Research

# Three Novel Genetic Markers That Could Predict Severe Coronary Artery Disease.

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Abstract

**Introduction:** This study will identify genetic predictors of coronary artery disease (CAD) severity and develop genomic profiles for accurate classification of high-risk patients in a clinical cohort.
**Methods:** 719 patients who underwent coronary angiography for myocardial infarction or suspected coronary artery disease were scored on the basis of the degree of CAD luminal stenosis and the number of diseased main coronary vessels. A systematic statistical analysis strategy was then used to assess the association of SNPs with the coronary artery disease severity scores.
**Results:** The analysis revealed two SNPs that were associated with severity of coronary artery stenosis and 3 SNPs associated with number of diseased proximal vessels.
Rs12526453 in the PHACTR1 gene on chromosome 6, and rs6725887 in the WDR12 gene on chromosome 2 were predictive of the severity of CAD luminal stenosis (OR=1.38 p=0.02 and OR=1.46 p= 0.048 respectively). Rs6725887 was again associated with CAD severity as determined by the number of diseased proximal vessels (OR=2.01, p=0.004). Two further SNPs were also associated with CAD severity as defined by the number of diseased proximal vessels: rs4977574 in proximity to CDK2A & CDK2B genes on chromosome 9p21.3 (OR=1.64 p=.005) and rs10953541 in the BCAP29 gene on chromosome 7 (OR=1.814, p=.004).
**Conclusion:** This study identified four SNPs to be predictive of CAD severity as assessed by coronary angiography. This discovery may pave the way for the development of a genetic diagnostic and screening tool for coronary atherosclerosis and may also aid in the identification of targets for future gene therapy.

## Introduction

Coronary artery disease (CAD) remains a leading cause of mortality worldwide. In 2005, CAD resulted in 7.5 million deaths [(1)] and these numbers continue to rise [(2)]. CAD is characterized by lipid deposition and atherosclerotic plaque formation in the coronary arteries. Plaque formation is a result of endothelial dysfunction, oxidative stress and inflammation [(3)].

CAD is a multifactorial disease with well documented environmental risk factors, in particular smoking, hypertension, hypercholesterolaemia, diabetes and obesity [(4)]. CAD is known to be heritable, with a history of premature CAD in parents conferring almost three times higher risk of CAD in their offspring [(5)]. The extent to which inheritance influences the risk of CAD varies greatly and so the genetic factors are thought to exert risk both directly and through gene-environment interactions [(6)]. Myocardial infarction results from the rupture of vulnerable plaques, thrombosis and occlusion of the coronary vessels. Plaque vulnerability is determined by the size and consistency of the atheromatous core, thickness of the fibrous cap covering the core, and ongoing inflammation within the cap [(7)].

Direct DNA sequencing for Mendelian forms of cardiovascular disease traits, such as familial hypercholesterolaemia, identified causative mutations in the low-density lipoprotein receptor (LDLR) gene [(8)].

Association study techniques such as the candidate gene approach, were initially taken to define the genetic basis of CAD. However, CAD traits show complex inheritance and the candidate genes identified often failed to replicate these associations in subsequent studies [(9)]. The advent of high-throughput genetic testing led to the development of the genome wide association study (GWAS) approach. GWAS identified the first notable genetic variant (located on chromosome 9p21.3) associated with CAD [(10)]. Recently, the GWAS database (www.genome.gov/26525384) has catalogued the results from several large-scale GWASs of CAD patients with 22 SNPs in 19 genes convincingly implicated in the risk of CAD traits [(11)].

Invasive coronary angiography is the gold standard for establishing the presence, location and severity of coronary artery disease as it provides excellent spatial and temporal resolution for the visualization of the coronary arterial tree [(12)]. Most studies investigating the genetic basis of coronary artery disease were based on the clinical diagnosis of CAD traits such as myocardial infarction or angina, but not coronary anatomy as assessed by angiography. It is established that myocardial infarction and acute coronary occlusion most frequently relate to plaques that cause mild to moderate coronary artery stenosis, due to the propensity of a vulnerable plaque to rupture [(13)].

Therefore, studies which identify genetic loci associated with MI, will identify genes which may influence plaque stability as well as atherogenesis and thrombosis.

Distinct morphological characteristics of CAD show different degrees of heritability. Proximal coronary artery disease has been shown to display higher degree of heritability in comparison to distal disease [14]. Therefore, a focus of this study was on the degree of stenosis and the plaque distribution in the four major epicardial coronary arteries; left main, left anterior descending, left circumflex and right coronary arteries.

This current study is unique in that it directly assessed for SNP association with CAD severity through evaluation of invasive coronary angiography. Four SNPs were found to be predictive of CAD severity as assessed by coronary angiography. Two SNPs were predictive of the severity of proximal coronary artery disease luminal stenosis and a further two SNPs were predictive of triple vessel disease.

This discovery furthers the potential of developing a genetic test for screening for the risk of severe and diffuse coronary atherosclerosis permitting early detection and effective prevention by the aggressive control of modifiable risk factors.

## Methods

### Study population

This study was conducted collaboratively between the cardiology department of the Gold Coast University Hospital, Queensland and The Genomics Research Centre at Griffith University, Queensland, Australia. The study was approved by the institutional ethics committee and all subjects gave informed written consent. 719 Caucasian subjects who underwent coronary angiography between 2006 and 2012 were selected. The study population was homogeneous consisting only of Caucasians.

### Coronary angiography

The indications for coronary angiography included acute coronary syndrome (diagnosed by clinical history, electrocardiographic findings and elevated cardiac troponin [15], pre-surgical cardiac assessment, work-up for valve surgery and stable angina. The full spectrum of clinically manifested coronary artery disease was represented in this population as follows:

ST elevation myocardial infarction (STEMI) (5.7%) and Non-ST elevation myocardial infarction (NSTEMI) (26.0%); unstable angina (5.0%); Stable Angina (21.1%); and other reasons (42.1%) including positive cardiac stress testing, positive functional imaging and pre-operative assessment. Coronary angiography was carried out using a Siemans Axiom Artis image intensifier (Siemens Inc., Sweden) according to the Judkins' technique using standard 6 French gauge right and left coronary catheters with the images acquired at a frame count of 15 per second. The orthogonal projections were made according to a standard institutional protocol and images of the four main coronary arteries: the left main coronary artery (LMCA), the left anterior descending artery (LAD), the left circumflex artery (LCx) and the right coronary artery (RCA) were acquired.

Two independent workers analyzed and reported the findings of angiography on two separate occasions for cross-validation. The lesions in the main coronary arteries were recorded as a percentage of the stenosis of the vessel, determined by visual estimation of the diameter of the narrowing compared to the proximal normal vessel. The number of diseased main vessels and the number of lesions in each main vessel were recorded. The reporters of the angiograms were blinded to the genotyping results of the patients.

### Risk factors

All clinical data was obtained during the index admission. Each patients had the standard coronary risk factors recorded. 62.7% of the study population were male, 50.5% were hypertensive, 57.8% had hypercholesterolaemia, 21.3% had diabetes, 5.3% were current smokers and 20.6% of patients had a positive family history of CAD (CAD in a male first-degree relative or father less than 55 years, or female first-degree relative or mother less than 65 years).

### Genotyping

5ml EDTA blood samples were collected from the femoral or radial artery at the time of arterial puncture for vascular access. Genomic DNA was extracted and purified from peripheral blood lymphocytes using a standard salting-out procedure [16]. 22 previously identified SNPs [17] were genotyped in the 719 patients. SNPs were selected from a search of the GWAS database for SNPs associated with CAD in 2011.

Genotypes for each SNP were determined by restriction-fragment 140 length polymorphism (RFLP) analysis of restriction enzyme-digested PCR products on agarose gels. A 20-"l PCR reaction mix contained 1× PCR buffer, 1.75 mM MgCl2, 0.2 mM dNTPs, 0.15 "M of each primer, 20–40 ng of genomic DNA and 1.5 U of GoTaq® (Promega). Thermocycler conditions were an initial denaturation at 95°C for 10 min, followed by 35 cycles of 95°C for 45 s, 60°C for 45 s, 72°C for 45 s and a final extension step of 72°C for 7 min. Specific primers were used to amplify each region containing the SNPs and restriction enzymes (NEB) chosen that would differentiate the two alleles after separation on 3% agarose gels.

## Statistical analysis

Coronary artery disease severity was categorized using two steps. In the first step, the vessel score was defined as the number of diseased main vessels with >50% luminal diameter stenosis. A point was allocated to each diseased vessel: single vessel disease (1 point), two vessel disease (2 points) or triple vessel disease (3 points). As the left main coronary artery (LMCA) divides into both the LCx and LAD, stenosis of the left main coronary artery (LMCA) was considered more severe and was attributed a score of 2 points. For further analysis of the number of vessels involved, the number of vessels was dichotomised into 0 (no vessels, n=226) and 1 (1,2 or 3 vessels, n=493).

Secondly, for the analysis of the severity of luminal stenosis, the severity was dichotomised into 0 (minimal disease, <30% stenosis) and 1 (significant disease, >50% stenosis in 1 or more proximal vessel).

This approach was aimed at identifying genetic markers associated with the morphological characteristics of CAD which have clinical significance and which demonstrate the highest heritability. Therefore by focusing our scoring on proximal disease this was intended to enhance the likelihood of identifying genetic predictors of heritability and severity.

For the primary exploratory analysis, a bivariate correlation analysis of all SNPs was performed against severity (as determined by the number of vessels involved and the severity of stenosis) using Spearman's statistic [18].

Further analysis was made from the severity score indices of luminal stenosis and number of diseased vessels. A logistic regression model was generated for the SNPs identified in the severity score analysis. Age, sex, family history, dyslipidaemia, hypertension, smoking and diabetes were factored in to the analysis as co-variates.

## Results

From the primary exploratory analysis 2 SNPS were shown to be associated with severity of stenosis: rs12526453 (OR=1.42 p = 0.01) and rs6725887 (OR=1.51 p = 0.031). Four SNPs were shown to be associated with triple vessel disease: rs10953541 (OR=1.77 p=0.005), rs12526453 (OR=1.52, p=0.014), rs4977574 (OR=1.66 p=0.003) and rs6725887 (OR=2.05 P=0.003).

Results of logistic regression analysis for the severity of coronary artery luminal stenosis showed rs12526453 in the PHACTR1 gene on chromosome 6, and rs6725887 in the WDR12 gene on chromosome 2 to be predictive for the severity of coronary artery disease after adjustment for cofactors (OR=1.38 p=0.02 and OR=1.46 p= 0.048, respectively).

Results of logistic regression analysis for the number of proximal coronary artery vessels involved again showed rs6725887 in the WDR12 gene to be associated with CAD severity (OR=2.01, p=0.004). Two further SNPs were also associated with CAD severity as defined by the vessel score: rs4977574 in proximity to CDK2A & CDK2B genes on chromosome 9p21.3 (OR=1.64 p=.005); and rs10953541 in the B-cell receptor-associated protein 29 (BCAP29) on chromosome 7 (OR=1.814, p=.004).

## Discussion

This study evaluated the association between 22 SNPs and CAD severity as assessed by the gold standard investigation of invasive coronary angiography. Results of the analysis have shown four SNPs in total to be associated with disease severity.

One of the strengths of our study design is that we identify cases based on angiographic evidence of both severity and the most heritable morphological characteristics of CAD.

Other case-control comparison studies often have variability in study enrollment, in particular, controls are chosen from healthy individuals in the general population whose coronary anatomy is not known and therefore may well be harbouring sub-clinical atherosclerosis. Each of the controls in this study has angiographic evidence of the percentage of luminal stenosis in their coronary arteries and the study design excludes individuals from the general population who may have CAD but are asymptomatic.

Variants of phosphatase and actin regulator 1 gene (PHACTR1) demonstrated association with early onset MI in a study published by the MI genetics consortium [19]. PHACTR1 has also been associated with severity of coronary stenosis in a Lebanese cohort (OR = 1.37).Rs9349379 in their study was associated with the severity of atherosclerosis in the Lebanese study but was not replicated in this study. Although rs12526453 did not show association in the Lebanese exploratory set it did show association in their replication set [20]. Multiple GWASs in the Chinese Han population have not shown rs12526453 and PHACTR1 to be associated with CAD or MI. In the Chinese studies the minor allele frequency (based on HapMap data) of rs12526453 was 0% and none of the 113 SNPs in or near PHACTR1 showed significant association ($p < 0.01$) for coronary artery disease [21,22] . Although their study sizes were small in comparison to the European studies, it suggests that PHACTR1 may not play a role in the Chinese population.

The pathophysiology of the PHACTR1 in coronary artery disease remains to be elucidated. PHACTR1 has been proposed to be a key regulator of endothelial cell function [23] and is a regulator protein of protein phosphatase 1 (PP1), an enzyme that regulates endothelial nitric oxide (eNO) [24]. eNO is an important modulator of coronary artery disease and has been shown to influence thrombogenicity and inflammation [25]. Mutations in the PHACTR1 may contribute to atherosclerotic plaque formation by leading to endothelial dysfunction. Our finding that rs12526453 is associated with coronary artery disease severity but not associated with MI is suggestive that the role of PHACTR1 is in atherogenesis (plaque formation) and not plaque rupture or thrombosis. Rs4977574 has previously been shown to be associated with coronary artery disease severity in an Italian study [26] and also with MI [19, 27]. Rs4977574 is located within the ANRIL gene that encodes a large non-coding RNA. The ANRIL gene pathophysiology is unknown but its role in CAD has been implicated in several expression studies.

The ANRIL promoter region contains binding sites for zinc-finger proteins that are critical for the transcription of CDKN2Aand CDKN2B and, in the mouse, deletion of the 9p21 orthologous non-coding region, including the 3′ region of ANRIL, affects proliferation of vascular cells with severely reduced expression of CDKN2A and CDKN2B[27]. At least 11 chromosomal regions have been identified as associated with the risk of MI beyond that of traditional risk factors, with each risk allele increasing the risk of MI by a relatively small margin (10-30%) [28]. Myocardial infarction and coronary occlusion most frequently evolve from mild to moderate stenosis whilst severe atherosclerotic coronary artery disease is less likely to be the cause of MI [7] . Severe disease is clinically significant due to its associated symptoms, morbidity and mortality, and also in light of the therapeutic options available, such as stent insertion or coronary artery bypass surgery.

Conventional risk factors do not explain the full extent of coronary artery disease pathophysiology. This study shows the importance of using the phenotype information gained from coronary angiography to begin to identify the mechanism of action of the risk genes identified in large scale GWASs. Data from coronary angiography will also improve the development and accuracy of genetic tools for identifying individuals at risk of severe coronary artery disease.

This data could eventually enable earlier prevention and would be a leap forward in the personalization of medicine based on one's genetic risk. The identification of novel genetic links and elucidation of their pathophysiology will be essential for the development for new therapies for the prevention and treatment of coronary artery disease.

## Conclusion

In conclusion, we have identified four SNPs: rs12526453 in the PHACTR1 gene on chromosome 6, rs6725887 in the WDR12 gene on chromosome 2, rs4977574 in the ANRIL gene on chromosome 9p21.3 and rs10953541 in the BCAP29 gene on chromosome 7, to be predictive of CAD severity as assessed by coronary angiography. This discovery may pave the way for the development of a genetic diagnostic and screening tool for coronary atherosclerosis. It may also help identify targets for future gene therapeutics against atherosclerotic coronary disease.

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