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The poor outcome in high molecular risk, hydroxycarbamide resistant/intolerant ET is not ameliorated by ruxolitinib

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Abstract

Essential Thrombocythemia (ET) patients at high-risk of thrombosis require cytoreductive treatment, typically with hydroxycarbamide. Many patients are resistant or intolerant to hydroxycarbamide (HC-RES/INT) and are at increased risk of disease progression. MAJIC-ET is a randomized phase 2 study comparing ruxolitinib (RUX) to best available therapy (BAT) in HC-RES/INT ET, which showed no difference between the two arms in rates of hematological response or disease progression. The impact of additional non-MPN driver mutations (NDM) on the risk of disease complications in HC-RES/INT ET patients is unknown. Since the presence of

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NDM may influence trial outcomes, we expand the primary MAJIC-ET analysis to serially evaluate NDM in MAJIC-ET patients using a targeted myeloid 32-gene panel. NDM at baseline were detected in 30% of patients, most frequently affecting *TET2* (11%) followed by *TP53* (6.4%) and *SF3B1* (6.4%). The presence of a NDM was associated with inferior 4-year transformation-free survival (TFS; 65.4% [95% CI 53.3 – 75%] vs. 82.8% [95% CI 73.2 – 89.1%], p=0.017). Specifically, *TP53* (p=0.01) and splicing factor (SF, *SF3B1, ZRSR2, SRSF2*; p<0.001), but not *TET2* mutations were associated with reduced TFS which was not mitigated by RUX treatment. Longitudinal analysis identified new mutations in 19.3% of patients; primarily affecting *TET2, TP53* and *SF3B1*. We report the first comprehensive mutational analysis of HC-RES/INT ET patients and highlight the clinical/prognostic utility of serial mutation analysis for NDM in HC-RES/INT ET, including the importance of SF and *TP53* mutations which identify HC-RES/INT ET patients at increased risk of disease transformation.

Essential Thrombocythemia (ET) is a myeloproliferative neoplasm (MPN) defined by thrombocytosis, increased risk of vascular thrombosis,[1,2] hemorrhage[3] and progression to myelofibrosis (MF)[4,5] and acute myeloid leukemia (AML).[4,5] Patients are risk-stratified to identify those who might benefit from cytoreduction to reduce the risk of vascular complications.[6] Resistance/intolerance to hydroxycarbamide (HC-RES/INT), a first-line cytoreductive treatment, develops in 20% of high-risk patients[7] with increased risk of disease progression and reduced survival.[8] New approaches are needed to predict disease transformation risk in these patients, together with development of therapies that reduce this risk.

Following the discovery of the Janus Kinase 2 *(JAK2*) mutation (*JAK2*V617F), present in ~50% of ET,[9] the first approved JAK1/JAK2 inhibitor, Ruxolitinib (RUX), is now widely used for treatment of myelofibrosis[10] and polycythemia vera.[11] The MAJIC-ET trial explored the role of RUX in HC-RES/INT ET, randomizing patients 1:1 to RUX or best available therapy (BAT), demonstrating similar rates of 1-year complete hematological response (CHR).[12] Mutational status was not comprehensively reported in this paper. This is important as ET patients (29-72%)[13,14] carry mutations in non-MPN driver genes (NDM). Inferior prognosis is associated with specific mutations at diagnosis.[14] The impact of NDM in HC-RES/INT ET is unknown, as is the effect of RUX on disease course in molecularly defined subgroups. We therefore evaluated mutational status of MAJIC-ET patients and correlated this with clinical outcomes.

Next generation sequencing (NGS) was performed at baseline (n=110) and serially if a later sample was available (see Supplemental Methods for NGS and statistical analysis methodology). Median follow-up was 55 months (95% confidence interval [CI], 49.9–60.4). *JAK2, CALR* and *MPL* mutations were present in 49.1%, 30% & 4.5% of patients, respectively and 16.4% of patients were "triple-negative" (TN). Baseline NDM were present in 30% (n=33) of patients with >1 present in 10% (Figure 1A), most frequently *TET2* (n=12), *TP53* (n=7) and *SF3B1* (n=7) genes (Figure 1B; Supplemental Table 1). Driver mutation variant allele frequency (VAF) was higher than NDM VAF in 66.67%, 87.5% and 20% of *JAK2, CALR* and *MPL*-mutated patients respectively (Figure 1C). Patients with NDM tended to be older with lower hemoglobin levels (Figure 1D, Supplemental Table 2).

TP53 mutations trended towards a higher frequency in TN (17.6%) than in *JAK2/CALR/MPL*-mutated patients (4.3%), p=0.073. In the primary analysis, driver mutation status did not correlate with CHR[12]. Since platelet count reduction is a key therapeutic goal, we performed a post-hoc analysis defining platelet response as <400 x 10^9/l at 1-year. RUX-treated *JAK2*V617F-mutated patients had significantly more platelet responses than *JAK2*V617F wild-type (WT) patients, a difference not seen for BAT-treated patients (Figure 1E). RUX discontinuation more often occurred in non-*JAK2*V617F-mutated patients (OR 3.9, 95% CI 1.2 – 13.1%, p=0.027) in whom treatment failure was the most frequent cause (41.7%, n=10/24) followed by treatment toxicity (33.3%, n=8/24). In contrast, in *JAK2*V617F-mutated patients, the commonest cause for RUX discontinuation was a transformation event (43.8%, n=7/16) followed by treatment failure (31.3%, n=5/16). NDMs did not influence hematological/symptom responses (Supplemental Table 3).

Transformation events occurred in 12.7% (Supplemental Table 3). *TP53*-mutated patients had inferior 4-year transformation-free survival (TFS) of 42.9% (95% CI 9.8–73.4%) versus 79.8% (95% CI 69.7–86.8%) for WT patients, p=0.011 (Figure 2A). Splicing factor (SF) mutations conferred a poorer 4-year TFS of 40% (95% CI 12.3–67%) versus 81.5% for WT patients (95% CI 71.4–88.3%; p=0.00039, Figure 2B); predominantly attributable to mutated-*SF3B1* (p=0.004). High molecular risk (HMR) mutations in this cohort (defined by SF and *TP53* mutations) conferred a poorer TFS (p<0.0001, Figure 2C) which was not ameliorated by RUX (Figure 2D). HMR mutations retained their negative impact on multivariable analysis (Figure 2E). Driver mutation VAF ≥50% and male gender independently conferred a poorer TFS, findings reported by other groups.[15,16] Mutated-*TET2* did not correlate with clinical outcomes, comparable to previous findings.[14]

Thrombotic events (19.1%, n=21/110) were not influenced by mutational status overall. This is in contrast to previous studies reporting a greater thrombotic risk in *JAK2*V617F-mutated patients.[4] A possible explanation is that this association is not seen in HC-RES/INT patients who have a longer disease course and have undergone treatment, often with multiple lines of therapy. Furthermore, the number of events here is small and should therefore be interpreted with caution. Hemorrhagic events (9.1%, n=10/110) were specifically associated with SF mutations, p=0.007 (Supplemental Table 3). Grade 3/4 hematological toxicities were not associated with mutational status. Overall survival at 4-years of 91.5% (95% CI 80.2-96.4%) in BAT and 83% (95% CI 70.4-90.5%) in RUX arms (p=0.22) was not influenced by mutational status.

1-year driver mutation molecular responses (MR) were rare (n=3), occurring exclusively in the RUX arm; a complete MR (CMR) in 2 patients (*JAK2*V617F-mutated and *CALR*-mutated) and one *CALR*-mutated partial MR (PMR). Longitudinal driver mutation analysis was performed in 54% (n=50/93); median analysis time 48 (24–60) months with no significant change in VAF at any time point (Supplemental Figure 1A & B). 1-year MR was lost in 2 patients (Supplemental Figure 1C & D) in association with clonal evolution of NDM in both cases. Longitudinal NDM analysis was possible in 52% (n=57/110); median analysis time 40 (6-60) months. New NDM, defined by identification at VAF ≥5%, were detected in 19.3% (n=11/57) at a similar frequency across treatment arms (Supplemental Table 4) and no significant correlations were detected with baseline NDM or clinical/

survival outcomes. However, a median follow-up time of 10.7 months (95% CI 9.05–12.4) after later NDM analysis is not sufficient time for survival analysis. These data highlight the clinical utility of serial molecular analysis in HC-RES/INT ET.

In this analysis, we identify NDM at baseline in 30% of patients, a higher frequency than most previous analyses, which may relate to this high-risk nature of this cohort.[13–15,17] *TP53* and *SF3B1* mutations were observed each at 6.4%, higher than previously reported in ET (~2 and 2-5% respectively).[13–15,18] This may relate to the fact that this study analyzes a particular high-risk cohort for which there is limited data published on mutation profiles for comparison. The frequent detection of *TP53* mutations in TN patients was unexpected but the numbers are too few (n=3) to draw firm conclusions. Disease transformation was specifically associated with SF (most commonly *SF3B1*) and *TP53* mutations, determining a HMR for this cohort. Although prevalence of non-*SF3B1* SF mutations in this cohort was low, we included these as HMR as they are established adverse risk mutations in MPNs.[15] However, this definition of HMR requires independent validation in larger cohorts before being applied in clinical practice. *TP53* mutations in MPNs have been associated with AML transformation[14,15] but have not been reported to increase myelofibrotic transformation in ET.[14,15] Myelofibrotic transformation has been reported in association with SF mutations in ET, most often mutated-*SF3B1,*[14,19] but a recent large MPN study, identified *SRSF2, ZRSR2* and *U2AF1* but not *SF3B1*[15] as myelofibrotic transformation predictors in ET. This contrasts with myelodysplastic syndromes where *SF3B1* mutations confer better survival[20–22] with lower risk of disease progression[20] suggesting disease context and co-mutations (primarily *JAK2*V617F here) are relevant.

Importantly, disease transformation in HMR patients was not mitigated by RUX which is noteworthy as there has been interest in the possibility that early intervention with JAK2 inhibition might attenuate disease progression. We observed a novel association between SF mutations and hemorrhagic events; this finding needs independent corroboration due to low event rate. We also found that *JAK2*V617F-mutated status correlated with improved platelet responses to RUX, and notably, more non-*JAK2*V617F mutated patients stopped RUX raising the possibility that *JAK2*V617F-mutated ET patients might selectively benefit from RUX.

In summary, we report for the first time, comprehensive mutational analysis of HC-RES/INT ET within the context of a prospective randomized clinical trial. We found a particularly high prevalence of *TP53* and splicing factor mutations, which was strongly predictive of subsequent disease transformation, and was not mitigated by RUX. This highlights the clinical/prognostic utility of serial mutation screening in HC RES/INT ET to allow identification of patients at risk of disease transformation.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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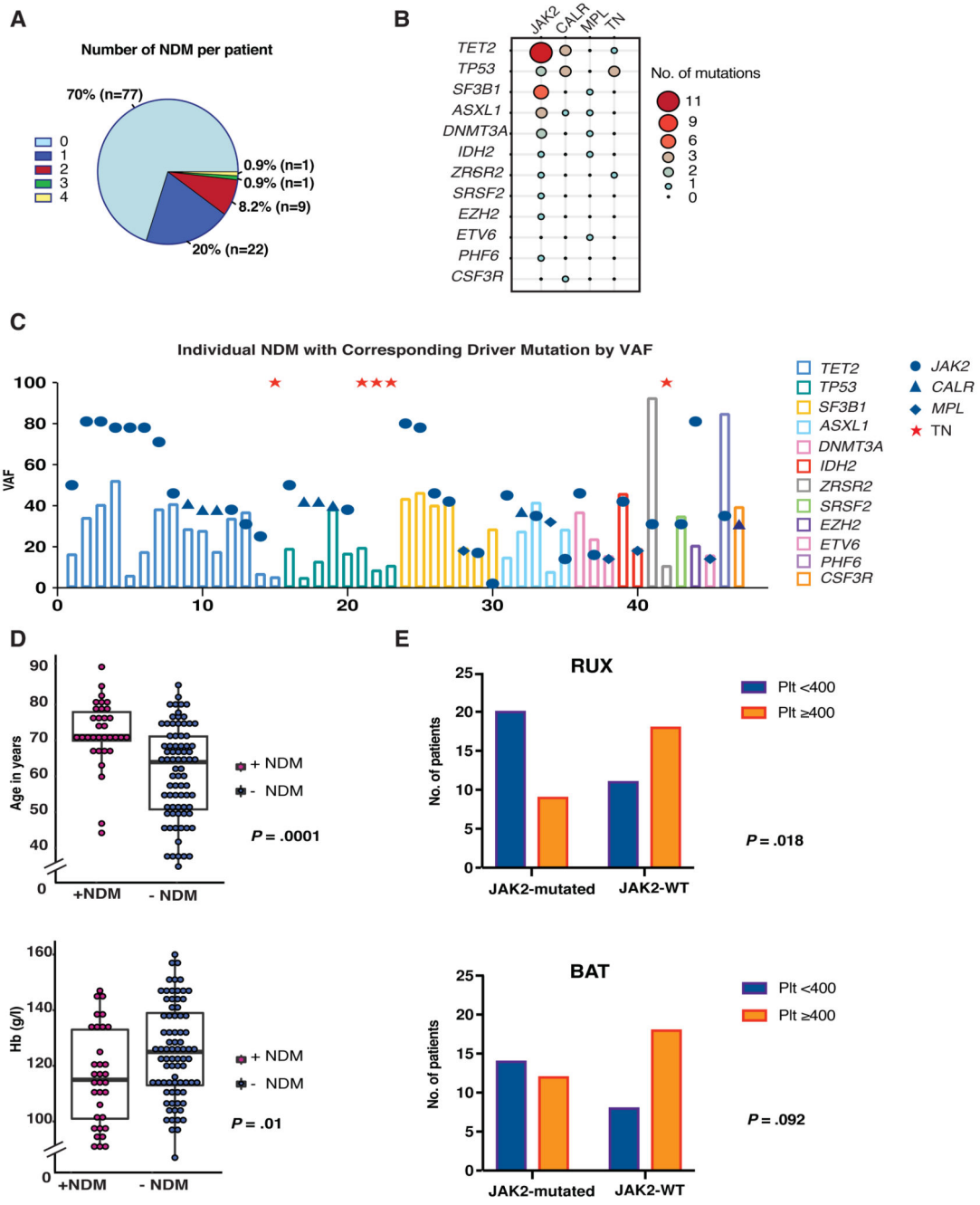

Figure 1. Baseline mutational analysis and correlation with clinical characteristics and treatment response.

(A) Pie chart showing number of NDM per patient. (B) Balloon plot showing association of driver mutations with NDM with size and colour of bubble corresponding to frequency of association; NDM were more often associated with *JAK2*V617F mutations. (C) Column and dot plot showing variant allele frequencies (VAF) of each NDM (column) with corresponding driver mutation (blue dot). Red star indicating TN patient; driver mutation VAF was higher in 66.67%, 87.5% and 20% of *JAK2*, *CALR* and *MPL*-mutated patients suggesting driver mutation acquisition first in these, although with the caveat that order of

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mutation acquisition can only be definitively assigned using single-cell methodologies.[23] (D) Dot and box plots of median age at trial entry in patients with NDM compared to patients without NDM; 71 versus 64 years, p=0.0001 (upper plot) and hemoglobin (Hb) level (mean Hb 115g/l) lower in patients with NDM compared to patients without NDM (mean Hb 125g/l), p=0.01 (lower plot). Dots represent each individual patient and each horizontal line and box represent the median for age/mean for Hb and interquartile ranges respectively using Mann-Whitney U test to compare median ages (non-normal distribution) and Student's t-test to compare Hb means (normal distribution). (E) Post hoc analysis of 1-year platelet count responses; significantly more patients on RUX who were *JAK2*-mutated achieved plt <400 than non *JAK2*-mutated patients (upper bar chart). This difference was not seen within the BAT arm (lower bar chart). BAT=best available therapy; *JAK2*=*JAK2*V617F; NDM=non-MPN driver mutation; Plt <400=platelet count of, 400 x 10^9/l; Plt ≥400=platelet count of ≥400 x 10^9/l; RUX=ruxolitinib; TN=Triple negative.

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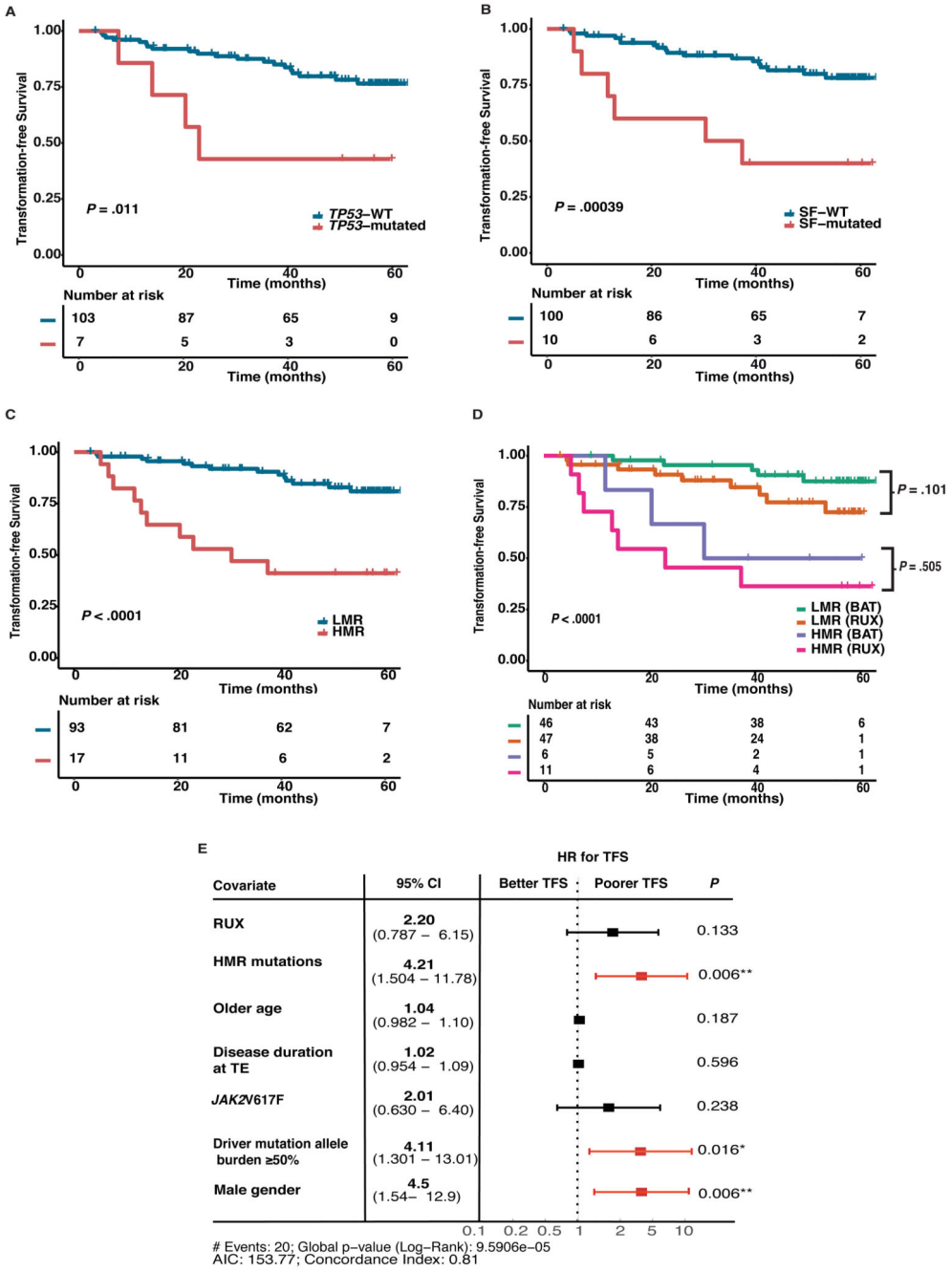

Figure 2. Kaplan-Meier curves of transformation-free survival (TFS) stratified by mutational statuses with survival estimates, reported at 4-years.

(A) *TP53* mutations were associated with inferior 4-year TFS; *TP53*-mutated (42.9% [95% CI 9.8 – 73.4%]) versus *TP53*-wild type (WT) patients (79.8% [95% CI 69.7 – 86.8%]), p=0.011. (B) SF mutations conferred a poorer 4-year TFS; SF-mutated (40% [95% CI 12.3 – 67%]) versus SF-WT (81.5% [95% CI 71.4 – 88.3%]), p=0.00039. (C) Comparing patients with HMR with LMR at 4-years; HMR 41.2% (95% CI 23.3-72.7%) versus LMR 84.6% (95% CI 76.9 – 93.1%), p<0.0001. (D) Stratifying patients with high risk molecular (HMR) mutations in this study by treatment arm demonstrates no amelioration of negative impact of

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HMR mutation with RUX treatment; patients with HMR on RUX had TFS at 4-years of 36.4% (95% CI 26.2 – 46.6%) and on BAT 50% (29.1 – 67.7%) (p=0.505 between these arms) as compared to those without these mutations (i.e. low molecular risk, LMR) with TFS at 4-years of 84.7% (95% CI 71.6 – 92%) on RUX and of 90.6% (95% CI 78.5 – 96%) on BAT (p=0.101 between these arms). The log-rank test was used to compare survival estimates between groups. (E) Forest plot showing multivariable cox model of TFS. Covariates significant on univariate analysis were included; *TP53* mutations, SF mutations, treatment arm, *JAK2*V617F mutation status, disease duration at trial entry (TE), age and gender. HMR mutations independently retained negative impact on TFS with a hazard ratio (HR) of 4.21, p=0.006. Treatment arm, *JAK2*V617F status, disease duration at TE and age were not significant but notably male gender was associated with a poorer TFS, HR 4.5, p=0.006. Driver mutation allele ≥50% was independently associated with a poorer TFS, HR 4.11, p=0.016. Age and disease duration at TE were categorized as continuous variables. CI=confidence interval; HR=hazard ratio; HMR=high molecular risk risk (SF and *TP53* mutations); LMR=low molecular risk (without SF or TP53 mutations); *JAK2*=*JAK2*V617F; NDM=non-MPN (myeloproliferative neoplasm) driver mutation; SF=splicing factor mutation (*SF3B1, ZRSR2, SRSF2*); WT=wild type.