age group (Table 7). We also measured the serum insulin level in this survey. The serum insulin level was almost constant except in the 20- to 29-year-old age group and the mean insulin level in this survey was 7.3 μU/ml (Table 8). The mean insulin level was slightly higher in women than in men.

Finally, we determined uric acid levels. The mean uric acid level in this survey was 5.4 mg/dl. The mean uric acid level was significantly higher in men than in women (Table 9). Although the level of uric acid in men was al-

Table 7. Fasting glucose (mg/dl) for each 10-year group in Japanese.

	All			Men			Women		
Age	*N*	Mean	S.D.	*N*	Mean	S.D.	*N*	Mean	S.D.
0–9	158	88	7	74	88	7	84	87	6
10–19	170	85	6	57	87	7	113	85	6
20–29	996	88	16	340	89	20	655	87	13
30–39	1,281	92	15	886	93	14	395	90	18
40–49	2,865	95	18	2,018	97	19	847	90	12
50–59	2,909	99	20	2,002	101	20	907	94	19
60–69	1,489	98	21	752	102	25	737	95	15
70–79	531	98	16	257	99	16	274	97	15
80–89	52	103	27	22	104	36	30	102	20
Total	10,451	95	19	6,408	98	20	4,042	92	16

Table 8. HbA1c for each 10-year group in Japanese.

	All			Men			Women		
Age	*N*	Mean	S.D.	*N*	Mean	S.D.	*N*	Mean	S.D.
0–9	155	4.7	0.2	72	4.7	0.2	83	4.7	0.2
10–19	171	4.7	0.3	58	4.7	0.3	113	4.6	0.3
20–29	1,147	4.6	0.4	374	4.6	0.6	772	4.6	0.3
30–39	1,261	4.7	0.5	871	4.7	0.5	390	4.7	0.4
40–49	2,536	4.9	0.6	1,844	4.9	0.7	692	4.8	0.5
50–59	2,676	5.1	0.7	1,879	5.1	0.7	797	5.1	0.7
60–69	1,141	5.2	0.8	614	5.3	0.9	527	5.2	0.6
70–79	443	5.3	0.7	209	5.3	0.7	234	5.4	0.8
80–89	52	5.4	0.8	22	5.4	1.0	30	5.3	0.6
Total	9,582	4.9	0.7	5,943	5.0	0.7	3,638	4.9	0.6

Table 9. Serum insulin (μU/ml) for each 10-year group in Japanese.

	All			Men			Women		
Age	*N*	Mean	S.D.	*N*	Mean	S.D.	*N*	Mean	S.D.
0–9	216	6.7	5.2	102	6.5	6.2	114	6.9	4.1
10–19	463	7.1	7.2	196	6.1	5.1	267	7.9	8.3
20–29	1,171	11.4	12.9	382	9.9	10.6	788	12.1	13.8
30–39	1,410	8.2	9.0	942	8.3	8.9	468	8.0	9.2
40–49	2,734	6.7	5.5	1,877	6.7	5.0	857	6.6	6.4
50–59	2,636	6.4	5.6	1,731	6.0	4.2	905	7.3	7.5
60–69	1,118	6.1	5.3	589	5.9	5.3	528	6.4	5.2
70–79	440	6.2	14.8	211	5.2	5.6	229	7.1	19.7
80–89	53	5.8	4.6	23	6.1	5.8	30	5.6	3.6
Total	10,241	7.3	8.0	6,053	6.8	6.2	4,186	8.0	10.0

most constant in all age groups, the uric acid level in women gradually increased according to age (Table 10).

Discussion

In this survey we found that the mean total cholesterol level in the Japanese general population increased by 5 mg/dl in the last 10 years. This increase, however, is attributed to the increase in HDL-cholesterol, but not to LDL-cholesterol. The triglyceride level has also increased in the last 10 years. This increase is attributed to the increase in middle-aged men, making us anticipate a further increase in the incidence of hypertriglyceridemia in the future. The significance of triglyceride as a risk factor for CHD has recently obtained more attention world-wide, and its relationship with hyperinsulinemia and glucose intolerance is emphasized (15,16). In the analysis by Yamamoto *et al.* on the survey in 1990, they concluded that the most important cause of hypertriglyceridemia is overweight. According to the survey conducted by the Ministry of Health, Labor and Welfare, the body mass index increased from 1980 to 2000 only in men, but not women. Therefore, the increase in triglyceride levels in Japanese men correlates with the increase of obese men. RLP-cholesterol is implicated as an atherogenic lipoprotein and our data showed a correlation of RLP-cholesterol with the triglyceride level. Therefore, we also should pay attention to the level of RLP-cholesterol. The importance of RLP-cholesterol in the prevention of CHD, such as being a marker for postprandial hyperlipidemia, should be determined in a future trial. Thus to reduce the triglyceride levels, we need to encourage lifestyle changes, such as more exercise and consuming a traditional Japanese diet instead of a modern 'western' diet in the Japanese general population, especially amongst men. Unless we can change our lifestyle in Japan, more people will die from cardiovascular disease in the 21st century.

In spite of the dramatic increase in the triglyceride level in men in the last 10 years, the HDL-cholesterol level also increased in the last 10 years. This is a somewhat unexpected finding, because hypertriglyceridemia is generally associated with a decrease in the HDL-cholesterol level. In this survey we changed the method of measuring HDL-cholesterol from the precipitation method to the enzymatic method. However, we have confirmed that this change of method does not affect the level of HDL-cholesterol. Therefore, we have at the moment no idea why both triglyceride and HDL-cholesterol increased in the last 10 years only in men.

Guidelines for the proper management of risk factors, and for targeting the prevention and treatment of atherosclerotic disease, have been established in the United States (17,18) and Europe (19). The Japan Atherosclerosis Society also published a guideline for the management of hyperlipidemia for the prevention of CHD in 2002. As in the American and European guidelines, the Japanese guideline also emphasized the importance of the management of high risk patients, such as patients with multiple risk factors or diabetes as well as those with established CHD (20). Although our survey shows no increase in LDL-cholesterol level, the triglyceride level was significantly increased in the last 10 years. Especially, the mean triglyceride level of men in their 40s is 150 mg/dl, indicating about half of the participants have hypertriglyceridemia. Because hypertriglyceridemia is one criteria of metabolic syndrome, our result implies that the number of the patients with metabolic syndrome will increase in Japan. Therefore, in the next survey in 2010, we will investigate the incidence of the metabolic syndrome in the general Japanese population after establishing guidelines for the management of metabolic syndrome in Japan. This survey also indicates that we, as the members of the Japan Atherosclerosis Society, have to make every effort to call more clinical attention to the

Table 10. Serum uric acid (mg/dl) for each 10-year group in Japanese.

	All			Men			Women		
Age	*N*	Mean	S.D.	*N*	Mean	S.D.	*N*	Mean	S.D.
0–9	0	–	–	0	–	–	0	–	–
10–19	3	6.7	0.7	3	6.7	0.7	0	–	–
20–29	410	4.7	1.4	137	6.1	1.3	273	4.0	0.8
30–39	927	5.6	1.5	714	6.0	1.3	213	4.0	0.9
40–49	2,425	5.5	1.5	1,763	6.1	1.3	662	4.1	0.9
50–59	2,459	5.5	1.4	1,762	6.0	1.3	697	4.3	0.9
60–69	1,141	5.2	1.4	618	5.8	1.3	523	4.5	1.0
70–79	296	5.1	1.5	152	5.8	1.4	144	4.4	1.1
80–89	25	4.9	1.6	8	5.0	0.9	17	4.9	1.8
Total	7,686	5.4	1.4	5,157	6.0	1.3	2,529	4.3	1.0

management of dyslipidemia for prevention of CHD.

Currently approximately 4 million people are taking statins for hyperlipidemia in Japan. In this survey about 5% of the participants were taking lipid-lowering drugs, most of which are supposed to be statins. The mean total cholesterol level of the participants without lipid lowering drugs was 209 mg/dl, which is slightly higher than the mean total cholesterol levels of all the participants. In this sense, the participants in this survey represent the general population in Japan. Use of lipid-lowering drugs such as statins would be more important for the treatment of high risk patients to prevent CHD.

In 2000, another survey was conducted by the Ministry of Health, Labor, and Welfare. In this study, more subjects were selected from rural, agricultural, and mountainous areas, and the results showed no rise in serum cholesterol in the last 10 years (from 1990 to 2000). In this study carried out by the members of the Japan Atherosclerosis Society, more subjects from urban areas were included. In both studies, the cholesterol levels were significantly lower in the agricultural and mountainous districts than in the districts including large cities like Tokyo and Osaka in 1980. In 1990, the difference in serum cholesterol levels was no longer significant between urban, rural, and mountain village areas. Therefore, it is not clear why these studies show a different trend in the cholesterol level. However, Kuzuya et al also found an increase in total cholesterol levels from 1989 to 1998 in Aichi Prefecture in the central region of Japan (21).

In this survey we also determined fasting glucose, insulin, and HbA1c levels of approximately 10,000 participants. We think that this is the largest survey of glucose metabolism in Japan. Our data indicate that the glucose and HbA1c levels gradually increased according to age in both sexes. However, the plasma insulin levels are almost constant in all age groups. We also showed that the uric acid level was significantly higher in men than in women. This is consistent with the data that the incidence of hyperuricemia and gout is higher in males than in females. Alcohol consumption would contribute to the higher level of uric acid in men. According to the database from the Ministry of Health, Labor, and Welfare (http://www.mhlw.go.jp/toukei/), the incidence of hyperuricemia in men and women is increasing in Japan. Because hyperuricemia is related to obesity, hypertension, and insulin resistance, and eventually to the incidence of CHD, controlling the uric acid level would be important for the prevention of CHD in Japan.

Thus this report tells us the importance of the prevention and treatment of hyperlipidemia for the prevention of CHD in Japan. We need to establish guidelines for lifestyle change to prevent the further increase of dyslipidemia in the future.

Acknowledgements: This study was supported by research grants for health sciences from the Japanese Ministry of Health and a grant from the Japan Atherosclerosis Society. We also thank Osaka Pharmaceutical Manufactures Association for supporting our work. We also thank Nobuo Shirahashi (Osaka City University Medical School) for his advice on statistical analysis.

Appendix

Research Group on Serum Lipid Survey 2000 in Japan

Chairman: Toru Kita, Department of Cardiovascular Medicine, Kyoto University Graduate School of Medicine

Principal investigators: Akira Yamamoto, National Cardiovascular Center, Osaka, Japan.

Yuji Matsuzawa, Department of Internal Medicine, Osaka University, Osaka, Japan.

Yasushi Saito, Department of Internal Medicine, Chiba University, Chiba, Japan.

Shin-ichi Oikawa, Department of Internal Medicine, Nippon Medical School, Tokyo, Japan.

Noriaki Nakaya, Fussa Hospital, Tokyo, Japan.

Jun Sasaki, International University of Health and Welfare, Fukuoka, Japan.

Hiroshi Mabuchi, Department of Internal Medicine, Kanazawa University, Ishikawa, Japan.

Nobuhiro Yamada, Department of Internal Medicine, Tsukuba University, Ibaraki, Japan.

Hiroshige Itakura, Ibaraki Christian University, Ibaraki, Japan.

Yuichi Ishikawa, Faculty of Health Sciences, Kobe University, Hyogo, Japan.

Tadayoshi Ouchi, Department of Geriatric Medicine, University of Tokyo. Tokyo, Japan

Hiroshi Horibe, Keisen Clinic, Hyogo, Japan.

Tamio Teramoto, Department of Internal Medicine, Teikyo University, Tokyo, Japan.

Hidenori Arai, Department of Geriatric Medicine, Kyoto University, Kyoto, Japan

Co-principal investigators (institutes): Kazuaki Shimamoto (Sappro Medical University), Takao Koike (Hokkaido University), Akizuki Morikawa (Asahikawa Red Cross Hospital), Makoto Tominaga (Yamagata University), Toshihiro Suda (Hirosaki University), Nobuyuki Sugawara (Mizusawa general hospital), Hideo Hamaguchi (Tsukuba University), Saburo Hori (St.Luka's International Hospital), Hideaki Bujo (Chiba University), Fumitaka Osuzu (National Defense Medical College), Koichi Kozaki (Tokyo University), Toshiro Murase (Toranomon Hospital), Katsuji Senda (Hamamatsu Social Insurance Hospital), Tomoo Okada (Nihon University), Akihiro Inazu and Toshinori Higashikata (Kanazawa University), Isamu Miyamori and Koji Oida (University of Fukui Faculty of Medical Sciences), Susumu Miyamoto (Himi Municipal Hospital), Akihisa Iguchi (Nagoya University), Nagahiko Sakuma (Nagoya City University), Taku Yamamura (National Cardiovascular Center), Shizuya Yamashita (Osaka University), Toshiko Kawakita and Atsuhiko Sato (Kyoto Center for preventive medicine), Mitsuhiro Yokoyama (Kobe University), Genshi Egusa (Egusa clinic), Masunori Matsuzaki (Yamaguchi University), Masayoshi Kihata (Chugoku Central Hospital), Hitoshi Kukida (Udazima Social Insurance Hospital), Shoji Kohori (National Hospital organization Kumamoto Medical Center), Kyosuke Yamamoto (Saga University Faculty of Medicine), Sadatoshi Birou (Kagosima University), Takao Ota (University of Ryukyus), Masato Ageta (Miyazaki Prefectural Nichinan Hospital)

References

(1) Anderson KM, Castelli WP, and Levy D: Cholesterol and mortality. 30 years of follow-up from the Framingham study. JAMA, 1987; 257: 2176–2180

(2) Stamler J, Wentworth D, and Neaton JD: Is relationship between serum cholesterol and risk of premature death from coronary heart disease continuous and graded? Findings in 356,222 primary screenees of the Multiple Risk Factor Intervention Trial (MRFIT). JAMA, 1986; 256: 2823–2828

(3) The Lipid Research Clinics Coronary Primary Prevention Trial results. I. Reduction in incidence of coronary heart disease. JAMA, 1984; 251: 351–364

(4) Holme I: Cholesterol reduction and its impact on coronary artery disease and total mortality. Am J Cardiol, 1995; 76: 10C–17C

(5) Ericsson CG, Hamsten A, Nilsson J, Grip L, Svane B, and de Faire U: Angiographic assessment of effects of bezafibrate on progression of coronary artery disease in young male postinfarction patients. Lancet, 1996; 347: 849–853

(6) Shepherd J, Cobbe SM, Ford I, Isles CG, Lorimer AR, MacFarlane PW, McKillop JH, and Packard CJ: Prevention of coronary heart disease with pravastatin in men with hypercholesterolemia. West of Scotland Coronary Prevention Study Group. N Engl J Med, 1995; 333: 1301–1307

(7) Sacks FM, Pfeffer MA, Moye LA, Rouleau JL, Rutherford JD, Cole TG, Brown L, Warnica JW, Arnold JM, Wun CC, Davis BR, and Braunwald E: The effect of pravastatin on coronary events after myocardial infarction in patients with average cholesterol levels. Cholesterol and Recurrent Events Trial investigators. N Engl J Med, 1996; 335: 1001–1009

(8) Levy RI and Moskowitz J: Cardiovascular research: decades of progress, a decade of promise. Science, 1982; 217: 121–129

(9) Vartiainen E, Puska P, Pekkanen J, Tuomilehto J, and Jousilahti P: Changes in risk factors explain changes in mortality from ischaemic heart disease in Finland. BMJ, 1994; 309: 23–27

(10) Marmot MG, Syme SL, Kagan A, Kato H, Cohen JB, and Belsky J: Epidemiologic studies of coronary heart disease and stroke in Japanese men living in Japan, Hawaii and California: prevalence of coronary and hypertensive heart disease and associated risk factors. Am J Epidemiol, 1975; 102: 514–525

(11) Johnson CL, Rifkind BM, Sempos CT, Carroll MD, Bachorik PS, Briefel RR, Gordon DJ, Burt VL, Brown CD, Lippel K, Kajiyama G, Kokubu T, Uzawa H, Mimura G, and Shimada O: Declining serum total cholesterol levels among US adults. The National Health and Nutrition Examination Surveys. JAMA, 1993; 269: 3002–3008

(12) Konishi T: Total Serum Cholesterol Levels in Normal Subjects in Japan. Jpn Circ J, 1965; 29: 505–510

(13) Sekimoto H, Goto Y, Goto Y, Naito C, Yasugi T, Okido M, Kuzuya F, Takeda R, Yamamoto A, and Fukuzaki H: Changes of serum total cholesterol and triglyceride levels in normal subjects in Japan in the past twenty years. Research committee on familial hyperlipidemia in Japan. Jpn Circ J, 1983; 47: 1351–1358

(14) Current state of and recent trends in serum lipid levels in the general Japanese population. Research Committee on Serum Lipid Level Survey 1990 in Japan. J Atheroscler Thromb, 1996; 2: 122–132

(15) Gaziano JM, Hennekens CH, O'Donnell CJ, Breslow JL, and Buring JE: Fasting triglycerides, high-density lipoprotein, and risk of myocardial infarction. Circulation, 1997; 96: 2520–2525

(16) Yamamoto A, Yamamura T, Kawaguchi A, Kameda K, and Matsuzawa Y: Triglyceride and glucose intolerance as a risk factor for coronary heart disease. Cardiology, 1991; 78: 185–193

(17) Lauer MS and Fontanarosa PB: Updated guidelines for cholesterol management. JAMA, 2001; 285: 2508–2509

(18) Grundy SM: United States Cholesterol Guidelines 2001: expanded scope of intensive low-density lipoprotein-lowering therapy. Am J Cardiol, 2001; 88: 23J–27J

(19) Wood D, De Backer G, Faergeman O, Graham I, Mancia G, and Pyorala K: Prevention of coronary heart disease in clinical practice: recommendations of the Second Joint Task Force of European and other Societies on Coronary Prevention. Atherosclerosis, 1998; 140: 199–270

(20) Hata Y, Mabuchi H, Saito Y, Itakura H, Egusa G, Ito H, Teramoto T, Tsushima M, Tada N, Oikawa S, Yamada N, Yamashita S, Sakuma N, and Sasaki J: Report of the Japan Atherosclerosis Society (JAS) Guideline for Diagnosis and Treatment of Hyperlipidemia in Japanese adults. J Atheroscler Thromb, 2002; 9: 1–27

(21) Kuzuya M, Ando F, Iguchi A, and Shimokata H: Changes in serum lipid levels during a 10 year period in a large Japanese population. A cross-sectional and longitudinal study. Atherosclerosis, 2002; 163: 313–320

Journal of Atherosclerosis and Thrombosis Vol. 12, No. 5

Original Article

Polymorphisms in Four Genes Related to Triglyceride and HDL-cholesterol Levels in the General Japanese Population in 2000

Hidenori Arai[1], Akira Yamamoto[2], Yuji Matsuzawa[3], Yasushi Saito[4], Nobuhiro Yamada[5], Shinichi Oikawa[6], Hiroshi Mabuchi[7], Tamio Teramoto[8], Jun Sasaki[9], Noriaki Nakaya[10], Hiroshige Itakura[11], Yuichi Ishikawa[12], Yasuyoshi Ouchi[13], Hiroshi Horibe[14], Tohru Egashira[15], Hiroaki Hattori[15], Nobuo Shirahashi[16], and Toru Kita[17] on behalf of the Research group on Serum Lipid Level Survey 2000 in Japan

[1] *Department of Geriatric Medicine, Kyoto University School of Medicine, Kyoto, Japan.*
[2] *National Cardiovascular Center, Osaka, Japan.*
[3] *Department of Internal Medicine, Osaka University, Osaka, Japan.*
[4] *Department of Internal Medicine, Chiba University, Chiba, Japan.*
[5] *Department of Internal Medicine, Tsukuba University, Ibaraki, Japan.*
[6] *Department of Internal Medicine, Nippon Medical School, Tokyo, Japan.*
[7] *Department of Internal Medicine, Kanazawa University, Ishikawa, Japan.*
[8] *Department of Internal Medicine, Teikyo University, Tokyo, Japan.*
[9] *International University of Health and Welfare, Tochigi, Japan.*
[10] *Fussa Hospital, Tokyo, Japan.*
[11] *Ibaraki Christian University, Ibaraki, Japan.*
[12] *Faculty of Health Sciences, Kobe University, Hyogo, Japan.*
[13] *Department of Geriatric Medicine, University of Tokyo, Tokyo, Japan.*
[14] *Keisen Clinic, Tokyo, Japan.*
[15] *Department of Advanced Technology and Development, BML, Inc., Tokyo, Japan.*
[16] *Osaka City University Medical School, Osaka, Japan.*
[17] *Department of Cardiovascular Medicine, Kyoto University Graduate School of Medicine, Kyoto, Japan.*

We studied the association of six common polymorphisms of four genes related to lipid metabolism with serum lipid levels. We selected single-nucleotide polymorphisms (SNPs) in the genes for cholesteryl ester transfer protein (*CETP*), lipoprotein lipase (*LPL*), hepatic lipase (*LIPC*), and apolipoprotein CIII (*APOC3*), and studied 2267 individuals randomly selected from the participants of Serum Lipid Survey 2000. There was a significant association of *CETP* polymorphism (D442G, Int14 +1 G → A, and TaqIB), *LPL* polymorphism (S447X), and *LIPC* polymorphism (–514 → CT) with HDL-cholesterol levels. We also found a significant association of *LPL* polymorphism (S447X) and *APOC3* polymorphism (SstI) with triglyceride levels. This is the largest database showing the association of common genetic variants in lipid metabolism with serum lipid levels in the general Japanese population. Further study is necessary to elucidate the role of these gene polymorphisms in cardiovascular events. *J Atheroscler Thromb, 2005; 12: 240–250.*

Key words; Hyperlipidemia, Polymorphism, Cholesterol ester transfer protein, Lipoprotein lipase, Triglyceride lipase, Apolipoprotein CIII

Address for correspondence: H. Arai, Department of Geriatric Medicine, Kyoto University School of Medicine, 54 Kawahara-cho, Shogoin, Sakyo-ku, Kyoto 606-8507, Japan.
E-mail: harai@kuhp.kyoto-u.ac.jp
Received April 1, 2005
Accepted for publication July 1, 2005

Introduction

Hyperlipidemia is a major risk factor for coronary artery disease (CAD) (1). In contrast to the sharp decline in both serum cholesterol levels and mortality from CAD in the United States and Western Europe, remarkable increases

in serum cholesterol levels as well as CAD mortality have been anticipated in the Asian-Pacific area due to industrialization and the modernization of lifestyle (2). The importance of lifestyle is also proved by the fact that Japanese who migrated to Hawaii and California, for example, showed higher levels of serum cholesterol and a higher incidence of CAD than people in Japan (3). Thus, dietary habits and other environmental factors affect serum cholesterol levels and CAD mortality in the population. However, genetic traits are also an important determinant of serum lipid levels.

Major mutations have been described coding for the low-density lipoprotein (LDL) receptor, apolipoprotein B, and so forth, affecting mainly serum LDL-cholesterol levels (4, 5). However, plasma triglyceride (TG) and high-density lipoprotein (HDL)-cholesterol levels are also considered established risk factors for CAD (6). Therefore, the association of common variants of candidate genes with changes in TG and HDL-cholesterol levels would be important determinants for CAD risk. Considering the recent prevalence of metabolic syndrome, it would be also intriguing to examine the effect of these genetic polymorphisms on the development of metabolic syndrome. So far in Japan, however, a large-scale analysis has not been performed on common gene variants related to lipid metabolism.

In 2000, we conducted a survey in the general Japanese population, involving 12,839 people from all over the country (7). We tried to examine the frequency of common polymorphisms of four genes related to lipid metabolism and show an association with serum lipid levels. Among the factors involved in lipid metabolism, we chose the following 4 genes because of the association with TG or HDL-cholesterol. Cholesteryl ester transfer protein (CETP) facilitates the transfer of cholesteryl ester from HDL to apolipoprotein B-containing lipoproteins (8). CETP is a key protein in reverse cholesterol transport and its deficiency is associated with hyperalphalipoproteinemia (9–11). Among several polymorphisms of the *CETP* gene, a G to A substitution at the 5' splice donor site of intron 14 (Int14 +1 G → A) and a missense mutation in exon 15 (D442G) are common mutations of hyperalphalipoproteinemia in Japanese (12, 13). The Int14 +1 G → A mutation results in a null allele: homozygotes with the mutation have no CETP in plasma and markedly elevated levels of HDL-cholesterol (10). The D442G mutation is near the carboxy terminal region of CETP shown to be essential for its function (14, 15). The TaqIB polymorphism of the *CETP* gene is one of the most studied polymorphisms worldwide. The B2 allele of the TaqIB polymorphism in intron 1 was associated with decreased CETP levels and high HDL-cholesterol levels (16) and with coronary heart disease risk in the Framingham Study (17). Therefore, we selected these three polymorphisms for our analysis.

Lipoprotein lipase (LPL) is one of the key enzymes in the metabolism of TG-rich lipoproteins. Among several polymorphisms of the *LPL* gene we chose S447X, which is common, having an allele frequency of approximately 20% in healthy individuals, and whose mutation is associated with a favorable lipid profile (18–20). Hepatic lipase (LIPC) is also a member of the lipase superfamily and plays an important role in the metabolism and modeling of both pro- and anti-atherogenic lipoproteins (21). Among the several polymorphisms we selected, -514C → T, located in the promoter region of the *LIPC* gene, has been demonstrated to influence LIPC activity levels (22). Apolipoprotein CIII (apoCIII) can inhibit LPL and reduces the uptake of TG-rich remnant particles and the SstI polymorphism of the *APOC3* gene has been shown to be associated with hypertriglyceridemia and CAD in various human populations (23–27). Therefore, we also examined these polymorphisms in the general Japanese population.

The aim of this study was, therefore, to examine the incidence of these gene polymorphisms and their contribution to lipid concentrations in the general Japanese population.

Methods

Designs and data collection

This work is part of the Serum Lipid Survey 2000 from various areas around Japan. The Ethics committee, graduate school and faculty of Medicine, Kyoto University approved the study protocol and all subjects provided written informed consent for the genetic analysis. The DNA samples were handled according to the guidelines from the Ministry of Health, Labor, and Welfare. In the Serum Lipid Survey 2000, a total of 12,839 subjects were recruited at 36 hospitals across the country. The subjects in the present study were participants in the survey at 9 hospitals from whom informed content for genotyping was sought. Of 12,839 subjects, 2267 (17.7%) with no lipid-altering medication were randomly selected for the present study. In some institutes, information on gender was not disclosed.

Laboratory methods

All serum and blood samples were obtained in the fasting state. All lipid and other analyses were conducted with venous blood samples within one week of collection at BML (Saitama, Japan). Serum cholesterol and TG levels were measured by enzymatic assay. HDL-cholesterol and LDL-cholesterol levels were measured enzymatically with a kit from Daiichi Kagaku Co. Ltd. (Tokyo, Japan). The results of lipid analyses were indirectly standardized according to the criteria of the CDC Lipid Standardization Program (25). DNA was extracted with a QIAamp DNA blood kit (Qiagen, Hilden, Germany).

Detection of gene mutations by Invader® assay

We used the Invader® assay to screen three known mutations of the *CETP* gene, one mutation of the *LIPC* gene, one mutation of the *LPL* gene, and one mutation of the *APOC3* gene, as previously described (26). In brief, the probe/Invader®/$MgCl_2$ mixture was prepared by combining 3 μl of primary probe/Invader® mix and 5 μl of 22.5 mM $MgCl_2$ per reaction. The primary probes/Invader® mixture contained 3.5 μmol/l wild primary probe, 3.5 μmol/l mutant primary probe, 0.35 μmol/l Invader® oligonucleotide, and 10 mmol/l MOPS. Eight microliters of primary probe/Invader®/$MgCl_2$ mixture as well was added into a 96-well plate. Seven microliters of 5 fmol/l synthetic target oligonucleotides, 10 μg/ml yeast tRNA (no target blank), and genomic DNA (15 ng/μl) were added, and denatured by incubation at 95°C for 10 min. After 15 μl of mineral oil (Sigma, St. Louis, MO, USA) was overlayed into each well, the plate was incubated isothermally at 63°C for 4 h in a DNA thermalcycler (PTC-200; MJ Research, Watertown, MA, USA) and then kept at 4°C until fluorescence was measured. The fluorescent intensities were measured using a fluorescence microtiter plate reader (Cytoflour 4000; Applied Biosystems) with excitation at 485 nm/20 nm (Wave length/Band width) and emission, at 530 nm/25 nm for FAM, and excitation at 560 nm/20 nm and emission, at 620 nm/40 nm for RED. The genotyping was based on calculations with the ratios of net counts with wild primary probe to net counts with mutant primary probe. The probes used in this study were designed and synthesized by Third Wave Technologies, Inc (Madison, WI).

Data analyses

Differences in means were evaluated using an analysis of variance. Multiple regression analysis was done to compare age- and sex-adjusted means. The χ^2-test was used to compare the incidence of each genotype. The analysis was performed with the statistical Package for Social Sciences (SPSS Japan Inc. ver. 11.5, Tokyo, Japan).

Results

We investigated the frequency and phenotypic association of the common polymorphisms of *CETP*, *LPL*, *LIPC*, and *APOC3* genes at the population level in 2,267 subjects. Table 1 summarizes the mean serum lipid levels in the participants in this study. The mean age, and total cholesterol, TG, HDL-cholesterol, and LDL-cholesterol levels in this population were similar to the values for all 12,839 participants in Serum Lipid Survey 2000. We also found that the medians of total, LDL-, and HDL-cholesterol levels did not differ appreciably from the means, thereby excluding gross right-hand tailing of the distribution (data not shown). These results indicate that the participants in the gene analysis are representative of the general Japanese population.

Table 1. Lipid profile and age of all the participants.

	All	Men	Women
T-Cho (mmol/l)	5.18 (0.021)	5.23 (0.046)	5.15 (0.046)
TG (mmol/l)	1.31 (0.024)	1.58 (0.050)*	1.11 (0.039)*
HDL-c (mmol/l)	1.53 (0.010)	1.38 (0.020)*	1.65 (0.017)*
LDL-c (mmol/l)	3.00 (0.020)	3.08 (0.044)*	2.93 (0.039)*
Age (years)	47.1 (0.58)	49.5 (0.87)*	45.3 (0.76)*
Men (%)	43		

Data are expressed as the mean (SEM).
* $p < 0.01$, men vs. women.

Table 2 summarizes the association of the gene polymorphisms with serum lipid levels in all the participants. Tables 3 and 4 show the analysis in male and female participants, respectively. Table 5 shows age- and sex-adjusted means with 95% CI. We found that Hardy-Weinberg equilibrium was the case for all the SNPs, supporting the assumptions of random mating in this population except *CETP* Int14 +1 G → A, for which no homozygote was found in this population.

The incidence of heterozygote mutations of D442G and Int14 +1 G → A of the *CETP* gene was 8.1 and 0.6 %, respectively. These mutations were associated with higher HDL-cholesterol levels. The heterozygous mutation of D442G was also associated with lower TG levels only in men. Although the incidence of the homozygous mutation of D442G and heterozygous mutation of Int14 +1 G → A was quite low and the difference was not significant, the TG levels tended to be higher. The incidence of B1B1, B1B2, and B2B2 genotypes of the *CETP* TaqIB polymorphism was 35.8, 48.4, and 15.8%, respectively. The B2 allele of the *CETP* TaqIB polymorphism was associated with higher HDL-cholesterol levels in all the participants, men, and women. Although the difference was not statistically significant, the participants with the B2 allele tended to have lower TG levels, which is different from the results with the homozygous mutation of D442G and heterozygous mutation of Int14 +1 G → A.

We then determined the polymorphisms of *LPL* S447X mutations in this population. The incidence of heterozygous and homozygous mutations in the *LPL* gene was 20.7 and 1.3%, respectively. The mutation of the LPL S447X site was associated with higher HDL-cholesterol and lower TG levels, although the difference in the level of HDL-cholesterol in men or of TG in women was not statistically significant, possibly due to the small sample number.

The incidence of the CC, CT, and TT genotypes of *LIPC* in the Japanese was 24.9, 50.4, and 24.7%, respectively. Overall, the T allele was associated with an increase in HDL-cholesterol levels. However, the difference was not

Table 2. Demographic and lipid profile of all the participants according to genotype.

CETP D442G (rs2303790)

Genotype	Age	%	HDL-c (mmol/l)	TG (mmol/l)	LDL-c (mmo/l)
wt	47	91.6	1.53 (0.001)	1.37 (0.025)	3.06 (0.021)
hetero	48.4	8.1	1.75 (0.004)	1.15 (0.061)	2.90 (0.075)
homo	46.5	0.2	1.81 (0.18)	1.60 (0.101)	3.19 (1.580)
			$p = 0.000$	$p = 0.071$	$p = 0.154$

CETP Int14 +1 G → A (rs5742907)

Genotype	Age	%	HDL-c (mmol/l)	TG (mmol/l)	LDL-c (mmo/l)
wt	47	99.4	1.54 (0.009)	1.36 (0.024)	3.06 (0.020)
hetero	58.7	0.6	2.12 (0.262)	1.72 (0.362)	3.08 (0.316)
			$p = 0.000$	$p = 0.241$	$p = 0.938$

CETP TaqIB (rs708272)

Genotype	Age	%	HDL-c (mmol/l)	TG (mmol/l)	LDL-c (mmo/l)
B1B1	46.8	35.8	1.50 (0.016)	1.36 (0.036)	3.00 (0.033)
B1B2	48.4	48.4	1.54 (0.013)	1.38 (0.038)	3.08 (0.030)
B2B2	48.2	15.8	1.66 (0.024)	1.25 (0.043)	3.08 (0.051)
			$p = 0.000$	$p = 0.160$	$p = 0.362$

LPL S447X (rs328)

Genotype	Age	%	HDL-c (mmol/l)	TG (mmol/l)	LDL-c (mmo/l)
wt	47.3	78	1.53 (0.011)	1.37 (0.029)	3.06 (0.023)
hetero	46.2	20.7	1.60 (0.020)	1.24 (0.043)	3.06 (0.046)
homo	48	1.3	1.63 (0.101)	1.08 (0.125)	3.29 (0.189)
			$p = 0.004$	$p = 0.032$	$p = 0.487$

LIPC 514CT (rs1800588)

Genotype	Age	%	HDL-c (mmol/l)	TG (mmol/l)	LDL-c (mmo/l)
CC	49.7	24.9	1.49 (0.018)	1.37 (0.046)	3.11 (0.040)
CT	45.6	50.4	1.53 (0.013)	1.33 (0.034)	3.03 (0.029)
TT	47.6	24.7	1.63 (0.020)	1.39 (0.050)	3.06 (0.040)
			$p = 0.000$	$p = 0.520$	$p = 0.255$

APOC3 SstI (rs5128)

Genotype	Age	%	HDL-c (mmol/l)	TG (mmol/l)	LDL-c (mmo/l)
S1S1	46.6	42	1.56 (0.015)	1.32 (0.039)	3.06 (0.032)
S1S2	47	45.8	1.54 (0.013)	1.34 (0.033)	3.03 (0.029)
S2S2	48.9	12.2	1.52 (0.025)	1.53 (0.070)	3.11 (0.060)
			$p = 0.413$	$p = 0.021$	$p = 0.434$

Data are expressed as the mean (SEM). Each p-value was based on an analysis of covariance.

significant in men. The TG levels do not seem to be affected by this SNP.

The incidence of the S1S1, S1S2, and S2S2 genotypes of the *APOC3* SstI polymorphism was 42.0, 45.8, and 12.2%, respectively. Although the HDL and LDL-cholesterol levels were similar for all the genotypes, the S2 allele was associated with higher TG levels in all the participants and in men, but not in women. Among the SNPs studied, no polymorphism was found to affect LDL-cholesterol levels. We also determined sex- and age-adjusted means in Table 5 by multiple regression analysis. Due to the limited sample number and large variability of data, a

Table 3. Demographic and lipid profile of male participants according to genotype.

CETP D442G (rs2303790)

Genotype	*n*	HDL-c (mmol/l)	TG (mmol/l)	LDL-c (mmo/l)
wt	351	1.36 (0.020)	1.60 (0.052)	3.11 (0.045)
hetero	26	1.60 (0.105)	1.19 (0.176)	2.98 (0.194)
		$p = 0.003$	$p = 0.035$	$p = 0.453$

CETP TaqIB (rs708272)

Genotype	*n*	HDL-c (mmol/l)	TG (mmol/l)	LDL-c (mmo/l)
B1B1	121	1.33 (0.034)	1.64 (0.087)	3.06 (0.073)
B1B2	203	1.36 (0.026)	1.55 (0.068)	3.11 (0.064)
B2B2	53	1.56 (0.063)	1.53 (0.147)	3.13 (0.107)
		$p = 0.001$	$p = 0.664$	$p = 0.758$

LPL S447X (rs328)

Genotype	*n*	HDL-c (mmol/l)	TG (mmol/l)	LDL-c (mmo/l)
wt	292	1.36 (0.022)	1.65 (0.060)	3.08 (0.047)
hetero	81	1.43 (0.048)	1.36 (0.082)	3.16 (0.112)
homo	4	1.51 (0.386)	0.95 (0.295)	2.80 (0.513)
		$p = 0.278$	$p = 0.029$	$p = 0.617$

LIPC 514CT (rs1800588)

Genotype	*n*	HDL-c (mmol/l)	TG (mmol/l)	LDL-c (mmo/l)
CC	99	1.32 (0.032)	1.66 (0.094)	3.08 (0.072)
CT	188	1.40 (0.032)	1.51 (0.075)	3.08 (0.069)
TT	90	1.40 (0.041)	1.60 (0.095)	3.08 (0.085)
		$p = 0.266$	$p = 0.499$	$p = 0.996$

APOC3 SstI (rs5128)

Genotype	*n*	HDL-c (mmol/l)	TG (mmol/l)	LDL-c (mmo/l)
S1S1	165	1.37 (0.031)	1.50 (0.073)	3.16 (0.072)
S1S2	173	1.40 (0.031)	1.58 (0.076)	3.00 (0.060)
S2S2	39	1.31 (0.054)	1.92 (0.162)	3.13 (0.138)
		$p = 0.473$	$p = 0.041$	$p = 0.196$

Data are expressed as the mean (SEM). Each *p*-value was based on an analysis of covariance.

Table 4. Demographic and lipid profile of female participants according to genotype.

CETP D442G (rs2303790)

Genotype	*n*	HDL-c (mmol/l)	TG (mmol/l)	LDL-c (mmo/l)
wt	440	1.58 (0.018)	1.128 (0.0412)	2.93 (0.041)
hetero	34	1.67 (0.074)	1.15 (0.092)	2.98 (0.140)
		$p = 0.002$	$p = 0.590$	$p = 0.306$

CETP TaqIB (rs708272)

Genotype	*n*	HDL-c (mmol/l)	TG (mmol/l)	LDL-c (mmo/l)
B1B1	183	1.58 (0.028)	1.13 (0.057)	2.93 (0.062)
B1B2	220	1.67 (0.026)	1.15 (0.066)	2.98 (0.059)
B2B2	72	1.75 (0.043)	0.92 (0.057)	2.85 (0.105)
		$p = 0.004$	$p = 0.127$	$p = 0.461$

LPL S447X (rs328)

Genotype	*n*	HDL-c (mmol/l)	TG (mmol/l)	LDL-c (mmo/l)
wt	369	1.62 (0.020)	1.14 (0.046)	2.95 (0.046)
hetero	102	1.73 (0.038)	0.99 (0.065)	2.85 (0.081)
homo	4	1.97 (0.164)	0.72 (0.177)	3.89 (0.321)
		$p = 0.010$	$p = 0.185$	$p = 0.054$

LIPC 514CT (rs1800588)

Genotype	*n*	HDL-c (mmol/l)	TG (mmol/l)	LDL-c (mmo/l)
CC	102	1.59 (0.041)	1.15 (0.089)	2.93 (0.086)
CT	249	1.63 (0.022)	1.04 (0.046)	2.90 (0.050)
TT	124	1.73 (0.037)	1.20 (0.091)	3.03 (0.090)
		$p = 0.014$	$p = 0.210$	$p = 0.406$

APOC3 SstI (rs5128)

Genotype	*n*	HDL-c (mmol/l)	TG (mmol/l)	LDL-c (mmo/l)
S1S1	207	1.65 (0.028)	1.05 (0.054)	2.90 (0.062)
S1S2	208	1.62 (0.026)	1.18 (0.067)	2.93 (0.059)
S2S2	60	1.75 (0.045)	1.08 (0.079)	3.03 (0.106)
		$p = 0.078$	$p = 0.272$	$p = 0.608$

Data are expressed as the mean (SEM). Each *p*-value was based on an analysis of covariance.

significant difference was not found in TG levels in *LPL* or *APOC3* polymorphisms.

To determine the contribution of *CETP* and *LPL* gene polymorphisms to hyperalphacholesterolemia (2.58 mmol/l or over) and hypoalphacholesterolemia (1 mmol/l or under), we divided all the participants into 3 groups according to HDL-cholesterol levels; 1 mmol/l or under, 1 to 2.58 mmol/l, and 2.58 mmol/l or over. We then assessed the incidence of each genotype. The incidence of hyper- and hypoalphacholesterolemia was 1.8 and 8.3%, respectively. Among the genes studied, we found 3 gene polymorphisms to be associated with the incidence of high HDL-cholesterol (2.58 mmol/l or over) (Table 6). Participants with the B2B2 genotype of *CETP* TaqIB had a higher incidence of high HDL-cholesterol levels than the others. Heterozygotes of the *CETP* D442G polymorphism had a higher incidence of higher HDL-cholesterol levels than individuals with the wild type. Homozygotes of the *LPL* S447X polymorphism had a higher incidence of higher HDL-cholesterol levels than the others.

Discussion

In this study we have demonstrated the frequency of six common polymorphisms of four genes related to lipid

Table 5. Age- and sex-adjusted means of all the participants according to genotype.

CETP D442G (rs2303790)

Genotype	HDL-c (mmol/l)			TG (mmol/l)			LDL-c (mmo/l)		
	mean	low	upper	mean	low	upper	mean	low	upper
wt	1.53	1.49	1.56	1.37	1.29	1.45	3.05	2.98	3.11
hetero	1.72	1.62	1.83	1.18	0.90	1.46	2.90	2.68	3.12
homo	1.91	1.70	2.13	1.00	0.42	1.55	2.75	2.30	3.20
	$p = 0.0005$			$p = 0.200$			$p = 0.210$		

CETP Int14 +1 G → A (rs5742907)

Genotype	HDL-c (mmol/l)			TG (mmol/l)			LDL-c (mmo/l)		
	mean	low	upper	mean	low	upper	mean	low	upper
wt	1.54	1.51	1.57	1.35	1.27	1.43	3.04	2.97	3.10
hetero	2.13	1.72	2.54	1.70	0.63	2.79	2.97	2.11	3.83
	$p = 0.0048$			$p = 0.514$			$p = 0.877$		

CETP TaqIB (rs708272)

Genotype	HDL-c (mmol/l)			TG (mmol/l)			LDL-c (mmo/l)		
	mean	low	upper	mean	low	upper	mean	low	upper
B1B1	1.47	1.42	1.51	1.41	1.30	1.54	3.03	2.93	3.13
B1B2	1.56	1.53	1.59	1.34	1.25	1.42	3.04	2.97	3.10
B2B2	1.65	1.59	1.71	1.26	1.09	1.41	3.05	3.00	3.12
	$p = 0.0001$			$p = 0.154$			$p = 0.873$		

LPL S447X (rs328)

Genotype	HDL-c (mmol/l)			TG (mmol/l)			LDL-c (mmo/l)		
	mean	low	upper	mean	low	upper	mean	low	upper
wt	1.53	1.49	1.56	1.38	1.30	1.47	3.03	2.96	3.10
hetero	1.60	1.54	1.65	1.24	1.09	1.39	3.07	2.95	3.19
homo	1.66	1.55	1.78	1.11	0.80	1.40	3.11	2.87	3.35
	$p = 0.033$			$p = 0.090$			$p = 0.546$		

LIPC 514CT (rs1800588)

Genotype	HDL-c (mmol/l)			TG (mmol/l)			LDL-c (mmo/l)		
	mean	low	upper	mean	low	upper	mean	low	upper
CC	1.48	1.46	1.51	1.33	1.26	1.40	3.05	3.00	3.10
CT	1.54	1.52	1.56	1.35	1.31	1.39	3.04	3.01	3.07
TT	1.59	1.57	1.62	1.37	1.30	1.44	3.02	2.97	3.07
	$p = 0.0076$			$p = 0.770$			$p = 0.530$		

APOC3 SstI (rs5128)

Genotype	HDL-c (mmol/l)			TG (mmol/l)			LDL-c (mmo/l)		
	mean	low	upper	mean	low	upper	mean	low	upper
S1S1	1.55	1.51	1.60	1.30	1.18	1.41	3.04	2.95	3.13
S1S2	1.54	1.50	1.57	1.38	1.29	1.46	3.03	2.96	3.10
S2S2	1.52	1.45	1.58	1.45	1.29	1.63	3.02	2.89	3.16
	$p = 0.4223$			$p = 0.180$			$p = 0.816$		

Data are expressed as the mean (95% confidence interval). Each p-value was based on an analysis of covariance.

metabolism and its incidence and association with serum lipid levels in the general Japanese population. Because this is the largest Japanese population ever analyzed, these data would be useful for future analyses on the general Japanese population.

The prevalence of the D442G and Int14 +1 G → A mutations is very high in the general Japanese population, with heterozygote frequencies of 7 and 1%, respectively

Table 6. Incidence of CETP TaqIB, D442G, and LPL S447X genetypes according to HDL levels.

CETP TaqIB				
Genotype		HDL-c (mmol/l)		
	1.0> (8.3%)	1.0 ≤, 2.58 > (89.9%)	2.58 ≤ (1.8%)	†
B1B1	72 (9.9%)	644 (88.8%)	9 (1.2%)	$p = 0.009$
B1B2	79 (8.2%)	870 (90.2%)	16 (1.7%)	
B2B2	15 (4.8%)	284 (91.6%)	11 (3.5%)	
CETP D442G				
WT	161 (8.7%)	1671 (89.8%)	29 (1.6%)	$p = 0.011$
Hetero	5 (3.6%)	125 (91.2%)	7 (5.1%)	
Homo	0 (0%)	2 (100%)	0 (0%)	
LPL S447X				
WT	134 (8.9%)	1354 (89.4%)	26 (1.7%)	$p = 0.002$
Hetero	21 (5.0%)	390 (93.3%)	7 (1.7%)	
Homo	2 (8.0%)	21 (84.0%)	2 (8.0%)	

Column percentage is shown on top. Each box shows the number of participants in each category and its percentage in each genotype.
† The χ^2-test was used.

(10, 11, 27, 28). Our large-scaled study showed similar frequencies of these mutations, with 8.1 and 0.6%, respectively, indicating that our study population represents the general Japanese population and confirmed that the frequency of these mutations is quite high in Japanese. Because these mutations are associated with lower levels of CETP activity (27), the plasma level of HDL-cholesterol is higher in heterozygotes and homozygotes. We have also confirmed that the incidence of the mutation D442G is higher in people with hyperalphalipoproteinemia (2.58 mmol/l or over).

A genetic *CETP* deficiency is the most important and common cause of hyperalphalipoproteinemia in Japanese and contributes to 60% of hyperalphacholesterolemia (29). However, the role of CETP in atherogenesis is still under debate. A study in the Japanese Omagari area has shown a relatively increased incidence of coronary atherosclerosis in patients with CETP deficiency (30). In the Copenhagen City Heart Study, increased HDL-cholesterol levels caused by mutations in *CETP* were associated with an increased risk of CAD in caucasian females (31). In contrast, the B2 allele of the TaqIB polymorphism is associated with a low CETP mass, higher HDL-cholesterol levels, and a decreased risk of coronary artery disease (17). The reason for this discrepancy is unknown. Dose effects of CETP mass or another genetic abnormality may explain the difference in risk for CAD. Hirano *et al.* showed that people with weak LIPC activity had a higher incidence of CAD (32). Therefore, it is possible that LIPC activity is involved in these differences. More studies are needed to determine the role of CETP in CAD in various populations with different genetic backgrounds.

Our study is consistent with others in terms of the allele frequency of the S447X polymorphism of the *LPL* gene (19, 20, 33). Recent studies showed that the X447 mutation is associated with a favorable lipid profile, and lower TG and higher HDL-cholesterol levels, and that it may confer protection against coronary artery disease (19, 20, 33). We also found a similar tendency in men and women. However, a significant change in HDL-cholesterol levels was found in the total population and women, but not in men. Because the X447 mutation is associated with stronger LPL activity, the TG levels were lower in heterozygotes and homozygotes as expected, although the difference was not significant in women. Homozygotes seem to have lower TG levels than heterozygotes, which reflects the gene dosage effect. Because carriers of S447X have a favorable lipid profile in terms of HDL-cholesterol and TG, and a decreased risk of CAD (35, 36), we should examine whether carriers of S447X have fewer coronary artery events.

In terms of *LIPC* gene polymorphisms, our data clearly indicate that the frequency of the TT genotype is significantly higher in Japanese than in Caucasians (37, 38). However, a higher frequency of the TT genotype is also reported in Koreans and Japanese (39–41). Therefore, this difference might partly explain the higher HDL-cholesterol levels in Asians.

Our results on the allele frequency of the Sst1 polymor-

phism of the *APOC3* gene were almost comparable to the data on Asian Indians (42), but not on Caucasians (43). Caucasians seem to have a lower allele frequency of S2. Although a association of higher TG levels with the S2 allele has been reported in studies carried out in Caucasians (44–46) and Asians (47–49), our data show that such an association was found in the total population and in men, but not in women. Few other studies, however, have found any significant association between the Sst1 polymorphism and hypertriglyceridemia (50–52). The linkage disequilibrium between this polymorphism and the causative mutation might be weakened or absent in some populations (44).

Our data clearly showed that the heterozygotes of the D442G mutation, homozygotes of the LPL S447X mutation, and people with the TaqIB2B2 genotype had a higher incidence of hyperalphalipoproteinemia with HDL-cholesterol levels of 2.58 mmol/l or over. Alcohol consumption and smoking can also affect the levels of HDL-cholesterol. Corbex et al showed that the HDL levels of people with certain polymorphisms of the *CETP* gene are modulated by alcohol consumption (53). Therefore, it might be necessary to take into account environmental factors for the effect of gene polymorphisms on HDL-cholesterol levels as well as on the risk of cardiovascular events.

In summary, we have provided the largest ever database of gene polymorphisms related to lipid metabolism in the general Japanese population. A prospective study is now under way to determine the contribution of these gene polymorphisms to cardiovascular risk in Japanese.

Acknowledgements: We thank Shizuya Yamashita (Osaka University) and Hideaki Bujo (Chiba University) for critical reading of the manuscript. This study was supported by research grants for health sciences from the Japanese Ministry of Health and a grant from the Japan Atherosclerosis Society. We also thank the Osaka Pharmaceutical Manufacturer's Association for supporting our work.

Appendix

Research Group on Serum Lipid Survey 2000 in Japan

Chairman: Toru Kita, Department of Cardiovascular Medicine, Kyoto University Graduate School of Medicine
Principal investigators: Akira Yamamoto, National Cardiovascular Center
Yuji Matsuzawa, Department of Internal Medicine, Osaka University
Yasushi Saito, Department of Internal Medicine, Chiba University
Shinichi Oikawa, Department of Internal Medicine, Nippon Medical School
Noriaki Nakaya, Fussa Hospital
Jun Sasaki, International University of Health and Welfare
Hiroshi Mabuchi, Department of Internal Medicine, Kanazawa University
Nobuhiro Yamada, Department of Internal Medicine, Tsukuba University
Hiroshige Itakura, Ibaraki Christian University
Yuichi Ishikawa, Faculty of Health Sciences, Kobe University
Tadayoshi Ouchi, Department of Geriatric Medicine, University of Tokyo
Hiroshi Horibe, Keisen Clinic
Tamio Teramoto, Department of Internal Medicine, Teikyo University
Hidenori Arai, Department of Geriatric Medicine, Kyoto University
Collaborators: Tohru Egashira and Hiroaki Hattori, Department of Advanced Technology and Development, BML, Inc.
Nobuo Shirahashi, Osaka City University Medical School

References

(1) Murray CJ and Lopez AD: Mortality by cause for eight regions of the world: Global Burden of Disease Study. Lancet, 349: 1269–1276, 1997
(2) Watanabe H, Yamane K, Fujikawa R, Okubo M, Egusa G, and Kohno N: Westernization of lifestyle markedly increases carotid intima-media wall thickness (IMT) in Japanese people. Atherosclerosis, 166: 67–72, 2003
(3) Marmot MG, Syme SL, Kagan A, Kato H, Cohen JB, and Belsky J: Epidemiologic studies of coronary heart disease and stroke in Japanese men living in Japan, Hawaii and California: prevalence of coronary and hypertensive heart disease and associated risk factors. Am J Epidemiol, 102: 514–525, 1975
(4) Rader DJ, Cohen J, and Hobbs HH: Monogenic hypercholesterolemia: new insights in pathogenesis and treatment. J Clin Invest, 111: 1795–1803, 2003
(5) Austin MA, Hutter CM, Zimmern RL, and Humphries SE: Familial hypercholesterolemia and coronary heart disease: a HuGE association review. Am J Epidemiol, 160: 421–429, 2004
(6) Gaziano JM, Hennekens CH, O'Donnell CJ, Breslow JL, and Buring JE: Fasting triglycerides, high-density lipoprotein, and risk of myocardial infarction. Circulation, 96: 2520–2525, 1997
(7) Arai H, Yamamoto A, Matsuzawa Y, Saito Y, Yamada N, Oikawa S, Mabuchi H, Teramoto T, Sasaki J, Nakaya N, Itakura H, Ishikawa Y, Ouchi Y, Horibe H, and Kita T: Serum Lipid Survey and its recent trend in the General Japanese Population in

2000. J Atheroscler Thromb, 12, 98–106, 2005
(8) Yen FT, Deckelbaum RJ, Mann CJ, Marcel YL, Milne RW, and Tall AR: Inhibition of cholesteryl ester transfer protein activity by monoclonal antibody. Effects on cholesteryl ester formation and neutral lipid mass transfer in human plasma. J Clin Invest, 83: 2018–2024, 1989
(9) Brown ML, Inazu A, Hesler CB, Agellon LB, Mann C, Whitlock ME, Marcel YL, Milne RW, Koizumi J, Mabuchi H, et al.: Molecular basis of lipid transfer protein deficiency in a family with increased high-density lipoproteins. Nature, 342: 448–451, 1989
(10) Inazu A, Brown ML, Hesler CB, Agellon LB, Koizumi J, Takata K, Maruhama Y, Mabuchi H, and Tall AR: Increased high-density lipoprotein levels caused by a common cholesteryl-ester transfer protein gene mutation. N Engl J Med, 323: 1234–1238, 1990
(11) Hirano K, Yamashita S, Funahashi T, Sakai N, Menju M, Ishigami M, Hiraoka H, Kameda-Takemura K, Tokunaga K, Hoshino T, et al.: Frequency of intron 14 splicing defect of cholesteryl ester transfer protein gene in the Japanese general population – relation between the mutation and hyperalphalipoproteinemia. Atherosclerosis, 100: 85–90, 1993
(12) Nagano M, Yamashita S, Hirano K, Kujiraoka T, Ito M, Sagehashi Y, Hattori H, Nakajima N, Maruyama T, Sakai N, Egashira T, and Matsuzawa Y: Point mutation (–69 G → A) in the promoter region of cholesteryl ester transfer protein gene in Japanese hyperalphalipoproteinemic subjects. Arterioscler Thromb Vasc Biol, 21: 985–990, 2001
(13) Sakai N, Yamashita S, Hirano K, Menju M, Arai T, Kobayashi K, Ishigami M, Yoshida Y, Hoshino T, Nakajima N, et al.: Frequency of exon 15 missense mutation (442D:G) in cholesteryl ester transfer protein gene in hyperalphalipoproteinemic Japanese subjects. Atherosclerosis, 114: 139–145, 1995
(14) Tall AR: Plasma cholesteryl ester transfer protein. J Lipid Res, 34: 1255–1274, 1993
(15) Wang S, Deng L, Milne RW, and Tall AR: Identification of a sequence within the C-terminal 26 amino acids of cholesteryl ester transfer protein responsible for binding a neutralizing monoclonal antibody and necessary for neutral lipid transfer activity. J Biol Chem, 267: 17487–17490, 1992
(16) Hannuksela ML, Liinamaa MJ, Kesaniemi YA, and Savolainen MJ: Relation of polymorphisms in the cholesteryl ester transfer protein gene to transfer protein activity and plasma lipoprotein levels in alcohol drinkers. Atherosclerosis, 110: 35–44, 1994
(17) Ordovas JM, Cupples LA, Corella D, Otvos JD, Osgood D, Martinez A, Lahoz C, Coltell O, Wilson PW, and Schaefer EJ: Association of cholesteryl ester transfer protein-TaqIB polymorphism with variations in lipoprotein subclasses and coronary heart disease risk: the Framingham study. Arterioscler Thromb Vasc Biol, 20: 1323–1329, 2000
(18) Stocks J, Thorn JA, and Galton DJ: Lipoprotein lipase genotypes for a common premature termination codon mutation detected by PCR-mediated site-directed mutagenesis and restriction digestion. J Lipid Res, 33: 853–857, 1992
(19) Kuivenhoven JA, Groenemeyer BE, Boer JM, Reymer PW, Berghuis R, Bruin T, Jansen H, Seidell JC, and Kastelein JJ: Ser447stop mutation in lipoprotein lipase is associated with elevated HDL cholesterol levels in normolipidemic males. Arterioscler Thromb Vasc Biol, 17: 595–599, 1997
(20) Groenemeijer BE, Hallman MD, Reymer PW, Gagne E, Kuivenhoven JA, Bruin T, Jansen H, Lie KI, Bruschke AV, Boerwinkle E, Hayden MR, and Kastelein JJ: Genetic variant showing a positive interaction with beta-blocking agents with a beneficial influence on lipoprotein lipase activity, HDL cholesterol, and triglyceride levels in coronary artery disease patients. The Ser447-stop substitution in the lipoprotein lipase gene. REGRESS Study Group. Circulation, 95: 2628–2635, 1997
(21) Bensadoun A and Berryman DE: Genetics and molecular biology of hepatic lipase. Curr Opin Lipidol, 7: 77–81, 1996
(22) Zambon A, Deeb SS, Hokanson JE, Brown BG, and Brunzell JD: Common variants in the promoter of the hepatic lipase gene are associated with lower levels of hepatic lipase activity, buoyant LDL, and higher HDL2 cholesterol. Arterioscler Thromb Vasc Biol, 18: 1723–1729, 1998
(23) Ordovas JM, Civeira F, Genest J, Jr., Craig S, Robbins AH, Meade T, Pocovi M, Frossard PM, Masharani U, Wilson PW, et al.: Restriction fragment length polymorphisms of the apolipoprotein A-I, C-III, A-IV gene locus. Relationships with lipids, apolipoproteins, and premature coronary artery disease. Atherosclerosis, 87: 75–86, 1991
(24) Anderson RA, Burns TL, Lee J, Swenson D, and Bristow JL: Restriction fragment length polymorphisms associated with abnormal lipid levels in an adolescent population. Atherosclerosis, 77: 227–237, 1989
(25) Johnson CL, Rifkind BM, Sempos CT, Carroll MD, Bachorik PS, Briefel RR, Gordon DJ, Burt VL, Brown CD, Lippel K, et al.: Declining serum total cholesterol levels among US adults. The National Health and Nutrition Examination Surveys. Jama, 269: 3002–3008, 1993
(26) Nagano M, Yamashita S, Hirano K, Ito M, Maruyama T, Ishihara M, Sagehashi Y, Oka T, Kujiraoka T, Hattori H, Nakajima N, Egashira T, Kondo M, Sakai N, and Matsuzawa Y: Two novel missense mutations in the CETP gene in Japanese hyperalphalipo-

proteinemic subjects: high-throughput assay by Invader assay. J Lipid Res, 43: 1011–1018, 2002
(27) Inazu A, Jiang XC, Haraki T, Yagi K, Kamon N, Koizumi J, Mabuchi H, Takeda R, Takata K, Moriyama Y, et al.: Genetic cholesteryl ester transfer protein deficiency caused by two prevalent mutations as a major determinant of increased levels of high density lipoprotein cholesterol. J Clin Invest, 94: 1872–1882, 1994
(28) Inazu A, Koizumi J, Haraki T, Yagi K, Wakasugi T, Takegoshi T, Mabuchi H, and Takeda R: Rapid detection and prevalence of cholesteryl ester transfer protein deficiency caused by an intron 14 splicing defect in hyperalphalipoproteinemia. Hum Genet, 91: 13–16, 1993
(29) Sakai N, Santamarina-Fojo S, Yamashita S, Matsuzawa Y, and Brewer HB, Jr: Exon 10 skipping caused by intron 10 splice donor site mutation in cholesteryl ester transfer protein gene results in abnormal downstream splice site selection. J Lipid Res, 37: 2065–2073, 1996
(30) Hirano K, Yamashita S, Nakajima N, Arai T, Maruyama T, Yoshida Y, Ishigami M, Sakai N, Kameda-Takemura K, and Matsuzawa Y: Genetic cholesteryl ester transfer protein deficiency is extremely frequent in the Omagari area of Japan. Marked hyperalphalipoproteinemia caused by CETP gene mutation is not associated with longevity. Arterioscler Thromb Vasc Biol, 17: 1053–1059, 1997
(31) Agerholm-Larsen B, Nordestgaard BG, Steffensen R, Jensen G, and Tybjaerg-Hansen A: Elevated HDL cholesterol is a risk factor for ischemic heart disease in white women when caused by a common mutation in the cholesteryl ester transfer protein gene. Circulation, 101: 1907–1912, 2000
(32) Hirano K, Yamashita S, Kuga Y, Sakai N, Nozaki S, Kihara S, Arai T, Yanagi K, Takami S, Menju M, et al.: Atherosclerotic disease in marked hyperalphalipoproteinemia. Combined reduction of cholesteryl ester transfer protein and hepatic triglyceride lipase. Arterioscler Thromb Vasc Biol, 15: 1849–1856, 1995
(33) Hata A, Robertson M, Emi M, and Lalouel JM: Direct detection and automated sequencing of individual alleles after electrophoretic strand separation: identification of a common nonsense mutation in exon 9 of the human lipoprotein lipase gene. Nucleic Acids Res, 18: 5407–5411, 1990
(34) Mattu RK, Needham EW, Morgan R, Rees A, Hackshaw AK, Stocks J, Elwood PC, and Galton DJ: DNA variants at the LPL gene locus associate with angiographically defined severity of atherosclerosis and serum lipoprotein levels in a Welsh population. Arterioscler Thromb, 14: 1090–1097, 1994
(35) Gagne SE, Larson MG, Pimstone SN, Schaefer EJ, Kastelein JJ, Wilson PW, Ordovas JM, and Hayden MR: A common truncation variant of lipoprotein lipase (Ser447X) confers protection against coronary heart disease: the Framingham Offspring Study. Clin Genet, 55: 450–454, 1999
(36) Peacock RE, Hamsten A, Nilsson-Ehle P, and Humphries SE: Associations between lipoprotein lipase gene polymorphisms and plasma correlations of lipids, lipoproteins and lipase activities in young myocardial infarction survivors and age-matched healthy individuals from Sweden. Atherosclerosis, 97: 171–185, 1992
(37) Anderson JL and Carlquist JF: Genetic polymorphisms of hepatic lipase and cholesteryl ester transfer protein, intermediate phenotypes, and coronary risk: do they add up yet? J Am Coll Cardiol, 41: 1990–1993, 2003
(38) Couture P, Otvos JD, Cupples LA, Lahoz C, Wilson PW, Schaefer EJ, and Ordovas JM: Association of the C-514T polymorphism in the hepatic lipase gene with variations in lipoprotein subclass profiles: The Framingham Offspring Study. Arterioscler Thromb Vasc Biol, 20: 815–822, 2000
(39) Yamakawa-Kobayashi K, Somekawa Y, Fujimura M, Tomura S, Arinami T, and Hamaguchi H: Relation of the -514C/T polymorphism in the hepatic lipase gene to serum HDL and LDL cholesterol levels in postmenopausal women under hormone replacement therapy. Atherosclerosis, 162: 17–21, 2002
(40) Somekawa Y, Umeki H, Kobayashi K, Tomura S, Aso T, and Hamaguchi H: Effects of hormone replacement therapy and hepatic lipase polymorphism on serum lipid profiles in postmenopausal Japanese women. J Clin Endocrinol Metab, 87: 4766–4770, 2002
(41) Hong SH, Song J, and Kim JQ: Genetic variations of the hepatic lipase gene in Korean patients with coronary artery disease. Clin Biochem, 33: 291–296, 2000
(42) Chhabra S, Narang R, Krishnan LR, Vasisht S, Agarwal DP, Srivastava LM, Manchanda SC, and Das N: Apolipoprotein C3 SstI polymorphism and triglyceride levels in Asian Indians. BMC Genet, 3: 9, 2002
(43) Hoffer MJ, Sijbrands EJ, De Man FH, Havekes LM, Smelt AH, and Frants RR: Increased risk for endogenous hypertriglyceridaemia is associated with an apolipoprotein C3 haplotype specified by the SstI polymorphism. Eur J Clin Invest, 28: 807–812, 1998
(44) Shoulders CC, Grantham TT, North JD, Gaspardone A, Tomai F, de Fazio A, Versaci F, Gioffre PA, and Cox NJ: Hypertriglyceridemia and the apolipoprotein CIII gene locus: lack of association with the variant insulin response element in Italian school children. Hum Genet, 98: 557–566, 1996

(45) Dallinga-Thie GM, van Linde-Sibenius Trip M, Rotter JI, Cantor RM, Bu X, Lusis AJ, and de Bruin TW: Complex genetic contribution of the Apo AI-CIII-AIV gene cluster to familial combined hyperlipidemia. Identification of different susceptibility haplotypes. J Clin Invest, 99: 953–961, 1997
(46) Paul-Hayase H, Rosseneu M, Robinson D, Van Bervliet JP, Deslypere JP, and Humphries SE: Polymorphisms in the apolipoprotein (apo) AI-CIII-AIV gene cluster: detection of genetic variation determining plasma apo AI, apo CIII and apo AIV concentrations. Hum Genet, 88: 439–446, 1992
(47) Ko YL, Ko YS, Wu SM, Teng MS, Chen FR, Hsu TS, Chiang CW, and Lee YS: Interaction between obesity and genetic polymorphisms in the apolipoprotein CIII gene and lipoprotein lipase gene on the risk of hypertriglyceridemia in Chinese. Hum Genet, 100: 327–333, 1997
(48) Hong SH, Park WH, Lee CC, Song JH, and Kim JQ: Association between genetic variations of apo AI-CIII-AIV cluster gene and hypertriglyceridemic subjects. Clin Chem, 43: 13–17, 1997
(49) Zeng Q, Dammerman M, Takada Y, Matsunaga A, Breslow JL, and Sasaki J: An apolipoprotein CIII marker associated with hypertriglyceridemia in Caucasians also confers increased risk in a west Japanese population. Hum Genet, 95: 371–375, 1995
(50) Price WH, Morris SW, Burgon R, Donald PM, and Kitchin AH: Apolipoprotein CIII polymorphism and coronary heart disease. Lancet, 2: 1041, 1986
(51) Marcil M, Boucher B, Gagne E, Davignon J, Hayden M, and Genest J, Jr: Lack of association of the apolipoprotein A-I-C-III-A-IV gene XmnI and SstI polymorphisms and of the lipoprotein lipase gene mutations in familial combined hyperlipoproteinemia in French Canadian subjects. J Lipid Res, 37: 309–319, 1996
(52) Bai H, Saku K, Liu R, Imamura M, and Arakawa K: Association between coronary heart disease and the apolipoprotein A-I/C-III/A-IV complex in a Japanese population. Hum Genet, 95: 102–104, 1995
(53) Corbex M, Poirier O, Fumeron F, Betoulle D, Evans A, Ruidavets JB, Arveiler D, Luc G, Tiret L, and Cambien F: Extensive association analysis between the CETP gene and coronary heart disease phenotypes reveals several putative functional polymorphisms and gene-environment interaction. Genet Epidemiol, 19: 64–80, 2000

High Frequency of a Retinoid X Receptor γ Gene Variant in Familial Combined Hyperlipidemia That Associates With Atherogenic Dyslipidemia

Atsushi Nohara, Masa-aki Kawashiri, Thierry Claudel, Mihoko Mizuno, Masayuki Tsuchida, Mutsuko Takata, Shoji Katsuda, Kenji Miwa, Akihiro Inazu, Folkert Kuipers, Junji Kobayashi, Junji Koizumi, Masakazu Yamagishi, Hiroshi Mabuchi

Objective—The genetic background of familial combined hyperlipidemia (FCHL) has not been fully clarified. Because several nuclear receptors play pivotal roles in lipid metabolism, we tested the hypothesis that genetic variants of nuclear receptors contribute to FCHL.

Methods and Results—We screened all the coding regions of the PPARα, PPARγ2, PPARδ, FXR, LXRα, and RXRγ genes in 180 hyperlipidemic patients including 60 FCHL probands. Clinical characteristics of the identified variants were evaluated in other 175 patients suspected of coronary disease. We identified PPARα Asp140Asn and Gly395Glu, PPARγ2 Pro12Ala, RXRγ Gly14Ser, and FXR −1g−>t variants. Only RXRγ Ser14 was more frequent in FCHL (15%, $P<0.05$) than in other primary hyperlipidemia (4%) and in controls (5%). Among patients suspected of coronary disease, we identified 9 RXRγ Ser14 carriers, who showed increased triglycerides (1.62±0.82 versus 1.91±0.42 [mean±SD] mmol/L, $P<0.05$), decreased HDL-cholesterol (1.32±0.41 versus 1.04±0.26, $P<0.05$), and decreased post-heparin plasma lipoprotein lipase protein levels (222±85 versus 149±38 ng/mL, $P<0.01$). In vitro, RXRγ Ser14 showed significantly stronger repression of the lipoprotein lipase promoter than RXRγ Gly14.

Conclusion—These findings suggest that RXRγ contributes to the genetic background of FCHL. (***Arterioscler Thromb Vasc Biol.* 2007;27:923-928.**)

Key Words: apolipoproteins ■ gene mutations ■ lipoprotein lipase ■ familial combined hyperlipidemia ■ nuclear receptors

Familial combined hyperlipidemia (FCHL) is the most common form of inherited hyperlipidemia. FCHL shows strong genetic susceptibility resembling an autosomal dominant disease,[1-3] but most of the underlying causal mechanisms remain to be elucidated. Lipoprotein lipase (LPL) has been implicated as one of the genes that modify the lipid phenotype in FCHL.[4,5] "Intra-individual variability" of the lipoprotein phenotype is often included as a criterion in diagnosis.[6] However, a recent prospective study of FCHL families suggests that this variability may even include normolipidemic periods in affected subjects.[7] This feature indicates that FCHL could be a "disease of regulation" rather than a genetic defect in certain peripheral components of lipid metabolism.

Nuclear receptors are transcription factors that can be activated by specific ligands. Recent studies have shown that nuclear receptors, especially retinoid X receptor (RXR) and its heterodimerization partners,[8] play important roles in maintenance of lipid homeostasis on their activation by a variety of ligands derived from dietary cholesterol and fatty acids.[9] The peroxisome proliferator-activated receptors (PPARs) family, the oxysterol sensor liver X receptor (LXR), and the bile acid sensor farnesoid X receptor (FXR) are all involved in control of plasma lipid concentrations.[10] Thus, we tested the hypothesis that variants of these nuclear receptors, ie, PPARα, PPARγ2, PPARδ, LXRα, FXR, and RXRγ, could constitute part of the genetic background of atherogenic dyslipidemia, particularly of FCHL.

Methods

Subjects

The study design consists of 2 parts. First, we screened for frequent variants in the nuclear receptor candidate genes among 180 patients with primary hyperlipidemia, including 60 unrelated patients with FCHL (clinical characteristics are presented in supplemental Table I, available online at http://atvb.ahajounals.org). Patients with familial

Original received August 28 2006; final version accepted January 6, 2007.

From the Departments of Lipidology (A.N., M.M., J. Kobayashi, H.M.) and Cardiovascular Medicine (M.K., M. Tsuchida, M. Takata, S.K., K.M., M.Y.), Graduate School of Medical Science, Kanazawa University, Japan; Center for Liver, Digestive, and Metabolic Diseases, Laboratory of Pediatrics (T.C., F.K.), University Medical Center Groningen, Groningen, the Netherlands; School of Health Sciences, Faculty of Medicine (A.I.), and Department of General Medicine (J. Koizumi), Kanazawa University Hospital, Japan.

Correspondence to Atsushi Nohara, Department of Lipidology, Graduate School of Medical Science, Kanazawa University, Takara-machi 13-1, Kanazawa 920-8641, Japan. E-mail a-nohara@med.kanazawa-u.ac.jp

Arterioscler Thromb Vasc Biol. **is available at http://www.atvbaha.org** **DOI: 10.1161/01.ATV.0000258945.76141.8a**

hypercholesterolemia and secondary hyperlipidemia were excluded. Diagnosis of FCHL was based on the fulfillment of all of the following three criteria: (1) Phenotype IIb, IIa, or IV hyperlipidemia according to the Fredrickson classification; (2) Presence of phenotype IIb, IIa, or IV hyperlipidemia in a first-degree relative and at least one family member with phenotype IIb; (3) Exclusion of familial hypercholesterolemia. Two hundred ninety-eight anonymous samples from healthy males were used as controls for frequency analysis of identified mutations. All blood samples in this study were obtained after an overnight fast.

Second, we evaluated the clinical impact of potentially relevant variants in another 175 patients who were suspected of having coronary artery disease based on any of the following reasons: ECG abnormalities, cumulative coronary risk factors, and/or chest symptoms. The group included 105 patients who had undergone coronary angiography. Patients with familial hypercholesterolemia were excluded because of their clear genetic background for hyperlipidemia. The extent and severity of atherosclerotic changes in coronary angiography were assessed by assigning scores to each of the 15 segments, according to the classification of the American Heart Association Grading Committee. The coronary stenosis index (CSI) was defined as the sum of the following scores[11]: A normal coronary angiogram was graded 0, stenosis of less than 25% was graded 1, 25% to 50% stenosis was graded 2, 50% to 75% stenosis was graded 3, and more than 75% stenosis was graded 4. CSI is a useful index for evaluating mild-moderate coronary atherosclerotic changes.

All the subjects and controls enrolled were inhabitants of the Hokuriku district of Japan. Written informed consent was obtained from each of the subjects. The study protocol was approved by the ethics committee of the Graduate School of Medical Science, Kanazawa University.

Laboratory Analyses

Total cholesterol (TC), triglycerides (TG), high-density lipoprotein (HDL)-cholesterol, apolipoproteins, glucose, and thyroid hormones were measured according to standard clinical laboratory techniques. HDL-cholesterol fractions were obtained by dextran sulfate-magnesium chloride precipitation and assayed using a commercial kit (Daiichi, Tokyo, Japan).[12] Separation of lipoproteins by ultracentrifugation was performed as described by Havel et al.[13] Plasma remnant-like particle (RLP)-cholesterol was determined by immunoabsorption using the commercial RLP-C JIMRO kit.[14] Plasma cholesteryl ester transfer protein (CETP) concentrations were determined by enzyme-linked immunosorbent assay using the monoclonal antibody TP2 and a rabbit polyclonal antibody raised against recombinant human CETP.[15] For LPL assessment, blood samples were obtained 10 minutes after an intravenous injection of 30 IU heparin/kg body weight. LPL activity was measured using radiolabeled triolein emulsion after hepatic lipase (HL) inhibition by SDS as previously described.[16] LPL mass was measured by sandwich enzyme-linked immunosorbent assay (ELISA) using specific monoclonal antibody against LPL (Daiichi Pure Chemicals Co Ltd, Tokyo, Japan).[17]

Genetic Analyses of Candidate Genes

Genomic DNA was isolated from peripheral white blood cells using standard phenol-chloroform extraction techniques. We screened all the coding regions of PPARα (NM_032644), PPARδ (NM_006238), PPARγ2 (NM_015869), LXRα (NM_005693), FXR (NM_005123), and RXRγ (NM_006917) genes with flanking exon-intron boundaries by polymerase chain reaction (PCR)-denaturing gradient gel electrophoresis (DGGE) using the DCode system (Bio-Rad), which is highly accurate in detecting changes in nucleic acids.[18] The structural organization and nucleotide sequences of these genes were retrieved from the gene database of NCBI. Lists of all GC-clamped primers used in DGGE analysis are available online (supplemental Table II). Samples with a variant detected by DGGE analysis were directly sequenced on an ABI310 analyzer (Applied Biosystems). PCR-restriction-fragment-length polymorphisms analysis on the RXRγ Ser14 variant was performed with the primers 5′-AGCCGAGAGAGGCGGTAATA-3′ (forward) and 5′-TACAGGTCCACGCAGTGAAG-3′ (reverse) in patients suspected of coronary artery disease. Digestion with *Alu*I resulted in a 76-bp fragment for Ser allele and a 120-bp fragment for Gly allele.

Cell Culture and Transfection Assays

Cos7 cells were grown in DMEM supplemented with 10% FCS, penicillin/streptomycin, sodium pyruvate, glutamine, and nonessential amino acids (Gibco BRL, Invitrogen). The medium was changed every 48 hours. Cos7 cells were transfected using FuGENE 6 reagent (Roche): 150 ng of the indicated LPL firefly luciferase reporter plasmid (a generous gift of Dr B. Staels, Institut Pasteur de Lille, France), that contains the proximal 466-bp of the human LPL promoter in front of the ATG cloned into the *Hind*III site of the pGL3 plasmid, was cotransfected with or without 100 ng of the human RXRγ expressing vector (a generous gift of Dr W. Lamph, Ligand Pharmaceuticals Inc, San Diego, Calif). After an overnight incubation, cells were incubated with medium containing 10% FCS with or without the retinoid LGD1069, (1 μmol/L, Sigma) and luciferase activity was assayed 48 hours later using an Orion luminometer (Berthold). Transfection studies were performed at least 3 times in triplicate. Transfection efficiency was monitored by cotransfection of 150 ng of a SV40-driven β-galactosidase expression plasmid. A positive RXRE TKpGL3 construct was made by cloning 3 copies of the direct repeat AGGTCA spaced by 5 nucleotides in the TKpGL3 plasmid.

Plasmid Site-Directed Mutagenesis

Nucleotide substitution was introduced in the plasmid expressing human RXRγ using the QuikChange Site-Directed Mutagenesis Kit (Stratagene, The Netherlands) and the primer 5′-CATGAAGTTTCCCGCAAGCTATGGAGGCTCCCCTGG C-3′ in which the nucleotide in bold indicates the mutation.

Statistical Analysis

The frequency distribution of genotypes was compared using standard χ^2 tests. Student t test was used for normally distributed parameters and the Kruskal–Wallis test was used for non-normally distributed parameters: triglycerides levels, LPL levels, and CSI. JMP 5.1.2 software (SAS Institute Inc) was used for statistical calculation.

Results

Identified Variants in Nuclear Receptor Genes

With PCR-DGGE analysis, we identified 4 variants with amino acid changes, ie, Asp140Asn and Gly395Glu in the PPARα gene, Pro12Ala in the PPARγ2 gene, Gly14Ser in the

TABLE 1. Frequencies of Nuclear Receptor Genes Variants Identified in This Study

	FCHL n=60	Other Hyperlipidemia n=120	General Population n=298	*P* Value
PPARαGly395Glu				
Glu395	3 (5%)	1 (0.8%)	6 (2%)	ns
PPARαAsp140Asn				
Asn140	2 (3%)	1 (0.8%)	2 (0.6%)	ns
PPARγ2Pro12Ala				
Ala12	5 (8%)	10 (8%)	20 (7%)	ns
FXR −1g−>t				
−1g/t	19 (32%)	34 (28%)	108 (36%)	ns
−1t/t	2 (3%)	6 (5%)	27 (9%)	ns
RXRγGly14Ser				
Ser14	9 (15%)	5 (4%)	15 (5%)	0.03

TABLE 2. Clinical Characteristics of Patients With RXRγ Variant

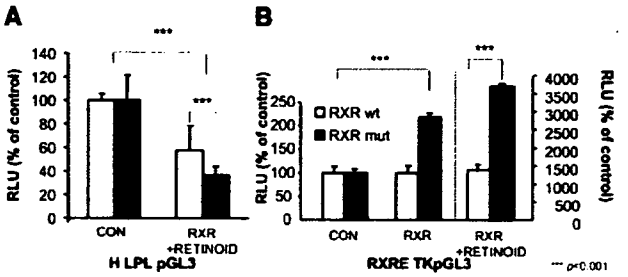
	RXRγ Gly14Ser		
	Gly/Gly	Gly/Ser	*P* Value
Number (M/F)	166 (78/88)	9 (5/4)	
Age, y	58±15	58±7	ns
BMI, kg/m^2	23.4±5	23.9±2	ns
Smoking, %	36	33	ns
Total cholesterol, mmol/L	5.98±1.4	5.96±1.55	ns
Triglycerides, mmol/L	1.62±0.82	1.91±0.42	$P<0.05$
HDL cholesterol, mmol/L	1.32±0.41	1.04±0.26	$P<0.05$
LDL cholesterol, mmol/L	3.94±1.27	4.07±1.45	ns
HDL2 cholesterol, mmol/L	0.78±0.28	0.54±0.10	$P<0.05$
HDL3 cholesterol, mmol/L	0.44±0.10	0.39±0.08	ns
ApoA-I, g/L	1.38±0.31	1.18±0.18	ns
ApoA-II, g/L	0.32±0.06	0.28±0.05	$P<0.05$
ApoB, g/L	1.31±0.38	1.35±0.31	ns
ApoC-II, g/L	0.06±0.02	0.05±0.02	ns
ApoC-III, g/L	0.11±0.05	0.10±0.03	ns
ApoE, g/L	0.06±0.02	0.05±0.01	ns
RLP cholesterol, mmol/L	0.15±0.10	0.21±0.10	$P<0.01$
CETP, mg/L	2.52±0.82	2.48±0.73	ns
Intraindividual lipoprotein phenotype variability, %	27	88	$P<0.01$
Fasting glucose, mmol/L	5.72±1.39	5.33±0.72	ns
HbA1c, %	5.6±1.0	5.8±1.0	ns
Fasting insulin, pmol/L	70.8±90.3	52.1±1.0	ns
HOMA-IR	2.28±2.1	2.19±1.7	ns
Diabetes, %	28	33	ns
HL activity, U/L	0.24±0.09	0.26±0.07	ns
LPL activiy, U/L	0.11±0.06	0.08±0.03	$P<0.05$
LPL mass, ng/mL	222±85	149±38	$P<0.01$
FT3, pmol/L	0.42±0.01	0.044±0.01	ns
FT4, pmol/L	15.2±5.15	13.3±2.57	ns
TSH, μU/mL	2.31±2.8	2.53±0.9	ns
Number (M/F)	100 (50/50)	5 (4/1)	
CSI	12.3±10	21.4±6	$P<0.05$
			mean±SD

BMI indicates body mass index; HOMA-IR, homeostasis model assessment; FT3, free triiodothyronine; FT4, free thyroxine; TSH, thyroid stimulating hormone.

RXRγ gene, and 1 nucleotide substitution in a flanking coding region, ie, FXR −1g−>t variant. The PPARγ2 Pro12Ala polymorphism has already been well-described,[19] whereas the others represent novel variants identified in this study. In humans, variants in the RXRγ gene have been associated with elevated triglyceride levels in familial type 2 diabetes, but none of these variants showed an altered coding sequence.[20] Therefore, this is the first description of a RXRγ variant with an amino acid substitution. In the PPARα gene, the Leu162Val variant has been reported in Western countries,[21] but this variant was not identified in this study. We also identified some silent nucleotide substitutions, ie, 891C−>G (rs13306747) and 1431C−>T (rs1724155) in the PPARγ2 gene, 1233C−>T (rs9658166) in the PPARδ gene, and 1134A−>G (rs1131379) in the LXRα gene. We did not identify variants with amino acid changes in the PPARδ and LXRα genes. We further investigated the variants with amino acid substitutions and the −1g−>t FXR variant, because of the likelihood that these induced altered physiological function.

Higher Frequency of RXRγ Variant in FCHL

We evaluated the frequencies of the 5 identified polymorphisms in subjects with FCHL, subjects with other forms of primary hyperlipidemia and in the general population (Table 1). Only the RXRγ Ser14 variant was found to be significantly more frequent in FCHL patients (15%) compared with that in other forms of primary hyperlipidemia (4%) or the general population (5%).



A, Cos7 cells were cotransfected with RXRγ wild-type or the Ser14 variant and activated with retinoid in presence of the LPL promoter. B, Cos7 cells were cotransfected with RXRγ wild-type or the Ser14 variant and activated with retinoid in presence of a positive RXRE cloned in the TKpGL3 plasmid.

Atherogenic Plasma Lipids Profiles and Coronary Atherosclerosis Associated With the RXRγ Ser14 Variant

To establish the impact of the identified RXRγ variant on metabolic parameters and on coronary atherosclerosis, we evaluated anthropometric parameters and laboratory data from 175 patients suspected of coronary disease. The RXRγ Ser14 variant was identified in 9 patients, all of whom were heterozygotes. Eight of the RXRγ Ser14 carriers had hyperlipidemia, while the remaining 1 demonstrated an isolated low HDL cholesterol level. Clinical characteristics of patients with or without the RXRγ Ser14 allele are shown in Table 2. There was no difference in age or body mass index between the two groups. In their lipid profiles, RXRγ Ser14 carriers had higher TG, lower HDL cholesterol especially in the HDL2 subfraction, and lower apolipoprotein A-II levels. There was no difference in CETP protein levels between the groups. Furthermore, we found that the RLP cholesterol level was significantly higher in the RXRγ Ser14 carriers than in the wild-type. Subjects with this variant also showed significantly lower LPL activities and protein levels in post-heparin plasma. Separation of lipoproteins demonstrated that the Ser14 carriers had higher TG levels in very low-density lipoprotein (VLDL) and low-density lipoprotein (LDL) fractions, higher cholesterol levels in VLDL, and lower cholesterol levels in HDL (supplemental Table III).

Two RXRγ Ser14 carriers were diagnosed as FCHL (22%), and 2 additional carriers were suspected of FCHL with hyperlipidemic siblings without information on first-degree relatives. Among non-carriers, 22 of 166 patients were diagnosed as FCHL (13%). One hundred twenty-five patients suspected of coronary disease showed hyperlipidemia and the intraindividual variability of lipoprotein phenotype was significantly more frequent in RXRγ Ser14 carriers (7 of 8 hyperlipidemic patients; 88%) than in wild-type (32 of 117 hyperlipidemic patients; 27%, Table 2).

There was no significant difference in the thyroid hormone levels between the two groups.

Four males and 1 female were identified as RXRγ variant carriers among 105 patients who underwent coronary angiography. The carriers of RXRγ Ser14 demonstrated significantly higher CSI than those with the wild-type (Table 2).

RXRγ Variant Represses More Efficiently the LPL Promoter Activity

Because RXRγ Ser14 carriers showed significantly lower LPL activities and protein levels in post-heparin plasma, we hypothesized that activated-RXRγ downregulates LPL gene expression by a transcriptional mechanism and that RXRγ variant is more effective in repressing the LPL promoter activity. Therefore, transfection assays were performed using the LPL promoter cotransfected with either wild-type RXRγ or the variant (Figure). Interestingly, RXRγ Gly14 significantly repressed (−40%) the LPL promoter activity, whereas the RXRγ Ser14 repressed even more strongly (−60%, $P<0.001$, Figure A). Moreover, the RXRγSer14 was a more potent activator of a positive RXRE cloned in front of a TKpGL3 plasmid (note the different scales in Figure B). Taken together, our results indicate that RXRγ downregulates human LPL gene expression, at least partially by a transcriptional mechanism, and that the newly identified RXRγ variant is a more potent repressor than the wild-type in this respect, as well as a more potent transactivator of a positive RXR response element.

Gain of Function Variant of PPARα and Increased LDL-C Levels

The carriers of the PPARα variant Gly395Glu tended to have higher frequency in the FCHL population, although not statistically significant. Four subjects were identified as PPARα Glu395 carriers in the coronary artery disease-suspected group and showed significantly higher LDL-cholesterol levels (supplemental Table IV). On in vitro functional analysis, Glu395 showed a moderately but significantly increased transcriptional activity compared with wild-type PPARα (supplemental Figure I, available online at http://atvb.ahajournals.org). The previously described Leu162Val variant of the PPARα gene has been shown to give gain of function in in vitro,[24] has been associated with raised LDL-cholesterol levels.[21,22] Our results appear to be in accordance with these previous reports.

Discussion

The main findings of the present study are the following: (1) identification of novel polymorphisms in plasma lipid levels-associated nuclear receptor genes, (2) a higher frequency of the RXRγ gene variant Gly14Ser in subjects with FCHL, (3) RXRγ Ser14 variant carriers showed more atherogenic dyslipidemia associated with coronary atherosclerosis, (4) the RXRγ variant showed a stronger response to its ligand in repression of the LPL promoter than the wild-type RXRγ.

RXRs are major heterodimerization partners of nuclear receptors such as PPARs, LXRs, and FXR. Three RXR isotypes have been identified: RXRα, RXRβ, and RXRγ. Synthetic RXR ligands induce hypertriglyceridemia through decreased clearance of VLDL by LPL-dependent pathways,[23,24] except in 1 study.[25] In contrast to the embryonic lethality observed in RXRα- and RXRβ-deficient mice, RXRγ-deficient mice develop apparently normal.[26] Yet, RXRγ-deficient mice showed reduced fasting plasma TG levels and increased skeletal muscle LPL activity when fed a high fat diet.[27] The human RXRγ gene is located on chro-

mosome 1q21-q23, ie, the so-called "FCHL locus",[28] and both linkage analysis and a twin study have indicated that the RXRγ gene is linked with dyslipidemia in Chinese and German families.[29,30]

To our knowledge, there are only few data concerning the physiological roles and targets of RXRγ in humans. The RXRγ gene is mainly expressed in skeletal muscles, central nervous system, skin, intestine, and lung. In the present study, LPL protein mass and activity were significantly decreased in RXRγ variant carriers. Because LPL is mainly expressed in adipose tissues and in skeletal muscles, we assume that this is attributable to the fact that the presence of the RXRγ variant affects LPL expression in skeletal muscles. RXRγ mRNA is detectable in adipose tissue only at a low level,[31] but it has been reported that RXRγ could replace RXRα in heterodimerization with PPARγ in adipose tissue.[32] Therefore, there is a possibility that RXRγ variant expression in adipose tissue contributes to the changes in LPL.

It has been reported that RXRγ-deficient mice show a 17% increase in serum thyroid hormone (T4) and a 20% increase in thyroid-stimulating hormone (TSH) levels.[33] In the present study, thyroid hormone levels did not appear to differ sufficiently between variant carriers and non-carriers to explain the differences observed in lipid levels.

It has been shown that low LPL levels contribute to disorders associated with TG-rich lipoprotein catabolism with low HDL, especially in HDL2,[34,35] and are associated with increased risk for future coronary disease.[36] Thus, the low LPL could well contribute to the increase in TG and the decrease in HDL-cholesterol levels in subjects with the RXRγ variant.

We assessed the functional consequence of the RXRγ Ser14 variant in vitro. The activation function-1 (AF-1) domain of RXRγ is located between amino acids 1 and 103, and is required for optimal ligand-dependent transactivation of RXR response element.[37] Fourteen amino acids are located within the AF-1 domain and are conserved among humans, mice, and chickens. In a transfection assay, RXRγ Ser14 repressed LPL promoter activity more strongly than the wild-type RXRγ. In addition, the Ser14 variant was a more potent inducer of a positive RXR response element. Therefore, we speculate that the Ser14 variant induces a better recruitment and/or stabilization of RXR cofactors. Further studies will be required to understand the precise molecular mechanism(s) involved in the LPL regulation by RXRγ Ser14.

Within the so-called FCHL locus, on chromosome 1q21-q23, several genes have been reported to be associated with the FCHL phenotype[28,30,38] and with type 2 diabetes.[39] First, the thioredoxin interacting protein gene was shown to be associated with combined hyperlipidemia in mice, but no disease-causing mutation has been found in humans so far.[40,41] Currently upstream stimulatory factor 1 (USF1) is considered the most promising candidate gene of FCHL.[42] In the USF1 gene, no amino acid substitution has been identified in the coding regions, but single nucleotide polymorphisms in the 3′untranslated region and in intron 7 have been reported to be associated with FCHL, metabolic syndrome, or type 2 diabetes mellitus quite reproducibly.[43–45] However, populations did not show any such association have also been reported.[46–48] These reports emphasize the complexity of phenotypic expression in multi-factorial diseases such as FCHL. RXRγ had been reported to show an association with TG and cholesterol levels on linkage analysis,[29,30] and we identified novel RXRγ variant that associated with atherogenic dyslipidemia. However, the changes in lipid levels attributable to the RXRγ variant alone were not sufficient to cause FCHL. Thus, we suggest the RXRγ gene variant to be a strong modifier rather than a causative gene in development of the FCHL phenotype.

In conclusion, the present study suggests that a variant of RXRγ gene contributes to genetic dyslipidemia, including FCHL, based on the increased frequency of this variant in FCHL, its association with an atherogenic lipid profile, and initial functional studies.

Acknowledgments

The authors thank Sachio Yamamoto for technical assistance. Drs William W. Lamph and Bart Staels are kindly acknowledged for the generous gift of plasmids.

Sources of Funding

This work has been supported by a scientific research grant from the Ministry of Education, Science, and Culture of Japan (No.17790603) and ONO Medical Research Foundation. Thierry Claudel was supported by Grant 2002B017 from the Netherlands Heart Foundation.

Disclosures

None.

References

1. Goldstein JL, Schrott HG, Hazzard WR, Bierman EL, Motulsky AG. Hyperlipidemia in coronary heart disease. II. Genetic analysis of lipid levels in 176 families and delineation of a new inherited disorder, combined hyperlipidemia. *J Clin Invest.* 1973;52:1544–1568.
2. Rose HG, Kranz P, Weinstock M, Juliano J, Haft JI. Inheritance of combined hyperlipoproteinemia: evidence for a new lipoprotein phenotype. *Am J Med.* 1973;54:148–160.
3. Nikkila EA, Aro A. Family study of serum lipids and lipoproteins in coronary heart-disease. *Lancet.* 1973;1:954–959.
4. Hoffer MJ, Bredie SJ, Snieder H, Reymer PW, Demacker PN, Havekes LM, Boomsma DI, Stalenhoef AF, Frants RR, Kastelein JJ. Gender-related association between the -93T−>G/D9N haplotype of the lipoprotein lipase gene and elevated lipid levels in familial combined hyperlipidemia. *Atherosclerosis.* 1998;138:91–99.
5. Hoffer MJ, Bredie SJ, Boomsma DI, Reymer PW, Kastelein JJ, de Knijff P, Demacker PN, Stalenhoef AF, Havekes LM, Frants RR. The lipoprotein lipase (Asn291−>Ser) mutation is associated with elevated lipid levels in families with familial combined hyperlipidaemia. *Atherosclerosis.* 1996;119:159–167.
6. Gaddi A, Galetti C, Pauciullo P, Arca M. Familial combined hyperlipoproteinemia: experts panel position on diagnostic criteria for clinical practice. Committee of experts of the Atherosclerosis and Dysmetabolic Disorders Study Group. *Nutr Metab Cardiovasc Dis.* 1999;9:304–311.
7. Veerkamp MJ, de Graaf J, Bredie SJ, Hendriks JC, Demacker PN, Stalenhoef AF. Diagnosis of familial combined hyperlipidemia based on lipid phenotype expression in 32 families: results of a 5-year follow-up study. *Arterioscler Thromb Vasc Biol.* 2002;22:274–282.
8. Mangelsdorf DJ, Evans RM. The RXR heterodimers and orphan receptors. *Cell.* 1995;83:841–850.
9. Shulman AI, Mangelsdorf DJ. Retinoid x receptor heterodimers in the metabolic syndrome. *N Engl J Med.* 2005;353:604–615.
10. Claudel T, Staels B, Kuipers F. The Farnesoid X receptor: a molecular link between bile acid and lipid and glucose metabolism. *Arterioscler Thromb Vasc Biol.* 2005;25:2020–2030.