Inappropriately low hepcidin levels in patients with myelodysplastic syndrome carrying a somatic mutation of SF3B1

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ABSTRACT

Somatic mutations of the RNA splicing machinery have been recently identified in myelodysplastic syndromes. In particular, a strong association has been found between SF3B1 mutation and refractory anemia with ring sideroblasts, a condition characterized by ineffective erythropoiesis and parenchymal iron overload. We studied the relationship between SF3B1 mutation, erythroid activity and hepcidin levels in myelodysplastic syndrome patients. Erythroid activity was evaluated through the proportion of marrow erythroblasts, soluble transferrin receptor and serum growth differentiation factor 15. Significant relationships were found between SF3B1 mutation and marrow erythroblasts ($P=0.001$), soluble transferrin receptor ($P=0.003$) and serum growth differentiation factor 15 ($P=0.035$). Serum hepcidin varied considerably, and multivariable analysis showed that the hepcidin to ferritin ratio, a measure of adequacy of hepcidin levels relative to body iron stores, was inversely related to the SF3B1 mutation ($P=0.013$). These observations suggest that patients with SF3B1 mutation have inappropriately low hepcidin levels, which may explain their propensity to parenchymal iron loading.

Introduction

The majority of patients with myelodysplastic syndrome (MDS) present with anemia, and most of them become transfusion dependent during the clinical course of the disease.1,2 Transfusion iron overload is, therefore, a common complication in MDS, although its impact on clinical outcome varies considerably in different MDS subtypes.3 The redistribution of transfusion iron from reticuloendothelial to parenchymal cells is modulated by hepcidin, a peptide that interacts with ferroportin inhibiting the release of iron from macrophages.4 Hepcidin production is enhanced by iron and inflammation, suppressed by anemia and hypoxia, and negatively modulated by the erythroid marrow activity. In mouse models of beta thalassemia, ineffective erythropoiesis is typically associated with downregulation of hepcidin, upregulation of ferroportin in the duodenum, and increased iron absorption.5 Interestingly, increased levels of growth differentiation factor 15 (GDF15) have been found in thalassemia, and GDF15 has been shown to inhibit hepcidin production in primary human hepatocytes.6 Although the role of GDF15 as negative erythropoietic regulator of hepcidin is still debated, these observations suggest a potential link between ineffective erythropoiesis, release of bone morphogenetic proteins like GDF15, suppression of hepcidin production, and parenchymal iron loading in the so-called iron loading anemias. Various pathogenetic mechanisms are responsible for anemia in MDS patients.7 Ineffective erythropoiesis is mainly found in patients with low-risk disease, typically in refractory anemia with ring sideroblasts,8 whereas reduced proliferation of the erythroid marrow is observed in those with advanced disease and excess of blasts.7 Recently, we identified somatically acquired mutations in SF3B1, a gene encoding a core component of the RNA splicing machinery, in MDS patients with ring sideroblasts.9,10 This genotype-phenotype correlation was also shown in subjects having a proportion of ring sideroblasts below the diagnostic threshold of 15%.11 These observations strongly support a causal relationship between SF3B1 mutations and ring sideroblasts,9 and suggest a link between SF3B1 mutations and both ineffective erythropoiesis and parenchymal iron loading.8,11 To investigate this link, we studied the relationship between SF3B1 mutation status, erythroid marrow activity, hepcidin level, and body iron status in a cohort of MDS patients.

Design and Methods

These investigations were approved by the Ethics Committee of the “Fondazione Istituto di Ricovero e Cura a Carattere Scientifico” (IRCCS) Policlinico San Matteo, Pavia, Northern Italy. The procedures followed were in accordance with the Helsinki Declaration of 1975, as revised in 2000, and samples were obtained after subjects provided informed consent.

We studied 76 patients with MDS or myelodysplastic/myeloproliferative neoplasm (MDS/MPN) followed at the Department of Hematology, “Fondazione IRCCS Policlinico San Matteo”, Pavia, Italy.
northern Italy. This cohort belongs to the patient population of our recent study on clinical significance of \textit{SF3B1} mutations.\textsuperscript{10} According to the 2001 and 2008 World Health Organization (WHO) classification criteria,\textsuperscript{11,12} 26 patients had refractory anemia with ring sideroblasts (RARS) or refractory cytopenia with multilineage dysplasia (RCMD) and ring sideroblasts (RCMD-RS), 22 patients had refractory anemia (RA) with unilineage dysplasia or RCMD, 23 patients had refractory anemia with excess blasts (RAEB) type 1 (RAEB-1) or RAEB type 2 (RAEB-2), and 5 patients had RARS associated with marked thrombocytosis (RARS-T).

Clinical and hematologic features of the patient cohort are reported in Table 1. Risk assessment was based on the WHO-based prognostic scoring system (WPSS)\textsuperscript{13} accounting for severity of anemia.\textsuperscript{3}

Soluble transferrin receptor (sTfR), a measure of total erythroid activity,\textsuperscript{14} was quantified using an immunonephelometric method (Dade Behring Marburg GmbH, Marburg, Germany) on a BN II System analyzer. Serum erythropoietin (Epo) was assayed by a solid-phase chemiluminescent immunometric method (Immulite 2000 Analyzer, Siemens Medical Solution Diagnostics, Los Angeles, CA, USA). The quantification of serum GDF15 was performed with DuoSet ELISA for human GDF15 (R&D Systems, Abingdon, UK). Serum ferritin was assayed using a nephelometric method (NLatex Ferritin, Siemens Healthcare Diagnostics Products, Marburg, Germany) on a BN II System analyzer.

Serum hepcidin-25 measurements were performed by a combination of weak cation exchange chromatography and time-of-flight mass spectrometry (WCTX-TOF MS).\textsuperscript{15} Peptide spectra were generated on a Microflex LT matrix-enhanced laser desorption/ionization TOF MS platform (Bruker Daltonics). Serum hepcidin-25 concentrations were expressed as nmol/L (nM). The lower limit of detection of this method was 0.5 nM; average coefficients of variation were 2.7% (intra-run) and 6.5% (inter-run). The reference range of serum hepcidin-25 is from less than 0.5 to 14.7 nM (median 4.5 nM) for men, from less than 0.5 to 12.3 nM (median 2.0 nM) for premenopausal women, and from less than 0.5 to 15.6 nM (median 4.9 nM) for postmenopausal women.

Mononuclear cells were separated from peripheral blood or bone marrow samples by standard density gradient centrifugation. Granulocyte and T-lymphocytes were isolated from peripheral blood as previously described.\textsuperscript{16} Genomic DNA was extracted from granulocytes, T lymphocytes, or mononuclear bone marrow cells by following standard protocols for human tissue. The coding exons of \textit{SF3B1} were screened using massively parallel pyrosequencing of DNA pools using the genome sequencer FLX 454 system (Roche, Branford, CT, USA) as previously described in detail.\textsuperscript{10} Numerical variables are summarized by median and range; categorical variables are described with count and relative frequency (%) of subjects in each category. Comparison of numerical variables between groups was carried out using a non-parametric approach (Mann-Whitney test or Kruskall Wallis ANOVA). Correlations between quantitative variables were assessed using Spearman’s rank correlation. Independent predictors of variables of interest were assessed using multivariable generalized linear regression models. Statistical analyses were performed using Stata 11.2 software (StataCorp LP, College Station, TX, USA).

Results and Discussion

Somatic mutations of \textit{SF3B1} were found in 21 of 76 (28%) patients. The proportion of positive patients was significantly higher in WHO categories defined by ring sideroblasts (17 of 51 or 55%) than in other categories (4 of 45 or 9%) (P=0.001). The median value for \textit{SF3B1} mutant allele burden was 45% (range 7%-64%).

The relationship between \textit{SF3B1} mutant allele burden and erythroid activity is illustrated in Figure 1, while additional information is provided in the Online Supplementary Appendix. There was a significant relationship between \textit{SF3B1} mutation and sTfR level (P=0.003), and between \textit{SF3B1} mutant allele burden and sTfR (r=0.38, P=0.001). A significant association between \textit{SF3B1} mutation and GDF15 levels was observed, patients carrying a \textit{SF3B1} mutation having higher values than those without mutation (P=0.053). In addition, a positive correlation was found between \textit{SF3B1} mutant allele burden and GDF15 (r=0.28, P=0.016).

Variable hepcidin levels were found in the patients studied (median value 7.1 nM, range from less than 0.5 to 92.0 nM). There was a significant relationship between hepcidin and serum ferritin (r=0.59, P<0.001). A significant interac-
tion between \textit{SF3B1} mutation and serum ferritin was observed in a multivariable linear regression model on serum hepcidin level ($P=0.005$), suggesting a blunted hepcidin response to iron overload in \textit{SF3B1}-mutated patients (reflected in the lower slope of the gray regression line in Figure 2).

We then calculated the hepcidin to ferritin ratio, which represents a measure of adequacy of hepcidin levels relative to body iron stores. Significantly lower values of hepcidin to ferritin ratio were found in patients with \textit{SF3B1} mutation compared with those without mutation ($P=0.004$), and a significant relationship was observed between hepcidin to ferritin ratio and \textit{SF3B1} mutant allele burden ($r=-0.37$, $P=0.001$) (Figure 1). The relationship between hepcidin to ferritin ratio and measurements of erythroid activity is illustrated in Online Supplementary Figure S1.

Multivariable analyses were performed in order to identify the most important factors affecting hepcidin level and hepcidin to ferritin ratio in MDS patients (Online Supplementary Table S1). In a multivariable analysis including hemoglobin, Epo, sTR, GDF15, bone marrow blasts, cytogenetic risk groups and \textit{SF3B1} mutant allele burden, the hepcidin to ferritin ratio was independently associated with \textit{SF3B1} mutant allele burden ($P=0.013$).

The above findings indicate that MDS patients carrying a somatic mutation of \textit{SF3B1} not only have a distinct clinical phenotype characterized by ring sideroblasts, but show also evidence of ineffective erythropoiesis and relative hepcidin deficiency, i.e. typical features of iron loading anemias. In particular, patients with \textit{SF3B1} mutation had high proportions of bone marrow erythroblasts and high levels of sTR, both measures of total erythroid activity, and slightly increased levels of GDF15, a measure of ineffective erythropoiesis.

The \textit{SF3B1} mutant allele burden was independently associated with hepcidin levels and the hepcidin to ferritin ratio. This suggests that expanded but ineffective erythropoiesis may suppress hepcidin production in MDS patients carrying a mutation of \textit{SF3B1}, and lead to hepcidin levels that are inappropriately low relative to body iron stores.

Consistent with this observation, in a previous study on hepcidin levels in MDS, WHO category remained a significant predictor of hepcidin in multivariate analyses adjusted for ferritin and transfusion history, and the lowest levels were indeed found in RARS patients.

It should be noted, however, that in our study the \textit{SF3B1} mutation retained an effect on hepcidin level independently of erythroid marrow activity, suggesting that the mutant
Recent studies show that SF3B1 mutation is associated with downregulation of essential mitochondrial gene networks and more specifically of ABCB7, and that SF3B1 haploinsufficiency leads to formation of ring sideroblasts. It has been previously shown that iron sequestration into mitochondria results in cytosolic iron depletion and increased cellular iron uptake. Therefore, the possibility exists that abnormal mitochondrial iron homeostasis has an effect on hepcidin production.

In conclusion, our study suggests that MDS patients carrying a somatic mutation of SF3B1 have inappropriately low hepcidin levels which may cause excessive reticuloendothelial iron release and parenchymal iron loading. This may be relevant for decision-making concerning treatment of iron overload in MDS patients. Taking into account life expectancy, clinical consequences of transfusion iron overload, and adequacy of hepcidin production, transfusion-dependent patients with refractory anaemia with ring sideroblasts appear to be those more likely to develop deleterious consequences of parenchymal iron overload. These patients may, therefore, benefit from iron chelation therapy, a very controversial issue in the MDS community. Despite considerable skepticism, recent studies have shown improvements in hematologic and hepatic parameters in MDS patients receiving iron chelation.

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