paracrine factor for OCL formation and that ActA is a rational target for reduction of the downstream, bone-specific effects of IL-3 on OCL stimulation in MMBD.

CONFLICT OF INTEREST

GDR is a consultant for Amgen and receives research support from Eli Lilly and Co. The other authors declare no conflict of interest.

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OPEN

Targeting proliferation of chronic lymphocytic leukemia (CLL) cells through KCa3.1 blockade

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Although the dependence of Ca^{2+} signaling and mitosis on K^+ channel activity in lymphocytes has been thoroughly examined,[1] the therapeutic significance of these findings for malignant hematological diseases is largely unexplored. Out of approximately 80 different K^+ channel genes in humans, T and B cells express the voltage-dependent K^+ channel, Kv1.3, and the Ca^{2+}-activated K^+ channel, KCa3.1. Expression levels of K^+ channels vary with lymphocyte maturation and activation state.[1,2] Accordingly, selective blockade of the predominant K^+ channel type allows lymphocyte subset specific inhibition of proliferation.[1,2] Given the importance of controlling Ca^{2+}-influx, there is growing interest in selective K^+ channel blockers to suppress cell proliferation in autoimmune diseases and cancer.[3–5]

Chronic lymphocytic leukemia (CLL) is a heterogeneous lymphoproliferative malignancy of clonally expanded $CD5^+CD19^+$ B cells.[6] CLL cells are presumably derived from an activated antigen-experienced precursor (IgD^+CD27^+).[7] While their majority in the peripheral blood is cell cycle arrested, CLL cells in lymphoid organs proliferate, delivering substantial amounts of tumor cells daily.[8] Critically, CLL cells in lymphoid niches are protected against cytotoxic effects of many chemotherapeutics and likely cause minimal residual disease and future relapse.[6]

If leukemic cell proliferation is driven by K^+ efflux, selective K^+ channel blockers could be of clinical benefit to attack B cell neoplasms. Accordingly, we first characterized K^+ channels in resting and proliferating primary CLL cells using *in vitro* stimulation with stromal cells and autologous $CD4^+$ T cells (T4), and then we correlated K^+ channel expression with proliferation markers in lymphoid tissue and peripheral CLL cells. We moreover showed the sensitivity of CLL cell proliferation on K^+ channel blockade in two different proliferation models. Patch-clamp analysis of primary CLL cells revealed a use-dependent, voltage-gated K^+ current, sensitive to the Kv1.3 specific blocker PAP-1 (Supplementary Figures S1B and C) and a Ca^{2+}-activated K^+ current, blocked by TRAM-34 (Supplementary Figure S1C).[1] In addition, we detected

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similar levels of Kv1.3 and KCa3.1 mRNA in resting CLL and normal B cells (Supplementary Figures S2A and B).

In B cells from healthy donors, PMA and ionomycin stimulation increases KCa3.1 expression as well as cell proliferation.[2] Nineteen hours PMA and ionomycin exposure caused up-regulation of the early activation marker CD69 (Supplementary Figures S2A$_i$ and B$_i$) and down-regulation of Kv1.3 mRNA in CLL and B cells (Supplementary Figures S2A$_{ii}$ and B$_{ii}$). However, contrary to B cells, 3-day phorbol 12-myristate 13-acetate (PMA) and ionomycin exposure did not induce proliferation in CLL cells coinciding with low KCa3.1 expression. Furthermore, we detected slightly elevated mRNA expression levels of Kv11.1 (herg1) channels in resting CLL cells compared with normal B cells, as described in previous works,[9] but did not detect mRNA traces in activated cells (Supplementary Figure S2C).

In contrast to PMA and ionomycin treatment, coculture of CLL cells with stromal cells and enriched with CD3/CD28-activated autologous T4 cells,[10] a surrogate for proliferative niches in lymphoid organs, induced up-regulation of the late activation markers CD80 and CD86 in CLL cells (Supplementary Figure S3A). Moreover, Ki-67$^+$ CLL cells, indicating proliferating cells, increased from barely detectable to about 11% in activated samples (Figures 1Aa and b). Consistent with the hypothesis that changes in K^+ channel profile and activation may be required for CLL proliferation, activated CLL cells up-regulated KCa3.1 channels (Figures 1B–D). CFSE labeling of CLL cells undergoing same culture conditions moreover demonstrated actual cell divisions after 2–3 more days in culture, also accompanied by CD80 and CD86 up-regulation (Supplementary Figures S3A and B) and similar changes in K^+ channel profile (Supplementary Figure S3C). CLL cells up-regulated KCa3.1 mRNA, whereas Kv1.3 levels remained low (Figure 1B, Supplementary Figure S3C). Whole cell patch-clamp recording of CLL cells revealed about 14 KCa3.1 and 110 Kv1.3 channels per cell before stimulation, 40 KCa3.1 and 290 Kv1.3 channels when cultured in control conditions without T4 cell enrichment and 395 KCa3.1 and 268 Kv1.3 channels when stimulated with activated T4 cells for 3 days culminating in a 11-fold increase of the KCa3.1/Kv1.3 ratio compared with control cultured CLL cells (Figure 1C, Supplementary Table S1). These K^+ channel per cell estimations are similar to those of early memory B cells (IgD$^+$CD27$^+$) from healthy individuals.[2] Fluorescent immuno-labeling in these cocultures also revealed higher fluorescence signals of KCa3.1 in Ki-67$^+$CD19$^+$ cells compared with control cells (Figure 1Da). Numerical evaluation of KCa3.1 fluorescence intensity (FI) showed significantly higher numbers in proliferating samples compared with controls (Figure 1Db).

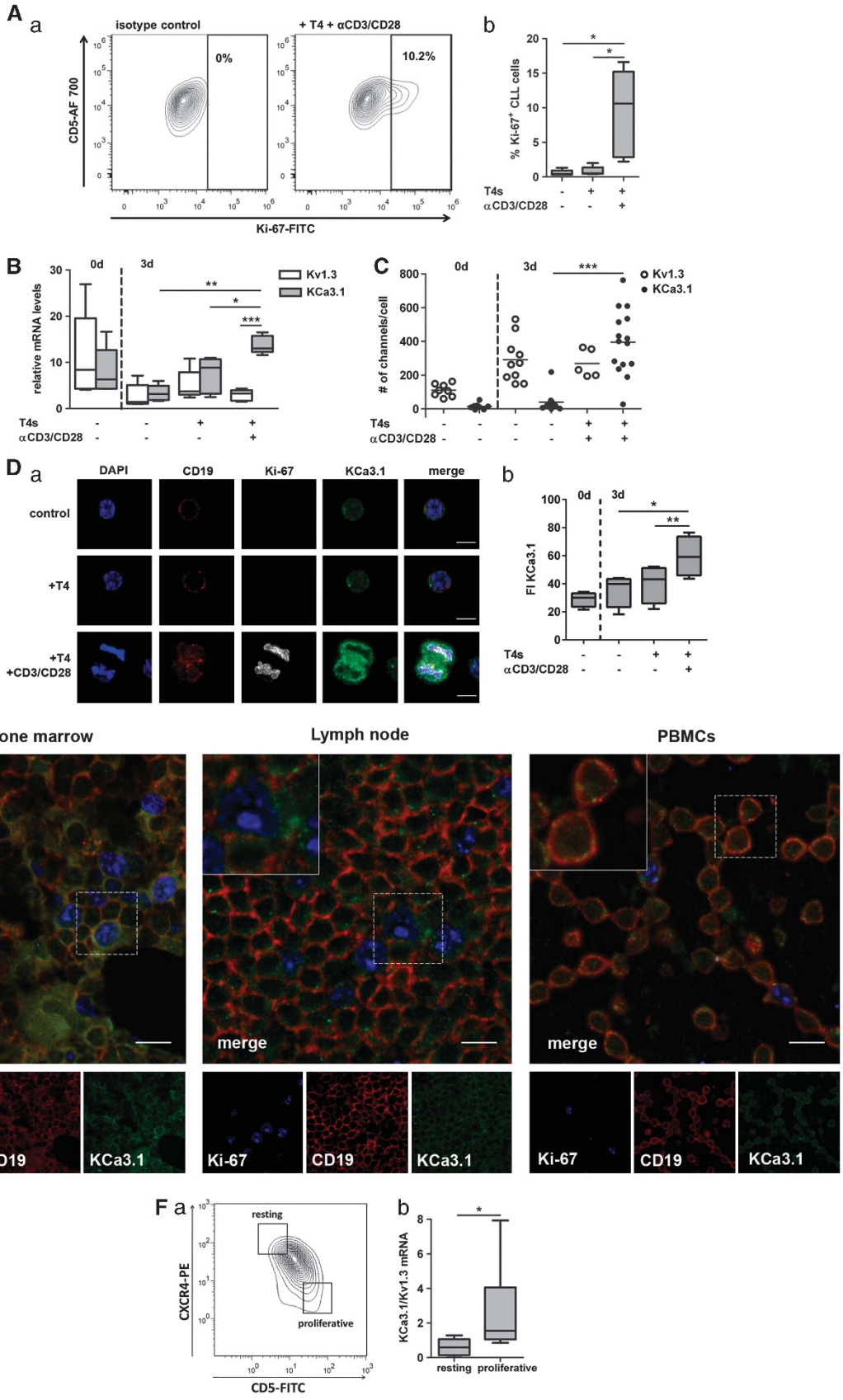
We estimated the association between KCa3.1 expression and proliferation markers in lymphoid tissues using immunohistochemistry. Although normal lymphoid tissues displayed Ki-67$^+$ cells well organized in germinal center structures (Supplementary Figures S4D and E), consecutive tissue sections from diffusely infiltrated CLL lymph nodes (Supplementary Figure S4A) lacked these defined organizations and exhibited diffuse Ki-67 as well as KCa3.1 expression. The nodular CLL infiltration of a representative tonsil (Supplementary Figure S4B) and a bone marrow sample (Supplementary Figure S4C) depicts an extraordinary high KCa3.1 expression in Ki-67^{high} areas of the CD19$^+$ CLL infiltrate. Co-localization studies using fluorophore-labeled antibodies to Ki-67 (blue), CD19 (red) and KCa3.1 (green) in these lymph nodes and bone marrow samples demonstrated that CD19$^+$ CLL cells exhibiting Ki-67 expression also exhibited a high KCa3.1 fluorescence signal, whereas a low KCa3.1 signal was found in Ki-67$^-$ CD19$^+$ CLL cells from the peripheral blood (Figure 1E), where their majority is quiescent. A higher KCa3.1 expression of CLL cells in lymphoid tissue exhibiting a substantial number of Ki-67$^+$ cells compared with CLL cells in the peripheral blood may reflect an enhanced activation status of CLL cells in lymphoid organs. This is in line with the assumption that cell contact with fibroblasts, T cells, stromal cells and other cell types has an activating effect on CLL cells in proliferative niches,[6] as mimicked in our coculture model used for *in vitro* experiments (also see Asslaber *et al.*[10]).

Thus, similar to lymphocytes from healthy donors,[1] CLL cells express Kv1.3 and KCa3.1, and like IgD$^+$CD27$^+$ early memory B cells,[2] up-regulate KCa3.1 expression in mitogenic environments *in vitro* and presumably also *in vivo*.

CLL cells recycle between proliferative niches in lymphoid organs and the peripheral blood.[6] Consequently, recently divided cells might also exhibit a specific K^+ channel pattern. Prior *in vivo* studies, using heavy water-labeling of CLL cells in patients, identified CXCR4^{dim}CD5^{bright} CLL cells in the peripheral blood as recently proliferated cells, whereas CXCR4^{bright}CD5^{dim} cells as resting.[11] FACS sorting of these two populations of CLL cells from the peripheral blood of CLL patients and subsequent qRT-PCR studies revealed an about 5-fold higher KCa3.1/Kv1.3 mRNA ratio in CXCR4^{dim}CD5^{bright} compared with CXCR4^{bright}CD5^{dim} CLL cells in our studies, supporting the assumption that KCa3.1 is up-regulated in proliferating CLL cells *in vivo* (Figure 1F).

Next, we tested the effect of KCa3.1 channel blockade on CLL cell proliferation. We previously described that pre-activation of CLL cells in peripheral blood monocyte (PBMC) cocultured with CD40L-expressing fibroblasts caused up-regulation of CD80 and CD86.[10] After 24 h, this went along with KCa3.1 mRNA up-regulation (Supplementary Figure S5A). Before activation of T cells in the PBMCs, blockers selective for KCa3.1 (TRAM-34, clotrimazole) or Kv1.3 (PAP-1, Psora-4) were added.[1] After an additional 48 h, clotrimazole reduced cell viability (determined by Annexin-V/7-AAD—negativity) most likely because of its inhibition of the cytochrome P450 enzymes, whereas TRAM-34, PAP-1 and Psora-4 lacked these effects

Figure 1. Activated CLL cells up-regulate KCa3.1 channels upon proliferation. (**A**a) Three-day cocultures of CLL cells with autologous T4 cells and α-CD3/CD28 beads induced proliferation in CLL cells determined by Ki-67 expression and flow cytometry (FC) (representative FC plot). (**A**b) Median expression levels of Ki-67 were at 10.6% (CLL + T4 + α-CD3/CD28 beads) compared with 0.4% (CLL only) ($P = 0.0388$) and 0.5% (CLL + T4) ($P = 0.0361$) ($N = 5$). (**B**) Proliferating CLL cells (+ T4 + α-CD3/CD28 beads) showed higher quantitative expression of KCa3.1 mRNA levels compared with CLL cells only ($P = 0.0013$) or CLL cells with T4 cells ($P = 0.0187$), resulting in predominant expression of KCa3.1 mRNA compared with Kv1.3 in proliferating CLL cells ($P = 0.0003$) ($N = 5$). (**C**) Whole cell patch clamp recording of CLL cells depicted up-regulation of KCa3.1 channels in the plasma membrane of three-day T4-stimulated cells compared with control cells cultured without T4s ($P < 0.0001$) (number of patients: $N = 3$). (**D**a) Immunofluorescence (IF) stainings of KCa3.1 on proliferating Ki-67$^+$ (gray) CD19$^+$ CLL cells (red) revealed a high KCa3.1 fluorescence signal (green) in samples with three-day α-CD3/CD28-stimulated T4 cells (lower row) compared with unstimulated controls with (middle row) and without (upper row) T4 cell enrichment, visualized by confocal microscopy ($N = 7$). Bars represent 5 μm. (**D**b) Quantitative analysis of KCa3.1 fluorescence intensity (FI) yielded increased KCa3.1 values in stimulated CLL cells compared with controls (CLL only: $P = 0.0121$; CLL + T4 cells: $P = 0.0028$) ($N = 4$). (**E**) FFPE-bone marrow ($N = 7$), -lymph node ($N = 3$) and -PBMC samples ($N = 2$) of patients ($N = 8$) co-stained with antibodies to Ki-67 (blue), CD19 (red) and KCa3.1 (green) were visualized via confocal microscopy. Small images below each merged multicolor picture depict single channel images. Bars represent 20 μm. (**F**a) CLL cells (CD5$^+$CD19$^+$) were FACS-sorted in two opposing populations according to their CD5 and CXCR4 expression pattern. The CD5^{bright}CXCR4^{dim} population corresponds to the proliferative CLL cells whereas the CD5^{dim}CXCR4^{bright} fraction represents resting cells. (**F**b$_i$) Subsequent qRT-PCR of these two populations revealed a 4.7-fold increase in KCa3.1/Kv1.3 ratio in the proliferative compared with the resting populations ($mean_{rest} = 0.5972$; $mean_{prolif} = 2.829$; $P = 0.0391$) ($N = 7$). *$P < 0.05$; **$P < 0.01$; ***$P < 0.001$.



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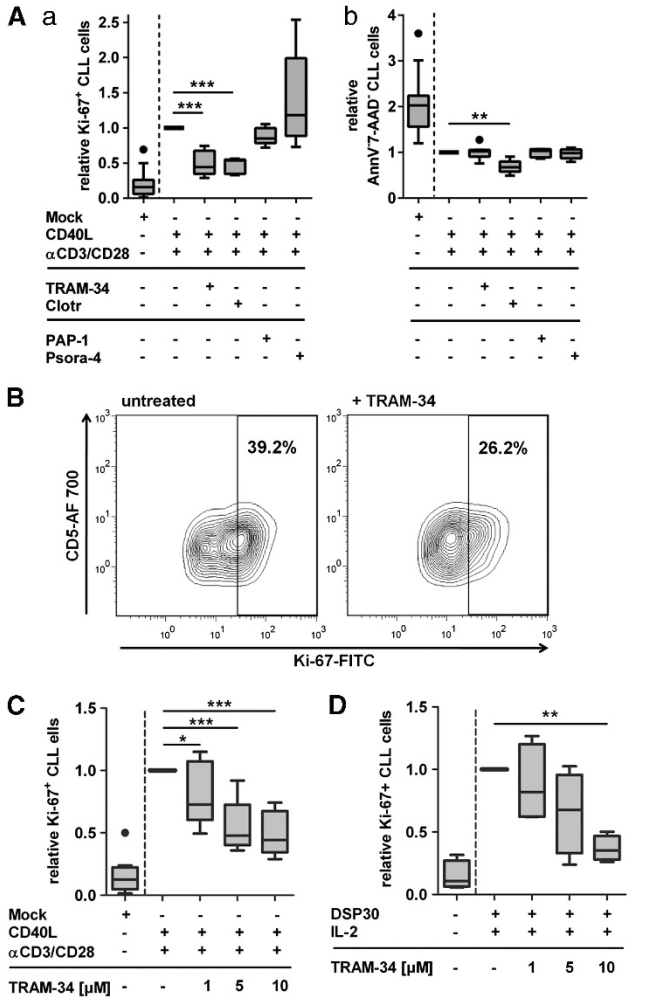

Figure 2. Proliferation of CLL cells is significantly diminished by KCa3.1 channel blockade. (A) CLL cells (in PBMCs containing T cells), 24 h pre-activated on a CD40L-expressing fibroblast layer, were treated with TRAM-34 (10 μM) ($N = 10$) or clotrimazole (10 μM) ($N = 5$) and PAP-1 (10 μM) ($N = 5$) or Psora-4 (10 μM) ($N = 5$), respectively, before T cell activation with α-CD3/CD28 beads. Forty-eight hours later, Ki-67 expression on $CD5^+CD19^+$ CLL cells was determined by FC. Values were normalized to untreated proliferating controls. (Aa) PAP-1 and Psora-4 did not decrease Ki-67 expression (PAP-1: $P = 0.8570$; Psora-4: $P = 0.6060$), whereas TRAM-34 ($P < 0.0001$) and clotrimazole ($P = 0.0004$) did. (Ab) Clotrimazole reduced CLL cell viability (Annexin-V^-7-AAD^- cells) ($P = 0.0092$), TRAM-34, PAP-1 and Psora-4 did not (TRAM-34: $P = 0.9501$; PAP-1: $P = 0.9918$; Psora-4: $P = 0.9715$). (B) Representative FC plots illustrating the effect of TRAM-34 (10 μM) on Ki-67 expression pre-gated on $CD5^+CD19^+$ CLL cells; (C) Dose-escalation of TRAM-34 using 1, 5 or 10 μM ($N = 10$) showed concentration-dependent reduction of Ki-67 expression on CLL cells: 1 μM: $P = 0.0419$; 5 μM: $P < 0.0001$; 10 μM: $P < 0.0001$; values were normalized to untreated proliferating controls. (D) Twenty-four hours CpG pre-stimulated CLL cells were treated with escalating concentrations of TRAM-34 before supplementation with IL-2 ($N = 4$). Ki-67 expression of CLL cells was reduced in a concentration-dependent manner (10 μM: $P = 0.0011$). Samples were normalized to activated controls. *$P < 0.05$; **$P < 0.01$; ***$P < 0.001$.

(Figure 2Ab, Supplementary Figures $S6A_{ii}$ and B_{ii}).[1,2] TRAM-34 and clotrimazole, but not PAP-1 or Psora-4 reduced Ki-67 expression in CLL cells, indicating involvement of KCa3.1 channels in cell cycle activation (Figure 2Aa, Supplementary Figure $S6A_i$). The reduction of Ki-67^+ CLL cells by TRAM-34 was concentration-dependent: 1 μM TRAM-34 reduced median Ki-67-expression by 27.3%, 5 μM by 52.2%, 10 μM by 55% (Figures 2B and C, Supplementary Figure $S6B_i$). TRAM-34 did not affect fibroblast proliferation (Supplementary Figure $S5B_i$) or CD40L-expression (Supplementary Figure $S5B_{ii}$) nor viability (Supplementary Figure $S5C_i$) or Ki-67 levels (Supplementary Figure $S5C_{ii}$) of T cells in this coculture. Likely mechanisms for T cell insensitivity to TRAM-34 are that (1) CLL-specific T4 cells are mainly effector memory cells,[12] regulating their membrane potential via Kv1.3[1] and (2) α-CD3/CD28 beads ignite supra-physiological responses overriding KCa3.1 blockade. Alternatively, activation of CLL cells with the CpG-motive DSP30, a potent TLR-9-directed mitogen for CLL cells, in combination with IL-2[13] increased Ki-67^+ CLL cells after 72 h. Addition of TRAM-34 after 24 h of pre-activation with DSP30, before IL-2 exposure, also revealed a concentration-dependent decrease of Ki-67^+ CLL cells (10 μM: − 63.4%) (Figure 2D, Supplementary Figure S6C). In contrast to our study, Leanza *et al.*[14] recently described an apoptotic effect of Kv1.3 blockers on CLL cells by blockade of mitochondrial Kv1.3 channels and concluded that Kv1.3 blockers are eligible therapeutics for CLL. Because CLL cells lacking a supportive microenvironment activate cell death instead of mitotic programs, culture conditions can influence drug sensitivities.[6] Accordingly, CLL cell culture in the absence of supporting cells in the Leanza study may favor Kv1.3 blockade-dependent apoptosis, whereas our 'lymphoid niche surrogate' failed to promote apoptosis following Kv1.3 blockade.

Supported by beneficial outcomes following K^+ channel blockade in animal models of human diseases[3,4] and clinical trials,[15] we advocate KCa3.1 channels as promising new therapeutic targets in CLL.

CONFLICT OF INTEREST

The authors declare no conflict of interest.

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Myelodysplastic/myeloproliferative neoplasms, unclassifiable (MDS/MPN, U): natural history and clinical outcome by treatment strategy

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Created in 2001 and retained in 2008, the World Health Organization (WHO) now recognizes a distinct category of myelodysplastic/myeloproliferative neoplasms (MDS/MPN) for those patients diagnosed with clinical, morphologic and laboratory features that overlap with those of both MDS and MPN.[1] Four hematopathologic diagnoses exist within this category: chronic myelomonocytic leukemia (CMML), atypical chronic myeloid leukemia BCR-ABL1 negative (aCML), juvenile myelomonocytic leukemia (JMML) and myelodysplastic/myeloproliferative neoplasm, unclassifiable (MDS/MPN-U).

Of the four 'overlap' MDS/MPN syndromes, MDS/MPN-U is the least well characterized.[2] It encompasses patients with features of both MDS and MPN at presentation that do not satisfy criteria for CMML, JMML or aCML. MDS/MPN-U is formally defined as patients with no preceding history of MDS or MPN, no recent cytotoxic growth factor therapy, no Philadelphia chromosome, *BCR-ABL1* fusion gene, *PDGFRA*, *PDGFRB* or isolated del(5q), t(3;3)(q21;q26) or inv(3)(q21q26), and with dysplastic features in $\geqslant 1$ hematopoietic cell line, $<20\%$ blasts in the blood and bone marrow, prominent myeloproliferative features (that is, platelet count $\geqslant 450 \times 10^9$/l or white blood cell count $\geqslant 13 \times 10^9$/l, with or without splenomegaly), or *de novo* disease with mixed myeloproliferative and myelodysplastic features that cannot be assigned to any other category of MDS, MPN or of MPS/MPN (Supplementary Table 1).[3] Although there are no identifying cytogenetic or molecular features of MDS/MPN-U, recurrent mutations are found within this category and notably include *JAK2-V617F* in approximately 25% of patients. In addition, *JAK2-V617F* mutations are present in up to 60% of MDS/MPN-U patients with refractory anemia with ringed sideroblasts and thrombocytosis (RARS-T), a pathologic diagnosis currently residing as a provisional entity within the MDS/MPN-U category.[4,5]

MDS/MPN-U is a rare diagnosis, making up $<5\%$ of all myeloid disorders.[6] Accordingly, clinical characteristics and the natural history of patients with MDS/MPN-U are not well established, although poor prognosis among patients with MDS/MPN-U (without RARS-T) has been suggested in small series to date.[5,6] No standard prognostic or treatment algorithms for MDS/MPN-U exist. Our aim was to evaluate patients with a confirmed diagnosis of MDS/MPN-U without RARS-T, to provide insights into the nature of this unique myeloid overlap syndrome with implications to appropriate treatment strategies.

All patients with MDS/MPNs from January 1987 to February 2013 at the University of Texas MD Anderson Cancer Center were reviewed. Patients seen before 2006 with a diagnosis of MDS/MPD or MPD-unclassifiable were reviewed by two independent hematopathologists and diagnoses were modified, when appropriate, according to the current WHO criteria.[3] Patients were excluded if the *de novo* presence of both myelodysplastic and myeloproliferative features at diagnostic presentation was unable to be confirmed. All patients with RARS-T were excluded as per the 2008 definition.[3] In total, 85 patients with a diagnosis of MDS/MPN-U, without RARS-T, were included. All patients were tested and were negative for the *BCR/ABL* translocation by fluorescence *in situ* hybridization and/or PCR, and *JAK2-V617F* was assessed by standard PCR technique. Peripheral and bone marrow monocyte counts were assessed in all patients, and all patients were classified according to the international prognostic scoring system

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