

Candidate Genes Involved in Cardiovascular Risk Factors by a Family-Based Association Study on the Island of Kosrae, Federated States of Micronesia

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Altered plasma levels of lipids and lipoproteins, obesity, hypertension, and diabetes are major risk factors for atherosclerotic cardiovascular disease. To identify genes that affect these traits and disorders, we looked for association between markers in candidate genes (*apolipoprotein AII* (*apo AII*), *apolipoprotein AI-CIII-AIV* gene cluster (*apo AI-CIII-AIV*), *apolipoprotein E* (*apo E*), *cholesteryl ester transfer protein* (*CETP*), *cholesterol 7 α -hydroxylase* (*CYP7a*), *hepatic lipase* (*HL*), and *microsomal triglyceride transfer protein* (*MTP*)) and known risk factors (triglycerides (Tg), total cholesterol (TC), *apolipoprotein AI* (*apo AI*), *apolipoprotein AII* (*apo AII*), *apolipoprotein B* (*apo B*), body mass index (BMI), blood pressure (BP), leptin, and fasting blood sugar (FBS) levels.) A total of 1,102 individuals from the Pacific island of Kosrae were genotyped for the following markers: *Apo AII/MspI*, *Apo CIII/SstI*, *Apo AI/XmnI*, *Apo E/HhaI*, *CETP/TaqIB*, *CYP7a/BsaI*, *HL/DraI*, and *MTP/HhpI*. After testing for population stratification, family-based association analysis was carried out. Novel associations found were: 1) the *apo AII/MspI* with *apo AI* and BP levels, 2) the *CYP7a/BsaI* with *apo AI* and BMI levels. We also confirmed the following

associations: 1) the *apo AII/MspI* with Tg level; 2) the *apo CIII/SstI* with Tg, TC, and *apo B* levels; 3) the *Apo E/HhaI* E2, E3, and E4 alleles with TC, *apo AI*, and *apo B* levels; and 4) the *CETP/TaqIB* with *apo AI* level. We further confirmed the connection between the *apo AII* gene and Tg level by a nonparametric linkage analysis. We therefore conclude that many of these candidate genes may play a significant role in susceptibility to heart disease. © 2002 Wiley-Liss, Inc.

KEY WORDS: lipids and lipoproteins; candidate genes; family-based association

INTRODUCTION

Altered plasma levels of lipids and lipoproteins, obesity, hypertension, and diabetes are major risk factors for atherosclerotic cardiovascular disease. Genetic and environmental factors are known to affect the incidence of these conditions. Although monogenic conditions can result in abnormalities, it is now thought that much of the population variance is due to the contribution of many genes and gene-environment interactions. Genes involved in lipid metabolic pathways have been investigated as candidates in many general population association studies, but the results from these studies are far from conclusive and often inconsistent, partly due to the common pitfalls in these types of association studies, such as inadequate sample size and population stratification. Attempts to identify genes responsible for these risk factors by association studies should be easier in studies of populations that are more homogeneous, both genetically and with regard to life style, assuming that significant phenotypic variation still exists. With this idea in mind we have initiated a comprehensive epidemiological and genetic study on the Pacific island of Kosrae, Federated States of Micronesia.

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Kosrae is located 2,500 miles northeast of Australia and was originally settled by a small number of founders (estimated to be 50), originating from Polynesia around 50 A.D. Westerners first visited Kosrae in 1824. During the 19th century the combined effects of typhoons and exposure of the native population to Western communicable diseases reduced the indigenous population from 3,000–6,000 to about 300 individuals by 1910. Historical and genealogical records indicate that these 300 survivors were the result of extensive admixture between native Kosraean females and male Caucasian whalers from New England and Europe who visited the island in the mid to late 19th century. As late as 1945 Kosraeans consumed a diet consisting mostly of fish, fruits, and vegetables, and the average individual was noted to be lean. After WWII Kosrae was designated a U.S. Trust Territory and this led to drastic lifestyle changes, with islanders becoming more sedentary and consuming large quantities of high-fat foods, such as Spam, turkey tails, and hamburgers, supplied through U.S. aid. These changes resulted in a large increase in the Kosraean population, accompanied by a dramatically increased prevalence of obesity, an outcome similar to that of other indigenous populations, such as the Pima Indians of Arizona and the Nahuans [Shmulewitz et al., 2001].

Concern over the epidemic of obesity led in 1994 to a screening of practically all adult Kosraeans not only for obesity, but also for other related conditions such as dyslipidemia, diabetes, and hypertension. In addition, blood was obtained for DNA analysis from each participant and an extensive family tree was constructed for the entire island. In the current study, eight markers in or near the following candidate genes previously implicated in lipoprotein abnormalities, apolipoprotein AII (*apo AII*), apolipoprotein AI-CIII-AIV gene cluster (*apo AI-CIII-AIV*), apolipoprotein E (*apo E*), cholesteryl ester transfer protein (*CETP*), cholesterol 7 α -hydroxylase (*CYP7a*), hepatic lipase (*HL*), and microsomal triglyceride transfer protein (*MTP*), were tested in a family-based association study for correlation to lipid and lipoprotein, body mass index (BMI), blood pressure (BP), leptin, and fasting blood sugar (FBS) levels. We also assessed the existence of population stratification on these marker/trait pairs and its implications on the association results.

MATERIALS AND METHODS

Sample Collection

A total of 2,188 adult Kosraeans (ages, 20–85 years) were studied for dyslipidemia, obesity, diabetes, and hypertension as part of a public health campaign to identify risk factors for noncommunicable diseases, begun in 1994. The study protocol was approved by the Institutional Review Board of Rockefeller University and the Kosrae Department of Health. Informed consent was obtained for all participants. Recruitment was through open meetings and radio announcements, and over 90% of eligible Kosraeans participated. Participants filled out questionnaires that included family data that were subsequently used to construct a family

tree of the population of the island. A detailed description of the study protocol; the measurement of lipids and lipoproteins, BMI, systolic blood pressure (SBP), diastolic blood pressure (DBP), leptin, and FBS levels; the prevalence of risk factors; the effects of covariates; and a factor analysis of these traits has been recently published [Shmulewitz et al., 2001]. Of relevance to the study, standard lipoprotein quantification requires ultracentrifugation of either fresh specimens or specimens kept overnight at 4°C, and cannot be performed on frozen plasma or serum. Due to the lack of adequate facilities on the island and the inability to ship serum overnight at 4°C, it was not possible to determine low-density lipoprotein (LDL) and high-density lipoprotein (HDL) cholesterol levels directly. Instead, their major apolipoprotein components, apolipoprotein B (apo B) and apolipoprotein AI (apo AI), respectively, were measured. The levels of apo AI and apo B are highly correlated with HDL cholesterol and LDL cholesterol, respectively, making them excellent surrogates [Bachorik et al., 1997]. Total cholesterol (TC) (Boehringer-Mannheim, Indianapolis, IN) and triglyceride (Tg) (Sigma Diagnostics, St. Louis, MO) levels were measured with commercially available enzymatic kits. Apo AI, apolipoprotein AII (apo AII), and apo B levels were determined using standard enzyme-linked immunosorbent assay (ELISA) techniques. FBS was measured by a finger stick with a glucometer. Leptin levels were determined by a commercially available radioimmunoassay kit (Linco, St. Louis, MO), with a lower detection limit of 0.5 ng/ μ l and 5% interassay coefficient of variation. Genomic DNA was extracted from whole blood that was previously frozen on dry ice by standard techniques and kept at –20°C until analysis. Genotyping was done on 1,102 Kosraeans, who comprised one major large branch of the family tree.

Genotyping

All 10 single nucleotide polymorphism (SNP) sites were genotyped by PCR amplification of specific fragments of genomic DNA followed by digestion with the respective restriction enzymes. The candidate genes and their related polymorphic sites are listed in columns 1 and 2 in Table I. The primers for DNA amplification are listed in column 3. A typical PCR reaction was done in a 20- μ l volume consisting of 10 mM Tris-HCl (pH 9.0 at 25°C), 50 mM KCl, 3 mM MgCl₂, 0.1% Triton X-100, 300 μ M deoxyribonucleoside triphosphates (dNTPs), 10 ng of genomic DNA, 50 pmol of each primer, and 0.5 unit of Taq polymerase. For *apo E/HhaI*, *apo CIII/SstI*, and *CETP/TaqIB*, 10% dimethyl sulphoxide (DMSO) was included in the reaction solution. The reaction was performed by first denaturing at 94°C for 4 min, followed with 35 rounds of denaturing for 30 sec at 94°C, annealing for 30 sec at an experimentally optimized annealing temperature and elongating for 30 sec at 72°C, and a final step of elongation at 72°C for 7 min. Restriction enzyme and buffer were subsequently added into the mix and incubated at 37°C for 4 hr. The products were separated on agarose gel, except for *apo E*, which was separated

TABLE I. List and Characteristics of Tested Polymorphic Sites in Kosraean Population

Gene cluster	Polymorphism	Amplification primers (5' to 3')	Location	Gene function	Reference
Apo AII	Apo AII/Mspl	CTTTGCATTCACAAAGGAAGTACTAG and TTCTTTTTCATAGAAATGACTAACAG	Apo AII (C2777A) 3'-untranslated	HDL component	[Tsao et al., 1985]
	Apo AII (CA)n	ACTGCTGTGGACCCAGCTGAAAAG and CCTGTCTCGGAACC-AAAGCTCCTG	Apo AII intron2		[Tsao et al., 1985]
Apo AI-CIII-AIV	Apo CIII/SstI	GATTCCTGCCGTGAGGTCTCAGGGCTGTGCT and CCTGGAGTCTGTCCAGTGCACGCCACA	Apo CIII (G3147C, 3'-UTR)	Apo CIII is VLDL and HDL component	[Dammerman et al., 1993]
	Apo AI/XmnI	GGAAACAGGGCCCTACACTGTG and GTCTGCAGCCCTTGCAGTCTGATC	Apo AI (C2500T)	Apo AI is major HDL component	[Shoulders et al., 1993]
	D11S1998	AGCCATCAACTAGCTTTTCCC and GGGAGGCACCAACAGATG	Within 3 cm of Apo AI		[www.gdb.org]
Apo E	Apo E/HhaI	GCGGGCAGGCCCGGCTGGCGG and CGCTCGGCCCTCGGGGCCCG	Apo E1112 (C3745T) Apo E158 (C3883T)	VLDL, LDL, chylomicron remnant clearance	[Hixson and Vernier, 1990]
CETP	CETP/TaqIB	CACTAGCCAGAGAGAGAGTG and CTGAGCCAGCCGCACACTAAC	Intron 1 of CETP	Lipid exchange between HDL and Tg-rich lipoproteins	[Fumeron et al., 1995]
CYP7a	Cyp7a/BsaI	AATGTTTTTCCCAGTTCCTTTTC and AATTAGCCATTGTTCATCTATTAG	CYP7a (A278C)	Rate-limiting step in bile acid synthesis	[Wang et al., 1998]
Hepatic lipase	HL/DraI	GGGGGAAGAAGTGTGTTACTCTAGGATCACC and CACAGGGACTGTGTCCATTCTCCG	HL (G-250A)	Tg hydrolysis	[Guerra et al., 1997]
MTP	MTP/HspI	GGATTTAAATTTAAACTGTTAATTCATAATCAC and AGTTTCACACATAAAGGACAATCATCTA	MTP (G-493T)	Assembly and secretion of VLDL	[Karpe et al., 1998]
LPL	LPL/RsaI	GCCGATACAAATCTTGGIG and CTGCTTCTTTTGGCTCTGACTGA	Lpl291, Asn to Ser	Hydrolysis of Tg in chylomicron and VLDL	[Reymer et al., 1995]
	LPL/MnlI	CTCTGATTCTGATGTGGCCCTGAGTIG and CTCCCTTAGGGTGC AAGCTCAGG	Lpl471, Ser to stop		[Hata et al., 1990]

on 6% acrylamide gel. The common alleles were defined as allele 1, except in *apo E/HhaI*, where allele 3 was the most common of the three alleles (E2, E3, and E4). The sizes of DNA fragments for all of the alleles are listed in Table II, column 2.

The two microsatellite markers, the *apo AII* intragenic dinucleotide repeat and D11S1998 near *apo AII-CIII-AIV*, were genotyped with fluorescent primers on the 373 system (Applied Biosystems, Foster City, CA). PCR was carried out in condition similar to that described above, except that the primer concentration was reduced to 5 pmol. Allele calling was done by two individuals independently with Genotyper; afterwards the data were exported to a database where discrepancies were resolved.

Statistical Analysis

In the family-based association study the quantitative traits were corrected for age and sex by linear regression, and outliers (values that fell more than 3 SD from the mean) were excluded. All of the quantitative variables had an approximately symmetric distribution, except for Tg, leptin, and FBS. To reduce skewness in the latter, a natural logarithm transformation was applied. However, in order to facilitate comparisons with other studies, we still reported the analysis result using Tg, since this has been done customarily. There was no essential difference between the results from Tg and log Tg.

A standard way to estimate association between genotype and trait is to regress the trait on the genotype and obtain estimates of the phenotypic means. In this process, the phenotypic means for the various genotypes are calculated, and assuming a normal distribution for the means and environmental noise, their significance is estimated. A prerequisite for this type of analysis is that the samples are independent. Although the

familial relationship of the Kosraean samples violates this assumption, the regression analysis is valid as long as the following two conditions are met: 1) the correlation among relatives is solely due to the genotype of the locus being tested, and 2) the genotype for every pedigree member is known [Ewens and Spielman, 1995]. Since the Kosraeans lived in a remarkably homogeneous environment, we can assume a uniform environment and we assumed that the first condition was met. For the second condition, since only about 45% of the population was typed, we used the known familial relationships in the Loki program [Heath, 1997] to estimate the genotype of the individuals. In doing so, we corrected for the familial (genetic) correlation between relatives. The Loki program uses a Markov chain Monte Carlo method to sample the unknown genotypes and gives an accurate description of the genotype distribution profile. In this study, 1 million sets of missing genotypes were generated, proportional to the probability given the observed genotype data and the pedigree structure. A regression analysis was performed as described earlier for each set of samples, and the estimates obtained from each regression were then averaged over the 1 million sets of samples. This provides estimates of the phenotypic means that have been averaged over the missing genotype data. For simplicity, the phenotypic mean of the most common genotype (11) was always set at zero, while the phenotypic means of the other genotypes (12 or 22) were estimated as deviations from that of 11. In addition to a point estimate, this process also allows estimation of the distribution of the phenotypic means. From this, 95% confidence intervals and the probability that a particular phenotypic mean differs from zero can be empirically estimated, without relying on assumptions of normality. For example, to estimate the *P* value of the phenotypic mean of genotype 12, which is smaller than the phenotypic mean of 11, we simply counted the proportion of samples in which the regression estimate of the phenotypic mean of 12 is < 0 . The Loki method takes advantage of the whole set of trait data and is robust in estimating *P* values in the sense that it does not rely on the normality of the estimates. The method easily allows for the addition of other factors into the regression model, and in this study, age and sex were added to the model. Individuals with Mendelian inconsistencies were excluded prior to the analysis.

MAPMAKER/SIBS 0.9 was used in the sibpair analysis [Kruglyak and Lander, 1995]. The Kosraean pedigree was too large to be analyzed with existing linkage software, so we split the Kosrae pedigree into two-generation nuclear families. Those families with two or more genotyped children were used in a sibpair analysis for the quantitative traits using the MAPMAKER/SIBS 0.9 program. Prior to running the MAPMAKER/SIBS 0.9, PEDMANAGER was used to check Mendelian inconsistencies. For each marker, we excluded those families whose member(s) showed any inconsistency. In general, the numbers of inconsistent individuals were very low.

The QTD program by Abecasis et al. [2000] was used to examine the evidence of population stratifica-

TABLE II. Allelic Characteristics of the Genotyped Markers in Kosraean Population

Polymorphism	Allele size (bp)	Allele frequency
Apo AII/MspI	1: 175 and 296	0.83
	2: 471	0.17
Apo CIII/SstI	1: 549	0.62
	2: 232 and 317	0.38
Apo AI/XmnI	1: 392	0.74
	2: 173 and 219	0.26
CETP/taq-1B	1: 174 and 361	0.62
	2: 535	0.38
Cyp7a/BsaI	1: 393	0.84
	2: 90 and 303	0.16
HL/DraI	1: 462	0.72
	2: 350	0.28
MTP/HhpI	1: 20 and 89	0.69
	2: 109	0.31
Apo E/HhaI	2: 83 and 91	0.02
	3: 35 and 48 and 91	0.81
	4: 39 and 48 and 72	0.17
		0.005
LPL/RsaI	1: 238	0.995
	2: 23 and 215	0.005
LPL/MnlI	1: 325	1
	2: 26 and 258	0

tion. The test evaluates whether there are subpopulation groupings where the following quantity varies in the absence of linkage: $(p-q) \times \mu$. The p and q are allele frequencies and μ is the phenotypic mean, so it reports stratification that affects the traits only. The same two-generation nuclear families used in the sibpair analysis were used in this analysis, since the complexity of the Kosraean pedigree exceeded the limit of the QTDT. This should give a good estimation, although it tends to overestimate the existence of stratification, since each sibship would have unique allele frequencies (determined by parental alleles) and phenotypes (determined by shared genetic and environmental components).

RESULTS

Family-Based Associations

We genotyped 10 polymorphisms and tested whether there were significant differences in phenotypic means for Tg, log Tg, TC, apo AI, apo AII, apo B, BMI, SBP, DBP, log leptin, and log FBS among all the genotypes in the eight polymorphisms that were common. These common polymorphisms included the *apo AII/MspI*, *apo CIII/SstI*, *apo AI/XmnI*, *apo E/HhaI*, *CETP/TaqIB*, *CYP7a/BsaI*, *HL/DraI*, and *MTP/HhpI*. Allele frequencies of these polymorphisms in the Kosraean population are listed in Table II. In Tables III and IV, we summarize all of the polymorphisms that displayed significantly different genotype-specific means at the significance level of $P < 0.01$.

For the *apo AII/MspI* polymorphism, compared to individuals with two common alleles (M1/M1), the mean Tg level in individuals with homozygous minor alleles (M2/M2) was increased by 56%. The difference was highly significant ($P < 0.0001$). The result was similar when the analysis was done using log Tg ($P = 0.008$), thus not caused by outliers. The Tg-raising effect

of the M2 allele seemed to be recessive, since the M1/M2 individuals displayed a Tg level similar to that in M1/M1. The M2 allele was also associated with a decreased level of apo AI. This effect was dosage dependent (3% decrease in the heterozygous M1/M2 and 7% decrease in the homozygous M2/M2) and reached significance for the homozygous M2 individuals ($P = 0.01$). Interestingly, both M1/M2 and M2/M2 subjects also demonstrated statistically significant decreases in DBP and SBP, compared to that of M1/M1 subjects. These results suggest that the M2 allele is a recessive allele associated with higher Tg levels, a codominant allele associated with lower apo AI levels, and a dominant allele associated with decreased BP.

For the *apo CIII/SstI* polymorphism, compared to individuals with two common alleles (S1/S1), the mean Tg level was 10% higher in S1/S2 individuals ($P = 0.004$) and 27% higher in S2/S2 individuals ($P = 0.0002$). A similar result was obtained when the analysis was done using log Tg, whereas log Tg was 4.5% higher in S1/S2 individuals ($P = 0.0008$) and 9.5% higher in S2/S2 individuals ($P < 0.0001$). The mean TC level was 2.0% higher in S1/S2 individuals and 6.4% higher in S2/S2 individuals ($P = 0.0006$). The apo B level displayed the same trend as the TC level. These results suggest that the S2 allele is a codominant allele associated with increased Tg levels and a recessive allele associated with increased TC and apo B levels.

For the *CETP/TaqIB* polymorphism, compared to individuals carrying two common alleles (T1/T1), the heterozygous individuals (T1/T2) showed a 2.7% increase in apo AI level ($P = 0.1$), and individuals carrying two rare alleles (T2/T2) showed a 6.1% increase in apo AI level ($P = 0.002$). This indicates that the rare allele is associated with increased apo AI levels.

For the *CYP7a/BsaI* polymorphism, compared to homozygous common allele carriers (B1/B1), individuals

TABLE III. Significant Genotype/Phenotype Associations, Part I*

Genotype/phenotypic mean	11		12		22		Published before?
	11	12	P-value	22	P-value		
<i>Apo AII/MspI</i>							
Tg	98.73	100.48	0.3	152.68	< 0.0001	Yes	
Log Tg	1.94	1.97	0.2	2.11	0.008	Yes	
Apo AI	115.99	112.45	0.02	107.79	0.01	No	
DBP	78.46	74.22	0.004	74.60	0.002	No	
SBP	121.70	116.27	0.009	116.18	0.006	No	
<i>Apo CIII/SstI</i>							
Tg	94.57	104.01	0.004	119.82	0.0002	Yes	
Log Tg	1.93	2.01	0.0008	2.11	< 0.0001	Yes	
TC	173.70	177.26	0.06	184.73	0.0006	Yes	
Apo B	85.89	87.71	0.09	92.27	0.001	Yes	
<i>CETP/TaqIB</i>							
Apo AI	112.76	115.85	0.01	119.64	0.002	Yes	
<i>CYP7a/BsaI</i>							
Apo AI	114.26	114.74	0.4	128.29	0.0008	No	
BMI	31.06	31.82	0.02	33.36	0.004	No	

*For all the polymorphisms, 1 refers to the common allele and 2 refers to the rare allele. Tg, TC, apo AI, and apo B are reported as mg/dl. DBP and SBP are reported as mmHg. The unit for BMI was kg/m². The phenotypic means of 12 and 22 were compared with those of 11, and the P-values at which differences happened by chance were shown. The analyses were done with the Loki method that corrected for age and gender, and took into account of the family.

TABLE IV. Significant Genotype/Phenotype Associations, Part II*

Genotype/phenotype mean	33		34		44		32		Agree with previous reports?
	33	34	<i>P</i> -value	44	<i>P</i> -value	32	<i>P</i> -value		
Apo E/HhaI									
Tg	97.15	105.05	0.01	98.81	0.4	102.26	0.3	Yes	
Log Tg	1.94	2.02	0.003	2.05	0.1	1.97	0.3	Yes	
TC	175.12	178.11	0.1	187.31	0.04	159.73	0.002	Yes	
Apo AI	116.54	111.07	< 0.0001	109.04	0.04	117.78	0.4	Yes	
Apo B	86.26	88.68	0.04	95.42	0.02	72.16	< 0.0001	Yes	

*The details for this table are similar to those of Table III, except that, for the *apo E/HhaI* polymorphism, the E3 is the common allele, and the phenotypic means of 34, 44, and 32 were compared to those of 33.

with two rare alleles (B2/B2) displayed a 12% increase in apo AI levels ($P = 0.0008$). Also, compared to B1/B1 individuals, BMI was increased by 2.4% for B1/B2 individuals ($P = 0.02$) and 7.4% for B2/B2 individuals ($P = 0.004$).

For the *apo AI/XmnI*, *HL/DraI*, and *MTP/HhpI* polymorphisms, no associations were found at the significance level we set ($P = 0.01$).

For the *apo E/HhaI* polymorphism, since there were not enough E2/E2 and E4/E2 individuals (0 and 6, respectively), we examined the effects of genotypes E3/E2, E3/E3, E4/E3, and E4/E4 on the lipid and other traits. We observed, in the above order, stepwise increases in apo B and TC levels, and stepwise decreases in apo AI levels (see Table IV). Compared to individuals of E3/E3, the mean apo B level was 16% lower in E3/E2 individuals, 2.7% higher in E4/E3 individuals, and 10% higher in E4/E4 individuals. Similarly, the mean TC level was the lowest in E3/E2 individuals and the highest in E4/E4 individuals. For the Apo AI level the trend was opposite. Compared to E3/E3 individuals, the mean apo AI level was 1.1% higher in E3/E2 individuals, 4.7% lower in E4/E3 individuals ($P < 0.0001$), and 6.4% lower in E4/E4 individuals. The mean Tg level was the lowest in E3/E3 individuals and was significantly higher in E4/E3 individuals ($P = 0.01$). Similar results were obtained when analysis was done with log Tg. Overall, these results are consistent with the generally established effects of E4, E3, and E2 on apo B levels and further clarified their influence on TC, apo AI, and Tg levels.

The N291S and S447X polymorphisms in *lipoprotein lipase* have been shown to influence the lipid and lipoprotein profile [Hata et al., 1990; Reymer et al., 1995; Fisher et al., 1997] and were chosen initially for the study. Yet the rare allele of N291S was not detected, and the rare allele frequency of S447X was 0.5%. As a result, these polymorphisms were not informative enough to be included in the study.

Linkage

In order to corroborate our results in the association analyses, we performed linkage analysis on all the traits for the *apo AII* and *apo AI-CIII-AIV* loci, using microsatellite markers of the *apo AII* dinucleotide repeat (intragenic; heterozygosity, 0.57) and D11S198 (within 3 cm of the *apo AI-CIII-AIV*; heterozygosity, 0.69). Out of the 223 nuclear families used in the sibpair analysis,

68 had 2 siblings, contributing 68 sibpairs; 46 had 3 siblings, contributing 138 sibpairs; 36 had 4 siblings, contributing 216 sibpairs; 34 had 5 siblings, contributing 340 sibpairs; 16 had 6 siblings, contributing 240 sibpairs; 17 had 7 siblings, contributing 357 sibpairs; and 6 had 8 siblings, contributing 168 sibpairs. The total number of sibpairs was 1,527. For the intragenic *apo AII* dinucleotide repeat, a single-point nonparametric analysis yielded a Z-score of 1.86 for Tg levels, corresponding to $P = 0.0314$; and a Z-score of 2.22 for log Tg, corresponding to $P = 0.0132$. For the D11S1998, no significant linkage was found. The result from linkage analysis further confirms that the *apo AII* locus or a gene in its vicinity is important for Tg levels.

Population Stratification

Our family-based association analysis can correct fully for population stratification, on the condition that all individuals were genotyped. Since we genotyped only approximately 45% of the individuals, it was possible that residual effects of population stratification still existed. To guard against this possibility, we used the QTDT program [Abecasis et al., 2000] to check the existence of population stratification on all the 80 possible marker/trait combinations (8 markers and 10 traits). The following two marker/trait combinations gave indications of population stratification at a significance level of $P < 0.01$: the *apoA II/MspI* vs. SBP ($P = 0.007$) and the *HL/DraI* vs. apo AII levels ($P = 0.004$). There was no evidence of population stratification for most of the associations we observed, such as the *apo AII/MspI* with Tg and apo AI levels, the *apo CIII/SstI* with Tg, TC, and apo B levels, the *CETP/TaqIB* with apo AI levels, and the *apo E/HhaI* with TC, apo AI, and apo B levels. In conclusion, the associations we found were not a result of stratification.

DISCUSSION

In this study we have selected common polymorphisms that have been shown to influence lipid and lipoprotein levels and tested their roles in modulating lipid, BMI, BP, leptin, and FBS levels in a family-based association study. We observed the following significant associations: *apo AII/MspI* to Tg, apo AI, and BP; *apo AI-CIII-AIV* to Tg, TC, and apo B; *apo E/HhaI* to Tg, TC, and apo B; *CETP/TaqIB* to apo AI; and *CYP7a/BsaI* to apo AI and BMI levels. Our study is the first to report that the *apo AII/MspI* polymorphism is associated with

apo AI and BP levels, and *CYP7a*/BsaI with apo AI and BMI levels.

The *apo AII*/MspI polymorphism has been reported to be associated with apo AII [Scott et al., 1985] and Tg levels [Ferns et al., 1986; Hong et al., 1998] in some studies, but not others [Kessling et al., 1988; Rajput-Williams et al., 1989; Dupuy-Gorce et al., 1996]. Recently, familial combined hyperlipidemia in a Finnish population was mapped to the chromosome 1q21-23 region that is close to, but does not include, *apo AII* [Pajukanta et al., 1998]. Moreover, a gene responsible for a combined hyperlipidemia phenotype in a mutant mouse strain was also mapped to the chromosome 3 region that is syntenic to human chromosome 1q21-23 [Castellani et al., 1998]. In our study, the M2 allele was associated with highly increased Tg levels, indicating that *apo AII*, or a gene close by, plays a strong role in influencing Tg levels in the Kosraean population. The effect of the allele seems to be recessive, since heterozygous individuals are unaffected. The linkage analysis further substantiates the conclusion. The study also indicates that the M2 allele associated with increased Tg levels is also associated with decreased apo AI levels. Decreased apo AI levels could be a secondary effect of elevated Tg levels, since elevated the Tg level is correlated with reduced apo AI levels in the general population, due to accelerated lipid exchange between HDL cholesterol and Tg from Tg-rich particles catalyzed by CETP. Furthermore, the Tg in HDL is hydrolyzed by hepatic lipase, decreasing the size of HDL, increasing the removal rate of HDL, and resulting in further reduction of HDL [Breslow, 2000]. In our study, however, the M2 allele is likely to have an independent effect on apo AI levels, since it behaved like a recessive allele in influencing Tg levels, yet like a codominant allele in influencing apo AI levels. We also found a statistically significant association of the *apo AII*/MspI polymorphism with DBP and SBP. This is the first report that links the *apo AII*/MspI polymorphism to BP.

The *apo AI-CIII-AIV* locus on chromosome 11 consists of *apo AI*, *apo AIV*, and *apo CIII*, the first two of which encode major constituents of intestinally derived lipoproteins, as well as HDL, and the last of which encodes a major constituent of very low-density lipoprotein (VLDL), chylomicron remnants, and HDL. Overexpression of *apo CIII* led to increased Tg levels in mouse models [Ito et al., 1990; Aalto-Setälä et al., 1992]. Many studies have reported an association between polymorphic sites in this locus and lipid parameters such as apo AI, apo B, apo CIII, Tg, HDL cholesterol, and LDL cholesterol [Dammerman et al., 1993; Cohen et al., 1994; Waterworth et al., 2000]. In a study with 18 Dutch families with familial combined hyperlipidemia, the minor alleles of the SstI (S2) and XmnI (X2) polymorphisms have been associated with high Tg and TC levels, and were enriched in FCHL patients [Dallinga-Thie et al., 1996, 1997]. Negative results for association were also reported [Marcil et al., 1996]; whether genetic heterogeneity plays a role in this discrepancy is unknown. Our study indicates that the *apo AI-CIII-AIV* cluster influences the levels of Tg, TC, and apo B in a dosage-dependent manner in the

Kosraean population. No significant linkage was detected using D11S1998 in the sibpair analysis. This could be due to information loss connected with the reduction of the original Kosraean pedigree into nuclear families; alternatively, the effect of D11S1998 might be too subtle to be picked up by the linkage analysis. The S2 allele has been associated with increased SBP in a study with 15 subjects that were S2 carriers and had type 2 diabetes mellitus and 48 controls [Tas and Abdella, 1994]. In our study no significant difference in BP was found.

Apo E is a ligand for LDL receptor (LDLR) and LDL receptor-related protein (LDLR) and mediates hepatic removal of VLDL, IDL, and chylomicron remnants. The E2, E3, and E4 polymorphism has been consistently associated with apo B and LDL cholesterol levels and accounts for 10% of variance of LDL cholesterol levels [Breslow, 2000]. Its effects on Tg, apo AI, and HDL cholesterol levels are not settled. In a recent large-scale study in a Caucasian general population [Frikke-Schmidt et al., 2000], *apo E* genotypes E2/E2, E3/E2, E4/E2, E3/E3, and E4/E3 (in this order) were found to correlate with stepwise increases in TC and apo B levels in both genders, and stepwise decreases in HDL cholesterol and apo AI levels in women, but not in men. In our study of the effects of E3/E2, E3/E3, E4/E3, and E4/E4 genotypes on the lipid profiles, we observed the four genotypes in the above order associated with stepwise increases in TC and apo B levels and stepwise decreases in apo AI levels. The results are highly consistent with those from the Caucasian population.

CETP promotes transfer of cholesterol ester from HDL to Tg-rich lipoproteins in exchange for Tg and is part of the reverse cholesterol transport pathway that is believed to play a protective role for atherosclerosis [Tall, 1993]. CETP activity is reported to be inversely associated with HDL cholesterol. The rare allele of *CETP*/TaqIB has been reported to be associated with lower plasma CETP activity [Freeman et al., 1990; Hannuksela et al., 1994; Kuivenhoven et al., 1997; Gudnason et al., 1999], higher HDL cholesterol levels [Kondo et al., 1989; Freeman et al., 1990; Bu et al., 1994; Hannuksela et al., 1994; Fumeron et al., 1995; Kuivenhoven et al., 1997; Gudnason et al., 1999], and higher apo AI levels [Kondo et al., 1989; Gudnason et al., 1999]. By contrast, other reports describe the absence of a relationship between *CETP* and HDL cholesterol [Cohen et al., 1994]. Our results are in agreement with that of Gudnason et al. [1999] and confirm that the rare allele is associated with increased apo AI levels.

CYP7a encodes a rate-limiting enzyme in bile acid biosynthesis. *CYP7a* has been linked to LDL cholesterol levels, and the *CYP7a*/BsaI polymorphism has been associated with LDL and HDL cholesterol levels [Wang et al., 1998]. In our study, the significant associations are those with the level of apo AI, a close surrogate of HDL cholesterol, and with BMI. We did not find association of *CYP7a* to the level of apo B, a close surrogate of LDL cholesterol. Our data is the first to suggest that the *CYP7a* is associated with apo AI levels and BMI.

Association studies are powerful and widely used, but in practice can be problematic. This is reflected from the

observation that many positive results are not replicated in later studies. Besides the common pitfall of inadequate sample size, the major confounding factors are population stratification due to ethnic admixture, multiple hypothesis testing, variable linkage disequilibrium with the causal SNPs, and population-specific modifiers. Compared to previous association analyses, this study has advantages that strengthen our findings. The study involved a large sample size consisting of 2,188 people phenotyped and 1,102 people genotyped. The study was done in an isolated population where the environment is highly homogeneous, thus increasing the probability of picking up subtle genetic effects. The family-based study corrected for the presence of ethnic admixture, reducing potential false positives arising from population stratification. Variable linkage disequilibrium with the causal SNPs can lead to varying results among different populations, so we have chosen those SNPs that are common and intragenic. In the case of multiple hypothesis testing, we have tested 8 markers, 10 phenotypes, and 2 genotypic comparisons in the study, constituting 160 tests, although in reality the number should be much less than 160, since many of the tests, such as Tg and log Tg, or DBP and SBP, are closely dependent. If we adopt the most stringent Bonferroni correction [Snedecor and Cochran, 1989], the threshold should be $0.05/160 = 0.0003$. Under this threshold we still found the following significant associations: *apo AII/MspI* with Tg levels, *apo AI-CIII-AIV* with Tg levels, and *apo E/HhaI* with apo AI and apo B levels. To balance the need for detecting subtle influences and minimizing false positives, we set the significance level at 0.01 for our analyses.

Population stratification is a common confounding factor that can lead to false positive results in association studies. The modern Kosraean population is derived from an admixture of Caucasians and Polynesians during the 19th century, so a traditional association study might be confounded by population stratification. Our family-based analyses could fully eliminate the interference of population stratification once all genotypes were known, but they were not available. As a result, we looked for the existence of population stratification. We chose the same significance value ($P < 0.01$) as we did in the association analyses for the same considerations. Only two polymorphism/trait combinations showed stratification: *Apo AII/MspI* vs. SBP and *HL/DraI* vs. apo AII levels. This indicates that in this ethnically admixed population, population stratification does not pose a major obstacle for association analysis. No evidence of population stratification was found for the associations of *apo AII/MspI* with Tg; apo AI with DBP levels; *apo CIII/SstI* with Tg, TC, and apo B levels; *CETP/TaqIB* with apo AI levels; and *apo E/HhaI* with apo AI and apo B levels; so these results, in all likelihood, represent real positive associations. Furthermore, the allele frequencies for *apo CIII/SstI*, *apo AI/XmnI*, *CETP/TaqIB*, *HL/DraI*, and *MTP/HhpI* polymorphisms in the Kosraean population were very similar to those found in Caucasians, further reducing the probability of stratification in these markers. In the Kosraean population, the allele frequencies of E2, E3, and E4 are

2%, 81%, and 17%, respectively. In Caucasians the frequencies are 8%, 77%, and 15%, respectively. So the E2 allele is dramatically underrepresented in the modern Kosraean population. Since the modern Kosraean population has descended from the admixture of Caucasian and indigenous Kosraeans, the most natural explanation is that indigenous Kosraeans had no E2 allele, and Caucasians contributed about 25% of total genome. This is consistent with a previous estimation that approximately 100 Caucasian whalers and 300 Kosraean females were the progenitors of the modern Kosraean population. Further studies with Y chromosome markers and mitochondria DNA markers are currently under way and will help clarify the situation.

CONCLUSION

Our study has shown for the first time the association between *apo AII/MspI* and apo AI and BP levels and the association between *CYP7a/BsaI* and apo AI and BMI levels. It also confirmed many previous published association studies, such as *apo AII/MspI* with Tg levels; *apo CIII/SstI* with Tg, TC, and apo B levels; *CETP/TaqIB* with apo AI levels; and *apo E/HhaI* with Tg, TC, and apo B levels. This study demonstrates the value of a family-based association analysis and the great potential of the Kosrae study.

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