Identification of risk factor for coronary artery disease using SNP association study

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연구실에서 함께 연구를 하며 지내온 붉은 입술 이혜자 선생님, 차분한 카리스마를 지닌 박미현 선생님, 부드럽고 순수한 성격의 김광중 선생님, 다정한 하고 친절한 한혜리 선생님, 항상 과묵하지만 착한 복정 선생님, 친동생 같은 예쁜 막내 오지희 선생님, 부지런하고 착한 반효정 선생님, 묵묵히 팀의 힘든 일을 많이 해주시는 김동준 선생님, 조용하고 침착한 김영진 선생님, 따뜻하고 밝은 홍은정 선생님, 신혼생활 중인 황주연, 고민지 선생님과 행정업무에 바쁘신 피성일 선생님에게도 감사의 뜻을 전합니다.

석사시절 많은 가르침을 주시고 연구의 길을 알려주신 오종원 교수님, 강수민 누나, 임호수 형, 장준석 형, 정호영 형, 최한별, 최한섭, 김훈, 이남경, 김연구, 김정희, 안대균, 김승진, 유지승 그리고 심성보 함께 했던 생활도 잊을 수 없으며 모두에게 감사 드립니다.

학위 과정 동안에 많은 기도와 격려를 해주신 사랑하는 부모님 그리고 누나,

매형께 진심으로 감사의 마음을 전하며, 항상 힘들때면 언제나 어깨를 빌려주는 친형 같은 성준 형, 언제나 말없이 믿고 힘이 되어준 혜림이, 항상 나를 친형처럼 따르는 태윤이, 항상 듬직하고 착한 원식이, 친구라면 뭐든 앞장서는 의리있는 준영이, 목사님이 되어 남을 위해 기도하는 창룡이, 힘들지만 말없이 열심히 살고 있는 작은 원식이에게도 진심으로 감사 드립니다.

늦게나마 본 학문의 연구가 좋아서 시작한 대학원 생활을 이제 박사 학위를 마지막으로 마무리를 지으려고 합니다. 힘든 시기도 많았고, 좋은 연구 결과에 기쁨을 가졌던 학위 과정 동안의 생활을 결코 잊을 수 없을 것 같습니다. 앞으로 연구하며 나아가야 할 길이 쉽지 않겠지만, 지금까지 관심을 가지고 격려와 조언을 아끼지 않으셨던 많은 분들의 사랑에 보답하도록 노력하겠습니다. 지속적인 조언과 기도를 부탁 드리며 끝까지 많은 지도 부탁 드립니다.

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차 승 헌 드림

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ABSTRACT

Identification of risk factor for coronary artery disease using SNP association study

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Genetic studies are expanding past the identification of rare ‘Mendelian’ diseases to encompass common complex disorders such as Hypertension, Diabetes, Cardiovascular disease (CVD), and Coronary Artery Disease (CAD). The recent completion of the International HapMap Project, many tools are in hand for genetic association studies seeking to test the common disease common variant hypothesis. Genetic association studies, in which the allele or genotype frequencies at markers are determined in affected individuals and compared with those of controls.

For this processing, two approaches were considered: in the first one, we identify the genetic variations of 31 CAD candidate genes selected by colocalization of positional and functional candidate genes. However, with over several hundreds single nucleotide polymorphisms (SNPs) identified by re-sequenced all exons and promoter regions of those genes using 24 unrelated Korean individual’s DNA samples.; in the second approach, we used for CCR2 gene and nonsynonymous SNPs (nsSNPs) selection in candidate genes for association test (i.e., the change in genetic code produces a change in function of the gene).

We identified a total of 409 SNPs including 40 nonsynonymous SNPs, 32 insetion/deletion (INS/DEL) and 4 microsatellites. Among 31 candidate genes, CCR2 gene were examined for the association with CAD male patients. A total of 13 genetic variants, including 1 deletion and 12 SNPs, were identified in the Korean population. Although we could not detect any association of CCR2 polymorphic markers with CAD, several SNP markers of CCR2 gene showed highly significant signals with the number of arteries with significant coronary artery stenosis in the CAD male patients. The most significant signal was detected at the SNP located at exon 2 (Asn260Asn; +780T/C) (odds ratio: 1.49, p=0.0005).

Among 40 nonsynonymous SNPs, 29 SNPs were examined for an association with CAD male patients. A significant association with CAD was observed in a polymorphism of the ADD1 gene (Gly460Trp; +29017G/T) (odds ratio: 0.71–0.81, P=0.01–0.04). The same polymorphism was also associated with the number of arteries with significant coronary artery stenosis in the CAD patients (P=0.01) as well as the increase in systolic blood pressure (P=0.02).

Furthermore, G allele of ADD1 gene (Gly460Trp; +29017G/T) was significantly associated with less MetS (odds ratio: 0.39–0.58, p=0.02), MetS with CAD (odds ratio: 0.68, p=0.03), and CAD only (odds ratio: 0.74–0.81, p=0.03). We found ADD1 Gly460Trp was associated with MetS severity (p for trend=0.02 when compared by using Mantel–Haenszel analysis), and that MetS is an independent risk factor for CAD (p for trend=0.001). The ADD1 Gly460Trp polymorphism was also associated with abnormal blood pressure (p=0.04) and blood glucose levels (p=0.03).

Collectively, these data suggest that ADD1 Gly460Trp polymorphism was significantly associated with an increased risk of CAD as well as MetS in the Korean

population. We also confirmed that the ADD1 Gly460Trp polymorphism is associated with abnormal blood pressure (p=0.02–0.04) and blood glucose levels (p=0.03).Moreover, our data showed that ADD1 and CCR2 can play a role in the pathogenesis of CAD, especially to the number of vessels in CAD.

Key words: Single nucleotide polymorphisms (SNPs), α-adducin 1 (ADD1), CC-Chemokine receptor-2 (CCR2), Coronary Artery Disease, Metabolic Syndrome

I. LITERATURE REVIEW

1. Genome-wide association studies

1.1. Overview

The international HapMap Consortium recently completed genotyping over 3.8 milion single nucleotide polymorphisms (SNPs) in three populations, and the results of studing patterns of linkage disequilibrium (LD) indicate that characterization of 300,000-500,000 tag SNPs is sufficient to provide good genomic coverage for linkage-disequilibrium based association studies in many populations (1). This would open up the possibility to systematically identify all possible gene variants in different human populations, associate their presence with individual phenotypes, including disease susceptibility, and determine the functional impact of such variation (1, 2).

The most abundant source of genetic variation in the human genome comprises SNPs. A single nucleotide polymorphisms (SNPs) are DNA sequence variations where one of four nucleotides -A, T, G or C- is substituted for another (for example, C for A). A genome-wide association study is a powerful approach that involves rapidly scanning markers across the complete sets of DNA, or genomes, of many people to find genetic variations associated with a particular disease. In addition, rapid advances in understanding the patterns of human genetic variation and maturing high-throughput, cost-effective methods for genotyping are providing powerful research tools for identifying genetic variants that contribute to health and disease (2, 3). These advances have set the stage for genome-wide association studies, in which a dense set of SNPs across the genome is genotyped to survey the most common genetic variation for a role

in disease or to identify the heritable quantitative traits that are risk factor for disease (4, 5). Such studies are particularly useful in finding genetic variations that contribute to common, complex diseases, such as diabetes, hypertension, heart disease and mental illnesses (6).

1.2. Linkage versus association studies

Genome-wide linkage analysis is the method traditionally used to identify disease genes, quantitative traits, and has been tremendously successful for mapping genes that underlie monogenic 'Mendelian' diseases (7). In some cases, genomic regions that show significant linkage to the disease have been identified, leading to the discovery of variants that contribute to susceptibility to diseases such as inflammatory bowel disease (IBD) (8-11), schizophrenia (12) and type 1 diabetes (13).

Linkage studies search for regions of the genome with a higher-than-expected number of shared alleles among affected individuals within a family. Closely related individuals tend to share large regions of the genome inherited from the same recent ancestor. However, for most common diseases, linkage analysis has achieved only limited success (14), and the genes discovered usually explain only a small fraction of the overall heritability of the disease. Most common diseases and clinically important quantitative traits have complex architectures, for which the phenotype is determined by the sum total of, and/or interactions between, multiple genetic and environmental factors (15). Therefore, any individual genetic variant will generally have a relatively small effect on disease risk. The typical frequencies of variants that underlie common disease are largely unknown, but common variants have been proposed to influence disease susceptibility (16-18).

Association studies using common allelic variants have been proposed as a powerful method for identifying common variants that underlie disease susceptibility (15). Association studies compare the frequency of alleles or genotypes of a particular variant between patients and controls. Candidate gene association studies have identified many of the genes that are known to contribute to susceptibility to common disease or selection of genes with a known or inferred biological function whose role makes it plausible that they may predispose to disease or the observed phenotype (19-22). Candidate gene studies based on having predicted the identity of the correct gene or genes, usually on the basis of biological hypotheses or the location of the candidate within a previously determined region of linkage. When the fundamental physiological defects of a disease are unknown, the candidate-gene approach will clearly be inadequate to fully explain the genetic basis of the disease.

Importantly, linkage analysis is more powerful than association analysis for identifying rare high-risk disease alleles, but association analysis is expected to be more powerful for the detection of common disease alleles that confer modest disease risks.

1.3. Direct versus indirect association

Linkage-based positional cloning and linkage disequilibrium-based whole genome association studies are statistical methods that make no prior assumptions about the identity of the genes to be found. This offers obvious advantages, but also poses serious obstacles to their application in unraveling of genotype–phenotype interactions involved in human complex disease (25). In contrast, candidate gene approaches are based on the study of genes selected through knowledge of the disease phenotype,

genetic studies in model organisms, or location (usually by a linkage study) (20).

Genes rarely act alone and interactions between different gene variants in a pathway or across different pathways need to be elucidated (candidate pathway approach). Studies of direct association target polymorphisms which are themselves putative causal variants. This type of study is the easiest to analysis and the most powerful, but limited by complete knowledge about functional variation and identification of candidate polymorphisms (23). A mutation in a codon which leads to an amino acid change is a candidate causal variant. However, it is likely that many causal variants responsible for heritability of common complex disorders will be non-coding. Thus, direct association studies only have the potential to discover some of the genetic causes of disease and disease-related traits (24).

Most current association mapping is indirect, with reliance on LD between a disease susceptibility allele and either a single marker allele or a multilocus haplotype. The systematization of the indirect approach is the aim of the HapMap Project, through a genome-wide study of haplotype-block structure in several populations. Indirect association studies are more difficult to analysis, and there is still debate as to the best methods. They are also less powerful than direct studies. The advantage of indirect association analysis is that it does not require prior determination of which SNP might be functionally important, but the disadvantage is that a much larger number of SNPs needs to be genotyped (25, 26). Both direct and indirect association testing currently can be applied effectively to candidate genes that have been implicated in disease pathogenesis by other means, as long as common variants have been comprehensively identified in the candidate gene, and the two approaches are not mutually exclusive (fig. 1).

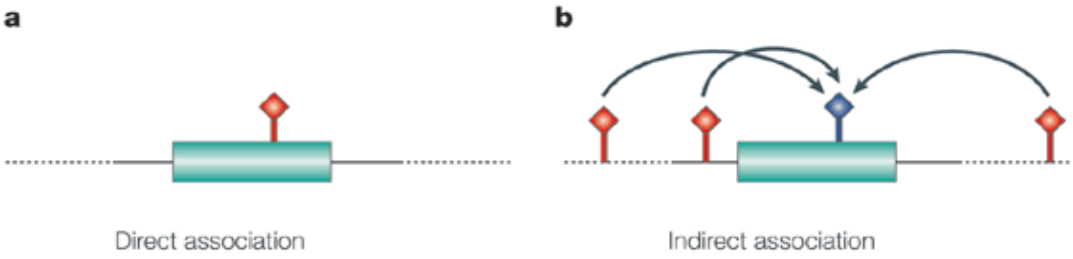


Fig. 1. Direct versus indirect association analysis. a, In direct association analysis, A case in which a candidate SNP (red) is directly tested for association with a disease phenotype. For example, this is the strategy used when SNPs are chosen for analysis on the basis of prior knowledge about their possible function, such as missense SNPs that are likely to affect the function of a candidate gene (green rectangle). **b,** The SNPs to be genotyped (red) are chosen on the basis of linkage disequilibrium (LD) patterns to provide information about as many other SNPs as possible. In this case, the SNP shown in blue is tested for association indirectly, as it is in LD with the other three SNPs. A combination of both strategies is also possible (2).

1.4. Whole genome-wide association

Advances in the field of genetics are highly dependent on enabling technologies to perform accurate high-resolution genomic analysis. Whole genome genotyping (WGG) technologies have recently emerged as attractive tools to genotype hundreds of thousands of SNP markers on a genome-wide scale (5). These SNP markers can be used in linkage disequilibrium-based (LD) association studies to find genomic regions harboring variants associated with increased disease incidence. LD is the nonrandom association between two or more alleles such that certain combinations of alleles are more likely to occur together on a chromosome than other combinations of alleles.

The WGG approach is sensitive in detecting multiple small gene effects often found in complex diseases and does not rely on prior identification of candidate genes or regions. Currently, a fixed set of genome-wide SNP markers appears attractive. SNP content for genome-wide association studies can be categorized based on random selection (27), tag SNPs, and functional (focused sets of genes or non-synonymous SNPs) marker sets. The International HapMap project has provided the haplotype block structure of the human genome, and enabled selection of tag SNPs for several populations (28). The haplotype map and corresponding tag SNPs provide a framework for discovering associations between genes and disease, and may enable SNP characterization to play a role in personalized medicine. These studies will result in a new generation of diagnostic markers and tools, enabling early disease or disease susceptibility detection.

2. Statistical analysis of marker associations

2.1 Overview

Association analysis of genetic polymorphisms has been most often performed in a case-control setting, with unrelated case subjects compared to unrelated control subjects. Significant differences in allele frequencies between cases and controls are taken as evidence for involvement of an allele in disease susceptibility. Case-control analysis is thus a well-validated technique for the discovery of alleles associated with human disease susceptibility (29). One of the limitations of linkage analysis is the difficulty of fine mapping the location of a gene influencing a complex disorder. There are not usually enough meioses within 1-2 megabases of the disease gene to detect recombination events; moreover, with the effects of phenotypic and genetic heterogeneity in complex diseases, critical recombination events cannot be identified with certainty. An advantage of using population data is that patterns of LD are the result of recombinations that have occurred in the past generations and therefore effectively increase the recombinant sample. There is also some evidence that marker-disease association studies are generally more powerful than transmission disequilibrium-based tests (30). LD mapping has been applied to many simple monogenetic human traits with success (31), although many properties of the technique have not been studied extensively. Applications and extensions of such mapping approaches to more complex human traits are in an early stage of development (32, 33). This is an important area of methodological research, as the use of LD mapping in appropriate populations may represent a means of dealing with some of the complexities and difficulties associated with standard mapping approaches to complex genetic traits (34). Mapping susceptibility loci for complex traits is thus critical to the success of current gene discovery projects in heart, lung and blood disease.

2.2 Single SNP-Disease Association analysis

2.2.1 Hardy–Weinberg Equilibrium

When the classical χ^2 goodness-of-fit test for Hardy-Weinberg equilibrium (HWE) is used on samples with related individuals, the type I error can be greatly inflated. In particular the test is inappropriate in population isolates where the individuals are related through multiple lines of descent. Calculation of HWE also serves as a crude quality check on the data; experience suggests that gross deviations from HWE often indicate genotyping errors or population admixture. However, the possibility that a deviation from HWE is due to a deletion polymorphism (35) or a segmental duplication (36) that could be important in disease causation should now be considered before discarding loci. Testing for deviations from HWE can be carried out using a Pearson goodness-of-fit test, often known simply as 'the χ^2 test' because the test statistic has approximately a χ^2 null distribution. However, that there are many different χ^2 tests. The Pearson test is easy to compute, but the χ^2 approximation can be poor when there are low genotype counts, and it is better to use a Fisher exact test, which does not rely on the χ^2 approximation (37).

2.2.2 Haplotype and genotype data

A haplotype is a combination of alleles at multiple linked loci that are transmitted together (fig. 2). Haplotype may refer to as few as two loci or to an entire chromosome depending on the number of recombination events that have occurred between a given set of loci. Underlying an individual's genotypes at multiple tightly linked SNPs are the two haplotypes, each containing alleles from one parent. True haplotypes are more

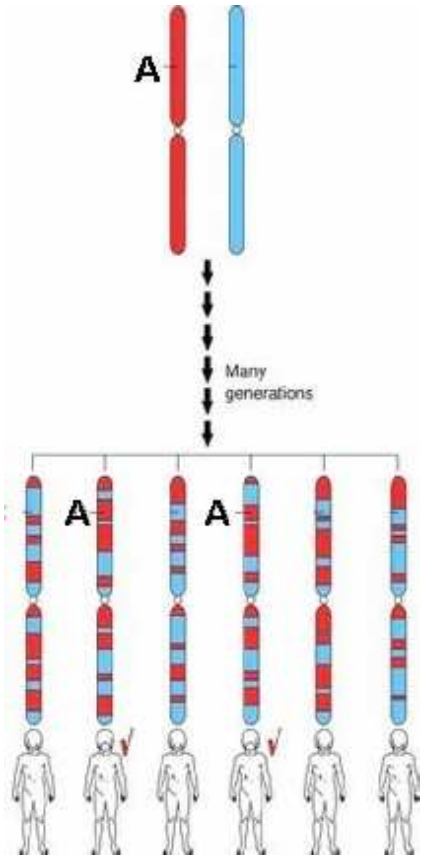


Fig. 2. Segments of haplotypes. This diagram shows two ancestral chromosomes being scrambled through recombination over many generations to yield different descendant chromosomes. If a genetic variant marked by the A on the ancestral chromosome increases the risk of a particular disease, the two individuals in the current generation who inherit that part of the ancestral chromosome will be at increased risk. Adjacent to the variant marked by the A are many SNPs that can be used to identify the location of the variant (http://www.hapmap.org/ originhaplotype).

informative than genotypes, but inferred haplotypes are typically less informative because of uncertain phasing. However, the information loss that arises from phasing is small when LD is strong. Note that phasing cases and controls together allows better estimates of haplotype frequencies under the null hypothesis of no association, but can lead to a bias towards this hypothesis and therefore a loss of power. Conversely, phasing cases and controls separately can inflate type 1 error rates. A similar issue arises in imputing missing genotypes.

2.2.3. Measures of LD

LD will remain crucial to the design of association studies until whole-genome resequencing becomes routinely available. Currently, few of the more than 10 million common human polymorphisms are typed in any given study. Among the measures that have been proposed for two-locus haplotype data, the two most important are D' and r^2 (38). The absolute value of D' is determined by dividing D by its maximum possible value, given the allele frequencies at the two loci. The case of $D'=1$ is known as complete LD. Values of $D'<1$ indicate that the complete ancestral LD has been disrupted. The magnitude of values of $D'<1$ has no clear interpretation. Estimates of D' are strongly inflated in small samples. Therefore, statistically significant values of D' that are near one provide a useful indication of minimal historical recombination, but intermediate values should not be used for comparisons of the strength of LD between studies, or to measure the extent of LD (4). The measure r^2 is in some ways complementary to D'. r^2 is equal to D^2 divided by the product of the allele frequencies at the two loci. Hill and Roberson deduced that E $[r^2]=1/1+4Nc$ where c is the recombination rate in morgans between the two markers and N is the effective

population size (39). This equation illustrates two important properties of LD. First, expected levels of LD are a function of recombination. The more recombination between two sites, the more they are shuffled with respect to one another, decreasing LD. Second, LD is a function of N, emphasizing that LD is a property of populations (15).

2.2.4 SNP tagging

SNP tagging method is based on the r^2 LD statistic and determines groupings of markers which are in tight correlation with an individual marker or markers (tagging markers) within a grouping. In anticipation of cost-effective SNP genotyping technologies and the availability of databases of a large number of candidate SNPs, many investigators are seriously considering genome-wide SNP scans with the hope of performing hypothesis-free disease association studies as opposed to hypothesis-driven candidate gene or region studies. Although the cost of SNP genotyping may be rapidly decreasing and without excessive loss of statistical power, it is still infeasible to genotype all available SNPs across the human genome (15).

2.3 Case–control association analysis

2.3.1 Overview

In a case-control study a group of affected subjects referred to as cases is compared to a group of unaffected subjects referred to as controls with respect to potential exposures of risk factors. If there is an association between the marker and the disease, the distribution of the genotypes is different within cases and controls.

There are two different reasons for an association which are of primary interest. The first reason is that the marker locus is itself a disease locus, thus the association is causal. The second reason is that the marker locus is in linkage disequilibrium with the disease locus because both are in close proximity on the genome. A further question is how marker loci to test for association are chosen (25). There are basically two approaches of genetic case-control studies with different amount of genotyping, the candidate gene approach and the genome scan. Most of the test statistics we describe here are developed for the candidate gene approach where one or several candidate genes are investigated in the study. These candidate genes are often chosen out of biological reasons because they code for some proteins which are expected to be functionally related to the phenotype of interest (20).

2.3.2 Case–control phenotype

Genotype and phenotype data are collected for case and control individuals. Both genotype and phenotype data often contain misclassification errors (40), adversely affecting statistical tests used to locate disease genes. The importance of studying phenotype errors in the context of genetic studies. They note that more than 1300 National Institutes of Health (NIH)-funded studies of complex genetic diseases have yielded fewer than 50 causative polymorphisms in humans (21, 41) More importantly, only 16%-30% of initially reported associations are confirmed without evidence of between-study heterogeneity or bias (41, 42). The problem of phenotype misclassification is particularly important, given the high error rates encountered in some studies (2).

The most analysis of SNP genotypes and case–control group at a single SNP is to

test the null hypothesis of no association between rows and columns of the $2 \times n$ contingency tables that contains the counts of the three genotypes among cases and controls. Here and elsewhere, n is the number of observed genotypes at the marker locus. However, there is a reduction in the power of the chi-square test and an increase in the minimum sample size needed to maintain constant asymptotic power at a fixed significance level (43). A key issue that arises then is a quantification of power loss in the presence of phenotype errors.

2.3.3 Continuous outcomes

Statistical tools for continuous (or quantitative) traits are linear regression and analysis of variance (ANOVA). ANOVA is analogous to the Pearson test in that it compares the null hypothesis of no association with a general alternative, whereas linear regression achieves a reduction in degrees of freedom from 2 to 1 by assuming a linear relationship between mean value of the trait and Genotype. In either case, tests require the trait to be approximately normally distributed for each genotype, with a common variance (14, 15, 21).

The linear models that are outlined above for continuous traits cannot be applied directly to case–control studies, because case–control status is not normally distributed and there is nothing to stop predicted probabilities lying outside the range 0–1. These problems are overcome in logistic regression, in which the transformation logit $(\pi) = \log (\pi / (1 - \pi))$ is applied to π_i, the disease risk of the ith individual. The value of logit (π_i) is equated to either β_0, β_1 or β_2, according to the genotype of individual i (β_1 for heterozygotes). The likelihood-ratio test of this general model, against the null hypothesis $\beta_0 = \beta_1 = \beta_2$, has 2 df, and for large sample sizes is equivalent to the Pearson

2-df test. Users can improve the power to detect specific disease risks, at the cost of lower power against some other risk models, by restricting the values of β_0, β_1 and β_2. Tests for recessive or dominant effects can be obtained by requiring that $\beta_0 = \beta_1$ or $\beta_1 = \beta_2$. So far, logistic regression has not brought much that is new for single-SNP analyses. However, logistic regression offers a flexible tool that can readily accommodate multiple SNPs, possibly with complex epistatic and environmental interactions or covariates such as sex or age of onset. One potential problem with regression-based analyses is that they assume prospective observation of phenotype given the genotype, whereas many studies are retrospective (44, 45).

3. Common Variant / Common Disease (CVCD) hypothesis

3.1. Overview

The most well-known hypothesis suggests that the disease-predisposing alleles occur frequently in the population and each of them contributes little to disease susceptibility. This is known as the common variant, common disease (CVCD) hypothesis (46). The alternative heterogeneity model suggests that rare alleles with strong phenotypic effects may underlie the genetic background for common diseases. According to the so-called neutral hypothesis, the allelic spectrum of common diseases is similar to that of the allelic spectrum of all the variants in the genome (15). The potential allelic spectra according to these three hypotheses are presented in fig. 3. In addition to the sample size requirements, the allelic spectrum of the disease in question is important in sampling strategies. Different patterns of decline in disease risk among relatives can be expected for diseases with major gene effects compared to multiple

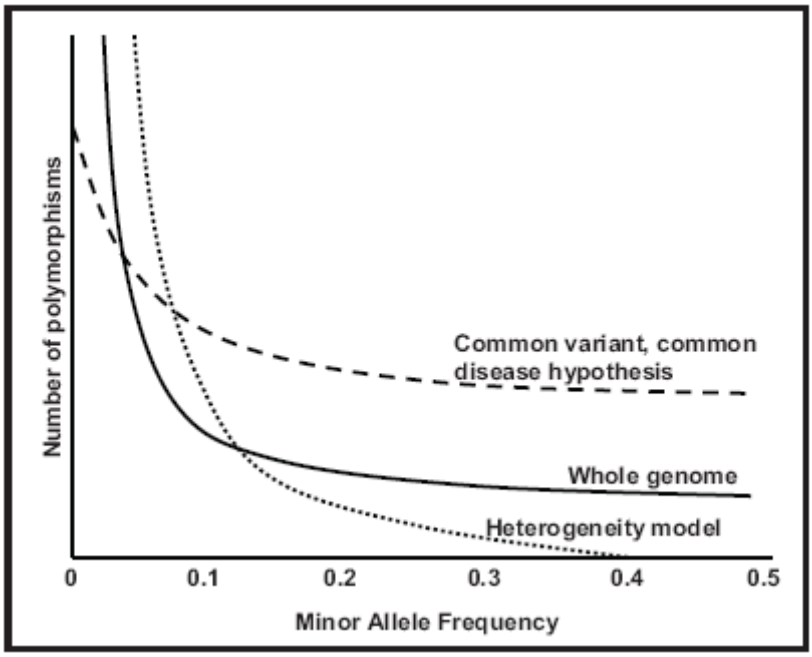


Fig. 3. The depiction of three hypothetical distributions of the allelic spectra of the common diseases. The common variant, common disease (CVCD) hypothesis suggests a large number of high frequency variants. According to the classical heterogeneity model, more rare variants are expected and, thus, more genetic heterogeneity is expected in the population. The solid line shows an estimated allelic spectrum in the whole genome, regardless of whether the variants are disease-causing or not. Modified from Wang et al (15).

genes with minor effects. In theory, the correlation of traits between related subjects should decrease linearly with distancing of relatedness, if few rare variants with additive and independent effects underlie the disease (15). In contrast, in the case of multiple alleles with minor and potentially interdependent effects, the risk may decline more rapidly with a decrease in relatedness. In addition, more genetic heterogeneity is expected in pedigrees when more distant relatives are included.

3.2. Complex disease

Complex diseases occur commonly in the population and are a major source of disability and death worldwide. They are thought to arise from multiple predisposing factors, both genetic and nongenetic, and joint effects of those factors are thought to be of key importance (47). Parkinson disease (PD) serves as an example of a complex disease. Other examples include Alzheimer disease, diabetes mellitus, nicotine and alcohol dependence, and several types of cancer (48). While major inroads have been made in identifying the genetic causes of rare Mendelian disorders, little progress has been made in the discovery of common gene variations that predispose to complex diseases (147, 148). The single gene variants that have been shown to associate reproducibly with complex diseases typically have small effect sizes or attributable risks. However, the joint actions of common gene variants within pathways may play a major role in predisposing to complex diseases (the paradigm of complex genetics), and the discovery of susceptibility genes and pathways may have sizeable public health benefits (109, 149).

3.3. Coronary artery disease

Coronary artery disease (CAD) is arteriosclerosis of the inner lining of the blood vessels that supply blood to the heart. CAD is a common form of heart disease and is a major cause of illness and death. CAD begins when hard cholesterol substances (plaques) are deposited within a coronary artery. The plaques can cause a tiny clot to form which can obstruct the flow of blood to the heart muscle. Symptoms of CAD include 1) chest pain (angina pectoris) from inadequate blood flow to the heart; 2) heart attack (acute myocardial infarction), from the sudden total blockage of a coronary artery; or 3) sudden death, due to a fatal rhythm disturbance (49). In many patients, the first symptom of CAD is myocardial infarction or sudden death, with no preceding chest pain as a warning. For this reason, doctors perform screening tests to detect signs of CAD before serious medical events occur. Screening tests are of particular importance for patients with risk factors for CAD. These risk factors include a family history of CAD at relatively young ages, an abnormal serum cholesterol profile, cigarette smoking, elevated blood pressure (hypertension), and diabetes mellitus (50).

3.4. Metabolic syndrome

The major characteristics of metabolic syndrome include insulin resistance, abdominal obesity, elevated blood pressure, and lipid abnormalities. The third National Cholesterol Education Program (NCEP) Adult Treatment Panel (ATPIII) defines the metabolic (or insulin resistance) syndrome as the presence in an individual of at least three of the following five risk factors (51). The metabolic syndrome is a major risk factor for cardiovascular disease (CVD) and type 2 diabetes. Although insulin resistance is also a key risk factor for CVD and type 2 diabetes, hyperinsulinemia is not

included as a potential risk factor by the ATP III; its definition was designed for use in clinical practice with adults, and insulin levels are not usually assessed in clinical practice (52).

II. SNP DISCOVERY OF CORONARY ARTERY DISEASE CANDIDATE GENES IN A KOREAN POPULATION

1. Introduction

Single nucleotide polymorphisms (SNPs) are single base pair positions in genomic DNA at which different sequence alternatives (alleles) exist in normal individuals in some population. SNPs are an abundant form of genetic variation, distinguished from rare variations by a requirement for the least abundant allele to have a frequency of 1% or more. Among those genetic variation have been used for the identification of disease-related genes and DNA markers of individual risk factor (53, 54).

Coronary artery disease (CAD) is the most common cause of death in industrialized countries, and the prevalence is increasing dramatically in developing countries. CAD is a complex multifactorial and polygenic disorder that is thought to result from an interaction between an individual's genetic background and various environment factors (55). Molecular genetic studies of rare, Mendelian forms of the disease have identified several mutations that cause premature CAD many of which affect levels of LDL and HDL cholesterol (56). Candidate gene studies build on existing knowledge by investigating variation in genes that are already implicated in the pathophysiology of a disease. However, candidate-gene studies of validated biological pathways that have the potential to identify new genes and pathways (60).

Therefore, we have undertaken a sequencing analysis of the disease-related genes to gain a better understanding of complex diseases such as CAD.

2. Materials and Methods

2.1. Selection of candidate SNPs and DNA samples

With the use of public database, including PubMed and **O**nline **M**endelian **I**nheritance in **M**an, we selected 31 candidate genes that have been characterized and suggested to be associated with CAD. 31 candidate genes distributed across 16 autosomes and X chromosome. We further selected coding regions (cSNPs) and some untranslated regions from the disease candidate genes. A total of 24 samples are unrelated Korean populations. To achieve this objective, at least two lines of investigation are needed: a) finding the primary function of genes involved in a particular pathway, and b) the published candidate gene association with CAD. The candidate genes were chosen on the basis of their potential relevance to the selected common diseases. A large number of SNPs in coding regions (cSNPs) and some UTR regions from the selected disease candidate genes were collected in publicly available dbSNP database (http:// www.ncbi .nlm.nih.gov /SNPs).

2.2. Direct sequencing for verification of the cSNPs

Genomic DNA for sequencing were isolated from 24 Korean DNA samples. Genotypes for the biallelic polymorphism were determined by polymerase chain reaction (PCR) with the following sequence specific primer. We designed polymerase chain reaction (PCR) primers using the Primer 3 program (58). Primer pairs were designed to produce about 500 base pairs by PCR, and their Tms ranged between 59°C and 62°C as far as possible. We sequenced the 24 Korean samples (21 males and 3 females, 35-42 years old of age), thereby permitting allele frequencies to be estimated

among 16 autosomes and X chromosome. The PCR reaction was performed with 20 ng of genomic DNA as the template in a 30$\mu\ell$ reaction mixture by using a AmpliTaq Gold (Applied Biosystems, Foster City, CA, USA) as follows: activation of Taq polymerase at 94 °C for 5minutes, 35 cycles of 94 °C for 30 seconds, 55-68 °C, and 72 °C for 45 seconds each were performed, finishing with a 10-minute step at 72 °C. For automated directing sequencing, the PCR products were amplified using the internal forward and reverse primer. Direct PCR sequencing reaction were performed using a PRISM BigDye Terminator v3.1 Cycle sequencing Kit. The DNA samples containing the extension products were added to Hi-Di formamide (Applied Biosystems, Foster City, CA). The mixture was incubated at 95 °C for 5 min, followed by 5 min on ice and then analyzed by ABI Prism 3730 DNA analyzer (Applied Biosystems, Foster City, CA). The results were analyzed using the software DNAstar (http://www.dnastar.com).

2.3. Korean-SNP database

The Korean_SNP database was constructed at National Genome Research Institute (National Institute of Health, Korea). All SNP allele frequency data described in this study are currently available at Korean-SNP database (http://ksnp.ngri.re.kr/ SNP/index.jsp) (59).

3. Results and Discussion

3.1 Comparison with SNPs and Ins/Del in NCBI D/B

CAD is similar to other common complex genetic disorders such as

atherosclerosis, diabetes and asthma (55). The detailed investigation of variation in functionally important regions of the human genome is expected to promote and cording region understanding of genetically complex disease. Candidate gene association studies have been regarded as effective tools to study complex traits. We identified between 409 SNPs of 31 CAD candidate genes, among 24 individuals Korean population. Among all SNPs discovered in this study, 76.35% of SNPs were known SNPs, whereas 19.68% of SNPs were novel. SNPs are an abundant form of genetic variation, distinguished from rare variations by a requirement for the least abundant allele to have a frequency of 1% or more. Interactions between genetics and environmental factors are thought to play key roles in the pathogenesis of CAD. Among those, genetic variation has been used for the identification of disease-related genes (Fig. 4).

3.2. The SNPs were categorized according to their locations in the gene region

We sequenced about 300 kb of genomic DNA regions of biological importance such as coding regions, splicing junctions, and promoter regions because the nucleotide variants in these regions have been hypothesized to be involved in the pathogenesis of complex disease (61-63). 409 SNPs identified, 84 SNPs were in coding region, 54 SNPs in regulatory region and 196 SNPs in intron region and 75 SNPs in UTR region. Of the 84 coding SNPs, 40 were non-synonymous substitutions. Overall, the percentage of SNPs distribution in these regions is different for the dbSNP (NCBI dbSNP build 112) (Fig. 5). First, in 40.4% of dbSNP fall into the intron

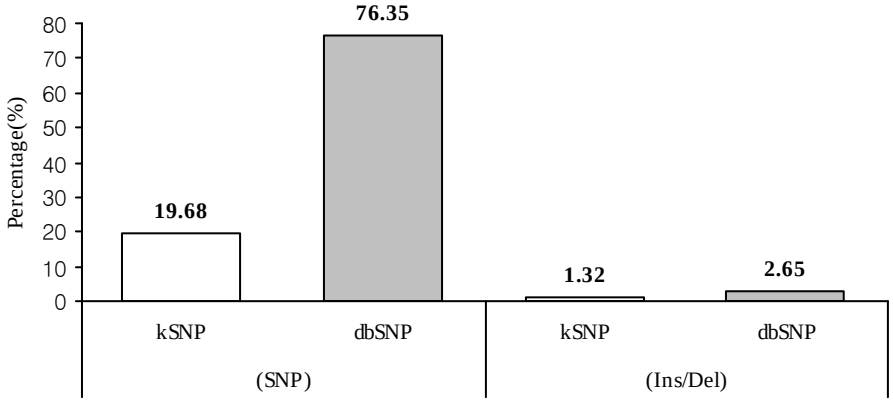


Fig. 4. Distribution of SNPs discovered in this study. 76.35% of SNPs were known SNPs, whereas 19.68% of SNPs were novel.

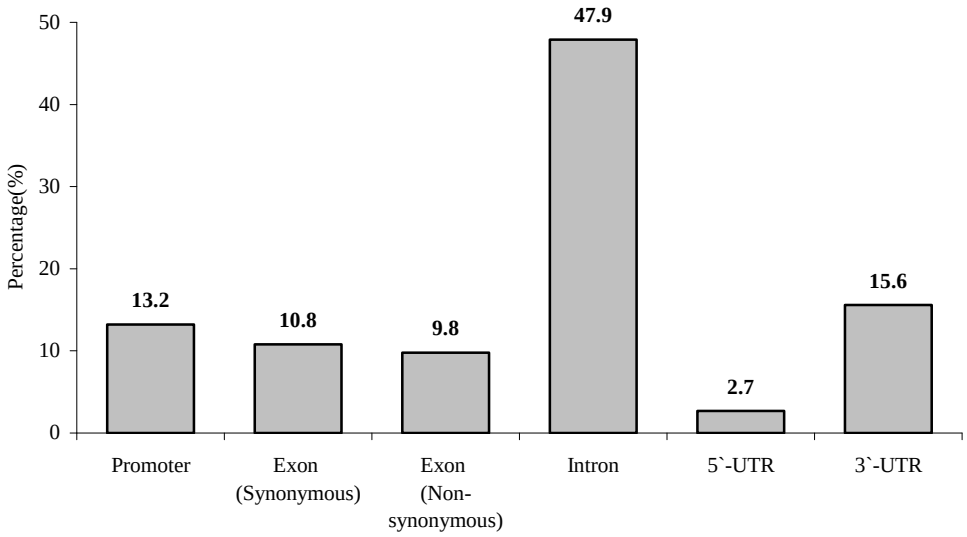


Fig. 5. Distribution of SNPs according to location.

region, compared to 47.9% in the KSNP. Second, the dbSNP have a higher proportion of SNP (46%) located in exon regions than does KSNP (20.6%). Third the KSNP have a higher proportion of SNP (31.5%) located in Promoter/UTR regions than does dbSNP (13.6%) (64). Patterns of SNP distribution in genomic regions observed here that the difference is probably because of the small sample size and smaller sets of genes.

3.3. The observed distributions of minor allele frequencies of 409 SNPs

To investigate the allele frequency distribution of the detected SNPs, we classified the minor allele frequency of the SNPs. According to this classification, 28.1% belonged to the ≤10% frequency group, 17.6% belonged to the 10-20% frequency group, 14.2% belonged to the 20-30% frequency group, 17.9% belonged to the 30-40% frequency group, and 22.2% belonged to the 40-50% frequency group. The observed frequency of SNPs is one per 1,138bp and common SNPs are one per 1,860bp. The average minor allele frequency is 19.3% in the Korean population. In addition, among 302 polymorphic SNPs, approximately two thirds (71.86%) had greater than 10% minor allele frequency in Korean population (fig. 6).

3.4. Distribution of SNPs according to mutation type

As general trend we observed that the most frequent type of mutation is a base change of either A/G or C/T. This is consistent with the experimental observation that cytosine demethylation is the most common mutational event (fig. 7). The detected SNPs were categorized according to nucleotide substitution as either transitions (C/T or G/A) or transversions (C/T, A/G, C/A, or T/G). There was a relative increase in the

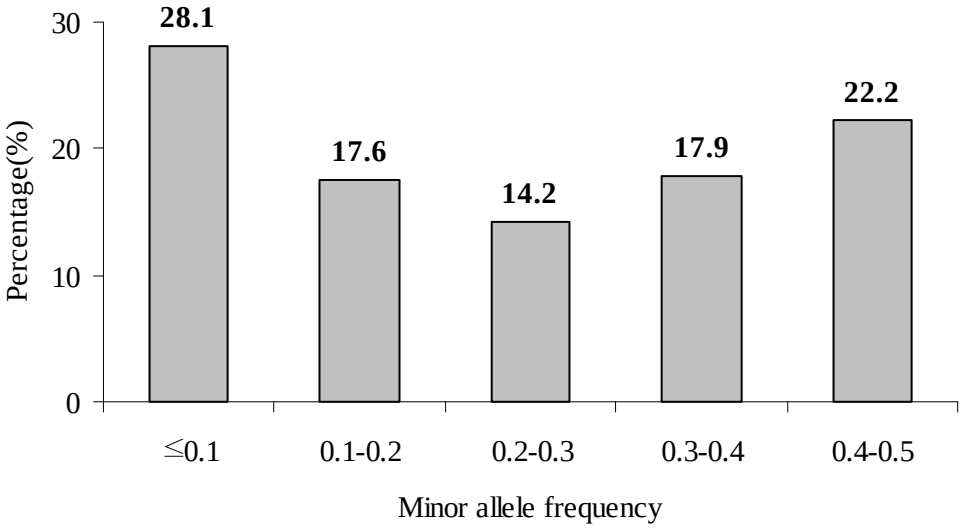


Fig. 6. Distribution of SNP minor allele frequencies for 409 SNPs discovered in 31 genes.

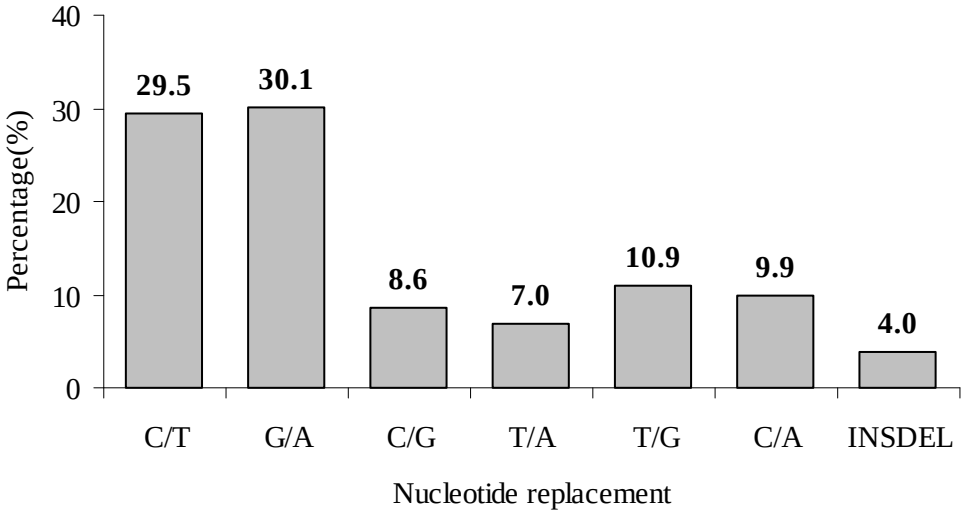


Fig. 7. Distribution of transitions and transversions among SNPs.

proportion of transitions over transversions. We also observe a relative increase in frequency of C/A and its reverse complement, T/G transversions compared with C/G and A/T transversions. The SNPs consisted of 59.6% transitions and 40.4% transversions. Transitions occurred 1.47 times more frequently than transversions. As for the mouse (roughly 66.7% transitions) and for human (64% transitions), our data set shows a similar bias towards transitions (67.83%) (66, 67). About 71% of all polymorphisms were transitions, which are known to be typical for human genes (65). However, biological data sets tend to have a strong bias toward transitions due to DNA methylation, chemical differences between bases, and differences in DNA repair efficiency for different types of nucleotide mismatches. The high frequency of transitions detected has been observed in previous SNP discovery programs (67, 68) and reflects the high frequency of the C to T mutation after methylation (69).

3.5 Ratio of transitions to transversions

When analyzing the ratio of transitions to transversions (Table 1), the ratio was found to be greater in coding (1.82) than in noncoding variants (1.24). The investigation of transition/transversion ratios has a long history of scientific interest in interspecies comparisons (70). Our data support the previously formulated hypothesis, that among non-synonymous polymorphisms radical amino acid alterations preferentially result from transversions (71). This is sensible under the assumption that conservative alterations are functionally more similar to synonymous substitutions than to radical alterations, as concluded above (72).

In summary, we showed that in coding regions conservative alterations are more similar to synonymous variants than to radical variants. Large-scale identification of

Table 1. Occurrence of SNPs according to functional region and type of nucleotide replacement

Type of substitution	Cording regions	Noncording Regions	Total
Transitions	71	72	143
Transversions	39	58	97
Ratio of transitions to transversions	1.82	1.24	1.47

genetic variants in CAD candidate genes will greatly facilitate for searching CAD disease genes. Therefore, our results will be very useful information for disease association studies of CAD.

III. ASSOCIATION OF CCR2 POLYMORPHISMS WITH THE NUMBER OF CLOSED CORONARY ARTERY VESSELS IN CORONARY ARTERY DISEASE

1. Introduction

Coronary artery disease (CAD) is one of the most common forms of heart disease and the leading cause of heart attacks. CAD begins when hard cholesterol substances (plaques) are deposited within a coronary artery. Recently, inflammation has been associated with the development of CAD (73). In the process of inflammation, chemokines play crucial roles for the recruitment and activation of leukocytes. In addition to their functions in many immune responses, chemokines have also been implicated in the pathogenesis of atherosclerosis (74). Especially, the expression of the CC chemokine MCP1 is upregulated in human atherosclerotic plaques, in arteries of primates on a hypercholesterolemic diet, and in vascular endothelial and smooth muscle cells exposed to minimally modified lipids (75). MCP-1 can play a role as a chemokine by binding to the Chemokine (C–C motif) receptor 2 (CCR2) (76). CCR2 mediates agonist-dependent calcium mobilization and inhibition of adenylyl cyclase (77). CCR2 gene is located in the chemokine receptor gene cluster region on chromosome 3 (3p21) (78). Two alternatively spliced transcript variants (NM_000647 and NM_000648), CCR2A and CCR2B, are expressed by the gene. The CCR2 gene comprises 3 exons spanning approximately 7 kb of genomic sequence. Furthermore, the importance of the CCR2 gene has been demonstrated previously that MCP-1/CCR2 interaction plays a role in the pathogenesis of atherosclerosis (73, 79). Therefore, in this study, we screened the polymorphisms of chemokine receptor CCR2 in the Korean

population and investigated the association of the CCR2 genetic variants with CAD.

2. Materials and Methods

2.1. Subjects and measures

2.1.1. Patient population

The study subjects included 750 male consecutive patients with symptomatic coronary artery disease who underwent coronary angiography at Yonsei Cardiovascular Hospital between January of 2004 and April of 2005. 717 male patients without evidence of significant coronary artery disease, demonstrated by treadmill test, stress MIBI scan or coronary angiography, were used as the control population. All the patients in the study were enrolled in the Cardiovascular Genome Center of Yonsei University, a government sponsored project supported by the Ministry of Health and Welfare, Republic of Korea.

Risk factors of coronary artery disease were defined as follows: (1) Hypertension defined as blood pressure N140/90 mm Hg at the time of examination, current history of taking blood pressure medications and/or history of hypertension, (2) Diabetes defined as FBS ≥126 mg/dl, Random blood sugar ≥200 mg/dl, and/or patients with history of diabetes currently taking diabetic medications, (3) Hypercholesterolemia defined as total cholesterolN240 mg/dl, LDL cholesterolN160 mg/dl and/or patients currently taking statins at the time of study enrollment. The patients were enrolled after receiving informed consent. Patients with any of the following were excluded from participation: valvular heart disease; peripheral vascular disease; significant systemic

disease; history of inflammatory disease; and history of congestive heart failure with LVEF b30%.

At the time of initial enrollment, patients underwent a complete physical examination, a baseline electrocardiogram, and laboratory assessment. The study was approved beforehand by the institutional ethics committee and the procedures followed were in accordance with the institutional guidelines.

2.1.2. Coronary angiography

Coronary angiographies were performed via the femoral artery. All the angiographies were interpreted by the consensus of two independent observers. Significant coronary artery disease was defined as ≥50% luminal stenosis of vessels more than 1.5 mm in diameter. CAD patients were classified as one vessel, two vessel, and three vessel disease according to the number of epicardial coronary arteries involved (Table 2).

2.2. Sequence analysis of the human CCR2 gene

We have sequenced all exons, including exon–intron boundaries and the promoter region (−1.5 kb 5′-flanking) of the CCR2 gene to identify genetic variants, including single nucleotide polymorphisms (SNPs) in 24 Korean DNA samples using the ABI PRISM 3700 DNA analyzer (Applied Biosystems, Foster City, CA, USA). Seven primer sets for the amplification and sequencing analysis were designed based on GenBank sequences (Ref. Seq. of CCR2 mRNA: NM_000647 and contig: NT_079509). Sequence variants were verified by chromatograms. (PCR primer information used in this study is available on request). DNA polymorphisms were

identified using the PolyPhred program (http://droog.gs.washington.edu/PolyPhred.html) after sequence chromatograms were base-called with the Phred program and assembled with Phrap (80).

2.3. Genotyping with fluorescence polarization detection

For genotyping of polymorphic sites, amplifying primers and probes were designed for TaqMan® (81). Primer Express® (Applied Biosystems) was used to design both the PCR Primers and the MGB TaqMan probes. One allelic probe was labeled with the FAM dye and the other with the fluorescent VIC dye. Typically, PCR was run in the TaqMan Universal Master mix without UNG (Applied Biosystems) at a primer concentration of 900 nM and a TaqMan MGBprobe concentration of 200 nM. The reaction was performed in a 384-well format in a total reaction volume of 5 μl using 20 ng of genomic DNA. The plate was then placed in a thermal cycler (PE9700, Applied Biosystems) and heated at 50 °C for 2 min and 95 °C for 10 min followed by 40 cycles of 95 °C for 15 s and 60 °C for 1 min. The TaqMan assay plate was transferred to a Prism 7900HT instrument (Applied Biosystems) where the fluorescence intensity of each well was read. Fluorescence data files from each plate were analyzed using automated software (SDS 2.1: Applied Biosystems).

2.4. Statistics

The $\chi 2$ tests were used to determine whether individual variants were in equilibrium at each locus in the population (Hardy–Weinberg equilibrium). We examined Lewontin's D′ (|D′|) and the linkage disequilibrium (LD) coefficient r^2 between all pairs of biallelic loci (82). Haplotypes of each individual were inferred

using the algorithm developed by Stephens et al. (PHASE), which uses a Bayesian approach incorporating a priori expectations of haplotypic structure based on population genetics and coalescent theory (83). Phase probabilities of all polymorphic sites for haplotypes were calculated for each individual using this software. Individuals with phase probabilities of less than 97% were excluded in analysis. The genotype distributions of CCR2 polymorphisms and haplotypes between patient groups, including coronary artery obstructive disease, myocardial infarction, hypertension and diabetes subjects and normal subjects, were analyzed with multiple logistic regression and multiple linear regression models by controlling for age (continuous value), body mass index (BMI, continuous value), systolic blood pressure (SBP), diastolic blood pressure (DBP), triglyceride (TG) and total cholesterol and smoking status (nonsmoker=0, ex-smoker=1, current smoker=2) as covariates. Haplotype associations were analyzed using the algorithm developed by Schaid et al. (Haplo.Score and Haplo.GLM) (84), controlling for age, BMI and smoking status as covariates.

3. Results and Discussion

3.1. Identification of CCR2 polymorphisms

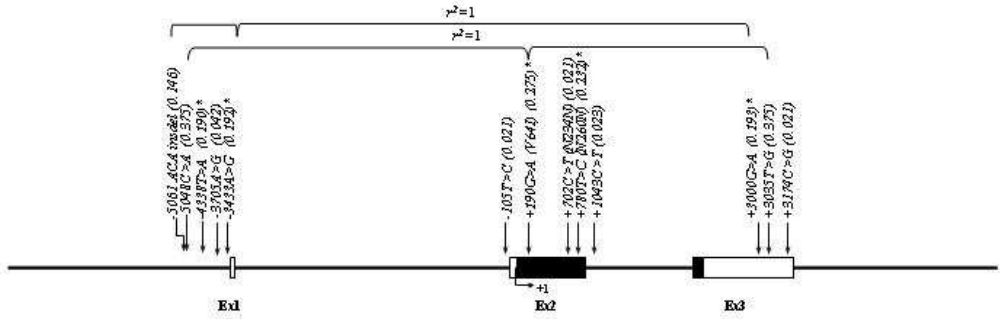
In order to screen the polymorphisms of CCR2 gene in the Korean population, we sequenced all exons, exon–intron boundaries and promoter regions of the gene using 24 genomic DNA samples randomly selected from the Korean population of an epidemiological cohort (Ansung and Ansan regions, Kyungki Province). We identified a total of 13 genetic variants including 1 deletion and 12 SNPs. Among 13 genetic variants (7 known and 6 new), 5, 2 and 6 variants were found in the promoter, intron

and exon, respectively. The positions and frequencies of genetic variants identified in CCR2 gene were shown in Fig. 8. All SNPs were in Hardy–Weinberg equilibrium. For association study, 5 SNP markers (2 SNPs;−4338T/A, −3433A/G) in promoter, 3 SNPs in exons including 1 non-synonymous SNP (+190G/A), 1 synonymous SNP (+780T/C) and 1 SNP (+3000G/A) at 3′-UTR region) were selected based on their linkage disequilibrium status, frequencies and locations.

3.2. Association of CCR2 polymorphisms with the number of coronary arteries with significant stenosis

For association study, we used 750 CAD male patients and 744 normal healthy male controls in unrelated Korean (Table 2). Initially, we investigated the association of CCR2 polymorphisms with CAD. However, no association was observed (Table 3). In order to determine any relationship with subphenotypes of CAD with CCR2 polymorphisms, further analysis was performed and significant associations were observed with the number of vessels with significant stenosis in CAD patients from four SNPs −4338T/A, −3433A/G, +780T/C, and +3000GNA (p=0.01–0.0005). The most significant signal was detected in a SNP at exon 2 (+780T/C, Asn260Asn) (odds ratio: 1.49, 95% CI: 1.19–1.87, p=0.0005) (Table 4). The association was also confirmed by haplotype association analysis (Table 4). Concordant to the results of SNP associations, ht-1 (TAGTG), composed of major allele SNPs, was found to be resistant to the increased number of closed vessel in CAD patients (odds ratio: 0.81, 95% CI: 0.67–0.97, p=0.02), and ht-3 (AGGCA), composed of mostly minor allele SNPs except one (+190G/NA), which did not show the association with the risk,

A. Map of *CCR2* (chemokine (C-C motif) receptor 2) on chromosome 3p21 (6.7 kb)



B. Haplotypes of *CCR2*

Hap.	-4338T>A	-3433A>G	+190G>A	+780T>C	+3000G>A	freq.
ht1	T	A	G	T	G	0.492
ht2	T	A	A	T	G	0.276
ht3	A	G	G	C	A	0.193
ht4	T	A	G	C	G	0.039

C. LD (|*D*| and r^2) measures among *CCR2* SNPs

		|D|				
		-4338T>A	-3433A>G	+190G>A	+780T>C	+3000G>A
r^2	-4338T>A	-	0.998	1	0.998	0.998
	-3433A>G	0.996	-	1	1	1
	+190G>A	0.09	0.091	-	1	1
	+780T>C	0.786	0.79	0.115	-	1
	+3000G>A	0.996	1	0.091	0.791	-

Fig. 8. Gene maps and haplotypes of CCR2 gene. Coding exons are marked by black blocks, and 5′ and 3′-UTRs by white blocks. The first base of the translation start site is denoted as nucleotide+1. Asterisks indicate polymorphisms genotyped in a larger population (n=1494). The frequencies of polymorphisms not subjected to larger scale genotyping were based on sequence data (n=24). A. Polymorphisms identified in CCR2 on chromosome 3p21 (Ref. Genome Seq. NT_079509). B. Haplotypes of CCR2. C. Linkage disequilibrium coefficients (|D′| and r^2) among CCR2 polymorphisms.

Table 2. Clinical profiles of the cardiovascular disease study subjects (n=1494)

	Control (n=744)			Case (n=750)			
	Mean		SD	Mean		SD	p-value
Age, year	52.43	±	9.88	55.73	±	8.31	<0.0001
SBP, mm Hg	112.42	±	10.97	119.47	±	17.43	<0.0001
DBP, mm Hg	74.14	±	7.41	77.14	±	9.97	<0.0001
BMI, kg/m^2	23.56	±	2.57	25.00	±	2.71	<0.0001
Glucose, mg/dl	86.41	±	10.17	99.08	±	32.59	<0.0001
T-cholesterol, mg/dl	187.94	±	27.86	181.01	±	39.68	0.00025
HDL, mg/dl	48.43	±	12.05	39.06	±	9.54	<0.0001
Triglycerides mg/dl	116.11	±	49.03	164.49	±	118.62	<0.0001
LDL, mg/dl	116.07	±	27.60	110.14	±	36.17	0.00120
$HOMA_{IR}$	1.55	±	1.54	2.39	±	2.49	<0.0001
Smoking (n)							
0		179			113		<0.0001*
1		195			466		
2		17			0		
3		350			171		
Disease							
HT				359 (47.9%)			
MI				363 (48.4%)			
DM				169 (22.6%)			
Number of diseased vessels							
One vessel, n				271 (36.1%)			
Two vessels, n				242 (32.3%)			
Three vessels, n				237 (31.6%)			

The values are mean ± SD. Values of p are obtained using Student's t test between CAD patients and controls.

*p-values are obtained using chi-square test between CAD patients and controls.

Table 3. Logistic analysis of CCR2 polymorphisms with the risk of CAD whilst controlling for age, BMI, smoke, SBP, DBP, TG and total cholesterol as covariate among cardiovascular disease and normal subjects

Gene	Loci	Position	AA change	Case	Control	Codominant		Dominant		Recessive	
						OR(95% CI)	*p*	OR(95% CI)	*p*	OR(95% CI)	*p*
CCR2	−4338T>A	Promoter	.	0.194	0.186	1.05(0.86–1.27)	0.65	1.09(0.86–1.37)	0.49	0.90(0.52–1.58)	0.72
	−3433A>G	Promoter	.	0.199	0.186	1.08(0.89–1.31)	0.43	1.10(0.87–1.39)	0.43	1.11(0.64–1.91)	0.71
	+190G>A	Exon2	Val64Ile	0.276	0.277	1.01(0.85–1.20)	0.91	1.02(0.82–1.28)	0.84	0.98(0.64–1.49)	0.91
	+780T>C	Exon2	Asn260Asn	0.235	0.230	1.01(0.84–1.22)	0.89	0.97(0.78–1.22)	0.80	1.27(0.77–2.07)	0.35
	+3000G>A	Exon3	.	0.198	0.187	1.07(0.88–1.29)	0.52	1.09(0.86–1.37)	0.49	1.05(0.61–1.81)	0.85
	ht1(TAGTG)	.	.	0.489	0.496	0.378	0.71	.	.	.	.
	ht2(TAATG)	.	.	0.277	0.275	−0.031	0.98	.	.	.	.
	ht3(AGGCA)	.	.	0.199	0.186	0.269	0.79	.	.	.	.

Table 4. Logistic analysis of CCR2 polymorphisms with the number of closed coronary artery vessels in CAD patients

Gene	Locus	Position	AA change	1	2	3	Codominant		Dominant		Recessive		HWE		
							OR (95%CI)	*p*	OR (95%CI)	*p*	OR (95%CI)	*p*	1	2	3
CCR2	−4338T>A	Promoter	.	0.223	0.195	0.156	1.40(1.09–1.78)	**0.007**	1.45 (1.09–1.93)	**0.01**	1.65(0.80–3.42)	0.18	0.759	0.229	0.487
	−3433A>G	Promoter	.	0.229	0.199	0.158	1.41(1.11–1.79)	**0.005**	1.48 (1.12–1.96)	**0.007**	1.65(0.83–3.25)	0.15	0.514	0.335	0.270
	+190G>A	Exon2	Val64Ile	0.273	0.263	0.288	0.95(0.77–1.17)	0.63	0.89 (0.68–1.16)	0.39	1.14(0.68–1.92)	0.62	0.748	0.923	0.296
	+780T>C	Exon2	Asn260Asn	0.275	0.233	0.182	1.49(1.19–1.87)	**0.0005**	1.59 (1.21–2.09)	**0.0009**	1.80(0.99–3.28)	0.06	0.615	0.164	0.323
	+3000G>A	Exon3	.	0.228	0.198	0.160	1.39(1.09–1.76)	**0.007**	1.45 (1.09–1.92)	**0.01**	1.68(0.85–3.31)	0.14	0.446	0.366	0.334
	ht-1	.	.	0.450	0.504	0.523	0.81(0.67–0.97)	**0.02**	0.77 (0.57–1.04)	0.09	0.72(0.53–0.99)	**0.04**	.	.	.
	ht-2	.	.	0.275	0.263	0.287	0.96(0.77–1.18)	0.68	0.90 (0.69–1.18)	0.44	1.14(0.68–1.91)	0.63	.	.	.
	ht-3	.	.	0.232	0.195	0.162	1.41(1.11–1.78)	**0.005**	1.45 (1.10–1.92)	**0.009**	1.81(0.91–3.59)	0.09	.	.	.

Genotype distributions and p-values for logistic analyses of three alternative models (co-dominant, dominant and recessive models) controlling for age, BMI, smoke,SBP, DBP, TG and total cholesterol as covariates are shown.

Bold characters indicate statistical significance of $p<0.05$.

showed the susceptibility to the increased number of closed vessels (odds ratio: 1.41, 95% CI: 1.11–1.78, p=0.005) (Fig. 8B). These results indicate that CCR2 contributes to the pathogenesis of CAD, especially to the number of vessels in CAD. It has been previously demonstrated that CCR2 Val64Ile polymorphism was associated with myocardial infarction and heart failure (85) and with reduced coronary artery calcification (86). In consistence with previous studies, our data suggest that CCR2 plays a crucial role in the pathogenesis of CAD, particularly in the number of vessels (angio) in CAD. However, while all associations with CAD phenotypes were located at CCR2 Val64Ile polymorphism in the previous studies for German population (85) and USA population (87), in this study for Korean population, CCR2 Val64Ile polymorphism was not associated with any CAD phenotypes including the number of vessels, indicating that there is clear ethnic difference in the CCR2 polymorphisms associated with CAD. Current study also did not detect the correlation between Val64Ile polymorphism and CAD possibly due to the very low allele frequency, providing additional instance of no correlation between Val64Ile polymorphism in CCR2 and CAD (88). However, CCR2 (+ 780T/C, Asn260Asn) polymorphism could increase the number of closed coronary vessels in our study.

IV. ASSOCIATION OF α-ADDUCIN Gly460Trp POLYMORPHISM WITH CORONARY ARTERY DISEASE IN A KOREAN POPULATION

1. Introduction

Common vascular diseases, including coronary artery disease (CAD), myocardial infarction, hypertension, and common congenital heart disease, are caused by multiple genetic and environmental factors (89, 90). In particular, CAD is one of the leading causes of death in Korea as well as in most industrialized countries. CAD begins when hard cholesterol substances (plaques) are deposited within a coronary artery. Association between CAD was partially mediated by insulin, HDL cholesterol and triglyceride levels, Body Mass Index (BMI) and blood pressure (91, 92). α-Adducin (ADD1) is a heterodimeric cytoskeleton protein consisting of an alpha-subunit and either a beta- or gamma-subunit. It is a protein of the cytoskeleton involved in the function of the Na+/K+ pump in the kidney (93, 95, 104, 126). The recently discovered these genes have been shown to be important in determining blood pressure levels, a major hypertension and CAD risk factor (94, 106, 131). Therefore, in order to investigate any possible association of CAD candidate genes, we examined the association of the polymorphisms of 31 CAD candidate genes.

2. Materials and Methods

2.1. Subjects and measures

The study subjects were recruited from the cardiovascular research center of

Yonsei University. Clinical symptoms and physical examination results of the 1284 unrelated Korean individuals (men only), including 749 CAD subjects and 535 normal healthy controls, were compatible. Normal subjects with a history of hypertension, hypercholesterolemia, diabetes, myocardial infarction, or metabolic syndrome were excluded. Risk factors of CAD were defined as follows: hypertension defined as blood pressure >140/90mmHg at the time of examination, current history of taking blood pressure medications or history of hypertension; diabetes defined as a fasting glucose level _126 mg/dl, random blood sugar _200mg/dl, or patients with history of diabetes currently taking diabetic medications; hypercholesterolemia defined as total cholesterol >240mg/dl, LDL cholesterol >160 mg/dl or patients currently taking statins at the time of study enrollment. The patients were enrolled after receiving informed consent. Patients with any of the following were excluded from participation: valvular heart disease, peripheral vascular disease, significant systemic disease, history of inflammatory disease, and history of congestive heart failure with left ventricular ejection fraction (LVEF) <30%. At the time of initial enrollment, patients underwent a complete physical examination, a baseline electrocardiogram, and laboratory assessment. The study was approved beforehand by the institutional ethics committee and the procedures followed were in accordance with the institutional guidelines. Characteristics, in terms of conventional risk factors, of the two subject groups are summarized in Table 1. In this study group, there were 359 hypertension patients. Among the 359 hypertension patients, 334 patients were taking antihypertensive medications at the time of enrollment. The other 25 patients were not taking medications at the time of enrollment and were diagnosed with the blood pressure criteria. We also observed higher cholesterol levels in the normal participants

Table 5. Clinical characteristics of the study population

	Control ($n=535$) Mean SD	Case ($n=749$) Mean SD	*P*-value
Age (years)	52.72 ± 9.88	55.74 ± 8.31	<0.0001
SBP (mmHg)	112.42 ± 10.97	119.47 ± 17.43	<0.0001
DBP (mmHg)	74.14 ± 7.41	77.14 ± 9.97	<0.0001
BMI (kg/m^2)	23.18 ± 2.57	25.01 ± 2.71	<0.0001
Glucose (mg/dl)	86.41 ± 10.17	99.08 ± 32.59	<0.0001
Total cholesterol (mg/dl)	187.94 ± 27.86	181.01 ± 39.68	0.00025
HDL (mg/dl)	48.43 ± 12.05	39.06 ± 9.54	<0.0001
Triglycerides (mg/dl)	116.11 ± 49.03	164.49 ± 118.62	<0.0001
LDL (mg/dl)	116.07 ± 27.60	110.14 ± 36.17	0.00120
$HOMA_{IR}$	1.55 ± 1.54	2.39 ± 2.49	<0.0001
Smoking (*n*)			
Non	138 (25.9%)	113 (15.0%)	<0.0001[a]
Ex	135 (25.4%)	466 (62.2%)	
Current	259 (48.7)	170 (22.8%)	
Disease			
Hypertension		359 (47.9%)	
Myocardial infarction		362 (48.3%)	
Diabetes mellitus		169 (22.6%)	
Hypercholesterolemia		42 (5.6%)	
Number of vessels			
One vessel, *n*		270 (36.1%)	
Two vessels, *n*		242 (32.3%)	
Three vessels, *n*		237 (31.6%)	

The values are mean ± SD. Values of P were obtained using Student's t test between CAD patients and controls. BMI, body mass index; DBP, diastolic blood pressure; SBP, systolic blood pressure. [a] P-values were obtained using χ^2 test between coronary artery disease patients and controls.

than in the CAD patients (Table 5). We believe that this observation is mainly due to the use of statins in CAD patients.

2.2. Coronary angiographic analysis

Coronary angiographies were performed via the femoral artery. Coronary angiography, however, was not performed in the healthy controls since our healthy controls were individuals with no evidence of coronary artery disease by symptoms, history or no invasive testing. All the angiographies were interpreted by the consensus of two independent observers. Significant CAD was defined as 50% or greater luminal stenosis of vessels more than 1.5mm in diameter. CAD patients were classified as 1-vessel or >1-vessel disease according to the number of epicardial coronary arteries involved. We did not analyze CAD as a quantitative trait by using percentage luminal stenosis because the number of coronary arteries involved better predicts disease severity than luminal stenosis. The results of angiography were used to classify the patients into two categories for some of the analyses: no significant CAD (normal subjects) versus significant CAD (patients with 1-vessel or >1-vessel disease).

2.3. Sequencing and genotyping of CAD candidate genes

We have sequenced all exons, exon–intron boundaries and the promoter region (approximately 1.0 kb) to identify genetic variations of candidate genes in 24 Korean DNA samples using an ABI Prism 3730 sequencer (Applied Biosystems, Foster City, California, USA). The Polyphred program (website:http://wwww.droog.gs.washington.edu/PolyPhred.html) was used to assemble the sequences and identify single nucleotide polymorphisms (SNPs) (96). All SNP data identified in this study are available at the

Korean SNP database (website:http://www.ngri.go.kr/SNP/). For genotyping of polymorphic sites, amplifying primers and probes were designed for the TaqMan assay (97). Primer Express (Applied Biosystems) was used to design both the PCR primers and the MGB TaqMan probes. One allelic probe was labeled with the FAM dye and the other with the fluorescent VIC dye. Typically, PCR was run in the TaqMan Universal Master mix without UNG (Applied Biosystems) at a primer concentration of 900nM and TaqManMGB-probe concentration of 200nM. The reaction was performed in a 384-well format in a total reaction volume of 5ml using 20 ng of genomic DNA. The plate was then placed in a thermal cycler (PE 9700; Applied Biosystems) and heated for 2 min at 50°C and for 10 min at 95°C, followed by 40 cycles of 95°C for 15 s and 60°C for 1 min. The TaqMan assay plate was transferred to a Prism 7900HT instrument (Applied Biosystems) where the fluorescence intensity of each well was read. Fluorescence data files from each plate were analyzed using automated software (SDS 2.2, Applied Biosystems).

2.4. Statistics

The χ^2 tests were used to determine whether individual variants were in equilibrium at each locus in the population (Hardy–Weinberg equilibrium). We employed a widely used measure of linkage disequilibrium between all pairs of biallelic loci, Lewontin's D′ (|*D*′|) and r^2 (98). Haplotypes and their frequencies were inferred using the software, HapAnalyzer, developed by Jung et al. (99). Quantitative clinical data were compared between patients with CAD and controls by the unpaired Student's t-test, whereas qualitative data were compared by the χ^2 tests. Genotype distribution of ADD1 SNPs among the CAD and the normal subjects was analyzed

with logistic regression models controlling for age as covariates. The association between genotypes or alleles of candidate genes and clinical variables was analyzed using one-way ANOVA. Statistical analysis for comparison between patients with CAD and normal subjects was performed using ANOVA followed by post hoc testing with Tukey's multiple comparisons test, using SPSS 12.0 software (SPSS Inc., Chicago, Illinois, USA). A level of $P<0.05$ was considered statistically significant.

3. Results

3.1. Identification of polymorphisms in 31 coronary artery disease candidate genes in the Korean population

In order to identify the genetic variations of CAD candidate genes in the Korean population, a total of 31 CAD candidate genes were selected for resequencing using 24 genomic DNA samples from 24 unrelated Koreans. All functional regions were sequenced, including all exons, exon–intron junctions and promoter regions (up to 1 kb upstream from the transcriptional start site) of the candidate genes. We identified a total of 445 genetic variants, 409 SNPs (323 known SNPs and 86 novel SNPs), 32 insertion/deletion polymorphisms and four microsatellites. Of the 445 genetic variants, 54 were in promoter regions, 14 in 5'-untranslated regions (UTRs), 84 in coding regions (44 synonymous cSNPs and 40 non-synonymous cSNPs), 221 in introns and 72 in 3'-UTRs. In the haplotype analysis, a total of 422 haplotypes, corresponding to an average of 13.6 haplotypes per gene, were inferred. The mean number of common haplotypes (frequency over 5%) per gene was 3.48 and, on average, common haplotypes represented 73.2%of chromosomes, indicating that a large proportion of

chromosomes were represented by the common haplotypes in our data set.

3.2. Association analysis of candidate genes with coronary artery disease in a Korean population

In order to investigate the functional polymorphisms of CAD candidate genes, association analysis was performed using non-synonymous cSNPs, which can result in altered amino acids in protein sequences. Among 40 non-synonymous cSNPs identified by sequencing 24 unrelated Korean DNA samples, we tested 29 non-synonymous cSNPs which have been successfully developed for the TaqMan assay. Some non-synonymous SNPs were excluded in genotyping due to complete linkage with other SNPs (correlation coefficient r^2= 1). Association analysis was carried out using 749 CAD subjects and 535 normal healthy controls. The allele frequencies of 29 non-synonymous cSNPs in Korean subjects (n=1284) are shown in Table 2. None of the non-synonymous cSNPs of candidate genes showed a significant association with CAD, except the ADD1 Gly460Trp (+29017G/T) polymorphism (P=0.007) (Table 6).

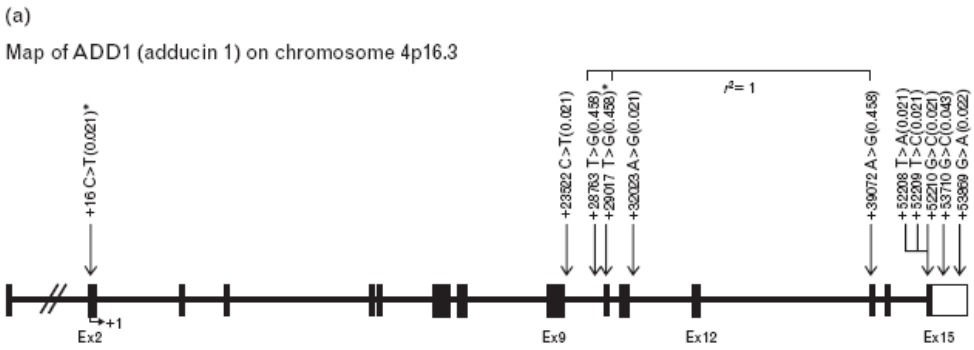
3.3. Polymorphisms of ADD1 gene and association of ADD1 Gly460Trp polymorphism with coronary artery disease in the Korean population

By resequencing the ADD1 gene using 24 unrelated Korean individuals' DNA samples, we identified a total of 11 SNPs, including six in coding regions (five non-synonymous SNPs and one synonymous SNP), two in 3'-UTRs and three in intron regions. The positions and frequencies of SNPs identified in the ADD1 gene are shown

Table 6. Genotypes and allele frequencies of 29 non-synonymous single nucleotide polymorphisms in Korean subjects

Gene Name	SNP ID (rs or new)	Position	Amino acid change	Allele	Allele frequencies determined by sequencing ($n = 24$)[a]		Allele frequencies determined by TaqMan assay ($n = 1284$)[b]		χ^2-test: *P*-value (749 CAD vs. 535 normal)[c]
ADD1	rs2295497	exon2	(CGT)Arg6Cys(TGT)	C/T	0.979(C)	0.021(T)	0.983(C)	0.017(T)	0.831
	rs4961	exon10	(GGG)Gly460Trp(TGG)	G/T	0.542(T)	0.458(G)	0.599(T)	0.401(G)	0.007*
ADRA1A	new	exon1	(ATC)Ile137Thr(ACC)	T/C	0.979(T)	0.021(C)	0.999(T)	0.001(C)	1.00
	rs3730287	exon1	(GGG)Gly247Arg(AGG)	G/A	0.979(G)	0.021(A)	0.968(G)	0.032(A)	1.00
	rs1048101	exon2	(CGC)Arg347Cys(TGC)	C/T	0.896(C)	0.104(T)	0.878(C)	0.122(T)	0.326
ADRB2	rs1042713	exon1	(AGA)Arg16Gly(GGA)	A/G	0.583(A)	0.417(G)	0.542(A)	0.458(G)	0.984
	rs1042714	exon1	(CAA)Gln27Glu(GAA)	C/G	0.854(C)	0.146(G)	0.906(C)	0.094(G)	0.892
ADRB3	rs4994	exon1	(TGG)Trp64Arg(CGG)	T/C	0.771(T)	0.229(C)	0.853(T)	0.147(C)	0.356
ARTS1	rs3734016	exon2	(GAG)Glu56Lys(AAG)	G/A	0.812(G)	0.188(A)	0.804(G)	0.196(A)	0.093
	rs26653	exon2	(CCT)Pro127Arg(CGT)	C/G	0.531(C)	0.469(G)	0.505(C)	0.495(G)	0.337
	rs26618	exon5	(ATA)Ile276Met(ATG)	A/G	0.792(A)	0.208(G)	0.743(A)	0.257(G)	0.951
	new	exon5	(ATA)Ile299Leu(CTA)	A/C	0.979(A)	0.021(C)	0.995(A)	0.005(C)	0.316
	rs2287987	exon6	(ATG)Met349Val(GTG)	A/G	0.896(A)	0.104(G)	0.960(A)	0.040(G)	1.00
	rs30187	exon11	(AGG)Arg528Lys(AAG)	G/A	0.562(G)	0.438(A)	0.518(G)	0.482(A)	0.242
	rs10050860	exon12	(GAC)Asp575Asn(AAC)	G/A	0.891(G)	0.109(A)	0.961(G)	0.039(A)	1.00
	rs27044	exon15	(CAA)Gln730Glu(GAA)	C/G	0.583(C)	0.417(G)	0.550(C)	0.450(G)	0.152
EDN1	rs5370	exon5	(AAG)Lys198Asn(AAT)	G/T	0.729(G)	0.271(T)	0.744(G)	0.256(T)	0.794
MTHFR	rs1801133	exon5	(GCC)Ala222Val(GTC)	C/T	0.562(C)	0.438(T)	0.563(C)	0.437(T)	0.845
	rs1801131	exon8	(GAA)Glu429AlA(GCA)	A/C	0.848(A)	0.152(C)	0.824(A)	0.176(C)	0.446
	new	exon12	(ACG)Thr653Met(ATG)	C/T	0.978(C)	0.022(T)	0.999(C)	0.001(T)	1.00
NOS1	new	exon19	(GAT)Asp966Val(GTT)	A/T	0.977(A)	0.023(T)	0.998(A)	0.001(T)	0.21
NOS2A	rs2297518	exon16	(TCG)Ser608Leu(TTG)	C/T	0.875(C)	0.125(T)	0.887(C)	0.113(T)	0.264
NPPA	rs5063	exon1	(GTG)Val32Met(ATG)	G/A	0.938(G)	0.062(A)	0.907(G)	0.093(A)	0.321
SELE	rs5368	exon9	(CAT)His468Tyr(TAT)	C/T	0.750(C)	0.250(T)	0.783(C)	0.217(T)	0.935
	rs5355	exon11	(CTC)Leu575Phe(TTC)	C/T	0.938(C)	0.062(T)	0.943(C)	0.057(T)	0.996
SLC14A2	new	exon2	(ACA)Thr33Ser(TCA)	A/T	0.979(A)	0.021(T)	0.986(A)	0.014(T)	0.561
	rs1484873	exon4	(GTC)Val132Ile(ATC)	A/G	0.688(G)	0.312(A)	0.800(G)	0.200(A)	0.695
	rs9960464	exon12	(CGA)Arg510Gln(CAA)	G/A	0.646(G)	0.354(A)	0.679(G)	0.321(A)	0.548
	rs3745009	exon20	(GCC)Ala880Thr(ACC)	G/A	0.604(G)	0.396(A)	0.564(G)	0.436(A)	0.711

CAD, coronary artery disease; SNP, single nucleotide polymorphism. [a] Allele frequencies determined by direct DNA sequencing using DNA samples from 24 Koreans. [b] Allele frequencies determined by Taqman assay using DNA samples from 1284 Koreans including 749 CAD patients and 535 normal controls. [c] P-values were obtained using Chi-square test between CAD patients (n=749) and controls (n=535).



(b)

Haplotypes of ADD1

Haplotype	+16 C>T	+23922 C>T	+28763 T>G	+29017 T>G	+32023 A>G	+39072 A>G	+52208 T>A	+52209 T>C	+52210 G>C	+53710 G>C	+53869 G>A	
ht1	C	C	T	G	A	C	T	T	G	G	G	0.5208333
ht2	C	C	G	T	A	G	T	T	G	G	G	0.3333334
ht3	C	C	G	T	A	G	T	T	G	C	G	0.0416667
ht4	C	T	G	T	A	G	T	T	G	C	A	0.0208333
ht5	C	C	G	T	A	G	A	C	C	G	G	0.0208333
ht6	C	C	G	T	A	G	T	T	G	G	A	0.0208333
ht7	T	C	G	T	A	G	T	T	G	G	G	0.0208333
ht8	C	C	T	G	G	C	T	T	G	G	G	0.0208333

Fig. 9. Gene map, haplotypes and linkage disequilibrium coefficient among ADD1 single nucleotide polymorphisms. (a) Coding exons are marked by black blocks and 5'- and 3'-untranslated regions (UTRs) by white blocks. The frequencies of polymorphisms without large-scale genotyping were based on sequencing data for SNP discovery (n=24). Asterisks (*) indicate SNPs that were genotyped in a larger-scale association test. The first base of the translation start site is denoted as nucleotide plus one (reference sequence of ADD1 on chromosome 4q16.3; NT_006081). (b) Haplotypes of ADD1 in the Korean population.

in Fig. 9a. Only two common (frequency >0.05) haplotypes (ht1 and ht2) of ADD1 were constructed (Fig. 9B). The linkage disequilibrium coefficients (|*D'*|) and r^2 among the SNPs were calculated for all of the study subjects (n=24) (data not shown).

As shown in Table 6 and Table 7, two non-synonymous cSNPs of the ADD1 gene (+16C/T and +29017G/T coding Arg6Cys and Gly460Trp, respectively) were tested for association with CAD in out study population. Two other non-synonymous cSNPs (+28763T/G and +39072A/G) were excluded from the genotyping due to complete linkage with +29017G/T (correlation coefficient r^2=1). Associations of ADD1 polymorphisms with the risk of CAD were analyzed using logistic analysis in patients and normal subjects. The G allele of ADD1 (+29017G/T) encoding Gly was significantly associated with decreased risk of CAD in co-dominant, dominant, and recessive models (odds ratio, OR= 0.71-0.81, *p*=0.01-0.04) (Table 3). On the other hand, the T allele of ADD1 Gly460Trp (+29017G/T) encoding Trp showed increased risk of CAD in all genetic models (OR= 1.23-1.39, data not shown). The genotype distribution in the study population was in agreement with the Hardy–Weinberg equilibrium (*p* >0.05). In the haplotype association analysis, two of the common haplotypes (freq=0.98) among the five haplotypes, ht1 [CT] (freq= 0.60) and ht2 [CG] (freq= 0.38), were also strongly associated with CAD (*p*= 0.009-0.040) (Table 7). The ht1 carrying risk T allele was associated with risk of CAD (OR= 1.24-1.40, *p*= 0.009-0.04), whereas the ht2 carrying G allele was protective of CAD (OR= 0.73-0.82, *p*= 0.02-0.05). These results show that the T allele of ADD1 Gly460Trp (+29017G/T) polymorphism is associated with risk of CAD.

Table 7. Logistic analysis of ADD1 polymorphisms in coronary artery disease patients and normal subjects

Locus[a]	Protein consequence	Genotype	Patients ($n=739$)	Control ($n=535$)	χ^2 test OR (95% CI)	P^b	Co-dominant		Dominant		Recessive	
							OR (95% CI)	P	OR (95% CI)	P	OR (95% CI)	P
		C/C	724 (96.7%)	514 (96.1%)								
+16C/T	Arg6Cys	C/T	25 (3.3%)	18 (3.4%)	1.13 (0.62–2.04)	0.69	0.77 (0.43–1.37)	0.37	0.79 (0.43–1.43)	0.43	NA	NA
		T/T	0 (0%)	1 (0.5%)								
		G/G	108 (14.5%)	104 (19.4%)								
+29017G/T	Gly460Trp	G/T	349 (46.8%)	254 (47.5%)	0.80 (0.68–0.94)	0.007	0.81 (0.69–0.95)	0.01	0.78 (0.62–0.99)	0.04	0.71 (0.53–0.96)	0.02
		T/T	289 (38.7%)	177 (33.1%)								
		ht1/ht1	292 (40.0%)	177 (33.1%)								
ht1 (CT)		ht1/–	349 (46.6%)	254 (47.5%)	1.26 (1.07–1.47)	0.005	1.24 (1.05–1.46)	0.009	1.40 (1.04–1.90)	0.03	1.28 (1.01–1.63)	0.04
		–/–	108 (14.4%)	104 (19.4%)								
		ht2/ht2	98 (13.1%)	94 (17.6%)								
ht2 (CG)		ht2/–	344 (45.9%)	253 (47.3%)	0.80 (0.68–0.94)	0.008	0.82 (0.70–0.96)	0.02	0.79 (0.63–0.99)	0.04	0.73 (0.53–0.99)	0.05
		–/–	307 (41.0%)	188 (35.1%)								

CI, confidence interval; NA, not available; OR, odds ratio. [a] Calculated from the translational start site. [b]Chi-square test.

Logistic regression models were used for calculating odds ratios (95% confidence interval) and corresponding P-values for each single nucleotide polymorphism site and three alternative models (co-dominant, dominant, and recessive) controlling age using SPSS.

3.4. Association of ADD1 Gly460Trp polymorphism with the number of vessels in coronary artery disease and blood pressure

We also investigated the role of ADD1 polymorphisms in the extent of vessel involvement in coronary artery disease. The ADD1 Gly460Trp (+29017G/T) genotypes and allele frequencies in CAD patients with 1-vessel or >1-vessel obstruction were compared with the control participants. Angiography revealed that 535 normal participants (41.7%) had no CAD, whereas one vessel disease (1-vessel disease) and two and three-vessel disease (>1-vessel disease) were diagnosed in 270 (21%) and 479 (37.3%) patients, respectively (Table 8). The proportion of patients carrying the TT genotype of ADD1 Gly460Trp (+29017G/T) was high in those with 1-vessel disease (39.0%) and >1-vessel disease (38.4%), but low among normal subjects (33.1%). Conversely, the GG genotype of the ADD1 Gly460Trp (+29017G/T) polymorphism (a protective allele for CAD) was high in normal subjects (19.4%) but low among subjects with >1-vessel disease (13.2%) (Table 4). The GG genotype of ADD1 Gly460Trp (+29017G/T) was significantly associated with a decreased extent of coronary disease (OR, 0.64–0.79; P=0.01), whereas the TT genotype was associated with increased extent of coronary disease (OR, 1.56; 95% CI 1.10–2.21; P=0.01; dominant model) of >1-vessel disease after adjustment for age (Table 9).

In addition, we also tested association of ADD1 polymorphisms with other risk factors of CAD such as body mass index, blood pressure, serum lipids and so on. Interestingly, the TT genotype (the risk allele of CAD) of ADD1 Gly460Trp was significantly associated with systolic blood pressure (SBP) in normal subjects (P=0.02) (Table 10) but not in the CAD patients (P=0.09). We further tested the association in the CAD-normotensive and CAD-hypertensive groups. We found a significant

Table 8. ADD1 Gly460Try (+29017G/T) polymorphism and extent of coronary artery disease

Genotype	Normal subject	1-vessel disease	>1-vessel disease
GG	104 (19.4%)	45 (16.7%)	63 (13.2%)
GT	254 (47.5%)	120 (44.3%)	232 (48.4%)
TT	177 (33.1%)	105 (39.0%)	184 (38.4%)
Total	535	270	479

Table 9. Association of ADD1 Polymorphisms and Extent of coronary artery disease

	Control versus 1-vessel disease						Control versus >1-vessel disease					
	Co-dominant		Dominant		Recessive		Co-dominant		Dominant		Recessive	
Locus	OR (95% CI)	*P*	OR (95% CI)	*P*	OR (95% CI)	*P*	OR (95% CI)	*P*	OR (95% CI)	*P*	OR (95% CI)	*P*
+16C/T	0.76 (0.35–1.66)	0.50	0.78 (0.35–1.74)	0.55	N/A	1.00	0.79 (1.03–1.06)	0.48	0.81 (0.41–1.58)	0.53	N/A	1.00
+29017T/G	0.85 (0.69–1.04)	0.11	0.77 (0.56–1.04)	0.09	0.85 (0.58–1.26)	0.42	0.79 (0.66–0.95)	0.01	0.80 (0.61–1.04)	0.92	0.64 (0.45–0.91)	0.01
ht1 (CT)	1.19 (0.97–1.47)	0.10	1.18 (0.80–1.74)	0.41	1.32 (0.97–1.79)	0.08	1.27 (1.06–1.52)	0.01	1.57 (1.11–2.22)	0.01	1.27 (0.97–1.65)	0.08
ht2 (CG)	0.85 (0.69–1.05)	0.13	0.77 (0.57–1.04)	0.09	0.89 (0.58–1.31)	0.51	0.80 (0.66–0.96)	0.02	0.79 (0.61–1.03)	0.08	0.65 (0.45–0.94)	0.02

Table 10. Association between Gly460Trp (+29017T>G) polymorphism and biochemical measures in control subjects

	Genotype			*P*-value		
Phenotype	T/T ($n=177$)	T/G ($n=254$)	G/G ($n=104$)	Co-dominant	Dominant	Recessive
SBP (mmHg)	114.1 ± 10.9	112.1 ± 10.5	110.5 ± 12.0	0.02	0.02	0.05
DBP (mmHg)	74.6 ± 7.2	74.2 ± 7.4	73.3 ± 7.8	0.37	0.34	0.19
BMI (kg/m^2)	23.3 ± 2.5	23.1 ± 2.5	23.2 ± 2.8	0.77	0.49	0.95
Blood glucose (mg/dl)	87.7 ± 12.7	85.4 ± 8.8	86.8 ± 8.0	0.07	0.08	0.71
Total cholesterol (mg/dl)	187.2 ± 25.5	188.3 ± 28.6	188.3 ± 30.0	0.91	0.65	0.88
HDL (mg/dl)	48.7 ± 12.2	48.3 ± 12.1	48.3 ± 11.7	0.94	0.74	0.90
Triglycerides (mg/dl)	119.7 ± 50.8	114.5 ± 47.7	113.9 ± 49.4	0.49	0.25	0.61
LDL (mg/dl)	114.7 ± 24.2	116.5 ± 28.0	117.3 ± 31.9	0.72	0.40	0.62
$HOMA_{IR}$	1.7 ± 2.0	1.5 ± 1.4	1.4 ± 0.8	0.34	0.25	0.30

Data are the means ± SD. BMI, body mass index; DBP, diastolic blood pressure; SBP, systolic blood pressure. P-values were obtained by ANOVA test.

association in the CAD hypertensive group (P=0.03), whereas association was still not observed in CAD-normotensive participants (P=0.35). The pattern of mean SBP, however, was changed in CAD patients with the highest mean SBP in the TG-heterozygote of the CAD-hypertensive group, but no change of mean SBP in the CAD -normotensive group. This result is most likely due to the patients taking the antihypertensive drug. On the other hand, no effect of ADD1 polymorphism was observed for differences in body mass index, serum lipids, or blood glucose levels. These data suggest that the ADD1 Gly460Trp polymorphism possibly influences the development of CAD by affecting blood pressure rather than other conventional risk factors of CAD.

4. Discussion

In this study, we examined the relationship between polymorphisms and CAD using genetic association analysis of candidate genes in CAD patients. Initially, we identified a total of 445 genetic variants, including 409 single nucleotide polymorphisms (SNPs) in the selected 31 CAD candidate genes for association study with CAD. We tested 29 non-synonymous SNPs identified in candidate genes for large-scale genotyping (n=1284), since non-synonymous SNPs can potentially alter the function of the encoded proteins, subsequently leading to the development of disease. Interestingly, only a polymorphism of the ADD1 gene (Gly460Trp) was associated with CAD (Table 6). The Gly460Trp polymorphism of the ADD1 gene was previously shown to be significantly associated with the presence of hypertension, cardiovascular disease and myocardial infarction (100–103). In our study, the Gly460Trp (+29017G/T) polymorphism of ADD1 was significantly associated with CAD and

systolic blood pressure in the Korean population. The G allele of ADD1 Gly460Trp (+29017G/T) polymorphism was significantly associated with decreased risk of CAD (OR, 0.71–0.81, *P*=0.01–0.04), whereas the T allele of ADD1 Gly460Trp (+29017G/T) polymorphism was associated with increased risk of CAD (OR, 1.23–1.40; *P*=0.01–0.04). In addition, the ht1 [CT] carrying risk T allele showed a significant association with increased risk of CAD (*P*=0.009–0.040) and the ht2 [CG] carrying protective G allele was associated with decrease risk of CAD (*P*=0.02–0.04) (Table 7). We also observed a significant association of the ADD1 Gly460Trp polymorphism with the number of closed coronary artery vessels in CAD patients (*P*=0.01) (Table 9). Interestingly, the severity of disease differed significantly with regard to the presence of the G allele of ADD1 Gly460Trp (+29017G/T). These results indicate that ADD1 Gly460Trp polymorphism was associated with CAD phenotypes, including the number of closed vessels and presence of coronary artery stenosis in the CAD patients.

In this study, we also hypothesized that the increased risk associated with the T allele of theADD1 gene polymorphism in development of CAD might be due to effects on blood pressure. Our results provide further support for this hypothesis. We observed that the T risk allele was also associated with SBP in the control subjects (*P*=0.02) in the Korean population (Table 10). We also tested the association of ADD1 with SBP in the CAD patients. We found a significant association in CAD-hypertensive group (*P*=0.03), whereas no association was observed in CAD-normotensive participants (*P*=0.35). The pattern of mean SBP, however, was changed in CAD patients with the highest mean SBP in TG-heterozygote. This result is most likely due to patients taking the antihypertensive drug. Consistent with our results, the same polymorphism

(Gly460Trp) of ADD1 has been shown to be involved in increased blood pressure (104-106). The mechanism by which ADD1 increases blood pressure is not known. ADD1 may, however, stimulate Na^+-K^+-ATPase, promoting sodium re-absorption by renal tubular cells (107-108, 116), since ADD1 is a protein of the cytoskeleton involved in the function of the sodium/potassium pump in the kidney. Alteration in renal sodium handling caused by the GG genotype of Gly460Trp of ADD1 will increase blood pressure sensitivity to sodium intake, resulting in increased risk for development of low-renin hypertension (109, 110). Several studies have shown a strong interaction betweenADD1 variants and salt intake in relation to blood pressure (104). Also, the frequency of the ADD1 Gly460Trp allele varies in different populations. The TT genotype was found in 59.9% of the subjects in our Korean population. These frequencies are very similar in East Asian populations such as Japanese and Chinese populations (100,111–113). Considering that the frequency of the TT genotype is higher in the East Asian population in contrast to a lower frequency in Caucasians (18% in north Italy, 20% in France, and 27% in Scotland) (100,114-116) the Gly460Trp allele variant may have a particularly significant influence on cardiovascular diseases among East Asian populations.

In summary, we demonstrated that the ADD1 Gly460Trp polymorphism is significantly associated with an increased risk of CAD in Korean men. We also confirmed that the same ADD1 Gly460Trp polymorphism is associated with blood pressure as well as the extent of closed coronary vessels in CAD patients. Our data show that genes involved in susceptibility to hypertension-related gene ADD1 are associated with CAD, supporting the hypothesis that genes associated with blood pressure can increase the risk of CAD.

V. The METABOLIC SYNDROME IS ASSOCIATED WITH THE Gly460Trp α-ADDUCIN POLYMORPHISM AND INCREASED RISK OF CORONARY ARTERY DISEASE

1. Introduction

The metabolic syndrome (MetS) is a complex, multifactorial disorder characterized by increased body weight, insulin resistance, elevated plasma triglyceride levels, low HDL-cholesterol, high blood pressure, and glucose homeostasis. Its prevalence in Western countries is increasing, largely because of risky behavior (117). The MetS is considered to be one of the most important risk factors for the development of cardiovascular disease (CVD) (118, 119) as it is associated with a three-fold increased risk of developing coronary heart disease (CHD), myocardial infarction, and stroke, and a three- to five-fold increased risk of death due to adverse cardiovascular events (120, 121). Patients with the MetS also have an enhanced propensity to develop arteriosclerosis prematurely (124). Since hypertension (a classic characteristic of the MetS) has also been associated with a two-fold higher risk of coronary artery disease (CAD) (122), it is important to identify the genes that independently confer susceptibility to MetS and to CAD. Moreover, since genetic ethnological studies have suggested that Hispanics and Asians are at greater risk of developing the MetS than other races (123), it is essential that the MetS-related polymorphisms in each ethnic group are identified.

Adducin is a heterodimeric cytoskeleton protein composed of three subunits denoted as α, β, and γ. These subunits are encoded by ADD1, ADD2, and ADD3, respectively, which are closely related genes that map to different chromosomes (125).

The α-subunit (ADD1), whose gene is located on chromosome 4, affects sodium and volume homeostasis by interacting with the epithelial sodium channel, the sodium-potassium-chloride co-transporter, and sodium, potassium adenosine triphosphatase (125-127). A series of studies performed in parallel has indicated that the Gly460Trp nucleotide polymorphism in ADD1, where glycine (Gly) at amino acid position 460 is substituted by tryptophan (Trp), is associated with elevated NA^+-K^+ pump activity (128), blood pressure (129), susceptibility to renal failure progression (130), and risk of salt-sensitive hypertension, vascular pathology, and CVD (131, 132). Here, we tested prospectively whether the ADD1 Gly460Trp polymorphism is associated with the MetS and/or CAD.

2. Materials and Methods

2.1 Subjects and measures

The study subjects were recruited from the Cardiovascular Research Center of Yonsei University. On the basis of their clinical symptoms and a physical examination, the 1453 unrelated male Korean individuals were divided into MetS (n=45), MetS with CAD (n=400), CAD only (n=340), and normal healthy control (n=668) groups. A subject was deemed to have the MetS on the basis of National Cholesterol Education Program (NCEP) III recommendations (133, 134), namely, if he had three or more of the following characteristics: (1) abdominal obesity (waist circumference >90 cm in men; the International Obesity Task Force criteria for Asian-Pacific populations were used to determine these waist circumference criteria (134); (2) triglycerides ≥1.7 mmol/1 (150 mg/dl); (3) HDL-cholesterol <1.0 mmol/1 (40 mg/dl); (4) blood pressure

≥130/85 mmHg or taking antihypertensive medication; (5) fasting glucose ≥6.1 mmol/1 (110 mg/dl). Normal subjects with a history of hypertension, hypercholesterolemia, CAD, diabetes, myocardial infarction, or the MetS were excluded. The CAD patients were enrolled after providing informed consent. Patients with the following characteristics were excluded from participation: valvular (heart) disease, peripheral vascular disease, significant systemic disease, history of inflammatory disease, and history of congestive heart failure with left ventricular ejection fraction (LVEF) <30%. The characteristics of the MetS, MetS with CAD, and CAD groups in terms of conventional risk factors are summarized in Table 11. For one analysis, the normal subject group was subdivided into subjects that lacked all five MetS criteria (n=313) and subjects that met one or two of these criteria (n=355) while the same MetS only group was divided into patients that satisfied three (n=41), four (n=4), or five (n=0) MetS criteria.

2.2 Coronary angiographic analysis

Coronary angiographies were performed *via* the femoral artery. All angiography results were interpreted by two independent observers until consensus was reached. Significant CAD was defined as ≥50% luminal stenosis of vessels >1.5 mm in diameter. CAD patients were classified into 1-, 2-, and 3-vessel disease groups depending on the number of epicardial coronary arteries involved.

2.3 Sequencing and genotyping of the ADD1 gene

To identify the genetic variation of the ADD1 gene in 24 Korean DNA samples, we used an ABI Prism 3730 sequencer (Applied Biosystems, Foster City, CA, USA)

to

Table 11. Clinical characteristics of control and patient groups

	Control (n=668)			MetS (n=45)			MetS With CAD (n=400)			CAD (n=340)		
	Mean	±	SD	Mean	±	SD	Mean	±	SD	Mean	±	SD
Age (year)	52.30	±	9.71	50.93	±	10.07	55.56	±	8.14*	55.9	±	8.50*
SBP (mmHg)	113.54	±	11.80	123.29	±	10.43*	119.53	±	16.78*	119.22	±	18.11*
DBP (mmHg)	75.39	±	8.37	81.4	±	8.718*	76.81	±	9.58‡	77.48	±	10.28†
BMI (kg/m^2)	23.36	±	2.60	26.67	±	3.50*	25.86	±	2.51*	24.02	±	2.59*
Glucose (mg/dL)	86.65	±	9.96	96.36	±	23.25†	106.63	±	40.16*	90.39	±	16.88*
Total cholesterol (mg/dL)	197.41	±	39.99	208.71	±	40.61	183.21	±	40.68*	178.38	±	38.51
HDL (mg/dL)	48.52	±	11.93	36.25	±	7.02*	34.57	±	6.98*	44.41	±	9.43*
Triglycerides (mg/dL)	142.84	±	106.84	229.49	±	95.75*	208.25	±	141.68*	113.72	±	48.20*
LDL (mg/dL)	120.74	±	31.99	129.89	±	37.88	109.21	±	36.80*	111.14	±	35.57*

The values shown are means ± SD. $^{*}p<0.0001$, $^{\dagger}p<0.001$, $^{\ddagger}p<0.05$, statistically significant compared to the control group, as determined by using Student's t-test. SBP, systolic blood pressure; DBP, diastolic blood pressure; BMI, body mass index; HDL, high density lipoproteins; LDL, low density lipoproteins

sequence all ADD1 exons, the exon-intron boundaries, and the 5' promoter region (~1.0 kb). The Polyphred program (http://wwww.droog.gs.washington.edu/PolyPhred.html) was used to assemble the sequences and identify single nucleotide polymorphisms (SNPs) (135). The list of SNPs identified by this study is available at the Korean SNP database (http://www.ngri.go.kr/SNP/). To genotype the polymorphic sites in other Korean subjects, amplifying primers and probes were designed and used in TaqMan assays (136). Primer Express (Applied Biosystems) was used to design both the PCR primers and the MGB TaqMan probes. One allelic probe was labeled with the FAM dye while the other was labeled with the fluorescent VIC dye. Typically, PCR was run in the TaqMan Universal Master mix without UNG (Applied Biosystems) at a primer concentration of 900 nM and a TaqMan MGB-probe concentration of 200 nM. The reaction was performed in a 384-well format in a total reaction volume of 5 μl using 20 ng of genomic DNA. The plate was then placed in a thermal cycler (PE 9700, Applied Biosystems), heated for 2 min at 50°C and then for 10 min at 95°C, after which 40 cycles of 95°C for 15 s and 60°C for 1 min were performed. The TaqMan assay plate was transferred to a Prism 7900HT instrument (Applied Biosystems) where the fluorescence intensity of each well was read. Fluorescence data files from each plate were analyzed by using automated software (SDS 2.2, Applied Biosystems).

2.4 Statistics

χ^2 tests were used to determine whether individual variants were in equilibrium at each locus in the population (Hardy–Weinberg equilibrium). Quantitative clinical

data of patients and controls were compared by using the unpaired Student's t-test, whereas qualitative data were compared by χ^2 tests. Genotype distribution of polymorphisms among the patients and the normal subjects was analyzed with logistic regression models controlling for age as a covariate. The association between genotypes of candidate genes and clinical variables was analyzed by using one-way ANOVA. We also examined how the number of MetS risk factors (from one to all five) in the CAD patients associate with the number of affected vessels and the degree of coronary artery stenosis by using the Mantel-Haenszel test for trends. For this, SPSS 12.0 software (SPSS Inc., Chicago, IL, USA) was employed. A level of $p<0.05$ was considered to be statistically significant.

2.5 SIFT and PolyPhen

For predictions on the effect of amino acid changing mutations, the stand-alone versions of two prediction programs were used: SIFT (v2.0; Sorting Intolerant From Tolerant) (150); and PolyPhen (command-line version) (151); in combination with the SwissProt/TrEMBL+PIR databases (downloaded October 25, 2004 from; ftp.expasy. org), and BLAST parameters with an expectation cutoff of 1E-04. We sorted the outcome of the two analyses in three categories: category 1 is 'Affected' by SIFT and 'Possibly/Probably Damaging' by Polyphen; category 2 is 'Tolerated' by SIFT and 'Possibly/Probably Damaging' by Polyphen or 'Affected' by SIFT and 'Benign' by Polyphen; category 3 is 'Tolerated' by SIFT and 'Benign' by Polyphen.

2.6 Korean SNP database

Most of the SNPs described in this study are listed in the Korean SNP database

(http://www.ksnp.ngri.re.kr/SNP/index.jsp), which was constructed in the Center for Genome Sciences (Korean National Institute of Health).

3. Results

3.1. Characteristics of the study population

The baseline characteristics of the patients and normal controls are summarized in Table 11. The MetS was diagnosed when a person satisfied three or more of the five NCEP criteria. The patients were divided into the MetS alone, the MetS with CAD, and the CAD alone groups.

3.2 ADD1 gene polymorphisms in the Korean population and the association between the ADD1 Gly460Trp polymorphism and the MetS

We sequenced the ADD1 gene in genomic DNA samples from 24 unrelated Koreans. All functional regions were sequenced, namely, all exons, exon-intron junctions, and the promoter region (up to 1 kb upstream from the transcriptional start site). In total, we identified 11 SNPs, five of which have not been identified previously. Of the 11 SNPs, six were in coding regions (1 synonymous cSNP and 5 non-synonymous cSNPs), three were at the exon-intron junctions, and two were in the 3'-UTR (Fig. 10).

To determine whether the five non-synonymous ADD1 cSNPs, which result in amino acid sequence changes, were associated with susceptibility or resistance to the MetS, we performed association analyses. For this, 1453 Koreans, of whom 45 had MetS alone, 400 had MetS with CAD, 340 had CAD alone, and 668 were normal

controls, were genotyped by TaqMan assays. Four of the non-synonymous cSNPs occurred with minor allele frequencies (<5%) (Fig. 1) and were not significantly associated with the MetS (data not shown). However, the frequency of the T allele of the non-synonymous Gly460Trp (+29017G/T) cSNP was 60% in the Korean population (Table 12). Analysis of the dbSNP database revealed that the frequency of the ADD1 Gly460Trp allele varies in different ethnic populations (Table 12).

We then performed logistic analysis with the (+29017G/T) non-synonymous ADD1 cSNP to determine its association with the MetS and/or CAD in the Korean population (Table 13). The GG allele of ADD1 (+29017G/T) encoding Gly was significantly associated with a decreased risk of MetS in co-dominant and dominant models (odds ratio, OR=0.39-0.58, p=0.02). The T allele encoding Trp was associated with a significantly increased risk of MetS and/or CAD (OR= 1.73-2.10, data not shown) in the recessive model for MetS patients with CAD (OR=0.68, p=0.03) and co-dominant and dominant models for CAD only patients (OR=0.74-0.81, p=0.03) (Table 13). Thus, the T allele of the ADD1 (+29017G/T) polymorphism is associated with an increased risk of developing MetS and/or CAD.

3.3 Relationship between the ADD1 (+29017G/T) genotype and the number of MetS criteria

A patient was defined as having MetS if he had at least three of five NCEP criteria. Many of the normal control subjects also had one or two of the five criteria. We determined the relationship between the ADD1 (+29017G/T) genotype and the number of metabolic risk factors in the MetS only and normal control subjects. As shown in Fig. 11, 20.7% of subjects with no criteria had the GG allele, but this

frequency decreased to 18.6%, 15.9%, 12.2%, and 0% as the number of criteria increased to one, two, three and four criteria, respectively. The TT genotype also

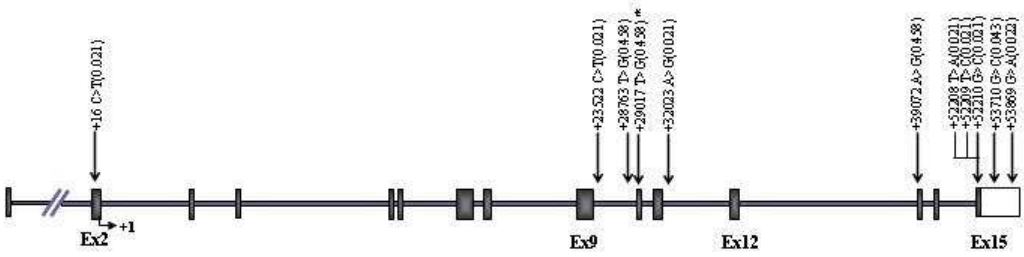


Fig. 10. Gene structure of ADD1 and location of SNPs found in Koreans. The coding exons are indicated by black blocks while the 5'- and 3'-untranslated region (UTR) is shown by a white block. The frequency of each polymorphism in the 24 Koreans is shown in brackets behind the arrow. The asterisk (*) indicates the SNP that was genotyped in 1453 Korean MetS and/or CAD patients and normal subjects. The first base of the translation start site is denoted as nucleotide +1 (the ADD1 reference sequence on chromosome 4q16.3 has the accession number NT_006081).

Table 12. Polymorphisms in the ADD1 gene and their frequencies in Korean and other ethnic populations

Gene	Locus[a]	rs#	Region	Protein Consequence	Genotype			HWE[b]	Korean[c]	African-American[c]	Caucasian[c]
					C/C*	C/R*	R/R*		(n=1453)	(n=50)	(n=50)
ADD1	+29017T/G	rs4961	Exon 10	Trp460Gly	531	682	240	0.380	0.400 (G)	0.109 (T)	0.167 (T)

*C/C, C/R, and R/R indicate homozygotes for the common allele (C/C), heterozygotes (C/R), and homozygotes for the rare allele (R/R), respectively.

[a]Calculated from the translational start site.

[b]*p*-value for Hardy-Weinberg equilibrium.

[c]Ethnic differences in rare allele frequency were determined by comparing our data with data in dbSNP (http://www.ncbi.nlm.nih.gov/SNP/).

tended to increase with the number of metabolic abnormalities. We also found a relationship between the ADD1 (+29017G/T) genotype and the severity of MetS (p for trend=0.02 when compared by using the Mantel–Haenszel analysis). Thus, the Gly460Trp polymorphism of the ADD1 gene was significantly associated with MetS severity.

3.4 Relationship between the number of MetS criteria and CAD severity

We also analyzed the association between the number of MetS risk factors (from zero to four) and the degree of coronary artery stenosis (i.e. the number of vessels affected) in the 740 CAD patients (400 MetS with CAD + 340 CAD only patients). As shown in Fig. 12, severe CAD was particularly prevalent among patients who satisfied many of the five MetS criteria. Indeed, the MetS criteria numbers correlated positively with the number of vessels affected (p for trend=0.001). Thus, MetS severity was associated with CAD severity. Since the T allele of the Gly460Trp polymorphism was also significantly associated with increased MetS severity (Fig. 11), these observations suggest that the ADD1 Gly460Trp genotype increases MetS severity and that this leads to an increased risk of severe CAD.

3.5 Association between the ADD1 Gly460Trp polymorphism and individual MetS criteria

We also tested the association of the ADD1 Gly460Trp polymorphism in the 668 normal control subjects with individual MetS criteria, namely, BMI, blood pressure, and serum lipid, triglyceride, and glucose levels. The T allele (the MetS and CAD risk allele) of ADD1 Gly460Trp was significantly associated with abnormal systolic blood

Table 13. Logistic analysis of ADD1 polymorphisms in normal subjects and MetS patients with or without CAD

Subjects	Genotype	Control	Patients	χ^2 test	Co-dominant		Dominant		Recessive	
				P^a	OR (95% CI)	*p*	OR (95% CI)	*p*	OR (95% CI)	*p*
Normal (n=668) versus MetS only (n=45)	G/G	128 (19.2%)	5 (11.1%)	0.02	0.58(0.37-0.91)	**0.02**	0.39(0.26-0.87)	**0.02**	0.52(0.20-1.34)	0.18
	G/T	318 (47.4%)	17 (37.8%)							
	T/T	222 (33.2%)	23 (51.1%)							
Normal (n=668) versus MetS with CAD (n=400)	G/G	128 (19.2%)	55 (13.8%)	0.03	0.83(0.69-1.00)	0.05	0.85(0.66-1.11)	0.24	0.68(0.48-0.97)	**0.03**
	G/T	318 (47.4%)	196 (49.0%)							
	T/T	222 (33.2%)	149 (37.3%)							
Normal (n=668) versus CAD only (n=340)	G/G	128 (19.2%)	52 (15.3%)	0.02	0.81(0.67-0.98)	**0.03**	0.74(0.56-0.97)	**0.03**	0.77(0.54-1.11)	0.16
	G/T	318 (47.4%)	151 (44.4%)							
	T/T	222 (33.2%)	137 (40.3%)							

OR, odds ratio; CI, confidence interval.

[a]Chi-square test.

Logistic regression models were performed by using SPSS to calculate the ORs (95% confidence intervals) and the corresponding *p*-values for the SNP in three alternative models (co-dominant, dominant, and recessive) that controlled for age. The common allele served as the referrent genotype for the heterozygote of the rare allele.

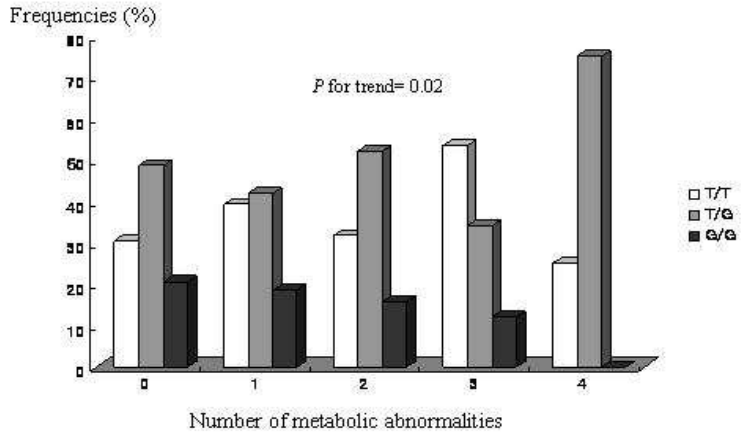


Fig. 11. Relationship between the ADD1 (+29017G/T) genotype and the number of metabolic abnormalities (0-4) in control (n=668) and MetS only (n=45) subjects. The MetS was diagnosed when a person satisfied three or more of five NCEP metabolic abnormality criteria.

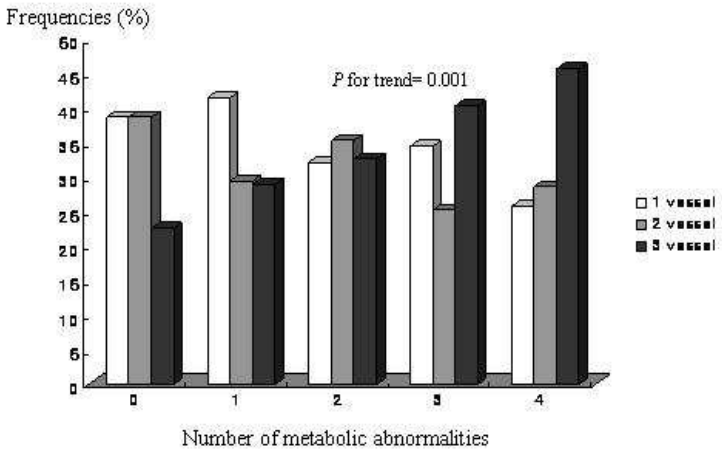


Fig. 12. Relationship between the number of metabolic abnormalities and CAD severity. The MetS with CAD and CAD only patients (n=740) were grouped according to the number of vessels affected and the number of NCEP criteria they satisfied

pressure and blood glucose levels (Table 14). Since the control subjects enrolled in the study were instructed not to take any Medicare drugs used for treating diabetes mellitus, high lipid levels, and high blood pressure, these observations suggest that the ADD1 Gly460Trp polymorphism mainly promotes CAD by affecting blood pressure (p=0.04) and blood glucose levels (p=0.03) rather than other conventional risk factors of CAD.

3.6 Obtaining non-synonymous SNP Tolerance Scores with SIFT and PolyPhen

we used two sequence homology–based tools [Sort Intolerant from Tolerant SIFT) and Polymorphism Phenotype (PolyPhen)] to predict the potential impact a non-synonymous SNP, which results in an amino acid substitution, may have on the activity of proteins encoded by genes involved in the steroid hormone metabolism and response pathway. The results of the SIFT and PolyPhen analyses are listed in Table 15. ADD1 Gly460Trp polymorphism was predicted to affect protein function by both programs. ADD1 Gly460Trp polymorphism (amino acid change) may affect the formation of heterodimers and NA^{+}-K^{+} pump endocytosis.

4. Discussion

Hispanics and Asians seem to be at greater risk of developing the MetS than other races (123), and it has been shown previously that the MetS is associated with CAD in European and South Asian men (123, 137) and an increased risk of developing CVD and diabetes (121, 138). Several studies have assessed how variations in the hypertension-related gene ADD1 associate with abnormal blood pressure, hypertension,

Table 14. Association between the Gly460Trp (+29017T>G) polymorphism and biochemical measures in control subjects

Phenotype	Genotype			*p*-value		
	T/T (n=177)	T/G (n=254)	G/G (n=104)	Co-dominant	Dominant	Recessive
SBP (mmHg)	115.42 ± 11.72	113.68 ± 11.67	113.02 ± 12.95	0.11	0.04	0.23
DBP (mmHg)	76.16 ± 8.39	75.69 ± 8.53	75.29 ± 8.72	0.62	0.39	0.46
BMI (kg/m^2)	23.64 ± 2.72	23.56 ± 2.80	23.51 ± 2.86	0.91	0.68	0.78
Blood Glucose (mg/dL)	88.77 ± 14.85	86.24 ± 9.19	87.08 ± 9.14	0.03	0.03	0.84
Total cholesterol (mg/dL)	200.93 ± 47.41	197.11 ± 36.07	195.10 ± 34.49	0.34	0.16	0.35
HDL (mg/dL)	47.88 ± 11.77	47.52 ± 12.20	47.95 ± 12.34	0.92	0.81	0.81
Triglycerides (mg/dL)	157.67 ± 121.87	145.37 ± 104.99	138.52 ± 87.10	0.21	0.10	0.25
LDL (mg/dL)	122.77 ± 33.39	120.75 ± 31.57	119.56 ± 32.62	0.63	0.37	0.52

The data shown are means ± standard deviation (SD). SBP, systolic blood pressure; DBP, diastolic blood pressure; BMI, body mass index. *p*-values were obtained by ANOVA

Table 15. SIFT and PolyPhen scores for functionally characterized Gly460Trp (+29017T>G) polymorphism

Gene (Protein ID)	dbSNP ID	AA change	SIFT score	SIFT Prediction	Plyphen score	Plyphen Prediction
ADD1 (NP_001110)	rs4961	Gly460Trp	0.03	Damaging	1.75	Possibly damaging

vascular pathology, and CVD (129, 131, 132). Here, we report that the Gly460Trp (+29017G/T) ADD1 gene variation is associated with the MetS and/or CAD in a Korean population. We also showed that this genetic variation was associated with higher numbers of MetS criteria. Furthermore, we observed that more severe CAD was associated with increased MetS severity.

We observed that the G allele of the ADD1 Gly460Trp (+29017G/T) polymorphism was significantly associated with decreased risk of MetS (OR= 0.26-0.91, p=0.02) and CAD with or without MetS (OR=0.48-0.98, p=0.03). Meanwhile, the T allele of the ADD1 polymorphism was associated with an increased risk of MetS and/or CAD. Notably, the G allele was more strongly associated with a decreased risk of MetS (OR=0.26-0.91) than with a decreased risk of CAD with or without MetS (OR=0.48-0.98). These observations are consistent with the considerable evidence showing that the Gly460Trp polymorphism of the ADD1 gene is significantly associated with the presence of hypertension, CVD, and myocardial infarction (131-132, 139-141). It is possible that mutations in the ADD1 gene enhance the risk of MetS and CAD because they affect the ability of ADD1 to regulate the NA^+-K^+ pump. Supporting this is that the T allele of the ADD1 Gly460Trp (+29017G/T) polymorphism is associated with higher NA^+-K^+ pump activity and impaired NA^+-K^+ pump endocytosis in renal tubular cells (126, 128).

We also observed that the T allele of the ADD1 Gly460Trp polymorphism is associated with increased numbers of MetS criteria (p for trend=0.02), and that higher numbers of MetS criteria in turn are associated with higher numbers of closed vessels in CAD (p for trend=0.001) (Fig. 11 and 12). Thus, the ADD1 Gly460Trp (+29017G/T) polymorphism appears to be an independent risk factor for MetS, while

MetS is closely associated with the occurrence of CAD. MetS is a multicausal process and thus it is likely that the ADD1 Gly460Trp polymorphism, along with other risk factors, may have an impact on CAD.

The frequency of the ADD1 Gly460Trp allele in East Asian populations is very similar to its frequencies in Japanese and Chinese populations (132, 141-144). Given that the frequency of the T allele is lower in Caucasians (18% in North Italy, 20% in France, and 27% in Scotland) (132, 145, 146), it is possible that the Gly460Trp allele variant may play a particularly significant role in the development of CVDs in East Asian people. However, most studies in humans suggest that polymorphism of the ADD1 gene cannot cause disease on its own.

Interestingly, Gly460Trp allele variant was predicted to affect protein function by both programs.

In summary, we demonstrated that the ADD1 Gly460Trp polymorphism was significantly associated with an increased risk of MetS and CAD in the Korean population. We also confirmed that the ADD1 Gly460Trp polymorphism is associated with abnormal blood pressure (p=0.04) and blood glucose levels (p=0.03). Moreover, our data showed that the ADD1 gene is involved in susceptibility to MetS, and that severe MetS is associated with severe CAD. This supports the hypothesis that genes associated with MetS can also increase the risk of developing CAD.

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ABSTRACT IN KOREAN

관상동맥 질환 관련 유전적 위험 요인 발굴

사람과 사람간의 유전적 차이는 99.9%가 동일하며 0.1%의 유전적 변이의 차이로 인해 개인간의 차이를 나타내게 된다. 이러한 사람의 DNA 염기서열에서 차이를 보이는 유전적 변이를 단일염기다향성 (Single Nucleotide Polymorphism: SNP)라고 한다. 좀 더 구체적으로 인구집단 (human population)에서 1% 이상의 빈도로 존재하는 2개의 대립 염기서열이 발생하는 위치를 SNP site라고 부른다. SNP와 같은 유전변이를 이용하여 복합형질 질환의 원인 유전자를 규명하고, 그들에 의한 질환과의 연관성 (association stydy) 분야는 그 질환의 치료 및 예방에 활용되어 맞춤의학의 시대를 앞당길 수 있다. 본 연구에서는 혈압에 관여하는 유전자 31개의 후보유전자를 선별하였다. 해당 유전자의 단백질을 생산에 관여하는 Exon, Promoter, 또는 Splice junction 부위의 염기 서열을 분석하여 총 409개의 SNP를 발굴하였고, 발굴된 SNP를 이용하여 각 후보 유전자에서 유전적 변이형 분포, haplotype의 종류 및 빈도, LD block의 pattern 등과 같은 기초적 분석을 하였다. 이러한 분석을 토대로 31개 유전자로부터 단백질 생성의 중요한 지역인 Coding 부위의 29개의 SNP sites (non-synonymous)를 선정하여 TaqMan 방법을 이용하여 관상동맥질환 및 정상군의 시료에서 대규모 genotyping을 수행하였고, 각 SNP 및 haplotype과 관상동맥질환과의 연관성 분석을 진행하였다. CCR2 유전자의 Exon에 위치한 Asn260Asn의 경우 심혈관 질환의 혈관의 막힘과 연관성이 있으며 (p=0.0005), 관상 동맥 질환의 악화에 관련이 있음을 확일 할 수 있었다. 또한 ADD1 유전자의

Gly460Trp의 경우 관상 동맥 질환 연관성 분석을 통해서 관상동맥 질환과 관련성 있음을 증명하였고 (p=0.01), 혈압의 증가와 관련있음을 밝힘으로써 (p=0.02), 질병 발생기전을 이해하는데 중요한 정보를 제공해줄 수 있고 더 나아가 예방 및 진단에 및 치료의 향상에 기여할 수 있을 것으로 본다. 또한 관상동맥 질환과 신진대사 증후군의 질환의 관계를 분석한 결과 신진대사 증후군의 경우 관상동맥 질환의 위험도 (p=0.02)를 증가시키는 것으로 나타났다. 따라서 질환과의 연관성 분석을 통한 합병증의 예방에도 기여할 수 있을 것으로 본다.

핵심단어: 고혈압, 관상동맥 질환, 신진대사 증후군, 질환연관성 분석, 단일염기다향성, 유전자, 형질