Prevalence of a new auto-activating colony stimulating factor 3 receptor mutation (CSF3R-T595I) in acute myeloid leukemia and severe congenital neutropenia

by Renee Beekman, Marijke Valkhof, Paulette van Strien, Peter Valk, and Ivo P. Touw

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Prevalence of a new auto-activating colony stimulating factor 3 receptor mutation (CSF3R-T595I) in acute myeloid leukemia and severe congenital neutropenia

Running title: Prevalence of CSF3R-T595I in AML

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IPT was the principal investigator and takes primary responsibility for the paper; RB, MV, and PvS performed laboratory work for this study; PJMV provided patient samples; IPT and RB coordinated the research and wrote the paper. The authors report no potential conflicts of interest.
Colony-stimulating factor 3 (CSF3), also known as granulocyte-colony stimulating factor (G-CSF), is the main growth factor driving neutrophil production under physiological conditions and during episodes of microbial infections. A tight but dynamic balance between signal activation and attenuation of CSF3R is essential to achieve a well-adjusted neutrophil output both under steady-state and infectious conditions (1, 2). CSF3 is used in the clinic to treat patients with severe congenital neutropenia (SCN), a bone marrow failure syndrome characterized by peripheral absolute neutrophil counts below 0.5 million per liter (3). Inherited or congenital mutations in ELANE and HAX1 are the most frequent cause of SCN (4). Acquisition of nonsense CSF3R mutations that truncate the carboxyl-terminus from the CSF3R protein is a common phenomenon in SCN patients (5). Studies in a variety of in vitro and in vivo models have established that these truncated CSF3R proteins confer elevated CSF3-induced proliferative responses to myeloid progenitors (5). CSF3R nonsense mutations have been reported in approximately 30% of SCN patients in the neutropenia phase, which increases to approximately 80% after progression to acute myeloid leukemia (AML) (6-8). In contrast, such CSF3R nonsense mutations have not been reported in de novo AML (9-11). We recently identified a new extracellular CSF3R mutation (T595I) in the leukemic blasts of a SCN patient after progression to AML (12). This mutation, located on the CSF3R allele that already carried the nonsense mutation, causes fully autonomous, i.e., colony-stimulating factor independent, proliferation of myeloid progenitors in semi-solid colony assays (12). Because most SCN patients receive life-long CSF3 therapy, we asked whether the acquisition of CSF3R-T595I mutations is unique for SCN/AML or whether this mutation can also be found in AML patients who did not receive CSF3 treatment. To address this, we investigated the prevalence of CSF3R-T595I mutations in 1446 consecutive de novo AML patients, none of which had a documented history of CSF3 treatment. The study was performed with permission of the Institutional Review Board of the Erasmus MC, registration number MEC-2008-387. Amplicons were generated from cDNA using 5'-GCTCAGAACCAGTCTTCTC-3' and 5'-CTGCTGTGAGCTGGGTCTG-3' primers and analyzed with denaturing high-performance liquid chromatography (dHPLC) using a WAVE device (Transgenomics, Omaha, NE, USA). Amplicons showing aberrant dHPLC patterns compared to wild type control were analyzed by Sanger sequencing using the 5'-GCTCAGAACCAGTCTTCTC-3' primer. We identified 5 AML patients who carried a CSF3R-T595I mutation identical to the SCN/AML case. Clinical, molecular and cytogenetic characteristics of these patients are listed in Table 1. In addition, we identified 2 patients with a CSF3R
mutation substituting a threonine at amino acid position 617 for an isoleucine (T617I) or an asparagine (T617N) in the CSF3R trans-membrane domain. Like T595I, T617N leads to ligand independent activation of CSF3R and has previously been reported as a germ line single nucleotide variation in hereditary chronic neutrophilia and in 2 out of 555 de novo AML patients (10, 13). As germline material was not available we cannot confirm whether the mutations listed in table 1 are acquired mutations. However, the fact that the T595I was acquired in the previously investigated SCN/AML patient (12) highly suggests that the identified mutations listed in table 1 were acquired as well. In none of the AML patients carrying CSF3R-T595I, T617N or T617I, CSF3R truncating mutations were present (Table 1).

Substitution of a threonine for an isoleucine at amino acid position 595 results in a structural change as well as an alteration in charge, i.e., from polar to hydrophobic. To investigate which of these features caused ligand independent activation, we deliberately constructed mutant CSF3R-T595V, in which substitution of threonine for valine introduces a hydrophobic residue that is structurally similar to threonine. Introduction of CSF3R-T595V into mouse bone marrow cells resulted in growth factor independent progenitor cell proliferation similar to CSF3R-T595I, strongly suggesting that hydrophobicity rather than an altered primary structure was responsible for the autonomous signaling properties of CSF3R-T595I (Figure 1, supplemental methods and Table S1). It is likely that CSF3R-T595I dimers or oligomers adopt a conformation that autonomously activates intracellular signaling, similar to the T617N mutation (13). In line with this, HeLa cell transfectants expressing CSF3R-T595I spontaneously accumulated STAT3 and STAT5 in the nucleus, a process that normally requires CSF3-induced activation of JAKs (data not shown) (5).

Although the CSF3R self-activating mutations are rare, important questions are how the CSF3R mutations contribute to the pathogenesis of AML and whether sustained administration of pharmacological dosages of CSF3 supports the evolution and expansion of clones harboring these mutations in SCN. Answering these questions will lead to a further understanding of the molecular mechanisms underlying leukemogenesis in these patients. The acquisition of the CSF3R-T595I mutation in the documented SCN/AML patient was a late event, detected in the AML that emerged 17 years after the initiation of CSF3 therapy, but absent in a bone marrow sample taken when the patient was treated with CSF3 for approximately 8 years (12). Furthermore, we were unable to detect CSF3R-T595I mutations in 21 SCN patients in the neutropenia phase and in 5 patients who underwent CSF3 therapy and progressed to acute leukemia (data not shown). These results, combined with the findings
in de novo AML patients argue against a relationship between the emergence of CSF3R-T595I mutations and CSF3 treatment. However, acquisition of this and other (T617I, T617N) mutations leading to autonomous proliferation of myeloid progenitors likely contributes to disease behavior and possibly has prognostic impact for AML (14).

CSF3R nonsense mutations remain the most prevalent acquired abnormalities identified to date in both ELANE-SCN and HAX1-SCN. Because clones harboring these mutations emerge before leukemia becomes overt, it is conceivable that abnormal signaling from the truncated CSF3R proteins aggravates the accumulating damage in SCN stem and progenitor cells caused by ELANE or HAX1 mutations. Elevated production of reactive oxygen species, known to be genotoxic and potentially carcinogenic, is one of the potential explanations as to why truncated CSF3R contribute to leukemic progression of SCN (15). Whether and how CSF3 therapy adds to this process remains to be elucidated.

References

**Table 1.** Clinical and cytogenetic characteristics of AML patients with CSF3R mutations.

<table>
<thead>
<tr>
<th>Case</th>
<th>WBC ((\times 10^9/\text{l}))</th>
<th>Age at diagnosis (yrs)</th>
<th>FAB class</th>
<th>Blast percentage in BM</th>
<th>Cytogenetic abnormalities</th>
<th>Molecular abnormalities *</th>
<th>CSF3R mutation</th>
<th>CSF3R truncating mutations $</th>
</tr>
</thead>
<tbody>
<tr>
<td>2187</td>
<td>48.9</td>
<td>50</td>
<td>M1</td>
<td>93</td>
<td>NA</td>
<td>None</td>
<td>T595I</td>
<td>No</td>
</tr>
<tr>
<td>3491</td>
<td>38</td>
<td>72</td>
<td>M1</td>
<td>95</td>
<td>46,XY</td>
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<td>T595I</td>
<td>No</td>
</tr>
<tr>
<td>14331</td>
<td>32.1</td>
<td>57</td>
<td>M2</td>
<td>37</td>
<td>46,XY</td>
<td>None†</td>
<td>T595I</td>
<td>ND</td>
</tr>
<tr>
<td>16252</td>
<td>NA</td>
<td>NA</td>
<td>NA</td>
<td>NA</td>
<td>NA</td>
<td>NA</td>
<td>T595I</td>
<td>No</td>
</tr>
<tr>
<td>19206</td>
<td>NA</td>
<td>NA</td>
<td>NA</td>
<td>NA</td>
<td>NA</td>
<td>NA‡</td>
<td>T617I</td>
<td>No</td>
</tr>
<tr>
<td>21997</td>
<td>NA</td>
<td>NA</td>
<td>NA</td>
<td>NA</td>
<td>NA</td>
<td>NA‡</td>
<td>T595I</td>
<td>No</td>
</tr>
<tr>
<td>21999</td>
<td>20.7</td>
<td>39</td>
<td>M2</td>
<td>58</td>
<td>46, XX</td>
<td>FLT3-ITD*†</td>
<td>T617N</td>
<td>No</td>
</tr>
</tbody>
</table>

WBC = white blood cell count, BM = bone marrow, CN = cytogenetic normal, NA = not available. ND = not determined. *Mutations in NPM1, FLT3ITD, FLT3TKD, N-RAS, K-RAS and CEBPA. "FLT3TKD and N-RAS mutations were not determined. ‡K-RAS mutations were not determined. $Investigated mutations are truncating mutations at amino acid positions 715, 717, 719, 725, 728 and 730.
Figure legend

Figure 1. Functional analysis of CSF3R-T595V mutant in myeloid progenitor cell assay. In vitro colony growth of Csf3r deficient murine hematopoietic progenitor cells expressing the wild type human CSF3R receptor (wt), the T595I and the T595V mutant, substituting a threonine at amino acid position 595 for an isoleucine or a valine respectively. CSF3R expressing constructs in pBABE-puro were generated as previously described (12). The T595V mutation was introduced in pBABE-puro using the QuikChange II XL Site-Directed Mutagenesis Kit (Agilent, Santa Clara, CA). Murine colony assays were performed as previously described (12). Colonies were grown in the presence of puromycin, either without growth factor (no GF) or with CSF3. The transduction efficiency was corrected for by dividing the number of CSF3-induced colonies by the number of CSF2 (granulocyte macrophage-colony stimulating factor, GM-CSF) induced colonies under puromycin selection as the CSF3R constructs confer puromycin resistance, but do not affect CSF2 induced colony growth. See Table S1 and the supplemental methods for further details.
Supplemental methods

Patient cell samples

Ficoll-gradient separated bone marrow cells from leukemic blasts of AML samples were used. All cell samples were obtained, frozen and anonymously stored according to established procedures for viable cell cryopreservation as previously described (1). cDNA preparation was performed as previously described (1).

CSF3R-T595I mutation analysis

Amplicons were generated using the 5'-GCTCAGAACCAGTCTTCTC-3’ and 5’-CTGCTGTGAGCTGGGTCTG-3’ primer. Cycling conditions were 30” at 95°C, 30” at 60°C and 30” at 72°C for 35 cycles. Amplicons were analysed on a denaturing high-performance liquid chromatography (dHPLC) using a WAVE device (Transgenomics, Omaha, NE, USA) at a temperature of 63.1°C. Amplicons showing an aberrant dHPLC pattern compared to the wildtype control were analysed by Sanger sequencing using the 5’-GCTCAGAACCAGTCTTCTC-3’ primer. Sanger sequencing was performed according to the manufacturer’s protocol (Applied Biosystems, Foster City, CA, USA). Patients carrying the T595I, T617I and T617N mutations were subsequently analysed for the presence of CSF3R truncating mutations (at amino acid position 715-730) by Sanger sequencing as previously described (2).

CSF3R constructs

CSF3R expressing constructs in pBABE-puro were generated as previously described (2). From pBABE-puro these constructs were ligated into pLNCX. Three different construct were used: wildtype, T595I and T595V. The T595V mutation was introduced in pBABE-puro at the position of the T595I mutation using the QuikChange II XL Site-Directed Mutagenesis Kit (Agilent, Santa Clara, CA) according to the manufacturer’s protocol, using the 5’-GTCCTCACCCTGATGGTCTTGACCCCAGAGG-3’ (sense) and 5’-CCTCTGGGGTCAAGACCATCAGGGTGAGGAC-3’ (antisense) primers.

Murine colony assays

Murine colony assays were performed as previously described (2).
### Supplemental Table

**Table S1. Colony numbers murine colony assay CSF3R mutants.** Murine lineage depleted Csf3r deficient bone marrow cells were retrovirally transduced with different CSF3R constructs. Myeloid colonies were grown under different growth factor conditions. All data are average values of 3 wells. GM-CSF colony growth was performed in the presence of puromycin, and was used to correct for the transduction efficiencies of the different CSF3R constructs. The delta method was used to calculate the standard deviation of the ratios. wt = wild type; sd = standard deviation.

<table>
<thead>
<tr>
<th>culture condition</th>
<th>mean number of colonies/well</th>
<th>mean number of colonies/well (corrected for number of GM-CSF colonies)</th>
</tr>
</thead>
<tbody>
<tr>
<td>no growth factor</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
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<tr>
<td></td>
<td>sd</td>
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<tr>
<td>G-CSF</td>
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<td>GM-CSF</td>
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</tr>
<tr>
<td></td>
<td>sd</td>
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### References