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CARDIOVASCULAR GENOMICS (R MCPHERSON, SECTION EDITOR)

# Functional Genomics of the 9p21.3 Locus for Atherosclerosis: Clarity or Confusion?

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**Abstract** The 9p21.3 locus was the first to yield to genome-wide association studies (GWAS) seeking common genetic variants predisposing to increased risk of coronary artery atherosclerotic disease (CAD). The 59 single nucleotide polymorphisms that show highest association with CAD are clustered in a region 100,000 to 150,000 base pairs 5′ to the cyclin-dependent kinase inhibitors *CDKN2B* (coding for $p15^{ink4b}$) and *CDKN2A* (coding for $p16^{ink4a}$ and $p14^{ARF}$). This region also covers the 3′ end of a long noncoding RNA transcribed antisense to *CDKN2B* (*CDKN2BAS*, aka ANRIL for antisense noncoding RNA at the ink4 locus) whose expression has been linked to chromatin remodeling at the locus. Despite intensive investigation over the past 7 years, the functional significance of the 9p21.3 locus remains elusive. Other variants at this locus have been associated with glaucoma, glioma, and type 2 diabetes mellitus, diseases that implicate tissue-resident macrophages. Here, we review the evidence that genetic variants at 9p21.3 disrupt tissue-specific enhancers and propose new insights to guide future studies.

**Keywords** 9p21.3 CAD risk locus · Enhancers · Atherosclerosis · Macrophages · Vascular smooth muscle cells · Cell proliferation · Functional genomics

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## Introduction

Coronary artery disease (CAD) is the leading cause of death in the world, according to World Health Organization statistics [1]. Although fewer than 5 % of individuals show symptoms of CAD before the age of 50 [2], intravascular ultrasound revealed that 1 in 6 adolescents and 85 % of persons over the age of 50 have measurable coronary atherosclerosis [3]. Modifiable risk factors such as high blood cholesterol, diabetes, high blood pressure, smoking, and obesity contribute to the risk of CAD [4], but genetic risk is equally important [5]. The notion that common genetic variants could contribute to the risk of CAD was borne out by the discovery of the first common CAD risk variant to be discovered by the genome-wide association study (GWAS) approach, located on chromosome 9 at 9p21.3 [6–8].

The 9p21 risk alleles are carried by 75 % of the world population (excluding black Africans) and confer risk for coronary atherosclerosis independently of known risk factors [6–8]. To date, more than 40 loci have been reported to contribute to CAD risk [9], but the 9p21.3 locus remains the second most significant (after *LPA*, encoding lipoprotein (a)). The population-attributable risk of CAD conferred by homozygosity for the 9p21.3 risk allele is 21 % [10]. It is important to realize that this is on the same order of magnitude as the population-attributable risk of hypertension (28 % in men and 29 % in women) [11] or of elevated total cholesterol (27 % in men and 34 % in women) [11]. The quest to identify the mechanism whereby the 9p21.3 confers risk for CAD has been ongoing for the past 7 years, and progress has been limited. Here, we will review what we know about the 9p21.3 locus and how evidence has gradually emerged to point to possible mechanisms.

## Atherosclerosis—a Common Inflammatory Disease of Arteries

CAD is an age progressive disease caused by atherosclerosis of the coronary arteries, an inflammatory condition in which the artery becomes occluded due to the accumulation of fatty material called plaque. Plaque is an aggregate of cholesterol and triglycerides taken up by tissue macrophages within the vessel wall to form foam cells [12]. Macrophage foam cells produce high levels of inflammatory cytokines and chemokines. That macrophages accumulate at atherosclerotic lesions is well documented [13], but the prevailing view that this is due to monocytes being recruited by local production of chemokines [14–20] has recently been challenged with evidence that recruited monocytes account for only about 15 % of the proliferating macrophages in atherosclerotic lesions [21]. Indeed, several recent studies indicate that tissue macrophages, while exhibiting distinct mature differentiated phenotypes, are endowed with the capacity for self-renewal similar to that of stem cells [22]. Genetic variants that influence macrophage self-renewal by affecting cell cycle control genes would be expected to affect the severity of atherosclerosis plaque formation.

## The 9p21.3 Genetic Risk Locus Promotes Atherosclerosis Rather Than Myocardial Infarction

We reported that the 9p21.3 risk alleles associated with angiographic CAD [8], whereas the deCODE Genetics group reported that it associated with myocardial infarction (MI) [7], and the Wellcome Trust Case Control Consortium found that it associated with CAD broadly defined (but predominantly recruited from patients with MI) [6]. It is well recognized that MI occurs on a substrate of coronary atherosclerosis. Thus, it was unclear whether these risk alleles promote the deposition of atherosclerotic lesions (CAD per se) or the formation of vulnerable plaque that are prone to rupture triggering thrombosis and MI. To address this question, we conducted a cross-sectional survey of 1714 patients with angiographically documented CAD and found that the risk allele associated with severity of CAD as measured by the number of occluded coronaries rather than MI among cases with pre-existing CAD [23•]. Shortly thereafter, the same finding was also reported by Patel et al. [24] who examined 2334 angiographic CAD cases. A recent meta-analysis comprising 21 studies and 33,673 subjects with angiographic data and MI status also confirmed that the 9p21.3 risk locus increases the burden of coronary atherosclerosis but not the risk of MI in the presence of underlying CAD [25]. Other vascular phenotypic manifestations of the 9p21.3 risk locus include abdominal aortic aneurysm [26–29], intracranial aneurysm [30, 31], the ankle-brachial pressure index, an indication of peripheral vascular disease [32], late onset Alzheimer's disease and vascular dementia [33], and ischemic stroke [34–37]. Thus, these findings are consistent with an effect of the 9p21.3 risk alleles to stimulate coronary atherosclerosis. Whether the effect is mediated through resident macrophages of the vessel wall or by modifying the cellular properties of arterial vascular smooth muscle cells (VSMCs) or a combination thereof remains unclear.

## The 9p21 Risk Locus Disrupts a Tissue-Specific Enhancer

The genetic risk locus at 9p21.3 consists of a cluster of 59 linked single nucleotide polymorphisms (SNPs) over a 53,202 bp region (Fig. 1). A growing consensus is that 1 or several of the 59 linked SNPs alter the DNA sequence and disrupt or create transcription factor binding sites that would alter the control of regional gene expression. The nearest protein coding genes are the cyclin-dependent kinase inhibitors *CDKN2A* (coding for $p16^{ink4a}$ and $p14^{ARF}$) and *CDKN2B* (coding for $p15^{ink4b}$) that lie about 100,000 base pairs upstream of the 9p21.3 locus. In addition, we find methylthioadenosine phosphorylase (*MTAP*) and much farther upstream nearly the entire type I interferon gene cluster.

We identified several functional enhancer elements at the 9p21 region [38•]. Deletion of the same region in the mouse genome was associated with reduced expression of *Cdkn2a* and *Cdkn2b* mRNAs, demonstrating the presence of regulatory enhancers at this locus [39••]. A recent study published in Nature by Kelly Frazer's group [40•] used the technique of chromatin conformation capture and identified short- and long-range interactions between sequences at the 9p21.3 locus and sequences in the vicinity of the genes encoding *CDKN2A* and *CDKN2B* and methylthioadenosine phosphorylase (*MTAP*) in the short range, and between *IFNW1* and interferon-α21 (*IFNA21*), in the long range, approximately 1 million base pairs upstream on chromosome 9. This finding is remarkable because it suggests that the influence of the enhancer sequences at 9p21.3 act at considerably greater distances than previously thought. Although these authors did not examine the expression of the type I interferon genes, a more recent study failed to find association between the circulating levels of various type 1 interferons, including IFNA21, and the 9p21 risk allele genotype in healthy individuals [41•]. Whereas these findings cast doubt as to the significance of the long-range interactions between the 9p21 risk alleles and distant enhancers, the effect of the 9p21.3 locus may reflect a tissue-specific enhancer or a disease-associated effect that has not yet been replicated in cultured cells. A recent study performed expression profiling of 9p21.3 genotyped monocyte-derived macrophages in culture and remarkably, found differential expression of genes not at

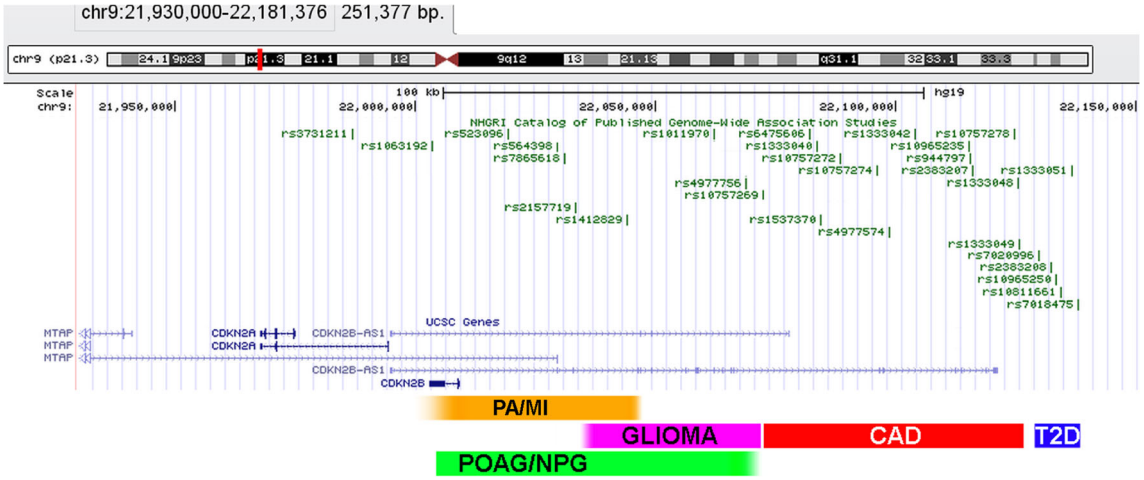


**Fig. 1** The 9p21.3 locus is a hotbed of haplotypes linked to several diseases: are these cell-specific enhancers? The coronary artery disease (CAD, *in red*) haplotype block spans 53,000 bp and includes 59 linked SNPs. Other GWAS loci include 1 for primary open angle glaucoma/normal pressure glaucoma (POAG/NPG) spanning 63,000 bp, another for glioma spanning 28,000 bp, and 1 for type 2 diabetes mellitus (T2D) spanning approximately 12,000 bp. It is noteworthy that the POAG/NPG and glioma haplotypes are partially dependent (eg, rs2157719 for glaucoma and rs4977756 for glioma have a D' of 0.905, $r^2$=0.718 and a LOD score of 31.79 according to HapMap). Similarly, the Glioma and CAD haplotypes are also partially dependent (rs4977756 of the glioma haplotype and rs1333049 of the CAD haplotype have a D' of 0.824, an $r^2$=0.422 and a LOD score of 16.53). In contrast, the T2D haplotype is independent of the other haplotypes (eg, rs1333049 and rs10811661 have a D' of 0.018, $r^2$=0 and LOD score of 0)

the 9p21.3 locus, but rather of the chemokines *CCL8* and *CCL2* and the lectins *CLEC4E* and *CLEC5A* [42•]. This study also did not find significant changes in type 1 interferon expression by 9p21.3 genotype.

## Haplotype Analysis Reveals Multiple Phenotypes at the 9p21.3 Locus

Tissue-specific enhancers are typically located at some distance from gene promoters [43]. If the 9p21.3 CAD risk locus disrupts sequences of a tissue-specific enhancer that control tissue-resident macrophage proliferation in the arterial wall and worsen coronary atherosclerosis, might other regions of the 9p21.3 locus affect cellular functions in other tissues?

When we further examined the 9p21.3 locus for association of specific haplotypes (groups of co-inherited SNPs) to different phenotypes we found 2 haplotypes, the well-known locus tagged by rs1333049 that associated with atherosclerosis and another tagged by rs518394 closer to *CDKN2B* that associated with MI, but only among cases of clinically significant (>50 % stenosis) angiographic CAD who were carriers of the rs1333049 non-risk allele [44•] (Fig. 1). Another surprise was that the MI haplotype was inversely associated with the severity of coronary atherosclerosis, suggesting that it exerts its effect on a minimal burden of plaque. A SNP linked to this region was previously associated with elevated platelet reactivity [45•]. Intriguingly, recent evidence points to a cross-talk between platelets and macrophages to clear blood borne bacterial pathogens, so that this dual haplotype may relate to an important component of the innate immune response [46].

Macrophages are the progeny of the myeloid lineage that includes Kupffer cells of the liver, tissue-resident macrophages of arteries, adipose tissue and pancreas, and microglia of the central nervous system. Genome-wide association studies have identified several diseases affected by polymorphisms at the 9p21.3 locus, including primary open angle glaucoma (PAOG) [47] and normal pressure glaucoma (NPG) [48, 49], glioma [50, 51], and type 2 diabetes (T2D) [52, 53] (Fig. 1). It is intriguing that in every case tissue macrophages are implicated in the disease process. Microglia worsen the outcome of glaucoma [49, 54, 55], glioma is often difficult to distinguish from gliosis [56], a condition in which microglia proliferate in response to pathogens [57], and in type 2 diabetes, where inflammatory macrophages proliferate in the vicinity of islets [58].

Mice hemizygous for *Mtap* (with 1 copy of *Mtap* deleted) were found to have more significant atherosclerotic lesions when bred on a susceptible background [59]. Whether this observation is the result of deficient MTAP function or reflects altered chromatin remodeling due to loss of one allele remains to be determined.

## Chromatin Remodeling at the 9p21.3 Locus

CTCF binding sequences are found between the *CDKN2A* gene and the antisense *ANRIL* transcript forming a bidirectional

promoter [60–62]. Expression of *ANRIL* and *CDKN2A* are both dependent on CTCF [61]. CTCF is a multifunctional transcription regulator that can not only activate 1 gene, but at the same time prevent inappropriate activation of another gene on the same chromosomal region. Thus, CTCF also acts as an insulator [63]. CTCF binds to unmethylated DNA target sequences. CTCF contains 11 zinc fingers and through its ability to bind DNA and homodimerize, CTCF can bring together distant sequences to form higher order chromatin structure [64]. CTCF has been implicated in long range intra- and even interchromosomal interactions [64, 65].

CTCF binds and activates the *CDKN2A* locus in a manner that is sensitive to DNA methylation [61, 62]. DNA methylation is not restricted to imprinted genes but is widely present across the genome [66, 67]. Importantly, increased genomic DNA methylation has been described in atherosclerotic lesions [68] and in peripheral lymphocytes of patients with CAD relative to controls [69]. The 9p21 CAD risk locus enhancer sequences located within the 3′ region of the *ANRIL* gene strongly upregulate transcription of *ANRIL*, and alter the levels of its multiple alternatively spliced isoforms [38•, 40•]. As a consequence, components of the polycomb repressor complex (PRC1 and PRC2) are recruited to the ANRIL transcript and silence gene expression from the *CDKN2A* locus, in part by histone 3 lysine 27 trimethylation by polycomb complex protein EZH2 [70, 71]. EZH2 also recruits the DNA methyltransferase DNMT1 [72] and this would also increase DNA methylation and inactivation of the *CDKN2A* locus. Zhuan et al. found that methylation of *CDKN2B* and elevated expression of *ANRIL* were associated with coronary artery disease in a Chinese angiographic study, but this was not tightly correlated to the 9p21 risk genotype [73]. This result suggests that allele-specific methylation is unlikely to participate in gene regulation at the 9p21.3 locus.

When cells differentiate or age they typically stop proliferating, and this requires activation of the cyclin dependent kinase inhibitors like $p16^{ink4a}$ [74]. Deletion of the 9p21.3 syntenic region in the mouse reversed cellular senescence of primary fibroblasts and VSMCs [39••]. Primary cultures of human arterial VSMCs showed reduced expression of $p16^{ink4a}$ and $p15^{ink4b}$ and increased cellular proliferation [75•]. Thus, the 9p21.3 locus appears to cause loss of *CDKN2A* and *CDKN2B* suppression. Activation of the *CDKN2A* locus requires displacement and eviction of PRC1 and PRC2, and the chromatin remodeling protein BRG1 plays a critical role in this process [76]. BRG1 interacts with CTCF and participates in remodeling long range interactions at the major histocompatibility complex locus [77]. BRG1 is also known to interact with several transcription factors, including GATA1 [78], STAT2 [79], and TEAD1 [80], so that these transcription factors are good candidates to regulate chromatin structure at the 9p21.3 locus.

## The Nature of the Altered Regulatory Sequences at the 9p21.3 Risk Locus

We reported that the risk allele of one of the SNPs (rs1333045) alters a TGFβ consensus sequence within an enhancer and reduced its function [38•]. Although we have not formally tested whether this is a true functional TGFβ site, this result suggests that sequences at or in the vicinity of this SNP are functional. A different hypothesis was proposed by the Frazer group. They suggested that another SNP (rs10757278) disrupts a putative binding site for the signal transducer of activated T cells 1 (STAT1), the transcription factor that mediates IFN-γ-inducible gene expression [40•]. However, our recent study showed that the upregulated expression of $p15^{ink4b}$ and $p16^{ink4a}$ in response to IFN-γ was not affected by the 9p21.3 risk allele, and in fact occurs largely by a posttranscriptional mechanism [81•]. Thus, a differential response to IFN-γ does not account for the 9p21 risk effect.

## TGFβ Signaling Inhibits Macrophage Foam Cell Formation

TGFβ is a cytokine that inhibits macrophage foam cell formation [82–84]. Low levels of plasma TGFβ are associated with a poor outcome in CAD [85]. Expression of a dominant negative mutant of the TGFβ receptor increases foam cell formation and worsens atherosclerosis [86]. Conversely, over-expression of TGFβ in macrophages reduces foam cell formation and atherosclerosis [87]. Together, these findings establish TGFβ as a potent anti-atherogenic factor.

What underlies the anti-atherogenic effect of TGFβ? Evidence suggests this comes about several ways. TGFβ suppresses the uptake and accumulation of oxidized LDL-cholesterol in macrophages by lowering the expression of the scavenger receptor CD36 [82, 83]. TGFβ also increases the export of cholesterol from macrophages by upregulating the expression of the cholesterol transporter ABCA1 [82, 84]. Macrophage proliferation also contributes to atherosclerosis. Ablation of the cell cycle inhibitor $p27^{Kip1}$ in hematopoietic progenitors accelerates macrophage proliferation and atherosclerosis [88]. Conversely, TGFβ blocks proliferation of human macrophages derived from myeloid cells stimulated by macrophage-colony stimulating factor (M-CSF) [89]. TGFβ has been shown to activate the expression of the cyclin-dependent kinase inhibitors *CDKN2B* ($p15^{ink4b}$) and *CDKN2A* ($p16^{ink4a}$) [90–92]. Of these TGFβ-responsive genes, only *CDKN2A* and *CDKN2B* are located in the vicinity and are under the direct control of the 9p21.3 genetic risk locus. Thus, genetic polymorphisms like rs1333045 that disrupt TGFβ-responsive elements at the 9p21.3 risk locus would be

predicted to interfere with TGFβ-mediated suppression of macrophage or VSMC proliferation and worsen atherosclerosis.

## TEAD Transcription Factors Interact with SMAD3 and Cooperate in TGFβ Signaling

TGFβ signaling activates SMAD transcription factors to regulate gene expression. TGFβ-responsive genes can be activated directly by SMADs through SMAD-responsive elements, or by other transcription factors via their interaction with SMAD proteins [93]. For example, TEAD transcription factors interact with SMAD3 and mediate TGFβ-dependent gene activation [94–96]. Recent evidence shows that TEAD/SMAD interaction controls proliferation of self-renewing cells [97] and that selective knockdown of TEAD transcription factors affects cellular senescence though a $p16^{inka}$-dependent pathway. Of particular interest, the activation of $p14^{ARF}$ by TGFβ2 in the eye is abrogated by deletion of the 9p21.3 risk locus syntenic region on mouse chromosome 4 [98•].

TEAD transcription factors are one of a small group of transcription factors that not only can regulate expression of genes in the proximity of TEAD-binding regulatory promoter/enhancer elements (in the short-range) [99], but can also activate genes at a distance by recruiting chromatin remodeling proteins [80, 100]. A recent study found that a chromatin remodeling protein is recruited to 2 TEAD binding sites within the 9p21.3 risk locus [100]. This result shows that TEAD factors are present at the 9p21.3 risk locus and may participate in its long-range regulation of gene expression.

## Conclusions

The mechanism whereby the 9p21.3 locus confers increased susceptibility to coronary atherosclerosis remains elusive. SNPs likely disrupt specific regulatory sequences within tissue-specific enhancers. Identifying these functional SNPs and the cells in which they are functional is a challenge not just for atherosclerosis but for other diseases that also associate with this locus.

### Compliance with Ethics Guidelines

**Conflict of Interest** Hsiao-Huei Chen, Naif A. M. Almontashiri, Darlène Antoine, and Alexandre F. R. Stewart declare that they have no conflict of interest.

**Human and Animal Rights and Informed Consent** This article does not contain any studies with human or animal subjects performed by any of the authors.

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- • Of importance
- •• Of major importance


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