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## 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<sup>high</sup>*, 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<sup>low</sup>*) (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<sup>high</sup>* is an independent prognostic factor for OS and EFS in the whole cohort and in patients carrying a normal karyotype.

Importantly, *TCF4<sup>high</sup>* 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<sup>low</sup>* 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.<sup>1,2</sup> Recently, Papaemmanuil and colleagues reported mutations in *TCF4* in MDS patients.<sup>3</sup> 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%)<sup>4</sup> and were associated with a poor prognosis,<sup>5</sup> 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>).<sup>6</sup> 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<sup>+</sup> control cells (hCD34<sup>+</sup>; 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.<sup>7</sup> Patients were divided into genetic risk groups according to the European LeukemiaNet (ELN) guidelines.<sup>8</sup>

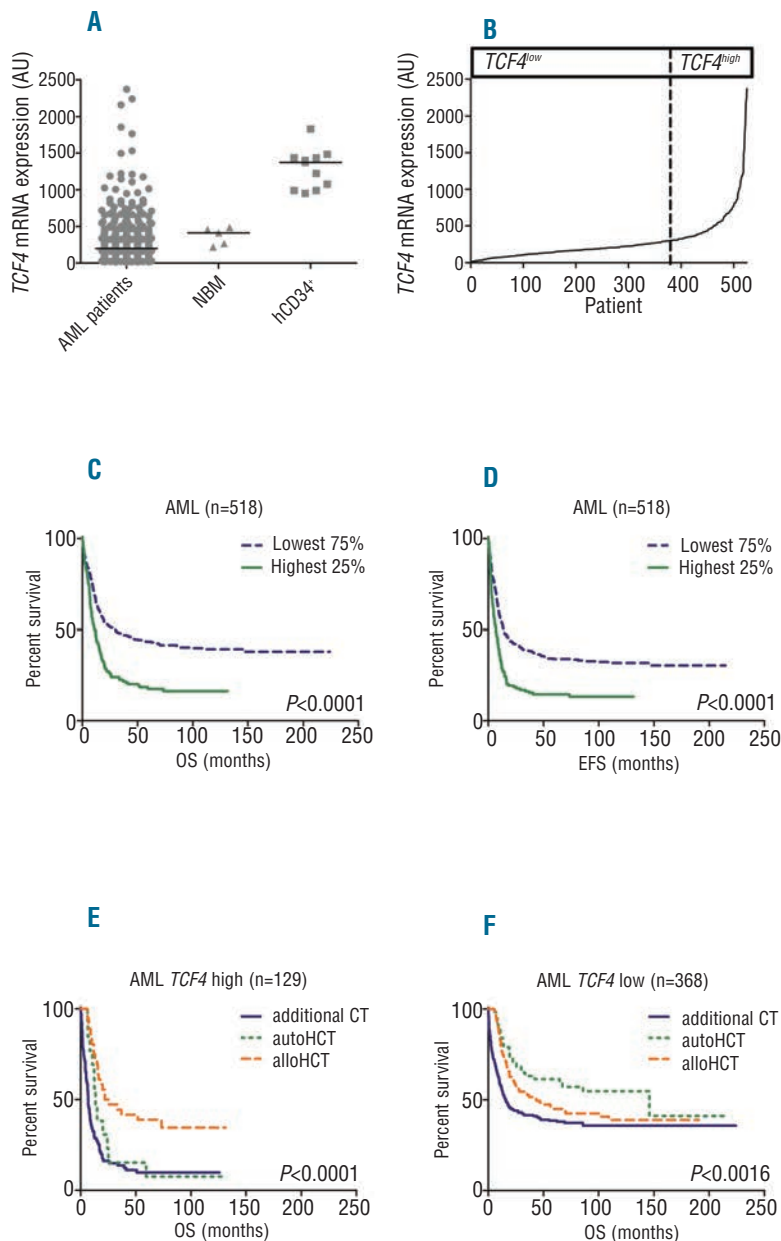
In the studied cohort of 525 AML patients, *TCF4* is differentially expressed in AML blasts compared to NBM and hCD34<sup>+</sup> (Figure 1A). To study the impact of *TCF4* expression levels on survival, the cohort was divided on the basis of differences in expression levels; expression below or above the median, tertiles, quartiles, quintiles, sextiles 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<sup>high</sup>*) and the lowest 75% *TCF4* expression (*TCF4<sup>low</sup>*) was used for further analysis. Characteristics of the patients in the *TCF4<sup>low</sup>* and *TCF4<sup>high</sup>* groups are described in *Online Supplementary Table S1*. *TCF4<sup>high</sup>* 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<sup>low</sup>* 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<sup>high</sup>* patients had a worse survival than patients classified as *TCF4<sup>low</sup>* (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).

Variable	Overall survival (n=506)				Event-free survival (n=506)			
	$\chi^2$ (Wald)	DF	P	HR (95% CI)	$\chi^2$ (Wald)	DF	P	HR (95% CI)
Favorable ELN risk group <sup>8</sup>	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 <sup>9</sup> )	11.02	1	0.001	1.59 (1.21 - 2.09)	14.78	1	<0.0001	1.66 (1.28 - 2.15)
<i>TCF4<sup>high</sup></i> 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<sup>8</sup>; 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<sup>+</sup> (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<sup>high</sup> (n=129) and TCF4<sup>low</sup> (n=389). (D) Same for event-free survival (EFS). (E) OS curves for TCF4<sup>high</sup> 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<sup>low</sup> 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<sup>7</sup> (OS:  $P=0.001$ ; EFS:  $P<0.0001$ ) (Online Supplementary Figure S3). In the multivariate Cox regression analysis, patients classified as TCF4<sup>high</sup> 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<sup>low</sup> 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<sup>high</sup> patients again showed a worse OS and EFS than TCF4<sup>low</sup> 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 independ-

ent 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<sup>high</sup> and TCF4<sup>low</sup> 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<sup>high</sup> patients who received alloHCT showed a superior survival compared to TCF4<sup>high</sup> 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,<sup>2</sup> 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.<sup>9</sup> In contrast, *Tcf4* expression appeared increased in rat-E1A-immortalized RK3E cells following  $\beta$ -catenin induced neoplastic transformation and aberrant expression of *Tcf4* promoted neoplastic transformation of RK3E cells.<sup>10</sup> These different observations might be explained by differences in cellular context, or by the different transcript variants of *TCF4*,<sup>11-14</sup> which could affect the function of the protein.<sup>10</sup> 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.<sup>3,4</sup>

*TCF4* has also been reported to be highly expressed in hematopoietic stem cells (HSC) and to show a decreased expression in committed progenitors.<sup>15</sup> 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.<sup>15</sup> 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<sup>+</sup> 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.

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