Effect of common methylenetetrahydrofolate reductase gene mutation on coronary artery disease in familial hypercholesterolemia

Effect of Common Methylenetetrahydrofolate Reductase Gene Mutation on Coronary Artery Disease in Familial Hypercholesterolemia

Running Head: MTHFR mutation accelerates CAD in FH

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ABSTRACT

Familial hypercholesterolemia (FH) is an autosomal dominant disorder characterized by primary hypercholesterolemia and premature coronary artery disease (CAD). However, the development of CAD in FH shows considerable inter-individual variations. An elevated level of plasma homocysteine (tHcy) has been recognized as an independent risk factor for CAD, and a MTHFR gene mutation, valine (V) was substituted for alanine (A), has been reported to be associated with elevated levels of tHcy in mutant homozygotes, i.e. VV. We studied 199 consecutive male heterozygous FH patients, 99 with CAD and 100 without CAD. In CAD group, genotype VV and V allele were significantly more frequent than in non-CAD group, 15% vs 7% in genotype ($p=0.035$) and 0.41 vs 0.30 in allele ($p=0.017$). The mean ages of onset in the CAD group were 50, 51, and 43 years, for genotypes AA, AV, and VV, respectively ($p<0.05$); the age of onset of CAD in genotype VV was significantly lower than in other two genotypes. Kaplan-Meier survivor curves indicated that the development of CAD was significantly accelerated by MTHFR mutation probably in a gene-dose dependent manner. Furthermore, only MTHFR genotype VV was shown to be an independent predictor of the early onset of CAD in the stepwise multiple regression analysis. The mean plasma tHcy level of genotype VV was significantly higher than those of other two genotypes. Thus, the MTHFR mutation appears to accelerate the onset of CAD through elevation of plasma tHcy levels in male heterozygous FH patients.

Key Words familial hypercholesterolemia, 5,10-methylenetetrahydrofolate

reductase, coronary artery disease

INTRODUCTION

During the last decade, mildly increased plasma homocysteine (tHcy) has been recognized as an independent risk factor for atherosclerotic disease including CAD (1). Recently, a common C to T mutation at nucleotide 677 of the 5,10-methylenetetra-hydrofolate reductase (MTHFR) gene, that converts alanine (A) to valine (V) and correlates with increased thermolability of MTHFR resulting in significantly higher tHcy levels in homozygotes, has been identified (2). Since then, > 20 case-control studies to investigate the relationship between the MTHFR genotype and CAD have been performed, and only 3 original reports (3-5), and 1 meta-analysis (6) reported the MTHFR genotype VV as a significant coronary risk factor. However, all other reports including large meta-analysis (7-11) failed to find a significant association, and more studies have been required to draw any conclusion. In the present study, we studied heterozygous Japanese FH males to investigate the effects of MTHFR gene mutation, as well as those of traditional coronary risk factors, on the development of their CAD. It is a unique approach to investigate the role of coronary risk factors in the group which have a major risk factor, FH, in common.

METHODS

Patient Selection: We enrolled 199 consecutive Japanese male patients with heterozygous FH who attended our hospital and were older than 26 years, the ages at when the youngest male FH heterozygotes showing CAD in our experiences. FH was diagnosed when either of two sets of criteria were met : 1) primary hypercholesterolemia (> 230 mg/dL in any age group) in a patient with tendon

xanthomas, or 2) primary hypercholesterolemia in any first-degree relative of a familial hypercholesterolemia patient (12,13). All clinical and laboratory data were obtained before the introduction of lipid-lowering therapy, except that plasma tHcy and serum lipoprotein(a) levels were determined during lipid-lowering therapy in 31 (9 received cholestyramine) and 82 subjects, respectively. Angiographic results were only used when obtained within 3 months of the introduction of lipid-lowering drug therapy. There was no patient receiving vitamin replacement including dietary folate supplement.

The patients were divided into two groups according to whether they showed symptomatic CAD (n=99) or did not (n=100). All enrolled subjects underwent a treadmill and / or a Master's double two-step exercise test at least twice a year. Symptomatic CAD was defined as the patient having had a myocardial infarction or a coronary artery bypass graft, or having symptomatic angina pectoris. Among the patients classified into the symptomatic CAD group, 8 patients diagnosed as having variant angina by coronary angiography were excluded because they did not full fill the entry criteria. All patients provided informed consent for participation in this study.

Assessment of Coronary Artery Disease: Coronary angiography was performed by standard techniques with multiple projections. Coronary angiograms were interpreted by at least two cardiologists without knowledges of the patient's clinical and laboratory findings. Stenosis ≥75% in diameter was considered significant. The extent of stenotic changes was assessed by the number of stenotic vessels (right coronary artery, left anterior descending artery, or left circumflex artery), ranged from 1 to 3. The severity of stenotic changes was assessed by a score assigned to each of 15 segments,

according to the classification of the American Heart Association Grading Committee. A normal coronary angiogram was graded 0; stenosis < 25% was graded 1; 25% to 50% stenosis was graded 2; 50% to 75% stenosis was graded 3; and ≥ 75% stenosis was graded 4. The coronary stenosis index was defined as the sum of these scores, with a maximal value of 60 (13). A coronary angiogram was performed in 91 of 99 patients with CAD.

Assessment of Conventional Risk Factors: Hypertension was considered to be present if antihypertensive treatment had been instituted or the blood pressure was > 160 mm Hg systolic or 95 mm Hg diastolic. The oral glucose tolerance test was assessed in all entry subjects after ingestion of 75 g glucose. Varying degrees of glucose intolerance were assessed by the criteria of the Japan Diabetes Society (14). The body mass index was calculated by wt (kg) / ht $(m)^2$. Subjects who smoked at least 10 cigarettes a day were classified as current smokers.

Lipoprotein and tHcy Analysis: All data concerning plasma lipoprotein and tHcy were measured after overnight fasting. Serum cholesterol, triglyceride, and high density lipoprotein cholesterol levels were determined by standard enzymatic methods (13). Low density lipoprotein cholesterol levels were calculated by use of the Friedewald formula. In 82 of the enrolled FH patients (46 CAD and 36 non-CAD patients), we could measure plasma tHcy levels as total plasma homocysteine combined with protein-bound and free fractions by high performance liquid chromatography.

MTHFR Genotyping: Genomic DNA was purified from peripheral white blood cells, and its in vitro amplifications were performed by polymerase chain reaction.

Analysis for the C-to-T mutation at nucleotide 677 in the MTHFR gene was carried out by the use of primers framing the mutation site (2). An initial denaturation step was carried out for 5 minutes at 94 degree, followed by 30 cycles of denaturation for 1 minute at 94 degree, annealing for 1 minute at 61 degree, and extension for 30 seconds at 72 degree. A final extension step was performed for 5 minutes at 72 degree. The two different alleles were designated A (alanine) and V (valine). The 198-bp fragment derived from the A allele is not digested by *Hinf I*, whereas the fragment of the same length from the V allele is digested by *Hinf I* into 175- and 23-bp fragments. The *Hinf I*-treated polymerase chain reaction fragments were electrophoresed in 3% agarose gel and stained with ethidium bromide .

Apolipoprotein E Genotyping: Analysis for the apolipoprotein E genotyping (ε2, ε3, ε4) was carried out by the use of an oligonucleotide described elsewhere, and a polymerase chain reaction -based method followed by *Hha I* digestion (15).

Statistical Analysis: All values are expressed as mean ± standard deviation unless otherwise stated. The frequency of occurrence of patients with hypertension, glucose intolerance and current smokers among different groups were compared by the chi-squared test. The levels of lipids, lipoprotein (a), and tHcy, numbers of diseased vessels, and coronary stenosis index were compared by parametric method (ANOVA or unpaired t-test) when the variable showed normal distribution, or by non-parametric method (Kruskal-Wallis or Mann-Whitney U test) when it did not. For the stepwise multiple regression analysis of the age of onset of CAD, the nominal variable (MTHFR genotype, apoE genotype, hypertension, cigarette smoking, glucose intolerance) was included as well as numerical variables {body mass index, serum levels of cholesterol,

triglyceride, high density lipoprotein cholesterol, lipoprotein (a)}. Kaplan-Meier curves free from CAD were compared by logrank test. All these analyses were performed using a computer system, Stat View Version 4.2 (Abacus Concepts, Berkeley, CA). A p value <0.05 was accepted as significant.

RESULTS

Characteristics of Study Patients: The clinical characteristics of FH patients with and without CAD are shown in Table I. There was no significant difference in the mean age of the patients between two groups. Also both groups showed similar lipid profiles except for high density lipoprotein cholesterol levels. Plasma tHcy levels of CAD group were significantly higher than those of non-CAD group.

Frequencies of MTHFR Genotype and Mutant Allele: The frequencies of MTHFR genotypes AA, AV, and VV are shown in Figure 1. Genotype distribution was significantly different between patients with and without CAD ($p<0.05$), and both genotype VV and V allele in CAD group were significantly more frequent than in non-CAD group ($p<0.05$).

Characteristics of CAD Patients According to MTHFR Genotype: The characteristics of FH patients with CAD by MTHFR genotype are shown in Table II. Significant differences among 3 genotypes were found in serum low density lipoprotein cholesterol levels. Genotypes AA and AV showed the highest and the lowest values, respectively. However, differences only between genotypes AA and AV reached statistical significance ($p<0.05$, Fisher's protected least significant difference method). Frequencies of non-lipid risk factors and the distribution of the

apo E allele were not different among MTHFR genotypes. In case of coronary angiography, both the number of diseased vessels and the coronary stenosis index were all similar values.

Development of CAD According to MTHFR Genotype: The ages at onset of CAD are shown in Figure 2. The mean age at which CAD manifested in patients with genotypes AA, AV and VV were 50, 51, and 43 years, respectively (p=0.028). The age of onset for genotype VV was significantly earlier than that for genotypes AA (p=0.018) and AV (p=0.010). Moreover, all 3 youngest CAD patients (26, 28, and 31 years) were classified as genotype VV. The 2 oldest-onset CAD patients (73 and 80 years) were genotype AA. The ages of FH patients without CAD are also shown in Figure 2. The oldest patients without CAD in genotypes AA, AV, and VV were 80, 68, and 48 years, respectively. Based on these data, we calculated Kaplan-Meier survivor curves. As shown in Figure 3, genotype VV appears to most strongly accelerate CAD resulting in its onset from their third decade of life. Although genotypes AV and AA showed similar curves until 50 years, genotype AV showed significantly rapid development after 60 years as compared to genotype AA (p=0.022). These results suggest the gene dose-dependent influence of MTHFR genotype.

To compare the effects of MTHFR genotype with those of other coronary risk factors, multiple regression analysis was performed using 10 variables {serum cholesterol, log transformed triglyceride, high density lipoprotein cholesterol, log transformed lipoprotein (a), log transformed body mass index, hypertension, current smoking, glucose intolerance, apo E genotype, and MTHFR genotype}. In this analysis, only the MTHFR genotype VV was a significant predictor of the onset age of

CAD (Table III).

Plasma tHcy Levels and MTHFR Genotype: Plasma tHcy levels were measured in 82 of 199 study patients (Table IV). In patients with genotype VV, plasma levels of tHcy were significantly higher than those with genotypes AA and AV.

DISCUSSIONS

The key finding of the present study are that common MTHFR gene mutation was significantly associated with CAD in male heterozygous FH, and that their CAD onset was significantly influenced by this mutation, possibly gene-dose dependently.

MTHFR Genotype and the Development of CAD: Among heterozygous FH patients, the onset of CAD usually occurs the third and fifth decades in men and women, respectively, and it develops progressively with age in both genders (13). However, there is a great amount of variation in their clinical expression of CAD (13,16-18). Previous study results have suggested that the type of underlying low density lipoprotein receptor gene mutations, gender, patient age, high density lipoprotein cholesterol levels, lipoprotein (a) levels, isoform of apolipoprotein E, smoking habits, and diabetes can significantly influence the development of CAD in heterozygous FH (16-22). In addition to them, our results suggest that the MTHFR genotype is a novel genetic risk factor.

Mildly elevated plasma tHcy levels associate with an increased risk for CAD (1, 23-25). Recently, common mutation in the MTHFR gene was identified, and was shown to be linked to mild hyperhomocysteinemia, probably through increased thermolability of this enzyme (2). Within the past 4 years, > 20 case-control studies

to investigate the relationship between MTHFR genotype and CAD have been reported (3-11). However, most of such reports failed to show a significant association (7-11), and further studies was required, especially in some specific subgroups. In the present study, we observed significant differences in both genotype and allele frequencies between FH patients with CAD versus those without CAD (Figure 1). Combining the results of Kaplan-Meier curves (Figure 3) and multiple regression analysis (Table 4), the mutant allele of MTHFR gene, designated V, significantly accelerates the onset of CAD probably in a gene-dose dependent manner. These observations suggest that the MTHFR gene mutation may play a greater role as a coronary risk factor when low density lipoprotein cholesterol levels are much higher than average level as observed in heterozygous FH. Only 1 previous report has evaluated the relationship between plasma tHcy levels, MTHFR genotypes, and clinical manifestations in FH (26). In that study, the authors concluded that plasma tHcy levels were increased in children whose parents had FH and cardiovascular disease as compared to those without parental cardiovascular disease, even though a marginal difference was noted in the frequency of MTHFR genotype VV between these 2 groups (18% vs 8%, p=0.07). In case of cardiovascular events, there was no previous studies which examined the effects of the MTHFR genotype. Thus, our study could demonstrate, for the first time, the significant role of the MTHFR genotype on the development of CAD in FH.

Unexpectedly, we found the significantly rapid CAD development in genotype AV only over 50, as compared to genotype AA (Figure 3). None of previous studies reported such potential significance of a heterozygote, AV. Although the reason for

this late-manifested difference remains unclear, possible explanation likely exists in the differential effects of the age-dependent alterations in tHcy metabolism, such as impaired renal function, on each MTHFR genotype.

MTHFR Genotype and Severity of CAD: Morita et al. (4) reported that the severity of angiographically defined coronary atherosclerosis might correlate with the MTHFR genotype. In the present study, both the number of vessels with significant stenosis and the coronary stenosis index were similar among the 3 genotypes, even though the mean age of onset of CAD in genotype VV was approximately 10 years younger than in the other genotypes. These observations suggest that MTHFR genotype play important role in the progression of CAD, as well as in its onset.

Distribution of Apo E Genotypes: Previous studies reported the apoE4 (28), or the apoE2 (29) allele as an independent risk factor for CAD in FH. Our results, however, support negative findings regarding the influence of apoE polymorphism as reported in other studies (17).

Study Limitations; We did not quantify the factors, like B vitamins, folate, and renal function, which might influence tHcy level. In order to determine the effects of these factors on the development of CAD in FH, further study is required.

Clinical Implications: The present study shows that determination of the MTHFR genotype provides significant information regarding the risk for CAD in male heterozygous FH patients. This genetic risk is inherited independently with the low density lipoprotein receptor gene. Thus, the variations in the development of CAD among FH patients, even when they are sharing the same low density lipoprotein receptor gene mutation, might be explained, at least partly, by the MTHFR genotype.

Recently, folate supplementation produced the MTHFR genotype-dependent reduction of plasma tHcy level (30). This could raise the possibilities that routine determination of tHcy levels and MTHFR genotype should be considered in the management of male heterozygous FH, and that tHcy-lowering dietary or drug therapy might have a greater role in primary or secondary prevention of CAD when introduced to patients with genotype VV.

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FIGURE LEGENDS

Figure 1. Distribution of MTHFR genotypes in patients with and without CAD

The distribution of MTHFR genotypes of CAD group was significantly different from that of non-CAD group (χ^2=6.06, df=2, p=0.046). Frequencies of VV genotype and V allele in CAD vs non-CAD groups were 15% vs 7% (p=0.035) and 0.41 vs 0.30 (p=0.017), respectively. The genotype distributions of CAD and non-CAD groups were both in Hardy-Weinberg equilibrium. CAD; coronary artery disease, MTHFR; 5,10-methylenetetrahydrofolate reductase

Figure 2. Ages of FH patients with and without CAD

The onset age of CAD (left panel) and the age at when the patients were confirmed as without CAD (right panel) were shown by box-plot. The boxes indicate the lower and upper quartiles; the center lines represent the median. The bars below and above the boxes indicate the 10% and 90% values, respectively. Each open circle indicates the value lower or higher than 10% or 90% values, respectively. The mean onset age of CAD for MTHFR genotype AA, AV, and VV were 50, 51, and 43 years, respectively (p=0.028). The onset age of genotype VV was significantly earlier than that of AA (Fisher's protected least significant difference, p=0.018) and AV (p=0.010). In non-CAD group (right panel), the mean ages for genotype AA, AV, and VV were 49, 47, and 41 years, respectively (p=0.27). CAD; coronary artery disease, FH; familial hypercholesterolemia

Figure 3. Kaplan-Meier survival curves showing probabilities of free from CAD.

Onset ages in CAD group were considered as the time of events. Ages of patients in non-CAD group, which indicated potential life time free from CAD, were considered as the time of the censored data. By logrank test, significant difference in survival curves was observed among these 3 genotype ($p<0.0001$), and also was observed between all of 3 pairs of genotypes ($p=0.022$ in AA vs AV, $p=0.0002$ in AV vs VV, and $p<0.0001$ in AA vs VV). CAD; coronary artery disease