the spliceosome (rare phenotype) might have some hereditary component.

ARCH is a quantitative trait much like blood pressure and height. Although ARCH has been defined so far as a binary attribute, the true nature of ARCH is described by the clonal diversity of the blood system. Genetic diversity is defined by the number of hematopoietic stem and progenitor cells in a single individual and their relative contribution to the mature blood pool. This genetic diversity can change with aging (ARCH) but also due to other causes (aplastic anemia, chemotherapy, etc).[3] Accordingly, one can estimate the hereditary contribution to the blood system genetic diversity (as a continuous measure). Because this parameter changes with age and probably with sex and other parameters, the study design should take all these potential contributors into account. For example, ARCH or genetic diversity at a young age might be correlated with a stronger genetic component.

In some cases, a phenotype can have a stronger genetic predisposition in a specific subpopulation. A good example is end-stage kidney disease in African Americans.[10] ARCH has not been well characterized, yet in many human subpopulations, such epidemiological studies could shed light on specific genetic predisposition.

Another interesting aspect that was observed in both studies is the fact that in few cases the preleukemic mutation was acquired in utero (both twins carried the same mutation). This observation again stresses the importance of the environment in the evolution of ARCH. Only after many years did the ARCH phenotype evolve despite the presence of the mutation in hematopoietic stem cells for at least 50 years (in some of the cases). In utero acquisition of preleukemic mutations might be even more common but could be missed because of the disconcordance of the ARCH phenotype.

Altogether, the important conclusions from these twin studies suggest that, among the elderly, CH is more strongly influenced by the environment, with the caveats described above. These studies suggest that the search for environmental contributors to ARCH is more pressing than genetic predispositions.

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Comment on Bridgford et al, page 287

MPL membrane domain sequencing goes deep

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In this issue of *Blood*, Bridgford et al[1] present the first systematic approach to uncover all possible activating single point mutations within and around the transmembrane domain (TMD) of the thrombopoietin receptor (TpoR; also known as MPL). Several canonical activating MPL mutations (S505N and W515K/L/A/R) have been discovered through structure-function studies and patient sequencing,[2-6] but there remain "triple-negative" essential thrombocythemia and primary and secondary myelofibrosis cases, as well as hereditary thrombocytosis cases, without established driver mutations (ie, no *JAK2*, *CALR*, or *MPL* variants). The TpoR is a cytokine receptor that can be easily activated by dimerization in several different orientations,[7,8] which is unusual, but can explain the multitude of mutations that lead to an active or partially active phenotype. However, which specific variants can activate the receptor and drive disease?

Bridgford et al address this question with deep mutational scanning to evaluate all possible single amino acid substitutions in the human TpoR TMD for their ability to confer cytokine-independent growth in Ba/F3 cells. The authors undertook an incredibly efficient examination of all possible 19 amino acid residues (saturation mutagenesis) at each of the 29 positions of the target (TpoR) juxtamembrane-transmembrane region (residues 488 to 516). In all, 580 mutants were screened (29 positions, 20 codons each including the stop codon) representing the 19 remaining amino acids at each position. An enrichment was detected for the classical S505N and W515 mutations, but also for novel mutations (S493C/L, L498W, V501S, L502S/C, L508Q) (see Figure 1A in Bridgford et al).

This strategy was also used to answer another important question: whether the constitutive signaling of a canonical MPL

S505N mutant could be enhanced by another mutation in *cis*. The deep sequencing approach applied to a template of MPL S505N instead of wild-type MPL led to the novel finding that a number of mutations in *cis* at H499 and other positions significantly augmented the constitutive signaling of the canonical activating MPL S505N mutation. The power of this saturation deep sequencing approach is impressive and has allowed the rapid identification and testing of novel double mutants.

The results highlight the specificity and conformational requirements for activating mutations. Generally, only 1 or 2 substitutions exerted an enhancing effect at a specific position. Most, but not all, of the single activating mutations were positioned on 1 helical face of the TMD, possibly using polar interactions to promote dimerization, although further studies are required to establish the precise structural requirements for activation. More than 90 different second-site mutations enhanced the activity of MPL S505N. Among these were novel but weak or nonactivating mutations at H499, G503, G509, and S493, and canonical activating mutations at W515 and other mutations around the W515.

Subtle differences were revealed whereby V501A was stronger at enhancing S505N, whereas V501S was more effective as a single mutation. Of great interest, although not developed extensively in this report, the deep sequencing approach on MPL S505N also uncovered second-site mutations that antagonize S505N activation. These data might offer leads to specific inhibitory strategies for MPL S505N.

Careful examination of databases containing published and unpublished data from exon 10 sequencing of 2452 MPN patients showed that some of the 7 novel identified variants and several double mutations had previously been detected in patients. These single and double mutants[9] were created at the complementary DNA level and tested in Ba/F3 cells, where they confirmed an active phenotype.

Random mutagenesis around the TMD of the erythropoietin receptor coupled to functional selection in Ba/F3 cells has been accomplished previously, but without the next-generation sequencing before and after selection,[10] which in this study allowed for testing of all possible mutants, with rigorous control of similar representation in the library, thus avoiding potential biases that might otherwise result. The deep mutagenesis and next-generation sequencing used here could be extended to any region of MPL and to other cytokine receptors, or indeed to any other signaling protein, either wild type or already mutated. The approach, in principle, allows selection for activators and inhibitors of signaling, provided selection is available. One limitation is that it does not control for defects in cell surface localization or expression of a particular mutant at the protein level. Nevertheless, it does offer a novel and powerful way to readily predict which mutations might lead to oncogenic activity. Last, but not least, the identification of a mutation in MPL in patients that allows constitutive signaling is very likely to mean the identification of a driver mutation, and such mutations need to be confirmed in functional assays. All in all, this work provides a ready reference for possible activating mutations in MPL's TMD, which is likely to prove useful both for diagnostic purposes and for further understanding of the structural requirements that lead to MPL activation.

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