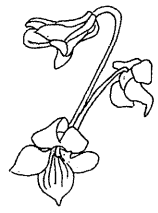
特集　創薬シーズとして期待される核酸医薬品 ～その展望と課題～

2．糖部架橋型核酸の医薬への応用



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核酸医薬の開発においては，優れた人工核酸の創製が必要不可欠である。核酸分子は，相補的な配列を持つ鎖と配列特異的に結合するという特性を元来有しているが，この特性を損なわず，あるいはむしろ高めつつ，生体内においても十分安定に存在できる人工核酸の開発が世界中で活発に展開されている。我々が開発してきた糖部架橋型核酸もその一つであり，糖部のコンホメーションをN型と呼ばれるRNA類似の構造に厳密に固定している。その結果，相補鎖RNAに対する高い結合親和性や生体内での優れた安定性を獲得するに至った。

この糖部架橋型人工核酸の一種である2',4'-BNA/LNA(2',4'-Bridged Nucleic Acid/Locked Nucleic Acid)は，RNAを標的とした核酸医薬として欧米を中心に臨床試験が展開されている。

1．はじめに

これまで低分子化合物が医薬品の中心的役割を担ってきたが，最近になってこれら低分子化合物を基盤とした新薬の開発にやや陰りが見られるようになってきた。

生命現象の解明が進むにつれ，タンパク質や核酸といった生体高分子同士の相互作用が疾病の発現に関わっていることが徐々に明らかとなってきた。しかし，低分子化合物を用いて，こうした生体高分子同士の相互作用を制御することは必ずしも容易ではないという点が，その一因として考えられる。

一方，核酸医薬の多くは，標的とするタンパク質の発現そのものを遺伝子レベルで抑制する。そのため，従来の低分子医薬では困難であった生体高分子間の相互作用の抑制という課題をも実現することが可能である。

本稿では，我々が開発してきた糖部架橋型人工核酸の創薬への応用例，特にRNAを標的とした核酸医薬開発の現状について概説したい。

2．RNAを標的とした核酸医薬に求められる特性

DNAに刻まれた遺伝情報は，**mRNA**(messenger RNA)へ転写され，さらにタンパク質へと翻訳される。一方，最近になってタンパク質へとコードされないRNA，いわゆるノンコーディングRNA(**ncRNA**)が，遺伝子の発現制御に大きく関わっていることも分かってきた[1)2)]。RNAを標的とした核酸医薬は，細胞の中でmRNAやncRNAと配列特異的に結合し，病気の原因となるタンパク質の発現を抑制することで機能する。(**図1**)。そのため，標的となるRNAと配列特異的にかつ強固に結合することが，核酸医薬には必要不可欠である。また生体内には核酸を分解する酵素が多

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生し，天然のDNAやRNAは速やかに分解をるため，核酸医薬の素材として利用すること難である。そのため，核酸医薬には生体内にる高い安定性が求められる。現在，臨床試験められている核酸医薬の多くは[3]，生体内の生を高めるためリン酸ジエステル結合をホスチオアート化するという工夫がなされてい一方，標的となる臓器への移行性（体内動態）御も核酸医薬の重要な課題と考えられてい

記のホスホロチオアート化を含め，さまざま学修飾により核酸医薬の体内動態を制御するは徐々に可能となりつつあるが[4]，これら核薬自身の化学修飾に加えて，核酸医薬を望み器に送達するためのデリバリー技術の確立がられている。

．架橋型人工核酸とは

97年，我々大阪大学のグループでは核酸位酸素原子と4'位炭素原子を架橋した新た工核酸，**2',4'-BNA**（2',4'-Bridged Nucleic）[5]，別名**LNA**（Locked Nucleic Acid）[6]，のに世界に先駆け成功した。（**図2**）。その合成いては，原著論文[5][7]のほか，既にいくつかのとしてまとめているので，そちらを参照してだきたい[8]～[10]。

橋型人工核酸の導入によって核酸の物性は大変化し，医薬素材としても非常に有望であるが分かってきた。例えば，標的RNAとの結和性に関する我々の実験から，12merのDNA',4'-BNA/LNAを6カ所導入した場合，約10も向上することが明らかとなった[11]。さら核酸を分解する酵素に対する耐性も大きく改た[12]。このうち前者については，当初の我々子設計通り，2',4'-BNA/LNAの糖部コンホションが厳密にRNAと同じN型に固定化さいることによる。一方，後者については，架分の立体的な効果から核酸分解酵素がリン酸ステル結合に接近することを阻害しているたあると考えられる。

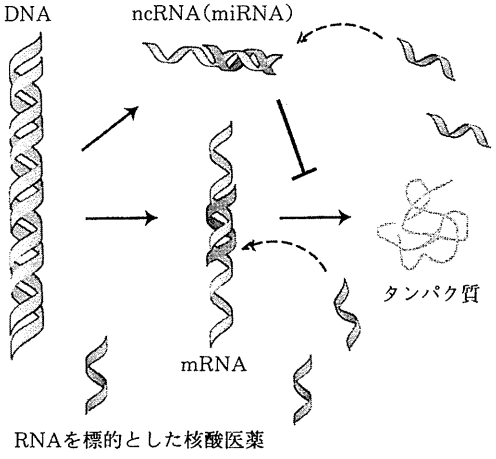

図1　RNAを標的とした核酸医薬の作用様式
細胞内において，RNAを標的とした核酸医薬は，メッセンジャーRNA（mRNA）やノンコーディングRNA（ncRNA）等の一本鎖RNAと配列特異的に結合し，それらRNAの機能を抑制することで遺伝子発現を制御する．
（筆者作成）

4．架橋型人工核酸を用いた核酸医薬開発の現状

架橋型人工核酸の開発は，我々の最初の報告の後に世界中で活発に行なわれてきた[9][10]。その中で，初代の架橋型人工核酸（2',4'-BNA/LNA）は既にデンマークのSantaris Pharma社を中心に臨床試験が進められている。（**図3**）[13]。そのうち代表的な2例を以下に紹介したい。

1）SPC3649（miravirsen）[14][15]

SPC3649はC型肝炎治療薬としてSantaris Pharma社により開発が進められている核酸医薬である。**HCV**（C型肝炎ウイルス）の増殖に深く関わっているmicroRNA（ncRNAの一種）であるmiR-122を標的としたもので，当初は，血中のコレステロール**LDL-C**（low density lipoprotein cholesterol）を低減する作用があるとして注目を集めていた[14]。これに加えて，最近の報告では，HCVに慢性感染したチンパンジーへの投与により，肝臓および血中でのHCV量を大幅に低減す

-BNA：2',4'-Bridged Nucleic Acid，**LNA**：Locked Nucleic Acid，**HCV**：C型肝炎ウイルス
-C：low density lipoprotein cholesterol

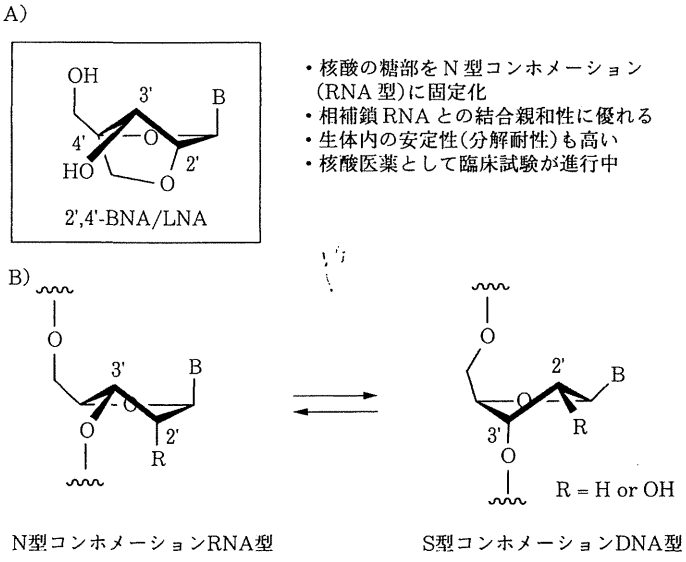

図2　A）架橋型人工核酸 2',4'-BNA/LNA の化学構造とその特徴
B）核酸（RNA，DNA）の糖部コンホメーション

核酸の糖部2'位と4'位の間に化学的な架橋を施すことで，通常はRNA型（N型）とDNA型（S型）の両方をとることができる糖部の構造を厳密にRNA型（N型）に固定化することができる．その結果，RNAを標的とした核酸医薬に相応しい優れた特性が得られる．

2',4'-BNA/LNA：2',4'-Bridged Nucleic Acid/Locked Nucleic Acid

（筆者作成）

ることが見出されている。またこの前臨床試験では，顕著な副作用は認められておらず，さらには最高で数カ月もの間効果が持続する等，極めて良好な結果が示されている[15]。現行のHCV治療薬としては，インターフェロンαやリバビリンが知られているが，これらは著効率が十分ではないという問題を残していたため，新たなメカニズムで作用する核酸医薬SPC3649には大きな期待が寄せられている。なお，このSPC3649については現在フェーズ2の臨床試験が進められている。（**図3**）[13]。

2）SPC4955[16]

一方，SPC4955は同じくSantaris Pharma社により，脂質異常症（高コレステロール血症）治療薬として開発されている核酸医薬である。これは，LDL-Cの主要構成成分である**ApoB**（apolipoprotein B）のmRNAを標的としたアンチセンス核酸であり，肝臓でのApoB合成を阻害することで血中LDL-Cの低減をもたらす。従来のアンチセンス核酸には，18～22mer程度の鎖長のオリゴヌクレオチドが主に用いられてきたが，2',4'-BNA/LNAアンチセンスにおいては，その優れたRNA結合親和性により，12～14mer程度の鎖長のオリゴヌクレオチドでも十分な効果を発揮することが示された。特に，*in vivo*での薬効は鎖長の長いアンチセンス核酸を凌ぐものであった。これは，従来のアンチセンス核酸の配列設計を根本的に見直すきっかけとなる重要な知見であるとともに，アンチセンス医薬のコスト低減につながるものと期待される。このSPC4955については現在フェーズ1段階にあり，今後の進展が期待される。（**図3**）[13]。

ApoB：apolipoprotein B

COMPOUND	TARGET	INDICATION	LEAD DISCOVERY	PRE-CLINICAL	PHASE I	PHASE II	PHASE III
miravirsen (SPC3649)	miR-122	Hepatitis C	○○○○○○○	○○○○○○○	○○○○○○○	○○○	
EZN-2968[1]	HIF-1a	Solid Tumors	○○○○○○○	○○○○○○○	○○○○○○○		
EZN-3042[1]	Survivin	Cancer	○○○○○○○	○○○○○○○	○○○○○		
EZN-4176[1]	Androgen Receptor	Cancer	○○○○○○○	○○○○○○○	○○○		
SPC4955	Apolipoprotein B	Hypercholesterolemia	○○○○○○○	○○○○○○○	○○○		
PCSK9 Program	PCSK9	Hypercholesterolemia	○○○○○○○	○○○○○○○			
Enzon Programs[1]	Beta-catenin, Her-3	Cancer	○○○○○				
Pfizer Programs[2]	Undisclosed	Various	○○○○○				
Shire Programs[2]	Undisclosed	Rare Genetic Disorders	○○○○○				
miRagen Programs[2]	Undisclosed	Cardiovascular Disease	○○○○○				
GSK Programs[2]	Undisclosed	Viral Diseases	○○○○○				

Santaris Compounds □ Partnerd Compounds □

[1]Santaris Pharma A/S retains commercial rights in Europe, US and rest of world partnered with Enzon
[2]Worldwide commercial rights partnered

図3 Santaris Pharma 社（デンマーク）における架橋型人工核酸の医薬品開発状況

Santaris Pharma 社では，架橋型人工核酸（2',4'-BNA/LNA）を用いた核酸医薬の開発を進めている．miravirsen（SPC3649）および SPC4955 以外にも，がんをはじめとした，いくつかの核酸医薬が臨床試験に進んでいる．

（文献 13 より転載）

5．おわりに

架橋型人工核酸を搭載したオリゴヌクレオチドは標的RNAとの結合親和性が大きく向上することから，特にRNAを標的とした核酸医薬素材として有望である。核酸医薬の問題点の一つにそのコストがあげられるが，2',4'-BNA/LNAの合成に関しては十分な最適化が進められてきた。さらに，上述のように，従来よりも短い鎖長で十分な薬効を示すことが明らかとなっており，コストの問題に関してはある程度の目処がついたと言ってもいいであろう。一方，核酸医薬が抱えるその他の問題として，標的臓器へのデリバリー技術が十分ではないという点があげられる。架橋型人工核酸をはじめとする各種の人工核酸の開発とともに，デリバリー技術が一段と成熟化することで，今後ますます核酸医薬の利用範囲は広まっていくであろう。

文　献

1) 河合剛太，金井昭夫 編："機能性Non-coding RNA". クバプロ，東京．2006.
2) Sayed D, Abdellatif M：MicroRNAs in development and disease. Physiol Rev **91**：827-887, 2011.
3) Yamamoto T, et al：Antisense drug discovery and development. Future Med Chem **3**：339-365, 2011.
4) Kuboyama T, et al：Stoichiometry-focused ^{18}F-labeling of alkyne-substituted oligonucleotides using azido ([^{18}F]fluoromethyl) benzenes by Cu-catalized Huisgen reaction. Bioorg Med Chem **19**：249-255, 2011.
5) Obika S, et al：Synthesis of 2'-*O*,4'-*C*-methyleneuridine and -cytidine. Novel bicyclic nucleosides having a fixed C3'-*endo* sugar puckering. Tetrahedron Lett **38**：8735-8738, 1997.
6) Singh SK, et al：LNA (locked nucleic acids)：synthesis and high-affinity nucleic acid recognition. Chem Commun：455-456, 1998.
7) Obika S, et al：2'-*O*,4'-*C*-Methylene bridged nucleic acid (2',4'-BNA)：synthesis and triplex-forming properties. Bioorg Med Chem **9**：1001-1011, 2001.
8) 小比賀 聡，今西　武：糖部架橋型核酸医薬の合成とその性質．"核酸医薬の最前線" 和田　猛 監修．シーエムシー出版，東京．p.55-66, 2009.
9) Rahman SMA, Imanishi T, Obika S：Synthesis of several types of bridged nucleic acids. Chem Lett **38**：512-517, 2009.
10) Obika S, et al：Bridged nucleic acids：development, synthesis and properties. Heterocycles **81**：1347-1392, 2010.
11) Obika S, et al：Stability and structural features of the duplexes containing nucleoside analogues with a fixed N-type conformation, 2'-*O*,4'-*C*-methyleneribonucleosides. Tetrahedron Lett **39**：5401-5404, 1998.
12) Imanishi T, Obika S：Syntheses and properties of novel conformationally restrained nucleoside analogues. J Syn Org Chem Jpn **57**：969-980, 1999.
13) Santaris Pharma 社ホームページ．http://www.santaris.com/product-pipeline
14) Elmén, et al：LNA-mediated microRNA silencing in non-human primates. Nature **452**：896-899, 2008.
15) Lanford RE, et al：Therapeutic silencing of microRNA-122 in primates with chronic hepatitis C virus infection. Science **327**：198-201, 2010.
16) Straarup EM, et al：Short locked nucleic acid antisense oligonucleotides potently reduce apolipoprotein B mRNA and serum cholesterol in non-human primates. Nucleic Acids Res **38**：7100-7111, 2010.

Original Article

Defining Patients at Extremely High Risk for Coronary Artery Disease in Heterozygous Familial Hypercholesterolemia

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Aim: **Heterozygous patients with familial hypercholesterolemia (FH) are known to be associated with a high risk of coronary artery disease (CAD), which is a major determinant of their clinical outcome. The prognosis of heterozygous FH patients substantially varies, being dependent on the level of their CAD risk, and their therapeutic regimen should be individualized. We assessed critical levels of LDL-cholesterol (LDL-C) and Achilles tendon thickness (ATT) to identify heterozygous FH patients at "very high" risk for CAD.**

Methods: **One hundred and nine heterozygous FH patients who had no history of CAD and had had their plasma lipid profile and ATT assessed before treatment were followed up until their first CAD event or 31 December 2010. Multivariable logistic regression models were used to analyze the correlation of LDL-C and/or ATT levels with the risk of developing CAD.**

Results: **During the follow-up period, 21 of the 109 patients had a CAD event, diagnosed by coronary angiogram. Individuals in the highest tertile of LDL-C had a CAD risk 8.29-fold higher than those in the lowest tertile. Individuals in the highest tertile of the ATT group had a 7.82-fold higher CAD risk than those in the lowest tertile. Those who had either LDL-C ≥ 260 mg/dL or ATT ≥ 14.5 had a 23.94-fold higher CAD risk than those with LDL-C < 260 mg/dL and ATT < 14.5 mm.**

Conclusions: **In heterozygous FH patients, LDL-C 260 mg/dL or higher and/or ATT 14.5 mm or thicker are useful markers for extracting patients at "very high" risk for CAD.**

J Atheroscler Thromb, 2012; 19:369-375.

Key words; Familial hypercholesterolemia, LDL cholesterol, Coronary artery disease, Coronary risk, Achilles tendon thickness

Introduction

Familial hypercholesterolemia (FH) is an autosomal dominant disorder characterized by hypercholesterolemia, skin and tendon xanthomas and a high risk of coronary artery disease (CAD) due to premature atherosclerosis[1)]. FH has the highest prevalence in genetic metabolic diseases, showing one per 300 to 500 heterozygous patients in the general population[1, 2)]. High low-density lipoprotein cholesterol (LDL-C) is the first symptom, appearing in heterozygous FH even from birth, and xanthomas in the Achilles tendon usually appear during or after the late 10s and are found in half of all patients by the age of 30[1)]. Coronary artery disease (CAD), which determines the prognosis of FH patients, appears during or after the third decade of life in men and the fifth decade in women[3-5)].

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Received: August 23, 2011
Accepted for publication: October 14, 2011

CAD mortality in heterozygous FH is several times higher than that in the general population[1, 6-8]; therefore, it is very important to prevent CAD in heterozygous FH patients. The prognosis of heterozygous patients of FH varies substantially, such that some develop CAD at their 20s while others may not develop CAD until their seventh decade; therefore, the therapeutic regimen should be individualized according to the patients' risk of CAD.

Various risk factors for CAD have been identified in heterozygous patients with FH, such as age, sex, LDL-C, triglyceride (TG), HDL-C, Achilles tendon thickness (ATT), smoking, a family history of CAD, hypertension, diabetes mellitus, Lp(a), homocysteine and so on[3, 9-12]. Among these parameters, LDL-C and ATT are simple, specific and non-invasive to measure, and can easily be used by primary care physicians to evaluate the CAD risk. We therefore estimated the CAD risks in accordance with LDL-C and ATT in heterozygous FH patients in order to identify those at extremely high risk.

Methods

Subjects

Of the patients referred to the lipid clinic at the National Cerebral and Cardiovascular Center (NCVC) from 1977 to 2007, 329 consecutive patients diagnosed as FH heterozygotes using previously described criteria[6] were subjected to this study. After diagnosis, the FH patients had medical checks according to the standard procedure for treating heterozygous FH in NCVC. The patients were subjected to a treadmill test for CAD screening just after their first visit to our clinic. Those who had a positive result on the treadmill test were subjected to a coronary angiogram (CAG), and diagnosed with CAD with 75% or more stenosis. Those who had a negative result on the treadmill or no significant stenosis by CAG were included in this study. Among the 329 FH patients, 229 were excluded: 53 had a past history of CAD, 160 had not had LDL-C measured before treatment, 76 had not had ATT thickness measured and 3 had TG more than 4.5 mmol/L, so 109 were followed up until their first CAD event or 31 December 2010. After the first visit to our clinic, dietary and drug treatment, including statins, was immediately started and continued.

During the course, those who had chest pain or a positive result on the treadmill test performed biennially were subjected to CAG, and diagnosed with CAD with 75% or more stenosis. Medical records of the patients were examined according to the analysis protocol approved by our institutional ethics committee (ID#M20-25-2).

Clinical and Laboratory Characteristics

Serum lipid and lipoprotein levels were measured at the time of initial diagnosis, before any lipid-lowering treatment. TC, TG and HDL-C levels were measured enzymatically with a commercial kit (Daiichi Pure Chemicals Co., Tokyo, Japan) using an automated analyzer (Hitachi model 704; Hitachi, Tokyo, Japan) in the clinical laboratory of the National Cerebral and Cardiovascular Center (NCVC). LDL-C was calculated by the Friedewald formula. ATT was measured by X-ray according to the method previously described[13]. Body mass index (BMI) was calculated as weight in kilograms divided by height in meters squared (kg/m^2). Hypertension was defined as the use of antihypertensive drugs or blood pressure ≥140 mmHg systolic or ≥90 mmHg diastolic or both at the first clinic visit (the criteria for hypertension of the Japanese Society of Hypertension Guidelines)[14]. Diabetes mellitus was defined according to the 2002 Guideline for the Treatment of Diabetes Mellitus of the Japanese Diabetes Society[15]. A family history of CAD was defined as positive by having within 2nd degree family members with CAD on the standardized questionnaire. Smoking was defined as positive by having a smoking habit at the first visit to NCVC on the patient report.

Statistical Analyses

Continuous variables are presented as the means ± SDs. Categorical data are presented as numbers and percentages. Unpaired Student's *t*-test and one-way analysis of variance (ANOVA) were used to assess differences between groups in continuous variables. Differences in categorical variables were assessed by the χ^2 test.

Multivariable logistic regression analysis after adjusting for age, sex, hypertension, diabetes mellitus, smoking, family history of CAD, and low HDL-C (<40 mg/dL) were used to analyze correlations of LDL-C levels or ATT levels and the development of CAD. LDL-C levels were categorized into tertiles: (1) LDL-C <206 mg/dL, (2) LDL ≥206 and <260 mg/dL, (3) LDL-C ≥260 mg/dL. ATT levels were also categorized into tertiles: (1) ATT <9.0 mm, (2) ATT ≥9.0 mm and <14.5 mm, (3) ATT ≥14.5 mm. All the confidence intervals were estimated at the 95% level and significance was set at $p<0.05$. All data were analyzed with the SPSS version 16.0 (SPSS Inc., Chicago, IL, USA) statistical software package.

Table 1. Clinical characteristics of 109 patients with heterozygous FH classified with or without CAD

	Total $n=109$	CAD(−) $n=88$	CAD(+) $n=21$	p value
Age (years)	41.9±16.2	39.7±16.7	50.9±10.5	<0.01
Sex (male), n (%)	43 (39.4%)	30 (34.1%)	12 (57.1%)	0.052
Achilles tendon thickness (mm)	12.6±5.4	11.5±4.5	17.4±6.3	<0.0001
Skin xanthomas, n (%)	25 (22.9%)	16 (18.2%)	9 (42.9%)	0.052
Arcus cornea, n (%)	45 (41.3%)	27 (30.7%)	16 (76.2%)	0.001
Total cholesterol (mg/dL)	321±68	309±56	368±92	<0.001
Triglyceride (mg/dL)	139±82	134±85	156±65	0.272
HDL-C (mg/dL)	51±15	51±15	50±15	0.747
LDL-C (mg/dL)	242±70	232±59	287±92	0.001
Smoking (past or current), n (%)	42 (38.5%)	28 (31.8%)	14 (66.6%)	0.003
Hypertension, n (%)	19 (17.4%)	10 (11.4%)	9 (42.9%)	0.003
Diabetes mellitus, n (%)	9 (8.2%)	5 (5.7%)	4 (19.0%)	0.186
Family history of CAD, n (%)	47 (43.1%)	37 (42.0%)	10 (47.6%)	0.411

Table 2. Clinical characteristics at first visit in heterozygous patients of FH classified by LDL-C Levels (Mean±SD)

LDL-C (mg/dL) categories	LDL-C <206 $n=36$	206≦LDL-C<260 $n=36$	LDL-C ≥260 $n=37$	p value
Age (years)	43.7±15.6	42.0±17.5	40.0±15.8	0.645
Sex (male), n (%)	14 (38.9%)	13 (36.1%)	15 (40.5%)	0.928
Body mass index (kg/m^2)	22.2±3.3	22.5±3.2	22.8±6.8	0.880
Total cholesterol (mg/dL)	258±28	308±20	394±55	<0.001
Triglyceride (mg/dL)	149±102	134±67	134±74	0.672
HDL-C (mg/dL)	54±16	51±15	47±14	0.100
Smoking (past or current), n (%)	15 (41.7%)	10 (27.8%)	15 (40.5%)	0.385
Hypertension, n (%)	5 (13.9%)	6 (16.7%)	3 (8.1%)	0.660
Diabetes mellitus, n (%)	2 (5.6%)	3 (8.3%)	2 (5.4%)	0.831
Family history of CAD, n (%)	17 (47.2%)	14 (38.9%)	16 (43.2%)	0.775
Achilles tendon thickness (mm)	10.7±4.2	12.5±5.5	14.6±5.8	0.282
CAD, n (%)	5 (13.9%)	3 (8.3%)	13 (35.1%)	0.02

Results

Characteristics of the Patients Subjected to Analysis of the Correlations of LDL-C and CAD

Among 109 patients, 21 (19.3%) developed CAD during the subsequent period. There was a significantly higher prevalence of hypertension, skin xanthomas, arcus cornea and smoking in the CAD (+) group. Mean age, ATT, TC and LDL-C were significantly higher in the CAD (+) group than in the CAD (−) group (**Table 1**).

LDL-C Levels and Development of CAD

The clinical characteristics of patients categorized into tertiles according to their LDL-C levels are shown in **Table 2**. They clearly show that parameters other than TC levels were not significantly different in each tertile. Higher LDL-C was associated with higher TC and the incidence of CAD.

To examine the influence of conventional coronary risk factors, logistic regression analyses for CAD were performed. The multivariable adjusted odds ratios (ORs) for CAD are shown in **Table 3**. Individuals in the highest tertile (LDL-C ≥260 mg/dL) had a 8.29-fold increased risk of CAD incidence compared with those in the lowest tertile (LDL-C <206 mg/dL) (adjusted odds ratio (OR) 8.29, 95 % CI 1.33-51.47, $p=0.023$). No significant increase in the odds of future CAD in the second tertile (206≦LDL-C<260 mg/dL) (adjusted OR 0.42, 95%CI 0.05-3.26, $p=0.409$).

Table 3. Multivariate-adjusted odds ratio for CAD by logistic regression analyses according to LDL-C

LCL-C categories	n	Odds Ratio	95% CI	*p* value
LDL-C < 206 mg/dL	36	1.0 (referent)	–	–
206≦LDL-C<260 mg/dL	36	0.42	0.05-3.26	0.409
LDL-C ≥ 260 mg/dL	37	8.29	1.33-51.47	0.023

Multivariable logistic regression models for CAD are adjusted for age, sex, hypertension, diabetes mellitus, smoking, family history of CAD, and low HDL-C (<40 mg/dL).

Table 4. Clinical characteristics at first visit in heterozygous patients of FH classified by ATT levels (mean ± SD)

ATT (mm) categories	ATT < 9 *n*=36	9≦ATT<14.5 *n*=37	ATT ≥ 14.5 *n*=36	*p* value
Age (years)	39.7 ± 18.3	39.4 ± 16.4	45.2 ± 13.5	0.177
Sex (male), n (%)	11 (30.6%)	13 (35.1%)	18 (50.0%)	0.207
BMI (kg/m^2)	22.3 ± 2.8	21.7 ± 2.8	23.1 ± 2.7	0.883
Total cholesterol (mg/dL)	293 ± 42	319 ± 66	350 ± 79	0.002
Triglycerides (mg/dL)	140 ± 106	134 ± 69	142 ± 67	0.505
HDL-C (mg/dL)	57 ± 14	47 ± 14	48 ± 15	0.916
LDL-C (mg/dL)	208 ± 44	245 ± 67	274 ± 78	0.003
Smoking habit, n (%)	9 (25.0%)	13 (35.1%)	14 (38.9%)	0.001
Hypertension, n (%)	4 (11.1%)	2 (5.4%)	8 (22.2%)	0.094
Diabetes mellitus, n (%)	1 (2.8%)	1 (2.7%)	5 (13.9%)	0.125
Family history of CAD, n (%)	16 (44.4%)	17 (46.0%)	14 (38.9%)	0.815
CAD, n (%)	2 (5.6%)	4 (10.8%)	15 (41.7%)	<0.001

ATT Levels and Development of CAD

The clinical characteristics of patients categorized into tertiles according to their ATT levels are shown in **Table 4**. Higher ATT levels were associated with higher TC and LDL-C levels, smoking and the incidence of CAD.

The multivariable adjusted OR for CAD is shown in **Table 5**. Individuals in the highest tertile group of ATT ≥ 14.5 mm had a 7.82-fold increased risk of CAD compared with those in the ATT <9.0 mm group (95%CI 1.28-47.7, p=0.001). No significant increase in the odds of future CAD in the group with 9≦ATT<14.5 mm (adjusted OR 1.42, 95%CI 0.18-11.14, p=0.740).

LDL-C and/or ATT Levels and Development of CAD

To estimate the future risk for CAD using the combination of LDL-C and ATT thickness, the patients were divided into 3 groups, (1) LDL-C <260 mg/dL and ATT <14.5 mm, (2) LDL-C <260 and ATT ≥ 14.5, or LDL-C ≥ 260 and ATT <14.5, (3) LDL-C ≥ 260 and ATT ≥ 14.5. OR for CAD was calculated for these groups and shown in **Table 6**. Those who had both LDL-C ≥ 260 and ATT ≥ 14.5 had a 20.62-fold increased risk of CAD compared with those with LDL-C <260 and ATT <14.5 (95%CI 2.91-145.89). Those with either LDL-C ≥ 260 or ATT ≥ 14.5 had a 23.62-fold increased risk of CAD compared with those with LDL-C <260 and ATT < 14.5 (95%CI 3.11-184.16).

Discussion

As the prognosis of heterozygous FH patients varies substantially, the therapeutic regimen should be determined according to the CAD risk of individual patients. High levels of LDL-C and ATT are clinical signs already found at a young age and can be measured easily and non-invasively by family physicians in primary care, so they can be good markers for estimating the future CAD risk of FH. In the present study, we demonstrated the critical levels of LDL-C and ATT for estimation of the CAD risk in Japanese heterozygous patients with FH.

Several studies on the Japanese population have indicated that the serum cholesterol level is correlated significantly with the risk of CAD[16, 17]. The increased CAD incidence seems exponential with the serum cholesterol level in the general population, and it can be

Table 5. Multivariate-adjusted odds ratio for CAD by logistic regression analyses according to ATT levels

ATT (mm) categories	n	Odds Ratio	95% CI	*p* value
ATT < 9 mm	36	1.0 (referent)	–	–
9≦ATT< 14.5 mm	37	1.42	0.18-11.14	0.740
ATT ≥ 14.5 mm	36	7.82	1.28-47.7	0.001

Multivariable logistic regression models for CAD are adjusted for age, sex, hypertension, diabetes mellitus, smoking, family history of CAD and low HDL-C (< 40 mg/dL).

Table 6. Multivariate-adjusted odds ratio for CAD by logistic regression analyses according to both ATT and LDL-C levels

LDL-C and ATT categories	n	Odds Ratio	95% CI	*p* value
LDL-C < 260, ATT < 14.5 mm	54	1.0 (referent)	–	–
LDL-C < 260, ATT ≥ 14.5 mm or LDL-C ≥ 260, ATT < 14.5 mm	37	23.94	3.11-184.16	0.002
LDL-C ≥ 260, ATT ≥ 14.5 mm	18	20.62	2.91-145.89	0.002

Multivariable logistic regression models for CAD are adjusted for age, sex, hypertension, diabetes mellitus, smoking, family history of CAD and low HDL-C (< 40 mg/dL).

considered low until it hits a certain "threshold". The findings of the relationship with LDL-C levels and the onset of CAD in FH patients seem to show a "right shift" of this profile as LDL-C 2-fold higher and CAD incidence more than 10-fold. Previous studies have reported that higher LDL-C is related with the higher risk factors for the development of CAD even in heterozygous FH patients[18, 19], whereas other factors, such as age, gender, hypertension, smoking, or other lipid abnormalities, such as low HDL-C and high TG reportedly contribute to the increased risk[3, 12, 18-22]. Bujo *et al.* reported that male gender, age over 50, smoking, hypertension, diabetes mellitus, TG > 150 mg/dL and HDL-C < 40 mg/dL were risk factors for CAD in FH by multicenter, cross-sectional analysis[3].

As we reported in a previous paper, drug treatment including statins may influence the outcome of CAD[5]. The name and dose of drugs prescribed to the patients during the course are listed in **Table 7**. Because all FH patients had had intensive drug therapy to prevent the development of atherosclerosis, no comparison could be made with those without drug therapy. It was also impossible to analyze the difference in drugs statistically because there were so many patterns of prescription and most patients changed the type and dose of drugs several times during the course.

LDL-C levels under drug treatment may also affect the outcome. The mean LDL-C under drug treatment did not increase the odds ratio for CAD (odds ratio: 0.983, 95%CI: 0.97-1.00); however the relationship between mean LDL-C in the pre-treatment period and CAD risk remained due to pre-exposure to high LDL-C before treatment, although the absolute risk of CAD might be decreased at any LDL-C level by intensive drug treatment during the course.

Table 7. Lipid-lowering drugs in heterozygous FH patients during the course

	Dose/day
cholestyramine	4-12 g
colestimide	0.5-3 g
probucol	250-1,000 mg
pravastatin	10-30 mg
simvastatin	5-20 mg
fluvastatin	20-60 mg
atorvastatin	5-40 mg
pitavastatin	1-4 mg
rosuvastatin	2.5-20 mg
fenofibrate	100-200 mg
bezafibrate	100-400 mg
ezetimibe	5-10 mg

Civerira *et al.* reported that heterozygous FH with tendon xanthomas has a 3.1-fold increased risk of premature CAD compared with those without it[23]. The Achilles tendon was reported to be thicker in FH patients with CAD than in those without CAD in both sexes[12]. Persistent high LDL-C causes cholesterol depositions in the tendons and results in tendon xanthomas[1]. Achilles tendon xanthomas have been used

as one of the criteria for clinical diagnosis of FH because of their high sensitivity and specificity[1, 24]. A strongly positive correlation was observed between ATT and cholesterol-year scores in FH patients[25, 26], suggesting that ATT reflects both the severity and duration of hypercholesterolemia. ATT is an important factor that can be measured quantitatively as the deposition of cholesterol in the tissue. The present study showed that ATT is a good marker for evaluating the risk for CAD, indicating that there is a strong correlation between the deposition of cholesterol in extravascular tissue and the stenosis of coronary arteries. ATT should be used not only as a diagnostic parameter for FH but also, and more importantly, as a prognostic factor that indicates the need for a more aggressive approach for patients at high risk.

In conclusion, LDL-C ≥260 mg/dL and ATT ≧14.5 mm or thicker are useful criteria for identifying patients at "very high" risk of CAD in Japanese heterozygous FH. Patients with either of these risk factors require more intensive cholesterol-lowering therapy and a more careful medical check-up for CAD.

Acknowledgments

This work was supported by Grants-in-Aid for Scientific Research from the Japanese Ministry of Health, Labor and Welfare (H23-seisakutansaku-ippan-004 and H20-nanji-ippan-011) and the Cardiovascular Research Foundation (Suita, Japan).

References

1) Goldstein JL, Hobbs H, Brown MS: Familial hypercholesterolemia. Edited by Scriver CR BA, Sly WS, Valle D, pp2863-2913, McGraw-Hill, New York, 2001
2) Mabuchi H, Nohara A, Noguchi T, Kobayashi J, Kawashiri MA, Tada H, Nakanishi C, Mori M, Yamagishi M, Inazu A, Koizumi J: Molecular genetic epidemiology of homozygous familial hypercholesterolemia in the Hokuriku district of Japan. Atherosclerosis, 2011; 214: 404-407
3) Bujo H, Takahashi K, Saito Y, Maruyama T, Yamashita S, Matsuzawa Y, Ishibashi S, Shionoiri F, Yamada N, Kita T: Clinical features of familial hypercholesterolemia in Japan in a database from 1996-1998 by the research committee of the ministry of health, labour and welfare of Japan. J Atheroscler Thromb, 2004; 11: 146-151
4) Mabuchi H, Koizumi J, Shimizu M, Takeda R: Development of coronary heart disease in familial hypercholesterolemia. Circulation, 1989; 79: 225-232
5) Harada-Shiba M, Sugisawa T, Makino H, Abe M, Tsushima M, Yoshimasa Y, Yamashita T, Miyamoto Y, Yamamoto A, Tomoike H, Yokoyama S: Impact of statin treatment on the clinical fate of heterozygous familial hypercholesterolemia. J Atheroscler Thromb, 17: 667-674
6) Mabuchi H, Miyamoto S, Ueda K, Oota M, Takegoshi T, Wakasugi T, Takeda R: Causes of death in patients with familial hypercholesterolemia. Atherosclerosis, 1986; 61: 1-6
7) Jensen J, Blankenhorn DH, Kornerup V: Coronary disease in familial hypercholesterolemia. Circulation, 1967; 36: 77-82
8) Stone NJ, Levy RI, Fredrickson DS, Verter J: Coronary artery disease in 116 kindred with familial type II hyperlipoproteinemia. Circulation, 1974; 49: 476-488
9) Yagi K, Hifumi S, Nohara A, Higashikata T, Inazu A, Mizuno KO, Namura M, Ueda K, Kobayashi J, Shimizu M, Mabuchi H: Difference in the risk factors for coronary, renal and other peripheral arteriosclerosis in heterozygous familial hypercholesterolemia. Circ J, 2004; 68: 623-627
10) Pisciotta L, Cortese C, Gnasso A, Liberatoscioli L, Pastore A, Mannucci L, Irace C, Federici G, Bertolini S: Serum homocysteine, methylenetetrahydrofolate reductase gene polymorphism and cardiovascular disease in heterozygous familial hypercholesterolemia. Atherosclerosis, 2005; 179: 333-338
11) Real JT, Martinez-Hervas S, Garcia-Garcia AB, Chaves FJ, Civera M, Ascaso JF, Carmena R: Association of C677T polymorphism in MTHFR gene, high homocysteine and low HDL cholesterol plasma values in heterozygous familial hypercholesterolemia. J Atheroscler Thromb, 2009; 16: 815-820
12) Hirobe K, Matsuzawa Y, Ishikawa K, Tarui S, Yamamoto A, Nambu S, Fujimoto K: Coronary artery disease in heterozygous familial hypercholesterolemia. Atherosclerosis, 1982; 44: 201-210
13) Mabuchi H, Ito S, Haba T, Ueda K, Ueda R: Discrimination of familial hypercholesterolemia and secondary hypercholesterolemia by Achilles' tendon thickness. Atherosclerosis, 1977; 28: 61-68
14) Ikeda N, Hasegawa T, Hasegawa T, Saito I, Saruta T: Awareness of the Japanese Society of Hypertension Guidelines for the Management of Hypertension (JSH 2000) and compliance to its recommendations: surveys in 2000 and 2004. J Hum Hypertens, 2006; 20: 263-266
15) Matsushima M: [Japan Diabetes Society clinical practice guideline]. Nippon Rinsho, 2002; 60 Suppl 9: 161-166
16) Nakamura Y, Yamamoto T, Okamura T, Kadowaki T, Hayakawa T, Kita Y, Saitoh S, Okayama A, Ueshima H: Combined cardiovascular risk factors and outcome: NIPPON DATA80, 1980-1994. Circ J, 2006; 70: 960-964
17) Okamura T, Kokubo Y, Watanabe M, Higashiyama A, Miyamoto Y, Yoshimasa Y, Okayama A: Low-density lipoprotein cholesterol and non-high-density lipoprotein cholesterol and the incidence of cardiovascular disease in an urban Japanese cohort study: The Suita study. Atherosclerosis, 2009; 203: 587-592
18) Hill JS, Hayden MR, Frohlich J, Pritchard PH: Genetic and environmental factors affecting the incidence of coronary artery disease in heterozygous familial hypercholesterolemia. Arterioscler Thromb, 1991; 11: 290-297
19) Ferrieres J, Lambert J, Lussier-Cacan S, Davignon J: Cor-

onary artery disease in heterozygous familial hypercholesterolemia patients with the same LDL receptor gene mutation. Circulation, 1995; 92: 290-295
20) Seed M, Hoppichler F, Reaveley D, McCarthy S, Thompson GR, Boerwinkle E, Utermann G: Relation of serum lipoprotein(a) concentration and apolipoprotein(a) phenotype to coronary heart disease in patients with familial hypercholesterolemia. N Engl J Med, 1990; 322: 1494-1499
21) Wiklund O, Angelin B, Olofsson SO, Eriksson M, Fager G, Berglund L, Bondjers G: Apolipoprotein(a) and ischaemic heart disease in familial hypercholesterolaemia. Lancet, 1990; 335: 1360-1363
22) Yanagi K, Yamashita S, Kihara S, Nakamura T, Nozaki S, Nagai Y, Funahashi T, Kameda-Takemura K, Ueyama Y, Jiao S, Kubo M, Tokunaga K, Matsuzawa Y: Characteristics of coronary artery disease and lipoprotein abnormalities in patients with heterozygous familial hypercholesterolemia associated with diabetes mellitus or impaired glucose tolerance. Atherosclerosis, 1997; 132: 43-51
23) Civeira F, Castillo S, Alonso R, Merino-Ibarra E, Cenarro A, Artied M, Martin-Fuentes P, Ros E, Pocovi M, Mata P: Tendon xanthomas in familial hypercholesterolemia are associated with cardiovascular risk independently of the low-density lipoprotein receptor gene mutation. Arterioscler Thromb Vasc Biol, 2005; 25: 1960-1965
24) Sullivan D: Guidelines for the diagnosis and management of familial hypercholesterolaemia. Heart Lung Circ, 2007; 16: 25-27
25) Schmidt HH, Hill S, Makariou EV, Feuerstein IM, Dugi KA, Hoeg JM: Relation of cholesterol-year score to severity of calcific atherosclerosis and tissue deposition in homozygous familial hypercholesterolemia. Am J Cardiol, 1996; 77: 575-580
26) Lehtonen A, Makela P, Viikari J, Virtama P: Achilles tendon thickness in hypercholesterolaemia. Ann Clin Res, 1981; 13: 39-44

Original Article

Multicenter Study to Determine the Diagnosis Criteria of Heterozygous Familial Hypercholesterolemia in Japan

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Aim: **Heterozygous patients of familial hypercholesterolemia (FH) are known to have a high risk of coronary artery disease (CAD). Early diagnosis and prompt treatment are necessary to prevent their CAD. In this study we tried to amend the Japanese diagnostic criteria of FH for general practitioners by examining each component of the current criteria.**
Methods: **A multicenter study was performed, which included 1356 dyslipidemic patients at 6 centers. Pretreatment demographic information including LDL-cholesterol (LDL-C), Achilles tendon thickness (ATT), family history of FH and premature CAD and the result of genetic analysis were analyzed.**
Results: **Of 1356 patients, 419 were diagnosed with FH by criteria in 1988, which were used as a golden standard. We tried to define FH according to 3 conventional major items, i.e., 1) LDL-C, 2) ATT and/or cutaneous nodular xanthomas (CX), 3) family history of FH and/or family history of premature CAD. We then determined the cutoff of LDL-C using the new criteria. When we used 180 mg/dL as the cutoff of LDL-C, 94.3% of FH patients and 0.85% of non-FH satisfied 2 or more criteria. When we used 190 mg/dL, 92.1% of FH and 0.85% of non-FH satisfied 2 or more criteria; therefore, we chose 180 mg/dL for the cutoff of LDL-C in the new criteria and proposed that the diagnosis of definite FH can be made if 2 or more criteria are satisfied.**
Conclusions: **We examined each component for the diagnosis of heterozygous FH in a multicenter study in Japan.**

J Atheroscler Thromb, 2012; 19:1019-1026.

Key words; Diagnosis criteria, Familial hypercholesterolemia, LDL cholesterol, Achilles tendon thickness, LDL receptor

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Received: April 9, 2012
Accepted for publication: June 26, 2012

Introduction

Familial hypercholesterolemia (FH) is a genetic disease caused by a mutation in genes related to low-density lipoprotein (LDL) metabolism. Heterozygous FH patients manifest high LDL cholesterol (LDL-C)

levels, skin and/or tendon xanthomas, and increased risk of premature coronary artery disease (CAD)[1]. High LDL-C levels are the first symptom that appears even from birth, while xanthomas on the Achilles tendon usually appear during or after the late 10s and CAD that determines the prognosis of FH patients appears during or after the third decade of life in men and the fifth decade in women[2-4]. Because morbidity and mortality of CAD in heterozygous FH are much higher than in the general population[1, 5-7], special attention should be paid to screen these patients and to prevent their atherosclerotic complications. For the diagnosis of FH, several criteria have been published throughout the world, including ours, reported in 1988[8]; however, appropriate diagnosis of FH by primary care physicians is not performed in general practice in Japan[9]. Therefore, it is very important to establish useful diagnostic criteria for primary care physicians to diagnose FH with high specificity and sensitivity.

Because FH patients are estimated to be more than 250,000, primary care physicians need to take care of most of them; therefore, the criteria should be as simple as possible for clinical usefulness and have high sensitivity and specificity. We have used diagnosis criteria for FH published in 1988 in Japan[8], which include hypercholesterolemia, presence of skin/tendon xanthoma and reduced LDL receptor activity as major items; however, it is difficult to measure LDL receptor activity in routine clinical practice and even lipid specialists do not measure its activity. Furthermore, it is not covered by Japan's health insurance system; therefore, it is necessary to make the current diagnostic criteria easy to use for general practitioners. Toward this end, we performed a multicenter collaborative study of 1397 patients with dyslipidemia.

Methods

Subjects

A total of 1397 patients with dyslipidemia, referred to the outpatient clinic of 6 hospitals (Kyoto University Hospital, Osaka University Hospital, Nippon Medical School Hospital, Chiba University Hospital, Kanazawa University Hospital, and National Cerebral and Cardiovascular Center Hospital), were the subjects to this study. Among these patients, 41 were excluded due to missing data. Consequently, 1356 patients with dyslipidemia were eligible for the present study. Most had been diagnosed with or without FH by lipid specialists at each hospital according to the criteria for FH reported in 1988, and genetic analysis was performed in 223 patients, some of whom were diagnosed with FH based only on mutations of the LDL receptor or PCSK9. The criteria were as follows: Major items included 3 items, (1) IIa or IIb phenotype at serum cholesterol level of 260 mg/dL or above; (2) Tendinous xanthoma or xanthoma tuberosum is present; (3) Reduced or abnormal receptor activity. Minor items included 3 items: (1) Xanthoma palpebratum; (2) Arcus juvenalis (<50 years); (3) Juvenile (<50 years) ischemic heart disease.

Table 1. Clinical characteristics of non-FH and FH patients in this cohort

	non-FH	FH	*p*
N	937	419	
Male (n, %)	453 (48.3%)	170 (42.7%)	<0.01
Age (y.o.)	58.3±16.3	52.9±18.6	<0.01
TC (mg/dL)	236±53	339±72	<0.01
LDL-C (mg/dL)	146±46	257±67	<0.01

Determination of Conventional Criteria for FH

In this study we tried to amend the current criteria. For the primary care setting, three major items, i.e. serum level of LDL-C, family history and specific physical findings of FH, were chosen as diagnostic items because all are easily assessed by general practitioners. Family history and specific physical findings were also separated in more detail. Finally, we set 5 items, (1) LDL-C, (2) specific physical findings: a) ATT, b) cutaneous nodular xanthomas (CX), (3) family history: a) family history of FH in 1st or 2nd degree relatives, b) family history of premature CAD in 1st or 2nd degree relatives. A family history of premature CAD was defined as having CAD before the age of 55 in males and 65 in females. First, we assessed the prediction for FH by the combination of physical findings and family history, and then we determined the cutoff point of LDL-C with the combination of the above-mentioned two items. LDL-C levels were calculated by the Friedewald formula. ATT levels were measured by X-ray according to the method previously described and determined as positive with 9 mm or more[10].

The data in the medical records of the patients were sent to the National Cerebral and Cardiovascular Center and examined. The study protocol was approved by the ethics committee of the National Cerebral and Cardiovascular Center (D#M20-25-2 for the multicenter trial and ID#M17-56-4 for the genetic analysis). The ethics committee of each hospital also approved the study protocol.

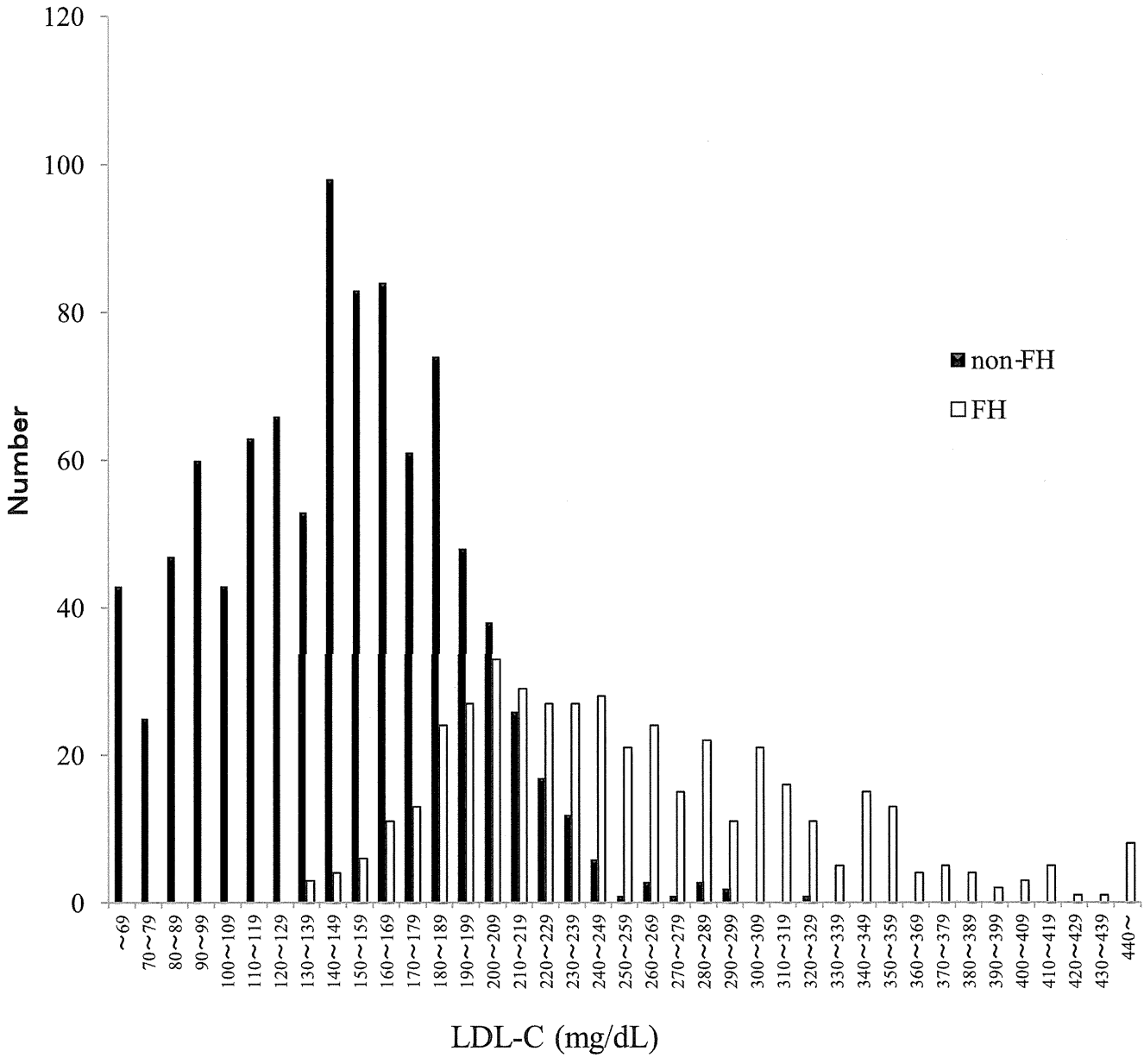

Fig. 1. Distribution of LDL-C levels before treatment in FH and non-FH patients. LDL-C levels were calculated by the Freidewald formula in patients with dyslipidemia diagnosed with FH or non-FH by specialists.

Statistical Analyses

Continuous variables are presented as the means ± SD. Categorical data are presented as numbers and percentages. Unpaired Student's *t*-test and one-way analysis of variance (ANOVA) were used to assess differences between groups in continuous variables. Differences in categorical variables were assessed by the χ^2 test.

Results

Among 1356 patients, 419 had been diagnosed with FH, while 937 with non-FH. Patient demographic data are shown in **Table 1**. FH patients were younger than non-FH patients. TC and LDL-C levels were 339 and 257 mg/dL in FH patients, respectively, and were significantly higher than in non-FH patients. The distribution of LDL-C levels in both groups is shown in **Fig. 1**. FH patients were divided into 3

Table 2. LDL-C levels in FH patients with or without genetic data

LDL-C (mg/dL)	FH (Total)	FH (Mut+)	FH (Mut−)	FH (no genetic data)	*p*-value
N	419	224	41	173	
Mean	257.4	266.2*	229.0*	252.9	
SD	67.39	69.85	60.14	63.70	
MEDIAN	244	253	216	241	0.003
IQ					
25%	205	213	189	203	
75%	300	308	244	295	

FH (Mut+): mutations in the LDL receptor or PCSK9, FH (Mut−): no mutations found, FH (no genetic data): no genetic analysis
*$p<0.005$ by Bonferroni

Table 3. Sensitivity and specificity in screening FH by physical findings and family history

	Specificity	Sensitivity
Physical findings		
ATT (+) (%)	98.6	64.1
CX (+) (%)	99.6	9.4
ATT (+) or CX (+) (%)	98.6	64.6
ATT(+) and CX(−)	99.6	11.7
Family history		
Family history of FH (+) (%)	93.6	98.2
Family history of CAD (+) (%)	96.3	28.3
Family history of FH (+) or CAD (+) (%)	91.7	98.7
Family history of FH (+) and CAD (+) (%)	98.2	27.4

ATT: Achilles tendon thickness, CX: Cutaneous nodular xanthomas
FH ($n=224$) was diagnosed by mutations in the LDL receptor and/or PCSK9. Non-FH ($n=937$) was diagnosed by specialists.

groups depending on their genetic data: FH with mutation(s) in LDL receptor or PCSK9, FH with no mutation(s) and FH with no genetic data. The mean and median of LDL-C along with SD and interquartile range of each group are shown in **Table 2**. LDL-C levels in FH with mutations were higher than those in FH without mutations.

We tried to define FH according to the screening standards as 3 major items, i.e., 1) LDL-C, 2) ATT and/or cutaneous nodular xanthomas (CX), 3) family history of FH and/or family history of premature CAD. We used LDL-C instead of total cholesterol, because LDL-C should better reflect the activity of the LDL receptor and is used for the goal of lipid management in the current Japanese guideline[8]. We incorporated "family history" as a major item because general practitioners were able to find FH by a family history of FH and/or premature CAD instead of LDL receptor activity. Sensitivity and specificity in screening FH by physical findings and family history are listed in **Table 3**. Based on these data, we decided to use 1) ATT or CX, and 2) family history of FH or CAD as 2 major items in addition to high LDL-C levels.

Next we tried to determine the cutoff levels of LDL-C. The percentage of the patients who satisfied each criterion according to LDL-C levels is listed in **Table 4**. Levels of 180 or 190 mg/dL are suggested as candidate cutoff levels. Therefore, the criteria for model 1 were set as those who satisfy 2 or more of the 3 criteria: 1) LDL-C 180 mg/dL or higher, 2) ATT (+) or CX (+), 3) Family history of FH or CAD, and for model 2, for which the cutoff point of LDL-C was changed to 190 mg/dl or higher, their sensitivity, specificity, and false positive and false negative rates were compared (**Table 5**). When we compared model 1 with model 2, higher sensitivity in model 1 than model 2 was obtained without any change in specificity, suggesting that 180 mg/dL is a better cutoff for LDL-C. The percentages were quite similar in FH with mutation (s) in LDL receptor or PCSK9, FH with no mutation (s) and FH with no genetic data. The diagnostic criteria of FH were then determined

Table 4. Percent satisfying each LDL-C level in non-FH and FH patients

	non FH	FH (All)	FH (Mut+)	FH (Mut−)	FH (No genetic data)
N	937	419	223	41	155
LDL-C ≥ 170 mg/dL (%)	30.5	94.5	96.0	85.4	94.8
LDL-C ≥ 180 mg/dL (%)	24.3	94.3	94.6	82.9	92.9
LDL-C ≥ 190 mg/dL (%)	16.6	92.1	93.7	75.6	89.7
LDL-C ≥ 200 mg/dL (%)	11.6	80.0	84.3	63.4	78.1

FH(Mut+): mutations in the LDL receptor or PCSK9, FH(−): no mutations found, FH (no genetic data): no genetic analysis

Table 5. Accuracy metrics of FH criteria using LDL-C cutoff levels of 180 or 190 mg/dL

	Sensitivity (%)	Specificity (%)	False positive (%)	False negative (%)
Model 1: Satisfying 2 or more of the following criteria: 1) LDL-C ≥ 180 mg/dL, 2) ATT(+) or CX(+), 3) Family history of FH or CAD	94.5	99.1	0.85	5.5
Model 2: Satisfying 2 or more of the following criteria: 1) LDL-C ≥ 190 mg/dL, 2) ATT(+) or CX(+), 3) Family history of FH or CAD	92.1	99.1	0.85	7.9

Table 6. Diagnostic criteria for adult (15 years or older) heterozygous FH

1	Hyper-LDL-cholesterolemia (LDL-C level before treatment: 180 mg/dL or more)
2	Tendon xanthoma (tendon xanthoma of the dorsal hands, elbows, and knees, or Achilles tendon thickening) or nodular xanthoma of the skin
3	Family history (relatives in the second degree): FH or premature CAD

-A diagnosis should be made after ruling out the possibility of secondary hyperlipidemia.
-Patients meeting 2 criteria should be regarded as having FH. Concerning those meeting 1 criterion, refer to Fig. 4. When FH is suspected, gene tests should be conducted to make a diagnosis.
-Nodular xanthoma of the skin does not include palpebral xanthoma.
-Patients with Achilles tendon thickening (9 mm or more) on radiography should be regarded as having xanthoma.
-When the LDL-C level is 250 mg/dL or more, FH should be strongly suspected.
-During drug therapy, the pretreatment lipid level should be employed as a reference value.
- CAD in males younger than 55 years old and females younger than 65 years old is defined as premature CAD.
-When a diagnosis of FH is made, the patient's family should also be investigated.
- LDL-C may be decreased after surgery, myocardial infarction, severe inflammation and so on. In these cases, LDL-C values before the diseases should be requested to give a diagnosis.
- To diagnose patients who have already been treated with statins, pretreatment levels of LDL-C should be requested; however, termination of statin treatment is not recommended to obtain pretreatment levels of LDL-C, even if the data are not available.

and are shown in **Table 6.**

Discussion

FH has the highest prevalence in genetic metabolic diseases, being heterozygous in one in 500 of the general population[1, 11]. Most young heterozygous FH patients have no symptoms other than high LDL-C levels, and those who have Achilles tendon thickness have no symptoms. The reason for undiagnosed FH patients to go to a clinic may be mainly divided into the following 4 situations: 1) a chance visit to a primary care physician due to flu or gastritis, etc., 2) recommendation of further medical examination due to high cholesterol at a health checkup, 3) transportation to the emergency room due to the development of acute coronary syndrome, 4) recommendation of medical consultation due to the presence of FH in his/

her family. The diagnostic criteria should be applied to these patients. Accordingly, conventional criteria are needed for the primary care setting.

Heterozygous FH patients show high levels of LDL-C, cutaneous and tendon xanthomas, and are complicated with myocardial infarction at young age by atherosclerosis due to intravascular exposure to high levels of LDL-C for many years. Because early diagnosis and treatment are recommended for these patients, the diagnostic criteria for FH have been reported in many countries including Japan[8, 12-17]. While some criteria give a satisfactory diagnosis of FH using specific items, others are adopting a scoring system. The Japanese criteria reported in 1988[8] were as follows. Major items included the following 3 items: (1) the patient shows the IIa or IIb phenotype at a serum cholesterol level of 260 mg/dL or above, in principle; (2) Tendinous xanthoma or xanthoma tuberosum is present; (3) Reduced or abnormal receptor activity is noted by LDL receptor analysis; however, for LDL receptor activity, even lipid specialists do not routinely measure activity. It would be even more difficult for primary care physicians to measure activity for the diagnosis of FH.

The cutoff level of serum cholesterol used in the first criterion in the criteria published in 1988 was 260 mg/dL; however, LDL-C is directly affected by dysfunction of the LDL receptor and is routinely measured in clinics by the direct method or Friedewald formula; therefore, we tried to use LDL-C as a cutoff level instead of total cholesterol. The presence of tendon and/or cutaneous nodular xanthomas was also used because of its convenience, high sensitivity and specificity. A family history of FH or premature CAD in 1st or 2nd degree relatives was proposed for the third criterion instead of measuring LDL receptor activity in the new diagnostic criteria. A family history of FH showed high sensitivity and specificity; however, primary care physicians may have difficulty obtaining this because it was not easy for them to reach a diagnosis of FH with the previous criteria. In the present study, accurate diagnosis of a family history of FH seemed to have been given because lipid specialists made the diagnosis at all the hospitals; however, the same result may not be applied to primary care physicians. Therefore, a family history of CAD, which may be easier to obtain, was added to the criteria. It should be noted that the sensitivity of a "family history of FH or CAD" was slightly higher than that of a "family history of FH". Accordingly, we chose a "family history of FH or premature CAD in 1st or 2nd degree relatives" as the third criterion.

The cutoff level of LDL-C for the diagnosis of FH should be set by its sensitivity and specificity in different cutoff points. The cutoff level of LDL-C for the diagnosis of FH was reported to be 190 mg/dL in Simon Broome[17], NICE[15] and 205 mg/dL in MEDPED[16]. In this study, 180 mg/dL was selected as the cutoff level together with the presence of xanthoma and the family history as the criteria for the diagnosis of FH because of its high sensitivity and specificity.

Reduced LDL receptor activity is direct evidence of FH and was used as one of the criteria in the previous version. Usually, LDL receptor activity is determined by the binding of fluorescent-labeled LDL to lymphocytes. The procedure of measuring LDLR activity is cumbersome and it is difficult to measure in routine clinical settings. Further, few companies can measure LDLR activity. Indeed, the specialists involved in this study measured LDLR activity only in 7 of 419 patients of FH, showing the sensitivity of the previous criteria as 60.9%. Therefore, in order to determine criteria sensitive enough to give a diagnosis of FH, the third item was changed from LDLR activity to family history.

There are some limitations in the present study. First, the patients analyzed in this study may have different characteristics from those followed by primary care physicians, because the physicians in this study are taking care of many FH patients and information about family history can be obtained more easily than by primary care physicians. Second, it is sometimes difficult for primary care physicians to take a complete family history, especially FH, and to diagnose ATT and/or the presence of CX, about which information can be missed in the primary care setting. Third, FH has been reported to have mutations in LDL receptor, PCSK9 and apolipoprotein B. Because mutations in PCSK9 may cause milder forms of FH, the sensitivity of the criteria may be reduced in these patients. Further study is required to address the applicability of the criteria for the primary care setting.

In conclusion, we have determined the cutoff of LDL-C for the diagnosis of FH by a multicenter study and proposed conventional diagnostic criteria by using high LDL-C, ATT and/or the presence of CX, and a family history of FH and/or CAD for primary care settings.

Acknowledgments

We thank Dr. Daisaku Masuda, Miyako Yuasa-Kawase (Osaka University), Mihoko Ogita (Kyoto University), Masa-aki Kawashiri (Kanazawa University) Minoru Takemoto (Chiba University), Taro Harada (Nippon Medical School) and Noriyuki Iwamoto

(National Cerebral and Cardiovascular Center) for their help with data acquisition and analysis. This work was supported by Grants-in-Aid for Scientific Research from the Japanese Ministry of Health, Labor and Welfare (H23-seisakutansaku-ippan-004 and Research on Measures for Intractable Diseases) and the Cardiovascular Research Foundation (Suita, Japan).

Disclosures

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Kowa Co. Ltd. – Honoraria
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Kaneka Medix Co. Ltd. – Research Grants
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MSD – Honoraria
Daiichi-Sankyo Co. Ltd. – Honoraria and Research Grants
Kowa Co. Ltd. – Honoraria
Otsuka Co. Ltd. – Research Grants
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Ono Pharmaceutical Co. Ltd. – Honoraria
Pfizer Co. Ltd. – Honoraria
Astellas Pharma Inc. – Honoraria
Takeda Pharmaceutical Co. Ltd. – Honoraria
Kowa Pharmaceutical Co. Ltd. – Honoraria
Novartis Pharma K.K. – Research Grants
MSD K.K. – Research Grants
Takeda Pharmaceutical Co. Ltd. – Research Grants
Daiichi-Sankyo Co. Ltd. – Research Grants
Eli Lilly Japan K.K. – Research Grants
Sanofi-Aventis K.K. – Research Grants
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Dainippon Sumitomo Pharma Co. Ltd. – Research Grants
Kyowa Hakko Kirin Co. Ltd. – Research Grants
Astellas Pharma Inc. – Research Grants
Shionogi & Co. Ltd. – Research Grants
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Daiichi-Sankyo Co. Ltd. – Research Grants
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Kowa Co. Ltd. – Honoraria, Advisory Board
Skylight Biotech Inc. – Advisory Board
Astellas Pharma Inc. – Research Grants
MSD – Research Grants
Kissei Co. Ltd. – Research Grants
Otsuka Co. Ltd. – Research Grants
Shionogi & Co. Ltd. – Research Grants

References

1) Goldstein JL HH, Brown MS: Familial hypercholesterolemia. Edited by Scriver CR BA, Sly WS, Valle D, pp2863-2913, McGraw-Hill, New York, 2001
2) Bujo H, Takahashi K, Saito Y, Maruyama T, Yamashita S, Matsuzawa Y, Ishibashi S, Shionoiri F, Yamada N, Kita T: Clinical features of familial hypercholesterolemia in Japan in a database from 1996-1998 by the research committee of the ministry of health, labour and welfare of Japan. J Atheroscler Thromb, 2004; 11: 146-151
3) Mabuchi H, Koizumi J, Shimizu M, Takeda R: Development of coronary heart disease in familial hypercholesterolemia. Circulation, 1989; 79: 225-232
4) Harada-Shiba M, Sugisawa T, Makino H, Abe M, Tsushima M, Yoshimasa Y, Yamashita T, Miyamoto Y, Yamamoto A, Tomoike H, Yokoyama S: Impact of statin treatment on the clinical fate of heterozygous familial hypercholesterolemia. J Atheroscler Thromb, 17: 667-674
5) Mabuchi H, Miyamoto S, Ueda K, Oota M, Takegoshi T, Wakasugi T, Takeda R: Causes of death in patients with familial hypercholesterolemia. Atherosclerosis, 1986; 61: 1-6
6) Jensen J, Blankenhorn DH, Kornerup V: Coronary disease in familial hypercholesterolemia. Circulation, 1967; 36: 77-82
7) Stone NJ, Levy RI, Fredrickson DS, Verter J: Coronary artery disease in 116 kindred with familial type II hyperlipoproteinemia. Circulation, 1974; 49: 476-488
8) Teramoto T, Sasaki J, Ueshima H, Egusa G, Kinoshita M, Shimamoto K, Daida H, Biro S, Hirobe K, Funahashi T, Yokote K, Yokode M: Diagnostic criteria for dyslipidemia. Executive summary of Japan Atherosclerosis Society (JAS) guideline for diagnosis and prevention of atherosclerotic cardiovascular diseases for Japanese. Journal of atherosclerosis and thrombosis, 2007; 14: 155-158

9) Teramoto T: Status of lipid-lowering therapy prescribed on recommendations in the 2002 report of the Japan Atherosclerosis Society Guideline for Diagnosis and Treatment of Hyperlipidemia in Japanese Adults: A study of the Japan Lipid Assessment Program (J-LAP). Current Therapeutic Research, 2005; 66: 80-95
10) Mabuchi H, Ito S, Haba T, Ueda K, Ueda R: Discrimination of familial hypercholesterolemia and secondary hypercholesterolemia by Achilles' tendon thickness. Atherosclerosis, 1977; 28: 61-68
11) Mabuchi H, Nohara A, Noguchi T, Kobayashi J, Kawashiri MA, Tada H, Nakanishi C, Mori M, Yamagishi M, Inazu A, Koizumi J: Molecular genetic epidemiology of homozygous familial hypercholesterolemia in the Hokuriku district of Japan. Atherosclerosis, 2011; 214: 404-407
12) Watts GF, Sullivan DR, Poplawski N, van Bockxmeer F, Hamilton-Craig I, Clifton PM, O'Brien R, Bishop W, George P, Barter PJ, Bates T, Burnett JR, Coakley J, Davidson P, Emery J, Martin A, Farid W, Freeman L, Geelhoed E, Juniper A, Kidd A, Kostner K, Krass I, Livingston M, Maxwell S, O'Leary P, Owaimrin A, Redgrave TG, Reid N, Southwell L, Suthers G, Tonkin A, Towler S, Trent R: Familial hypercholesterolaemia: a model of care for Australasia. Atheroscler Suppl, 2011; 12: 221-263
13) Catapano AL, Reiner Z, De Backer G, Graham I, Taskinen MR, Wiklund O, Agewall S, Alegria E, Chapman MJ, Durrington P, Erdine S, Halcox J, Hobbs R, Kjekshus J, Perrone Filardi P, Riccardi G, Storey RF, Wood D: ESC/EAS Guidelines for the management of dyslipidaemias: the Task Force for the management of dyslipidaemias of the European Society of Cardiology (ESC) and the European Atherosclerosis Society (EAS). Atherosclerosis, 2011; 217 Suppl 1: S1-44
14) Damgaard D, Larsen ML, Nissen PH, Jensen JM, Jensen HK, Soerensen VR, Jensen LG, Faergeman O: The relationship of molecular genetic to clinical diagnosis of familial hypercholesterolemia in a Danish population. Atherosclerosis, 2005; 180: 155-160
15) Wierzbicki AS, Humphries SE, Minhas R: Familial hypercholesterolaemia: summary of NICE guidance. BMJ, 2008; 337: a1095
16) Williams RR, Hunt SC, Schumacher MC, Hegele RA, Leppert MF, Ludwig EH, Hopkins PN: Diagnosing heterozygous familial hypercholesterolemia using new practical criteria validated by molecular genetics. Am J Cardiol, 1993; 72: 171-176
17) Risk of fatal coronary heart disease in familial hypercholesterolaemia. Scientific Steering Committee on behalf of the Simon Broome Register Group. BMJ, 1991; 303: 893-896