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Chronic myeloproliferative neoplasms

**U2AF1** mutation types in primary myelofibrosis: phenotypic and prognostic distinctions

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It is now well established that approximately 90% of patients with primary myelofibrosis (PMF) express one of three “driver” mutations (JAK2, CALR, and MPL) that are often mutually exclusive and shown to be phenotypically and prognostically relevant [1]. In addition, targeted sequencing has revealed other mutations or DNA variants in more than 80% of patients with PMF, the most frequent being ASXL1 (36%), TET2 (18%), SRSF2 (18%), and U2AF1 (16%) [2]. ASXL1 and SRSF2 mutations have consistently been shown to be prognostically detrimental in PMF [1], while other mutations, such as SF3B1 and U2AF1, were phenotypically characterized by ring sideroblasts [3] and anemia/thrombocytopenia [4], respectively. The unique association between U2AF1 mutations and anemia and/or thrombocytopenia has also been demonstrated in myelo-dysplastic syndromes (MDS) [5]. In both PMF and MDS, U2AF1 mutations were associated with inferior survival, which, however, might have been accounted for by their...
association with other previously recognized risk factors, including anemia and thrombocytopenia [4, 6, 7]. In the current study, we classified U2AF1 mutations in PMF into the two main mutation variants, Q157 and S34, in order to examine differences in phenotype and prognostic relevance. The rationale for grouping U2AF1 mutations into these two mutation variants include (i) almost all PMF patients harbor one of these two mutations, (ii) classification in the two specific U2AF1 subtypes has already been assigned in MDS, and (iii) there is laboratory information that suggests functional differences between U2AF1-splice site interactions for S34 vs. Q157 variants [8, 9].

The current study was approved by the institutional review board (IRB). Diagnoses and treatment approaches were in accordance with what was considered standard of care at the time of initial diagnosis or first referral [10]. Samples for mutation analysis included archived DNA from bone marrow or peripheral blood collected at time of patient referral, under IRB-approved protocols. Prognostically relevant mutations were screened by previously described methods, which included target-capture next generation sequencing [2] or standard PCR techniques and bidirectional sequencing [11]; specifically for U2AF1, we amplified two areas with known mutations that included residues S34 and Q157. Briefly, two separate PCR reactions were performed and the primers used were as follows: (for S34) Forward: 5′-GGTTGCTTAATACACGGGAAGA−3′; Reverse: 5′-AGTCGATCACCTGCCTCACT-3′; (for Q157) Forward: 5′-GCCTCGTGTGCATTCTCTG-3′; Reverse: 5′-CTTTTCAGTTTCGCGTGAG-3′. PCR amplification conditions were the same for both U2AF1 reactions and included a primary denaturation of 95 °C for 2 min, followed by 35 cycles of: 95 °C for 30 s, 57 °C for 45 s, and 72 °C for 45 s; and ended with a final extension of 72 °C for 5 min. Products were purified before sequencing. Statistical analyses considered clinical and laboratory parameters obtained at time of diagnosis or first referral, which coincided, in all instances, with time of sample collection for mutation analysis. Conventional methods were used for statistical analysis (JMP® Pro 13.0.0 software; SAS Institute, Cary, NC). Survival analysis was computed from the date of diagnosis or first referral to date of event. Patients receiving allogeneic stem cell transplant were censored at the time of their transplantation. Date of leukemic transformation replaced date of death, as the uncensored variable, for estimating leukemia-free survival. Cox proportional hazard regression model was used for multivariable analysis.

A total of 491 patients (median age 64 years; 62% males) were annotated for U2AF1 mutations. Driver mutation distributions were 60% JAK2, 19% type 1/like CALR, 4% type 2/like CALR, 7% MPL and 10% triple negative. Dynamic international prognostic scoring system (DIPSS) distributions were 10% high, 38% intermediate-2, 36% intermediate-1 and 16% low [12]. Spleen was palpable in 75% of patients and measured >10 cm below the left costal margin in 33%. Cytogenetic risk categories, according to the recently revised system[13], were very high risk in 7%, unfavorable in 16% and favorable in 78%. Mutational frequencies were 16% for U2AF1, 38% for ASXL1, 14% for SRSF2, 4% for EZH2 and 5% for IDH1/2. In order to assist with detailed examination of the previously recognized relationship between U2AF1 mutations and cytopenia, four separate categories of anemia and three of thrombocytopenia were considered; anemia was absent in 17% of patients while it was classified as being severe (transfusion dependent or <8 g/dl in women or <9 g/dl in men) in 33%, moderate (hemoglobin <10 g/dl in women and <11 g/dl in men) in 20% and mild (hemoglobin below the sex-adjusted lower limit of normal) in 30%; reference ranges for hemoglobin, at our institution, were 13.5−17.5 g/dl for men and 12.0−15.5 g/dl for women. Platelet count was 450×10(9)/l or more in 18% of patients, between 100 and 450×10(9)/l in 60% and below 100×10(9)/l in 22%.

Among the 77 (16%) patients with U2AF1 mutations, 50 (65%) involved Q157 ± other concomitant variants, 26 (34%) involved S34 ± other concomitant variants not including Q157 and one involved neither (see Fig. 1 for details). For the purposes of comparing the phenotypic and prognostic differences between distinct U2AF1 mutational types, we grouped all Q157 variants together, regardless of the presence of concomitant other U2AF1 mutations. Accordingly, comparison of the three major U2AF1 mutational categories (414 unmutated vs. 50 mutated for Q157 ± other concomitant variants vs. 26 mutated for S34 ± other concomitant variants not including Q157) disclosed the following significant associations: both Q157 and S34 variants with anemia (Fig. 1; p < 0.01), JAK2 and MPL mutations (Fig. 1; p < 0.01) and absence of marked splenomegaly defined by spleen palpable at >10 cm below the costal margin (p = 0.02). In addition, Q157, but not S34, was associated with thrombocytopenia (Fig. 1; p = 0.02), older age (p < 0.01), ASXL1 mutations (p < 0.01; Fig. 1) and constitutional symptoms (p < 0.01). Multiple logistic regression analysis confirmed the association between Q157 mutation and anemia, thrombocytopenia, driver mutational status, ASXL1 mutations and absence of marked splenomegaly; significance was lost for age and constitutional symptoms (data not shown). Significant associations were not evident for cytogenetic risk categories (p = 0.93), circulating blast percentage (p = 0.47), leukocyte count (p = 0.6), or EZH2 (p = 0.28) or IDH1/2 mutations (p = 0.44).

After a median follow-up of 3.9 years, 338 (69%) deaths, 51 (10%) leukemic transformations and 33 (7%) stem cell transplants were recorded. Overall survival was significantly shorter for patients with U2AF1 Q157 mutations,
compared to those with \textit{U2AF1} S34 mutations or \textit{U2AF1} unmutated cases (Fig. 2a, b); the survival effect was most evident in younger patients, where the information is important for making treatment decisions (Fig. 2b). The results were unchanged when analysis was limited to patients who harbored only Q157 variant (\(n = 43\)), only S34 variant (\(n = 25\)) or were unmutated for any \textit{U2AF1} mutation (\(n = 414\)); HRs (95\% CI) were 2.3 (1.6–3.2) for Q157 vs. \textit{U2AF1} unmutated, 2.1 (1.2–4.0) for Q157 vs. S34 and 1.1 (0.6–1.7) for S34 vs. \textit{U2AF1} unmutated. The prognostic relevance of \textit{U2AF1} Q157 mutations was not accounted for by age (\(p = 0.01\)), anemia (\(p = 0.007\),

\begin{figure}[h]
\centering
\includegraphics[width=\textwidth]{fig1}
\caption{\textit{U2AF1} mutation types among 491 patients with primary myelofibrosis and comparison of \textit{U2AF1} Q157 vs. \textit{U2AF1} S34 vs. \textit{U2AF1} unmutated categories, in regards to anemia, thrombocytopenia, driver mutational status, and \textit{ASXL1} mutations.}
\end{figure}

\begin{figure}[h]
\centering
\includegraphics[width=\textwidth]{fig2}
\caption{a) Survival data on 490 patients with primary myelofibrosis stratified by \textit{U2AF1} mutation types. b) Survival data on 370 transplant-age patients with primary myelofibrosis (age 70 years or younger), stratified by \textit{U2AF1} mutation types.}
\end{figure}
thrombocytopenia ($p < 0.001$), driver mutational status ($p < 0.001$) or ASXL1 mutations ($p < 0.001$). Multivariable analysis confirmed the independent prognostic contribution of $U2AF1$ Q157 but not S34 mutation type, both in the context of genetic risk factors and DIPSS (Supplemental Table 1). $U2AF1$ mutational status did not affect leukemia-free survival ($p = 0.52$). The overall results in terms of both phenotypic and prognostic differences were not affected by the removal of patients with dual mutations or those with $Y158E159dup$.

$SF3B1$, $SRSF2$, and $U2AF1$ constitute the three major spliceosome genes mutated in myeloid malignancies with the most frequent mutations occurring, respectively, in the K700, P95, and Q157/S34 hotspot codons [14]. These mutations are usually heterozygous and mutually exclusive and their mechanism of action involves altered 3' splice site recognition. In myeloid neoplasms, $U2AF1$ mutations might contribute to impaired erythroid differentiation and lineage-specific alteration of mRNA splicing transcripts [15]. Considering differences between $U2AF1$-splice site interactions for S34 vs. Q157 variants [8, 9], it is important to examine the clinical implications of different mutation types. The majority of $U2AF1$ mutations in MDS affect the S34 codon (approximately 72 vs. 30% for Q157 mutation type, in one study) [7], which is opposite of the pattern seen in the current study for PMF patients (65% for Q157 vs. 34% for S34) [5]. The observations in MDS and PMF are also different in regards to the age distribution of $U2AF1$ mutated patients; younger in MDS and older in PMF [5–7]. Similarly, we did not find $U2AF1$ mutation associations with +8 or 20q- cytogenetic abnormalities, as had been previously reported in MDS (data not shown) [5, 7]. On the other hand, in both MDS and PMF, $U2AF1$ mutations were associated with anemia, thrombocytopenia, and inferior survival [6, 7]. In MDS, thrombocytopenia was specifically associated with the S34 and anemia with the Q157 $U2AF1$ mutation types [5]. By contrast, in the current study with PMF patients, both mutation types were associated with anemia and the association with thrombocytopenia was most evident for the Q157 mutation type. Regardless, the current study establishes $U2AF1$ Q157, but not S34, as a candidate mutation for inclusion in future genetics-based risk stratification models; in this regard, it is noteworthy to recall our previously reported observations with ASXL1 mutations, where mutation type did not appear to matter [16]. Our observations in the current study also underscore the need to stratify $U2AF1$ mutated patients according to mutation types, especially in clinical trials involving splice modulating agents [17].

**Author contributions:** All authors have reviewed and approved the manuscript. C.A.H. reviewed pathology data. R.P.K. reviewed cytogenetic data. N.G. and A.P. contributed patients and assisted in study design and concept. T.L.L. and C.M.F. were in charge of molecular studies and analysis. A.T. developed the study concept and design, contributed patients, assisted in data extraction, performed statistical analysis and wrote the paper.

**Compliance with ethical standards**

**Conflict of interest** The authors declare that they have no conflict of interest.

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Chronic lymphocytic leukemia

Sustained long-lasting responses after lenalidomide discontinuation in patients with chronic lymphocytic leukemia

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Toxicity prompting treatment discontinuation remains a major challenge in the management of chronic lymphocytic leukemia (CLL), both with chemoimmunotherapy and with novel biological agents [1–3]. Toxicity-related treatment discontinuation was reported in up to 60% of CLL patients treated with the combination of fludarabine, cyclophosphamide, and rituximab [4, 5]. In patients treated with chemoimmunotherapy, treatment discontinuation can result in lower response rates and shorter time to next treatment (TTNT) [6]. Toxicity-related treatment discontinuation has been reported in about 10 to 20% of CLL patients treated with ibrutinib [7] and up to 40% of those treated with idelalisib plus rituximab [8]; typically, following discontinuation, the disease rapidly progresses with aggressive biological and clinical features and an overall unfavorable outcome [7, 8].

Lenalidomide is an immunomodulatory agent with well-established clinical benefits in CLL [9]. Unfortunately, its development has been slowed by the results of a phase III front-line clinical trial comparing single-agent lenalidomide with single-agent chlorambucil in elderly patients with CLL, which was stopped early because of higher rate of toxicity-related deaths in the lenalidomide arm [10].

Because lenalidomide affects the interaction between CLL cells and their immune microenvironment [11], we hypothesized that the clinical effects of lenalidomide persist after treatment discontinuation.

Two hundred eight patients with CLL received lenalidomide-based therapy on clinical trials at The University of Texas MD Anderson Cancer Center between December 2005 and October 2015. One hundred twenty patients received lenalidomide as front-line treatment and 88 as salvage therapy. Lenalidomide was given as a single agent in 75 patients and in combination with an anti-CD20 monoclonal antibody (rituximab or ofatumumab) in 123. Of these 208 patients, 43 (21%) had treatment discontinued because of toxicity. To test our hypothesis, we analyzed the long-term outcomes of these patients. This study has been...