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AUTHOR CONTRIBUTIONS

PH performed the cell culture, genotyping and next-generation sequencing (NGS) experiments, interpreted the results and wrote the manuscript. ACM and SL collected clinical data and contributed in follow-up of the patient. RB coordinated HSC collection and contributed in the material collection. RT designed NGS assays. LS performed cytological and flow cytometry analysis of patient samples. DB and CM performed the standard molecular and chimerism analysis. FF contributed in the material collection and sample preparation. MM and OL contributed in the follow-up of the patient. FD designed the research, interpreted the results and wrote the manuscript. All authors contributed in manuscript review.

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Thrombopoietin receptor is required for the oncogenic function of CALR mutants

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Myeloproliferative neoplasms (MPNs) are diseases characterized by the pathologic expansion of myeloid cells of the hematopoietic lineage. The three ‘classical’ MPNs include polycythemia vera (PV, increase in erythrocytes), essential thrombocythemia (ET, increase in platelets) and primary myelofibrosis (PMF, usually elevated platelet counts associated with fibrotic deposition in the bone marrow).1 MPNs are essentially clonal diseases driven by somatic mutations in hematopoietic stem and progenitor cells. So far, three genes have been identified that can drive the disease phenotype when mutated.2 Activating mutations in Janus Kinase 2 (JAK2) and the thrombopoietin receptor (MPL) have been known for a close to a decade and their mechanism of action has been extensively studied.3,4 Recently, we and others identified somatic mutations in the CALR gene in 25–35% of ET and PMF patients.5,6 CALR encodes the calreticulin protein that functions as a chaperone in the endoplasmic reticulum (ER).7 Calreticulin performs critical quality control functions by binding the sugar residues of N-glycosylated, immature and unfolded proteins, preventing their trafficking to the Golgi and allowing folding mechanisms to operate.8 Moreover, the negatively charged C-terminal end of calreticulin allows it to bind calcium ions and act as a calcium buffer in the ER, thereby playing an important role in calcium-mediated intracellular signaling.9

The CALR mutations associated with MPNs occur exclusively in the last exon of the gene (exon 9). These mutations are insertions and/or deletions that result in a ‘frameshift’ to a specific alternative reading frame, leading to the synthesis of a novel C-terminal peptide in the mutants that consists predominantly of positively charged amino acids. Despite the considerable heterogeneity of CALR mutations at the deoxyribonucleic acid (DNA) level, the translation from the alternative reading frame results in a relatively uniform C-terminal amino-acid sequence of the mutant CALR protein. We have previously shown that expression of the most prevalent mutant CALR (del52) can induce cytokine independence in Ba/F3 cells. This is associated with JAK2-mediated constitutive

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activation of the signal transducer and activator of transcription 5 (STAT5), which is the same signaling pathway activated by the other mutated genes driving the MPN phenotype—JAK2 and MPL. To understand the mechanism of action of mutant CALR and to identify the most differentially expressed genes, we performed gene expression analysis by transcriptome sequencing of the previously

![Figure 1](image-url)
Frameshift mutations in exon 9 of mouse Calr

Unmodified (wild type)

AAEKQMKDKQDEEQRLKEEDEKKRKEEDEEDEEDEEDEEDEEESPGAKDIL

In frame (-3/+3)

AEKQMKDKQDEEQRLKEEDEKKRKEEDEEDEEDEEDEEDEEESPGAKDIL

Alternative reading frame 1 (Disease associated; -1/+2)

CREADEGQAG*GAEA*GRRRGQEA*RGRRS*G*RG***QR*R*G*RR*EGGR*GRIPWPSQG*AVEATPPAFRAQLR

Alternative reading frame 2 (-2/+1)

Unmodified (wild type)

AAEKQMKDKQDEEQRLKEEDEKKRKEEDEEDEEDEEDEEDEEESPGAKDIL

In frame (-3/+3)

AEKQMKDKQDEEQRLKEEDEKKRKEEDEEDEEDEEDEEDEEESPGAKDIL

Alternative reading frame 1 (Disease associated; -1/+2)

CREADEGQAG*GAEA*GRRRGQEA*RGRRS*G*RG***QR*R*G*RR*EGGR*GRIPWPSQG*AVEATPPAFRAQLR

Alternative reading frame 2 (-2/+1)
published parental Ba/F3 cell lines. Strikingly, parental Ba/F3 cells transformed by the expression of CALR-del52 showed high levels of murine endogenous c-mpl mRNA expression (Figure 1a). This was confirmed by quantitative polymerase chain reaction (PCR) analysis of c-mpl expression in these cells (Figure 1b). However, in further experiments, transduction of parental Ba/F3 cells with the mutant CALR did not lead to upregulation of c-mpl expression or cytokine independence. This implied that the CALR mutant does not induce the transcription of the thrombopoietin receptor and the upregulation observed previously was purely a stochastic event. Therefore, we hypothesized that the oncogenic activity of the mutant CALR is dependent on the thrombopoietin receptor (MPL). Indeed, cotransduction of cells with retroviruses expressing mutant CALR (Type 1 and Type 2) and human MPL resulted in consistent transformation of the cells. Overexpression of wild-type CALR did not induce any cytokine independence in the cells even in the presence of MPL (Figure 1c). Moreover, cells expressing both MPL (retrovirus with green fluorescent protein marker) and a mutant CALR had a clear selective advantage over cells expressing only the mutant CALR, when cultured in IL-3-free medium (Figure 1d). This is also evident in dose–response curves of the double-transduced cells to increasing concentrations of IL-3 (Figure 1e).

We also performed CRISPR/Cas9-mediated modification of the murine endogenous Calr locus in Ba/F3 cells. The Ba/F3 parental and Ba/F3-MPL cells were transfected with plasmids expressing the Cas9 protein and a guide RNA targeting the exon 9 of murine Calr. The cells were singularized by serial dilution and cultured with and without IL-3 (Figure 2b). DNA was obtained from the colonies growing out of the single cells and a fragment size assay was performed to assess the mutational status of the endogenous Calr gene. Colonies carrying a mutation in the Calr gene were sequenced (Supplementary Table 1). Those colonies showing multiple peaks in the PCR product-size assay by fragment analysis were not analyzed further by sequencing, as they probably did not arise from a single cell (Figure 2c). In many cases, mutations were seen in both alleles of the Calr gene. In the presence of IL-3, frameshift mutations were detected in Calr gene in all three frames (in variable combinations within the two alleles) in both Ba/F3 parental (Figure 2d) and Ba/F3-MPL (Figure 2e) cells. However, in the absence of IL-3, not a single colony grew in Ba/F3 parental cells. Importantly, in Ba/F3-MPL cells, every colony had at least one allele mutated to the disease-associated reading frame when cultured in the absence of IL-3 (Figure 2f and Supplementary Table 1), underlining the absolute requirement of MPL for the ability of mutant CALR to transform Ba/F3 cells. Although the novel peptide at the C-terminal end of mutant mouse Calr is not completely identical to the human ortholog, it is still able to induce Ba/F3 transformation in the presence of MPL.

These data are in complete accordance with two recent studies that have shown that mutant CALR induces JAK-STAT activation downstream of MPL receptor. Marty et al.13 showed that the CALR-del52 induces thrombocytosis, leading to PMF, in a bone marrow transplantation assay, and that this is dependent on MPL-mediated JAK2 activation. Chachou et al. demonstrate that the CALR mutants directly interact with MPL and induce activation of the receptor. Moreover, both studies show that the expression of MPL is required for the transformation of Ba/F3 cells by CALR mutants.

Our data also imply that MPL is indispensable for the transformation of Ba/F3 cells by the CALR mutants. In an exceptional case, where the parental Ba/F3 cells could be transformed by mutant CALR, this was only possible by a stochastic event leading to selection of those cells that strongly upregulated the expression of endogenous MPL. In fact, this rare stochastic event led us to identify the mechanism of action by which CALR mutants can induce ligand-independent activation of the JAK-STAT signaling pathway in a completely unbiased approach. This ability of mutant CALR to activate MPL would explain the occurrence of CALR mutations specifically in ET and PMF. Both these diseases manifest as increase in thrombocyte numbers, and thrombocyte differentiation is induced by the activation of the thrombopoietin receptor (MPL). Furthermore, we performed CRISPR/Cas9-induced mutagenesis of the murine endogenous Calr locus. Our data showed that mutant Calr can likely mediate clonal advantage in only those hematopoietic progenitors that express MPL—hematopoietic stem cells and megakaryocytic progenitors. The bone marrow transplantation experiments reported by Marty et al.13 demonstrated the same concept in vivo. Moreover, activation of MPL leads to further downstream activation of the receptor-associated JAK2. This explains the ability of mutant CALR to activate JAK-STAT signaling as we had proposed previously and the efficacy of JAK2 inhibitors seen in both PMF patients.

CONFLICT OF INTEREST

The authors declare no conflict of interest.

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Role of CD20 expression and other pre-treatment risk factors in the development of infusion-related reactions in patients with CLL treated with obinutuzumab

Infusion-related reactions (IRRs) elicited by therapeutic antibodies are commonly observed in the treatment of hematologic malignancies. In contrast to the currently approved type 1 anti-CD20 monoclonal antibodies (mAb) rituximab and ofatumumab, obinutuzumab is different by virtue of being a type 2 anti-CD20 mAb, obinutuzumab is different by virtue of being a type 2 antibody with increased capacity to induce direct cell death.\(^1\)\(^-\)\(^5\)

The fragment crystallisable (Fc) portion is glycoengineered (afucosylated), which increases its binding affinity to FcγRIIIA and FcγRIIIB,\(^1\)\(^,\)\(^2\) enhancing antibody dependent cellular cytotoxicity and phagocytosis with less complement activation.

In the CLL11 trial (NCT01010061, ‘stage 2’), patients with previously untreated chronic lymphocytic leukemia (CLL) and co-morbidities were randomized to receive either rituximab or obinutuzumab with chlorambucil.\(^6\),\(^7\)

In the head-to-head comparison of the two antibody-containing regimens, obinutuzumab treatment resulted in a statistically significant improvement in efficacy endpoints. However, this improvement was accompanied by an increase in IRRs, with higher overall incidence and severity when compared with the rituximab-treated arm but largely limited to the first infusion. These IRRs had clinical importance resulting in a 7% discontinuation rate in the obinutuzumab-treated cohort, compared with <1% for rituximab. Predisposing biologic and clinical risk factors for IRRs are not well defined. In an attempt to better understand the profile of patients with CLL at particular risk of IRRs, we used this large clinical data set to perform a multivariate analysis.

Patients treated with a first infusion of obinutuzumab (n = 331) or rituximab (n = 326) were included. Plausible baseline risk factors were identified a priori and included parameters of disease burden, patient-specific factors such as age, concurrent medications and co-morbidities, FcγR genotype and baseline laboratory values. The primary outcome, development of IRR with the first infusion, was defined as the occurrence of related signs and symptoms during or within 24 h of administration.

Multivariable logistic regression models were fit to assess associations between patient characteristics and early IRR. The robustness of the model generated was then internally verified using bootstrapping techniques. A landmark analysis was performed to evaluate the impact of early IRR on progression-free survival (PFS) in both groups and Kaplan–Meier curves were used to illustrate the estimated conditional progression-free probabilities. All statistical analyses were conducted in SAS (SAS Institute, Cary, NC, USA).

The incidence of any grade IRR with the first infusion (all grades) was 65% (214/331) in the obinutuzumab-treated cohort and 27% (88/326) in the rituximab-treated cohort. Severe IRR (grade ≥3) events were seen in 20% (65/331) of patients treated with obinutuzumab and 3% (10/326) in the rituximab arm; there were no fatal IRRs. The features at baseline associated with increased risk of developing an IRR from obinutuzumab or rituximab were: higher density of CD20 expression on CD19+CD5+ CLL cells, increased CD16 (FcγRIIIB) expression on circulating CD56+ natural killer (NK) cells, palpable splenomegaly, higher absolute lymphocyte count (ALC) in peripheral blood (PB), neutropenia, a higher affinity FcγR genotype (VV or VF versus FF) and the presence of an underlying respiratory co-morbidity. Odds ratios (ORs) and 95% confidence intervals (CIs), based on complete case analysis, are presented in Table 1.

Since IRRs were more frequent and severe in obinutuzumab-treated patients, we next focused on this group exclusively. If patients treated with rituximab were excluded from the data set, the variable that contributed most to the probability of developing an IRR was the density of CD20 expression on CLL cells in PB as expressed by the mean fluorescent intensity (MFI) generated by flow cytometric analysis pre-treatment (OR 3.6, 95% CI 1.6–7.9). In addition, MFI of CD16 (FcγRIIIA) expression on circulating CD56+ natural killer (NK) cells, palpable splenomegaly, higher absolute lymphocyte count (ALC) in peripheral blood (PB), neutropenia, a higher affinity FcγR genotype (VV or VF versus FF) and the presence of an underlying respiratory co-morbidity. Odds ratios (ORs) and 95% confidence intervals (CIs), based on complete case analysis, are presented in Table 1.

To externally validate the importance of CD20 MFI as a risk factor, data from any available prior trials involving...