Commentary

Additive effects of gene regulatory variants in multifactorial disease

Lois M. Mulligan

Division of Cancer Biology and Genetics, Cancer Research Institute and Department of Pathology & Molecular Medicine, Queen's University, Kingston, Canada

Correspondence to: Dr. Lois M. Mulligan. Division of Cancer Biology and Genetics, Cancer Research Institute, Botterell Hall Rm A315, Queen's University, Kingston, ON, K7L 3N6, Canada. Email: mulligal@queensu.ca.

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The human genome contains extensive sequence variation, the vast majority of which lies in non-coding regions and is not known to be associated with any phenotype. However, as our understanding of the regulatory roles of sequences traditionally thought of as "non-coding" increases, it is clear that this pool of genomic variation may have mechanistic contributions to an array of complex genetic diseases. Multifactorial inheritance patterns underlie the vast majority of clinically relevant diseases of genetic origin. These conditions generally arise from the accumulated effects of multiple disease-associated variants. While familial risks in these diseases are clear, their genetics can be complex, and the significance of individual variants, and any overall pattern or clustering of mutations that give rise to the disease phenotype, difficult to interpret. As a result, it is not easy to predict risk or prognosis with any degree of certainty for the majority of the population.

Increasingly, patterns of genetic variations that affect expression or change functional outcomes by altering the regulation of key genes or gene networks are seen as important contributors to complex genetic diseases. In addition to well-characterized deleterious gene mutations that alter protein function or expression, variants in intronic or untranslated 5' or 3' sequences can affect the functions of regulatory elements and modulate expression of an otherwise normal gene sequence. Mutations in cis-acting regulatory sequences have been associated with a number of developmental diseases (1), however, recent studies have also shown that polymorphic variants within gene enhancer or repressor motifs can alter transcription factor or modifier binding and significantly change gene expression (2). In their recent studies, Chatterjee *et al*. (3) have evaluated a panel of polymorphic variants at the *RET* (rearranged during transfection) gene locus and assess their potential contributions to Hirschsprung disease (HSCR), a developmental disorder characterized by the failure of gut innervation. The authors describe approaches to identify key developmental regulatory elements and validate the biological effects of common sequence variants in these motifs that affect *RET* gene expression and function, and contribute to this complex disease phenotype.

RET is a receptor tyrosine kinase with important roles in development, maturation and maintenance of a number of human tissues, most notably of neural crest-derived cells (4). RET is required for migration of subsets of neural crest cells toward their target organs, and for proliferation, differentiation and survival of these cells once they reach their destinations (5,6). Under normal conditions, RET is activated by binding of glial cell line derived neurotrophic factor (GDNF) family ligands and a cell surface coreceptor (4). Loss of RET signals, through inactivating mutations or decreased RET expression, leads to reduced proliferation and survival of neural cells of the enteric nervous system, resulting in the failure of gut innervation seen in HSCR. Although RET is strongly associated with HSCR, only a minority of cases have detectable mutations in the *RET* coding sequence, and non-coding variants predicted to affect enhancer elements within introns or upstream of the coding

sequence have been predicted to alter *RET* expression (7-9). Chatterjee *et al*. (3) have sought to validate the role of *RET* non-coding variants in HSCR and assess the mechanisms by which they contribute to the disease phenotype. The authors investigated *RET* gene intronic and upstream regions for potential regulatory elements and common single nucleotide polymorphisms and identified three variants (rs2506030, rs7069590, rs2435357) within putative enhancer sequences (termed RET-7, RET-5.5 and RET+3, respectively) that could significantly reduce the functions of these elements. This group had previously shown that two of these variants individually were HSCR risk-alleles (7,8), but here they also demonstrated that "high-risk" haplotypes across these loci impaired binding of several transcription factors and reduced enhancer function *in vitro*. The RET+3 element is a known SOX10 binding motif (7), but Chatterjee *et al*., also validated binding of RARB at RET-7 and GATA2 at RET+3 and elegantly demonstrated that disruption of any of these enhancer motifs specifically blocked transcription factor binding and led to reduced *RET* expression.

Interestingly, the authors showed that the RET-7, RET-5.5 and RET+3 enhancer elements had variable activity at important developmental time points in neural lineages, suggesting overlapping developmental contributions. Thus, *in vivo RET* expression is promoted through the effects of transcription factor binding at multiple enhancers that exert these effects at different stages in the developing embryo. The high-risk HSCR haplotypes represent a "perfect storm" of risk genotypes-decreasing the effects of multiple enhancers, each in a specific developmental window, thus reducing *RET* expression in these key developmental stages, and conferring an overall reduction in biological potency, contributing to the HSCR phenotype. Further, the authors predict that decreased RET expression disrupts a feed-back loop and leads to reduced expression of SOX10 and GATA2, further enhancing the effects of these HSCR risk-variants. Feedback loops affecting receptor tyrosine kinase signaling are important regulatory mechanisms, and disruption of these loops through alteration of enhancer function can have broad effects on downstream signals (10,11).

To validate their *in vitro* findings, Chatterjee *et al*. (3) explore the effects of RET depletion on gene expression in the developing gut using a *RET* knockout animal model and wild-type control (12). The authors confirmed reductions in *SOX10* and *GATA2* seen *in vitro*, but also noted much broader changes in gene expression patterns for components of RET downstream signaling cascades, RET ligand and coreceptor, and other RET regulatory molecules. The authors suggest that RET is the key regulator of downstream signals but also a target of its own regulatory network. Importantly, the RET null model used lacks enteric ganglia and elements of the enteric nervous system in the colon (12) and thus the cellular composition of the gut tissues is altered relative to control. Recent studies have also shown that RET is required for hematopoietic stem cell survival and expansion and that RET depletion may affect the composition and maintenance of hematopoietic progenitors and lineage distribution in peripheral tissues and affect the gut immune environment (13,14). While the gene expression changes in the RET regulatory network in the embryonic gut observed in this study may be direct responses to RET loss and feedback loop reductions in important transcription regulators, it may also in part reflect these broader cellular changes. It will be interesting and exciting in future to see the authors tease apart cell autonomous effects of reduced *RET* expression from more global tissue-wide effects of RET-dependent changes in tissue cell type composition and the immune microenvironment to identify the mechanisms that affect overall gene expression changes in the RET regulatory network.

Chatterjee *et al*. (3) provide compelling evidence for high-risk susceptibility enhancer haplotypes that decrease *RET* expression in gut development contributing to HSCR. This is an important advance in a field where opportunities for genetic diagnosis and disease prediction have been very limited. However, identification of high-risk HSCR haplotypes is still some way from clinical application. These susceptibility haplotypes are over represented in HSCR patients, but notably, are also found in normal populations, suggesting that reduced expression of *RET* is required for HSCR but is not sufficient for full expression of the disease phenotype. The authors predict that there are a number of additional regulatory/enhancer sequences within the *RET* gene and its upstream sequences that likely contribute to control of *RET* expression that should also be explored. Together, these data demonstrate that tissue homeostasis is very sensitive to levels of RET activity and suggest its expression and function may be regulated through multiple cell autonomous and paracrine mechanisms.

Sequence variants that affect cis-acting regulatory elements are increasingly being recognized as important contributors to modulation of gene expression (2). In this study, Chatterjee *et al*. (3) apply both genetic and functional approaches to demonstrate that common

variants in regulatory elements can be important players in multifactorial disease by affecting expression of the primary disease-associated gene but also by modulating expression of components of its regulatory and signaling networks. The importance of characterizing integrated regulatory processes has been well understood for decades in the cancer field, where the effects of networks of oncogenes and tumour suppressor genes balance cell growth and survival to block the growth and spread of cancer (15). However, genetic contributions to multifactorial inheritance patterns have often been regarded in isolation, without consideration for the interdependence of regulatory systems. The work described in this study (3) offers new perspectives on integrating genetic and functional studies to identify the key signaling networks and important regulatory elements that contribute to the disease phenotype, and which will, in future, provide diagnostic and/or therapeutic opportunities to address complex inheritance patterns.

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