[Full Paper]

The parameters related to small dense low-density lipoprotein evaluated by non-denaturing polyacrylamide gel electrophoresis method in dyslipidemic subjects: with reference to the Japanese guideline for prevention of atherosclerotic cardiovascular diseases

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SUMMARY

To prevent the development of atherosclerotic diseases, the Japanese guideline for prevention of atherosclerotic cardiovascular diseases (released from Japan Atherosclerosis Society in 2007) is clinically referred. In addition, the importance of evaluating not only the level but also the quality of low-density lipoprotein (LDL) has been advocated. Small dense LDL (sdLDL) is regarded as representation of the quality of LDL. With the Lipoprint™ LDL subfraction system, utilizing non-denaturing polyacrylamide gel electrophoresis method which can evaluate several sdLDL-related parameters such as the area percentage of sdLDL, mean LDL particle size (mean LDL-PS), relative mobility in the form of the relative flotation rate (Rf), and LDL size-based phenotypic patterns. We attempted to assess the Japanese guideline for prevention of atherosclerotic cardiovascular diseases in terms of sdLDL-related parameters. We studied with 46 asymptomatic and cardiovascular disease-free dyslipidemic subjects (23 men, aged 30–58 years olds; 23 women, 31–58 years olds), who were within the primary prevention level. From the increased number of categories in the guideline, the following trends were observed; the smaller LDL-PS ($p=0.002$), the larger of area percentage of sdLDL ($p=0.042$), an increase in Rf ($p=0.068$), and an increase in the presence of pattern B (atherogenic phenotype) ($p=0.031$). These results suggested that, with the Lipoprint™ system, the usage of Japanese guideline may clinically reflect well the sdLDL-related parameters, in particular the mean LDL-PS, the area percentage of sdLDL and the pattern B.

Key words: small dense LDL, LDL particle size, Rf, type B pattern, dyslipidemia.

INTRODUCTION

Elevated circulating levels of low-density lipoprotein cholesterol (LDL-C) are associated with coronary artery

disease (CAD). To prevent the development of CAD, the evaluation of LDL-C is thought crucial effort. Recently, small dense LDL (sdLDL), a qualitative abnormal form of LDL that is easily oxidized and leads to atherogenic, has got-

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Abbreviations: LDL, low-density lipoprotein; sdLDL, small dense low-density lipoprotein; LDL-PS, LDL-particle size; Rf, relative flotation rate; CAD, coronary artery disease; LDL-C, low-density lipoprotein cholesterol; HDL-C, high-density lipoprotein cholesterol; NMR, nuclear magnetic resonance; GGE, gradient gel electrophoresis; HDL, high-density lipoprotein; VLDL, very low-density lipoprotein; TC, total cholesterol; TG, triglyceride; HbA_{1c}, hemoglobin A_{1c}; HPLC, high performance liquid chromatography.

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ten a great attention. Since sdLDL is reported to be associated with hypertension¹⁾, diabetes mellitus²⁾ and metabolic syndrome³⁾. Therefore, sdLDL has been acknowledged as one of the risk factors for CAD, that may be in the superiority of LDL-C levels^{4, 5)}.

In 2007, the Japanese guideline for prevention of atherosclerotic cardiovascular diseases was released by the Japan Atherosclerosis Society⁶⁾. The Japanese guideline divided primary prevention groups into the subgroups as categories 1, 2 and 3⁶⁾. Patients with dyslipidemia are classified by the number of risk factors, and the management target value of LDL-C to prevent CAD is determined in each category⁶⁾. Although the guideline was based on some evidence obtained in Japan, it remains necessary to discuss atherosclerotic risk factors from additional points of view including the quality of LDL. In spite of this necessity and the nationwide distribution of guideline, it is not easy to measure sdLDL in a daily clinic, and its relationship with the Japanese guideline has not been examined. Then, the aim of this study was to focus into the guideline of sdLDL.

Several analytical methods for the LDL-related quality have been developed. Gradient gel electrophoresis (GGE) is a major electrophoretic method of measuring LDL particles size (LDL-PS)⁷⁻⁹⁾. Both the peak and mean of LDL-PS are used, though the two are substantially similar. GGE can determine the peak of LDL-PS with the migration of standards of known diameters. Also, the phenotype of LDL particles can be determined: 'Type A' for a large buoyant LDL (peak at LDL-PS of >25.5 nm) and 'Type B' for a predominance of sdLDL (peak of <25.5 nm)¹⁰⁾. The patients with 'Type B' are 2- to 3-fold more set up to develop CAD than those with 'Type A'¹⁰⁾. As another method, nuclear magnetic resonance (NMR) spectroscopy is used to determine both LDL-PS and the molecular size of LDL subclasses^{11, 12)}. LDL-PS was determined by the molecular size percentage of respective LDL subclass relative to their diameters. With NMR spectroscopy, a relationship between lipoprotein subclasses and the severity of CAD has been clarified¹¹⁾. As an additional method, the use of non-denaturing polyacrylamide gel electrophoresis for LDL subfractions, with the LipoprintTM, has been reported^{13, 14)}. For the peak of LDL-PS with the non-gradient gel electrophoretic method, the relative electrophoretic mobility as the relative flotation rate (Rf) is determined¹⁵⁾. Taking the baseline reference point (Rf=0.0) for very low-density lipoprotein (VLDL) and the leading reference point (Rf=1.0) for high-density lipoprotein (HDL), Rf is calculated from the migration mobility between VLDL and LDL. The Rf value of 0.4 corresponds to the LDL diameter as 25.5 nm with GGE¹⁵⁾, thus the this value greater than 0.4 suggests the presence of sdLDL¹⁵⁾. The LipoprintTM system can be separated into seven LDL subfractions (from LDL-1 to LDL-7) based on Rf values, and can provide several sdLDL-related parameters such as Rf, the area percentage of

sdLDL, and phenotypic pattern B (defined as a mean LDL-PS of less than 26.5 nm). It has been reported that the mean LDL-PS is associated with the advanced grade of CAD¹⁶⁾. These parameters are useful for clinical significance but they may indeed to show some differences. Our study's aim includes a simultaneous comparison of these parameters. Thus, the present study investigated the relationship between the Japanese guideline and several sdLDL-related parameters in dyslipidemic subjects is evaluated with non-denaturing polyacrylamide gel electrophoresis.

SUBJECTS AND METHODS

A total of forty-six patients with dyslipidemia, recruited consecutively from our lipidemia clinic, were enrolled in the current study, approved by the Ethics Committee of the Kyoto Medical Center. All patients are taken the informed consent. None was treated with anti-dyslipidemic drugs or had suffered with CAD, corresponding to the primary prevention in the guideline⁶⁾. Medical histories were confirmed via interviews. After overnight fasted, body mass index (BMI) was calculated as weight, measured using a body fat analyzer (OMRON, Kyoto, Japan) that divided by squared height (kg/m²). Blood pressure was measured three times at 10-min intervals using a mercury sphygmomanometer (OMRON, Kyoto, Japan) as systolic blood pressure (SBP) and diastolic blood pressure (DBP), and the average was calculated from all the data. Venous blood samples were then taken. Plasma glucose levels, serum total cholesterol (TC), triglyceride (TG) levels, HDL-C levels and LDL-C levels were determined by enzymatic methods. Blood hemoglobin A_{1c} (HbA_{1c}) was measured by high performance liquid chromatography (HPLC) (ADAMSTM A1c; HA-8160, ARKRAY, Inc., Kyoto, Japan). Serum insulin was determined using the chemiluminescent immunoassay (ABBOTT JAPAN Co., Ltd., Tokyo, Japan).

Determination of LDL subfractions and particle size

Both the area percentage of sdLDL and the mean LDL-PS were measured with the LipoprintTM system (Quantimetrix Inc., Redondo Beach, CA, USA)^{13, 14)}. Briefly, 25 µl of serum sample and 200 µl of loading gel were applied on 3.3% polyacrylamide gel tube and mixed well several times. Next, the samples were photopolymerized at room temperature for 30 min and electrophoresed for 65 min (3 mA/gel tube). After the electrophoresis, a densitometric scanning was conducted with a ScanMaker i900 (MICROTEK Co., Carson, CA, USA) and the lipoprotein subfractions were calculated with an iMac personal computer (Apple Computer Inc., Cupertino, CA, USA). All LDL subfractions were calculated with a Rf between the VLDL fraction as Rf=0.0, and the HDL fraction as Rf=1.0. The first peak in LDL subfractions was chosen as the peak of LDL. When no sharp peak is found in the LDL subfraction, we chose the central peak of LDL. LDL-1 and LDL-2 are defined as large LDL,

and LDL-3 to LDL-7 are defined as sdLDL. Then, the mean of LDL-PS fraction was obtained automatically. The area percentage of sdLDL is expressed as a ratio to all lipoproteins. A mean LDL-PS over 26.8 nm was defined as the large particle, buoyant dominant 'pattern A', that between 26.8 nm and 26.5 nm as 'intermediate (I)', and that under 26.5 nm as 'pattern B'.

Statistical analyses

Data are expressed as means±standard deviations. ANOVA was used to test the differences and trends in parameters among categories. The Cochran-Armitage test was used to examine the tendency for the presence of pattern B by category. Differences in frequency of age, sex, and current smoking habit were calculated with the chi-square test. A regression analysis, adjusted for age, sex and BMI, was used to find a bivariable-correlation. Insulin and TG levels were log arism-transformed. The p value less than 0.05 was evaluated as statistically significant. All statistical analyses were performed with the Statistical Package of Social Science (SPSS for Windows, SPSS Inc., Chicago, IL).

RESULTS

The clinical characteristics of the patients with dyslipidemia by category are listed in Table 1. Age and sex did not differ significantly among the tested categories. In the tested categories, BMI (p for trend=0.001), SBP (p for trend<0.001), DBP (p for trend<0.001), glucose (p for trend=0.001), HbA_{1c} (p for trend<0.001), and (log-)TG (p for trend=0.001) were increased.

Table 1. Clinical characteristics of patients with dyslipidemia from the category of a primary prevention.

Parameter	Category of the primary prevention		
	1	2	3
Number	16	16	14
Age (years)	39±8	46±10	46±8
Sex (men) (%)	37.5	56.3	57.1
Current Smoking (%)	0	43.8	35.7
BMI (kg/m ²) ^a	22.3±2.4	24.7±2.8	26.3±3.3
SBP (mmHg) ^a	111±13	124±11	139±17
DBP (mmHg) ^a	64±10	79±12	82±11
Glucose (mmol/L) ^a	5.0±0.5	5.2±0.6	8.3±3.8
HbA _{1c} (%) ^a	4.9±0.4	5.2±0.3	7.4±1.4
Insulin (pmol/L)	28±12	41±23	53±62
LDL-C (mmol/L)	3.28±0.98	3.93±1.73	3.10±0.96
HDL-C (mmol/L)	1.78±0.44	1.53±0.36	1.50±0.41
Triglyceride (mmol/L) ^a	0.87±0.36	1.67±0.72	1.83±0.92

BMI: body mass index, SBP: systolic blood pressure, DBP: diastolic blood pressure, HbA_{1c}: hemoglobin A_{1c}, LDL-C: low-density lipoprotein cholesterol, HDL-C: high-density lipoprotein cholesterol.

Data are number and/or mean±standard deviation.

^a p<0.05: trend test using ANOVA or chi-square within-group in the respective parameter.

Data of the sdLDL-related parameters by category are listed in Table 2. In the tested categories on the Japanese guideline, the following trends were observed: the smaller mean LDL-PS (p=0.002), the larger of area percentage of sdLDL (p=0.042), the increased Rf (p=0.068), and the increased rate of pattern B (from 25.0 to 64.3%, p=0.031).

There was a significant correlation, adjusted for age, sex and BMI, between mean LDL-PS and the area percentage of sdLDL ($r=-0.956$, $p<0.001$), Rf ($r=-0.609$, $p<0.001$), and Pattern B ($r=-0.850$, $p<0.001$). There was also a correlation between the area percentage of sdLDL and the Rf value ($r=0.635$, $p<0.001$) as well as Pattern B ($r=0.769$, $p<0.001$), and between the Rf value and Pattern B ($r=0.518$, $p=0.004$). Furthermore, there was a relationship between mean LDL-PS and SBP ($r=-0.405$, $p=0.029$) as well as (log-)TG ($r=-0.526$, $p=0.003$). The area percentage of sdLDL correlated with SBP ($r=0.452$, $p=0.014$) and (log-)TG ($r=0.479$, $p=0.009$). The Rf value correlated with HbA_{1c} ($r=0.477$, $p=0.009$) and (log-)TG ($r=0.502$, $p=0.005$), and the Pattern B correlated with (log-)TG ($r=0.374$, $p=0.046$).

DISCUSSION

This study found that with an increase in the number of categories in the Japanese guideline for prevention of atherosclerotic cardiovascular diseases, sdLDL-related parameters, evaluated using non-denaturing polyacrylamide gel electrophoresis method, changed as follows: mean LDL-PS became smaller, area of sdLDL increased, and pattern B occurred more often. Although there were significant correlations between all sdLDL-related parameters in the present study, the mean LDL-PS, area of sdLDL and phenotypic pattern seem to be better than Rf itself for assessing the categories of the guideline. The Rf was examined by choosing the central part of the LDL peak, and our population might include some cases in which it was relatively difficult to observe a sharp peak. This might partly explain the weak correlation with the Rf (this would be a limitation to determining the Rf with the Lipoprint™ system).

Table 2. Data of sdLDL-related parameters according to the category of a primary prevention.

Parameter	Category of the primary prevention		
	1	2	3
Mean particle size (nm) ^a	27.00±0.50	26.51±0.52	26.22±0.59
Area percentage (%) ^a	3.1±5.0	7.3±6.9	9.2±7.7
Rf	0.31±0.02	0.33±0.04	0.34±0.04
Phenotypic classification			
Pattern A+I (number)	12	10	5
Pattern B (number) ^b	4	6	9

sdLDL: small dense low-density lipoprotein.

Data are number or mean±standard deviation.

^a p<0.05: trend test using ANOVA within-group by parameter.

^b p<0.05: Cochran-Armitage test was performed between the pattern A+I and B.

SdLDL has been highlighted as an independent predictor of CAD even in Japanese, who have lower levels of LDL-C than Westerners¹⁷⁾. The present study has shown the relationship between the Japanese guideline for prevention of CAD and the quality of LDL. An inverse relationship between sdLDL and TG levels has been reported¹⁸⁾. Increased TG levels can generate TG-rich lipoproteins that give rise to sdLDL through lipolysis¹⁸⁾. Increased levels of sdLDL have also been reported to be a useful marker of obesity¹⁹⁾, hypertension¹⁾, and diabetes mellitus²⁾. Our correlation analyses have further revealed the relationship between mean LDL-PS and SBP or TG levels. Moreover, there were the relationships among the area percentage of sdLDL and both SBP and TG levels, and among the Rf and both HbA_{1c} and TG levels. This study showed that all sdLDL-related parameters deteriorated for category 3 as a plausible. Therefore, category 3 found to include the patients with obesity, diabetes mellitus and hypertension.

In addition to the methods of evaluating the LDL-related quality presented in the INTRODUCTION, a few markers related to sdLDL have started to be used clinically in Japan. First examination, the HPLC analysis is performed to classify and quantify lipoproteins on the basis of differences in particle size²⁰⁾. Second, the sdLDL-cholesterol concentration is assayed using the heparin-magnesium precipitation method^{21, 22)}. The data obtained with these methods seem essentially similar to those obtained with the LipoprintTM system, but the respective methods have advantages. Direct comparisons between these methods are expected in the future.

In conclusion, the increased number of categories in the guideline, sdLDL-related parameters taken from non-denaturing polyacrylamide gel electrophoresis method, was gradually increased in the tested asymptomatic dyslipidemic subjects within the primary prevention level: mean LDL-PS, the area percentage of sdLDL and the pattern B may be more useful tool for increased sdLDL particles. These results also suggest that using the Japanese guideline may be some equivalence for consideration of the circumstances of sdLDL and lead to the prevention of atherosclerotic diseases in a daily clinical practice.

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