High expression of transcription factor 4 (*TCF4*) is an independent adverse prognostic factor in acute myeloid leukemia that could guide treatment decisions

Mutations in transcription factor 4 (*TCF4*) have recently been described in myeloid dysplastic syndromes (MDS) and acute myeloid leukemia (AML). We analyzed the impact of *TCF4* mRNA expression on clinical outcome in AML patients (n=525). Patients with high *TCF4* expression (*TCF4*^{high}, defined as the 25% highest *TCF4* expressors) had a significantly worse overall survival (OS) and event-free survival (EFS) than patients with lower *TCF4* expression (*TCF4*^{low}) (5-year OS 18% *vs.* 44%, *P*<0.0001; 5-year EFS 15% *vs.* 34%, *P*<0.0001, respectively). This was confirmed in an independent cohort (n=436). Multivariate analysis showed that *TCF4*^{high} is an independent prognostic factor for OS and EFS in the whole cohort and in patients carrying a normal karyotype.

Importantly, *TCF4*^{high} patients benefited most from an allogeneic hematopoietic cell transplantation (HCT), compared to an autologous HCT or additional chemotherapy (CT) (5-year OS 39%, 8%, 10%, *P*<0.0001; 5-year EFS 31%, 0%, 10%, *P*=0.001, respectively), while *TCF4*^{low} patients seemed to benefit most from an autologous HCT, compared to allogeneic HCT or additional CT (5-year OS: 61%, 45%, 39% *P*=0.002; 5-year EFS: 42%, 32%, 34%, *P*=0.102, respectively).

We demonstrate that high expression of *TCF4* is an independent adverse prognostic factor in AML that could guide treatment decisions.

TCF4 plays a role in a variety of developmental processes, including hematopoiesis. TCF4 is part of the basic helix-loop-helix (bHLH) class 1 family, also called E-proteins. These E-proteins recognize an E-box DNA binding site (CANNTG), which are present in a variety of tissue-specific enhancers.[1,2] Recently, Papaemmanuil and colleagues reported mutations in *TCF4* in MDS patients.[3] A total of 9 mutations were found in 7 of the 738 (0.9%) sequenced MDS patients. The *TCF4* mutations were found in various MDS subclasses. Mutations in *TCF4* have also been reported for AML cases (0.5%)[4] and were associated with a poor prognosis,[5] suggesting a potential role of TCF4 in the pathogenesis of these myeloid malignancies. Here we report that *TCF4* mRNA expression levels are an independent prognostic factor in AML patients.

TCF4 expression values measured using Affymetrix HGU133 plus 2.0 arrays were derived from a database which contains a cohort of 525 AML patients treated according to HOVON protocols (AML -04, -04A, -29, -32, -42, -43; available at *http://www.hovon.nl*).[6] Both bone marrow aspirates or peripheral-blood samples (at the time of diagnosis) have been analyzed. Blasts and mononuclear cells were purified by Ficoll–Hypaque (Nygaard) centrifugation and cryopreserved. The AML samples contained 80-100% blast cells after thawing, regardless of the blast count at diagnosis. To determine the *TCF4* expression, an average of 5 probe sets (which bind at different locations of the gene) were used. The microarray expression data were confirmed by qPCR (*Online Supplementary Figure S1*). In addition, the *TCF4* expression levels of healthy CD34^{+} control cells (hCD34^{+}; n=11) and mononuclear cell fractions derived from normal bone marrow (NBM; n=5) were available. A second, independent cohort of 436 AML patients was used for validation.[7] Patients were divided into genetic risk groups according to the European LeukemiaNet (ELN) guidelines.[8]

In the studied cohort of 525 AML patients, *TCF4* is differentially expressed in AML blasts compared to NBM and hCD34^{+} (Figure 1A). To study the impact of *TCF4* expression levels on survival, the cohort was divided on the basis of differences in expression levels; expression below or above the median, tertiles, quartiles, quintiles, sixtiles and septiles. In all these cohorts, univariate analysis showed that high expression of *TCF4* was associated with poor outcome. The highest expressors of *TCF4* showed a more than 2-fold shorter 5-year OS than the lowest expressors (*Online Supplementary Figure S2*). Since we found that *TCF4* expression is not normally distributed and because approximately 25% of the patients showed a much higher expression (Figure 1B), a distribution of the cohort based on the highest 25% (*TCF4*^{high}) and the lowest 75% *TCF4* expression (*TCF4*^{low}) was used for further analysis. Characteristics of the patients in the *TCF4*^{low} and *TCF4*^{high} groups are described in *Online Supplementary Table S1*. *TCF4*^{high} patients more often had high-risk cytogenetic abnormalities (*P*<0.0001), FLT3-ITD (*P*<0.0001) and their morphology more frequently corresponded with M0 or M1 FAB-subgroups (*P*<0.0001). *TCF4*^{low} patients were more likely to have biallelic *CEBPA* mutations (*P*=0.011). No associations between *TCF4* expression and age, sex, white blood cell (WBC) count, or other cytogenetic or molecular abnormalities could be identified.

Survival analysis according to the Kaplan-Meier method showed that *TCF4*^{high} patients had a worse survival than patients classified as *TCF4*^{low} (5-year OS 18% *vs.* 44%, *P*<0.0001; 5-year EFS 15% *vs.* 34%, *P*<0.0001, respectively) (Figure 1C and D). We confirmed the impact of *TCF4*

Table 1. Multivariate Cox's regression survival analysis. Factors predicting overall survival and event-free survival in acute myeloid leukemia patients of the first cohort with available complete data of all cytogenetic and molecular parameters (n=506).

	Overall survival (n=506)				Event-free survival (n=506)			
Variable	χ^2 (Wald)	DF	*P*	HR (95% CI)	χ^2 (Wald)	DF	*P*	HR (95% CI)
Favorable ELN risk group[8]	40.11	3	<0.0001		36.75	3	<0.0001	
Intermediate-I ELN risk group	16.55	1	<0.0001	1.92 (1.40 - 2.63)	13.12	1	<0.0001	1.72 (1.28 - 2.30)
Intermediate-II ELN risk group	9.36	1	0.002	1.65 (1.20 - 2.28)	9.05	1	0.003	1.58 (1.17 - 2.12)
Adverse ELN risk group	39.36	1	<0.0001	3.01 (2.13 - 4.24)	36.49	1	<0.0001	2.72 (1.97 - 3.76)
Age (>60 years)	18.06	1	<0.0001	1.81 (1.41 - 2.52)	9.82	1	0.002	1.57 (1.18 - 2.08)
WBC (>100 *10^{9})	11.02	1	0.001	1.59 (1.21 - 2.09)	14.78	1	<0.0001	1.66 (1.28 - 2.15)
TCF4^{high} expression	16.07	1	<0.0001	1.65 (1.29 - 2.11)	14.86	1	<0.0001	1.59 (1.26 - 2.02)

OS: overall survival; EFS: event-free survival; ELN: European LeukemiaNet[8]; DF: degrees of freedom; HR: Hazard Ratio; CI: Confidence Interval; WBC: white blood cell count.

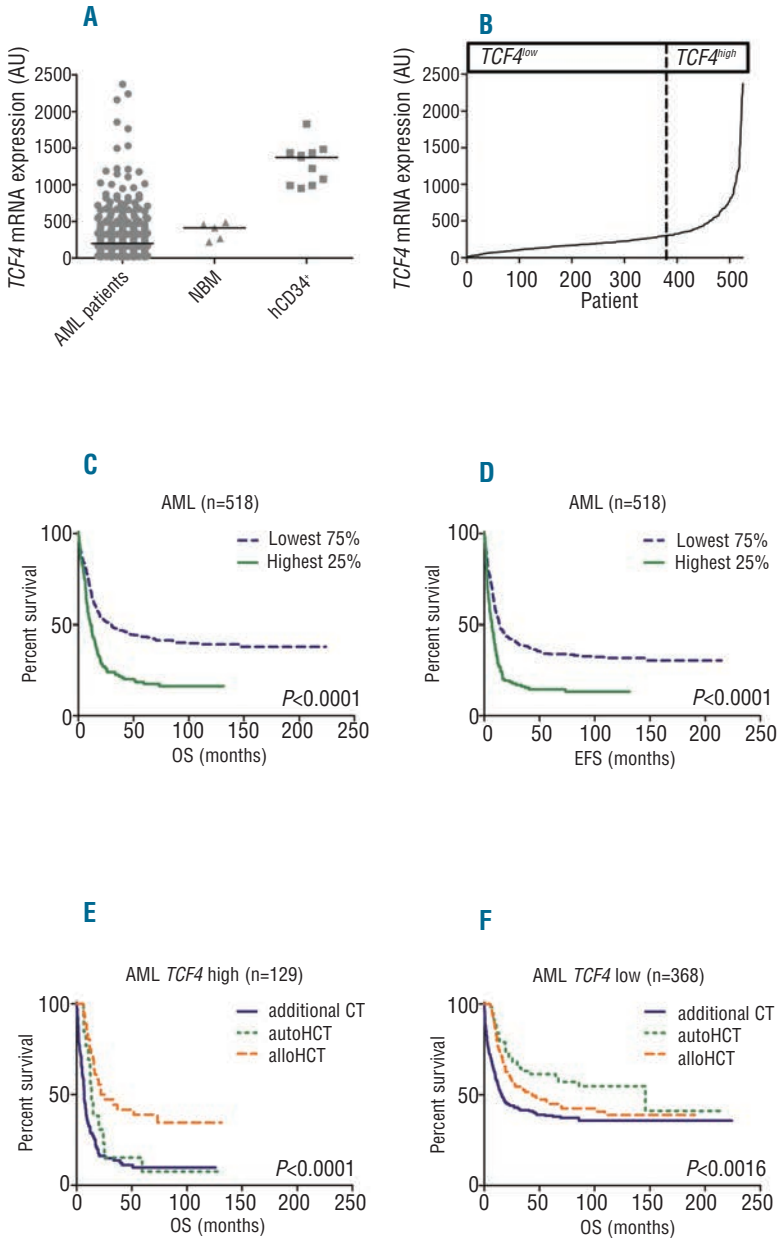

Figure 1. ***TCF4*** **expression and survival curves in the first cohort. (A) Expression of *TCF4* in AML patients (n=525), NBM (n=5) and hCD34^{+} (n=11). (B) *TCF4* expression ranked from lowest to highest expression (n=525). (C) Overall survival (OS) curves for AML patients with available follow-up data (n=518) stratified by *TCF4^{high}* (n=129) and *TCF4^{low}* (n=389). (D) Same for event-free survival (EFS). (E) OS curves for *TCF4^{high}* AML patients with available follow up and consolidation treatment data (n=129) stratified for conditioning with alloHCT (n=36), autoHCT (n=13) or additional CT (n=80). (F) OS curves for *TCF4^{low}* AML patients with available follow up and consolidation treatment data (n=386) stratified for conditioning with alloHCT (n=99), autoHCT (n=57) or additional CT (n=212).**

expression levels on survival in the second cohort of 436 AML patients[7] (OS: *P*=0.001; EFS: *P*<0.0001) (*Online Supplementary Figure S3*). In the multivariate Cox regression analysis, patients classified as *TCF4^{high}* had a significantly higher risk of death (HR 1.7, CI: 1.3–2.1; P<0.0001), relapse or not obtaining a CR than *TCF4^{low}* patients (HR 1.6, CI: 1.3–2.0; *P*<0.0001) (Table 1A). In addition, multivariate Cox regression analysis revealed *TCF4* expression, as a continuous variable per 100 arbitrary units (AU), was a significant predictor of OS and EFS (HR 1.04, CI: 1.01-1.07, *P*=0.024; HR 1.05, CI: 1.02-1.08, *P*=0.002, respectively) (*Online Supplementary Table S2A*). When selecting for AML patients with a normal karyotype, *TCF4^{high}* patients again showed a worse OS and EFS than *TCF4^{low}* patients (5-year OS 21% *vs.* 41%, *P*<0.0001; 5-year EFS 18% vs. 33%, *P*<0.0001, respectively) (*Online Supplementary Figure S4*). In the multivariate Cox regression analysis of normal karyotype AML patients, *TCF4* expression is also an independent predictor of survival (OS: HR 1.7, CI: 1.2-2.5, *P*=0.003; EFS: HR 1.7, CI: 1.2–2.4, *P*=0.005) (*Online Supplementary Table S2B*). Also as a continuous variable, *TCF4* expression remained an independent prognostic factor in this cohort (OS: HR 1.07 (per 100 AU), CI: 1.02-1.13, *P*=0.004; EFS: HR 1.08 (per 100 AU), CI: 1.03-1.13, *P*=0.003) (*Online Supplementary Table S2C*).

Interestingly, survival analysis according to the Kaplan-Meier method showed that *TCF4^{high}* and *TCF4^{low}* patients of the first cohort demonstrated a different survival benefit depending on the consolidation treatment they received, i.e, an additional cycle of chemotherapy (CT), autologous or allogeneic hematopoietic cell transplantation (autoHCT, alloHCT, respectively) (OS: Figure 1E and F; EFS: *Online Supplementary Figure S5*). *TCF4^{high}* patients who received alloHCT showed a superior survival compared to *TCF4^{high}* patients who received autoHCT or who received additional CT (5-year OS 39%, 8%, 10%, *P*<0.0001; 5-year EFS 31%,

0%, 10%, *P*=0.001, respectively). In contrast, patients classified as *TCF4*^{low} showed a trend towards significant superior survival after autoHCT, compared to *TCF4*^{low} patients who received alloHCT or additional CT (5-year OS: 61%, 45%, 39% *P*=0.002; 5-year EFS: 42%, 32%, 34%, *P*=0.102, respectively). Moreover, this difference in outcome, depending on type of consolidation treatment between the *TCF4*^{high} and the *TCF4*^{low} patients, was confirmed in multivariate Cox regression analysis (*Online Supplementary Table S3*). In the second cohort, only 7 patients in the *TCF4*^{high} group received autoHCT, hampering validation of our observations in this subgroup. Nevertheless, also in this cohort, consolidation treatment with alloHCT (n=44) resulted in significantly better OS for *TCF4*^{high} patients compared to *TCF4*^{high} patients who received additional chemotherapy (n=58) (5-year OS 41% *vs.* 8%, respectively; *P*<0.0001). Furthermore, in this cohort *TCF4*^{low} patients who received autoHCT (n=52) showed a superior OS compared with those patients who received alloHCT (n=86) or additional CT (n=186) (5-year OS 61%, 48% *vs.* 26%, respectively; *P*<0.0001), confirming the observations from the first cohort.

The biological role of TCF4 is poorly understood,[2] and contrasting observations are described in the literature. For example, enforced expression of members of the bHLH class A family, including TCF4, suppresses colony-forming efficiency of various cell lines due to upregulation of p21, p15 and p16, suggesting that these bHLH proteins act as negative regulators of cell growth.[9] In contrast, *Tcf4* expression appeared increased in rat-E1A-immortalized RK3E cells following β-catenin induced neoplastic transformation and aberrant expression of *Tcf4* promoted neoplastic transformation of RK3E cells.[10] These different observations might be explained by differences in cellular context, or by the different transcript variants of *TCF4*,[11-14] which could affect the function of the protein.[10] Possibly, TCF4 can either stimulate or inhibit cell growth, depending on its environment, which might indicate that an aberrant expression is not only a prognostic marker, but also a pathological feature. This would be in line with the report of mutations in *TCF4* in MDS and AML.[3,4]

TCF4 has also been reported to be highly expressed in hematopoietic stem cells (HSC) and to show a decreased expression in committed progenitors.[15] Since the frequency of *TCF4* mutations is relatively low (0.5% in AML), obviously not all patients with high expression of *TCF4* can have mutated *TCF4*. Interestingly, in MLL-AF9-mediated transformation of progenitor cells, *TCF4* has been shown to be up-regulated.[15] In the first cohort, patients with high *TCF4* expression are significantly more classified in the M0 or M1 FAB-subgroups than *TCF4*^{low} patients, suggesting that the leukemic cells of the *TCF4*^{high} patients derive from more immature cells. In addition, *TCF4* expression of patients in the *TCF4*^{high} group is comparable to the level of *TCF4* expression of hCD34^{+} cells. Furthermore, when looking at the *CD34* mRNA expression in the first cohort, 73.3% of the *TCF4*^{high} patients show a high *CD34* expression (above the median), compared to 42.1% of the *TCF4*^{low} patients. When including *CD34* expression in the multivariate Cox regression analysis, *CD34* expression is an independent prognostic factor in OS and EFS; nevertheless *TCF4* expression also remains an independent prognostic factor (*data not shown*).

Our observations report on the prognostic relevance of the level of *TCF4* expression in AML and demonstrate that high *TCF4* expression is associated with a worse survival. In addition, the *TCF4* expression levels seem to provide additional information in the response to treatment. Before considering *TCF4* expression levels in clinical decision-making, additional validation studies, also to define optimal cut-off levels, are needed. Further mechanistic studies are warranted on the role of TCF4 in myeloid diseases.

Florentien E.M. in 't Hout,[1,2] Bert A. van der Reijden,[1] Davide Monteferrario,[1,2] Joop H. Jansen,[1] and Gerwin Huls[1,2]

[1]Department of Laboratory medicine, Laboratory of Hematology, RadboudUMC; and [2]Department of Hematology, RadboudUMC, Nijmegen, The Netherlands

Correspondence: gerwin.huls@radboudumc.nl

doi:10.3324/haematol.2014.110437

Acknowledgments: the authors would like to thank Dr. P. Valk for providing cDNA.

Key words: TCF4, AML, treatment.

Information on authorship, contributions, and financial & other disclosures was provided by the authors and is available with the online version of this article at www.haematologica.org.

References

1. Ellenberger T, Fass D, Arnaud M, Harrison SC. Crystal structure of transcription factor E47: E-box recognition by a basic region helix-loop-helix dimer. Genes Dev. 1994;8(8):970-80.
2. Massari ME, Murre C. MINIREVIEW Helix-Loop-Helix Proteins: Regulators of Transcription in Eucaryotic Organisms. Mol Cell Biol. 2000;20(2):429-40.
3. Papaemmanuil E, Gerstung M, Malcovati L, Tauro S, Gundem G, Van Loo P, et al. Clinical and biological implications of driver mutations in myelodysplastic syndromes. Blood. 2013;122(22):3616-27.
4. Cancer Genome Atlas Research Network. Genomic and epigenomic landscapes of adult de novo acute myeloid leukemia. N Engl J Med. 2013;368(22):2059-74.
5. Makishima H, LaFramboise T, Przychodzen JP, Yoshida K, Ruffalo M, Gómez-Seguí I, et al. Clinical "MUTATOME" Of Myelodysplastic Syndrome; Comparison To Primary Acute Myelogenous Leukemia. ASH Annual Meeting Abstract. Blood. 2013;Abstract 518.
6. Wouters BJ, Löwenberg B, Erpelinck-Verschueren CA, van Putten WL, Valk PJ, Delwel R, et al. Double CEBPA mutations, but not single CEBPA mutations, define a subgroup of acute myeloid leukemia with a distinctive gene expression profile that is uniquely associated with a favorable outcome. Blood. 2009;113(13):3088-91.
7. Kharas MG, Lengner CJ, Al-Shahrour F, Bullinger L, Ball B, Zaidi S, et al. Musashi-2 regulates normal hematopoiesis and promotes aggressive myeloid leukemia. Nat Med. 2010;16(8):903-8.
8. Döhner H, Estey EH, Amadori S, Appelbaum FR, Büchner T, Burnett AK, et al. Diagnosis and management of acute myeloid leukemia in adults: recommendations from an international expert panel, on behalf of the European LeukemiaNet. Blood. 2010;115(3):453-74.
9. Pagliuca A, Gallo P, De Luca P, Lania L. Class A helix-loop-helix proteins are positive regulators of several cyclin-dependent kinase inhibitors' promoter activity and negatively affect cell growth. Cancer Res. 2000;60(5):1376-82.
10. Kolligs FT, Nieman MT, Winer I, Hu G, Van Mater D, Feng Y, et al. ITF-2, a downstream target of the Wnt/TCF pathway, is activated in human cancers with beta-catenin defects and promotes neoplastic transformation. Cancer Cell. 2002;1(2):145-55.
11. Corneliussen B, Thornell A, Hallberg B, Grundström T. Helix-loop-helix transcriptional activators bind to a sequence in glucocorticoid response elements of retrovirus enhancers. J Virol. 1991;65(11):6084-93.
12. Henthorn P, Kiledjian M, Kadesch T. Two distinct transcription factors that bind the immunoglobulin enhancer microE5/kappa 2 motif. Science. 1990;247(4941):467-70.
13. Liu Y, Ray SK, Yang XQ, Luntz-Leybman V, Chiu IM. A splice variant of E2-2 basic helix-loop-helix protein represses the brain-specific fibroblast growth factor 1 promoter through the binding to an imperfect E-box. J Biol Chem. 1998;273(30):19269-76.
14. Skerjanc IS, Truong J, Filion P, McBurney MW. A splice variant of the ITF-2 transcript encodes a transcription factor that inhibits MyoD activity. J Biol Chem. 1996;271(7):3555-61.
15. Krivtsov AV, Twomey D, Feng Z, Stubbs MC, Wang Y, Faber J, et al. Transformation from committed progenitor to leukaemia stem cell initiated by MLL-AF9. Nature. 2006;442(7104):818-22.