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Spliceosome mutations are common in persons with myeloproliferative neoplasm-associated myelofibrosis with RBC-transfusion-dependence and correlate with response to pomalidomide

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Myelo-proliferative neoplasm (MPN)-associated myelofibrosis (PMF) is a clonal disorder characterised by decreased blood cell concentrations, bone marrow fibrosis and a risk of transformation to acute myeloid leukaemia (AML). Persons with this cancer who become RBC-transfusion-dependent have a poor prognosis with limited therapy options to reverse their anemia (1). Most persons with MPN-associated myelofibrosis have *driver* mutations in *JAK2, CALR* or *MPL* resulting in aberrant JAK/STAT signaling (2). Non-driver mutations are common in spliceosome components, epigenetic regulators and notably in *ASXL1, EZH2, IDH1/2, SRSF2* and *U2AF1*^{Q157}, which are associated with adverse outcomes and are termed high-molecular risk mutations (3, 4).

RESUME was a randomized, double-blind, parallel group, controlled trial, assessing rates of RBC-transfusion-independence in subjects receiving pomalidomide or

placebo (5). Although response rates were similar, the study identified some differences between responders in the pomalidomide and placebo cohorts, including some subjects with a sustained response to pomalidomide, not observed in the placebo cohort. We reasoned the mutational landscape of subjects in the RESUME study might correlate with these pomalidomide responses.

Baseline DNA samples with corresponding clinical data were available for 205 of the 252 randomized subjects. Median age was 70 years (range, 44–90 years). Additional subject co-variates are reported and displayed in Table S1. We performed targeted DNA sequencing on samples from the 205 subjects using a Fluidigm Access Array 28 gene panel followed by next generation sequencing. Specific methods and analysis pipelines are described in the Supplement.

485 mutations were detected (Table S2). 198 of the 205 subjects (97%; 95% Confidence Interval [CI], 95, 99%) had a mutation in ≥1 targeted gene. 2 mutations were detected in 40% (33, 46%) and ≥3 in 39% (32, 46%; Figure 1A). 7 subjects had no detectable mutation. *JAK2*^{V617F}, *CALR* and *MPL* mutations were identified in 136 (66% [59, 72%]), 29 (14% [8, 19%]) and 14 (7% [4, 11%]) of the subjects (Figure 1A). There was no detectable driver mutation in 26 of the subjects with primary myelofibrosis (13% [8, 18%]; Table S1). Non-driver mutations were detected in 81% of subjects (75, 86%) with a higher frequency in males (88% [83, 93%] *versus* 61% [48, 74%]; *P*=0.0001). 45% of subjects (38, 52%) had a spliceosome mutation including *U2AF1* (22% [16, 28%]); *SF3B1* (12% [8, 16%]) *SRSF2* (7% [3, 14%]) and *ZRSR2* (8% [4, 12%]). More spliceosome mutations were detected in men than women (52% [44, 60%] *versus* 27% [15, 38%]; *P*=0.001; Table S1). Spliceosome

mutations were mutually exclusive in 85/92 subjects. 7 subjects had > 1 spliceosome mutation. (Figure S1). Spliceosome mutations were also less common in subjects with prior polycythemia vera (17% [2, 32%]) compared to subjects with prior essential thrombocythaemia (39% [22, 55%]) and those with primary myelofibrosis (51% [43, 59%]; *P*=0.074 and *P*=0.002; Table S1). Mutations in *ASXL1 (*30% [24, 36%]); *TET2* (16% [10, 20%]), *DNMT3A* (5% [2, 8%]) and *EZH2* (5% [2, 8%]) were detected at rates similar to those reported(3, 6). 46% *(*40, 53%) of subjects had a high molecular risk mutation. Subjects with *JAK2*^{V617F} were significantly more likely than subjects with a *CALR* mutation to have a non-driver mutation (72% [64, 79%] *versus.* 35% [(17, 52%] *P*=0.0001), a spliceosome mutation (47% [36, 53%] *versus*. 28% [6, 36%]; *P*=0.055), in particular a *U2AF1* mutation (26% [17, 32%] v*ersus* none; *P*=0.002) and a high molecular risk mutation (49% [41, 58%] *versus* 24% [8, 40%]; *P*=0.014). Certain mutations were positively correlated including *JAK2*^{V617F} and *U2AF1* (Odds Ratio [OR], 2.1 [0.9, 4.4]; *P*=0.07), *ASXL1* and *EZH2* (OR 4.4 [1.3, 15.7]; *P*=0.022) and *SF3B1* and *TP53* (OR=5 [1.1, 22.2]; *P*=0.035; Figure S1).

Median follow-up was 12 months (range, 11-14 months). 52 subjects died (25%) including 8 transforming to AML. 1-year survival was 76% (70, 80%). There were no significant associations between mutation topography and survival including high-risk mutations (Figure 1B). An exception was *TP53* mutation which was associated with a 1-year leukemia-free survival (LFS) of 47% (29, 63%) *versus*. 76% (70, 81%) in those without a *TP53* mutation (*P*=0.0085; Figure 1C).

There was no significant correlation between the probability of becoming RBC-transfusion-independent and driver mutation state in either cohort (Table S1). However, subjects with a non-driver mutation were less likely to achieve a 50 percent decrease in RBC-transfusions (OR=0.38 [0.18, 0.8]; *P*=0.01). When analysed by treatment arm, this reduction was only seen in subjects receiving pomalidomide (OR=0.3 [0.1, 0.7]; P=0.009; Figure 2A) but not in those receiving placebo (OR=0.9 [0.2, 3.4]; P=0.84; Figure 2A). In the entire cohort we detected a correlation between having a spliceosome mutation and a lower probability of becoming RBC-transfusion-independent in all subjects (OR=0.5 [0.2, 1.1]; *P*=0.096). However, this association was significant only in subjects receiving pomalidomide (OR=0.33 [0.11, 0.94] P=0.038 *versus* OR=1.1 [0.29, 4.3] P=0.87; Figure 2B). This association was largely attributable to a lower rate of RBC-transfusion-independence in subjects with a *U2AF1* mutation receiving pomalidomide (OR=0.1 [0.021, 1.1]; *P*=0.06 *versus* placebo OR=0.9 [0.2, 4.8]; *P*=0.89; Figure 2B). A similar correlation with receiving pomalidomide was detected when the endpoint was a 50 percent decrease in RBC-transfusions in subjects with a spliceosome mutation (OR=0.35 [0.15, 0.84]; *P*=0.018; *versus* OR=1.2 [0.4, 3.2]; *P*=0.79; Figure 2A). No other specific mutation or combination significantly correlated with either response category. Re-calculated RBC-transfusion-independence rates in the pomalidomide and placebo cohorts, censoring subjects with splicing factor mutations, showed no treatment effect on RBC-transfusion-independence rates (22% [12, 31%] *versus* 15% [3, 27%;, *P*=0.40) or 50 percent reduction in RBC-transfusions (35% [34, 70%] *versus* 32% [8, 64%], *P*=0.75). Censoring for non-driver mutations also did not distinguish transfusion independence rates 24% [8, 40%] *versus* 9% [7, 26%],

P=0.29) or 50 percent reduction in RBC transfusions 52% [34, 70%] *versus* 36% [8, 64%], *P*=0.39).

Although considerable data indicate a correlation between high-risk mutations and survival, we found no such correlation (3). There are some likely explanations. 1st, all of our subjects were high-risk with 2 independent adverse risk co-variates, anemia and RBC-transfusion-dependence and 2nd, median follow-up was only 1-year. Of note, we detected a correlation between *TP53* mutation and LFS. *TP53* mutation is not typically classified as a high-risk mutation in MPN-associated myelofibrosis but this may need reconsideration (7).

The conclusion of the RESUME study was there was no significant difference in response rates to pomalidomide and placebo in achieving RBC-transfusion-independence or a 50 percent reduction in RBC-transfusion frequency. This conclusion was perplexing as there were several subjects in the pomalidomide cohort responding to pomalidomide, who lost their response when pomalidomide was stopped and regained their response when pomalidomide was re-started. There were no similar subjects in the placebo cohort. Randomization is intended to balance cohorts for known and unknown (*latent*) co-variates correlated with outcome(s). When numbers of subjects randomized is small, such as the 252 subjects in the RESUME study, the substantial likelihood of an imbalance for ≥1 co-variate is presumed to be encompassed in the *P*-value. However, this adjustment is imperfect. We found a correlation between likelihood of response to pomalidomide and non-driver mutations including spliceosome mutations, a correlation not found in the placebo cohort. However, after censoring for non-driver mutations or

spliceosome mutations, receiving pomalidomide still had no significant impact on rates of RBC-transfusion-independence or a 50 percent reduction in RBC-transfusions. The study was not powered to detect such difference and these analyses were not pre-specified. Consequently, our conclusions are therefore hypothesis-generating and require validation. Our data also suggest a need for mutation topography analyses and stratification pre-randomization in intervention trials in MPN-associated myelofibrosis.

Conflicts of interest

Commented [OC1]: Need to confirm with authors

None.

Author contributions

OC designed and analyzed experiments and prepared the typescript; JO performed the statistical analysis and contributed to preparing the typescript. NB and GW performed bioinformatic analysis; GB designed the sequencing panels, processed samples and performed experiments; AH contributed to the design of the panels and advised on data analyses; AT, HKA, GB, TD, HG, QJ, J-JK, RM, FP, VR, GS, AV, DZ, MFM and JZ contributed samples. RPG supervised the project and helped prepare the typescript. AJM conceived and supervised the project, designed experiments and help prepare the typescript. All authors read and approved the typescript.

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Figure Legends

Figure 1. The mutational landscape of transfusion dependent myelofibrosis

(A)Frequency of mutations detected at baseline by targeted gene sequencing (n= 205 patients). Each column represents an individual patient. Only genes with pathogenic mutations detected in at least 5 patients are shown. Each colour corresponds to mutation type. VUS=variant of uncertain significance (see methods). (B) Kaplan-Meier curve of leukemia-free survival (LFS) stratified by HMR at 1-year; HMR did not influence 12-month LFS; HMR-mutated (71% [95% CI 63–78%]) versus HMR-wild type (WT) patients (78% [95% CI 71–84%]), p=0.13. (C) Kaplan-Meier curve of LFS stratified by *TP53* at 1-year showing a poorer LFS in *TP53*-mutated patients (47% [95% CI 29–63%]) versus *TP53*-WT (76% [95% CI 70–81%]), p=0.0085. HMR (high molecular risk - ASXL*1, EZH2, SRSF2 U2AF1* Q157)

Figure 2. Impact of mutational status on transfusion responses

(A) Forest plot depicting influence of mutational status on achievement of 50% reduction in red blood cell transfusion requirement; patients with SF mutations were less likely to achieve this in the POM arm only (OR=0.35 [0.15, 0.84]; *P*=0.018). This association with poorer transfusion responses in SF-mutated patients was restricted to POM-treated patients. (B) Forest plot depicting the influence of mutational status on achievement of red blood cell transfusion independence (RBC-TI) by treatment arm; patients with SF mutations were less likely to achieve RBC-TI in the POM arm

(OR=0.33 [0.11, 0.94] P=0.038) but not in the placebo arm; OR=1.1 [0.29, 4.3] P=0.87 predominantly attributable to patients with *U2AF1* mutations; OR=0.1 [0.021, 1.1]; *P*=0.06). Univariate logistic regression was performed for each group and outcomes with significant ($p<0.05$) odd ratios (OR) (denoted by *) were adjusted for male gender which was independently significant on univariate analysis.