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CHRONIC MYELOPROLIFERATIVE NEOPLASMS

RAS-pathway mutations are common in patients with ruxolitinib refractory/intolerant myelofibrosis: molecular analysis of the PAC203 cohort

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TO THE EDITOR:

The treatment of myelofibrosis (MF), a myeloproliferative neoplasm (MPN) driven by JAK-STAT pathway activating mutations, evolved with the advent of JAK inhibitors. The first-in-class agent, ruxolitinib (RUX), a JAK1/2 inhibitor, is now standard for treatment of splenomegaly and MF-associated symptoms [1]. However, MF is a biologically and clinically heterogeneous disease with certain difficult to treat patient subgroups. In particular, disease- or treatment-associated thrombocytopenia is associated with adverse outcomes [2, 3] and often requires RUX dose reductions or interruptions which may limit treatment efficacy. Thrombocytopenic patients who discontinue RUX have a median survival of less than 1 year [3].

Pacritinib (PAC), a JAK2/IRAK1/ACVR1 inhibitor that spares JAK1, has shown clinical benefit in thrombocytopenic MF in the PERSIST-1 and -2 trials [4, 5]. PAC203 was a randomized dose finding study of PAC in primary or secondary MF patients who were refractory or intolerant to RUX (RUX-ref/int), including patients with moderate and severe thrombocytopenia. Patients were randomized 1:1:1 (PAC 100 mg once daily [QD], 100 mg twice daily [BID] or 200 mg BID) stratified by baseline platelet count. This study established PAC 200 mg BID as the optimal efficacious and safe dose [6] and PAC is now FDA approved for the treatment of patients with MF who have thrombocytopenia.

Previous studies established the adverse prognostic implications of certain somatic gene mutations in MF; specifically mutations in epigenetic (*ASXL1*, *EZH2*) [7], splicing factor (*SRSF2*, *U2AF1*) [7] and *IDH1*/*IDH2* genes [7] are associated with disease progression and shortened survival. Reduced likelihood of RUX response has been associated with ≥3 mutations [8] but not with mutation type [9, 10]. Shorter time to RUX failure has been reported in those with *ASXL1*/*EZH2* mutations [9] and reduced time to RUX discontinuation in patients with ≥3 mutations [8]. Specific cytokine signatures have been correlated with RUX resistance [11] suggesting possible biologically relevant pathways (e.g. NFκB) mediating resistance. The mutation profiles of RUX-ref/int thrombocytopenic MF patients have not been well delineated. This represents a group with a major unmet need for effective management strategies, and a better understanding of their mutation profiles will assist the application of precision medicine in this challenging group.

We therefore performed mutational analysis on a subgroup the PAC203 cohort (110 patients at baseline and 42 patients at 24 weeks follow-up using a 32-gene TruSeq Custom Amplicon Panel (see Supplementary Methods). Furthermore, we interrogated cytokine profiles to understand the relationship between inflammatory signatures and clinico-genomic profiles in this cohort.

Characteristics of this group was representative of the overall PAC203 cohort [6]. Median follow-up time was 213 (95% confidence interval [CI]: 189–236) days. The median age was 68 (37–87) years, the median duration of prior exposure to RUX was 1.59 years (range 0–11 years) with 72.7% reporting prior exposure to non-RUX therapies (range 1–5 lines). Primary myelofibrosis (PMF) was the most prevalent disease category (56.4%, 62/110), followed by post-polycythemia vera MF (PPV-MF) in 29.1% (32/110) and post-essential thrombocythemia MF (PET-MF) in 14.5% (16/110). Thrombocytopenia was common: median baseline platelet count was 63 $\times 10^9$/L, with 38.2% (42/110) <50 $\times 10^9$/L. Baseline hemoglobin was <10 g/dL in 64.5% (71/110) of the cohort.

MPN driver mutation frequency was as expected for MF [10, 12]; *JAK2*V617F mutation was present in 77.3% (85/110), *CALR*-mutation in 12.7% (14/110; type 1: $n = 11$, type 2: $n = 3$), *MPL*-mutation in 8.2%, and "triple-negative" in 1.8% of cases, Fig. 1A. *JAK2*V617F variant allele frequency (VAF) was ≥50% in 68.2% (58/85) with VAF < 20% present in just 5.9% ($n = 5$) of patients. Non-MPN driver mutations (NDM) were present in 76.4% ($n = 84$) with ≥3 NDMs in 20.9% (23/110) of patients. Analogous to previous reports, the most prevalent NDMs were in *ASXL1* and *TET2* genes (in 29.1%, $n = 32$,

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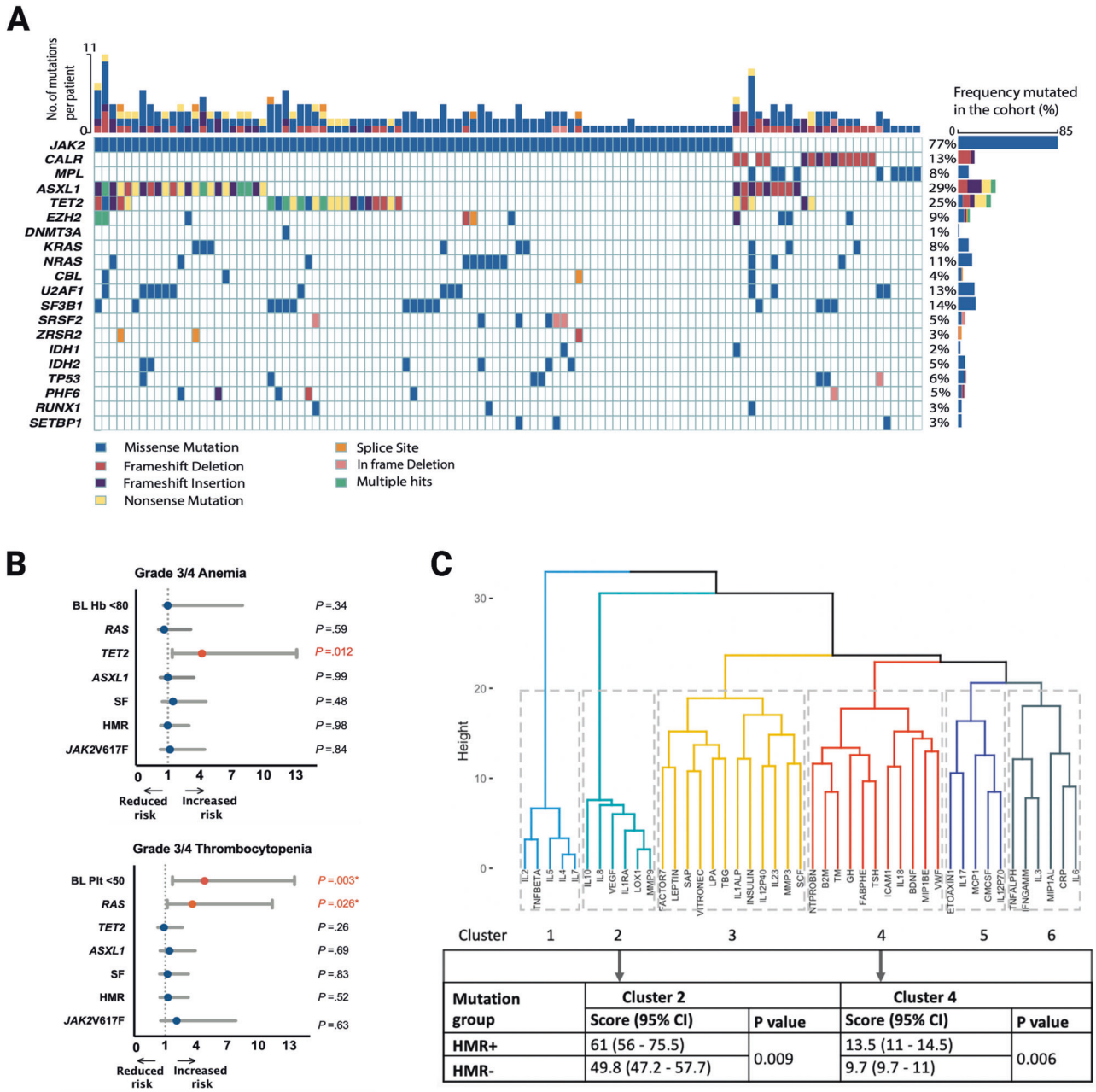

Mutation group	Cluster 2		Cluster 4	
	Score (95% CI)	P value	Score (95% CI)	P value
HMR+	61 (56 - 75.5)	0.009	13.5 (11 - 14.5)	0.006
HMR-	49.8 (47.2 - 57.7)		9.7 (9.7 - 11)	

Fig. 1 The mutation and cytokine profiles in the PAC203 cohort. A Waterfall plot of mutation distribution in the PAC203 cohort. **B** Forest plot illustrating the results of logistic regression analyses of mutation statuses and baseline hemoglobin count associated with the likelihood of grade 3/4 anemia; *TET2*-mutated patients were more likely to experience grade 3/4 anemia independent of baseline hemoglobin level; odds ratio (OR) 4.5, 95% CI 1.4–13.9, $P = 0.009$ (upper panel). Forest plot illustrating the results of logistic regression analyses of mutation statuses and baseline platelet count associated with the likelihood of grade 3/4 thrombocytopenia; *KRAS/NRAS*-mutated patients were more likely to experience grade 3/4 anemia after adjustment for baseline platelet level; OR 3.65, 95% CI 1.2–11.3, $P = 0.026$. (lower panel). Univariate logistic regression was performed for each variable. Significant *P* values (<0.05) highlighted in red and OR denoted by (*) were adjusted. JAK2V617F JAK2 V617F-mutated; HMR high molecular risk mutation [*IDH1/2, SRSF2, ASXL1, EZH2, U2AF1*Q157]; SF splicing factor mutation [*SF3B1*, *U2AF1*, *SRSF2*, *ZRSR2*], *ASXL1 ASXL1*-mutated; *TET2 TET2*-mutated, *RAS KRAS/NRAS*-mutated, BL Plt <50 baseline platelet level $<50 \times 10^9$/L. **C** Cluster dendrogram of cytokine levels in ruxolitinib refractory / intolerant study cohort with cluster scores 2 and 4 highlighted in the table for high molecular risk positive (HMR+) and negative (HMR−) patients.

and 26.4%, $n = 29$, of patients respectively) (Fig. 1A, Supplementary Table S1). Splicing factor (SF) gene mutations were mutually exclusive and detected in 34.5% ($n = 38/110$) of patients, which included *SF3B1* [13.6%, $n = 15$], *U2AF1* [12.7%, $n = 14$], *SRSF2* [5.5%, $n = 6$], *ZRSR2* [2.7%, $n = 3$]. Patients with SF mutations were more often categorized as PMF (76.3%) rather than PET-MF (15.8%) or PPV-MF (7.9%), $P = 0.001$ (Supplementary Table S2A). SF-mutated patients had lower baseline hemoglobin level (Hb <8 g/dL in 39.5% as compared with 16.9% in SF-wild type [WT], $P = 0.009$) and were more likely to be red cell transfusion dependent at trial entry (RCC-D) as compared with SF-WT patients (42.1% vs 22.2% respectively, $P = 0.012$; Supplementary Table S2A). *SF3B1*-mutated patients had higher trial entry platelet counts (platelet count $>100 \times 10^9$/L) in 66.7% vs. 28.7% in *SF3B1*-WT patients, $P = 0.004$).

Table 1. *RAS/CBL*-mutated patient baseline clinical and mutation characteristics.

	***RAS/CBL*-mutated, *N* = 23 *n* (%)**	***RAS/CBL*-WT, *N* = 85 *n* (%)**	***P* value**
Age in years, median (range)	69 (56–85)	68 (37–87)	0.42
Male gender, *n* (%)	14 (60.9)	50 (57.5)	0.77
MF diagnosis, *n* (%)			
Primary MF	13 (56.5)	49 (56.3)	
PPV MF	8 (34.8)	24 (27.8)	
PET MF	2 (8.7)	14 (16.1)	0.6
Prior ruxolitinib, *n* (%)			
Failure	16 (69.6)	64 (73.6)	0.64
Intolerance	18 (78.2)	59 (67.8)	0.33
Prior ruxolitinib duration in months, median (range)	29 (1.7–131.4)	16.6 (1.7–119)	0.38
Platelet count × 10^9/L, median (range)	59 (14–402)	63.5 (13–910)	0.47
Platelet count <50, ×10^9/L, *n* (%)	10 (43.5)	32 (36.8)	0.58
Hemoglobin <10 g/dL, *n* (%)	19 (82.6)	52 (59.8)	0.13
RBC transfusion dependent, *n* (%)	10 (43.5)	22 (25.2)	0.25
Platelet transfusion dependent *n* (%)	4 (17.4)	5 (5.7)	0.07
Peripheral blasts, median (range)	2 (1–5)	2 (0–17)	0.65
White blood cells, ×10^9/L, median (range)	6.6 (1.2–107.7)	6.8 (1.1–103.4)	0.85
Spleen Volume (cm^3) by MRI/CT, median (range)	2589 (458–5520)	2240 (262–4994)	0.22
Driver mutation status			
*JAK2*V617F	18 (78.2)	67 (77)	
CALR	2 (8.7)	12 (13.8)	
MPL	2 (8.7)	7 (8)	
Triple negative	1 (4.4)	1 (1.2)	0.7
JAK2 V617F allele burden ≥50%	12 (66.7)	46 (68.7)	1
HMR mutation	15 (65.2)	33 (37.9)	0.02
NDM ≥3	13 (56.5)	8 (9.2)	0.0001

WT wild-type, *MF* myelofibrosis, *PPV* post polycythemia, *PET* post essential thrombocythemia, *RBC* red blood cell, *HMR* high molecular risk, *NDM* non-myeloproliferative neoplasm driver mutation.

High molecular risk mutations (HMR; *IDH1/2, SRSF2, ASXL1, EZH2, U2AF1*Q157) [7] were present in 43.6% (48/110) and ≥2 HMR mutations were present in 15.4%, a prevalence similar to other high-risk enriched MF cohorts [12, 13]. No clinical parameters were associated with a HMR mutation (Supplementary Table S2B). Strikingly, RAS-pathway mutations, *KRAS/NRAS/CBL* (*RAS/CBL*-MT), were found at a higher frequency than previously described in MF cohorts [13, 14] in 20.9% of patients ($n = 23$; *RAS* $n = 20$, *CBL* $n = 3$; Fig. 1A, Table 1). These mutations were sub-clonal in the majority with a median VAF of 10% (range 1.9–95%). *RAS* mutations occurred in known mutation hotspots; the most prevalent was in codon G12 ($n = 12/20$) [13]. *RAS/CBL*-MT patients had a significantly higher frequency of NDMs (≥3 in 56.5% vs. 9.2% for *RAS/CBL*-WT patients, $P = 0.0001$, Table 1) and a co-mutated HMR mutation (65.2% vs 37.9% for *RAS/CBL*-WT patients, $P = 0.02$, Table 1). *KRAS/NRAS/CBL* and *TP53* ($n = 7$ patients) mutations were mutually exclusive in this cohort.

In patients with both molecular and 24-week clinical data, there were no significant correlations between driver or NDM mutation status (including specific analyses relating to HMR and *RAS/CBL*-MT status) and SVR or TSS response, although numbers of events for analysis were low. Grade 3/4 anemia occurred more often during the study period in *TET2*-mutated patients (odds ratio [OR] 4.2, 95% CI 1.4–13, $P = 0.012$), Fig. 1B. Grade 3/4 thrombocytopenia occurred more frequently in *RAS/CBL*-MT patients (OR 3.64, 95% CI 1.2–11.3, $P = 0.026$, Fig. 1B), including after adjusting for baseline platelet strata (< vs. ≥50 ×10^9/L). The presence of ≥3 NDMs was associated with an increased risk of infections (OR 7.59, 95% CI 2.45–23.4, $P = 0.0001$).

Follow-up molecular analysis at week 24 was performed in 38.2% ($n = 42/110$). No significant driver or NDM molecular responses (≥50% reduction in VAF) were detected. At least one new NDM was acquired in 7.1% (3/42) including *CBL* [2], *TET2* [1], *TP53* [1], *U2AF1* Q157 [1]. No associations were observed between follow-up mutation analyses and outcomes.

Unsupervised clustering identified 6 cytokine clusters at baseline, Fig. 1C. Elevated cluster 2 ($P = 0.009$) and 4 ($P = 0.006$) scores were associated with HMR mutations. Higher cluster 2 scores were also associated with driver mutation VAF ≥50%. The pro-inflammatory cytokines in cluster 2 linked to HMR mutations (HMR+) represented a cluster regulated by the NFκB pathway. The presence of a HMR mutation was particularly associated with significantly higher IL-8 levels (40.5 pg/ml) as compared with absence of an HMR mutation (24.5 pg/ml), $P < 0.0001$. Elevated tumor necrosis factor-alpha (TNF-α) was also associated with HMR mutations; TNF-α was 61 pg/ml in HMR+ vs. 48.5 pg/ml for HMR−, $P = 0.009$. Although RAS-pathway mutations were not associated with specific cluster scores, these patients did have higher levels of the NFκB-associated cytokine IL12P40 (1.1 ng/ml) as compared with *RAS/CBL*-WT patients (0.6 ng/ml), $P = 0.001$. There was no association between cytokine cluster scores and exposure to RUX.

We report the mutation landscape in RUX-ref/int cytopenic MF, showing enrichment for HMR mutations and, in particular, a higher frequency of *RAS*-pathway mutations (20.9%) than previously reported in MF cohorts (to date at a frequency of 6–8.1%) [13, 14]. *RAS* and HMR mutation co-occurrence has previously been described, which we also observed [14]. *RAS*-pathway mutations

often showed low allele burden and correlated with the presence of multiple NDMs, consistent with presence of *RAS*-pathway mutations in patients undergoing genetic evolution. Although mutation data was not available prior to RUX treatment in this cohort, recent single cell genetic analyses in myelofibrosis show *RAS*-pathway mutations were one of most common emergent mutations after exposure to RUX [15]. Activating mutations of the *RAS*-pathway have also been reported to correlate with reduced likelihood of spleen and symptom responses in patients with myelofibrosis treated with dual JAK1/2 inhibitors [13]. *RAS*-pathway mutations in MF have also been associated with shorter survival and progression to leukemia [14]. The PAC203 cohort therefore represents a genetically high risk group of patients.

Importantly, we report for the first time a relationship between HMR and *RAS* mutations and a pro-inflammatory cytokine signature. This signature mirrors a previously described RUX resistant cytokine profile [11] involving NFκB signaling. A potential underlying mechanism may be that the inflammatory microenvironment creates a selective pressure promoting the evolution of subclones carrying HMR and RAS-pathway mutations. We speculate that this combination of cell-intrinsic genetic properties of the clone, and cell-extrinsic inflammatory microenvironment might collectively confer JAKi resistance. Therapeutic strategies, including dual blockade of JAK2 and NFκB, may prove beneficial for treatment of MF. As a JAK2/IRAK1 inhibitor, PAC targets both pathways, as IRAK1 is upstream of NFκB signaling suggesting a potential role in those with HMR and *RAS*-pathway mutations. Although no specific TSS or SVR responses were observed in these patients on PAC203, the numbers of patients available for analysis was low, and the follow-up period may not have been sufficient to capture responses in this subgroup. Other strategies, including combinations targeting JAK and MEK/ERK pathways together with inflammatory pathways, for example through bromodomain inhibition, could be an effective strategy to mitigate clonal evolution in high-risk patients.

In summary, the PAC203 cohort encompasses a molecularly high-risk group, with a high incidence of HMR and *RAS* pathway mutations that may be associated with JAK1/2 inhibitor resistance. Our findings will help inform the application of precision medicine for this group of patients with a major unmet need for new therapeutic strategies.

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AUTHOR CONTRIBUTIONS

JMOS designed and analyzed experiments, performed the statistical analysis and prepared the typescript. AJM conceived and supervised the project, designed experiments and prepared the typescript. KH and HD performed experiments. All authors read and approved the typescript.

COMPETING INTERESTS

AJM has consulted for Novartis, Bristol Myers Squibb/Celgene, and AbbVie, has received research funding from Novartis, Bristol Myers Squibb/Celgene, and CTI BioPharma, has received honoraria from Novartis and CTI BioPharma, and has served on the speaker's bureau for Novartis. AG has acted in a consulting or advisory role for AbbVie, BMS, Constellation Pharmaceuticals, CTI BioPharma Corp., Novartis, PharmaEssentia, and Sierra Oncology. SB is employed by and holds stock in CTI BioPharma Corp. CNH has received honoraria from AbbVie, CTI BioPharma, Geron, Janssen, and Novartis; has served in consulting/advisory capacity for AOP, Celgene/BMS, Constellation Pharmaceuticals, CTI BioPharma, Galecto, Geron, Gilead, Janssen, Keros, Promedior, Roche, Shire, Sierra Oncology, and Novartis; has served on a speakers bureau for AbbVie, BMS, CTI BioPharma, Geron, Sierra Oncology, and Novartis; and has received research funding from BMS, Constellation Pharmaceuticals, and Novartis. STO has consulted for AbbVie, Blueprint Medicines, Celgene/BMS, Constellation Pharmaceuticals, CTI BioPharma, Disc Medicine, Geron, Incyte, and PharmaEssentia; and has received research funding from Actuate Therapeutics, Blueprint Medicines, Celgene/BMS, Constellation Pharmaceuticals, CTI BioPharma Corp., Incyte, Kartos Therapeutics, Sierra Oncology, and Takeda.

ADDITIONAL INFORMATION

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