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SHORT COMMUNICATION

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Polymorphisms of the apolipoprotein and angiotensin converting enzyme genes in young North Karelian patients with coronary heart disease

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Abstract The genes encoding apolipoproteins (apos) A-I, B, C-III and E as well as that encoding the angiotensin converting enyzme (ACE) have been proposed as candidate genes for coronary heart disease (CHD). We determined the common polymorphisms of the apo genes, previously found to influence serum lipid levels at the population level, and the insertion/deletion polymorphism of the ACE gene, recently reported to reflect the risk of myocardial infarction, in 82 very young (mean, 41 years) North Karelian Finns with symptomatic CHD and 50 controls of similar age. Patients with familial hypercholesterolemia had been excluded from this material. None of the polymorphisms examined, including the apo A-I promoter *Msp*I, apo C-III *Sst*I and apo B *Xba*I restriction fragment polymorphisms, a common variation of apo E (ε2, ε3 and ε4 alleles) and an ACE insertion/deletion (I/D) polymorphism, was significantly associated with the risk of premature CHD. Patients with CHD had a higher mean serum LDL cholesterol/HDL cholesterol ratio than controls (3.15 ± 1.30 vs 2.72 ± 0.98, $P < 0.05$), but no significant associations between the common apo gene polymorphisms and serum lipid levels were disclosed in either group. It is possible that other genetic loci than those proposed to be associated with accelerated atherosclerosis may be more important as risk factors of symptomatic CHD at the age of 40 years.

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Introduction

Family and twin studies have indicated that there is a strong genetic component in the etiology of coronary heart disease (CHD) (for review, see Goldstein and Brown 1984; Sing and Moll 1990). The familial component is particularly strong in early onset CHD, declines steeply with advancing age and is only negligible in patients that present with CHD after middle age (Rissanen 1979). Common polymorphisms of the apolipoprotein (apo) genes have been found to influence serum lipid levels, and some of these variations have been proposed to increase the risk of CHD (Humphries 1988; Lusis 1988). However, these associations still remain somewhat controversial in that their validity has not been confirmed in all populations. In the Finnish population the apo ε4, apo C-III *Sst*I S2 and apo B *Xba*I X2 alleles have been found to be associated with elevated serum LDL cholesterol and/or serum triglyceride levels, both in healthy adults (Aalto-Setälä et al. 1987, 1988; Ehnholm et al. 1986) and children (Aalto-Setälä et al. 1991; Lehtimäki et al. 1990). In addition, the apo E ε4 allele has been reported to predispose to atherosclerosis and CHD in many populations (for review, see Utermann 1987; Davignon et al. 1988), including the Finns (Kuusi et al. 1989; Nieminen et al. 1992). A common G to A variation in the promoter region of the apo A-I gene was shown to influence circulating high density lipoprotein (HDL) concentrations (Jeenah et al. 1990; Pagani et al. 1990), thereby rendering this polymorphism as another potential regulator of CHD risk. Recently, an insertion/deletion (I/D) polymorphism in intron 16 of the gene encoding angiotensin-converting enzyme (ACE) was shown to be associated with individual risk (Cambien et al. 1992) and parental history (Tiret et al. 1993) of myocardial infarction. This finding has not yet been confirmed in other studies, however. We undertook a study of the significance of these gene polymorphisms as determinants of CHD risk using three strict criteria in patient selection: ethnic homogeneity, young age (< 45 years) at presentation, and definite exclusion of familial hypercholesterolemia (FH).

Materials and methods

Patients, controls, and lipid assays

Recruitment of the patients and controls as well as the diagnostic criteria have been described in detail previously (Koivisto et al. 1993). In short, 55 consecutive patients aged 45 years or less admitted because of acute myocardial infarction (AMI group) and 35 consecutive patients examined because of exercise-related retrosternal pain or dyspnea (angina pectoris or AP group) at the Central Hospital of North Karelia, Joensuu, were enrolled for the present study. In the AP group the diagnostic workout included subjective maximal exercise testing, 99mTc-emission tomography during exercise and two-dimensional echocardiography. Our previous study revealed 8 patients with FH in these two groups (Koivisto et al. 1993) and these were excluded from the present study. The remaining 82 patients were combined into a single CHD group (78 men and 4 women, mean age 40.8 years). The control subjects of similar age (n = 50, 42 men and 8 women, mean age 38.7 years) were selected from people undergoing routine health examinations at the Joensuu Occupational Health Center. They did not show any signs of cardiovascular disease upon health questionnaire, clinical examination, electrocardiographic exercise testing and two-dimensional echocardiography. Analysis of the birth places of patients and controls showed that in both groups more than 75% of the subjects were born in the North Karelia province. Isolation of the serum lipoprotein fractions by sequential ultracentrifugation followed by assays for cholesterol and triglyceride concentrations by enzymatic techniques were carried out during the previous study (Koivisto et al. 1993).

DNA analysis

The determination of the apo B *Xba*I and apo C-III *Sst*I restriction fragment length polymorphisms (RFLPs) was carried out by the Southern blot technique (Aalto-Setälä et al. 1991). The X2 and S2 alleles denote alleles with the polymorphic sites present, and the X1 and S1 alleles those without the polymorphic sites. The apo A-I promoter *Msp*I polymorphism was assayed by a PCR (polymerase chain reaction) technique described previously (Pagani et al. 1990); the allele with A at the variable site is designated as M1 and that with G as M2. The I/D polymorphism of intron 16 of the ACE gene was determined according to Rigat et al. (1992), with confirmation of the DD genotype by the technique described by Shanmugam et al. (1993).

For determination of the apo E genotypes, a test kit (AB Sangtec Medical, Bromma, Sweden) based on a non-isotopic modification of the mini-sequencing technique of Syvänen et al. (1990) was used. In short, the target DNA fragment is first amplified using PCR primers P1 and P2 corresponding to nucleotide positions 3712–3734 and 3943-3922 of the apo E gene (for nucleotide numbering, see Paik et al. 1985). The P2 primer is biotinylated at its 5′ end (Bengtström et al. 1990) allowing immobilization of the amplified DNA on microtitration plate wells coated with streptavidin. The amplified DNA is rendered single-stranded and the variable nucleotides are identified by primer extension reactions carried out in two separate wells. In these reactions, the incorporation of a dinitrophenyl (DNP)-labeled nucleotide into the DNA is directed by two alternative detection step primers (D112, nucleotides 3724–3744; and D158, nucleotides 3863–3882) hybridizing to the regions immediately upstream of the variable first nucleotide (C or T) of codons 112 or 158. The type of nucleotide incorporated is detected with an anti-DNP-alkaline phosphatase conjugate followed by a colorimetric reaction measurable in a microtiter plate reader.

Statistical methods

Chi-square analysis was used to test allelic variation in the two study groups (CHD patients and controls). The t-test was used for statistical evaluation of the serum lipid data.

Results and discussion

We did not see any significant differences in the allelic frequencies determined by the biallelic apo A-I promoter *Msp*I, apo C-III *Sst*I, apo B *Xba*I or ACE gene I/D polymorphisms between the patients and controls (Table 1). The slight difference in the apo ε4 allelic frequency between the two groups (Table 1) did not reach statistical significance either. If patients with hyperlipidemias (serum cholesterol

Table 1 Genotype distributions (numbers of subjects) and allele frequencies of the apolipoprotein *(apo)* and angiotensin converting enzyme *(ACE)* gene polymorphisms in young North Karelian coronary patients and controls. *(RFLP* restriction fragment length polymorphism; *I/D* insertion/deletion)

Genotype/allele	Patients	Controls
Apo A-I promotor *Msp*I RFLP[a]		
M1M1	4	2
M1M2	22	9
M2M2	55	39
Allele M1	0.185	0.130
Allele M2	0.815	0.870
Apo C-III *Sst*I RFLP		
S1S1	62	37
S1S2	19	12
S2S2	1	1
Allele S1	0.872	0.860
Allele S2	0.128	0.140
Apo B *Xba*I RFLP[a]		
X1X1	29	20
X1X2	41	20
X2X2	11	10
Allele X1	0.611	0.600
Allele X2	0.389	0.400
Apo E common variation[a]		
ε2ε3	5	3
ε2ε4	1	0
ε3ε3	42	33
ε3ε4	30	11
ε4ε4	2	3
Allele ε2	0.038	0.030
Allele ε3	0.744	0.800
Allele ε4	0.219	0.170
ACE I/D polymorphism		
I/I	12	10
D/I	43	22
D/D	27	18
Allele I	0.409	0.420
Allele D	0.591	0.580

[a] Data on one or two patients lacking, for technical reasons or running out of samples

Table 2 Examples of serum lipid levels (mmol/l, mean ± SD) in young North Karelian coronary patients and controls, grouped according to their apo genotypes. Note that some of the rarest genotypes were not taken into account. In the case of apo E, genotypes with or without the apo ε4 allele were compared with each other

Gene/genotype	*n*	Total cholesterol	LDL-cholesterol	HDL-cholesterol	Triglycerides
Patients	All	6.09 ± 1.34	3.74 ± 1.07	1.27 ± 0.40	2.12 ± 1.49
ApoA-I M1M2	22	6.06 ± 1.11	3.81 ± 1.36	1.33 ± 0.36	1.81 ± 1.42
ApoA-I M2M2	55	6.09 ± 1.39	3.69 ± 1.02	1.26 ± 0.43	2.23 ± 0.62
ApoC-III S1S1	62	6.12 ± 1.35	3.82 ± 1.09	1.27 ± 0.42	2.01 ± 1.30
ApoC-III S1S2	19	6.02 ± 1.37	3.45 ± 1.02	1.25 ± 0.37	2.55 ± 1.99
ApoB X1X1	29	6.06 ± 1.33	3.93 ± 1.16	1.30 ± 0.51	1.87 ± 0.87
ApoB X1X2	41	5.96 ± 1.28	3.58 ± 1.36	1.24 ± 0.32	2.23 ± 1.61
ApoB X2X2	11	6.61 ± 1.65	3.78 ± 1.10	1.35 ± 0.39	2.26 ± 0.68
ApoE ε2ε3 + ε3ε3	47	6.11 ± 1.41	3.86 ± 1.12	1.29 ± 0.37	1.97 ± 1.38
ApoE ε3ε4 + ε4ε4	32	6.14 ± 1.28	3.60 ± 1.65	1.24 ± 0.46	2.43 ± 1.65
Controls	All	5.63 ± 1.05	3.40 ± 0.88	1.33 ± 0.35	1.62 ± 1.31
ApoA-I M1M2	9	6.30 ± 1.25	3.83 ± 0.85	1.25 ± 0.27	2.11 ± 1.69
ApoA-I M2M2	39	5.42 ± 0.92	3.29 ± 1.06	1.36 ± 0.36	1.46 ± 1.18
ApoC-III S1S1	37	5.70 ± 1.08	3.41 ± 0.79	1.34 ± 0.37	1.58 ± 1.09
ApoC-III S1S2	12	5.53 ± 0.96	3.43 ± 1.06	1.29 ± 0.31	1.81 ± 1.90
ApoB X1X1	20	5.59 ± 0.95	3.38 ± 0.94	1.38 ± 0.36	1.77 ± 1.81
ApoB X1X2	20	5.48 ± 1.01	3.33 ± 0.86	1.30 ± 0.35	1.48 ± 0.76
ApoB X2X2	10	6.02 ± 1.36	3.59 ± 0.85	1.26 ± 0.32	1.59 ± 1.02
ApoE ε2ε3 + ε3ε3	36	5.56 ± 1.05	3.38 ± 0.81	1.35 ± 0.34	1.40 ± 0.71
ApoE ε3ε4 + ε4ε4	14	5.80 ± 1.09	3.44 ± 1.08	1.26 ± 0.38	2.23 ± 2.20

> 7 mmol/l and/or triglycerides > 2 mmol/l) were omitted from analysis, the frequency of the ACE allele D in the remaining patients ($n = 43$) remained unchanged (0.59) and similar to that in controls.

Not unexpectedly, serum total and LDL cholesterol levels tended to be higher and HDL cholesterol levels lower in patients than controls (Table 2); the difference between serum cholesterol levels in the AP patients (6.39 ± 1.10) and controls (5.63 ± 1.15) was statistically significant ($P < 0.01$). However, no statistically significant genotype-related associations between the gene polymorphisms and serum lipid levels were found in patients or controls, although the trends toward elevated serum cholesterol levels in the apo B X2X2 genotype and elevated serum triglyceride levels in the apo C-III S1S2 genotype were consistent, and reminiscent of our previous data (Aalto-Setälä et al. 1987, 1988).

Our previous study in children and adolescents suggested that the apo C-III S2 allele occurs significantly more often in eastern (11.1%) than in southwestern (6.1%) Finland (Aalto-Setälä et al. 1991). In accord with this, the S2 allele frequency in 179 apparently healthy subjects (mean age 40 years) living in southern Finland was recently found to be 8.1% (H. Miettinen and K. Kontula, unpublished), a figure significantly ($P < 0.05$) lower than that (13.3%) in our whole North Karelian cohort ($n = 132$, Table 1). The clinical significance, if any, of this west-to-east difference in the frequency of the S2 allele remains obscure since serum lipid levels, for decades higher in eastern Finland, today show no significant differences across the country (Salomaa et al. 1990).

We paid special attention to the selection of the patients for our association study in order optimally to unravel genetic influences on the development of premature CHD. First, the population of North Karelia was chosen because this subregion ranks number one in CHD mortality rate in Finland (Pyörälä et al. 1985). Second, in this province patients with FH, a single-gene disease that may mask more subtle genetic influences on CHD, could be virtually excluded because of the fact that two specific mutations, easily detectable by a PCR assay, account for more than 90% of the FH-causing genes (Koivisto et al. 1993). Third, a family study on North Karelians (Rissanen 1979) had demonstrated that the younger the patient was at the diagnosis of myocardial infarction, the greater was the genetic component in his disease. Therefore, we restricted our study to very young CHD patients.

Even with these restrictions in the selection of our study population, we were unable to find any associations between CHD risk and a number of polymorphisms that in earlier studies were related to variations of serum lipid levels and/or risk of CHD in Finns or other populations. The I/D polymorphism of the ACE gene was likewise uninformative in this respect. We cannot offer any firm explanation for the fact that we did not see any relation between the apo ε4 allele and elevated serum cholesterol levels, an association that has been documented in earlier cross-sectional studies in Finland (Ehnholm et al. 1986;

Lehtimäki et al. 1990). Therefore, the lack of an association between the apo E polymorphism and the presence of CHD in our study may not be an unexpected finding, even though genetic variation of apo E may also regulate risk of CHD by mechanisms other than those affecting serum lipid levels. The major limitation of the present study was the relatively low number of subjects examined. Even considering this, our data suggest that at the age of 40 years genes regulating vascular tone, blood coagulation and fibrinolysis may be more important than those promoting atherosclerotic changes as determinants of risk of symptomatic CHD.

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References

Aalto-Setälä K, Kontula K, Sane T, Nieminen M, Nikkilä E (1987) DNA polymorphisms of apolipoprotein A-I/C-III and insulin genes in familial hypertriglyceridemia and coronary heart disease. Atherosclerosis 66:145–152

Aalto-Setälä K, Tikkanen MJ, Taskinen M-R, Nieminen M, Holmberg P, Kontula K (1988) XbaI and c/g polymorphisms of the apolipoprotein B gene locus are associated with serum cholesterol and LDL-cholesterol levels in Finland. Atherosclerosis 74: 47–54

Aalto-Setälä K, Viikari J, Åkerblom H, Kuusela V, Kontula K (1991) DNA polymorphisms of the apolipoprotein B and A-I/C-III genes are associated with variations of serum low density lipoprotein cholesterol level in childhood. J Lipid Res 32:1477–1487

Bengtström M, Jungell-Nortamo A, Syvänen A-C (1990) Biotinylation of oligonucleotides using a water soluble biotin ester. Nucleosides Nucleotides 9:123–127

Cambien F, Poirier O, Lecerf L, Evans A, Cambou J-P, Arveiler D, Luc G, Bard J-M, Bara L, Ricard S, Tiret L, Amouyel P, Alhenc-Gelas F, Soubrier F (1992) Deletion polymorphism in the gene for angiotensin-converting enzyme is a potent risk factor for myocardial infarction. Nature 359:641–644

Davignon J, Gregg RE, Sing CF (1988) Apolipoprotein E polymorphism and atherosclerosis. Arteriosclerosis 8:1–21

Ehnholm C, Lukka M, Kuusi T, Nikkilä E, Uterman G (1986) Apolipoprotein E polymorphism in the Finnish population: gene frequencies and relation to lipoprotein concentrations. J Lipid Res 27:227–235

Goldstein JL, Brown MS (1984) Genetics of cardiovascular disease. In: Braunwald E (ed) Heart disease, 2nd edn. W. B. Saunders, Philadelphia, pp 1606–1640

Humphries SE (1988) DNA polymorphisms of the apolipoprotein genes – their use in the investigation of the genetic component of hyperlipidaemia and atherosclerosis. Atherosclerosis 72: 89–108

Jeenah M, Kessling A, Miller N, Humphries S (1990) G to A substitution in the promoter region of the apolipoprotein AI gene is associated with elevated serum apolipoprotein AI and high density lipoprotein cholesterol concentrations. Mol Biol Med 7: 233–241

Koivisto U-M, Hämäläinen L, Taskinen M-R, Kettunen K, Kontula K (1993) Prevalence of familial hypercholesterolemia among young North Karelian patients with coronary heart disease: a study based on diagnosis by polymerase chain reaction. J Lipid Res 34:269–278

Kuusi T, Nieminen MS, Ehnholm C, Yki-Järvinen H, Valle M, Nikkilä EA, Taskinen M-R (1989) Apoprotein E polymorphism and coronary artery disease: increased prevalence of apolipoprotein E-4 in angiographically verified coronary patients. Arteriosclerosis 9:237–241

Lehtimäki T, Moilanen T, Viikari J, Åkerblom HK, Ehnholm C, Rönnemaa T, Marniemi J, Dahlen G, Nikkari T (1990) Apolipoprotein E phenotypes in Finnish youths: a cross-sectional and 6-year follow-up study. J Lipid Res 31:487–495

Lusis AJ (1988) Genetic factors affecting blood lipoproteins: the candidate gene approach. J Lipid Res 29:397–429

Nieminen MS, Mattila KJ, Aalto-Setälä K, Kuusi T, Kontula K, Kauppinen-Mäkelin R, Ehnholm C, Jauhiainen M, Valle M, Taskinen M-R (1992) Lipoproteins and their genetic variation in subjects with and without angiographically verified coronary artery disease. Arteriosclerosis 12:58–69

Pagani F, Sidoli A, Giudici GA, Barenghi L, Vergani C, Baralle FE (1990) Human apolipoprotein A-I gene promoter polymorphism: association with hyperalphalipoproteinemia. J Lipid Res 31:1371–1377

Paik Y-K, Chang DJ, Reardon CA, Davies GE, Mahley RW, Taylor JM (1985) Nucleotide sequence and structure of the human apolipoprotein E gene. Proc Natl Acad Sci USA 82:3445–3449

Pyörälä K, Epstein FH, Kornitzer M (1985) Changing trends in coronary heart disease mortality; possible explanations. Cardiology 72:5-10

Rigat B, Hubert C, Corvol P, Soubrier F (1992) PCR detection of the insertion/deletion polymorphism of the human angiotensin converting enzyme gene (DCP1) (dipeptidyl carboxypeptidase). Nucleic Acids Res 20:1433

Rissanen A (1979) Familial occurrence of coronary heart disease: effect of age at diagnosis. Am J Cardiol 44:60–66

Salomaa V, Korhonen HJ, Tuomilehto J, Vartiainen E, Pietinen P, Kartovaara L, Gref C-G, Nissinen A, Puska P (1990) Serum cholesterol distribution, measurement frequency and cholesterol awareness in three geographical areas of Finland. Eur Heart J 11:294–301

Shanmugam V, Sell KW, Saha BK (1993) Mistyping ACE heterozygotes. PCR Methods Appl 3:120–121

Sing CF, Moll PP (1990) Genetics of atherosclerosis. Annu Rev Genet 24:171–187

Syvänen A-C, Aalto-Setälä K, Harju L, Kontula K, Söderlund H (1990) A primer-guided nucleotide incorporation assay in the genotyping of apolipoprotein E. Genomics 8:684–692

Tiret L, Kee F, Poirier O, Nicaud V, Lecerf L, Evans A, Cambou J-P, Arveiler D, Luc G, Amouyel P, Cambien F (1993) Deletion polymorphism in the angiotensin-converting enzyme gene associated with parental history of myocardial infarction. Lancet 341:991–992

Utermann G (1987) Apolipoprotein E polymorphism in health and disease. Am Heart J 113:433–440