Clinical significance of TFR2 and EPOR expression in bone marrow cells in myelodysplastic syndromes

UNIVERSITÀ DEGLI STUDI DI TORINO

Clinical Significance of TFR2 and EPOR Expression in Bone Marrow Cells in Myelodysplastic Syndromes.

Running title: TFR2 and EPOR expression in MDS

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Abstract

Myelodysplastic syndromes (MDS) are heterogeneous hematopoietic disorders characterized by bone marrow failure, cytopenias and a tendency to transform into acute myeloid leukemia. Anemia and transfusional need can be major problems for patients with MDS. Erythropoiesis stimulating agents (ESAs) therapy is a standard treatment for the anemia of most patients with MDS; however not all MDS patients respond to ESAs and a majority of patients eventually relapse after an initial response. This study aimed to identify the clinical impact of TFR2 and EPOR expression in bone marrow cells at diagnosis in MDS patients. We report the expression pattern of TFR2α and TFR2β in various subtype of MDS in comparison to that of EPOR. We provide evidence that TFR2 expression could be a potential molecular marker associated with response to erythropoietin (Epo) treatments in MDS patients and that lower TRF2 expression is associated with the poorest survival in MDS.

Myelodysplastic syndromes (MDS) are a group of clonal disorders characterized by ineffective bone marrow hematopoiesis, peripheral blood cytopenias and substantial risk for transformation into acute myeloid leukemia (Tefferi *et al*, 2009).

Although scoring systems are used to predict the prognosis of MDS (Greenberg *et al*, 1997; Greenberg *et al*, 2012), they provides information that unfortunately do not always coincide with clinical outcome. Percentage of blasts (>10%) and unfavorable cytogenetic abnormalities are the strongest predictors for poor outcome and are associated with high risk or disease progression to acute leukemia. Those patients, if possible, should undergo to allogenic stem cell transplantation or, if not eligible, to hypomethylating agents treatment.

In lower-risk MDS patients, the main clinical problem is chronic anemia. Anemia responds in 30–50% of the cases to erythropoiesis-stimulating agents (ESAs). Some prognostic factors of erythroid response to ESAs have been well identified, with better response rates in patients with no or limited red blood cell (RBC) transfusion requirement, low baseline serum EPO level and no-aberrant myeloid blast at flow cytometry (Park *et al*, 2008). For all these reason, the clinical care of MDS patients is still challenging, mainly due to MDS phenotypic heterogeneity and lack of well-established markers that effectively monitor MDS natural history. Therefore, in the lower-risk MDS subset, predicting at diagnosis patients with risk of treatment failure are pivotal to personalizing treatments in order to improve the quality of life and to prolong survival.

Transferrin receptor 2 (TFR2), homologous to TFR1, is a protein mutated in hemocromatosis type 3 and contributes to regulate hepcidin in the liver (Ramos *et al*, 2011). It is also expressed in erythroid cells (declining as the erythroid progenitors mature) and in myeloid malignant disorders (Kawabata *et al*, 2001). TFR2 associates with erythropoietin receptor (EPOR) (Forejtnikova *et al*, 2010) and is required for efficient erythropoiesis (Nai *et al*, 2015; Nai *et al*, 2014). Unlike TFR1, the TFR2 gene gives rise to two isoforms referred to as TFR2α (full-length) and a shorter TFR2β (Kawabata *et al*, 1999).

This retrospective study aimed to investigate whether TFR2 isoforms are differentially expressed in patients with MDS, and if so, whether TFR2 is associated with patient's clinical outcomes. Moreover, in the same cohort of patients, we focused on EPOR expression level since it was previously reported that TFR2 could act as an escort protein for this receptor, being required for EPOR efficient cell surface expression (Forejtnikova *et al*, 2010). Bone marrow (BM) aspirates were obtained from 6 individuals with non-

malignant hematological disorders (4 males, 2 females, median age of 57 years ranging from 45 to 73) and from 42 treatment-naive patients at the diagnosis of MDS. Diagnosis of MDS was made according to the World Health Organization (WHO) criteria (Vardiman *et al*, 2009). The patient group consisted in 28 male and 14 female with a median age of 71 years (range 49-85) and a WHO 2008 distribution as follows: RA n= 19, RARS n=2, RCMD n=6, RAEB-1 n=8, RAEB-2 n=7.

After informed consent and ethical approval (EMATO/TFR2-RE, code 92/2015) were obtained, RNA was extracted from total bone marrow cells collected at diagnosis and TFR2α, TFR2β and EPOR expression were quantified by quantitative Real-Time PCR, in comparison to that of RNA Universal (Stratagene), normalized for ABL1. Primer sequences were: a) 5'TTTCCACCAGGGCAGACTCT3', b) 5'TCCCGAAGGCTGGTTTG3' for TFR2α; c) 5'AGTCCCCACCTCTCCCCGCT3', d) 5'GTGTTGGGGTGAGCCGGATC3' for TFR2β; e) 5'GAGCGTACAGAGGGTGGAGA3', f) 5'AGGATGACCACGAGGATGAG3' for EPOR. The relative gene expression was calculated using the equation, $2^{-\Delta\Delta Ct}$. Statistical analyses were performed using GraphPad Prism software. Comparisons between groups were performed by means of Mann–Whitney U-test (nonparametric analysis), and P values <0.05 indicated a significant difference.

In MDS patients TFR2α and TFR2β showed higher variability in expression (TFR2α 6.08±5.58; TFR2β 3.15±1.16) than in non-malignant BM cells (TFR2α 8.23±2.97; TFR2β 3.51±0.47). Due to MDS morphological and clinical heterogeneity, we evaluated TFR2α and TFR2β expression in the different WHO subgroups (Figure 1A-B). Among the different MDS subtypes, the expression of TFR2α and TFR2β was significantly lower in RAEB2 (TFR2α 4.44±2.11; TFR2β 2.15±0.59) when compared with controls ($p<0.05$ and $p<0.005$, respectively). A similar expression level was also seen in RARS but the limited number of patients with this condition precludes any statistical analysis. TFR2α and TFR2β expression was not correlated with total white blood cells, neutrophils and platelets counts, age at diagnosis and no significant differences were observed between sex (not shown).

We next compared TFR2 expression with that of EPOR. Similarly to TFR2, EPOR expression in bone marrow cells varied more widely in MDS patients (18.00±2.90) than in non-malignant individuals (17.31±1.62) and was statistically lower in RAEB2 (8.18±0.99, $p<0.005$) (Figure 1C). In addition, conducting a Spearman's correlation analysis on the mRNA expression of both TRF2 isoforms and EPOR, we found that TFR2α and TFR2β expression is positively correlated with EPOR expression (Figure 1D-E).

To assess the clinical implication of TFR2 and EPOR expression in bone marrow cells, we categorized MDS patients into four expression sub-groups according to TFR2 and EPOR expression relative to the mean of the non-malignant BM and we analyzed the erythroid response in the cohort of patients that underwent to Epo treatment. We noticed that only patients with at least one of the TFR2 isoforms and EPOR levels comparable to normal controls reached an increase in hemoglobin level of ≥ 1.5 g/dl after 12 weeks of treatment. Instead, all non-responders, with the exception of 2 patients high-expressing TFR2/EPOR, had low levels of TFR2 or EPOR mRNA (Figure 2A).

We finally tested the effects of TFR2α, TFR2β and EPOR expression on survival in RAEB1-2 and RCMD (Figure 2B-C-D). Comparisons between Kaplan-Meier curves were carried out by log-rank test. In the first year of follow-up, patients with very low/low TFR2α or TFR2β expression levels had a significantly worse overall survival (OS) than those with normal/high TFR2 expression ($p<0.05$ and $p<0.01$ respectively). No significant difference was noted between patients with low or normal/high EPOR expression, although there was a tendency for poorer survival in the very low/low EPOR expression group.

Ever since it was demonstrated that TFR2 is a component of EPOR complex (Forejtnikova *et al*, 2010), there has been interest on understanding its extra-hepatic function. The TFR2 erythroid function has been recently described in normal erythropoiesis in mouse models lacking systemic or hematopoietic TFR2 (Nai *et al*, 2015; Nai *et al*, 2014). Considering the natural history of the disease, MDS are suitable models for studying how TFR2 expression change in clonal hematologic disorders and how it is related to EPOR. Moreover, as iron overload can have an adverse effect on hematopoietic precursors, knowing how the expression of an iron-related gene is modulated is important to understand how iron metabolism and hematopoiesis interact. We showed that TFR2α, TFR2β and EPOR have a much more variable pattern of expression in MDS compared to normal and that, differently compared to what previously reported (Kawabata *et al*, 2001), their expression are significantly lower in high risk MDS like RAEB2. Therefore, the lack of TFR2 could impair the entire myeloid lineage differentiation, as it does in human erythroid progenitors delaying erythroid terminal differentiation (Forejtnikova *et al*, 2010).

The data presented here demonstrate that the reduction of TFR2 isoforms mRNA is associated with poorer survival in patients with high grade MDS. This is in agreement with the work of Nakamaki et al. (Nakamaki *et al*, 2004) that found that *de-novo* acute myeloid leukemia patients with high levels of both TFR2 isoforms survived significantly longer.

Furthermore, we established a positive correlation between TFR2 and EPOR mRNA expression implying a possible co-regulation or interplay at transcriptional level of these two interacting proteins. Finally, we reported that TFR2 and EPOR expression could provide information on Epo treatment response since this was achieved only in individuals with both TFR2 and EPOR mRNA levels similar to normal. High level of expression seems to be also deleterious, probably due to an altered receptor signalling.

Limitations of our study include the relatively small number of patients and hence limited amount of cases used for the treatment-response analysis. Nevertheless, the level of bone marrow TFR2 mRNA seems to offer additional information on predicting Epo treatment response. However, before considering TFR2 and EPOR expression levels in clinical decision making, additional validation studies, are needed, also to define optimal cut-off levels.

In conclusion, our data provide evidence suggesting that TFR2 and EPOR expression could be used as potential molecular markers for a better management of MDS patients clinical course. To our knowledge, this is the first study to analyze TFR2 clinical value in MDS patients.

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FIGURES LEGENDS.

Figure 1. TFR2 and EPOR gene expression in newly diagnosed MDS BM samples.
(A-C) Real time analysis of TFR2α, TFR2β and EPOR transcripts in subtypes of MDS according to WHO classification, compared to non-malignant BM samples.
**p-value<0.01. The lines indicate mean value of each group.
(D-E) Spearman's correlation analysis on the mRNA expression of TFR2α, TFR2β and EPOR in our cohort of MDS patients. R and p values are indicated. Color and shape code of each individual point in the graphs are the same reported in figure 1 A-C.

Figure 2. Potential value of TFR2 in the clinical diagnostic application of MDS patients.
(A) Clinical and laboratory parameters of myelodysplastic patients treated with erythropoietin. WHO = World Health Organization classification of myelodysplastic syndromes, Dosage = erythropoietin dosage, RA = refractory anemia, RCMD = refractory cytopenia with multilineage dysplasia, RAEB-1 = refractory anemia with excess of blasts type 1, RAEB-2 = refractory anemia with excess of blasts type 2, RARS = refractory anemia with ringed sideroblasts, Hb = hemoglobin, ΔHb = hemoglobin variation after 12 weeks of treatment. Patients that responded to Epo treatment are highlighted in red. MDS patients were categorized into four expression sub-groups according to TFR2 and EPOR expression relative to the mean of the non-malignant BM cells, as follows: "very low" group when TFR2 and EPOR levels were less than minus two standard deviations (SD) from the mean of non-malignant BM; "low" between minus two and minus one SD; "normal" between minus and plus one SD; "high" more than one SD. (B-C-D) Kaplan-Meier survival curves for MDS RAEB1-2/RCMD patients based on TFR2α, TFR2β or EPOR expression level. *p-value<0.05 analyzed by log-rank test.

Figure 1.

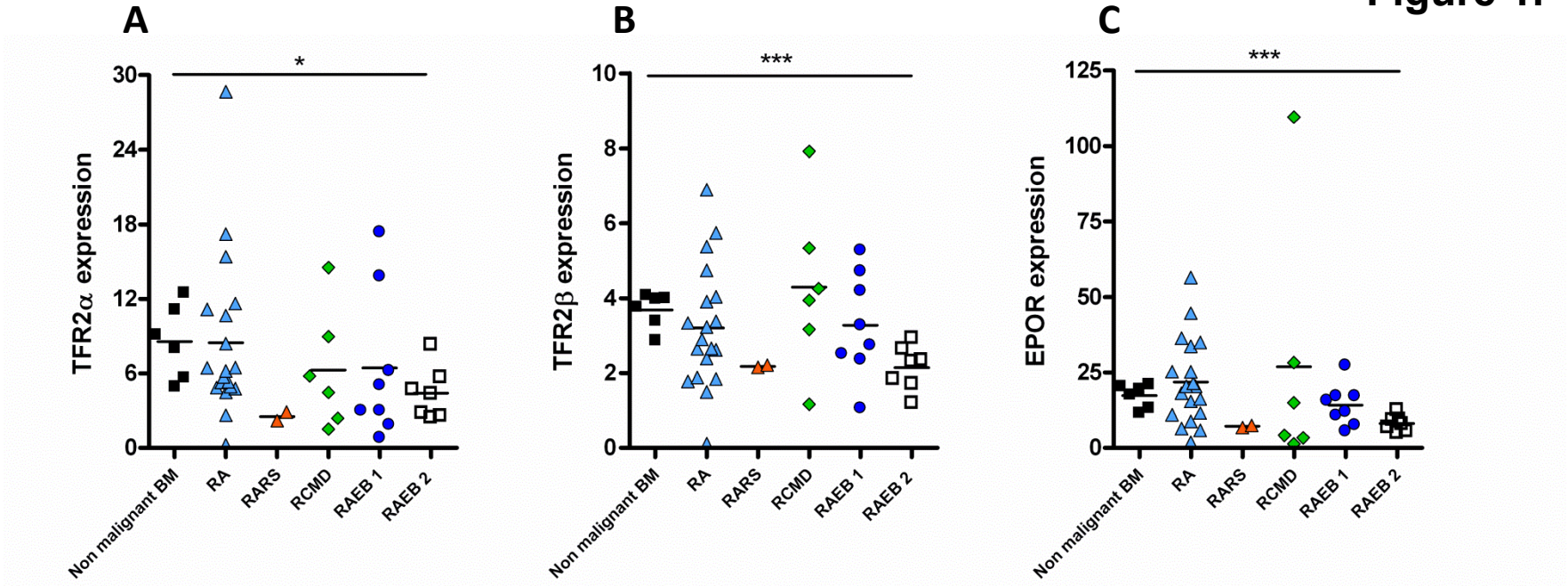
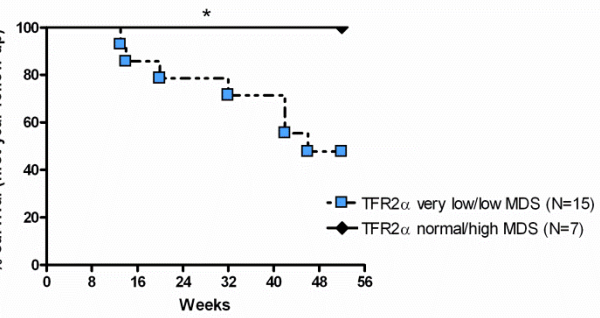

A
B
C
*
***
***
TFR2α expression
TFR2β expression
EPOR expression
Non malignant BM
RA
RARS
RCMD
RAEB 1
RAEB 2
D
E
Spearman R= 0.72
p<0.0001
Spearman R= 0.52
p<0.005
EPOR expression
(relative to mean of non-malignant BM)
TFR2α expression
(relative to mean of non-malignant BM)
TFR2β expression
(relative to mean of non-malignant BM)

Figure 2.

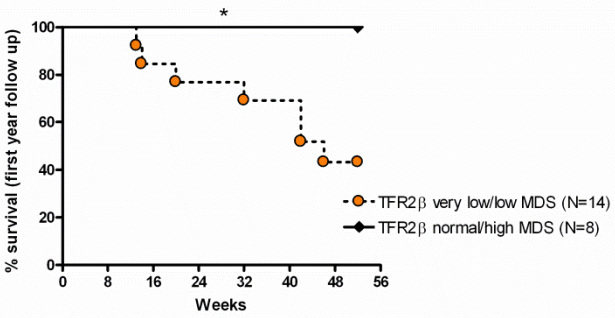
A

Case #	WHO	Dosage	Transfusion need	Hb (g/dL)	ΔHb (g/dL)	TFR2α	TFR2β	EPOR
1	RA	40000 U/week	no	10,5 -> 12,4	**1,9**	low	normal	normal
6	RA	40000 U/week	no	9,3 -> 9,9	0,6	high	high	high
12	RA	40000 U/week	no	8,8 -> 9,2	0,4	very low	very low	very low
13	RARS	40000 U/week	3RCB units/month	8,2 -> 8,8	0,6	low	very low	very low
17	RA	40000 U/week	no	9,3 -> 12,4	**3,1**	normal	very low	normal
25	RCMD	40000 U/week	no	9,7 -> 10	0,3	very low	very low	very low
41	RA	40000 U/week	no	9,2 -> 10,5	1,3	high	high	high
2	RAEB 1	80000 U/week	no	9,7 -> 10,6	0,9	low	very low	low
10	RAEB 1	80000 U/week	3RCB units/month	6,8-> 6,7	-0,1	very low	low	very low
11	RA	80000 U/week	3RCB units/month	7,5 -> 8,1	0,6	low	normal	low
14	RAEB 1	80000 U/week	4RCB units/month	7,3 -> 6,8	-0,5	very low	very low	very low
26	RCMD	80000 U/week	no	9,1 -> 13,3	**4,2**	normal	high	normal
29	RAEB 2	80000 U/week	2RCB units/month	7,9 -> 8,3	0,4	very low	very low	very low
34	RA	80000 U/week	no	8,3 -> 11,2	**2,9**	normal	high	normal
37	RCMD	80000 U/week	4 RCB units/month	7-> 7,2	0,2	very low	normal	very low
39	RAEB 1	80000 U/week	no	10,1 -> 12,2	**2,1**	high	normal	normal
40	RA	80000 U/week	2 RCB units/month	8,5-> 7	-1,5	normal	normal	low

B



C



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