26 Fukuhara S. Measuring HRQOL of patients with skin disease. *Manual of the DLQI and the Skindex29 Japanese Version*. Shorinsha, Tokyo 2004. (In Japanese.)
27 Higaki Y, Kawamoto K, Kamo T, Ueda S, Arikawa J, Kawashima M. Measurement of the impact of atopic dermatitis on patients' quality of life: a cross-sectional and longitudinal questionnaire study using the Japanese version of Skindex-16. *J Dermatol* 2004; **31**: 977–982.
28 Takahashi N, Suzukamo Y, Nakamura M *et al.* Japanese version of the Dermatology Life Quality Index: validity and reliability in patients with acne. *Health Qual Life Outcomes* 2006; **4**: 46.
29 Tamaki K, Saeki H, Kadono T *et al.* Serum TARC/CCL17 levels as a disease marker of atopic dermatitis. *Jpn J Dermatol* 2006; **116**: 27–39. (In Japanese with English abstract.)
30 Japanese FK 506 Ointment Study Group. Clinical guidance for treatment of patients with atopic dermatitis by tacrolimus ointment 0.1% and 0.03%. *Jpn J Clin Dermatol* 2003; **57**: 1217–1234. (In Japanese.)
31 Sudilovsky A, Muir JG, Bocobo FC. A comparison of single and multiple applications of halcinonide cream. *Int J Dermatol* 1981; **20**: 609–613.
32 Bleehen SS, Chu AC, Hamann I, Holden C, Hunter JA, Marks R. Fluticasone propionate 0.05% cream in the treatment of atopic eczema: a multicentre study comparing once-daily treatment and once-daily vehicle cream application versus twice-daily treatment. *Br J Dermatol* 1995; **133**: 592–597.
33 Koopmans B, Lasthein Andersen B, Mork NJ, Austad J, Suhonen RE. Multicentre randomized double-blind study of locoid lipocream fatty cream twice daily versus locoid lipocream once daily and loco-base once daily. *J Dermatol Treat* 1995; **6**: 103–106.
34 Long CC, Finlay AY. The finger-tip unit – a new practical measure. *Clin Exp Dermatol* 1991; **16**: 444–447.
35 Long CC, Finlay AY, Averill RW. The rule of hand: 4 hand areas = 2 FTU = 1 g. *Arch Dermatol* 1992; **128**: 1129–1130.
36 Shimao S. Problems regarding side effects of corticosteroid therapy in the field of dermatology. *Nishinihon J Dermatol* 1978; **40**: 5–24. (In Japanese with English abstract.)
37 Takeda K, Harada T, Asato T, Ishimoto A, Nomoto M, Kuramoto M. Review on systemic effects of topical corticosteroids – with special reference to adrenocortical suppression following percutaneous absorption. *Igaku No Ayumi* 1977; **101**: 817–829. (In Japanese.)
38 Japanese FK 506 Ointment Study Group. Phase III comparison study on FK 506 ointment – comparison study for atopic dermatitis (trunk and extremities) between FK 506 ointment and betamethasone valerate ointment. *Nishinihon J Dermatol* 1997; **59**: 870–879. (In Japanese.)
39 The Japanese Dermatologocal Association. For patients using or going to use tacrolimus ointment (Protopic ointment). *Jpn J Dermatol* 2003; **113**: 2080–2083. (In Japanese.)
40 Hanifin JM, Paller AS, Eichenfield L *et al.* Efficacy and safety of tacrolimus ointment treatment for up to 4 years in patients with atopic dermatitis. *J Am Acad Dermatol* 2005; **53**: S186–S194.
41 Reitamo S, Rustin M, Harper J *et al.* A 4-year follow-up study of atopic dermatitis therapy with 0.1% tacrolimus ointment in children and adult patients. *Br J Dermatol* 2008; **159**: 942–951.
42 Arellano FM, Wentworth CE, Arena A, Fernandez C, Paul CF. Risk of lymphoma following exposure to calcineurin inhibitors and topical steroids in patients with atopic dermatitis. *J Invest Dermatol* 2007; **127**: 808–816.
43 Margolis DJ, Hoffstad O, Bilker W. Lack of association between exposure to topical calcineurin inhibitors and skin cancer in adults. *Dermatology* 2007; **214**: 289–295.
44 Ellis C, Luger T, Abeck D *et al.* International Consensus Conference on Atopic Dermatitis II (ICCAD II): clinical update and current treatment strategies. *Br J Dermatol* 2003; **148** (Suppl 63): 3–10.
45 Wollenberg A, Reitamo S, Girolomoni G *et al.* Proactive treatment of atopic dermatitis in adults with 0.1% tacrolimus ointment. *Allergy* 2008; **63**: 742–750.
46 Breneman D, Fleischer AB Jr, Abramovits W *et al.* Intermittent therapy for flare prevention and long-term disease control in stabilized atopic dermatitis: a randomized comparison of 3-times-weekly applications of tacrolimus versus vehicle. *J Am Acad Dermatol* 2008; **58**: 990–999.
47 Kawashima M, Hayashi N, Nogita T, Yanagisawa K, Mizuno A. The usefulness of moisturizers for maintenance of remission in atopic dermatitis. *Jpn J Dermatol* 2007; **117**: 1139–1145. (In Japanese with English abstract.)
48 Kawashima M, Harada S. A study of the effect on itch and quality of life of atopic dermatitis patients treated by standard therapy with anti-allergic drug. *Jpn J Clin Dermatol* 2006; **60**: 661–667. (In Japanese.)
49 Yanai K, Tashiro M, Okamura N *et al.* Non-sedating second-generation antihistamines: the penetration through blood-brain barrier by PET. *Nishinihon J Dermatol* 2009; **71**: 3–6. (In Japanese.)
50 Hide M, Furue M, Ikezawa Z *et al.* Guidelines for the diagnosis and treatment of urticaria and angioedema. *Jpn J Dermatol* 2000; **115**: 703–715. (In Japanese with English abstract.)
51 Zuberbier T, Bindslev-Jensen C, Canonica W *et al.* EAACI/GA^{2}LEN/EDF guideline: management of urticaria. *Allergy* 2006; **61**: 321–331.
52 Igarashi A, Nakagawa H, Takigawa M *et al.* Guidance for the use of Cyclosporin MEPC for atopic dermatitis. *Jpn J Clin Dermatol* 2009 (in press). (In Japanese.)
53 Sheehan MP, Atherton DJ. A controlled trial of traditional Chinese medicinal plants in widespread non-exudative atopic eczema. *Br J Dermatol* 1992; **126**: 179–184.

54 Sheehan MP, Rustin MH, Atherton DJ *et al.* Efficacy of traditional Chinese herbal therapy in adult atopic dermatitis. *Lancet* 1992; **340**: 13–17.
55 Fung AYP, Look PCN, Chong L-Y, But PPH, Wong E. A controlled trial of traditional Chinese herbal medicine in Chinese patients with recalcitrant atopic dermatitis. *Int J Dermatol* 1999; **38**: 387–392.
56 Kobayashi H, Ishii M, Takeuchi S *et al.* Efficacy and safety of a traditional herbal medicine, hochu-ekki-to in the long-term management of kikyo (delicate constitution) patients with atopic dermatitis: a 6-month, multicenter, double-blind, randomized, placebo-controlled study. *Evid Based Complement Alternat Med* 2008; [Jan 31 [Epub ahead of print]].
57 Kobayashi M. Investigation of scratching behavior in atopic dermatitis patients. *Jpn J Dermatol* 2000; **110**: 275–282. (In Japanese with English abstract.)
58 Brunsting LA. Atopic dermatitis (disseminated neurodermatitis) of young adults. *Arch Derm Syph* 1936; **34**: 935–957.
59 Nagai Y, Hayasaka S, Kadoi C. Cataract progression in patients with atopic dermatitis. *J Cataract Refract Surg* 1999; **25**: 96–99.
60 Taniguchi H, Ohki O, Yokozeki H *et al.* Cataract and retinal detachment in patients with severe atopic dermatitis who were withdrawn from the use of topical corticosteroid. *J Dermatol* 1999; **26**: 658–665.
61 Takehara K, Iizuka H, Ito M *et al.* Survey of health damage caused by inadequate treatment for atopic dermatitis. *Jpn J Dermatol* 2000; **110**: 1095–1098. (In Japanese with English abstract.)
62 Jekler J, Larko O. Phototherapy for atopic dermatitis with ultraviolet A(UVA), low-dose UVB and combined UVA and UVB. two paired comparison studies. *Photodermatol Photomed* 1991; **8**: 151–156.
63 Der-Petrossian M, Seeber A, Honigsmann H, Tanew A. Half-side comparison study on the efficacy of 8-methoxypsoralen bath PUVA versus narrow-band ultraviolet B phototherapy in patients with severe chronic atopic dermatitis. *Br J Dermatol* 2000; **142**: 39–43.
64 Stern RS, Lunder EJ. Risk of squamous cell carcinoma and methoxsalen (psoralen) and UV-A radiation (PUVA). *Arch Dermatol* 1998; **134**: 1582–1585.
65 Morita A. New ultraviolet phototherapy for atopic dermatitis. *Allergol Immunol* 2004; **11**: 1097–1103. (In Japanese.)
66 Saida T, Manabe M, Takenouchi T *et al.* Guidelines for management of skin cancer. *Jpn J Dermatol* 2007; **117**: 1855–1925. (In Japanese.)

APPENDIX

Table A1. Measures of severity

Mild: skin involvement by mild eruption[†] only
Moderate: <10% surface area involvement by eruption with severe inflammation (severe eruption[‡])
Severe: ≥10% but <30% skin involvement by severe eruption
Very severe: ≥30% of body involvement by severe eruption

[†]Mild eruption: primarily mild erythema, dryness and scales. [‡]Severe eruption: primarily erythema, papules, erosion, infiltration and lichenification. Cited from Guidelines for the treatment of atopic dermatitis 2008 by the Research Group granted by The Ministry of Health, Labor and Welfare.

Table A2. Evaluation criteria for degree of pruritus (Behavioral Rating Scores)

Score	Itching during the day
0	None
1	Mild itching, not annoying and not troublesome
2	Moderate itching, annoying and troublesome, may interfere with daily activities
3	Severe itching, very annoying, substantially interfering with daily activities
4	Very severe itching, interfering with daily activities
Score	**Itching during the night**
0	None
1	Mild itching, not annoying or interfering with sleep
2	Moderate itching, annoying and troublesome, may interfere with sleep
3	Severe itching, very annoying, substantially interfering with sleep
4	Very severe itching, interfering with sleep

Original Article

Association of *Chlamydophila pneumoniae* DNA in Peripheral Blood Mononuclear Cells and IgA Antibody with Atherosclerotic Diseases

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Abstract An association has been demonstrated between *Chlamydophila pneumoniae* (*C. pneumoniae*) infection and atherosclerosis, but data on the relationship between *C. pneumoniae* DNA in peripheral blood mononuclear cells (PBMC) and antibodies to this organism are lacking. We investigated the *C. pneumoniae* DNA in PBMC by polymerase chain reaction (PCR) and *C. pneumoniae* IgG and IgA antibodies by enzyme-linked immunosorbent assay of 168 patients with atherosclerotic diseases and 27 controls (healthy control subjects). *C. pneumoniae* DNA was detected for 48/168 (29%) atherosclerosis patients, IgG for 79 (47%), and IgA for 98 (58%), whereas the corresponding numbers for the controls were 11 (41%), 13 (48%), and 7 (26%). There was no significant difference of the *C. pneumoniae* DNA positivity rate between the atherosclerosis patients and the controls. However, the *C. pneumoniae* IgA-positive rate was significantly higher for carotid atherosclerosis patients who had *C. pneumoniae* DNA in their PBMC than for those without it (74% vs. 18%, $P < 0.05$). Among the patients with coronary artery disease, the *C. pneumoniae* IgA antibody positive rate was significantly higher for the patients with DNA than for those without it (68% vs. 18%, $P < 0.05$). Our results suggest that a high *C. pneumoniae* IgA antibody titer and *C. pneumoniae* DNA positivity are associated with an increased risk of atherosclerotic diseases due to endovascular *C. pneumoniae* infection.

Key words : *Chlamydophila pneumoniae* ; Atherosclerosis ; Peripheral blood mononuclear cells ; Polymerase chain reaction ; Antibody

Introduction

Chlamydophila pneumoniae (*C. pneumoniae*), an obligatory intracellular pathogen, is a common cause of respiratory tract infection[1)2)]. Several studies have already shown the presence of *C. pneumoniae* in blood stream of healthy volunteers and have indicated that more than half of the adult population has been exposed to this organism, with infection and reinfection occurring during their lifetime[1)~3)].

Atherosclerosis is a highly prevalent disease, and it is currently the greatest cause of morbidity and mortality in developed societies. Many risk factors have long been identified as contributing to the development of atherosclerosis that manifests as coronary artery disease (CAD) and myocardial infarction (MI). More recently, the possibility has been raised that infectious agents may trigger a cascade of biological and biochemical reactions leading to inflammation, atherogenesis, and vascular thrombosis. A serological association between *C. pneumoniae* and CAD was

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first demonstrated by Saikku *et al.* in 1988[4]. This association has been confirmed by subsequent studies[5], although several authors[6)~8)] have failed to find any association. We[9] previously studied a relationship between *C. pneumoniae* infection and the effect of lipid-lowering drugs on the carotid atherosclerosis (CA) of hypercholesterolemic patients.

C. pneumoniae infection reduced the effect of lipid-lowering therapy on CA, indicating that this organism may play a role in the progression of atherosclerosis. Moreover, *C. pneumoniae* has been detected in atherosclerotic tissues by polymerase chain reaction (PCR), immunohistochemistry, electron microscopy, culture, and other techniques[10].

C. pneumoniae infection generally starts in the respiratory tract, and the organisms within alveolar macrophages are probably spread systemically through the bloodstream[11)12)]. The elementary body, the metabolically inactive extracellular stage of the life cycle of chlamydiae, has never been found circulating freely in the blood, but monocytes/macrophages may carry *C. pneumoniae* from the lungs to the arterial walls. In vitro studies have indicated that *C. pneumoniae* can infect and reproduce within human endothelial cells, smooth muscle cells, and macrophages, which are key cell types involved in the process of atherosclerosis[13)14)]. Also, recent studies have detected *C. pneumoniae* DNA in the peripheral blood mononuclear cells (PBMC) of patients with CAD[15]. Because serology alone cannot diagnose vascular infection, direct detection methods based on examination of peripheral blood components may be more useful as markers of infection.

The aim of this study is to evaluate the association between *C. pneumoniae* and atherosclerosis to investigate the prevalence of *C. pneumoniae* DNA within PBMC and *C. pneumoniae* antibodies from patients with various atherosclerotic diseases.

Methods

Subjects

Between May 2001 and July 2002, 168 patients with various atherosclerotic diseases (109 men and 59 women, mean age 67 ± 9 years) and 27 controls (6 men and 21 women, mean age 59.0 ± 7.1 years) were enrolled at Kyushu University Hospital.

Selection of Subjects

All of the patients with atherosclerotic diseases admitted to Kyushu University Hospital (Fukuoka, Japan) were considered eligible for the present study. The type of atherosclerotic disease was CA for 68 patients, stable angina (SA) for 29, acute coronary syndrome (ACS) including acute myocardial infarction and unstable angina for 39, old myocardial infarction (OMI) for 32. The patients with CA had no history of CAD, but they all had an abnormal carotid intima-media thickness (IMT) and/or plaque on ultrasonography (defined as a generalized or focal IMT $\geq$ 1.1 mm, respectively)[8]. Patients with ACS had ischemic chest pain and typical changes on their electrocardiogram (ECG) and/or increased cardiac enzyme levels. Angina patients without clinical evidence of ischemia within the previous one month were defined as having SA. All of the patients with SA, ACS, or OMI (n = 100) underwent coronary angiography.

Exclusion criteria were acute infection, exacerbation of chronic infectious or inflammatory diseases, and severe liver or renal disease.

Informed consent for collection of blood or tissues was obtained from the patients (or their closest relatives). Information on each participant was compiled from the medical records and from a questionnaire about the personal medical history and lifestyle. The design of this study was approved by the Ethics Committee and the Data Protection Committee of Kyushu University Hospital (Fukuoka, Japan).

Selection of Controls

The control subjects were chosen from among, asymptomatic outpatients with hyperlipidemia who had no cardiac or infectious diseases. The absence of atherosclerosis in the controls was assessed as follows : normal 12-lead ECG, normal findings on echocardiography, < 25% stenosis of the carotid arteries on Doppler ultrasonography, and normal lower limb arteries on physical examination. A history of cardiac disease meant exclusion from the control group.

Laboratory Tests

Peripheral venous blood specimens and serum samples were obtained from all participants and stored at − 80℃ until analysis.

C. pneumoniae DNA was isolated from PBMC using the Smitest EX-R&D (Genome Science Laboratories, Fukushima, Japan) in accordance with the manufacturer's recommendations. PCR for the detection of *C. pneumoniae* was done using a *C. pneumoniae*-specific pair of primers (CP1/CP2 and CPC/CPD)[16].

C. pneumoniae IgG and IgA antibodies were measured with enzyme-linked immunosorbent assay (ELISA) kits (Hitazyme *C. pneumoniae*, Hitachi Chemical Co., Ltd., Tokyo, Japan), as described previously[8)9)17)~19)]. The IgG and IgA were positive when indices were ≥ 1. 10)[17]. When the IgG and IgA antibody detection rates by ELISA were compared with those for the microimmunofluorescence method, sensitivity was 90. 4% for IgG and 84. 6% for IgA, while specificity was 89.9% for IgG and 86.7% for IgA[18]. The rate of agreement between ELISA and Western blotting was 80.0% for IgG and 87.5% for IgA[19]. All laboratory tests were done in a blinded fashion.

Statistical Analysis

The mean levels of numerical variables were compared by the Mann-Whitney U test, while categorical variables were compared by the chi-square test or Fisher's exact test, as was appropriate. A P value < 0.05 was considered to indicate statistical significance.

Results

The characteristics of the participants are summarized in Table 1.

Compared with the controls, the patients with atherosclerosis were significantly older and were more likely to be men, to be current smokers, and to have a history of hypertension. *C. pneumoniae* DNA was found in the PBMC of 11 controls (41%), while IgG was positive in 13 controls (48%) and IgA was detected in 7 controls (26%).

The DNA detection rates of the patients with atherosclerosis and controls showed no significant difference, but the IgA positive rate was significantly higher in the patients with atherosclerosis. In contrast, the IgG positive rate was not significantly different between the two groups.

C. pneumoniae DNA was detected in the PBMC of 19 patients (28%) with CA, while IgG for *C. pneumoniae* was found in for 30 patients (44%) and IgA was detected in 32 patients (47%). *C. pneumoniae* DNA was detected in the PBMC of 10 patients (35%) with SA, while IgG for *C. pneumoniae* was found in 14 patients (48%) and IgA was detected in 15 patients (52%). *C. pneumoniae* DNA was detected in the PBMC of 9 patients (23%) with ACS, while IgG for *C. pneumoniae* was found in 16 patients (41%) and IgA was detected in 24 patients (62%). *C. pneumoniae* DNA was detected in the PBMC of 9 patients (28%) with OMI, while IgG for *C. pneumoniae* was found in 14 patients (44%) and IgA was detected in 20 patients (63%). The IgA positive rate of the patients with CAD (SA, ACS, and OMI) was significantly higher than the controls ($p < 0.05$. $p < 0.01$, $p < 0.01$, respectively).

The relationship between the *C. pneumoniae* DNA and IgG or IgA antibodies of the patients with atherosclerosis is shown in Table 2.

IgG for *C. pneumoniae* with atherosclerosis was found in 79 patients (42%) and IgA was

detected in 98 patients (58%), while IgG was positive in 13 controls (48%) and IgA was detected in 7 controls (29%). There was no significant different between the two groups. IgA positivity was significantly more common in the patients with atherosclerotic diseases (58%) than in the controls (29%) ($P < 0.05$). Among the patients with atherosclerotic diseases, the *C. pneumoniae* IgA antibody positive rate was significantly higher in the patients with DNA than in those without it (71% vs. 18%, $P < 0.05$).

The associations between *C. pneumoniae* DNA and antibody for *C. pneumoniae* IgG or IgA among the patients with each type of atherosclerotic disease and the controls are shown in Table 3.

The *C. pneumoniae* IgA-positive rate was significantly higher for CA patients who had *C. pneumoniae* DNA in their PBMC than for those without it (74% vs. 18%, $P < 0.05$). In contrast, the *C. pneumoniae* IgG- positive rate was not significantly higher for CA patients who had *C. pneumoniae* DNA than for those without it (47% vs. 64%). Among the patients with CAD (SA + ACS + OMI), the *C. pneumoniae* IgA antibody positive rate was significantly higher for the

Table 1 Characteristics of 168 patients with atherosclerotic diseases and 27 controls

Variables	Atherosclerotic diseases					Controls
	Total	CA	CAD			
			SA	ACS	OMI	
	(n=168)	(n=68)	(n=29)	(n=39)	(n=32)	(n=27)
Age, years	67 ± 9**	65 ± 8**	69 ± 8**	68 ± 10**	66 ± 11*	59 ± 7
Male	109 (65)**	25 (37)	20 (69)**	24 (62)**	28 (82)**	6 (22)
Current Smoker	65 (36)*	23 (34)*	11 (38)*	19 (49)**	12 (38)*	3 (11)
Diabetes mellitus	61 (36)	24 (32)	9 (31)	15 (39)	13 (41)	7 (26)
Hypertension	92 (55)**	35 (52)**	16 (55)**	22 (56)**	19 (59)**	5 (19)
C.pneumoniae DNA in PBMC	48 (29)	19 (28)	10 (35)	9 (23)	9 (28)	11 (41)
C.pneumoniae IgG ≥ 1.10	79 (47)	30 (44)	14 (48)	16 (41)	14 (44)	13 (48)
C.pneumoniae IgA ≥ 1.10	98 (58)**	32 (47)	15 (52)*	24 (62)**	20 (63)**	7 (26)

Values are represented as the mean ± SD or number (%).
CA, carotid atherosclerosis; SA , stable angina; ACS, acute coronary syndrome; OMI, old myocardial infarction
Chlamydophila pneumoniae ; *C. pneumoniae*
*P < 0.05 vs. controls
**P < 0.01 vs. controls

Table 2 Relationship between *C. pneumoniae* DNA and IgG or IgA seropositivity ; atherosclarosis patients versus controls

C. pneumoniae antibody	Atherosclerotic disease patients			Controls		
	Total (n=168)	DNA + (n= 47)	DNA − (n=121)	Total (n=27)	DNA + (n=11)	DNA − (n=16)
C. pneumoniae IgG ≥ 1.10	79 (42)	27 (59)	41 (34)	13 (48)	7 (64)	6 (36)
C. pneumoniae IgA ≥ 1.10	98 (58)*	33 (71)*	53 (44)	7 (29)	2 (18)	5 (31)

Values are represented as numbers (%).
DNA +, DNA positive ; DNA−, DNA negative
*P < 0.05 vs. controls

Table 3 Relationship between *C. pneumoniae* DNA and seropositivity by type of atherosclerosis

C. pneumoniae antibody	CA		CAD		Controls	
	DNA + (n= 19)	DNA − (n=49)	DNA + (n=28)	DNA − (n=72)	DNA + (n=11)	DNA − (n=16)
C. pneumoniae IgG ≥ 1.10	9 (47)	21 (43)	18 (64)	26 (36)	7 (64)	6 (38)
C. pneumoniae IgA ≥ 1.10	14 (74)*	18 (37)	19 (68)*	40 (56)	2 (18)	5 (31)

Values are represented as numbers (%).
DNA +, DNA positive ; DNA −, DNA negative
*P < 0.05 vs. controls

patients with DNA than for those without it (68% vs. 18%, $P < 0.05$). However, the *C. pneumoniae* IgG positive rate was not significantly higher for the patients who had *C. pneumoniae* DNA than for those without it (64% vs. 64%).

Discussion

The present case-control study adds new information to the growing pool of data regarding the association between atherosclerosis and *C. pneumoniae* infection. We demonstrated that a high *C. pneumoniae* IgA antibody titer and *C. pneumoniae* DNA positivity are associated with an increased risk of various atherosclerotic diseases. When we investigated whether or not detection of *C. pneumoniae* DNA (in PBMC and atherosclerotic lesions) or antibodies was associated with atherosclerosis, we found a strong association between *C. pneumoniae* DNA in PBMC and advanced atherosclerosis. In fact, both *C. pneumoniae* DNA and the *C. pneumoniae* IgA positive rate were significantly higher for patients with CAD than for controls and the IgA seropositive rate was significantly higher for patients with advanced atherosclerosis than for the controls.

Previous studies[20)~23)] have found *C. pneumoniae* DNA in PBMC at significantly higher rates in patients with atherosclerosis than in controls, but these reports have also shown that the prevalence of circulating *C. pneumoniae* DNA varies widely, being 8.8-59.4% of patients with atherosclerosis and 0.0-46.1% of controls. This wide variation of the *C. pneumoniae* positive rate was found both by using different methods and when independent investigators used similar methods. *C. pneumoniae* generally infects the respiratory tract initially, and may then be disseminated systemically by infected macrophages[11)12)]. A recent interesting study[24)] clearly reported that *C. pneumoniae* could not survive in macrophages for long term. Other group also demonstrated that lymphocytes have an important role as host cells for *C. pneumoniae*[25)]. Our results indicate that detection of chlamydiae in the bloodstream may be a general phenomenon among the adult population rather than being related to the stage of atherosclerosis, so the detection of *C. pneumoniae* DNA may not be a valid marker of current infection.

We found no correlation between the *C. pneumoniae* DNA positivity and seropositivity or antibody titers, as was previously observed in other studies[22)23)], even though IgA-positive patients were significantly more likely to have *C. pneumoniae* DNA in their PBMC than were IgA-negative patients. Most of the previous studies[22)23)], as well as our study, have shown a higher prevalence of *C. pneumoniae* antibodies than *C. pneumoniae* antigen. Therefore, current infection with *C. pneumoniae* may be indicated by the presence of antibodies in an antigen-positive patient. From our study, measurement of *C. pneumoniae* antibody seemed to be a more sensitive diagnostic method for infection than the detection of *C. pneumoniae* DNA by PCR. The presence of *C. pneumoniae* in atherosclerotic tissues is beyond doubt, but it is difficult to determine whether it is a primary cause of disease or a secondary invader, as well as whether it behaves innocently or aggressively in the latter case. The present results suggested that *C. pneumoniae* may be a secondary invader of atherosclerotic lesions and not the primary cause of atherosclerosis.

Our previous study[9)] showed that *C. pneumoniae* infection reduced the effect of lipid-lowering therapy on carotid atherosclerosis and that this organism may play a role in the progression of the atherosclerosis of hypercholesterolemic patients with advanced CA, although no association was found between *C. pneumoniae* seropositivity and mild atherosclerosis (such as early CA) in the general population[8)]. In the present study, we also found a significantly greater prevalence of IgA seropositivity in patients with more advanced atherosclerosis than in the controls, indicating that *C. pneumoniae* IgA positivity may be

associated with advanced atherosclerosis. Future research should take into account the fact that the lesions of atherosclerosis are not sterile, and studies such as clinical antibiotic intervention trials will be necessary.

The main limitation of the present study was its case-control design. Although care was taken to avoid potential biases, it is well known that retrospective studies are often unable to reproduce the associations detected in case-control studies, so further prospective studies are needed to confirm our findings.

In conclusion, a high *C. pneumoniae* IgA antibody titer and *C. pneumoniae* DNA positivity are associated with atherosclerosis but further studies are required to confirm whether or not chronic *C. pneumoniae* infection is actually an independent risk factor for atherosclerosis.

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References

1) Grayston JT, Campbell LA, Kuo CC, Mordhorst CH, Saikku P, Thom DH and Wang SP : A new respiratory tract pathogen : Chlamydia pneumoniae strain TWAR. J Infect Dis 161 : 618-625, 1990.
2) Kuo CC, Jackson LA and Campbell LA et al. : *Chlamydia pneumoniae* (TWAR). Clin Microbiol Rev 8 : 451-461, 1995.
3) Shimizu C, Nabeshima S, Kikuchi K, Furusyo N, Kashiwagi S and Hayashi J : Prevalence of antibody to Chlamydia pneumoniae in residents of Japan, the Solomon Islands, and Nepal. Am J Trop Med Hyg 67 : 170-175, 2002.
4) Saikku P, Leinonen M, Mattila K, Ekman MR, Nieminen MS, Makela PH, Huttunen JK : Valtonen V : Serological evidence of an association of a novel Chlamydia, TWAR, with chronic coronary heart disease and acute myocardial infarction. Lancet 2 : 983-986, 1988.
5) Danesh J, Collins R and Peto R : Chronic infections and coronary heart disease : is there a link? Lancet 350 : 430-436, 1997.
6) Danesh J, Whincup P, Walker M, Lennon L, Thomson A, Appleby P, Gallimore JR and Pepys MB : Low grade inflammation and coronary heart disease : prospective study and updated meta-analyses. BMJ 321 : 199-204, 2000.
7) Danesh J, Whincup P, Lewington S, Walker M, Lennon L, Thomson A, Wong YK, Zhou X and Ward M : Chlamydia pneumoniae IgA titres and coronary heart disease. Prospective study and meta-analysis. Eur Heart J 23 : 371-375, 2002.
8) Maeda N, Sawayama Y, Tatsukawa M, Shimizu C, Kashiwagi S and Hayashi J : Chlamydia pneumoniae seropositivity and early carotid atherosclerosis in a suburban Japanese population. Atherosclerosis 164 : 313-319, 2002.
9) Sawayama Y, Tatsukawa M, Okada K, Maeda N, Shimizu C, Kikuchi K and Hayashi J : Association of Chlamydia pneumoniae antibody with the cholesterol-lowering effect of statins. Atherosclerosis 171 : 281-285, 2003.
10) Taylor-Robinson D and Thomas BJ : Chlamydia pneumoniae in arteries : the facts, their interpretation, and future studies. J Clin Pathol 51 : 793-797, 1998.
11) Moazed TC, Kuo CC, Grayston JT and Campbell LA : Evidence of systemic dissemination of Chlamydia pneumoniae via macrophages in the mouse. J Infect Dis. 177 : 1322-1325, 1998.
12) Gupta S and Camm AJ : Chlamydia pneumoniae and coronary heart disease. BMJ 314 : 1778-1779, 1997.
13) Godzik KL, O'Brien ER, Wang SK and Kuo CC : In vitro susceptibility of human vascular wall cells to infection with Chlamydia pneumoniae. J Clin Microbiol 33 : 2411-2414, 1995.
14) Gaydos CA, Summersgill JT, Sahney NN, Ramirez JA and Quinn TC : Replication of Chlamydia pneumoniae in vitro in human macrophages, endothelial cells, and aortic artery smooth muscle cells. Infect Immun 64 : 1614-1620, 1996.
15) Smieja M, Mahony J, Petrich A, Boman J and Chernesky M : Association of circulating Chlamydia pneumoniae DNA with cardiovascular disease : a systematic review. BMC Infect Dis 2 : 21, 2002.
16) Tong CY and Sillis M : Detection of Chlamydia pneumoniae and Chlamydia psittaci in sputum samples by PCR. J Clin Pathol 46 : 313-317, 1993.
17) Kishimoto T, Matsushima T, Morikawa T and

Kawagoe K : Assay of specific anti-Chlamydia pneumoniae antibodies by ELISA. 3. Setting the serological criteria. Kansenshogaku Zasshi 73 : 457-466, 1999 (article in Japanese, abstract in English).

18) Numazaki K, Ikebe T and Chiba S : Detection of serum antibodies against Chlamydia pneumoniae by ELISA. FEMS Immunol Med Microbiol 14 : 179-183, 1996.

19) Kishimoto T, Kubota Y, Matsushima T, Izutsu H, Matsumoto A, Soejima R, Numazaki K, Chiba S, Yamazaki T, Sasaki N, Kaku M, Shimada J, Iwasaki E, Baba M, Koori Y, Aihara M, Chikumi H, Kosaba S, Nonaka Y, Ouchi K, Yamamoto T, Kashiwagi S, Kawayama T, Ohizumi K, Nagai H et al. : Assay of specific anti-Chlamydia pneumoniae antibodies by ELISA. 2. studies on clinical usefulness and serological diagnostic standards. Kansenshogaku Zasshi 70 : 830-839, 1996 ; (article in Japanese, abstract in English).

20) Freidank HM, Lux A, Dern P, Meyer-Konig U and Els T : Chlamydia pneumoniae DNA in peripheral venous blood samples from patients with carotid artery stenosis. Eur J Clin Microbiol Infect Dis 21 : 60-62, 2002.

21) Boman J, Soderberg S, Forsberg J, Birgander LS, Allard A, Persson K, Jidell E, Kumlin U, Juto P, Waldenstrom A and Wadell G : High prevalence of Chlamydia pneumoniae DNA in peripheral blood mononuclear cells in patients with cardiovascular disease and in middle-aged blood donors. J Infect Dis 178 : 274-277, 1998.

22) Wong YK, Dawkins KD and Ward ME : Circulating Chlamydia pneumoniae DNA as a predictor of coronary artery disease. J Am Coll Cardiol 34 : 1435-1439, 1999.

23) Sessa R, Di Pietro M, Schiavoni G, Santino I, Cipriani P, Romano S, Penco M and del Piano M : Prevalence of Chlamydia pneumoniae in peripheral blood mononuclear cells in Italian patients with acute ischaemic heart disease. Atherosclerosis 159 : 521-525, 2001.

24) Wolf et al. : Infect. Immune., 73 : 4560-4570, 2005.

25) Haranaga S, Yamaguchi H, Friedman H, Izumi S and Yamamoto Y : Chlamydia pneumoniae infects and multiplies in lymphocytes in vitro. Infect. Immume., 69 : 7753- 7759, 2001.

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PBMC 中の *Chlamydophila pneumoniae* DNA と *Chlamydophila pneumoniae* 抗体との関連

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Chlamydophila pneumoniae (*C. pneunomiae*) 感染は動脈硬化に関与しているといわれているが, 末梢血中の *C. pneunomiae* DNA と *C. pneunomiae* 抗体との関連を示した報告は少ない. 私共は 168 例の動脈硬化性疾患群と 27 例のコントロール群に対して, ELISA 法による *C. pneunomiae* 抗体測定と同時に, PCR 法を用いて末梢血中の *C. pneunomiae* DNA を測定し比較検討した. 動脈硬化性疾患群において, *C. pneunomiae* の DNA の検出率は 48/168 (29%), IgG 抗体陽性率は 79/168 (47%), IgA 抗体陽性率は 98/168 (58%) であった. 一方, コントロール群では, DNA の検出率は 11/27 (41%), IgG 抗体陽性率は 13/27 (48%), IgA 抗体陽性率は 7/27 (26%) であった. DNA の検出率は, 両群に有意差は認めなかったが, 末梢血中の DNA 陽性例における IgA 抗体陽性率は, 動脈硬化性疾患群 (74%) では, コントロール (18%) と比較して有意に高く ($p < 0.05$), その中の冠動脈疾患 (急性冠症候群, 安定狭心症, 不安定狭心症) においても, コントロールと比較して有意に高かった ($p < 0.05$). これらの結果より *C. pneunomiae* における IgA 抗体高値かつ DNA 陽性の場合は, 血管内 *C. pneunomiae* 感染による動脈硬化性疾患発症の危険因子に深く関与している可能性が示唆された.

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The longitudinal quantitative assessment by transient elastography of chronic hepatitis C patients treated with pegylated interferon alpha-2b and ribavirin

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ABSTRACT

The aim of this study was to assess the association between liver stiffness measured by transient elastography (FibroScan®) and the efficacy of pegylated interferon alpha-2b plus ribavirin combination treatment for patients with chronic hepatitis C virus (HCV) infection. We prospectively studied 145 Japanese patients with chronic HCV infection. FibroScan was done at baseline, at the end of treatment, and at 48 and 96 weeks after the end of treatment. The FibroScan values were significantly decreased for sustained virological response (SVR) patients (the mean rate of change; −16.2%, −32.2% and −43.5%) in comparison with non-SVR patients (−7.2%, −2.1% and +17.3%) at the end of treatment ($P = 0.0127$), and 48 weeks ($P < 0.0001$) and 96 weeks ($P < 0.0001$) after the end of treatment. Among the non-SVR patients, the FibroScan values were significantly decreased for patients with biochemical response (BR) (−17.9%, −30.0% and −27.1%) in comparison with non-BR (−4.1%, +6.4% and +30.6%) at the end of treatment ($P = 0.0270$), and 48 weeks ($P < 0.0001$) and 96 weeks ($P < 0.0001$) after the end of treatment. The FibroScan values may predict a progressively better clinical outcome for patients with successful virological and biochemical responses.

1. Introduction

Hepatitis C virus (HCV) infection is a main cause of chronic viral hepatitis worldwide. Chronic hepatitis can lead to cirrhosis and hepatocellular carcinoma (HCC) (Seeff, 2002; Hayashi et al., 2000). Antiviral treatment with interferon (IFN) for chronic HCV infection can induce viral clearance and biochemical and histological improvement (Davis et al., 1989; Hayashi et al., 1994). Pegylated interferon (PEG-IFN) alpha in combination with ribavirin (RBV), which aims at viral eradication (Poynard et al., 2002a; Furusyo et al., 2008), has contributed to a reduction in the relapse rate and a significant increase in the rate of sustained virological response (SVR) compared with standard IFN monotherapy (Hayashi et al., 1994; Zeuzem et al., 2000; Lindsay et al., 2001). IFN treatment has been reported to be responsible for the regression of liver fibrosis in patients with SVR (Shiratori et al., 2000; Furusyo et al., 1997). Even if a virological response with IFN treatment was not obtained, the deterioration of compensated cirrhosis was prevented and the development of HCC was inhibited (Nishiguchi et al., 1995; Veldt et al., 2004; Kashiwagi et al., 2003), thereby increasing the survival rate (Poynard et al., 2002a).

Liver biopsy had long remained the gold standard for staging fibrosis. However, liver biopsy is no more considered as a perfect methodology because of the invasive nature of the procedure, sampling error and inter-observer variability (Regev et al., 2002; Manning and Afdhal, 2008). Therefore, further testing strategies are needed for assessment of the liver status of patients with liver diseases.

Transient elastography (FibroScan®; Echosens, Paris, France) has been proposed as a promising, rapid, noninvasive and reproducible method for measuring liver stiffness (Sandrin et al., 2002). We previously reported a clinical assessment of FibroScan among patients with chronic hepatitis B and C (Ogawa et al., 2007). The values measured by FibroScan (FibroScan values) have been significantly correlated with histopathological staging of percutaneous liver biopsy and have been shown to be more accurate than biochemical scores such as the aspartate aminotransferase (AST)-to-platelet ratio (APRI) and markers of liver fibrosis (e.g. hyaluronic acid and type IV collagen), which are products of the degradation or synthesis of the extracellular matrix.

It has been repeatedly observed that at least two biomarkers FibroTest® (Biopredictive, Paris, France) and FibroScan (Manning and Afdhal, 2008; Calès et al., 2008; Friedrich-Rust et al., 2008; Poynard et al., 2008; Shaheen et al., 2007) have the same

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diagnostic value as a 20–25 mm liver biopsy. In case of discordance between FibroTest and liver biopsy, half of failures are attributable to FibroTest and half to the biopsy (Poynard et al., 2004; Ngo et al., 2006). Moreover, FibroTest has already demonstrated a similar prognostic value as biopsy (Ngo et al., 2006). In a few countries, biomarkers are recommended by health authorities as first line test in patients with chronic hepatitis C, and biopsy only if biomarkers are not interpretable (Manning and Afdhal, 2008).

The aim of the present long-term prospective study was to evaluate the association between liver stiffness measured by FibroScan and the efficacy of combined PEG-IFN alpha-2b plus RBV treatment for patients with chronic HCV infection.

2. Materials and methods

2.1. Patients

We prospectively studied a total of 145 patients infected with chronic HCV infection. Of the 145 patients, 19 (13.1%) refused antiviral treatment because of financial problems or anxiety about the possibility of adverse effects and low initial fibrosis degree (low FibroScan values); however, 4 (21.1%) of them underwent liver biopsy. The 19 untreated patients were followed for the same 3-year period as the treated patients. Of the remaining 126 patients, 118 (93.7%) underwent liver biopsy, all of whom were treated with PEG-IFN alpha-2b plus RBV treatment between January 2005 and July 2006 and followed up for 2 years after the end of treatment.

All patients satisfied the following criteria: (1) chronically infected with HCV and (2) a history of an increased alanine aminotransferase (ALT) level for over 6 months. Exclusion criteria for the study were: (1) positivity for antibody to human immunodeficiency virus (HIV) or positivity for both hepatitis B surface antigen and anti-HCV; (2) clinical or biochemical evidence of hepatic decompensation; (3) excessive active alcohol (i.e. ethanol) consumption (>60 g/day) or drug abuse; (4) suspected hepatocellular carcinoma; or (5) treatment with antiviral or immunosuppressive agents prior to enrollment. Patients who fulfilled the above criteria were recruited at Kyushu University Hospital.

Informed consent was obtained from all patients before enrollment. The study was conducted in accordance with the ethical guidelines of the Declaration of Helsinki and the International Conference on Harmonization of guidelines for good clinical practice.

2.2. Clinical and laboratory assessment

Clinical parameters included aspartate aminotransferase, ALT, platelet count, type IV collagen, prothrombin time, HCV genotype and HCV RNA. We also calculated APRI, using 45 IU/L as the upper limit of the normal AST range (ULN), as previously recommended for evaluating liver fibrosis (Wai et al., 2003):

$$\text{APRI} = \frac{\text{AST (/ULN)} \times 100}{\text{platelet count } (\times 10^9/\text{L})}$$

Body mass index (BMI) was calculated as weight in kilograms/height in square meters. Serum levels of AST, ALT, type IV collagen and HCV RNA, platelet counts, prothrombin time and HCV genotype were measured by standard laboratory techniques at a commercial laboratory (MBC Laboratory, Tokyo, Japan).

2.3. Transient elastography (FibroScan)

FibroScan was done in the right lobe of the liver through the intercostal spaces with the patient lying in the dorsal decubitus position with the right arm in maximal position. The tip of the probe transducer was covered with coupling gel and placed on the skin, between the ribs at the level of the right lobe of the liver. The operator, assisted by an ultrasonic time-motion image, located a liver portion at least 6 cm thick and free of large vascular structures. Once the measurement area had been located, the operator pressed the probe button to start acquisition. The elasticity was automatically calculated by the apparatus and the data were shown as kiloPascal (kPa). All examinations were performed by four accomplished operators (EO, KT, HT, and SO) of our department who individually experienced over 100 examinations. Only liver stiffness measurements obtained with at least six successful acquisitions and a success rate of at least 60% were considered reliable. The validity of FibroScan values depends on an interquartile range of all successful measurements (IQR/M) of less than 30% of median values (Poynard et al., 2008; Lucidarme et al., 2008). The mean IQR/M of the present study was 21.6% and no case with IQR/M > 30% was found. The first measurement of liver stiffness by FibroScan was performed within 2 weeks before liver biopsy examination.

2.4. Liver histology and quantification of liver biopsy

Liver biopsy was done for 122 (84.1%) of the 145 chronic HCV infected patients and was performed by experienced hepatologists with a 16-G disposable needle (Bard® Monopty®; C.R. Bard, Covington, GA) under ultrasound guidance. The median liver biopsy length was 18 mm (minimal length was 15 mm). Liver biopsy specimens were fixed in formalin and paraffin was embedded. All biopsy specimens were analyzed by two experienced pathologists who were blinded to the clinical data. For each specimen, the stage of fibrosis and the grade of activity were established according to the following criteria (Bedossa and Poynard, 1996). Fibrosis was staged on a 0–4 scale as follows: F0 = no fibrosis, F1 = portal fibrosis without septa, F2 = portal fibrosis and few septa, F3 = numerous septa without cirrhosis, and F4 = cirrhosis. The grading of activity, including the intensity of the necroinflammation, was scored as follows: A0 = no histological activity, A1 = mild activity, A2 = moderate activity, and A3 = severe activity.

2.5. Therapeutic protocol

All patients were treated with a weight-based, 1.5 μg/kg weekly dose of subcutaneous PEG-IFN alpha-2b (PegIntron A®; Schering-Plough, Osaka, Japan). In combination with PEG-IFN alpha-2b, RBV (Rebetol®; Schering-Plough) was given orally at a daily dose of 600–1000 mg based on bodyweight (600 mg for patients weighing < 60 kg, 800 mg for those weighing 60–80 kg, and 1000 mg for those weighing > 80 kg). The length of treatment was 48 weeks for genotype 1b and 24 weeks for genotype 2. The above durations and dosages are those approved by the Japanese Ministry of Health, Labor and Welfare. Patients were considered to have RBV-induced anemia if the hemoglobin level decreased to < 100 g/L. In such cases, a reduction in the dose of RBV was required. Some patients also had PEG-IFN alpha-2b induced psychological adverse effects or a decrease of white blood cell and platelet count. In such cases, a reduction in the dosage of PEG-IFN alpha-2b was required. Both PEG-IFN alpha-2b and RBV were discontinued if the hemoglobin level, white blood cell count, or platelet count fell below 85 g/L, 1×10^9/L, or 2.5×10^9/L, respectively. The treatment was discontinued if severe general fatigue, hyperthyroidism, interstitial pneumonia, or severe hemolytic problems developed, continuation of treatment was judged not to be possible by the attending physician, or the patient desired discontinuation of treatment. All patients received at least 80% or more of the target dosage of PEG-IFN alpha-2b and 60% or over of the RBV, because the condition under the sufficient dosage were needed to estimate between treatment response and liver disease progression accurately.

Table 1
Baseline characteristics of 145 patients with chronic HCV infection.

Characteristics	Non-treated (n = 19)	PEG-IFN alpha-2b combination with RBV treatment		
		SVR (n = 57)	Non-SVR (n = 69)	P-value*
Male/Female (n)	9/10	30/27	25/44	0.0655
Age (years)	63.8 ± 9.2	52.7 ± 13.2	60.3 ± 9.3	0.0003
Body mass index (kg/m^2)	22.2 ± 3.4	23.1 ± 3.1	22.8 ± 3.1	0.5912
Aspartate aminotransferase (AST) (IU/L)	61.2 ± 29.5	65.0 ± 39.2	65.8 ± 42.4	0.9192
Alanine aminotransferase (IU/L)	69.7 ± 50.9	88.3 ± 73.7	72.7 ± 53.2	0.1962
Platelet count (10^9/L)	154 ± 52	155 ± 45	154 ± 51	0.9294
Type IV collagen (ng/mL)	180 ± 68	172 ± 69	192 ± 88	0.1750
AST/Platelet count	1.39 ± 0.89	1.37 ± 0.99	1.35 ± 1.19	0.9100
Prothrombin time (%)	94.4 ± 15.9	90.2 ± 8.7	88.2 ± 13.1	0.3414
HCV genotypes 1b/2 n	19/0	34/23	65/4	<0.0001
Serum HCV RNA level (kIU/mL)	3207 ± 1542	1565 ± 1645	2014 ± 1455	0.1062
Liver histology				
Stage of fibrosis				0.7225
F0 (%)	1 (25.0)	7 (13.2)	7 (10.8)	
F1 (%)	0 (0.0)	10 (18.9)	17 (26.2)	
F2 (%)	1 (25.0)	19 (35.8)	16 (24.6)	
F3 (%)	2 (50.0)	11 (20.8)	15 (23.1)	
F4 (%)	0 (0.0)	6 (11.3)	10 (15.4)	
Grade of activity				0.7243
A0 (%)	0 (0.0)	0 (0.0)	0 (0.0)	
A1 (%)	1 (25.0)	17 (32.1)	21 (32.3)	
A2 (%)	2 (50.0)	34 (64.2)	39 (60.0)	
A3 (%)	1 (25.0)	2 (3.8)	5 (7.7)	
Not determined	15	4	4	

Data are shown by the mean ± standard deviation. PEG-IFN, pegylated interferon; RBV, ribavirin.
* P-values were analyzed between SVR and non-SVR patients.

2.6. Efficacy of treatment

SVR was defined as serum HCV RNA undetectable at 24 weeks after the end of treatment. This efficacy variable, SVR, was defined as non-detectable HCV RNA as measured by the COBAS® Amplicor® HCV Monitor Test (version 2.0), and the results were labeled as positive or negative. The lower limit of detection was 50 IU/mL (0.5 kIU/mL) (Lee et al., 2000).

2.7. Assessment of biochemical response (BR) among non-SVR patients

We evaluated BR among non-SVR patients after the end of treatment. Patients who had continuous ALT levels under 30 IU/L every month for 96 weeks after the end of treatment were defined as BR.

2.8. Determination of HCV RNA level and HCV genotype

During the treatment period, HCV RNA was analyzed by the COBAS® Amplicor® HCV Monitor assay (version 2.0; Roche Diagnostics, Tokyo, Japan), with a lower limit of quantitation of 5000 IU/mL and an outer limit of quantitation of 5,100,000 IU (5100 kIU)/mL. The COBAS® Amplicor® HCV Monitor assay (version 2.0) is a semi-automated nucleic acid amplification assay, consisting of manual sample preparation and automated reverse transcription (RT), amplification and detection steps on the COBAS® Amplicor® Analyzer. HCV genotype was determined by RT-PCR using universal and type-specific primers from the putative C gene of the HCV genome, according to Okamoto et al. (1992) and the genotype was classified into the type 1b or type 2a or 2b based on Simmonds et al. (1994).

2.9. Statistical analysis

Statistical analysis was done with BMDP statistical software for the IBM 3090 system computer (BMBD Statistical Software, Inc., Los Angeles, CA). Continuous data were expressed as mean values, mean ± standard deviation (SD) of the mean. The paired t-test, unpaired t-test, Mann–Whitney U-test or Kruskal–Wallis non-parametric analysis of variance was used for the analysis. The area under the receiver operating characteristic curve (AUROC) analysis was done to evaluate the relationship between histological findings and FibroScan values. The cutoff values were selected from the receiver operating characteristic (ROC) curve to maximize total sensitivity and specificity. A P-value less than 0.05 was regarded as statistically significant.

3. Results

3.1. Characteristics of patients

The major clinical and biochemical parameters of the patients at entry (baseline) are summarized in Table 1. The mean FibroScan values were significantly higher in the treated group (10.2 kPa) than in the non-treated group (7.6 kPa) (P = 0.0406). Of the 126 treated patients, 57 (45.2%) achieved SVR. The median age was significantly younger in the SVR group (52.7 years) than in the non-SVR group (60.3 years) (P = 0.0003). The rate of SVR was higher for patients with genotype 2 (85.2%) than for those with genotype 1b (34.3%) (P < 0.0001). No significant differences between the SVR and non-SVR groups were found for gender, BMI, AST, ALT, platelet count, type IV collagen, APRI, prothrombin time, or serum HCV RNA level.

3.2. Relationship between liver fibrosis and FibroScan values

Fig. 1a and b shows the distribution of FibroScan values according to fibrosis stage and activity grade, respectively. The median values of the patients were 4.8 kPa, 7.1 kPa, 8.5 kPa, 12.8 kPa and 19.5 kPa for F0, F1, F2, F3 and F4, respectively. The FibroScan values were significantly correlated with fibrosis stage (r = 0.807, P < 0.0001) and were also significantly increased in accordance with

Table 2
Optimal cutoff of FibroScan values for the determination of histological fibrosis stage in 122 biopsy-received patients with chronic HCV infection.

	Histological fibrosis stage by liver biopsy			
	$F=1$ (F0 vs. F1)	$F=2$ (F1 vs. F2)	$F=3$ (F2 vs. F3)	$F=4$ (F3 vs. F4)
Number	15/27	27/36	36/28	28/16
Cutoff value[a] (kPa)	6.1	7.2	10.3	14.9
AUROC	0.81	0.66	0.88	0.94
Sensitivity (%)	63.0	66.7	89.3	100.0
Specificity (%)	86.7	66.7	77.8	82.1
Positive predictive value (%)	89.5	66.7	75.8	76.2
Negative predictive value (%)	56.5	60.0	90.3	100.0
Positive likelihood ratio	4.72	2.00	4.02	5.60

AUROC, area under the receiver operating characteristic curve.
[a] The opitimal cutoff value is the one that gives the higher total sensitivity and specificity.

the grade of activity for the patients ($r=0.343$, $P<0.0001$). Table 2 shows the optimal liver stiffness cutoff values obtained for sensitivity and specificity. In analyses of adjacent fibrosis stages (F0 vs. F1, F1 vs. F2, F2 vs. F3 and F3 vs. F4), four threshold FibroScan values were identified: ≥6.1 kPa for F1 (sensitivity 63.0%, specificity 86.7%); ≥7.2 kPa for F2 (sensitivity 66.7%, specificity 66.7%); ≥10.3 kPa for F3 (sensitivity 89.3%, specificity 77.8%) and 14.9 kPa for F4 (sensitivity 100%, specificity 82.1%). The corresponding AUROC were 0.81 for F0 vs. F1, 0.66 for F1 vs. F2, 0.88 for F2 vs. F3 and 0.94 for F3 vs. F4.

3.3. *The longitudinal FibroScan values for the PEG-IFN alpha-2b plus RBV combination treatment (Fig. 2a and b)*

The baseline mean FibroScan values were 10.3 ± 4.8 kPa, 10.0 ± 5.5 kPa, and 7.6 ± 3.9 kPa for SVR ($n=57$), non-SVR ($n=69$), and non-treated patients ($n=19$), respectively. For SVR patients, the mean FibroScan values were 8.3 kPa, 6.6 kPa and 5.4 kPa at week 0 and at 48 and 96 weeks after the end of treatment, respectively. For non-SVR patients, the mean FibroScan values were 9.0 kPa, 9.5 kPa and 11.4 kPa at week 0 and at 48 and 96 weeks after the end of treatment, respectively.

The changes of the FibroScan values of each patient at the end of treatment, 48 weeks and 96 weeks after the end of treatment were calculated with the entry values as the estimated standard. Significant differences were found between SVR (−16.2%, −32.2% and −43.5%) and non-SVR patients (−7.2%, −2.1% and +17.3%) in the mean rate of change of FibroScan values between each testing point ($P=0.0127$, $P<0.0001$ and $P<0.0001$, respectively). For the untreated patients, the mean FibroScan values increased to 8.3 kPa, 9.8 kPa and 10.6 kPa (+12.9%, +38.0% and +49.1%, respectively) at each testing point. Significant differences were also found between treated patients (SVR and non-SVR) and untreated patients in the mean rate of change of FibroScan values.

3.4. *The longitudinal FibroScan values classified according to the BR and non-BR of non-SVR patients (Fig. 3a and b)*

Of the 69 non-SVR group patients, 16 (23.2%) achieved BR. The baseline mean FibroScan values were 9.3 ± 5.2 kPa and 10.2 ± 5.6 kPa for BR ($n=16$) and non-BR ($n=53$) patients, respectively. For BR patients, the mean FibroScan values were 7.4 kPa, 6.2 kPa and 6.7 kPa at week 0 and at 48 and 96 weeks after the end of treatment, respectively. For non-BR patients, the mean FibroScan

Table 3
Differences of the mean rate of changes of FibroScan values after PEG-IFN alpha-2b plus RBV treatment, classified by age group and gender.

	n	PEG-IFN alpha-2b combination with RBV treatment					
		Week 0	P-value*	Week 48	P-value*	Week 96	P-value*
SVR patients							
Age group			0.7995		0.4128		0.3874
<60 years	22	−16.3		−30.7		−42.1	
≥60 years	35	−15.9		−34.6		−45.9	
Gender			0.4517		0.6278		0.1740
Men	30	−17.7		−31.1		−46.3	
Women	27	−14.4		−33.4		−40.5	
Fibrosis stage			0.6653		0.2369		0.0927
≤F2	36	−16.1		−31.1		−42.0	
≥F3	17	−18.3		−37.4		−50.0	
Non-SVR patients							
Age group			0.7995		0.3478		0.3998
<60 years	27	−6.4		−6.9		11.5	
≥60 years	42	−7.8		1.1		20.9	
Gender			0.3218		0.1625		0.4507
Men	25	−10.8		−9.7		11.8	
Women	44	−5.3		2.3		20.4	
Fibrosis stage			0.6720		0.4893		0.4309
≤F2	40	−7.3		−5.0		13.9	
≥F3	25	−9.7		1.1		23.2	

PEG-IFN, pegylated interferon; RBV, ribavirin; SVR, sustained vilorogical response; week 0, at the end of treatment; week 48, 48 weeks after the end of treatment; week 96, 96 weeks after the end of treatment. The rates of changes of FibroScan values of each patient at the end of treatment, 48 weeks and 96 weeks after the end of treatment were calculated by the entry values as the estimated standard.
* P-values were analyzed at each point between age groups, between genders and between fibrosis stages.

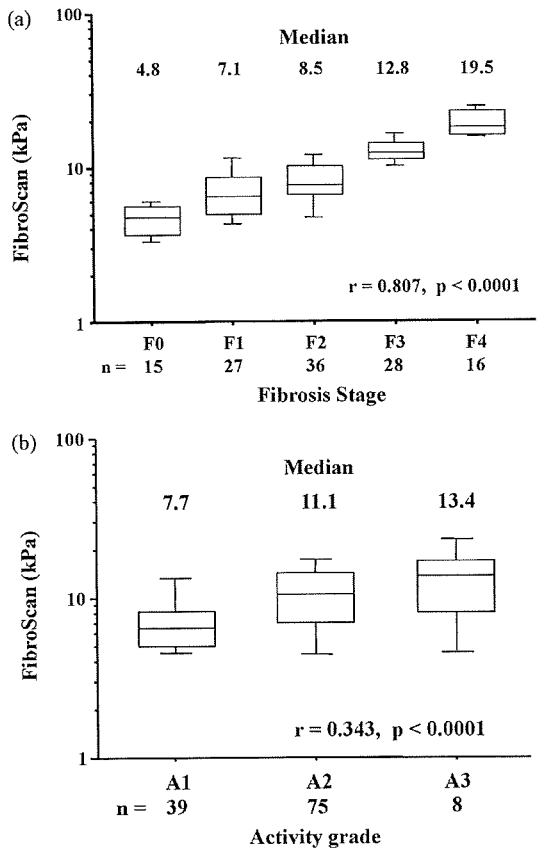

Fig. 1. FibroScan values for each fibrosis stage (a) and activity grade (b) of 122 biopsy-received patients with chronic hepatitis C virus infection. The vertical axis is a logarithmic scale. The bottom and the top boxes are the first and third quartiles, respectively. The length of the box represents the interquartile range within which 50% of the values are located. The lines through the middle of the boxes represent the median. The error bars are the minimum and maximum values.

values were 9.5 kPa, 10.5 kPa and 12.8 kPa at week 0 and at 48 and 96 weeks after the end of treatment, respectively. For BR patients, the mean rate of change of FibroScan values indicated improvement (−17.9%, −30.0% and −27.1% at weeks 0, 48 and 96 after the end of treatment, respectively) with antiviral treatment, in spite of there not being a virological effect. On the other hand, for non-BR patients, the mean rate of change of FibroScan values showed worsening over time (−4.1%, +6.4% and +30.6% at weeks 0, 48 and 96 after the end of treatment, respectively). A significant difference was found between BR and non-BR patients in the mean rate of change of FibroScan values in each period ($P = 0.0270$, $P < 0.0001$ and $P < 0.0001$, respectively). Moreover, although a significant difference was found between non-BR (+6.4%) and non-treated patients (+38.0%) at week 48 after the end of treatment ($P < 0.0001$), no significant difference was found between non-BR (+30.6%) and non-treated patients (+49.1%) at week 96 after the end of treatment ($P = 0.0835$) in the mean rate of change of FibroScan values. In other analyses, no significant difference was found between the longitudinal rate of change of FibroScan values and age (<60 and ≥60), gender or fibrosis stage (≤F2 and ≥F3) for both SVR and non-SVR patients, as shown in Table 3.

4. Discussion

The present prospective study consists of a demonstration of the association between liver stiffness measured by FibroScan and the efficacy of PEG-IFN alpha-2b plus RBV treatment. Recent reports have shown that treatment for chronic hepatitis C is associated with

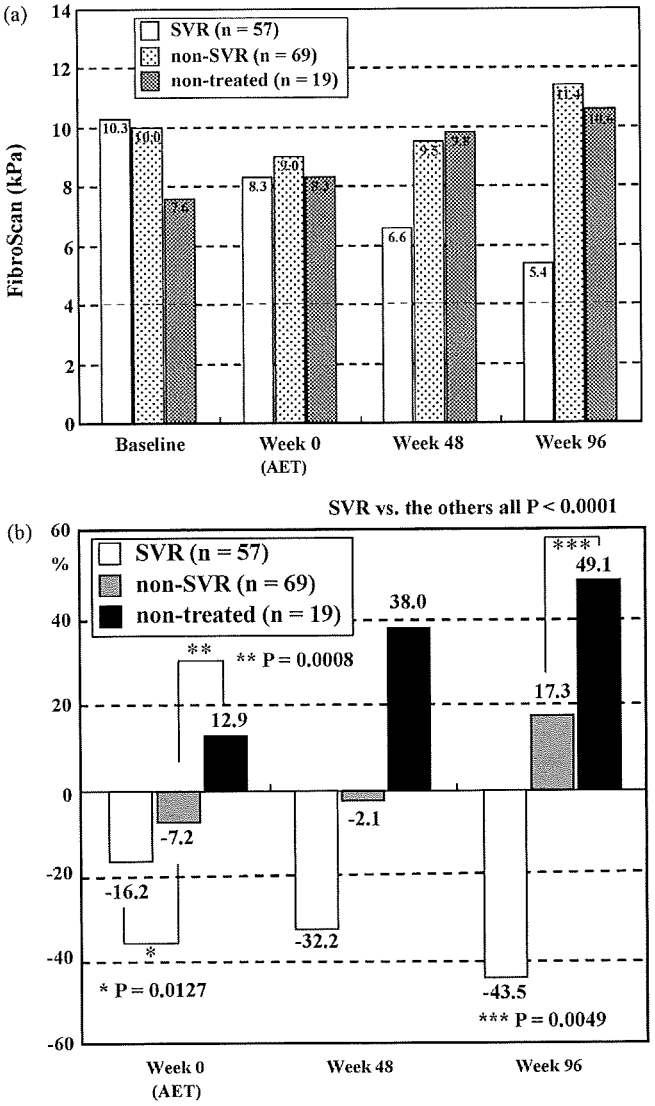

Fig. 2. The longitudinal mean FibroScan values (a) and the mean rate of change of FibroScan values (b) of 126 treated patients after pegylated interferon alpha-2b plus ribavirin combination treatment and 19 non-treated patients. The rates of change of the FibroScan values of each patient at the end of treatment and at 48 and 96 weeks after the end of treatment were calculated using the entry values as the estimated standard. The length of treatment was 48 and 24 weeks for HCV genotypes 1 and 2 patients, respectively. Weeks 0, 48 and 96 for the treated patients indicate the end of treatment and 48 and 96 weeks after the end of treatment, respectively. Weeks 0, 48 and 96 for the non-treated patients indicate 48, 96 and 144 weeks from entry, respectively. SVR, sustained virological response; AET, at the end of treatment.

an improvement of FibroScan values at the end of treatment and 6 months later, whatever the virological response (Vergniol et al., 2008, 2009). However, we investigated the association between an efficacy of the antiviral treatment and FibroScan values related to both virological and biochemical response for a longer period after the treatment than the previous reports. Consequently, we demonstrated that the liver elasticity of SVR patients markedly improved over time and that the liver elasticity of non-SVR patients with biochemical response also improved.

In PEG-IFN alpha plus RBV treatment for chronic hepatitis C, we previously reported that it was necessary to administer ≥80% of the target dosage of PEG-IFN alpha-2b plus ≥60% of the target dosage of RBV throughout the treatment in order to achieve virological efficacy in Japanese patients (Furusyo et al., 2008). Each patient in the present study was analyzed under this treatment dosage.

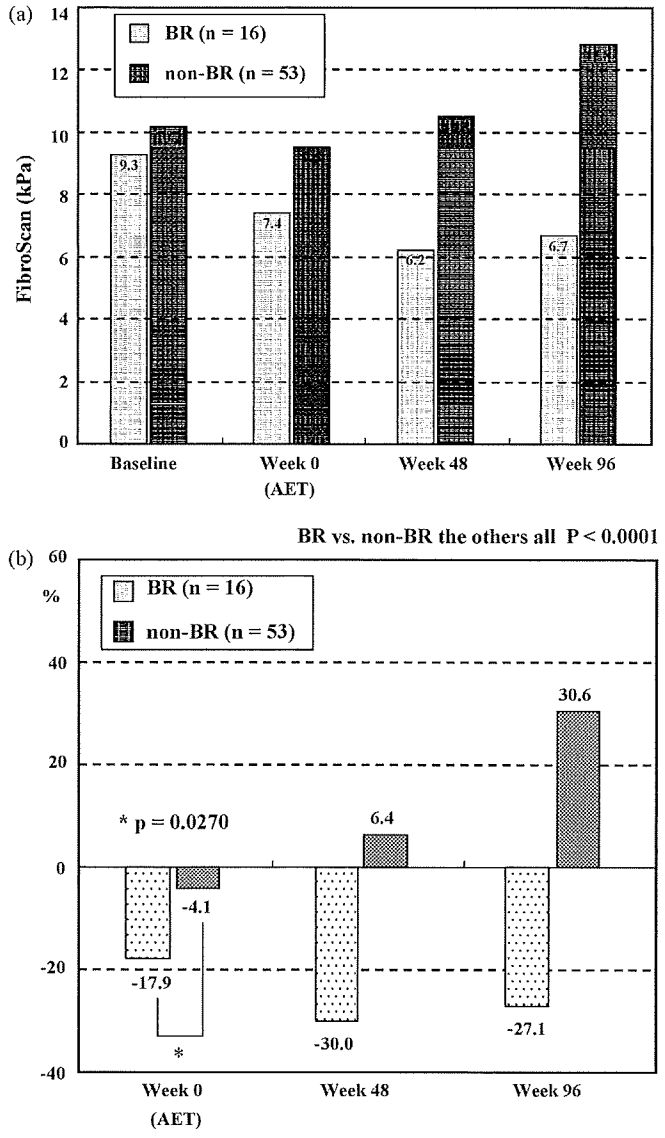

Fig. 3. The longitudinal mean FibroScan values (a) and the mean rate of change of FibroScan values (b) of the non-sustained virological response patients after pegylated interferon alpha-2b plus ribavirin combination treatment, classified by biological response (BR). The rates of change of the FibroScan values of each patient at the end of treatment and at 48 and 96 weeks after the end of treatment were calculated using the entry values as the estimated standard. Weeks 48 and 96 indicate 48 and 96 weeks after the end of treatment, respectively. AET, at the end of treatment.

FibroScan represents a novel clinical methodology based on ultrasound. This can survey a larger sample of the liver parenchyma than liver biopsy and thus more accurately estimates liver fibrosis across a wide range of liver disorders (Ziol et al., 2005; Ogawa et al., 2007). According to meta-analysis, FibroScan could be performed with excellent diagnostic accuracy at distinguishing liver cirrhosis (F4) or no liver cirrhosis (≤F3), with a mean AUROC of 0.94, and at distinguishing ≥F2 and ≤F1 with a mean AUROC of 0.84 (Friedrich-Rust et al., 2008). The optimal cutoff values for the diagnosis of F4 and ≥F2 suggested from the summary ROC are 13.01 kPa and 7.65 kPa, respectively (Friedrich-Rust et al., 2008). Thus, prior studies have already shown that FibroScan can distinguish absent or mild fibrosis from advanced fibrosis in both HCV-mono-infected and HIV-/HCV-co-infected individuals, but it seems to be less accurate at differentiating between intermediate stages. However, Macías et al. (2008) suggested that the usefulness of FibroScan could be enhanced using two different cutoff values (6.0 kPa and 9.0 kPa) to identify with ≤F1 and ≥F2, respectively, in HIV- and HCV-co-infected patients.

Biomarkers such as FibroTest demonstrated similar results as FibroScan in patients treated with IFN and RBV (Poynard et al., 2002b, 2003; Ngo et al., 2006; Patel et al., 2009) and the combination of FibroTest and ActiTest® (which is a modification of the FibroTest) give not only the fibrosis estimate but also the activity estimate (Poynard et al., 2003). FibroScan has never been demonstrated better than FibroTest for F4 vs. ≤F3 according to evidence-based data (Castéra et al., 2005; Shaheen et al., 2007; Manning and Afdhal, 2008; Poynard et al., 2008; Calès et al., 2008).

While natural history of liver fibrosis progression can vary depending on gender and alcohol consumption, age is the main risk factor for liver fibrosis (Poynard et al., 1997). We recommended that all our patients stop drinking alcohol while under treatment and during follow-up. In our study, the baseline mean value of FibroScan was 7.6 kPa, almost the same as the F2 stage, with a value of 10.5 kPa, nearly equal to the F3 stage, after 3 years in non-treated patients (median age 63.8 years). Poynard et al. (1997) suggested that the rate of liver fibrosis progression of untreated patients was highest in individuals older than 50 years (at a rate of 0.333 stage/year). The findings obtained in the present study were similar to those of the above report.

Many previous reports have shown that IFN treatment achieves biochemical and histological improvement with viral suppression by patients with chronic hepatitis C (Poynard et al., 2002a; Furusyo et al., 1997; Cammá et al., 1998; Bruno et al., 2007; Furusyo et al., 2008). Several potential mechanisms have been hypothesized for the anti-fibrotic effect of IFN, including that IFN alpha can directly reduce fibrogenesis. Shiratori et al. (2000) showed that the fibrosis stage improved from −0.60 at <3 years of follow-up to −0.88 at >3 years follow-up (a rate of −0.28/years) for SVR patients treated with non-pegylated IFN monotherapy. However, Everson et al. (2008) showed that the fibrosis stage improved −1.00 between before treatment and 6 months after the end of treatment of SVR patients receiving PEG-IFN alpha monotherapy. Poynard et al. (2002a) showed that the mean fibrosis stage 6 months after the end of treatment was 1.9 ± 0.9 (SD) for SVR patients with compensated cirrhosis treated with PEG-IFN alpha monotherapy or in combination with RBV. There was no significant difference between PEG-IFN alpha plus RBV and PEG-IFN alpha alone for the 48-week regimen as to an anti-fibrotic effect (Poynard et al., 2002a). Therefore, PEG-IFN alpha itself may have a stronger anti-fibrotic effect than non-pegylated IFN and RBV, due to a pharmaco-dynamic advantage.

In the present study, we demonstrated a dramatic reduction of FibroScan values in both SVR and BR patients. Firstly, our results confirmed, from the viewpoint of liver fibrosis, that IFN treatment is of long-term benefit for chronic hepatitis C patients. Moreover, such reduction was observed at an early stage of PEG-IFN alpha plus RBV treatment by SVR and BR patients. It is probable that such early reduction of FibroScan values means not only the improvement of fibrosis but also of inflammation in the liver as a result of treatment, because FibroScan values were significantly correlated with the grade of activity in the liver in the present study. Compared with the rate of change of FibroScan values between non-advanced fibrosis ($F \leq 2$) and advanced fibrosis ($F \geq 3$), no significant differences were found at each testing point. Secondly, another important result was that FibroScan values decreased even for BR patients without HCV clearance by treatment. The findings of the present study can explain why the rate of development of HCC is lower in BR patients treated with IFN. Because HCC tends to develop in patients with advanced liver fibrosis (cirrhosis), BR patients who have a reduction of fibrosis probably will not as quickly develop HCC in the future. Thirdly, although the virological treatment itself may produce good

results in terms of a short-term anti-fibrotic effect of the liver, the FibroScan values of non-BR patients became progressively worse in the long-term course after the antiviral treatment. In the case of a high serum ALT level for non-SVR patients, an additional antiviral retreatment has to be considered. In fact, we recommended antiviral treatment for the studied non-treated patients after the completion of this study, and then most of them have started to receive IFN treatment. A long-term IFN treatment regimen was effective in a smaller trial of the anti-fibrotic effect (Arase et al., 2004); therefore, we believe that treatment with careful attention to IFN-related adverse effects could be usefully introduced to help patients avoid progressing to liver fibrosis and HCC. Thus, the FibroScan is a very interesting tool for the follow-up of chronic HCV carriers, as it is easier to use than liver biopsy in clinical settings. These results are of great interest for the understanding of the effects of IFN treatment and of HCV-related liver disease.

In conclusion, our study shows that transient elastography (FibroScan) is a useful tool for the longitudinal assessment of IFN treatment of chronic hepatitis C patients.

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References

Arase, Y., Ikeda, K., Tsubota, A., Suzuki, F., Suzuki, Y., Saitoh, S., Kobayashi, M., Akuta, N., Someya, T., Hosaka, T., Sezaki, H., Kobayashi, M., Kumada, H., 2004. Interferon therapy for 2 years or longer reduces the incidence of hepatocarcinogenesis in patients with chronic hepatitis C viral infection. Intervirology 47, 355–361.

Bedossa, P., Poynard, T., 1996. An algorithm for the grading of activity in chronic hepatitis C. The METAVIR Cooperative Study Group. Hepatology 24, 289–293.

Bruno, S., Stroffolini, T., Colombo, M., Bollani, S., Benvegnù, L., Mazzella, G., Ascione, A., Santantonio, T., Piccinino, F., Andreone, P., Mangia, A., Gaeta, G.B., Persico, M., Fagiuoli, S., Almasio, P.L., Italian Association of the Study of the Liver Disease (AISF), 2007. Sustained virological response to interferon-alpha is associated with improved outcome in HCV-related cirrhosis: a retrospective study. Hepatology 45, 579–587.

Calès, P., Boursier, J., de Lédinghen, V., Halfon, P., Bacq, Y., Leroy, V., Dib, N., Oberti, F., Sawadogo, A., Rousselet, M.C., Lunel, F., 2008. Evaluation and improvement of a reliable diagnosis of cirrhosis by blood tests. Gastroenterol. Clin. Biol. 32, 1050–1060.

Cammá, C., Di Marco, V., Lo Iacono, O., Almasio, P., Giunta, M., Fuschi, P., Vaccaro, A., Fabiano, C., Magrin, S., Di Stefano, R., Bonura, C., Pagliaro, L., Craxí, A., 1998. Long-term course of interferon-treated chronic hepatitis C. J. Hepatol. 28, 531–537.

Castéra, L., Vergniol, J., Foucher, J., Le Bail, B., Chanteloup, E., Haaser, M., Darriet, M., Couzigou, P., de Lédinghen, V., 2005. Prospective comparison of transient elastography, Fibrotest, APRI, and liver biopsy for the assessment of fibrosis in chronic hepatitis C. Gastroenterology 128, 343–350.

Davis, G.L., Balart, L.A., Schiff, E.R., Lindsay, K., Bodenheimer Jr., H.C., Perrillo, R.P., Carey, W., Jacobson, I.M., Payne, J., Dienstag, J.L., 1989. Treatment of chronic hepatitis C with recombinant interferon alpha. A multicenter randomized, controlled trial. Hepatitis Interventional Therapy Group. N. Engl. J. Med. 321, 1501–1506.

Everson, G.T., Balart, L., Lee, S.S., Reindollar, R.W., Shiffman, M.L., Minuk, G.Y., Pockros, P.J., Govindarajan, S., Lentz, E., Heathcote, E.J., 2008. Histological benefits of virological response to peginterferon alpha-2a monotherapy in patients with hepatitis C and advanced fibrosis or compensated cirrhosis. Aliment. Pharmacol. Ther. 27, 542–551.

Friedrich-Rust, M., Ong, M.F., Martens, S., Sarrazin, C., Bojunga, J., Zeuzem, S., Herrmann, E., 2008. Performance of transient elastography for the staging of liver fibrosis: a meta-analysis. Gastroenterology 134, 960–974.

Furusyo, N., Hayashi, J., Ueno, K., Sawayama, Y., Kawakami, Y., Kishihara, Y., Kashiwagi, S., 1997. Human lymphoblastoid interferon treatment for patients with hepatitis C virus-related cirrhosis. Clin. Ther. 19, 1352–1367.

Furusyo, N., Kajiwara, E., Takahashi, K., Nomura, H., Tanabe, Y., Masumoto, A., Maruyama, T., Nakamuta, M., Enjoji, M., Azuma, K., Shimono, J., Sakai, H., Shimoda, S., Hayashi, J., Kyushu University Liver Disease Study (KULDS) Group, 2008. Association between the treatment length and cumulative dose of pegylated interferon alpha-2b plus ribavirin and their effectiveness as a combination treatment for Japanese chronic hepatitis C patients: project of the Kyushu University Liver Disease Study Group. J. Gastroenterol. Hepatol. 23, 1094–1104.

Hayashi, J., Ohmiya, M., Kishihara, Y., Tani, Y., Kinukawa, M., Ikematsu, H., Kashiwagi, S., 1994. A statistical analysis of predictive factors of response to human lymphoblastoid interferon in patients with chronic hepatitis C. Am. J. Gastroenterol. 89, 2151–2156.

Hayashi, J., Furusyo, N., Ariyama, I., Sawayama, Y., Etoh, Y., Kashiwagi, S., 2000. A relationship between the evolution of hepatitis C virus variants, liver damage, and hepatocellular carcinoma in patients with hepatitis C viremia. J. Infect. Dis. 181, 1523–1527.

Kashiwagi, K., Furusyo, N., Kubo, N., Nakashima, H., Nomura, H., Kashiwagi, S., Hayashi, J., 2003. A prospective comparison of the effect of interferon-alpha and interferon-beta treatment in patients with chronic hepatitis C on the incidence of hepatocellular carcinoma development. J. Infect. Chemother. 9, 333–340.

Lee, S.C., Antony, A., Lee, N., Leibow, J., Yang, J.Q., Soviero, S., Gutekunst, K., Rosenstraus, M., 2000. Improved version 2.0 qualitative and quantitative AMPLICOR reverse transcription-PCR tests for hepatitis C virus RNA: calibration to international units, enhanced genotype reactivity, and performance characteristics. J. Clin. Microbiol. 38, 4171–4179.

Lindsay, K., Trepo, C., Heintges, T., Shiffman, M.L., Gordon, S.C., Hoefs, J.C., Schiff, E.R., Goodman, Z.D., Laughlin, M., Yao, R., Albrecht, J.K., Hepatitis Interventional Therapy Group, 2001. A randomized, double-blind trial comparing pegylated interferon alfa-2b to interferon alfa-2b as initial treatment for chronic hepatitis C. Hepatology 34, 395–403.

Lucidarme, D., Foucher, J., Le Bail, B., Vergniol, J., Castera, L., Duburque, C., Forzy, G., Filoche, B., Couzigou, P., de Lédinghen, V., 2008. Factors of accuracy of transient elastography (fibroscan) for the diagnosis of liver fibrosis in chronic hepatitis C. Hepatology 49, 1083–1089.

Macías, J., Recio, E., Vispo, E., Rivero, A., López-Cortés, L.F., Ríos, M.J., Merino, D., González, M., Barreiro, P., de Lédinghen, V., Quereda, C., Pineda, J.A., 2008. Application of transient elastometry to differentiate mild from moderate to severe liver fibrosis in HIV/HCV co-infected patients. J. Hepatol. 49, 916–922.

Manning, D.S., Afdhal, N.H., 2008. Diagnosis and quantitation of fibrosis. Gastroenterology 134, 1670–1681.

Ngo, Y., Munteanu, M., Messous, D., Charlotte, F., Imbert-Bismut, F., Thabut, D., Lebray, P., Thibault, V., Benhamou, Y., Moussalli, J., Ratziu, V., Poynard, T., 2006. A prospective analysis of the prognostic value of biomarkers (FibroTest) in patients with chronic hepatitis C. Clin. Chem. 52, 1887–1896.

Nishiguchi, S., Kuroki, T., Nakatani, S., Morimoto, H., Takeda, T., Nakajima, S., Shiomi, S., Seki, S., Kobayashi, K., Otani, S., 1995. Randomised trial of effects of interferon-alpha on incidence of hepatocellular carcinoma in chronic active hepatitis C with cirrhosis. Lancet 346, 1051–1055.

Ogawa, E., Furusyo, N., Toyoda, K., Takeoka, H., Otaguro, S., Hamada, M., Murata, M., Sawayama, Y., Hayashi, J., 2007. Transient elastography for patients with chronic hepatitis B and C virus infection: non-invasive, quantitative assessment of liver fibrosis. Hepatol. Res. 37, 1002–1010.

Okamoto, H., Sugiyama, Y., Okada, S., Kurai, K., Akahane, Y., Sugai, Y., Tanaka, T., Sato, K., Tsuda, F., Miyakawa, Y., Mayumi, M., 1992. Typing hepatitis C virus by polymerase chain reaction with type-specific primers: application to clinical surveys and tracing infectious sources. J. Gen. Virol. 73, 673–679.

Patel, K., Benhamou, Y., Yoshida, E.M., Kaita, K.D., Zeuzem, S., Torbenson, M., Pulkstenis, E., Subramanian, G.M., McHutchison, J.G., 2009. An independent and prospective comparison of two commercial fibrosis marker panels (HCV FibroSURE and FIBROSpect II) during albinterferon alfa-2b combination therapy for chronic hepatitis C. J. Viral Hepat. 16, 178–186.

Poynard, T., Bedossa, P., Opolon, P., 1997. Natural history of liver fibrosis progression in patients with chronic hepatitis C. Lancet 349, 825–832.

Poynard, T., McHutchison, J., Manns, M., Trepo, C., Lindsay, K., Goodman, Z., Ling, M.H., Albrecht, J., 2002a. Impact of pegylated interferon alfa-2b and ribavirin on liver fibrosis in patients with chronic hepatitis C. Gastroenterology 122, 1303–1313.

Poynard, T., Imbert-Bismut, F., Ratziu, V., Chevret, S., Jardel, C., Moussalli, J., Messous, D., Degos, F., GERMED cyt04 Group, 2002b. Biochemical markers of liver fibrosis in patients infected by hepatitis C virus: longitudinal validation in a randomized trial. J. Viral Hepat. 9, 128–133.

Poynard, T., McHutchison, J., Manns, M., Myers, R.P., Albrecht, J., 2003. Biochemical surrogate markers of liver fibrosis and activity in a randomized trial of peginterferon alfa-2b and ribavirin. Hepatology 38, 481–492.

Poynard, T., Munteanu, M., Imbert-Bismut, F., Charlotte, F., Thabut, D., Le Calvez, S., Messous, D., Thibault, V., Benhamou, Y., Moussalli, J., Ratziu, V., 2004. Prospective analysis of discordant results between biochemical markers and biopsy in patients with chronic hepatitis C. Clin. Chem. 50, 1344–1355.

Poynard, T., Ingiliz, P., Elkrief, L., Munteanu, M., Lebray, P., Morra, R., Messous, D., Bismut, F.L., Roulot, D., Benhamou, Y., Thabut, D., Ratziu, V., 2008. Concordance in a world without a gold standard: a new non-invasive methodology for improving accuracy of fibrosis markers. PLoS ONE 3, e3857.

Regev, A., Berho, M., Jeffers, L.J., Milikowski, C., Molina, E.G., Pyrsopoulos, N.T., Feng, Z.Z., Reddy, K.R., Schiff, E.R., 2002. Sampling error and intraobserver variation in liver biopsy in patients with chronic HCV infection. Am. J. Gastroenterol. 97, 2614–2618.

Sandrin, L., Tanter, M., Gennisson, J.L., Cathelilne, S., Fink, M., 2002. Shear elasticity probe for soft tissues with 1-D transient elastography. IEEE Trans. Ultrason. Ferroelectr. Freq. Control 49, 436–446.

Seeff, L.B., 2002. Natural history of chronic hepatitis C. Hepatology 36, S35–S46.

Shaheen, A.A., Wan, A.F., Myers, R.P., 2007. FibroTest and FibroScan for the prediction of hepatitis C-related fibrosis: a systematic review of diagnostic test accuracy. Am. J. Gastroenterol. 102, 2589–2600.

Shiratori, Y., Imazeki, F., Moriyama, M., Yano, M., Arakawa, Y., Yokosuka, O., Kuroki, T., Nishiguchi, S., Sata, M., Yamada, G., Fujiyama, S., Yoshida, H., Omata, M., 2000. Histologic improvement of fibrosis in patients with hepatitis C who have sustained response to interferon therapy. Ann. Intern. Med. 132, 517–524.

Simmonds, P., Alberti, A., Alter, H.J., Bonino, F., Bradley, D.W., Brechot, C., Brouwer, J.T., Chan, S.W., Chayama, K., Chen, D.S., Choo, Q.L., Colombo, M., Cuypers, H.T.M., Date, T., Dusheiko, G.M., Esteban, J.I., Fay, O., Hadzyannis, S.J., Han, J., Hatzakis, A., Holmes, E.C., Hotta, H., Houghton, M., Irwine, B., Kohara, M., Kolberg, J.A., Kuo, G., Lau, J.Y.N., Lelie, P.N., Maertens, G., McOmish, F., Snedecar, G.W., 1994. A proposed system for the nomenclature of hepatitis C viral genotypes. Hepatology 19, 1321–1324.

Veldt, B.J., Saracco, G., Boyer, N., Cammà, C., Bellobuono, A., Hopf, U., Castillo, I., Weiland, O., Nevens, F., Hansen, B.E., Schalm, S.W., 2004. Long term clinical outcome of chronic hepatitis C patients with sustained virological response to interferon monotherapy. Gut 53, 1504–1508.

Vergniol, J., Foucher, J., Mamou, K., Castéra, L., Bernard, P.H., Couzigou, P., de Lédinghen, V., 2008. Non-invasive evaluation of liver fibrosis using FibroScan in long-term sustained-virological responder patients after HCV treatment. Gastroenterol. Clin. Biol. 32, 1061–1063.

Vergniol, J., Foucher, J., Castéra, L., Bernard, P.H., Tournan, R., Terrebonne, E., Chanteloup, E., Merrouche, W., Couzigou, P., de Lédinghen, V., 2009. Changes of non-invasive markers and FibroScan values during HCV treatment. J. Viral Hepat. 16, 132–140.

Wai, C.T., Greenson, J.K., Fontana, R.J., Kalbfleisch, J.D., Marrero, J.A., Conjeevaram, H.S., Lok, A.S., 2003. A simple noninvasive index can predict both significant fibrosis and cirrhosis in patients with chronic hepatitis C. Hepatology 38, 518–526.

Zeuzem, S., Feinman, S.V., Rasenack, J., Heathcote, E.J., Lai, M.Y., Gane, E., O'Grady, J., Reichen, J., Diago, M., Lin, A., Hoffman, J., Brunda, M.J., 2000. Peginterferon alfa-2a in patients with chronic hepatitis C. N. Engl. J. Med. 343, 1666–1672.

Ziol, M., Handra-Luca, A., Kettaneh, A., Christidis, C., Mal, F., Kazemi, F., de Lédinghen, V., Marcellin, P., Dhumeaux, D., Trinchet, J.C., Beaugrand, M., 2005. Noninvasive assessment of liver fibrosis by measurement of stiffness in patients with chronic hepatitis C. Hepatology 41, 48–54.

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The association between fatal vascular events and risk factors for carotid atherosclerosis in patients on maintenance hemodialysis: Plaque number of dialytic atherosclerosis study

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ABSTRACT

Atherosclerotic vascular diseases are a major cause of morbidity and mortality for end-stage renal disease patients. We followed prospectively 226 hemodialysis patients by carotid ultrasonography to determine if ultrasonographic markers are predictive of the prognosis of these patients. The end-point was death or completion of the five-year follow-up period. Fatal cerebrovascular and cardiovascular events were the most common cause of death. By multivariate analysis, diabetes mellitus (DM) ($P=0.005$), plaque number (PN) by ultrasonography ($P=0.023$), age ($P=0.001$), calcium–phosphate product ($P=0.049$), and serum albumin ($P=0.009$) were extracted as independent risk factors. The five-year increase of PN was significantly greater for DM patients than for non-DM patients. Moreover, PN was an independent marker of a fatal event, irrespective of DM status. Our results suggest that PN may be a useful predictor of the long-term prognosis of hemodialysis patients.

1. Introduction

Atherosclerotic vascular diseases are a major cause of morbidity and mortality for end-stage renal disease patients [1]. The traditional risk factors for atherosclerotic disease, such as hypertension, dyslipidemia, diabetes mellitus (DM), and smoking, are well known for the general population. Our previous study revealed that the prevalence of carotid atherosclerosis by ultrasonography was significantly higher in hemodialysis patients than in the general population [2]. Other authors have reported non-traditional risk factors such as inflammation [3,4] and the hemodialysis procedure itself [5–8].

Diabetic nephropathy has become increasingly common among patients on maintenance hemodialysis in Japan [1]. The number of patients with DM undergoing hemodialysis was about 41.3% of all patients to who were newly introduced hemodialysis in 2004, and has exceeded the number of patients with chronic renal failure since 1998. The number of patients with diabetic nephropathy on hemodialysis each year is about 14,000 in Japan [1]. DM patients undergoing hemodialysis have more advanced carotid artery lesions than non-DM patients [2].

High-resolution B-mode ultrasonography has made possible the noninvasive evaluation of common carotid artery intima-media thickness (CCA-IMT). CCA-IMT has become widely accepted as a marker of generalized atherosclerosis [9–12] and an association has been made with the occurrence of future vascular events.

The aim of this five-year prospective study was to reconfirm the role of the traditional risk factors for atherosclerosis by ultrasonographic measurement of the number of plaques (plaque number: PN), CCA-IMT, and plaque score (PS) [12] as predictors of long-term risk. We also hoped to clarify the relationship between the putative risk factors and the progression of carotid atherosclerosis in Japanese hemodialysis patients, which is important for more precise assessment of the prognosis.

2. Methods

2.1. Study design

This prospective study was done to evaluate the five-year follow-up of 226 hemodialysis patients. Throughout this study, all

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hemodialysis patients received the best medical and surgical care available at the time.

2.2. Recruitment and follow-up

The profile of the patients in 2000 is described in our previous report [2]. Each patient was on regular dialysis (4–5 h three times per week) at baseline of the study (duration: 108.4 ± 82.7 months, range: 1–348 months). The dialysate contained 140 mEq/l of sodium, 2.0 mEq/l of potassium, 3.0 mEq/l of calcium, 1.0 mEq/l of magnesium, 110 mEq/l chloride, 30 mEq/l of bicarbonate, and 100 mg/dl of glucose. The lost to follow-up, deceased, and surviving patients are summarized in Fig. 1. The 226 hemodialysis patients (124 male and 102 female; mean age 60.4 ± 13.2 years, range: 22–86 years) were assessed at two dialysis units in Fukuoka Prefecture. Of the 226 hemodialysis patients, 30 were unavailable for follow-up, including 23 who were transferred to other hospitals and 7 who underwent kidney transplantation after the day of examination by carotid ultrasonography. Of the 124 male patients, 28 (22.6%) had DM, 112 (90.3%) had hypertension, 12 (9.6%) had hyperlipidemia, and 50 (40.3%) had a history of a vascular event. Of the 102 female patients, 23 (22.5%) had DM, 83 (81.4%) had hypertension, 12 (11.8%) had hyperlipidemia, and 32 (31.4%) had a history of a vascular event. Hyperlipidemia was seen in 24 (10.6%) patients including 12 (4 male, 8 female) who were untreated, and 12 (8 male, 4 female) who were treated with a statin based antihyperlipidemia drug.

Over the course of the study, 73 of the 226 patients (32.3%) died within the five-year period, with a mean follow-up of 2.29 (range: 0.03–4.98 years) and 167.2 person-years. The 123 surviving patients have a follow-up of five-year period, and 614.9 person-years of follow-up. The 30 lost to follow-up had a mean follow-up of 4.70 (range: 0.50–5.00 years) and 102.8 person-years of follow-up. There were 884.9 person-years of follow-up in this study, and a mean survival period of 3.92 years. All patients were Japanese and informed consent was obtained. The study was done in accordance with the principles of the Declaration of Helsinki.

2.3. End-points

Briefly, the first end-points of the study were a fatal event (vascular event including cerebrovascular and cardiovascular events, infection, malignancy, cardiac failure, or another illness as the main cause of death). Of the 73 who had died, 20 patients (27.4%) died of a vascular event (15 of cerebrovascular and 5 of cardiovascular events) which were defined as the second end point, 15 (20.5%) of infectious disease (8 of pneumonia, 4 of sepsis, 2 of shunt infection, one of cerebral abscess), 8 (11.0%) of malignancy (4 of digestive cancer, 2 of leukemia, one of spine cancer, one of kidney cancer), 7 (9.6%) of sudden death, and 23 (31.5%) of other causes (6 of cardiac failure, 4 of electrolyte abnormality, 2 of ileus, 2 of gastrointestinal bleeding, one of hepatic failure, one of thrombosis, one of acute pancreatitis, 4 patients who changed hospitals for whom the cause of death was unknown, and 2 of accidents).

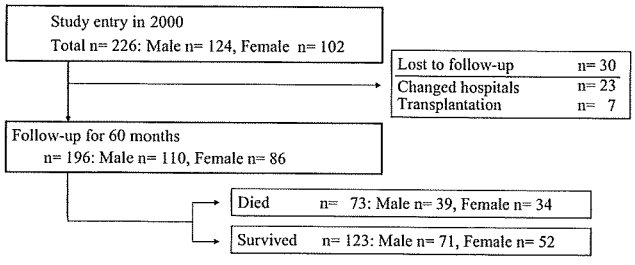

Fig. 1. Flow chart of 226 hemodialysis patients. Thirty patients were unable to be followed. The main causes of death among the 73 patients who suffered a fatal event were vascular events, infection, heart failure, and malignancy.

2.4. Medical history and lifestyle

Data were compiled from medical records and a questionnaire that included personal medical history, family history, and lifestyle habits. Pre-dialysis and post-dialysis blood pressure was measured three times per week at rest for five weeks in 2000, after which the mean blood pressure was calculated for each patient. Hypertension was defined as mean systolic pressure ≥140 mmHg, mean diastolic pressure ≥90 mmHg, or treatment with antihypertensive medications. Hyperlipidemia was defined as either total cholesterol ≥220 mg/dl or receiving lipid-lowering therapy. DM was defined as treatment with anti-diabetic agents or insulin or a past history of DM. Body mass index (BMI) was calculated as the weight in kilograms divided by the height in square meters. A 12-lead electrocardiogram was recorded, and evidence of left ventricular hypertrophy (LVH) was assessed using the Sokolow–Lyon criteria.

2.5. Laboratory parameters

All blood samples were obtained immediately before hemodialysis and stored at −20 °C until analysis. Total cholesterol, triglycerides, and creatinine (enzymatic method), high-density lipoprotein cholesterol (homogeneous assay method), albumin (bromcresol green method), phosphorus (Fiske-Subbarow method), magnesium (xylidyl blue method), intact parathyroid hormone (immunoradiometric assay), Qualitative C-reactive protein (CRP) (turbidimetric immunoassay), and the hemoglobin, hematocrit, and white blood cell count (autoanalyzer) were measured by a commercial laboratory (CRC, Fukuoka, Japan). Qualitative CRP ≥0.5 mg/dl was defined as positive. Low-density lipoprotein cholesterol was calculated according to the Friedewald formula. All assays were done blinded to clinical data and the results of ultrasound examination.

2.6. Carotid ultrasound

Of the 123 surviving patients, 10 declined to undergo carotid ultrasonography in 2005, so data from these patients were only used for comparison of characteristics at baseline and for drawing the survival curve.

Thus, the study included 113 patients who had carotid ultrasonography between 2000 and 2005. Carotid artery lesions were assessed by high resolution B-mode ultrasonography with a 7.5 MHz linear array probe (SSD-1700, Aloka, Tokyo, Japan), as described previously. All examinations of the carotid arteries were done by the leading author, a well trained physician, without any knowledge of patient history or risk factor profile. Each subject was examined in the supine position in a semi-dark room. Both longitudinal and transverse images of the right and left carotid arteries were obtained in the anterior oblique, lateral, and posterior oblique planes. CCA-IMT was defined as the distance between the lumen–intima interface and the media–adventitia interface on B-mode images. Using the probe at an antero-oblique angle, the far wall of the carotid artery was visualized bilaterally in the common carotid artery (CCA-IMT: 20–50 mm proximal to the bifurcation of blood flow), the carotid bulb (0–20 mm proximal to the bifurcation of flow), and the internal and external carotid arteries (ICA and ECA: 0–20 mm distal to the bifurcation).

CCA-IMT was measured at 20, 25, and 30 mm proximal to the bifurcation of flow at the far wall of the right and left common