. Waist-to-hip ratio >0.90 in men or >0.85 in women, or BMI ≥30 kg/m^2
2. Serum triglycerides ≥150 mg/dL (1.7 mmol/L) or HDL-cholesterol <35 mg/dL (0.9 mmol/L) in men and <39 mg/dL (1.0 mmol/L) in women
3. Blood pressure ≥140/90 mmHg
4. Urinary albumin excretion rate ≥20 mg/min or albumin : creatinine ratio >30 mg/g

NCEP-ATIII[5]

Any three or more of the following criteria :

1. Waist circumference >102 cm in men and >88 cm in women
2. Serum triglycerides ≥150 mg/dL (1.7 mmol/L)
3. Blood pressure : ≥130/85 mmHg
4. HDL-cholesterol : <40 mg/dL (1.04 mmol/L) in men and <50 mg/dL (1.29 mmol/L) in women
5. Serum glucose : ≥110 mg/dL (6.1 mmol/L)(≥5.6 mmol/L may be applicable)

Definition and diagnostic criteria of MetS in Japanese[1]

Waist circumference ≥90 cm in women or ≥85 cm in men plus two or more of the followings :

1. HDL-C <40 mg/dL, or TG ≥150 mg/dL
2. BP ≥130/85 mmHg or person is receiving drug treatment for hypertension
3. FPG ≥110 mg/dL

IDF[6]

Waist circumference ≥80 cm in women or ≥90 cm in men (for South Asians and Chinese) plus two or more of the followings :

1. HDL-C <50 mg/dL (1.29 mmol/L) in women or <40 mg/dL (1.03 mmol/L) in men ; or specific treatment for this lipid abnormality
2. TG ≥150 mg/dL (1.7 mmol/L) ; or specific treatment for this lipid abnormality
3. BP ≥130/85 mmHg ; or treatment of previously diagnosed hypertension
4. FPG ≥100 mg/dL (5.6 mmol/L) ; or previously diagnosed type 2 diabetes

WHO : World Health Organization, NCEP-ATIII : Adult Treatment Panel III of the National Cholesterol Education Program, IDF : International Diabetes Federation, MetS : metebolic syndrome, IFG : impaired fasting glucose, IGT : impaired glucose tolerance, HDL-C : HDL-cholesterol, BP : blood pressure, FPG : fasting plasma glucose, TG : triglycerides.

diabetes. Although the exact answer may vary depending on the diagnostic criteria used and the target population selected, it is instructive to determine whether individuals with MetS have been reported to be at high risk for these disorders in the previous studies.

Subjects with T2DM

In an analysis of data from 428 participants with newly diagnosed type 2 diabetes mellitus (T2DM), who were CVD-free at study entry, NCEP-MetS at baseline was found to be associated with an increased risk of CVD incidence in the 5 years following diagnosis of T2DM[9]. In the Botnia study, in which a total of 4483 subjects aged 35–70 years were participated, WHO-MetS was seen in 10% of subjects with normal glucose tolerance (NGT), in 50% of subjects with impaired fasting glucose (IFG) or impaired glucose tolerance (IGT), and 80% in subjects with T2DM. MetS was found to be an independent risk factor for CVD in a subpopulation of individuals with NGT, IFG/IGT, and T2DM[10].

Subjects without History of CVD and T2DM

Is MetS an independent risk factor for CVD in individuals without CVD or T2DM? Analysis of a population-based, prospective cohort study including 1209 Finnish men (aged 42–60 years at baseline) who were initially without CVD, cancer, or diabetes, has provided a positive answer to this question. WHO-MetS was found to be associated with 2.6–3.0-fold higher mortality from CVD and with 1.9–2.1-fold higher mortality from all-causes, although the NCEP-MetS was a weaker predictor of CVD and all-cause mortality[11]. Najarian *et al*. have reported that MetS is

more prevalent (28%) than diabetes (10%) and that it is a significant independent risk factor for stroke in people without diabetes[12]. Wison *et al.* have reported in another study that NCEP-MetS is associated with increased risk for CVD and T2DM in middle-aged adults who were without CVD or T2DM at baseline and were followed-up over 8 years[13].

Subjects with Only Minor Hemodynamic and Metabolic Abnormalities

The utility of the concept of MetS may be judged by whether subjects diagnosed with MetS have a higher risk of CVD than those without. As described above, presence of MetS may increase CVD-associated morbidity in the subjects with diabetes and hypertension, therefore, more attention should be paid to such individuals. On the other hand, however, patients with established diabetes, hypertension, or dyslipidemia may periodically visit the clinic or hospital as an outpatient, and these conditions may be already being treated. We do know that the subjects with diabetes or hypertension are already at higher risk for CVD regardless of their MetS status.

The results of the above-mentioned study by Wilson *et al.*[13] may seem to be good enough to conclude with confidence that MetS is a predictor for CVD even in low risk subjects. It should be noted, however, that in their study, increased blood pressure, defined as ≥130/85 mmHg or on therapy, was present in 91% of men and in 89% of women with MetS in the study, corresponding to more than twice its prevalence in men (43%) and in women (34%) without MetS. In their study, in addition, the relative risk of MetS for CVD was adjusted only by age.

Thus, it should be questioned whether MetS may really increase the risk for future CVD events in subjects with low risk factors for CVD. For example, it should be tested whether MetS will independently predict the future development of CVD in individuals with either optimal, normal, or high normal blood pressure (blood pressure <140/90 mmHg) and with either normal or impaired fasting glycemia (FPG <126 mg/dL) at baseline. This point has not been well evaluated in Japan, and should be analyzed in future studies.

Why does MetS Increase CVD Incidence?

Although the underlying causes of MetS are not completely clear, several adipocytokines present in the visceral fat, including leptin[14], adiponectin, plasminogen activator inhibitor-1 (PAI-1), tumor necrosis factor (TNF-alpha), and interleukin (IL)-6, may explain the link between MetS and CVD. Leptin may play a crucial role in the regulation of body fat, food intake and energy expenditure. Adiponectin, an adipocyte-specific protein, may be one of the key players in the development of MetS, as deficiency of this adipocytokine induces severe diet-induced insulin resistance[15]. A decrease in these plasma concentration of adiponectin is associated with a higher risk for T2DM and CVD. Of note, adiponectin levels may be modulated by lifestyle habits, such as smoking[16], dietary factors[17], and physical exercise[18]. Relative to subcutaneous fat, visceral fat secretes relatively higher amounts of PAI-1 and TNF-alpha. Increased PAI-1 levels are associated with subsequent development of cardiovascular events[19]. TNF-alpha may also play a role in the insulin resistance[20]. Modulation of adipocytokine secretion may explain the observed link between obesity and a higher incidence of CVD. Normalizing the secretion of these adipocytokines, by lifestyle modification or, perhaps by certain drugs, may resolve the clustering of atherogenic risk factors and subsequently reduce the future CVD events.

Lifestyle Habits and MetS

Individuals with MetS who undertake lifestyle modification show less development of diabetes[21]. Thus, dietary and exercise intervention could be implemented by primary healthcare systems. So what is known about the relationship between lifestyle habits (e.g. cigarette smoking, alcohol intake, supplement use) and MetS?

Cigarette Smoking

Several previous studies have shown that smoking reduces insulin sensitivity[22,23]. Furthermore, we have reported that the prevalence of MetS is increased more than two-fold in current-smokers as compared with never-smokers[24]. Weitzman *et al.* have reported that environmental tobacco smoke may also increase the incidence of MetS[25]. They found that exposure to tobacco smoke, whether by active or passive smoking, is associated with a fourfold increase in the risk of the MetS among overweight adolescents. The finding that long-term use of nicotine gum may also increase insulin resistance[26] supports the notion that nicotine is one of the tobacco ingredients that reduces insulin sensitivity. However, it may be too optimistic to presume that stopping smoking will immediately ameliorate insulin resistance because, after multivariate adjustment, a history of cigarette smoking has been found to be still associated with increased prevalence of MetS even 5 years after an individual has quit[24,27].

Alcohol Intake

As described above, active and passive cigarette smoking may increase the prevalence of MetS. What is known about the relationship between alcohol and MetS? By analyzing cross-sectional data from the Third National Health and Nutrition Examination Survey, Freiberg *et al.* have reported that mild to moderate alcohol consumption is associated with a lower prevalence of MetS[28]. Nevertheless, we are unable to conclude emphatically that alcohol per se is not a contributor to MetS, as the mode of association between alcohol intake and MetS might differ according to both ethnicity[29] and the type of beverage consumed[28]. Furthermore, we are not sure where the boundaries lie between moderate and heavy drinking in the Japanese population in terms of MetS.

Antioxidant Use

By analyzing the data from the Third National Health and Nutrition Examination Survey, Ford *et al.* have reported that, after multivariate adjustment, participants with the metabolic syndrome had significantly lower concentrations of retinyl esters, vitamin C, and carotenoids, and vitamin E[30]. They also reported an inverse association between serum concentrations of vita-

min D and the prevalence of MetS[31].

Intake of antioxidants, by contrast, does not appear to have an effect on cardiovascular outcomes[32,33], although some studies have reported conflicting results[34,35]. It is not surprising that a healthy balanced diet with a frequent intake of vegetables, fruits, fish, pasta and rice and a low intake of fried food is protective against MetS[36].

Management of MetS

The American Diabetes Association (ADA) and the European Association for the Study of Diabetes (ESAD) have published a Joint Statement[37,38], in which it is stressed that the utility of the concept of MetS as the new risk marker for CVD requires further investigation. There is a discussion that clinicians should evaluate and treat all CVD risk factors regardless of whether a patient meets the diagnosis criteria for MetS[38]. Several investigators have questioned whether MetS is really an established clinical entity[39].

Individuals with established dyslipidemia, hypertension, and diabetes are often periodically followed-up at medical institutions in Japan. Even without diagnosis of MetS, patients with these risk factors are already considered to be at high risk for CVD and are often undergoing therapy to ameliorate these disorders in some medical institutes. Thus, an important question would appear to be whether individuals diagnosed with MetS are at substantially higher risk for CVD, especially if the extent of each of their MetS components is mild enough such that on its own it would not seem to require active intervention.

We should certainly continue to pursue whether some drugs, such as peroxisome proliferator-activated receptor (PPAR) agonists, are effective in reducing the incidence of MetS and/or reducing the development of CVD and T2DM in individuals with MetS. On the other hand, lifestyle modification is the cornerstone of MetS treatment and costs less[40]. The clinical importance of diagnosing MetS seems to depend not only on which definition is used, but also on how this syndrome is comprehended and managed by healthcare practitioners.

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Metabolic Syndrome May Not Associate With Carotid Plaque in Subjects With Optimal, Normal, or High-Normal Blood Pressure

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Abstract—Much evidence indicates that metabolic syndrome is a risk factor for the development of cardiovascular disease, but whether metabolic syndrome is an independent risk factor for early atherosclerosis in the individuals with only minor hemodynamic abnormalities, if any, is not well investigated. Here we have investigated the association between metabolic syndrome and carotid atherosclerosis in individuals with blood pressure of <140/90 mm Hg. Between 1994 and 2003, 8143 subjects underwent general health screening including carotid ultrasonography. Of 8143 individuals, 5661 individuals without antihypertensive medications who had blood pressure of <140/90 mm Hg were considered to have optimal, normal, or high-normal blood pressure. After adjustment for age, systolic blood pressure, body mass index, total and high-density lipoprotein cholesterol, triglycerides, fasting glucose, and smoking status, metabolic syndrome was not found to be an independent risk factor for carotid plaque (odds ratio: 1.65; 95% CI; 0.72 to 3.76 in women and odds ratio: 0.95; 95% CI: 0.70 to 1.28 in men) or for carotid intima-media thickening (odds ratio: 0.56; 95% CI: 0.18 to 1.72 in women and odds ratio: 0.93 95% CI: 0.62 to 1.38 in men) in these subjects. Thus, presence of metabolic syndrome may not increase the prevalence of carotid atherosclerosis independent of other cardiovascular risk factors in Japanese individuals with optimal, normal, or high-normal blood pressure. (***Hypertension*. 2006;48:411-417.**)

Key Words: metabolism ■ carotid arteries ■ atherosclerosis ■ risk factor ■ hypertension, arterial

Metabolic syndrome (MetS) is a cluster of metabolic and hemodynamic abnormalities linked with insulin resistance.[1-4] Several diagnostic criteria have been advocated for MetS, of which those proposed by the World Health Organization[5] and the National Cholesterol Education Program[1] are used most frequently. Epidemiological studies have shown that MetS is not a rare occurrence[6] and is a risk factor for cardiovascular disease (CVD)[7] and stroke.[8] The coexistence of MetS in individuals with established CVD risk factors, such as diabetes and hypertension, has been shown to increase the risk of mortality and morbidity from CVD,[9-11] and, thus, such individuals should undergo lifestyle advice and/or active treatment to dissolve the clustering of these abnormalities to reduce the risk of future cardiovascular events.

On the other hand, however, subjects with such established risk factors for CVD are already regarded to be at higher risk for CVD irrespective of the presence or absence of MetS. Therefore, it should be tested whether the concept of MetS can be used to identify individuals at higher risk for future CVD events when the extent of their hemodynamic and metabolic abnormalities is only mild, if present at all. Here, by analyzing the cross-sectional data from individuals who underwent general health screening, we have investigated the impact of MetS on carotid atherosclerosis in the subjects with optimal, normal, or high-normal blood pressure (BP). We also performed a similar analysis in the subpopulations that additionally had, if present, only mild abnormalities in glucose metabolism.

Methods

Study Subjects

The study was approved by the Ethical Committee of Mitsui Memorial Hospital. Between September 1994 and December 2003, 8143 subjects underwent general health screening, including carotid ultrasonography at the Center for Multiphasic Health Testing and Services, Mitsui Memorial Hospital. BP, taken at the center, was classified according to the Sixth Report of the Joint National Committee on Detection, Evaluation, and Treatment of High Blood Pressure[12]: optimal BP: systolic BP (SBP) <120 mm Hg and diastolic BP (DBP) <80 DBP; normal BP: SBP 120 to 129 DBP or DBP 80 to 84 mm Hg; and high-normal BP: SBP 130 to 139 mm Hg or DBP 85 to 89 mm Hg. Fasting glucose levels were classified according to the American Diabetes Association criteria[13]: normal fasting glucose: FPG <100 mg/dL; and impaired fasting glucose: FPG ≥110 and <126 mg/dL. Individuals who were taking antidiabetic medication were not included.

Definition of MetS

We used a modified version of the National Cholesterol Education Program Adult Treatment Panel III (NCEP ATPIII)[1] and MetS was

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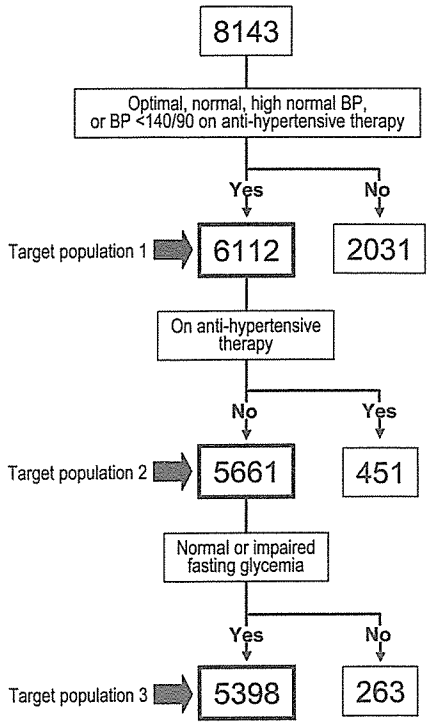

Figure 1. Flow chart showing the 3 target populations.

diagnosed when ≥3 of the following components were present: triglycerides ≥150 mg/dL; high-density lipoprotein cholesterol <40 mg/dL in men or <50 mg/dL in women; fasting plasma glucose (FPG) ≥110 mg/dL; SBP ≥130 mm Hg, DBP ≥85 mm Hg, or taking an antihypertensive medication; and body mass index (BMI) ≥25.0 kg/m^2 (the waist circumference was not available in this study). This BMI cutoff point was chosen instead of other previously used values[14] because of the discrepancy in BMI between white and Japanese populations in terms of morbidity.[15]

Selection of Subpopulation

We selected 3 types of target population as follows. Of the 8143 individuals, those with BP of <140/90 mm Hg were selected for target population 1 (n=6112). In addition, of the 6112 individuals in target population 1, those without antihypertensive medications (individuals with optimal, normal, and high-normal BP) were selected for target population 2 (n=5661). Furthermore, of the 5661 individuals in target population 2, those with either normal fasting glucose or impaired fasting glucose were selected for target population 3 (n=5398; Figure 1).

Carotid Ultrasonography

Carotid artery status was studied using high-resolution B-mode ultrasonography (Sonolayer SSA270A, Toshiba) equipped with a 7.5-MHz transducer as described previously.[16] Plaque was defined to be present when there is ≥1 clearly isolated focal thickening of the intima-media layer with thickness of ≥1.3 mm at the common or internal carotid artery or the carotid bulb. Carotid wall intima-media thickening was said to be present when intima-media thickness, which was measured at the far wall of the distal 10 mm of the common carotid artery, was ≥1.0 mm.[16]

Statistical Analysis

Comparisons of categorical and continuous variables were made by using χ^2 and Student t tests, respectively. Logistic regression analysis was used to obtain adjusted odds ratios and their 95% CIs to predict the presence of carotid plaque or carotid intima-media thickening. Statistical analyses were carried out by using StatView (version 5.0; SAS Institute Inc). Results are expressed as mean±SD. A value of $P<0.05$ was taken to be statistically significant.

Results

Association Between MetS and Carotid Atherosclerosis in Individuals With BP of <140/90 mm Hg (Target Population 1)

The age of the subjects ranged from 21 to 88 years (women, 22 to 87 years; men, 21 to 88 years). In this population, MetS was found in 81 (4%) of 2143 women and 511 (13%) of 3969 men (Table 1). In women, carotid plaque was found in 22 (27%) of the 81 MetS-positive subjects, which was significantly greater than that observed in the MetS-negative subjects (280 of 2062 [14%]; $P<0.001$ by χ^2 test). Similarly, in men, carotid plaque was found in 158 (31%) of the 511 MetS-positive subjects, which was significantly greater than that observed in the MetS-negative subjects (890 of 3458 [26%]; $P=0.013$ by χ^2 test; Figure 2; Table 2). When multivariate logistic regression analysis was performed after adjusting for age, the association between MetS and carotid plaque was statistically significant in both genders (Table 2). After full adjustment for additional CVD risk factors, including total cholesterol, smoking status, and components of the MetS (BMI, SBP, total cholesterol, high-density lipoprotein cholesterol, triglycerides, and FPG), however, this relationship was statistically significant in women, but not in men. After full adjustment, MetS was not significantly associated with carotid intima-media thickening in either gender (Table 3).

Association Between MetS and Carotid Atherosclerosis in Individuals With Optimal, Normal, or High-Normal BP (Target Population 2)

Of the 6112 individuals in target population 1, 451 individuals were taking antihypertensive medication. To investigate the association between MetS and carotid atherosclerosis in individuals with optimal, normal, and high-normal BP, we omitted these individuals. In this population, the age of the subjects ranged from 21 to 88 years (women, 22 to 87 years; men, 21 to 88 years), and MetS was found in 64 (3%) of 2034 women and 410 (11%) of 3627 men (Table 4). Carotid plaque was found in 11 (17%) of the 64 MetS-positive female subjects and in 103 (25%) of the 410 MetS-positive male subjects. The carotid plaque prevalence was not significantly different from that observed in the MetS-negative subjects (249 of 1970 [13%] women; $P=0.29$; 769 of 3217 [24%] men; $P=0.59$; Figure 2 and Table 2). After full adjustment, MetS was not significantly associated with carotid plaque or carotid intima-media thickening in either gender (Table 3).

Association Between MetS and Carotid Atherosclerosis in Individuals With Optimal, Normal, or High-Normal BP, Together With Normal or Impaired Fasting Glycemia (Target Population 3)

Of the 5661 individuals in target population 2, 263 individuals whose FPG levels were ≥126 mg/dL were omitted in the target population 3. In this population, the age of the subjects ranged from 21 to 88 years (women, 22 to 87 years; men, 21 to 88 years), and MetS was found in 57 (3%) of 2000 women

TABLE 1. Baseline Characteristics of the Target Population 1

Variables	Women			Men		
	Carotid Plaque (−) (n=1841)	Carotid Plaque (+) (n=302)	*P*	Carotid Plaque (−) (n=2921)	Carotid Plaque (+) (n=1048)	*P*
Age, y	54.1±10.0	63.5±8.7	<0.0001	53.4±10.0	62.8±9.5	<0.0001
BMI, kg/m^2	21.5±2.9	21.6±2.8	0.58	23.6±2.7	23.5±2.5	0.20
SBP, mm Hg	113±13	118±14	<0.0001	118±12	121±12	<0.0001
DBP, mm Hg	70±8	71±9	0.031	74±8	74±8	0.89
Optimal normal BP, n (%)	1200 (65)	141 (47)	<0.0001	1475 (50)	384 (37)	<0.0001
Normal BP, n (%)	334 (18)	62 (21)	0.32	684 (23)	280 (27)	0.033
High normal BP, n (%)	240 (13)	57 (19)	0.0065	596 (20)	208 (20)	0.70
On antihypertension medication, n (%)	67 (4)	42 (14)	<0.0001	166 (6)	176 (17)	<0.0001
Total cholesterol, mg/dL	214±35	224±33	<0.0001	204±32	206±32	0.048
High-density lipoprotein cholesterol, mg/dL	71±17	70±17	0.51	55±5	55±15	0.79
Triglycerides, mg/dL	91±58	97±53	0.095	142±106	134±100	0.043
Uric acid, mg/dL	4.6±0.9	4.8±1.1	<0.0001	6.1±1.2	6.1±1.2	0.88
Fasting glucose, mg/dL	90±14	91±13	0.24	99±21	101±22	0.0020
Hemoglobin A1C, %	5.1±0.5	5.3±0.5	<0.0001	5.3±0.7	5.5±0.7	<0.0001
Metabolic syndrome, n (%)	59 (3)	22 (7)	0.0006	353 (12)	158 (15)	0.013
Smoking status						
Never, n (%)	1534 (83)	264 (87)	0.19	934 (32)	281 (27)	<0.0001
Former, n (%)	97 (5)	11 (4)		841 (29)	388 (37)	
Current, n (%)	210 (11)	27 (9)		1146 (39)	379 (36)	

χ^2 test was used for categorical variables, and *t* test was used for continuous variables.

and 315 (9%) of 3398 men (Table 5). Carotid plaque was found in 11 (19%) of the 57 MetS-positive female subjects and in 77 (24%) of the 315 MetS-positive male subjects. The carotid plaque prevalence was not significantly different from that observed in the MetS-negative subjects (244 of 1943 [13%] women; P=0.13; 723 of 3083 [23%] men; P=0.69; Figure 2 and Table 2). After full adjustment, MetS was not found to be significantly associated with carotid plaque or carotid intima-media thickening in either gender (Table 3).

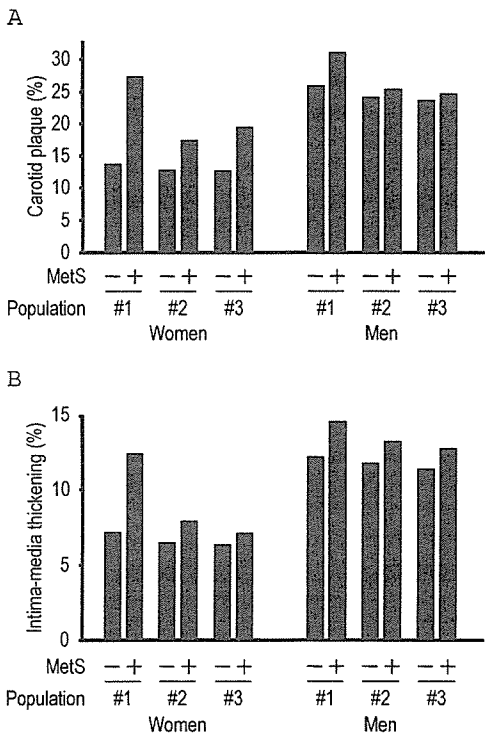

Figure 2. Prevalence of carotid plaque and carotid intima-media thickening according to the presence or absence of metabolic syndrome in the 3 target populations.

Discussion

Here, we have assessed whether MetS, as diagnosed by modified ATPIII criteria, is an independent risk factor for carotid atherosclerosis in the individuals with optimal, normal, or high-normal BP. Within this population, MetS was not found to be an independent risk factor for carotid atherosclerosis in either gender after the full-adjustment for age and other risk factors for CVD.

In several previous studies, the association between MetS and CVD has been assessed not only in individuals with diabetes[17,18] but also in nondiabetic subjects. For example, in a prospective cohort study of Finnish men who did not have CVD or diabetes at enrollment, MetS was found to be associated with a 2.6- to 3.0-fold higher mortality from CVD.[19] Another study found that MetS was associated with increased risk for CVD in middle-aged adults who did not have CVD or type 2 diabetes mellitus at enrollment.[20] These findings indicate that MetS may increase the future risk for CVD and stroke irrespective of the presence or absence of diabetes. On the other hand, Juutilainen et al[21] have reported that after adjusting for confounding factors, the presence of MetS predicted CVD mortality in nondiabetic women but not in nondiabetic men during the 18-year follow-up.

These studies may seem to substantiate the idea that MetS may be a risk for future CVD mortality, at least in women with low risk profiles. However, hypertension (defined as

TABLE 2. Logistic Regression Analysis With Metabolic Syndrome as an Independent Variable and the Carotid Plaque as a Dependent Variable

Variables	Women		Men	
	Odds Ratio (95% CI)	*P*	Odds Ratio (95% CI)	*P*
Target population 1				
Unadjusted	2.37 (1.43–3.94)	0.0008	1.29 (1.06–1.58)	0.013
Adjusted for age	2.11 (1.22–3.64)	0.0076	1.39 (1.12–1.73)	0.0030
Adjusted for age, TC, smoking status	2.04 (1.18–3.52)	0.011	1.33 (1.07–1.66)	0.011
Adjusted for age, TC, smoking status, BMI, SBP, TG, HDL-C, FPG	2.67 (1.39–5.15)	0.0033	1.14 (0.87–1.49)	0.34
Target population 2				
Unadjusted	1.43 (0.74–2.78)	0.29	1.07 (0.84–1.35)	0.59
Adjusted for age	1.29 (0.63–1.63)	0.48	1.21 (0.94–1.56)	0.15
Adjusted for age, TC, smoking status	1.23 (0.60–2.50)	0.58	1.14 (0.88–1.48)	0.31
Adjusted for age, TC, smoking status, BMI, SBP, TG, HDL-C, FPG	1.65 (0.72–3.76)	0.24	0.95 (0.70–1.28)	0.72
Target population 3				
Unadjusted	1.67 (0.85–3.26)	0.14	1.06 (0.81–1.38)	0.69
Adjusted for age	1.54 (0.75–3.19)	0.24	1.25 (0.94–1.68)	0.13
Adjusted for age, TC, smoking status	1.47 (0.71–3.04)	0.30	1.18 (0.88–1.58)	0.28
Adjusted for age, TC, smoking status, BMI, SBP, TG, HDL-C, FPG	1.84 (0.79–4.27)	0.15	0.98 (0.69–1.38)	0.91

TC indicates total cholesterol; TG, triglycerides; HDL-C, high-density lipoprotein cholesterol.

≥160/95 mm Hg or on therapy) was prevalent in >65% of the women in the study by Juutilainen et al,[21] but their results were not adjusted for BP. Similarly, Isomaa et al[18] have reported that MetS is a predictor of cardiovascular mortality and morbidity in subjects with normal glucose tolerance. However, their results were adjusted for sex and age but not for BP, although 23% to 24% of their target population included subjects with hypertension (defined as >160/90 mm Hg or on therapy). Furthermore, Wilson et al have reported that in >8 years of follow-up, MetS was found to be associated with increased risk for CVD with odds ratios of 2.3 and 2.9 in middle-aged women and men, respectively, who lacked CVD or type 2 diabetes mellitus at baseline. Again, this result was adjusted only for age, although prevalence of hypertension (defined as ≥130/85 mm Hg or on therapy) was more prevalent in individuals with MetS (89% in women and 91% in men) than in those without MetS (34% in women and 43% in men).

TABLE 3. Logistic Regression Analysis With Metabolic Syndrome as an Independent Variable and the Carotid Intima-Media Thickening as a Dependent Variable

Variables	Women		Men	
	Odds Ratio (95% CI)	*P*	Odds Ratio (95% CI)	*P*
Target population 1				
Unadjusted	1.85 (0.93–3.66)	0.78	1.23 (0.94–1.60)	0.13
Adjusted for age	1.53 (0.73–3.21)	0.26	1.37 (1.03–1.81)	0.030
Adjusted for age, TC, smoking status	1.54 (0.73–3.24)	0.26	1.28 (0.96–1.70)	0.094
Adjusted for age, TC, smoking status, BMI, SBP, TG, HDL-C, FPG	0.87 (0.37–2.05)	0.75	0.98 (0.69–1.39)	0.89
Target population 2				
Unadjusted	1.23 (0.49–3.12)	0.66	1.14 (0.84–1.55)	0.39
Adjusted for age	1.04 (0.38–2.82)	0.94	1.36 (0.98–1.88)	0.063
Adjusted for age, TC, smoking status	1.05 (0.39–2.86)	0.92	1.25 (0.90–1.73)	0.19
Adjusted for age, TC, smoking status, BMI, SBP, TG, HDL-C, FPG	0.56 (0.18–1.72)	0.31	0.93 (0.62–1.38)	0.72
Target population 3				
Unadjusted	1.13 (0.40–3.16)	0.82	1.14 (0.80–1.62)	0.46
Adjusted for age	0.94 (0.31–2.83)	0.91	1.43 (0.99–2.07)	0.060
Adjusted for age, TC, smoking status	0.95 (0.31–2.88)	0.92	1.31 (0.90–1.91)	0.16
Adjusted for age, TC, smoking status, BMI, SBP, TG, HDL-C, FPG	0.44 (0.13–1.52)	0.20	1.00 (0.64–1.57)	1.00

TC indicates total cholesterol; TG, triglycerides; HDL-C, high-density lipoprotein cholesterol.

TABLE 4. Baseline Characteristics of the Target Population 2

Variables	Women			Men		
	Carotid Plaque (−) (n=1774)	Carotid Plaque (+) (n=260)	*P*	Carotid Plaque (−) (n=2755)	Carotid Plaque (+) (n=872)	*P*
Age, y	53.8±9.9	63.3±8.9	<0.0001	53.1±10.0	62.6±9.8	<0.0001
BMI, kg/m^2	21.4±2.9	21.5±2.7	0.52	23.6±2.7	23.4±2.5	0.13
SBP, mm Hg	112±13112±13	116±14	<0.0001	118±12	120±12	<0.0001
DBP, mm Hg	70±8	70±9	0.21	74±8	74±8	0.18
Optimal normal BP, n (%)	1200 (68)	141 (54)	<0.0001	1475 (54)	384 (44)	<0.0001
Normal BP, n (%)	334 (19)	62 (23)	0.056	684 (25)	280 (32)	<0.0001
High normal BP, n (%)	240 (13)	57 (22)	0.0003	596 (22)	208 (24)	0.17
Total cholesterol, mg/dL	214±35	225±33	<0.0001	204±32	207±33	0.061
High-density lipoprotein cholesterol, mg/dL	71±17	71±17	0.63	55±15	55±15	0.88
Triglycerides, mg/dL	90±59	94±49	0.34	142±107	134±107	0.043
Uric acid, mg/dL	4.5±0.9	4.8±1.1	<0.0010	6.1±1.2	6.1±1.2	0.83
Fasting glucose, mg/dL	90±14	91±12	0.32	99±20	101±22	0.0095
Hemoglobin A1C, %	5.1±0.5	5.3±0.4	<0.0001	5.3±0.8	5.5±0.7	<0.0001
Metabolic syndrome, n (%)	53 (3)	11 (4)	0.28	307 (11)	103 (12)	0.59
Smoking status						
Never, n (%)	1471 (83)	226 (87)	0.27	871 (32)	235 (27)	<0.0001
Former, n (%)	97 (5)	11 (4)		781 (28)	316 (36)	
Current, n (%)	206 (12)	23 (9)		1103 (40)	321 (37)	

χ^2 test was used for categorical variables, and *t* test was used for continuous variables.

It has been suggested recently by several investigators that clinicians should evaluate and treat all cardiovascular risk factors regardless of whether a patient meets the diagnosis criteria for MetS.[22,23] In fact, individuals with established dyslipidemia, hypertension, and diabetes may be periodically followed-up at medical institutions and undergo medical therapy or lifestyle modification to ameliorate each of these disorders, regardless of the presence or absence of MetS. On

TABLE 5. Baseline Characteristics of the Target Population 3

Variables	Women			Men		
	Carotid Plaque (−) (n=1745)	Carotid Plaque (+) (n=255)	*P*	Carotid Plaque (−) (n=2598)	Carotid Plaque (+) (n=800)	*P*
Age, y	53.8±9.9	63.3±8.9	<0.0001	52.8±10.0	62.7±10.0	<0.0001
BMI, kg/m^2	21.4±2.9	21.6±2.7	0.37	23.5±2.6	23.3±2.5	0.21
SBP, mm Hg	112±13	116±14	<0.0001	118±12	120±12	<0.0001
DBP, mm Hg	70±8	70±9	0.26	74±8	74±8	0.44
Optimal normal BP, n (%)	1180 (68)	139 (55)	<0.0001	1409 (54)	346 (43)	<0.0001
Normal BP, n (%)	329 (19)	60 (24)	0.078	637 (25)	258 (32)	<0.0001
High normal BP, n (%)	236 (14)	56 (22)	0.0004	552 (21)	196 (25)	0.052
Total cholesterol, mg/dL	214±36	225±33	<0.0001	204±32	207±34	0.023
High-density lipoprotein cholesterol, mg/dL	71±17	71±17	0.69	55±15	56±16	0.49
Triglycerides, mg/dL	90±58	94±50	0.26	140±105	132±108	0.067
Uric acid, mg/dL	4.5±0.9	4.8±1.1	0.0014	6.1±1.2	6.1±1.1	0.69
Fasting glucose, mg/dL	89±9	90±10	0.047	95±10	96±10	0.11
Hemoglobin A1C, %	5.0±0.4	5.2±0.4	<0.0001	5.2±0.4	5.3±0.4	<0.0001
Metabolic syndrome, n (%)	46 (3)	11 (4)	0.13	238 (9)	77 (10)	0.69
Smoking status						
Never, n (%)	1448 (83)	221 (87)	0.33	829 (32)	220 (28)	0.0008
Former, n (%)	93 (5)	11 (4)		745 (29)	284 (36)	
Current, n (%)	204 (12)	23 (9)		1024 (39)	296 (37)	

χ^2 test was used for categorical variables, and *t* test was used for continuous variables.

the other hand, if individuals who do not have overt hemodynamic or metabolic abnormalities are at higher risk for atherosclerotic diseases when they have MetS, this concept may be useful for isolating individuals at higher risk for atherosclerotic diseases from those with low-risk profiles. For these reasons, we selected individuals with low-risk profiles to assess the usefulness of the MetS concept, and the odds ratio of MetS for carotid atherosclerosis has been calculated after adjusting for other conventional risk factors.

In the current study MetS was not found to be an independent predictor for either carotid plaque or carotid intima-media thickening in the individuals with optimal, normal, or high normal BP; however, we cannot jump to the conclusion that the concept of MetS is insignificant in such population. We may also have to investigate the possible association between MetS and other conditions, such as lacunar infarctions and arterial stiffness. These points should be analyzed in future studies.

The strength of our study is that we could abstract and analyze the data of individuals with optimal, normal, and high-normal BP from a large set of cross-sectional data of those who had undergone general health screening. On the other hand, our study has some limitations. For example, waist circumference data were not available in the study sample; thus, we used BMI as a surrogate of waist circumference, as has been done in several previous studies.[24,25] Although the ability of MetS to predict CVD may differ according to the criteria used[26] and ethnicity,[27] waist circumference may be a more suitable risk factor component for the definition of MetS because of its strong correlation with computed tomography measurements of abdominal fat.[28] In the near future, we are planning to validate the use of other MetS criteria, including waist circumference data, which have now been collected at our institute since 2005, in terms of isolating individuals with higher risk for early atherosclerosis from those with low-metabolic/hemodynamic risk profiles. Because our study was cross-sectional in nature, whether or not MetS, as defined here, would be useful in predicting future cardiovascular events cannot be determined. This point should also be assessed in future studies.

Perspectives

In the current study, by analyzing the cross-sectional data from individuals who had optimal, normal, or high-normal BP, we showed that MetS defined by modified ATPIII criteria was not an independent predictor for either carotid plaque or carotid intima-media thickening. A body of evidence exists that supports the notion that MetS is a risk factor for atherosclerotic disease. On the other hand, clinicians may have to evaluate and treat all of the atherogenic risk factors regardless of whether a patient meets the diagnosis criteria for MetS. If individuals with only mild hemodynamic and metabolic abnormalities are considered to be at substantially higher risk for CVDs when they have MetS, the concept of MetS would be useful in avoiding the underestimation of the cardiovascular risk in such subjects. Although our data did not support that MetS was an independent risk factor for carotid atherosclerosis in individuals without hypertension, whether the presence of MetS in such subjects would increase the possibility of the future development of CVD should be investigated in longitudinal studies.

Disclosures

None.

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OBSERVATIONS

Association Between Smoking, Hematological Parameters, and Metabolic Syndrome in Japanese Men

Cigarette smoking increases the risk for metabolic syndrome (1), and it may also affect hematological parameters (2). Because certain hematological parameters may be associated with metabolic syndrome (3), we have investigated whether the mode of association between smoking and metabolic syndrome varies according to hematological parameters.

Among individuals who had undergone a general heath screening test between 1994 and 2003, 27,972 subjects (9,729 never smokers [52.8 ± 10.7 years], 7,242 former smokers [54.8 ± 9.9 years], and 11,001 current smokers [50.4 ± 9.8 years]) answered in full a questionnaire concerning their smoking habits and were enrolled in the current study. Metabolic syndrome was defined as the presence of three or more of the following: *1*) fasting glucose ≥110 mg/dl, *2*) blood pressure ≥130/85 mmHg, *3*) triglycerides ≥150 mg/dl, *4*) HDL cholesterol <40 mg/dl, and *5*) BMI ≥25 kg/m^2. The interquartile cutoff points were 4,700, 5,500, and 6,600 cells/μl for white blood cell (WBC) count and 14.4, 15.1, and 15.7 g/dl for hemoglobin level.

Compared with the never smokers, the WBC count and hemoglobin level were significantly higher in the current smokers (5,200 ± 1,200 vs. 6,400 ± 1,800 cells/μl, $P < 0.0001$, and 14.8 ± 1.0 vs. 15.2 ± 1.0 mg/dl, $P < 0.0001$, respectively). After adjusting for age and total cholesterol level, logistic regression analysis showed that current smokers had a higher incidence of metabolic syndrome with an odds ratio (OR) of 1.59 (95% CI 1.47–1.73) compared with never smokers. Compared with the lowest quartile (Q), the incidence of metabolic syndrome was significantly more frequent in the higher quartiles of the WBC count (Q2, OR 1.73 [95% CI 1.54–1.95]; Q3, 2.50 [2.23–2.80]; and Q4, 3.80 [3.41–4.24]) and in those of the hemoglobin level (Q2, 1.65 [1.47–1.86]; Q3, 2.41 [2.15–2.70]; and Q4, 4.05 [3.63–4.53]).

The association between current smoking and metabolic syndrome was found to be statistically significant in lower quartiles of the WBC count (Q1, OR 1.40 [95% CI 1.10–1.79] and Q2, 1.36 [1.13–1.64]) but not in the higher ones (Q3, 1.02 [0.87–1.18] and Q4, 1.04 [1.89–1.21]). By contrast, the association between current smoking and metabolic syndrome was statistically significant regardless of the hemoglobin level (Q1, 1.50 [1.19–1.88]; Q2, 1.53 [1.27–1.84]; Q3, 1.43 [1.21–1.67]; and Q4 1.25 [1.09–1.43]). These results suggest that the association between smoking and metabolic syndrome may be heavily confounded by certain factors that increase the circulating WBC count.

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ORIGINAL ARTICLE

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Interferon-α/β upregulate IL-15 expression in vitro and in vivo: analysis in human hepatocellular carcinoma cell lines and in chronic hepatitis C patients during interferon-α/β treatment

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Abstract Type I interferon (IFN) possesses antiviral and antitumor activities and also having an immune regulatory effect, activating cellular immune response and upregulating several cytokines. Recent study has shown that type I IFN upregurates the dendritic cell production of IL-15 capable of activating natural killer cells and $CD8^+$ memory T lymphocytes. However, it is still unknown if type I IFN induces IL-15 production in non-immune cells and if type I IFN affects IL-15 production in vivo. The present study investigated the effect of type I IFNs on IL-15 expression in hepatocellular carcinoma (HCC) cell lines in vitro and in patients with chronic hepatitis C in vivo. When three HCC cell lines, Huh7, HepG2, and JHH4 were cultured in vitro, IFN upregulation of IL-15 expression was observed at both the mRNA and protein levels. In experiments using Huh7 cells, upregulation of IL-15 expression occurred within 24 h of the start of IFN stimulation, and both IFN-α and -β dose-dependently increased IL-15 production in the range from 100 U/ml to 10,000 U/ml of concentration. IFN-β showed stronger activity in IL-15 production induction in vitro than IFN-α. For in vivo examination, sera were obtained from 21 chronic hepatitis C patients treated with IFN and 29 healthy individuals, and the serum IL-15 level was quantified by ELISA. The serum IL-15 level of chronic hepatitis C patients before IFN treatment was similar to that of the healthy controls and significantly increased only during the IFN administration period. These results confirm that IFN-α/β induce IL-15 production and also suggest that IL-15 may be associated with type I IFN-induced immune response.

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Introduction

Interferon (IFN)-α and -β are categorized as type I IFN and possess antiviral activity useful for the treatment of chronic hepatitis C. The hepatitis C virus (HCV), the pathogen of hepatitis C, causes a persistent infection in 80% of patients exposed and leads to chronic hepatitis and hepatic fibrosis that progresses in some patients to liver cirrhosis and hepatocellular carcinoma (HCC) [1–5]. A sustained elimination of serum HCV RNA is observed in 30–40% of patients administered IFN-α or -β [6–11]. Type I IFNs also have antitumor activity and are used for the treatment of chronic myelogenous leukemia and renal cell carcinoma. Moreover, IFN treatment decreases the HCC carcinogenesis rate of chronic hepatitis C patients [12–14].

Type I IFN directly affects cells to induce the antiviral proteins 2′–5′ oligo adenyl synthetase, Mx protein and PKR protein kinase [15], and also affects tumor cells to elicit a cell-cycle arrest or an apoptosis [16–18]. Recently, it has been reported that both type I IFN-induced antiviral defense and tumor suppression are related to p53 gene expression [19]. It has also been shown that type I IFNs activate cytolytic T lymphocytes (CTL) and natural killer cells (NK) [20–22], and that they upregulate production of several T cell-derived cytokines, such as IL-1β, IL-6 and TNF-α [11, 23, 24]. This suggests that the antiviral and antitumor activity of type I IFN is associated with the immune system in vivo. However, the immunological mechanisms of type I IFN are still largely unknown.

It has been reported that type I IFN upregurates IL-15 expression from dendritic cells in vitro [25]. IL-15 is a

four-helix bundle cytokine related to IL-2, and its receptor consists of a unique α-chain and shared IL-2 receptor β- and γ-chains. In contrast to IL-2, which is mainly expressed in activated T cells, IL-15 is produced by various cells such as monocytes/macrophages, epitherial and fibroblast cells, placenta, skeletal muscle, heart, lung, kidney, and liver, but not by normal resting or activated T cells [26]. It is considered that IL-15 is essential for NK and NK-T cell development [27–29] and that it is capable of promoting proliferation, long-term survival and activation of CD8 memory T cells [30–32], suggesting that IL-15 plays a pivotal role in protective immune response. Thus, it is possible that IL-15 may be involved in type I IFN-induced immune response, but the relationship remains to be clarified. To better understand the immunobiological function of type I IFNs and to develop new therapeutic methods, it is important to investigate implications of how type I IFNs affect IL-15 production.

In this study, we attempted to determine if and how IFN-α and -β upregulate IL-15 production in human HCC cell lines. These results confirmed that IFN-α/β induce IL-15 production and show the first evidence of IFN-α/β induced IL-15 production in non-immune cells. Our study also indicated that serum IL-15 levels increase in chronic hepatitis C patients during the IFN-α or -β administration period. These data suggest the clinical significance of IL-15 in type I IFN-induced immune response.

Materials and methods

Cell culture and IFNs

HCC cell lines Huh7, HepG2 and JHH4 were maintained in Dulbecco's modified Eagle's medium supplemented with 10% heat-inactivated FBS and antibiotic agents (100 U/ml penicillin G and 100 μg/ml streptomycin) in a humidified atmosphere of 5% CO_2 at 37°C. In all experiments, 2×10^5 cells were seeded in a 6-well cell-culture plate and cultured for 24 h to allow the cells to stick to the culture plate and enter their logarithmic growth phase. After 24 h of preincubation, the medium was changed to fresh medium with or without IFNs. Natural human IFN-α (Sumiferon) and natural human IFN-β (Feron) were kindly provided by Sumitomo Pharmaceutical (Japan) and Dai-Ichi Pharmaceutical (Japan), respectively.

Patients with chronic HCV infection and controls

Twenty-one Japanese patients (12 men and 9 women; age range, 31–76 years; mean, 52.2 years) with chronic hepatitis C were studied. All patients were positive for serum HCV RNA and had elevated serum alanine aminotransferase (ALT). No patient was positive for hepatitis B surface antigen (HBsAg) or anti-human immunodeficiency virus (HIV) antibody. Twenty-nine healthy volunteers (16 men and 13 women; age range, 42–74 years; mean, 58.1 years) negative for serum HCV RNA, HBsAg, and anti-HIV antibody and without a clinical history or symptoms of liver disease were recruited as controls. Before any treatment was given, a liver biopsy was done for chronic hepatitis C patients and histological changes were evaluated. Quantification of serum HCV RNA was done for all patients before treatment by competitive polymerase chain reaction (PCR), as described previously [33]. Eleven chronic hepatitis C patients were given 6 million units of natural IFN-α (Sumiferon, Sumitomo Pharmaceutical Co., Japan) by intramuscular injection daily for 14 days, then three times weekly for 22 weeks. Another ten chronic hepatitis C patients were given 6 million units of natural IFN-β (Feron, Dai-ichi Pharmaceutical Co., Japan) by intravascular drip infusion daily for 56 days. The serum ALT level of the chronic hepatitis C patients treated with IFN was tested monthly during the observation period. A qualitative HCV-RNA examination of the serum from the chronic hepatitis C patients was done 6-months after the cessation of IFN treatment. Response to IFN treatment was classified as follows: virological responders were defined as patients in whom serum HCV-RNA was negative at 6 months after the cessation of IFN treatment and biochemical responders were defined as patients in whom serum ALT was continuously normal for 6 months after the cessation of IFN treatment. Informed consent was obtained from all patients and healthy volunteers.

RNA extraction and cDNA synthesis

Total RNA was extracted from cultured cells and liver biopsy samples using the RNeasy Mini kit (QIAGEN, Germany) and treated with RNase-Free DNase Set (QIAGEN, Germany) to remove contaminated DNA, according to the manufacturer's instruction. A concentration of isolated RNA was measured by spectrophotometer, and 200 ng of total RNA was applied to reverse transcription using Superscript II RNase H^- Reverse Transcriptase (Invitrogen, Carlsbad, CA, USA) with 50 ng of random hexamers according to the manufacturer's instructions. The complementary DNA (cDNA) solution obtained was used for the subsequent PCR.

Preparation of DNA standards

To prepare DNA standards for real-time PCR, IL-15 and β-actin genes were amplified from cDNA by PCR. The forward and reverse primers for human IL-15 were hIL-15F2: 5′-GCAGGGCTTCCTAAAACAGA-3′and hIL-15R2: 5′-GTTGTTTGCTAGGATGATCAG-3′, and those for human β-actin were hβ-actinF1: 5′-GGTCACCCACACTGTGCCCAT-3′ and hβ-actinR1: 5′-GGATGCCACAGGACTCCATGC-3′. For the PCR

of the IL-15 gene, 0.5 μmol of Tris-HCl (pH 8.4), 1.25 μmol of KCl, 37.5 nmol of $MgCl_2$, 5 nmol of dNTPs, 10 pmol each of the forward and reverse primers, 0.5 U of Platinum Taq DNA (Invitrogen, Carlsbad, USA), and 1 μl of cDNA solution were mixed with distilled water to a 50 μl final volume in a 0.5 ml tube. The mixture was incubated in a thermal cycler at 94°C for 3 min, followed by 36 cycles at 94°C for 1 min, 55°C for 1 min, 72°C for 1 min, and 72°C for a final 3 min. The PCR for β-actin was performed using the same mixture condition as the PCR for IL-15, except for the primer sets. Thermocycling conditions for the β-actin PCR consisted of an initial 94°C for 3 min, followed by 20 cycles at 94°C for 1 min, 57°C for 1 min, 72°C for 1 min, and an additional 72°C for 3 min. PCR products were applied to electrophoresis on 1% agarose gel. Specific amplification was verified according to the predicted size of each amplicon (IL-15 PCR product, 240 bp; β-actin PCR product, 350 bp). IL-15 and β-actin PCR products were then extracted from the gel using the MinElute Gel Extraction Kit (QIAGEN, Germany), according to the manufacturer's instruction. The concentration of the gel-extracted PCR products was measured by spectrophotometer. Finally, the gel-extracted IL-15 and β-actin PCR products were diluted with 0.1 × Tris-EDTA buffer.

Real-time PCR

Human IL-15 and β-actin mRNAs were quantified by real-time PCR. The primer sets for real-time PCR of the IL-15 and β-actin genes were the same used in normal PCR for a standard preparation, as described above. PCR was done using the Light Cycler (Roche, Mannheim, Germany) with LightCycler-FastStart DNA Master SYBR Green I (Roche, Mannheim, Germany). The PCR condition for IL-15 was as follows: after an initial denaturing at 95°C for 10 min, the amplification was done by 40 cycles of denaturing at 95°C for 10 s, annealing at 55°C for 10 s, and extension at 72°C for 10 s. The PCR condition for β-actin was as follows: after an initial denaturing at 95°C for 10 min, the amplification was done by 40 cycles of denaturing at 95°C for 10 s, annealing at 57°C for 10 s, and extension at 72°C for 10 s. The amplified products were monitored directly by measuring the increase of the dye intensity of the SYBR Green I that binds to the double-strand DNA amplified by PCR. The copy number of mRNA in the cDNA samples was calculated using standard amplification curves.

Assay for IL-15 concentration

The IL-15 concentration of supernatants collected from the cell cultures and serum samples was determined by use of a human IL-15 ELISA kit (Genzyme, Cambridge, MA, USA), according to the manufacturer's instructions. The absorbance at 450 nm (reference at 540 nm) was measured. The assay was done in duplicate.

Statistical analysis

Statistical analysis was done using the StatView software package (SAS Institute Inc., Cary, NC, USA). Unpaired Student's *t*-test was used to assess the statistical significance of differences in pre-treatment serum IL-15 levels between sera from controls and chronic hepatitis C patients. Paired Student's *t*-test was used to compare the serially assayed serum IL-15 of chronic hepatitis C patients. The χ^2 test was used for gender comparison of the controls and chronic hepatitis C patients. Ages differences between the controls and chronic hepatitis C patients were compared by unpaired Student's *t*-test. $P < 0.05$ was considered significant.

Results

IFN-α and -β upregulation of IL-15 production in Huh7 cells

To determine if IFN-α and -β upregulate IL-15 transcription in a HCC cell line, IL-15 mRNA expression level was quantified by RT-PCR in Huh7 cells cultured for 72 h with various concentrations of IFN-α or -β. The β-actin mRNA expression level was also determined for use in adjusting the IL-15 mRNA expression level (Fig. 1a). In comparison with the controls, the IL-15 mRNA level increased in Huh7 cells cultured with IFN-α or -β. This IL-15 increase in the Huh7 cells cultured with both IFNs was dose-dependent in the range from 100 U/ml to 10,000 U/ml of IFN concentration. The IL-15 transcription induction activity was higher in IFN-β than in IFN-α, when compared at the same concentration. These data suggest that IFN-α/β upregulated IL-15 gene transcription in this Huh7 cell line.

To verify IL-15 upreguration of type I IFNs at the protein level, IL-15 concentration in the supernatant of the Huh7 culture was determined by ELISA. Huh7 cells were cultured with or without IFNs at various concentrations. After a 72 h-culture, the IL-15 concentration in the supernatant was examined by ELISA. Figure 1b shows the IL-15 concentration in the culture supernatant of each condition. As expected from the results of IL-15 mRNA quantification, the IL-15 concentration increased in comparison with the controls in the supernatants of the Huh7 cells cultured with IFN-α or -β. The IL-15 concentration dose-dependently increased in both the IFN-α and -β cultures, and the concentration was higher in the IFN-β than in the IFN-α culture.

The Huh7 cell number was determined by flow cytometry after a 72-h culture period to eliminate the possibility that the increase in IL-15 concentration in

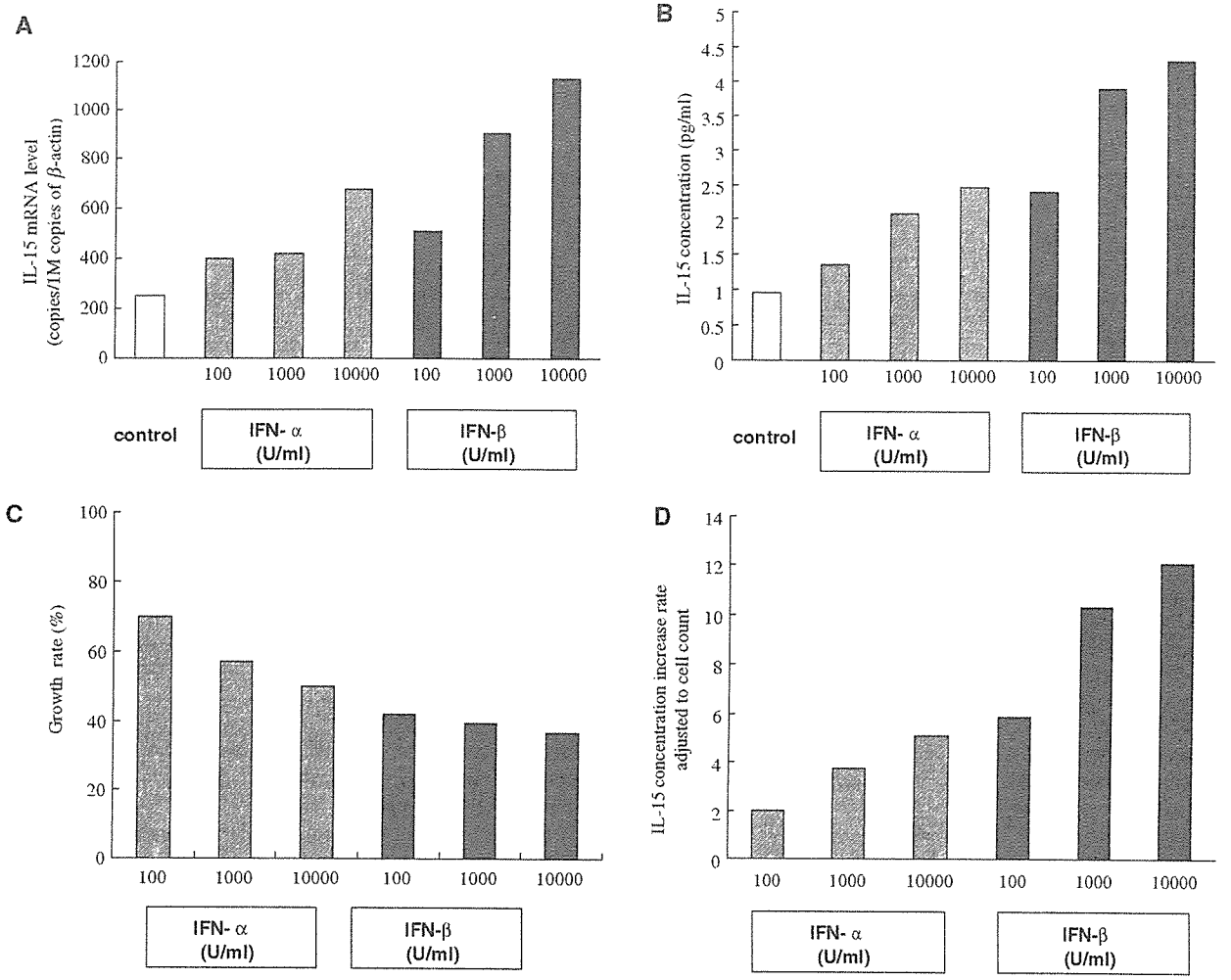

Fig. 1 The effects on IL-15 expression in Huh7 cells of IFN-α and -β at various concentrations. Huh7 cells were cultured for 72 h with or without IFN-α/β at consentrations of 100, 1,000, or 10,000 U/ml. **a** The IL-15 Expression level and the β-actin gene were quantified by reverse-transcription real-time PCR. The IL-15 mRNA expression level was adjusted to the β-actin mRNA expression level. **b** The IL-15 concentration in the culture supernatant was quantified by ELISA. **c** Cell numbers were counted by flow cytometry after detachment with trypsinization, and the cell growth rate of IFN-treated cells in comparison with the controls was calculated. **d** The supernatant IL-15 concentration was adjusted to the cell number, and the IL-15 production increase rate of IFN-treated cells in comparison with the controls was calculated. All experiments were repeated three times and representative results are depicted

Huh7 culture with IFN-α/β might have been caused by an increase in cell number. The cell count data showed that type I IFNs suppressed Huh7 proliferation in a dose dependent manner and that the suppression was stronger in Huh7 cultured with IFN-β than with IFN-α (Fig. 1c). Moreover, the IL-15 concentration of the culture supernatant was adjusted to the cell number of the corresponding culture, and the ratio of IFN-treated conditions to controls was calculated (Fig. 1d). Because the adjustment reflects the IL-15 production level of each cell, it could be shown conclusively that IFNs promote IL-15 production from Huh7 cells. Thus, these results confirmed that IFN-α/β upregulate IL-15 production from Huh7 cells.

We also examined the IL-15 mRNA expression level of Huh7 cells cultured with IFN at different time points. Huh7 cells were cultured with or without 1,000 U/ml of IFN-α/β, the cells were harvested at 24, 48 and 72 h, and the IL-15 mRNA expression level was determined (Fig. 2a). A control culture without IFN showed almost the same IL-15 mRNA expression level throughout the period of observation. In the cells cultured with IFN-α, the IL-15 mRNA level increased at 24 h, and maintained this level to 72 h In the cells cultured with IFN-β, the IL-15 mRNA level also increased at 24 h. However, an even higher level was noted at 72 h. These data suggest that upregulation of IL-15 transcription occurs within 24 h after the start of stimulation by either IFN-α or -β, but that the manner of IL-15 induction may differ between IFN-α and -β.

The IL-15 concentration of the supernatant was determined, by ELISA, in a Huh7 culture with or

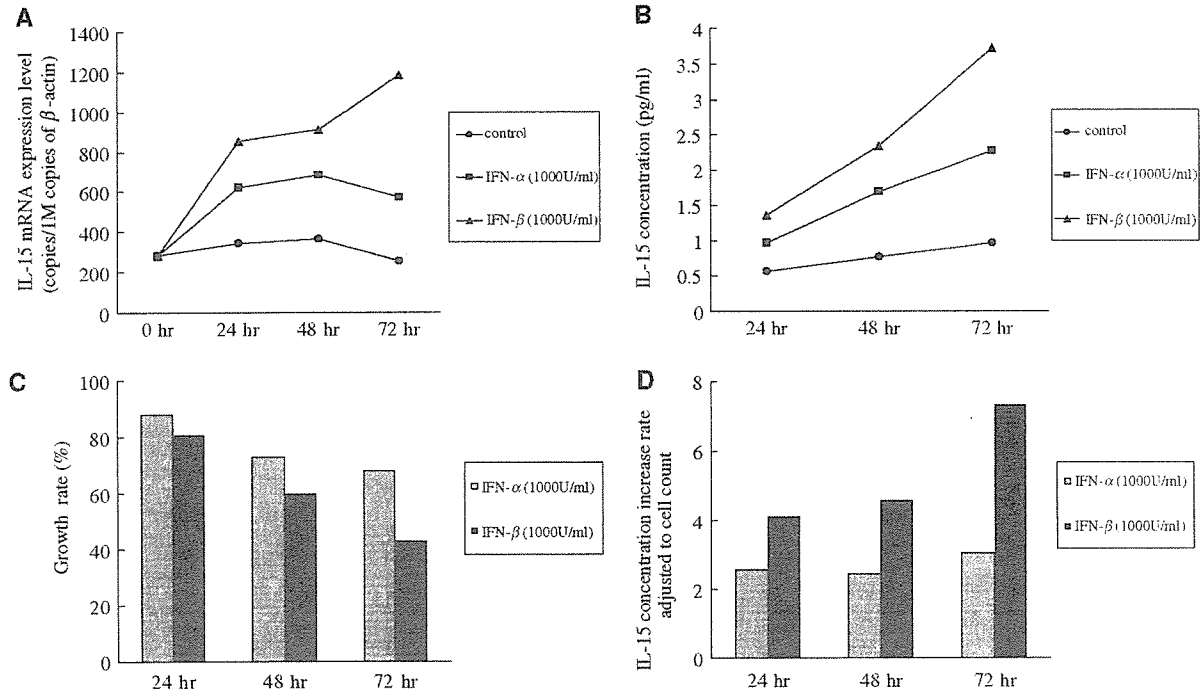

Fig. 2 The effects of IFN-α and -β on IL-15 expression in Huh7 cells at various time points. Huh7 cells were cultured with or without 1,000 U/ml IFN-α/β for 24, 48, or 72 h. **a** IL-15 and β-actin gene expression levels were quantified by reverse-transcription real-time PCR. The IL-15 mRNA expression level was adjusted to the β-actin mRNA expression levels. **b** The IL-15 concentration in the culture supernatant was quantified by ELISA. **c** Cell numbers were counted by flow cytometry after detachment with trypsinization, and the cell growth rate of IFN-treated cells in comparison with the controls was calculated. **d** The supernatant IL-15 concentration was adjusted to the cell number, and the IL-15 production increase rate of IFN-treated cells in comparison with the controls was calculated. All experiments were repeated three times and representative results are depicted

without 1,000 U/ml of IFN at 24, 48, and 72 h. At each time point, the IL-15 concentration was higher in Huh7 cultured with IFN than in the control (Fig. 2b). The Huh7 cell-growth rate was determined by cell count, and the data showed that Huh7 cell-growth was time-dependently suppressed by IFNs (Fig. 2c). To compare the IL-15 production level in each cell among different culture conditions, we adjusted the IL-15 concentration of the culture supernatant to the cell number. Figure 2d shows the IL-15 production increase rate of IFN-treated cells in comparison with the controls. At each time point, an increase in the IL-15 level was observed, even after adjustment to the cell number, and the value was higher in the culture with IFN-β than in that with IFN-α. As observed in mRNA quantification, the increase in IL-15 production from cells cultured with IFN-β continued at 72 h. These results confirm that both IFN-α and -β increase IL-15 production from Huh7 cells within 24 h and that the IFN-β activity is stronger than that of IFN-α.

IFN-α and -β upregulattion of IL-15 production in other HCC cell lines

To clarify if type I IFNs upregulate IL-15 expression in other HCC cell lines, we quantified IL-15 mRNA expressed in HepG2 and JHH4 cells cultured with or without type I IFNs. The cells were cultured with or without IFN-α or -β at a concentration of 1,000 U/ml and, after a 72-h culture, the IL-15 mRNA expression level was determined by RT-PCR. Figure 3a shows the IL-15 mRNA levels of HepG2 and JHH4 cells after culture with or without type I IFNs. In both types of cells, the IL-15 mRNA expression level was increased by type I IFNs and, at same concentration, IFN-β showed stronger IL-15 mRNA expression induction activity than IFN-α.

The IL-15 concentration of the supernatant and the number of cells were determined by ELISA and flow cytometry, respectively, in the experiments on HepG2 and JHH4 cultured for 72 h with or without type I IFNs at a concentration of 1,000 U/ml (Fig. 3b, c). The IL-15 concentration in the supernatant of both types of cells cultured with type I IFNs, increased in comparison with their respective controls and was higher in cells cultured with IFN-β than IFN-α. A suppression of cell growth was observed in both types of cells cultured with type I IFNs, and the suppression activity of IFN-β was stronger than that of IFN-α. Figure 3d shows the cell count-adjusted IL-15 level, which reflects IL-15 production from each cell. The adjusted IL-15 data clearly showed that HepG2 and JHH4 cells cultured with IFN-β produced more IL-15 than those cultured with IFN-α. These results indicate that type I IFNs upregulate IL-15