Severe congenital neutropenia and chronic neutrophilic leukemia: an intriguing molecular connection unveiled by oncogenic mutations in *CSF3R*

Ivo P. Touw, and Renée Beekman

Department of Hematology, Erasmus University Medical Center, Rotterdam, The Netherlands

E-mail: i.touw@erasmusmc.nl *doi:10.3324/haematol.2013.090571*

Acquired mutations in the colony-stimulating factor 3 receptor gene (*CSF3R*), truncating the cytosolic region of the CSF3R protein, were discovered almost two decades ago in severe congenital neutropenia (SCN) patients receiving CSF3 treatment to alleviate neutropenia. These *CSF3R* mutations are thought to drive clonal expansion by overriding CSF3 hypo-responsiveness of hematopoietic stem and progenitor cells (HSPCs) and are associated with leukemic progression in SCN patients. Furthermore, malignant transformation in one SCN patient coincided with acquisition of an additional auto-activating *CSF3R* mutation, supporting the hypothesis that perturbed CSF3 signaling contributes to leukemic transformation of SCN. While acquisition of *CSF3R* mutations had so far mainly been observed in SCN patients receiving CSF3 therapy, Maxson and colleagues[1] discovered that both truncating and auto-activating *CSF3R* mutations are frequently present in chronic neutrophilic leukemia (CNL) and atypical chronic myeloid leukemia (aCML). Furthermore, this study provided preliminary evidence for clinical utility of tyrosine kinase inhibitors (e.g. dasatinib or ruxolitinib) to eradicate *CSF3R* mutant clones. Here, we discuss the biological and clinical significance of these new findings in the light of the unanticipated and intriguing connection between SCN and aCML/CNL, diseases that are respectively characterized by a severe paucity or an excess of neutrophils.

CSF3R *mutations in severe congenital neutropenia*

Severe congenital neutropenia is a genetically heterogeneous disorder with a variable inheritance.[2] Mutations in *ELANE*, the gene encoding neutrophil elastase, are the most frequent cause of autosomal dominant and sporadic SCN.[2] CSF3 (also known as granulocyte colony-stimulating factor, G-CSF) is successfully used to treat SCN, alleviating the severe neutropenia and reducing infection-related mortality in over 90% of the patients.[3] A major complication in SCN patients is the high risk of developing myelodysplastic syndrome (MDS) and acute myeloid leukemia (AML).[4,5] This leukemic transformation correlates with the acquisition of mutations in *CSF3R*.[6-8] These are mostly nonsense mutations, causing the truncation of approximately 100 amino acids of the cytoplasmic domain of the receptor.[6-8] Activation of CSF3R truncation mutants with CSF3 induces a hyperproliferative response of myeloid progenitors *in vivo* and results in sustained STAT5 activation, an essential step in the clonal expansion of HSPCs expressing these mutant receptors.[9-11] Furthermore, activation of truncated CSF3Rs results in excessive production of reactive oxygen species (ROS).[12] The time between the first detection of *CSF3R* mutations and the diagnosis of MDS/AML varies greatly, i.e. between several months to up to more than ten years, indicating that additional aberrations play a role in leukemic transformation. Whole exome sequencing on serial hematopoietic samples of an SCN patient who progressed to MDS/AML 17 years after the start of G-CSF treatment identified 12 non-synonymous somatic mutations.[13] Two of these mutations, in *LLGL2* and *ZC3H18*, were acquired in a CSF3R mutant clone in the early SCN phase, 15 years before overt leukemia. This clone, carrying the delta 715 *CSF3R* truncating mutation (*CSF3R*-d715), expanded in time, whereas clones harboring functionally similar but structurally distinct *CSF3R* mutations (d717, d725, d730) became extinct. These latter clones did not harbor additional mutations that were found in the leukemic clone, which may explain why they disappeared over time. Additionally, a new mutation (*CSF3R*-T595I) in the extracellular domain of CSF3R on the already affected *CSF3R*-d715 allele was present in the AML phase, resulting in autonomous proliferation of myeloid progenitors.[13] These findings suggest that leukemic progression of SCN is a multistep process in which clones with mutations arising early in the SCN phase (*CSF3R*-d715, *LLGL2*, *ZC3H18*) selectively expand and acquire additional mutations (e.g. in *SUZ12*, *ASXL1*, *RUNX1*, *CSF3R-T595I*) at later stages of leukemia development. The question as to whether CSF3R mutations cause leukemic progression of SCN, e.g. by evoking DNA damage as a result of elevated ROS production, or merely drive clonal expansion of the affected HSPCs still has to be resolved.[14]

CSF3R *mutations in chronic neutrophilic leukemia and atypical chronic myeloid leukemia*

Chronic neutrophilic leukemia and aCML are myeloproliferative diseases with an unfavorable prognosis, i.e. a median survival between 14 and 21 months after initial diagnosis.[15,16] Until recently, the molecular pathogenesis of these diseases was unknown and the diagnosis based on morphological aspects, clinical criteria and exclusion of known genetic entities like the Philadelphia translocation indicative of CML, or JAK2 mutations indicative of myeloproliferative neoplasms (MPN).[17,18] Maxson and colleagues sequenced 1862 genes, representing all kinases, phosphatases, cytokine receptors and selected adapter proteins and identified *CSF3R* mutations in 16 of 27 patients with CNL or aCML.[1] The most prevalent mutation was a missense mutation in *CSF3R*, annotated as T618I, replacing a threonine by an isoleucine in the membrane proximal extracellular region of the CSF3R protein. This is the same mutation as the auto-activating CSF3R-T595I mutation found in SCN/AML; the difference in nomenclature is due to inclusion of the signal peptide of 23 amino acids in the amino acid numbering by Maxson *et al.*[1,13] Significantly, 5 CNL patients also had *CSF3R* truncation mutations which were present on the same allele as the CSF3R-T618I mutation, identical to what was reported in SCN/AML.[13] However, the prevalence of auto-activating membrane proximal mutations (14 of 27) exceeded the number of distal truncating mutations (6 of 27) in CNL and aCML, while in SCN the

opposite is observed (R Beekman and IP Touw, 2013, unpublished data). There is some controversy about the occurrence of *CSF3R* mutations in aCML when strict WHO-defined criteria are applied, and on the same basis, the frequency of auto-activating *CSF3R* mutations in CNL is estimated to exceed 80%.[19]

Why do SCN/AML and CNL share identical CSF3R mutations?

CSF3R mutations are rare or absent in MDS, chronic myelomonocytic leukemia (CMML), primary myelofibrosis (PMF) and *de novo* AML,[19-23] which makes the frequent involvement of these mutations in the pathogenesis of SCN/AML and CNL exceptional. Although it can easily be seen why isolated *CSF3R* auto-activating mutations lead to the uncontrolled expansion of myeloid progenitors in CNL and SCN/AML, it remains puzzling how the combination of *CSF3R* auto-activating and truncating mutations unite the molecular pathogenesis of these conditions. Specifically, it is unclear why one-third of the CNL patients also had acquired *CSF3R* truncation mutations. Multiple studies have shown that perturbed JAK/STAT signaling promotes the clonal expansion of pre-leukemic *CSF3R* mutant clones in SCN patients,[9,11,24,25] which is in line with the fact that clones with *CSF3R* truncation mutations in SCN patients usually appear before presentation of overt leukemia. Similar information on the timing of acquisition of these mutations in CNL is missing, because pre-neoplastic blood or bone marrow samples from these patients are usually not available. A scenario of a CSF3-driven clonal expansion phase cannot immediately be envisaged in CNL, unless patients would first go through a stage of CSF3 hyporesponsiveness and bone marrow failure from which HSPCs escape through the acquisition of *CSF3R* truncating mutations. Although there is still no experimental evidence for this, the example of mutations in *JAK2* and *NRAS* in MDS, MPN and the MDS/MPN overlap syndrome provides an analogy of how mutations affecting cytokine signaling may contribute to the disease transition from a myelosuppressive state towards a myeloproliferative neoplasm.[26] Another implication of the clonal evolution model is that *CSF3R* mutations in CNL may be preceded by other defects. However, this remains to be determined, as the candidate approach adopted to identify mutations in CNL and aCML[1] by Maxson and colleagues did not identify the full spectrum of abnormalities. Until now, mutations in *SETBP1*, encoding a nuclear protein that interacts with *Hoxa9* and *Hoxa10* promoters and enhances the self-renewal of HSPCs in mice[27] were found to be most frequently co-expressed with *CSF3R* mutations in CNL.[19] Recurrent *SETBP1* mutations were also detected in secondary AML but not in *de novo* AML,[28,29] suggesting that they may be driving the transition from a pre-leukemic towards a leukemic state. Notably, *SETBP1* mutations were reported to elevate the ability of SETBP1 to immortalize HSPCs,[28] but whether and how they co-operate with *CSF3R* mutations is unknown. Still, the possibility remains that other abnormalities, e.g. in epigenetic regulators, precede the acquisition of *CSF3R* mutations, as has been demonstrated in MPN with *JAK2*-V617F mutations.[30] These mutations might provide yet another missing link between SCN/AML, CNL and aCML.

Clinical implications

The discovery of *CSF3R* mutations in CNL and aCML was part of an integrated approach involving the use of small molecule tyrosine kinase inhibitors and interfering RNAs.[1] The results clearly indicate that the *CSF3R* auto-activating and truncating mutations affect distinct signaling pathways which can be targeted by different inhibitors. The JAK1/JAK2 inhibitor ruxolitinib effectively reduced proliferative signaling from the auto-activating CSF3R-T618I mutant in an *in vitro* model and a preliminary study showed the potential of ruxolitinib to reduce neutrophil levels in a CNL patient. Whether ruxolitinib, either as a single treatment or combined with, for example, hydroxyurea will lead to durable and meaningful responses in CNL needs to be addressed in future clinical studies. The reported finding that dasatinib selectively inhibits clones expressing truncated CSF3Rs merits further studies to analyze the combined effect of ruxolitinib and dasatinib on CNL and aCML clones carrying both types of CSF3R mutations. Furthermore, the effectiveness of dasatinib on clones carrying the truncated CSF3R may not only be of clinical significance for CNL and aCML, but also for SCN. Future studies will explore how dasatinib and other inhibitors affect G-CSF signaling from wild type and truncated CSF3Rs. This will reveal how inhibitors can be employed *in vivo* to selectively eradicate clones harboring truncated CSF3Rs in SCN patients, to reduce the risk of malignant transformation.

Ivo Touw is Professor at the Department of Hematology at the Erasmus University Medical Center (Erasmus MC), Rotterdam, The Netherlands. His main research interests are the mechanisms involved in the pathogenesis of bone marrow failure syndromes, and their transformation to myelodysplasia and acute myeloid leukemia. Renée Beekman did her PhD training at the Erasmus MC. She currently works as a post doc researcher at the August Pi i Sunyer Biomedical Research Institute (IDIBAPS) in Barcelona, Spain. She has received fellowships from the EU (Marie Curie) and The Netherlands Organisation for Scientific Research (Rubicon). Her main interests are the epigenetic and genetic mechanisms underlying hematologic malignancies.

Financial and other disclosures provided by the author using the ICMJE (www.icmje.org) Uniform Format for Disclosure of Competing Interests are available with the full text of this paper at www.haematologica.org.

References

1. Maxson JE, Gotlib J, Pollyea DA, Fleischman AG, Agarwal A, Eide CA, et al. Oncogenic CSF3R mutations in chronic neutrophilic leukemia and atypical CML. N Engl J Med. 2013;368(19):1781-90.
2. Dale DC, Link DC. The many causes of severe congenital neutropenia. N Engl J Med. 2009;360(1):3-5.
3. Dale DC, Bonilla MA, Davis MW, Nakanishi AM, Hammond WP, Kurtzberg J, et al. A randomized controlled phase III trial of recombinant human granulocyte colony-stimulating factor (filgrastim) for treatment of severe chronic neutropenia. Blood. 1993;81(10):2496-502.
4. Rosenberg PS, Alter BP, Bolyard AA, Bonilla MA, Boxer LA, Cham B, et al. The incidence of leukemia and mortality from sepsis in patients with severe congenital neutropenia receiving long-term G-CSF therapy. Blood. 2006;107(12):4628-35.
5. Rosenberg PS, Zeidler C, Bolyard AA, Alter BP, Bonilla MA, Boxer LA, et al. Stable long-term risk of leukaemia in patients with severe congenital neutropenia maintained on G-CSF therapy. Br J Haematol. 2010;150(2):196-9.
6. Dong F, Brynes RK, Tidow N, Welte K, Löwenberg B, Touw IP. Mutations in the Gene for the Granulocyte Colony-Stimulating–Factor

Receptor in Patients with Acute Myeloid Leukemia Preceded by Severe Congenital Neutropenia. N Engl J Med. 1995;333(8):487-93.
7. Dong F, Dale DC, Bonilla MA, Freedman M, Fasth A, Neijens HJ, et al. Mutations in the granulocyte colony-stimulating factor receptor gene in patients with severe congenital neutropenia. Leukemia. 1997;11(1):120-5.
8. Germeshausen M, Ballmaier M, Welte K. Incidence of CSF3R mutations in severe congenital neutropenia and relevance for leukemogenesis: Results of a long-term survey. Blood. 2007;109(1):93-9.
9. Hermans MH, Ward AC, Antonissen C, Karis A, Lowenberg B, Touw IP. Perturbed granulopoiesis in mice with a targeted mutation in the granulocyte colony-stimulating factor receptor gene associated with severe chronic neutropenia. Blood. 1998;92(1):32-9.
10. Hermans MH, Antonissen C, Ward AC, Mayen AE, Ploemacher RE, Touw IP.. Sustained receptor activation and hyperproliferation in response to granulocyte colony-stimulating factor (G-CSF) in mice with a severe congenital neutropenia/acute myeloid leukemia-derived mutation in the G-CSF receptor gene. J Exp Med. 1999;189(4):683-92.
11. Liu F, Kunter G, Krem MM, Eades WC, Cain JA, Tomasson MH, et al. Csf3r mutations in mice confer a strong clonal HSC advantage via activation of Stat5. J Clin Invest. 2008;118(3):946-55.
12. Zhu QS, Xia L, Mills GB, Lowell CA, Touw IP, Corey SJ. G-CSF induced reactive oxygen species involves Lyn-PI3-kinase-Akt and contributes to myeloid cell growth. Blood. 2006;107(5):1847-56.
13. Beekman R, Valkhof MG, Sanders MA, van Strien PM, Haanstra JR, Broeders L, et al. Sequential gain of mutations in severe congenital neutropenia progressing to acute myeloid leukemia. Blood. 2012;119(22):5071-7.
14. Beekman R, Touw IP. G-CSF and its receptor in myeloid malignancy. Blood. 2010;115(25):5131-6.
15. Bohm J, Schaefer HE. Chronic neutrophilic leukaemia: 14 new cases of an uncommon myeloproliferative disease. J Clin Pathol. 2002;55(11):862-4.
16. Hernandez JM, del Canizo MC, Cuneo A, Garcia JL, Gutierrez NC, Gonzalez M, et al. Clinical, hematological and cytogenetic characteristics of atypical chronic myeloid leukemia. Ann Oncol. 2000;11(4):441-4.
17. Bennett JM, Catovsky D, Daniel MT, Flandrin G, Galton DA, Gralnick H, et al. The chronic myeloid leukaemias: guidelines for distinguishing chronic granulocytic, atypical chronic myeloid, and chronic myelomonocytic leukaemia. Proposals by the French-American-British Cooperative Leukaemia Group. Br J Haematol. 1994;87(4):746-54.
18. Galton DA. Haematological differences between chronic granulocytic leukaemia, atypical chronic myeloid leukaemia, and chronic myelomonocytic leukaemia. Leuk Lymphoma. 1992;7(5-6):343-50.
19. Pardanani A, Lasho TL, Laborde RR, Elliott M, Hanson CA, Knudson RA, et al. CSF3R T618I is a highly prevalent and specific mutation in chronic neutrophilic leukemia. Leukemia. 2013;27(9):1870-3.
20. Beekman R, Valkhof M, van Strien P, Valk PJ, Touw IP. Prevalence of a new auto-activating colony stimulating factor 3 receptor mutation (CSF3R-T595I) in acute myeloid leukemia and severe congenital neutropenia. Haematologica. 2013;98(5):e62-3.
21. Forbes LV, Gale RE, Pizzey A, Pouwels K, Nathwani A, Linch DC. An activating mutation in the transmembrane domain of the granulocyte colony-stimulating factor receptor in patients with acute myeloid leukemia. Oncogene. 2002;21(39):5981-9.
22. Link DC, Kunter G, Kasai Y, Zhao Y, Miner T, McLellan MD, et al. Distinct patterns of mutations occurring in de novo AML versus AML arising in the setting of severe congenital neutropenia. Blood. 2007;110(5):1648-55.
23. Mehta HM, Glaubach T, Long A, Lu H, Przychodzen B, Makishima H, et al. Granulocyte colony-stimulating factor receptor T595I (T618I) mutation confers ligand independence and enhanced signaling. Leukemia. 2013 Jun 6. doi: 10.1038/leu.2013.164. [Epub ahead of print]
24. McLemore ML, Poursine-Laurent J, Link DC. Increased granulocyte colony-stimulating factor responsiveness but normal resting granulopoiesis in mice carrying a targeted granulocyte colony-stimulating factor receptor mutation derived from a patient with severe congenital neutropenia. J Clin Invest. 1998;102(3):483-92.
25. Touw IP, Bontenbal M. Granulocyte colony-stimulating factor: key (f)actor or innocent bystander in the development of secondary myeloid malignancy? J Natl Cancer Inst. 2007;99(3):183-6.
26. Bacher U, Schnittger S, Kern W, Weiss T, Haferlach T, Haferlach C. Distribution of cytogenetic abnormalities in myelodysplastic syndromes, Philadelphia negative myeloproliferative neoplasms, and the overlap MDS/MPN category. Ann Hematol. 2009;88(12):1207-13.
27. Oakley K, Han Y, Vishwakarma BA, Chu S, Bhatia R, Gudmundsson KO, et al. Setbp1 promotes the self-renewal of murine myeloid progenitors via activation of Hoxa9 and Hoxa10. Blood. 2012;119(25):6099-108.
28. Makishima H, Yoshida K, Nguyen N, Przychodzen B, Sanada M, Okuno Y, et al. Somatic SETBP1 mutations in myeloid malignancies. Nat Genet. 2013;45(8):942-6.
29. Piazza R, Valletta S, Winkelmann N, Redaelli S, Spinelli R, Pirola A, et al. Recurrent SETBP1 mutations in atypical chronic myeloid leukemia. Nat Genet. 2013;45(1):18-24.
30. Shih AH, Abdel-Wahab O, Patel JP, Levine RL. The role of mutations in epigenetic regulators in myeloid malignancies. Nat Rev Cancer. 2012;12(9):599-612.

Light chain amyloidosis: the heart of the problem

Giampaolo Merlini, and Giovanni Palladini

Amyloidosis Research and Treatment Center, Fondazione IRCCS Policlinico San Matteo and Department of Molecular Medicine University of Pavia, Pavia, Italy

E-mail: gmerlini@unipv.it *doi:10.3324/haematol.2013.094482*

In this issue of Haematologica, Dinner *et al.* report the outcome of a pilot study of an oral regimen of lenalidomide in combination with dexamethasone and low-dose melphalan in 25 patients with light chain amyloidosis (AL), most of them with cardiac involvement.[1] The treatment proved to be toxic and unable to improve survival in patients with advanced amyloid cardiomyopathy and poor performance status. High rates of early cardiac deaths (42%) and of cardiac arrhythmias (33%), probably triggered by high-dose dexamethasone, drug myelotoxicity limiting the duration of treatment to 3 cycles, and a mere 9% cardiac response rate, contributed to a highly disappointing median overall survival of 1.75 months in patients with late-stage heart damage. The outcome of this study highlights the urgent need for novel approaches in order to improve the prospects of AL amyloidosis patients with advanced cardiomyopathy.

Light chain amyloidosis is the most common systemic amyloidosis, with an incidence of 10 patients per million per year, resulting in approximately 5,000 new patients/year in the European Union. It is sustained by a usually small, indolent, plasma cell clone whose biological features, shared by less proliferative plasma cell dyscrasias, make it more sensitive to chemotherapy. The clonal plasma cells synthesize an excess of light chains (LC) with specific mutations and unique structural features causing systemic proteotoxicity. Interactions of the misfolded LCs with cells, extracellular matrix components and other constituents commonly found in amyloid deposits, such as serum amyloid P (SAP), and other molecules, play an important role in the disease process and are potential targets for therapy. AL amyloidosis is also the most severe systemic amyloidosis because over 70% of patients present with cardiac involvement, which results in the development of rapidly progres-