The 9p21.3 risk locus for coronary artery disease: A 10-year search for its mechanism

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Received 1 January 2017; revised 3 March 2017; accepted 5 March 2017; Available online 25 April 2017

Abstract

The 9p21.3 risk locus is the first locus to be associated with an increased risk of coronary artery disease (CAD)-related events and many other phenotypes. This locus contains 59 single nucleotide polymorphisms (SNPs) in a region with multiple long range enhancers and long non-coding RNAs (lncRNAs) that affect the expression of neighbouring genes, cyclin-dependent kinase 2A and 2B (CDKN2A and CDKN2B), which are required for controlling vascular smooth muscle cell proliferation and ageing. Several studies have attempted to identify the precise mechanism by which this locus exerts its pathogenic effect to increase the risk of CAD-related events. In this review, we will highlight the major advances in our understanding of the genotype-phenotype correlation at the mechanistic and phenotypic levels. The high population attributable risk of the 9p21.3 risk locus, mechanistic knowledge acquired thus far, and ongoing research efforts could facilitate the design of novel therapeutic molecules to reduce the risk of CAD and its related events.

Keywords: 9p21.3; CDKN2A and CDKN2B; Coronary artery disease; Risk locus; Smooth muscle cell

Introduction

The major cause of CAD is atherosclerosis, which results from the plaque build-up and narrowing of the inner walls of the coronary arteries that supply heart muscle with blood, thereby leading to limited blood flow and eventual ischaemia. Plaque primarily comprises fat (cholesterol and fatty acids)-laden macrophages, vascular smooth muscle cells (VSMCs), cellular debris, and minerals, such as calcium. The white blood cell (WBC) component of plaque produces inflammatory cytokines that recruit inflammatory cells to the site of
the plaque. Although this process is meant to be protective, it further contributes to the plaque size and inflammation.\(^1\) CAD is a common and complex chronic disease with traditional and genetic risk factors. The traditional risk factors of CAD include age, gender, obesity, dyslipidemia, diabetes, hypertension and smoking. These factors act independently or in concert with each other to increase the risk of CAD. Controlling for the known risk factors of CAD, such as smoking, randomized clinical trials have shown that hypercholesterolaemia\(^2\) is associated with an approximately 30–40% reduction in clinical events, such as myocardial infarction and subsequent death.\(^3\) The other 40–60% of the risk of CAD is heritable according to epidemiological, twin and family studies. The heritability of CAD is the component of the CAD risk explained by genetic factors.\(^4\) In a landmark case-control study, CAD was shown to have strong heritability, ranging from 56% (when patients with monogenic heart disease are excluded) to 63% (including patients with monogenic heart diseases).\(^5\) Moreover, first-degree relatives showed a higher risk index for ischemic heart disease and stroke (3 and 1, respectively) than second-degree relatives (1 and 0.5, respectively). The heritability in this study was calculated using Falconer’s method to determine the heritability of certain traits or phenotypes based on the difference between twins. Among first-degree relatives with CAD, heritability was estimated at approximately 100% in patients under age 46; whereas, heritability ranged from 15% to 30% in late onset cases of CAD.\(^6\) The younger the CAD patient at the diagnosis of the first event of MI, the more common was CAD in his relatives of parents and siblings.

**The pathophysiology of atherosclerosis**

Atherosclerosis is an asymptomatic chronic late onset disease. Atherosclerotic plaques are divided into stable or unstable plaques. Stable plaques are often asymptomatic and contain collagen-rich extracellular matrix that is primarily produced primarily smooth muscle cells. The collagen-rich extracellular matrix (ECM) generates a stabilizing fibrous cap that separates the plaque from the lumen of the vessel. By contrast, unstable plaques are rich in foam cells and collagen-poor ECM, making the unstable and vulnerable to rupture. The rupture of the plaque and release of its thrombogenic components trigger thrombosis and formation of blood clots that occlude arteries and induce MI.\(^7\)

**Cell proliferation in atherosclerosis**

The process of cell proliferation is regulated by a group of proteins called cyclin-dependent kinases (CDKs) and cyclin-dependent kinase inhibitors (CKIs). Activation of cell cycle phase-specific CDKs drives progression through this phase. For example, the CKIs p15 and p16 interact with and inactivate CDK4 and CDK6, preventing them from binding and activating cyclin D. Inactive cyclin D cannot phosphorylate the cytoplasmic retinoblastoma protein (RB) and release the sequestered E2 promoter binding factor 1 (E2F1) to enter the nucleus, thereby activating transcription of genes involved in the progression from G1 to S phase.\(^8,9\)

Epigenetic modifications, such as hypermethylation, were enriched in the aorta and PBMCs, playing causative roles in the process of atherosclerosis development in a mouse model of CAD.\(^10\) Hypermethylation is significantly associated with the risk of CAD.\(^11,12\) Risk factors, such as dyslipidemia and hyperhomocysteinemia, are key mediators of the hypermethylation observed in patients with CAD.\(^10,12\) Hypermethylation of the upstream regions of the TGFBR3 genes was significantly enriched in those patients.\(^11\) Perturbed expression of CDKN2A and CDKN2B has been associated with several tumours.\(^13–15\) Methylation of CDKN2A and CDKN2B is associated with CAD in humans.\(^16\) Excessive cell proliferation within the walls of the arteries contributes to the enlargement of the plaque and restenosis after angioplasty.\(^17\) VSMCs and macrophages are the main proliferating cell types in human atherosclerotic plaques.\(^18\)

**TGFβ and VSMC proliferation in atherosclerosis**

Proliferation of VSMCs is critical for repair and healing after vascular injury or insult. However, if the insult persists, as in atherosclerosis, then the mitogenic stimulus continues and the proliferation of VSMCs becomes atherogenic.\(^19\) VSMCs produce ECM, which stabilizes the plaque, and the migration and proliferation of arterial smooth-muscle cells enlarge the atherosclerotic lesion.\(^11\) TGFβ inhibits VSMC migration and proliferation and induces collagen-rich ECM production.\(^20,21\) In the walls of normal vessels, VSMCs primarily express type II TGFβ receptor (TGFBR2), as opposed to type I TGFβ receptor, which is produced in VSMCs from atherosclerotic vessels.\(^22\) In response to TGFβ, normal VSMCs expressing type II TGFβ receptor greatly induce the expression of contractile proteins and minimal production of the ECM. By contrast, diseased VSMCs expressing type I TGFβ receptor increase the expression of collagen rich-ECM but fail to induce the expression of contractile proteins in response to TGFβ. If fresh VSMCs are maintained in TGFβ-containing media, then these cells maintain type II TGFβ receptor.\(^22\) Low concentrations of TGFβ increase the proliferation of VSMCs, consistent with the fact that low levels of plasma TGFβ are associated with a poor outcome in CAD.\(^23,24\) Conversely, at higher concentrations, TGFβ inhibits VSMC proliferation\(^25\) and reduces atherosclerosis.\(^25\) The levels of plasma TGFβ are reduced at sites of lesion development in the intima of the coronary arteries and human aorta.\(^26,27\) In sum, these studies demonstrate that TGFβ is critical for maintaining the contractility of VSMCs and inhibiting their proliferation and migration to the intima of arteries. Therefore, impaired or a lack of TGFβ signalling is atherogenic. Mutations in the TGFBR1 and TGFBR2 genes that cause congenital heart disease and arterial aneurysms are associated with increased VSMC proliferation, increased collagen expression and reduced contractile protein (SMC z-actin, β-myosin, and calponin) expression.\(^28,29\)

TGFβ induces the expression of p16 and p15 through Smad proteins, such as Smad2 and Smad3, to control the cell cycle and induce cellular senescence.\(^8,30,31\) Smad3 interacts with the TEA-domain (TEAD) family of transcription factors, particularly TEAD3 and TEAD4.\(^32\) The TEAD family has a
common N-terminal domain that enables these proteins to
bind a specific DNA element called M-CAT (5'-CATC-3')
and a transactivation domain to interact with co-activators,
such as Smad proteins. TEAD transcription factors play key
roles in the expression of cardiac, smooth and skeletal muscle-
specific genes (such as SMC α-actin and β-myosin).33 These
factors play a major role in tumour suppression and cell cycle
control.34 The major role of TEAD factors in VSMCs and
cardiac development explains their involvement in congenital
and developmental heart diseases.35

9p21.3 CAD risk locus as the first hit of a GWAS

Using microarrays of SNPs to genotype large numbers of
cases and controls, the first common genetic variants at
chromosome 9p21.3 conferring a risk for CAD were identi-
fied.36 This risk locus has also been associated with other
diseases, such as type 2 diabetes,37 CAD-associated MI, abdominal aortic and intracranial aneurysm.38 Several other
large GWASs have confirmed this association with CAD.39,40 The minor allele frequency (MAF) in different
populations is as follows: European (50%), Sub-Saharan African (50%), African American (24%), Asian (47%),
and Han Chinese (37%). The mechanism whereby these ge-
etic variants contribute to the risk of CAD has remained
evasive.41 The 9p21 risk alleles predict the severity of CAD
according to the burden of arterial atherosclerosis: the 9p21 risk allele was more frequently observed in patients
with a narrowing of 3 coronary arteries than in those with
a single affected artery.42 No significant association with
the frequency of the 9p21.3 risk locus was observed be-
tween CAD patients with or without MI,42 suggesting
that the 9p21.3 risk locus functions through plaque
development and not rupture. The 9p21.3 locus contains 59
linked SNPs located 100,000 base pairs upstream of the cell
cycle suppressor genes CDKN2A (codes for p16 and p14)
and CDKN2B (codes for p15). This locus overlaps with the
3’ region of ANRIL (antisense noncoding RNA at the ink4
locus non-coding gene). The 9p21.3 risk variants over-
lapping the 3’ region of ANRIL are associated with the
induced expression of different splicing isoforms of ANRIL
and reduced expression of CDKN2A and CDKN2B.43 Polycomb
repressor complex 1 and 2 (PRC1 and PRC2) and polycomb complex protein EZH2 are recruited to
ANRIL, which in turn leads to the recruitment of DNA
methyltransferase (DNMT1), further increasing DNA
methylation and inactivation of the CDKN2A locus.44
However, the increased expression of the ANRIL transcript
and methylation of the CDKN2A and CDKN2B loci were
significantly associated with CAD in angiographic-defined
patients, but not those with the 9p21.3 risk locus.16

Several studies have demonstrated reduced expression of
p16 and p15 in the presence of the 9p21.3 CAD risk locus in
aortic smooth muscle cells (AoSMCs).43,45,46 The reduced
expression of p16 and p15 at the risk locus is associated
with increased HAoSMC proliferation and a failure to
enter senescence. Indeed, p16 and p15 are well-known
tumour suppressors and cellular senescence markers
that function through the retinoblastoma pathway. Thus, the
9p21 risk allele may promote the deposition of atheroscle-
rotic plaques in the coronary arteries, likely through
accumulation of fat-laden foam cells and proliferation of
VSMCs in the intima, rather than the weakening of the
extracellular matrix to cause plaque rupture and myocardial
infarction.

The regulation of gene expression at the 9p21.3 CAD risk
locus

The 9p21.3 risk locus is as complex as the associated
phenotypes. The 9p21.3 risk locus contains several enhancers
with defined risk haplotypes linked to distinct phenotypes,
suggesting that the 9p21.3 risk locus exerts tissue- and
disease-specific effects.37,48 For example, the CAD risk
haplotype tagged by rs1333049 is associated with CAD and
atherosclerosis burden but not with MI in patients with
CAD compared to patients without CAD, suggesting a
phenotype-specific enhancer effect.49

Gene transcript profiling showed no association of the
CAD risk variants with genes in the vicinity of 9p21.3 in
donor macrophages.50,51 Primary HAoSMCs from
atherosclerotic plaques showed reduced expression of p16
and p15 proteins and increased proliferation when heterozygous for the risk allele (65). Knockout of the
9p21.3 orthologous sequences in mice resulted in a
significant reduction of CDKN2A and CDKN2B
eexpression and increased aortic smooth muscle cell
proliferation as well as failure to enter senescence, with
the strongest effect observed in aortic tissues.46
Consistent with these findings, we showed that the 9p21.3 CAD risk locus is associated with reduced expression of p15 and p16, increased proliferation of HAoSMCs and failure to enter senescence.52
Given these data, the increased proliferation and reduced expression p15 and p16 are the established
biological phenotypes linked to the 9p21.3 CAD risk locus
(Figure 1). Overall, these data suggest phenotype- and
tissue-specific effects of the variants at the 9p21.3 locus.

The VSMC proliferation observed with the 9p21.3 risk
locus is involved in the pathogenesis of atherosclerosis
and plaque growth. However, to intervene with the pathogenesis of this locus, we have to determine the mechanisms by which
the p15 and p16 levels are reduced to affect VSMC prolifera-
tion. Several attempts have been made to identify this
mechanism. Knockout of the mouse orthologue has provided
a model to study the disease in an animal context.40
This model supported previous findings of reduced p15 and p16
expression in patients with CAD.43 Notably, 45% of the
knockout mice that developed tumours and neoplasms were
not reported to associate with the 9p21.3 CAD risk locus
according to GWASs. In an attempt to determine the
mechanism, another study claimed that interferon-γ long-
range induction of p15 and p16 as well as other genes is dis-
rupted by the risk variant that disrupts STAT1 binding at the
locus.48 However, our work did not support this hypothesis
and showed that p15 and p16 were induced by interferon-γ,
independent of the 9p21.3 risk locus.32 We used a larger
sample size and many different types of cells. Consistent
with our findings, Erridge et al. (2013) showed that the
interferon-γ signalling to the interferon family of genes is
not disrupted by the presence of the 9p21.3 risk locus.30

Pilbrow et al. (2013) showed that expression of genes
involved in the TGFβ pathway was affected by the 9p21.3
risk locus in multiple human tissues. Using the knockout mouse model of the 9p21.3 risk locus, Loinard et al. (2014) showed reduced TGFβ-dependent Smad2 signalling associated with reduced p15 expression as well as increased proliferation in AoSMCs in the knockout mice. Moreover, these authors also showed that these mice were susceptible to aneurysm and plaque rupture; these effects were preventable using CDK inhibitors. Our unbiased scanning approach for the disruption of transcription factor binding at the 9p21.3 locus identified TEAD3 as a mediator of TGFβ induction of p16 (p15 was not affected), which is disrupted by the presence of the risk locus. Our findings are consistent with those of Pilbrow et al. (2013) with regard to the involvement of the TGFβ pathway in the risk mechanism. However, our work did not support the findings of Loinard et al. (2014), as we showed contrasting findings. This contradiction could reflect species differences between mice and humans and also models differences. These previous studies used a knockout mouse model, whereas we used primary HAoSMCs that carry CAD-linked SNPs, which is more biologically and clinically relevant to the 9p21.3 risk locus. The reduction in the level of p15 expression may reflect the post-transcriptional effect of the ANRIL locus and its transcript. Our recent work provided a novel mechanism by which TEAD3 and TEAD4 induce p16 expression and mediate TGFβ induction of p16 at the 9p21.3 locus.

Conclusion

A peptide that activates TEADs factors leading to control of the cell cycle and suppression of gastric cancer growth in vivo has been developed. Future studies can employ similar strategies to prevent or mitigate atherosclerosis in CAD patients who are not homozygotes for the 9p21.3 risk allele. Notably, the mechanism by which the 9p21.3 risk locus affects the expression of CDKN2A and smooth muscle proliferation does not explain or account for the ability of 9p21.3 to confer a risk of coronary artery and aortic calcification, and this mechanism awaits future studies.

Author’s contribution

NA wrote initial and final draft of the review. NA has approved the final draft and is responsible for the content of the manuscript.

Conflict of interest

The author has no conflict of interest to declare.

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**How to cite this article:** Almontashiri NAM. The 9p21.3 risk locus for coronary artery disease: A 10-year search for its mechanism. *J Taibah Univ Med Sc* 2017;12(3): 199–204.