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Perspectives

The Role of Noncoding "Junk DNA" in Cardiovascular Disease

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The complete sequencing of the human genome has revealed many mysteries on how aberrations in gene structure and regulation contribute to disease. One of the most unforeseen and potentially revolutionary findings since the complete annotation of the human genome is the complexity of noncoding DNA. What was once considered "junk DNA" now holds the keys to many novel gene regulatory mechanisms, and genetic variation within these regions likely accounts for a major portion of disease susceptibility. Until recently, the biochemical mechanisms linking noncoding DNA, which does not encode any proteins, to the pathogenesis of disease were largely unknown, but there has been a torrent of new information in this field that has the prospect of changing how we diagnose and treat patients.

An interesting example of how noncoding DNA can regulate gene expression and increase cardiovascular disease risk was recently reported by Visel et al. in *Nature (1)*. Many previous studies have identified a strong association between a genetic locus on 9p21 with cardiovascular disease outcomes, but the cause for this association was unknown. In some populations, as many as 25% of individuals harbor the risk allele, which increases cardiovascular risk as much as 3-fold. The report by Visel et al. *(1)* suggests that a distal noncoding DNA element located in 9p21 controls vascular gene expression and may account for its association with cardiovascular disease. Mice with an orthologous deletion of a 70-kbp segment in chromosome 4, which corresponds to the 9p21 region in humans, had increased weight gain and mortality on a high-fat diet. Deletion of this region also caused the selective loss of expression of 2 adjacent genes that lie approximately 100 kbp outside of the deletion. The 2 genes affected by the deletion are the cyclin-dependent kinase inhibitor genes *Cdkn2a*[2] (cyclin-dependent kinase inhibitor 2A) and *Cdkn2b* (cyclin-dependent kinase inhibitor 2B), which showed decreased expression in vascular tissue as well as in other tissues. The deletion was not found to affect the expression of any other neighboring genes. In accordance with the previously described role for *Cdkn2a/b* in cell division, decreased expression of these genes led to unchecked cell proliferation. Primary aortic smooth muscle cells and embryonic fibroblasts from homozygous mice with the 70-kbp deletion showed a 2- to 3-fold higher proliferation rate in cell culture, with no signs of senescence, even after many cell passages. The mice with the deletion also showed a marked increase in tumor cell development from several different cell types.

Linking distal-acting control elements to the expression of specific genes is often a challenge. Data from this study, however, convincingly demonstrated the presence of a cis-regulatory element within the 9p21 risk interval that regulates the transcription of *Cdkn2a/b*. C57BL/6 and 129/Sv mice have different single-nucleotide polymorphisms in their genes encoding Cdkn2a and Cdkn2b, and when these 2 mice strains were crossed, heterozygous mice produced equal amounts of mRNA from each gene variant, as measured by allele-specific PCR analysis. In contrast, when heterozygous mice were produced by crossing C57BL/6 mice with 129/Sv mice missing the 70-kbp interval on chromosome 4, mRNA for Cdkn2a/b was produced only from the C57BL/6 allele without the deletion. This result indicates that the 9p21 risk interval contains a distal-acting, cis-regulatory element that acts on the *Cdkn2a/b* gene locus, because a trans-acting element from the intact 70-kbp segment from the C57BL/6 allele would have been expected to rescue the expression from the 129/Sv allele with the deletion. Although the supposition still remains to be proved, individuals who harbor the risk allele in 9p21 presumably have a mutation or a polymorphism in a cis-regulatory element that interferes with the ability of this control region to act locally to increase the transcription of the *CDKN2A* [cyclin-dependent kinase inhibitor 2A (mel-

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[2] Genes: *Cdkn2a*, cyclin-dependent kinase inhibitor 2A [*Mus musculus* gene]; *Cdkn2b*, cyclin-dependent kinase inhibitor 2B [*Mus musculus* gene]; *CDKN2A*, cyclin-dependent kinase inhibitor 2A (melanoma, p16, inhibits CDK4) [human gene]; *CDKN2B*, cyclin-dependent kinase inhibitor 2B (melanoma, p16, inhibits CDK4) [human gene].

Table 1. Distal noncoding DNA regulatory elements.

Regulatory element	Abbreviation	Regulatory function	Composition
Enhancer	—	Increase transcription	DNA sequence
Repressor/silencer	—	Decrease transcription	DNA sequence
Insulator	—	Shield genes from distal inhibitory signals	DNA sequence
MicroRNA	miRNA	Decrease translation, increase mRNA degradation	Small ncRNA
Small interfering RNA	siRNA	Increase mRNA degradation	Small ncRNA
Telomere-specific small RNA	tel-sRNA	Regulate cell cycle	Small ncRNA
Piwi-interacting RNA	piRNA	Repress retrotransposons	Small ncRNA
Promoter-associated small RNA	PASR	Regulate transcription	Small ncRNA
Enhancer RNA	eRNA	Increase transcription	ncRNA

anoma, p16, inhibits CDK4)] and *CDKN2B* [cyclin-dependent kinase inhibitor 2B (melanoma, p16, inhibits CDK4)] genes.

The identity of the cis-regulatory element in 9p21 is still unknown. A previous study showed, however, that a conserved noncoding region within 9p21 increases the expression of heterologous promoters *(1)*, a finding consistent with the presence of an enhancer. Enhancers are specific noncoding DNA sequences that act in cis on nearby genes but can be located several thousand base pairs upstream or downstream from the transcription start site (Table 1). Enhancers are believed to alter local chromatin structure by binding specific proteins, which then facilitate gene transcription by promoting the attachment of RNA polymerase II and other necessary transcription factors. Alternatively, the control element in 9p21 could also be an insulator DNA sequence, which shields genes from distal negative regulatory control elements (Table 1). Besides enhancers and insulators, other noncoding regulatory DNA sequences that produce noncoding RNAs (ncRNAs)[3] have recently been described *(2)* (Table 1). Some types of enhancers, in fact, have been found to produce ncRNAs called "eRNAs" that can increase gene expression. Such eRNAs and other ncRNAs, however, would be expected to work in trans, although it has been proposed that some ncRNAs may remain tethered to their site of transcription and affect only local gene expression. A candidate eRNA called "ANRIL," which is already known to affect cell proliferation, has been identified in the 9p21 interval *(3)*, but whether it is responsible for the association of 9p21 with cardiovascular disease is not known at this time. MicroRNAs (miRNAs) are another class of ncRNAs that recently have generated a lot of interest because of their widespread effect on gene regulation. miRNAs are short oligonucleotides, usually about 22 bp in length, that can form a complex with their target mRNAs via complementary base pairing. They typically promote mRNA degradation and/or suppression of translation *(4)*. For several diseases, including cardiovascular disease, miRNAs have been shown to either contribute to or play a central role in the pathogenesis of disease and are now being investigated as possible therapeutic agents because of their ability to modulate gene expression *(4)*. As many as a third of the genetic loci linked to a wide variety of diseases in genomewide association studies have been found in noncoding regions of DNA, which suggests that many of the recently described noncoding DNA control elements may be involved in disease pathogenesis and thus may serve as potential risk markers.

Another interesting finding from the study by Visel et al. *(1)* is that the deletion of the 70-kbp interval on chromosome 4 in mice did not appreciably affect plasma lipids. This result is consistent with human studies in which patients with the risk allele did not appear to have either increased LDL cholesterol or low HDL cholesterol, despite their higher incidence of cardiovascular disease. It is well known that many individuals who do develop cardiovascular disease do not appear to be at risk according to their lipoprotein profile, suggesting that the development of atherosclerosis may depend not just on increased LDL but also on how arterial vessels respond to the injury or inflammation caused by the infiltration of LDL. One way vessels are known to respond to LDL infiltration is to proliferate smooth muscle cells, which then migrate from the media into the intima. A hypothesis that remains to be investigated is that decreased *CDKN2A/B* expression in patients with the risk allele may make smooth muscle cells from these patients hyperresponsive to prolif-

[3] Nonstandard abbreviations: ncRNA, noncoding RNA; eRNA, enhancer RNA; miRNA, microRNA.

erative signals generated by LDL in atherosclerotic plaques.

Understandably, most of our focus on the genetic susceptibility of cardiovascular disease has been on genes that affect lipid metabolism and other known risk factors that can be readily interrogated with plasma biomarkers or with simple physiological tests, such as blood pressure. A recent study of 101 well-documented single-nucleotide polymorphisms associated with cardiovascular disease has shown, however, that they do not significantly improve our ability to predict cardiovascular events after adjustment for plasma lipids *(5)*. This finding is perhaps not surprising given that besides genetic variation, many additional dietary and lifestyle factors, as well as the other commonly measured cardiovascular risk markers, can also affect plasma lipid concentrations. Therefore, another implication of the study by Visel et al. *(1)* is that it may be more fruitful to focus on genetic factors that control the response of the vessel wall to LDL infiltration and on the subsequent inflammation and other pathologic processes it engenders. Except perhaps for C-reactive protein, we currently do not have good biomarkers for the vascular injury response, and it may not be fully tractable without the use of genetic tests.

Thomas Edison once said, "To invent, you need a good imagination and a pile of junk." Fortunately, we still have our "junk DNA" and, we hope, new diagnostic tests based on noncoding DNA control elements, such as the one identified on the 9p21 interval, that will add someday to our ability to predict cardiovascular disease risk.

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References

1. Visel A, Zhu Y, May D, Afzal V, Gong E, Attanasio C, et al. Targeted deletion of the 9p21 non-coding coronary artery disease risk interval in mice. Nature 2010;464:409–12.
2. Lindberg J, Lundeberg J. The plasticity of the mammalian transcriptome. Genomics 2010;95:1–6.
3. Kim TK, Hemberg M, Gray JM, Costa AM, Bear DM, Wu J, et al. Widespread transcription at neuronal acitivity-regulated enhancers. Nature 2010;465:182–7.
4. Vickers KC, Remaley AT. MicroRNAs in atherosclerosis and lipoprotein metabolism. Curr Opin Endocrinol Diabetes Obes 2010;17:150–5.
5. Paynter NP, Chasman DI, Paré G, Buring JE, Cook NR, Miletich JP, Ridker PM. Association between a literature-based genetic risk score and cardiovascular events in women. JAMA 2010;303:631–7.