Transcription factor 4 (*TCF4*) expression predicts clinical outcome in *RUNX1* mutated and translocated acute myeloid leukemia

Transcription factors play an essential role in hematopoiesis. Aberrations in transcription factors may contribute to the development and maintenance of leukemia. Runt related transcription factor 1 (RUNX1, a.k.a. AML1, CBFα2 or PEBP2αB) is a transcription factor which belongs to the core-binding factor (CBF) family. RUNX1 directly contacts DNA, but the binding affinity for DNA significantly increases after dimerizing with its cofactor, core binding factor-β (CBFβ). RUNX1 plays a key role in definitive hematopoiesis, and disruption of RUNX1 contributes to malignant transformation.[1] In acute myeloid leukemia (AML), *RUNX1* mutations (*RUNX1*^{mut}) are frequently found and are associated with a poor prognosis.[1-3] Furthermore, more than a dozen different chromosomal translocations have been described that involve either *RUNX1* or its partner *CBFβ*. Of these, the most common translocations are the t(8;21)(q22;q22), leading to a fusion protein *RUNX1-RUNX1T1*, and the inv(16)(p13;q22)) leading to a CBF-MYH11 fusion protein. These aberrations are found in approximately 12-15% and 8-10% of adult AML respectively.[1] Interestingly, CBF translocations are associated with a favorable prognosis.[1] For almost a decade, t(8;21) (*RUNX1-RUNX1T1*) and inv(16) (*CBFβ-MYH11*) have been incorporated as a separate AML entity with favorable prognosis in the World Health Organization (WHO) classification of myeloid neoplasms and acute leukemia. In addition, in the European Leukemia Net (ELN) recommendations of diagnosis and management of AML they are incorporated as important markers of good prognosis. In the recent WHO classification update in 2016, AML with a *RUNX1*^{mut} has been added as a provisional entity and this category of AML has also been classified in the ELN recommendations as a poor prognostic group. The exact working mechanism via which RUNX1^{mut} operates has not yet been elucidated. Previously, we found that the expression level of transcription factor 4 (*TCF4*, also known as *E2-2* and *ITF2*) is an independent prognostic factor in AML, after correction for ELN risk group (2010), age and white blood cell count. Patients with high *TCF4* mRNA expression (highest quartile) have a worse survival and benefit more from a more aggressive treatment approach compared to patients with low *TCF4* expression.[4] In accordance, *TCF4* expression significantly contributes to expression signatures linked with poor AML outcome.[5] TCF4 is implicated in erythroid-megakaryocytic differentiation, B- and T-cell development and is crucial for plasmacytoid DC (pDC) development.[6,7] In blastic plasmacytoid dendritic cell neoplasms (BPDCN) TCF4 has been shown to be the master regulator of the oncogenic program.[7] A role for TCF4 in malignant hematopoiesis is further indicated by

Table 1. Multivariate Cox regression analysis.

A		Excluding TCF4				Including TCF4	
OS; Variable	Wald	HR (95% CI)	*P*	df	Wald	HR (95% CI)	*P*
TCF4 highest 25%				1	8.86	1.61 (1.18 - 2.21)	0.003
RUNX1 mutation	6.29	1.77 (1.13 - 2.76)	0.012	1	2.30	1.43 (0.90 - 2.28)	0.129
WBC >100x10^9/L	13.17	2.02 (1.38 - 2.95)	<0.001	1	12.10	1.96 (1.34 - 2.87)	0.001
B		**Excluding TCF4**				**Including TCF4**	
OS; Variable	**Wald**	**HR (95% CI)**	***P***	**df**	**Wald**	**HR (95% CI)**	***P***
TCF4 highest 25%				1	9.09	1.49 (1.15 - 1.93)	0.003
t(8;21) present	4.13	0.57 (0.33 - 0.98)	0.042	1	2.75	0.63 (0.37 - 1.09)	0.098
WBC >100x0^9/L	11.06	1.81 (1.28 - 2.58)	0.001	1	10.83	1.80 (1.27 - 2.56)	0.001
C		**Excluding TCF4**				**Including TCF4**	
OS; Variable	**Wald**	**HR (95% CI)**	***P***	**df**	**Wald**	**HR (95% CI)**	***P***
TCF4 highest 25%				1	9.05	1.49 (1.15 - 1.92)	0.003
inv(16) present	14.06	0.40 (0.25 - 0.65)	<0.001	1	12.85	0.42 (0.26 - 0.67)	<0.001
WBC >100x10^9/L	11.20	1.81 (1.28 - 2.59)	0.001	1	10.71	1.80 (1.27 - 2.55)	0.001

(A) Overall survival (OS); left *RUNX1* status and white blood cell count (WBC) included in the model; and right TCF4 expression, *RUNX1* status and WBC included in the model. (B) OS; left t(8;21) and WBC included in the model; and right *TCF4* expression, t(8;21) and WBC included in the model. (C) OS; left inv(16) and WBC included in the model; and right *TCF4* expression, inv(16) and WBC included in the model.

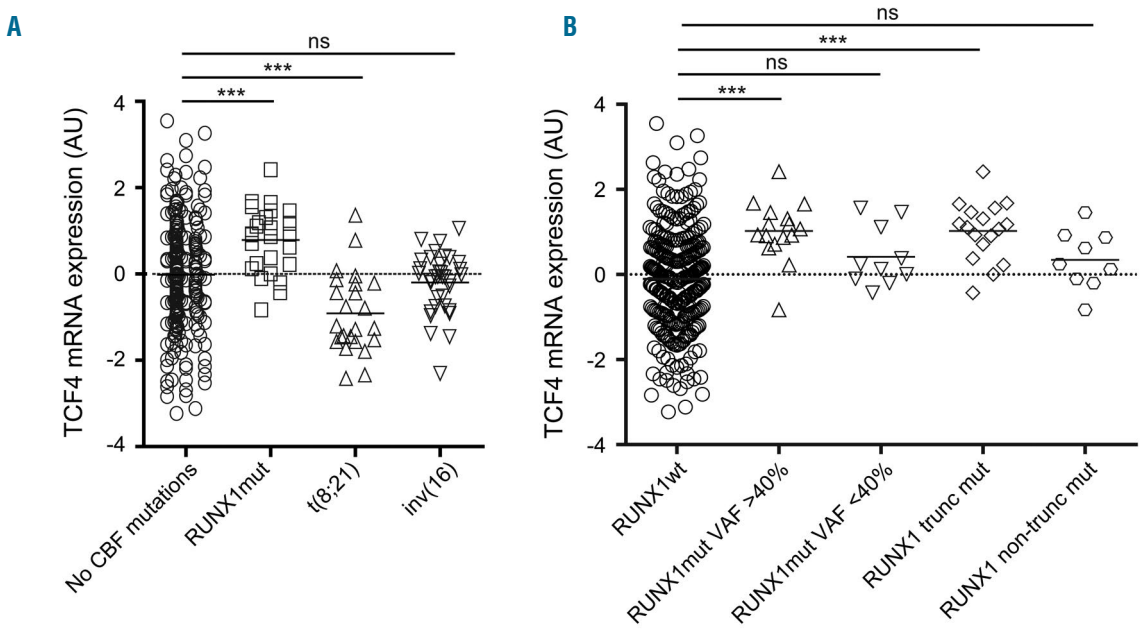

Figure 1. *TCF4* expression in acute myeloid leukemia (AML) patients with (A) no core binding factor (CBF) mutations (n=245), *RUNX1* mutations (n=26), t(8;21) (n=24) and inv(16) (n=35). (B) *RUNX1* wild-type (n=306), *RUNX1*^{mut} variant allele frequency (VAF) ≥40 (n=16), *RUNX1*^{mut} VAF <40% (n=10), truncated *RUNX1*^{mut} (n=17), non-truncated *RUNX1*^{mut} (n=9). AU: arbitrary unit; ***: *P*<0.0001; ns: not significant.

the finding that mutations in *TCF4* are found in AML and myelodysplastic syndromes (MDS), albeit at low frequencies.[4] Furthermore, high *TCF4* expression is associated with self-renewal properties[8] and is downregulated during differentiation unless progenitors obtain transformed properties.[8-10]

We analyzed the *TCF4* expression in an independent cohort of 436 AML patients, of which 330 cases with a known *RUNX1* status. Patients with a *RUNX1*^{mut} (n=26 [7.9%]), had a significantly higher *TCF4* expression while t(8;21) patients had a significantly lower *TCF4* expression compared to patients without a *CBF* aberration (Figure 1A; mean 0.79 *vs.* -0.02, *P*<0.001 and -0.91 *vs.* -0.02, *P*<0.001, respectively). Patients with an inv(16) (affecting the *CBFβ* gene, which serves as a dimerization partner for RUNX1), had a *TCF4* expression comparable to patients without a CBF aberration (Figure 1A, mean -0.20 *vs.* -0.02, *P*=0.374). Patients with *RUNX1*^{mut} with a variant allele frequency (VAF) ≥40% (n=16) showed a higher *TCF4* expression compared to patients with a *RUNX1* mutation with a VAF <40% (n=10; Figure 1B; mean 1.02 *vs.* 0.42, *P*=0.061). Furthermore, *RUNX1*^{mut} mutations that were more likely to alter protein function (truncating mutations (n=17)) showed a significantly higher impact on *TCF4* expression than non-truncated *RUNX1*^{mut} mutations (n=9; Figure 1B; mean 1.02 *vs.* 0.35, *P*=0.024). To corroborate these findings, we analyzed the publicly available Vizome dataset (*http://www.vizome.org/ aml/*). In 484 AML patients, there were significantly more *RUNX1*^{mut} patients in the high TCF4 (highest 25%) expressing group compared to the low *TCF4* expressing group (lowest 25%) (27.3% *vs.* 2.5%, *P*<0.0001). Furthermore, others have shown a correlation between *RUNX1* mutations and *TCF4* expression.[2,3,11]

In line with previous work,[4] in univariate analyses, patients in the present cohort with a high *TCF4* expression (highest 25%) showed an inferior overall survival (OS) and event free survival (EFS) compared to patients with a low *TCF4* expression (lowest 75%) (Figure 2A, *Online Supplementary Figure S1A* and *Online Supplementary Table S1*; 5-year OS 23% *vs.* 38%, *P*=0.001; 5-year EFS 13% *vs.* 31%, *P*<0.0001). As expected, the presence of a *RUNX1*^{mut} mutation correlated with inferior survival, whereas a t(8;21) or inv(16) correlated with better survival (Figure 2B-D, *Online Supplementary Figure S1B-D* and *Online Supplementary Table S1*; 5-year OS 14% *vs.* 38%, *P*=0.014, 5-year OS 49% *vs.* 33%, *P*=0.035; 5-year OS 63% *vs.* 31%, *P*<0.0001; respectively). In addition, we analyzed *TCF4* expression and *RUNX1* status is the context of well-known prognostic factors, including white blood cell count (WBC), cytogenetics, age and presence of *CEBPα*, *NPM1* or *FLT3*-ITD mutations in a multivariate Cox regression model. Also here we found that *TCF4* expression is an independent prognostic factor, either divided in highest 25% and 75% lowest expression or as continues variable, in and out of the context of *RUNX1* status (*Online Supplementary Table S2A-D*). We did not consider t(8;21) in this context, since it has already been incorporated in the cytogenetic risk groups. Therefore, we performed another multivariate analyses merely to test if the predictive effect of *RUNX1*^{mut} or t(8;21) is dependent on *TCF4* expression, since both these *RUNX1* aberrations and *TCF4* expression are correlated and have an impact on univariate survival. A multivariate Cox regression analysis including WBC, *RUNX1* mutational status and/or *TCF4* expression (highest quartile/lowest 75%) was performed. Cytogenetics, molecular markers and age were not included in this multivariate model, because of their correlation with *TCF4* expression or *RUNX1* status (*Online Supplementary Table S3A*). When *TCF4* expression was not taken into account, *RUNX1* status and WBC were both independent prognostic variables for OS (Table 1A; *RUNX1*^{mut} hazard ratio [HR] 1.77, 95% confidence interval (CI): 1.13-2.76, *P*=0.012) and

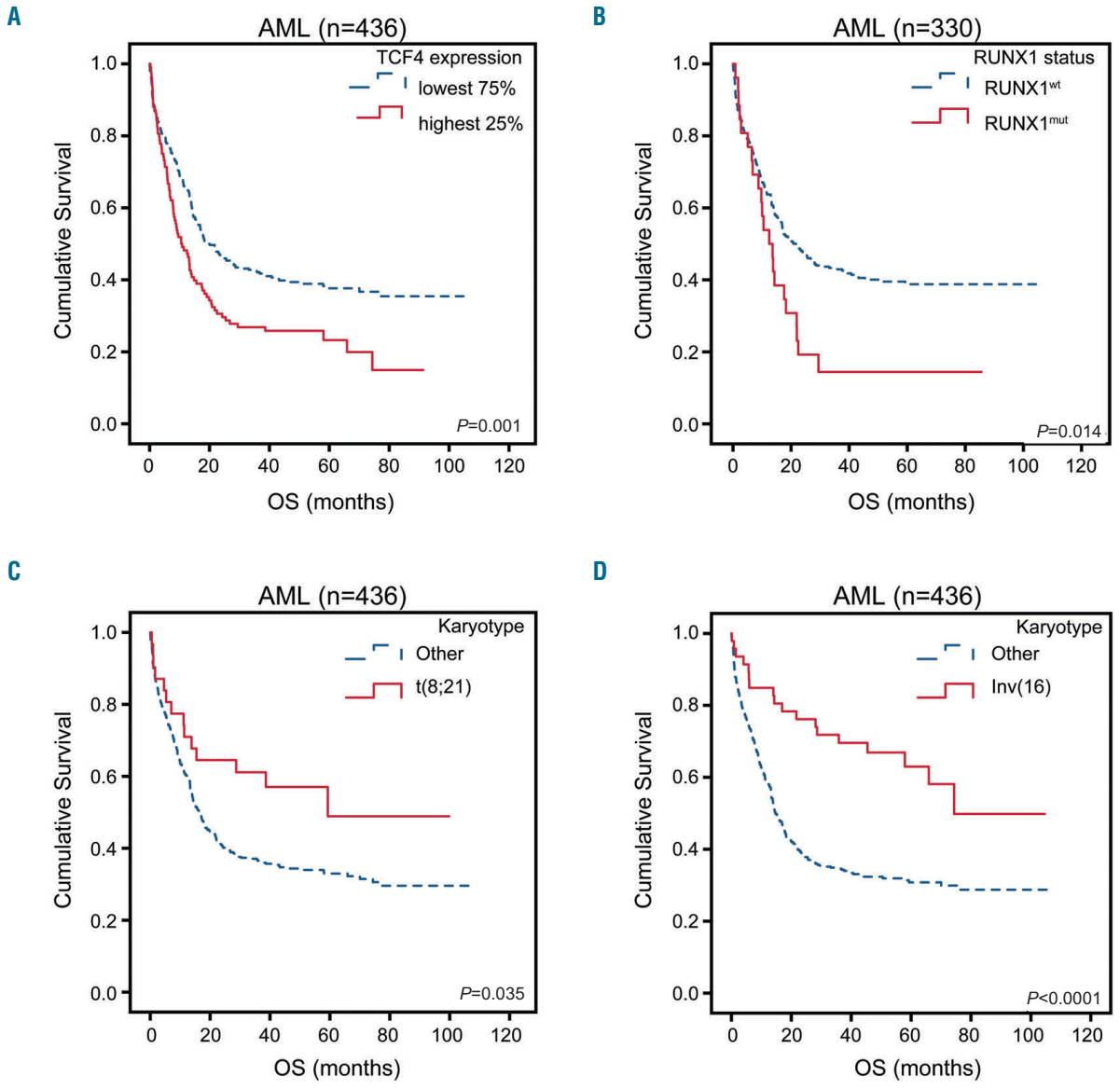

Figure 2. Overall survival (OS) curves for acute myeloid leukemia patients with available data stratified on (A) *TCF4* expression, lowest 75% (n=324), highest 25% (n=108); (B) *RUNX1* mutational status, *RUNX1* wild-type (n=304), *RUNX1* mutation (n=26); (C) presence (n=31) or absence of t(8;21) (n=405); (D) presence (n=47) or absence of inv(16) (n=389).

EFS (*Online Supplementary Table S3B*). However, when *TCF4* expression was added to this model, WBC and *TCF4* expression showed a significant effect on OS, while the *RUNX1* mutational status lost its significance and had a diminished HR of more than 10% (indicating there is a direct correlation between these variables) in both OS (Table 1A; *RUNX1*^{mut} HR 1.43, 95% CI: 0.90-2.28, *P*=0.129) and EFS (*Online Supplementary Table S3B*). This indicates that the significance of *RUNX1*^{mut} is dependent on *TCF4* expression. In contrast, when adding *RUNX1*^{mut} to an equation with *TCF4* expression and WBC, the addition of *RUNX1* status had no noteworthy impact on the significance or HR of *TCF4* expression (*Online Supplementary Table S4A-B*). Analyzing *RUNX1* translocation in the multivariate model showed that also the prognostic impact of t(8;21) diminished strongly when *TCF4* expression was included (Table 1B and *Online Supplementary Table S3C*), however less pronounced than *RUNX1*^{mut}. Again, the addition of t(8;21) had no noteworthy impact on the significance or HR of *TCF4* expression (*Online Supplementary Table S4C-D*). In contrast, the effect of the inv(16) remained significant on OS and EFS independently of the presence of *TCF4* expression (Table 1C, *Online Supplementary Table S3D* and *Online Supplementary Table S4E-F*). This is in accordance with the independency of inv(16) for *TCF4* expression.

Aberrations of *RUNX1* are widely used in the clinic for the biological and prognostic classification of AML. The pathomechanisms explaining the different outcome of the different *RUNX1* aberrations, however, is not yet elucidated. We and others show that *TCF4* is highly expressed in *RUNX1*^{mut} AML,[2,3,11] while there is a low expression in t(8;21) AML patients.[4,12] This corresponds

with the poor prognosis of high *TCF4* (highest 25%) expression and *RUNX1*^{mut} and the favorable prognosis of low *TCF4* expression and t(8;21) AML patients.[4] Interestingly, we show in a multivariate analysis that *TCF4* expression prevails over *RUNX1* status in predicting outcome in AML. Possibly, RUNX1, being a transcription factor, has a direct effect on the *TCF4* promoter, which is different between RUNX1 wild-type, *RUNX1*^{mut} and RUNX1-RUNX1T1. In primary AML, RUNX1 has been shown to bind to the *TCF4* promoter in *RUNX1* wild-type and *RUNX1*^{mut} cells, however there was no AML-ETO binding on the *TCF4* promoter in AML-ETO primary cells (*Online Supplementary Figure S2*).[13] In contrast, in Kasumi cells, *RUNX1* did bind to the *TCF4* promoter.[14] TCF4 has been described as one of the most dominant factors involved in self-renewal.[8-10,15] In granulocyte-monocyte progenitors (GMP), transduced with various other oncogenes, *TCF4* was up-regulated and proposed to be part of the 'leukemia initiation signature.[8] In addition, RUNX1 is not the only factor influencing *TCF4* expression, since only 5-10% *RUNX1* mutations are found in AML patients, while 25% of AML patients have a high *TCF4* expression. For example, in AML and mouse models with translocations of the mixed lineage leukemia gene (*MLL*) and its most common partner AF9 (*MLL-AF9*), *TCF4* was identified as a direct transcriptional target of MLL-AF9.[15] In MLL-AF9 positive cells, *TCF4* was strongly up-regulated[9,10,15] and identified to be part of the "self-renewing signature".[9] Moreover, in MLL-rearranged AML cells, down-regulation of *TCF4* or upregulation of its natural inhibitor, prolonged the survival in transplantation experiments.[15] The TCF4-induced enhanced stemness may contribute to the poor performance of AML patients with high *TCF4* expression. Various oncogenes like *BCL2, MYC, TCL1A* and *TCL1B* have been reported as downstream targets of TCF4.[7] To what extent these downstream targets are moderators in survival and the effects of *RUNX1* mutations remains to be studied.

In conclusion, we found a correlation between *TCF4* expression and *RUNX1* aberrations, *TCF4* being upregulated in *RUNX1*^{mut}, and downregulated in *RUNX1* translocated AML (t(8;21)). *TCF4* expression appeared to mediate the prognostic outcome of *RUNX1* aberrations. This indicates that TCF4 might be an important downstream target of RUNX1. Studies to identify the biological mechanism are warranted.

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