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SRSF2 and *U2AF1* mutations in primary myelofibrosis are associated with *JAK2* and *MPL* but not calreticulin mutation and may independently reoccur after allogeneic stem cell transplantation

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Spliceosome gene mutations are among the 50–60 driver mutations underlying myelodysplastic syndromes (MDSs).[1] *U2AF1* mutations for example have been reported to occur in up to 16% of primary myelofibrosis (PMF), and was found to be associated with anemia and thrombocytopenia in PMF.[2] We could show that spliceosome gene mutations are already present in early stages of PMF before fibrosis and cytopenia become manifest.[3] Recently, a negative association between mutations of calreticulin (*CALR*) and spliceosome genes has been described.[4]

CALR is a Ca^{2+}-binding protein, which was found in 2013 to be mutated in *JAK2*- or *MPL*-unmutated PMF and essential thrombocythemia.[5,6] Mutations were mutually exclusive of *JAK2* or MPL mutations. *JAK2*-mutated and triple-negative patients were shown to have significantly shorter survival periods in comparison to those with somatic frameshift mutations in the *CALR* gene.[4,5] Tefferi *et al.*[4] described significantly lower frequency of spliceosome mutations in *CALR*-mutated cases and attributed the lower incidence of anemia to the lower frequency of *U2AF1* mutations.

Up to now allogeneic hematopoietic stem cell transplantation (AHSCT) represents the only curative treatment modus for patients with PMF.[7] Selection of patients suitable for this kind of treatment is performed according to prognostic scoring and tolerable risks of individual patients. Data of Heuser *et al.*[8] suggest a better overall survival for *CALR*-mutated PMF patients after AHSCT.

In this study, we analyzed 69 patients with PMF grades of fibrosis 2–3 (Table 1) who have undergone allogenic stem cell transplantation for *JAK2*, *MPL*, *CALR* and spliceosome gene mutations (*SRSF2*, *U2AF1* and *SF3B1*) using bone marrow trephines and pyrosequencing as described.[9]

CALR was rarely combined with splice factor gene mutations (10.5% of all *CALR*-mutated cases; negative correlation, $P = 0.0418$) and these combinations were restricted to *SF3B1*. Combined mutations with *U2AF1* and *SRSF2* could not be found at all. The frequency of accompanying splice factor gene mutations in *CALR*-mutated patients was significantly lower than that in patients without a *CALR* mutation (21/50, 42%; $P = 0.04$) or in those with a *JAK2* mutation (18/41, 44%; $P = 0.04$). *U2AF1* was the most frequent splice factor gene mutation associated with JAK^{V617F}. In PMF, splice factor gene mutations were associated significantly more often with a *JAK2* mutation than with a *CALR* mutation ($P < 0.00005$; Fisher's exact tests).

In our cohort 7 patients (10%) revealed neither *JAK2* nor *MPL* or *CALR* mutation. In the 'triple-negative' subgroup of PMF, exclusively mutations of *SRSF2* occurred (n=2), but no alterations of *U2AF1* and *SF3B1* could be observed (Table 1).

Because of the low number of *MPL*-mutated cases in this series additional samples of PMF with bone marrow fibrosis grade 2–3 and known *MPL* mutation ($n = 20$, all *JAK2* exon 14 wild type) were investigated for combination with splice factor gene mutations. Among the 20 *MPL*-mutated cases 10 samples exhibited splice factor gene mutations (50%). Three samples revealed mutation of *U2AF1* (15%), six of *SRSF2* (30%) and one of *SF3B1* (5%), respectively. Consequently, MPL-mutated PMF cases appear to carry splice factor gene mutations with a similar frequency as *JAK2*-mutated cases. *CALR*-mutated cases behave different from *JAK2*- and *MPL*-mutated cases in that splice factor gene mutations occur significantly rarer ($P < 0.005$ for *MPL*) and only combinations with *SF3B1* could be found.

After a median follow-up of 18 months four patients suffered molecular and histopathological relapse. Interestingly, the recurrent disease was different from the primary MPN and differences with regard to histopathology as well as to molecular aberrations could be observed. In two patients the bone marrow displayed reduced myelofibrosis (< MF2) but cytopenia was still evident (Patient 3 and 4 in Table 2). *JAK2* mutation was no longer detectable in these cases but splice factor gene mutations persisted and as shown for *SRSF2* to a similar allelic burden as in the bone marrow before AHSCT. Bone marrow biopsy in one case (patient 3) still revealed atypical megakaryocytes and myelofibrosis (MF1), consistent with relapse of PMF but with reduction of fibrosis (Table 2). In another case with persisting *U2AF1* mutation after AHSCT histology showed a different picture more reminiscent of MDS with excess of blasts, and megakaryocytes did not show the atypia anymore characteristic for PMF (patient 4, Table 2). In patient No 1 *JAK2* mutation was still detectable, but with a reduced allelic burden (15%) compared with the primary biopsy (80%), whereas *SRSF2* mutation remained on an identical level of 50% (Table 2). In this case myelofibrosis was also diminished 1 year after AHSCT (MF grade 3 to MF grade 1) but increased again to MF grade 2 after 4 years. In addition, there was one case with persisting *JAK2* mutation and loss of splice factor gene mutations (*U2AF1*) after AHSCT (patient 2).

Our results show that PMF with high-risk scores eligible for AHSCT represents a molecularly heterogeneous disease despite uniform histopathology of bone marrow with atypical megakaryocytic proliferation and evident myelofibrosis. Cytopenia which is used for risk stratification and which is the dominant cause to treat patients with AHSCT appears to be associated in a considerable proportion of PMF cases with splice factor gene mutations. In these cases fibrotic obliteration of bone marrow spaces seems not to be the only cause of cytopenia. Splice factor gene mutations are significantly more frequently combined with *JAK2* and *MPL* mutations than with *CALR* mutation. Furthermore, different hematopoietic clones proliferate in PMF giving rise to a molecular mosaic. After AHSCT of PMF relapses may uncover the underlying clonal mosaic and different diseases may emerge. Despite reduction of myelofibrosis and eradication of the $JAK2^{V617F}$ clone cytopenia may persist. The molecular mosaic in myeloproliferative neoplasms has been demonstrated to be the result of both independent clones

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Table 1. PMF patients treated by allogeneic stem cell transplantation

	Patients with AHSCT	*Patients with splice factor gene mutations*	*Patients with follow-up biopsies (n = 52) and molecular relapse*
Number of patients (%)	69	23 (33%)	4 (7.7%)
Median age, years (range)	65.5 (33–75)	66 (44–76)	64 (61–76)
Male	42	15	3
Female	27	8	1
JAK2^{V617F} (%)	41 (59.4%)	18 (78.2%) 5 *JAK2/SRSF2* 10 *JAK2/U2AF1* 3 *JAK2/SF3B1*	3
MPL (exon 10)	2 (2.9%)	1 (1.4%) 1 *MPL/SF3B1*	0
Calreticulin (exon 9) (%)	19 (27.5%)	2 (8.7%) 2 *CALR/SF3B1*	0
SRSF2 (exon 1) (%)	7 (10.1%)	7 (21.8%) 5 *SRSF2/JAK2* 2 *SRSF2*/TN	2
U2AF1 (exons 2, 6) (%)	9 (13.0%)	10 (43.5%) 10 *U2AF1/JAK2*	1
SF3B1 (exons 14, 15)	6 (8.7%)	6 (26.1%) 3 *SF3B1/JAK2* 1 *SF3B1/MPL* 2 *SF3B1/CALR*	0
Median time of follow-up biopsy (months)	13	14	18
Myelofibrosis grade 2 and 3 (%)	69 (100%)	23 (100%)	2 (50%)

Abbreviation: AHSCT, allogeneic hematopoietic stem cell transplantation; PMF, primary myelofibrosis; TN, triple negative.

Table 2. Molecular relapses after allogeneic stem cell transplantation of PMF

	P1	*P1+AHSCT*	*P2*	*P2+AHSCT*	*P3*	*P3+AHSCT*	*P4*	*P4+AHSCT*
JAK2	V617F 80%	V617F 15%	V617F 50%	V617F 14%	V617F 14%	WT	V617F 27%	WT
SRSF2	P95H 50%	P95H 50%	WT	WT	P95H 30%	P95H 40%	WT	WT
U2AF1	WT	WT	Q157P 40%	WT	WT	WT	Q157P 40%	Q157P 40%
Myelofibrosis	MF3	MF2	MF3	MF3	MF3	MF1	MF3	MF0
Hemoglobin (g/dl)	9.2	11.5	7.1	8.4	9.4	10.1	8.4	8.6
Leukocytes (×10^3/μl)	13.7	13.8	3.8	0.2	4.4	3.9	21.6	1.7
Thrombocytes (×10^3/μl)	42	112	72	12	115	21	32	9

Abbreviations: +AHCST, after allogeneic hematopoietic stem cell transplantation; MF 0–3, grade of myelofibrosis; P, patient; PMF, primary myelofibrosis; WT, wild type.

proliferating in parallel as well as clonal evolution with stepwise acquisition of different mutations by a single neoplastic clone.[10] Molecular monitoring of patients having undergone AHSCT for PMF should not be restricted to *JAK2*, *MPL* or *CALR*, but all mutations present in the primary fibrotic neoplastic myeloproliferation should be included to interpret abnormal blood values after AHSCT. The apparently better prognosis of *CALR*-mutated PMF.[4,5] including cases treated with AHSCT[8] may at least in part be attributable to a less likely association with splice factor gene mutations.

CONFLICT OF INTEREST

The authors declare no conflict of interest.

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Protein kinase N3 deficiency impedes PI3-kinase pathway-driven leukemogenesis without affecting normal hematopoiesis

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Protein kinase N family genes encode AGC-type serine–threonine kinases (PKN1, PKN2 and PKN3) that interact with Rho family proteins to regulate cytoskeletal organization and gene expression.[1–3] Whereas *PKN1* and *PKN2* are ubiquitously expressed, *PKN3* expression is low in most normal human tissues but high in some malignancies.[3] PKN3 catalytic activity is regulated by RhoC and by the PI3-kinase (PI3K) signaling pathway.[4,5] *PKN3* inactivation inhibits growth of PI3K-driven prostate and breast cancer xenografts.[4,5] Its role in hematopoietic malignancies has not yet been described. Since the PI3K pathway is hyper-activated in many types of leukemia,[6–8] PKN3 may be a useful target for treating leukemia patients.

We analyzed *PKN1, PKN2* and *PKN3* expression in human T-cell acute lymphoblastic leukemia (T-ALL), a subtype that is often driven by hyper-active PI3K signaling.[8,9] We analyzed 7 control bone marrow samples and 117 primary T-ALL samples from a previously published microarray data set.[10] *PKN1* and *PKN2* were expressed at similar levels in control bone marrow and T-ALL specimens, but *PKN3* was expressed at significantly higher levels in human T-ALL relative to normal bone marrow (Figures 1a–c). We next characterized *Pkn3* expression in normal mouse hematopoietic stem cells (HSCs; $CD150^+CD48^-Lineage^-Sca1^+c\text{-}Kit^+$), lineage-restricted progenitors (RPs; $CD48^+Lineage^-Sca1^+c\text{-}Kit^+$), granulocyte-monocyte progenitors (GMPs) and thymocytes relative to unfractionated bone marrow. *Pkn3* was expressed in all cell populations, and it was most highly expressed in immature HSCs, RPs and GMPs (Figure 1d). These data raise the question of whether *PKN3* regulates normal hematopoiesis and T-cell leukemogenesis.

We used genetically engineered mice to test whether *Pkn3* regulates normal hematopoiesis and T-cell leukemogenesis *in vivo*. A conditional *Pkn3* allele ($Pkn3^{fl}$) was generated by flanking exons 2–6 with LoxP sites (Supplementary Figure S1A). Cre-mediated deletion of exons 2–6 caused a frame shift after codon 5 and a premature translational stop signal at codon 20. Germ line-deleted $Pkn3^{-/-}$ mice did not express transcripts from exons 2–6 or the PKN3 protein (Supplementary Figures S1B and C), consistent with our expectation that this deletion yields a null allele. $Pkn3^{-/-}$ mice were born in expected Mendelian ratios. They were viable, fertile and healthy, so *Pkn3* is not essential for normal development or homeostasis.

To test whether *Pkn3* regulates HSC function and hematopoiesis we generated $Pkn3^{fl/fl}$; *Mx1-Cre* mice and initiated poly-Inosine: poly-Cytosine (pIpC) treatments 6 weeks after birth to induce Cre expression (10 μg pIpC/dose for three doses). We genotyped HSC-derived colonies and confirmed that this pIpC dose was sufficient to delete both *Pkn3* alleles in all HSCs (data not shown). At 8 weeks after pIpC treatment $Pkn3^{fl/fl}$; *Mx1-Cre* mice had normal peripheral blood counts relative to littermate (Cre-negative) controls (Figures 1e–g). Control and *Pkn3*-deleted mice had similar numbers of HSCs in their bone marrow (Figure 1h). To assess the effects of embryonic *Pkn3* deletion, we generated $Pkn3^{fl/fl}$; *Vav-Cre* mice and measured HSC numbers at 6–8 weeks after birth. There was no significant difference in HSC numbers between control and $Pkn3^{fl/fl}$; *Vav-Cre* mice (Figure 1i).

We used competitive transplantation assays to test whether *Pkn3* regulates HSC function. We transplanted 300 000 control or $Pkn3^{fl/fl}$; *Vav-Cre* donor (CD45.2+) bone marrow cells along with 300 000 wild-type competitor (CD45.1+) bone marrow cells into lethally irradiated CD45.1+ mice. Control and $Pkn3^{fl/fl}$; *Vav-Cre* bone marrow reconstituted at similar levels overall (Figure 1j), and there were no differences in myeloid, B-cell or T-cell reconstitution (Figure 1k). Donor HSC frequencies were similar among recipients of control and $Pkn3^{fl/fl}$; *Vav-Cre* marrow (Figure 1l). Based on these data, *Pkn3* does not appear to be essential for normal hematopoiesis.

We next tested whether *Pkn3* contributes to changes in HSC function and leukemogenesis that occur following *Pten* deletion.[11,12] *Pten* encodes a lipid phosphatase that negatively regulates the PI3K pathway.[13] *Pten* deletion impairs HSC function, and *Pten*-deleted mice develop myeloproliferative neoplasms (MPN) and T-ALL.[11,14] These phenotypes can be completely attenuated by simultaneously deleting *Rictor* to inactivate mTORC2 and its substrate, AKT.[14] Since the PI3K pathway activates PKN3 in some cancers,[4] we tested whether *Pkn3* deletion can prevent HSC mobilization, MPNs, impaired HSC function and T-ALL in *Pten*-deleted mice.

We first characterized *Pkn3* expression in control and *Pten*-deleted hematopoietic cells. *Pten* deletion caused a significant reduction in *Pkn3* expression in HSCs and in MPN (~50 and 75%, respectively; Figure 2a). In contrast, *Pten* deletion did not significantly alter *Pkn3* expression in thymocytes or T-ALL cells. Thus, the effects of PI3K pathway activation on *Pkn3* expression are cell-type specific. This raises the question of whether genetic interactions between *Pten* and *Pkn3* are also cell-type specific.

To test whether *Pkn3* deletion rescues the HSC mobilization and MPN phenotypes, we generated $Pten^{fl/fl}$; $Pkn3^{fl/fl}$; *Mx1-Cre* (*Pten/*$Pkn3^{\Delta/\Delta}$) mice, as well as Cre-negative (control), $Pten^{fl/+}$; $Pkn3^{fl/fl}$; *Mx1-Cre* ($Pkn3^{\Delta/\Delta}$) and $Pten^{fl/fl}$; $Pkn3^{fl/+}$; *Mx1-Cre* ($Pten^{\Delta/\Delta}$) littermate mice. We deleted *Pten* and *Pkn3* 6 weeks after birth and analyzed spleen weights and HSC numbers 2 weeks later. We found that both $Pten^{\Delta/\Delta}$ and *Pten/*$Pkn3^{\Delta/\Delta}$ mice had enlarged spleens, and spleen HSC numbers were similarly increased in both $Pten^{\Delta/\Delta}$

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