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Combined assessment of WT1 and BAALC gene expression at diagnosis may improve leukemia-free survival prediction in patients with myelodysplastic syndromes

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ABSTRACT

Several genes with relevant pathogenetic and prognostic value have been identified in patients with myelodysplastic syndromes. Overexpression of WT1 at diagnosis has been associated with increased progression to acute myeloid leukemia and reduced leukemia free survival. Conversely, few data are available on the prognostic value of BAALC gene overexpression in AML and MDS patients. We evaluated the prognostic value of the combined expression of WT1 and BAALC genes at diagnosis in 86 MDS patients who had been stratified according to IPSS and R-IPSS scoring systems. Our results suggest that in the whole group of patients, low levels of both WT1 and BAALC were associated with a favorable outcome (3-year LFS 74.5%, median not reached), whereas patients presenting high expression levels of both genes had the worst prognosis (3-year LFS 0%, median 12 months, $p < 0.001$). More specifically, molecular profiling was especially useful for intermediate 1 and intermediate-2/high risk groups. This study suggests that evaluating WT1 and BAALC gene expression at diagnosis may improve standard risk stratification and possibly refine the therapeutic approach for MDS patients.

1. Introduction

Myelodysplastic syndromes (MDS) are clonal hematopoietic stem cell disorders characterized by ineffective hematopoiesis, peripheral cytopenias, and increased risk of progression to acute myeloid leukemia (AML) [1–4].

Several prognostic indexes, mainly based on diagnosis according to WHO classification, number and degree of cytopenias, transfusional need, blast count and karyotype have been developed in the last decade [5–8]. Although currently applied scores show good accuracy in predicting survival and risk of leukemic evolution, it is not unusual that patients who are allocated into the same risk group may present different outcomes, especially those who are considered at "intermediate-risk".

Several additional parameters, such as serum lactate dehydrogenase, ferritin, beta 2-microglobulin, and the degree of marrow fibrosis have been studied in order to refine outcome prediction [9], but their independent prognostic value is still uncertain.

Recurrent non-random somatic mutations or aberrant expression of the genes involved in signal transduction pathways, transcriptional regulation, RNA splicing, as well as epigenetic regulation have been identified in MDS [10–16]. Such genetic alterations have also been observed in the majority of MDS patients with normal karyotype. The reported prognostic impact of some of these alterations supports the hypothesis of a "biological predestination" of these clonal disorders [17–21].

WT1 overexpression has been associated with increased progression rate to AML and reduced leukemia-free survival (LFS) [22,23]. Few data are available on the prognostic value of BAALC gene overexpression in AML [24–27] and prognostic impact of BAALC gene overexpression in MDS patients has been recently evaluated together with other 3 genes [28]. BAALC expression analysis is routinely performed in our institution as part of the

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molecular assessment in AML at diagnosis [29]. In our center, BAALC expression correlates with adverse cytogenetic in AML patients older than 65 (unpublished data). Moreover a correlation between high BAALC levels and high risk karyotype has been reported by Thol et al. in MDS patients [28]. We considered the difficulty to study karyotype in MDS patients, due to low mitotic rate and inadequate number of metaphases. Therefore, based on these consideration, we speculated that BAALC overexpression might be a surrogate marker of poor risk cytogenetic alterations and decided to evaluate its prognostic value in MDS patients, analyzed alone or in combination with well-known and validated molecular, cytogenetic and clinical prognostic markers.

Table 1
Patient features and factors affecting leukemic evolution and LFS.

		Num. (%)	Leukemic evolution (%)	p (univ.)	p (multiv.)	Median LFS (months)	3-year LFS (%)	p (univ.)	p (multiv.)
All Patients		86	29 (33.7)	-	-	34	42.7	-	-
Sex	Male	49 (57)	16 (32.7)	0.810	-	31	45.9	0.991	-
	Female	37 (43)	13 (35.1)		-	34	39.6		-
WHO Classification	MDS associated with isolated del(5q)	3 (3.5)	0 (0)	0.000	0.014§ 0.065*	NR	100	0.000	0.473§ 0.428*
	Refractory Anemia	27 (31.4)	3 (11.1)			NR	71.2		
	Refractory Cytopenia with Multi-lineage Dysplasia	12 (14)	4 (33.3)			NR	58.7		
	Refractory Anemia with Excess Blasts-1	19 (22.1)	6 (31.6)			34	46.1		
	Refractory Anemia with Excess Blasts-2	21 (24.4)	16 (76.2)			12	0		
	Other	4 (4.7)	0 (0)			NR	100		
Karyotype	Good Risk	61 (79.2)	21 (34.4)	0.009	0.832§ 0.776*	31	43.8	0.004	0.456§ 0.446*
	Intermediate	6 (7.8)	0 (0)			NR	100		
	Poor Risk	10 (13)	7 (70)			16	16.7		
IPSS	Low-Risk	22 (25.6)	3 (13.6)	0.002	0.617§ 0.624*	NR	74.2	0.001	0.231§ 0.213*
	Intermediate-1	27 (31.4)	6 (22.2)			NR	56.9		
	Intermediate-2/High Risk	28 (32.6)	16 (57.1)			21	20.4		
R-IPSS	Very Low/Low Risk	24 (27.9)	4 (16.7)	0.021	0.304§ 0.259*	NR	60.4	0.004	0.005§ 0.006*
	Intermediate	31(36)	9 (29)			NR	54.9		
	High/Very High Risk	22 (25.6)	12 (54.5)			16	17.0		
WT1 levels	<1000	56 (65.1)	9 (16.1)	0.000	0.000§ -	NR	64.8	0.000	0.001§ -
	>1000	30 (34.9)	20 (66.6)			12	8.5		
BAALC levels	<1000	56 (65.1)	11 (19.6)	0.000	0.332§ -	NR	60.8	0.001	0.021§ -
	>1000	30 (34.9)	18 (60)			21	17.9		
Molecular Profile	WT1 <1000 and BAALC <1000	44 (51.2)	5 (11.4)	0.000	- 0.000*	NR	74.5	0.000	- 0.000*
	WT1 <1000 and BAALC >1000	12 (13.9)	4 (33.3)			34	41.6		
	WT1 >1000 and BAALC <1000	12 (13.9)	6 (50)			25	19.4		
	WT1 >1000 and BAALC >1000	18 (20.9)	14 (77.8)			12	0		

We performed a retrospective analysis on 86 MDS patients to evaluate the potential prognostic value of WT1 and BAALC expression. Our study suggests that the combined assessment of WT1 and BAALC expression at diagnosis might usefully integrate IPSS and R-IPSS based prognostic definition and could be helpful in selecting therapy.

2. Materials and methods

2.1. Patient selection and molecular analysis

From 2007 to 2012 152 MDS patients were referred to our center. Only in 86 patients the diagnostic and prognostic work up included cytogenetics and molecular analysis on fresh bone marrow samples at diagnosis and a complete clinical follow up was available. These patients form the study cohort. In the other 66 patients data were incomplete for at least one of the following reasons: decision of the hematologist to not perform molecular studies (advanced age, heavy comorbidity), diagnosis in a peripheral center and secondary referral to our division, failure of molecular study, absence of clinical follow up. Molecular analysis on marrow samples was performed after obtaining written informed consent, according to the Helsinki declaration.

The following molecular studies were carried out: evaluation of FLT3-ITD, TKD-FLT3 mutations, and determination of WT1 and BAALC gene expressions. FLT3 gene analysis was performed as described elsewhere [30]. WT1 expression was evaluated by theWT1 ProfileQuant® (ELN*) kit (IPSOGEN) following the manufacturer's instructions. A value of 1000WT1/ABL copies $\times 10^4$ was considered abnormal after being compared with samples from healthy donors [29,31]. BAALC expression was evaluated by the BAALC ProfileQuant®(ELN*) kit (IPSOGEN) following the manufacturer's instructions.[32] The value of 1000 BAALC/ABL copies $\times 10^4$ was arbitrarily used as the cutoff level [29]. None of the patients displayed any kind of FLT3

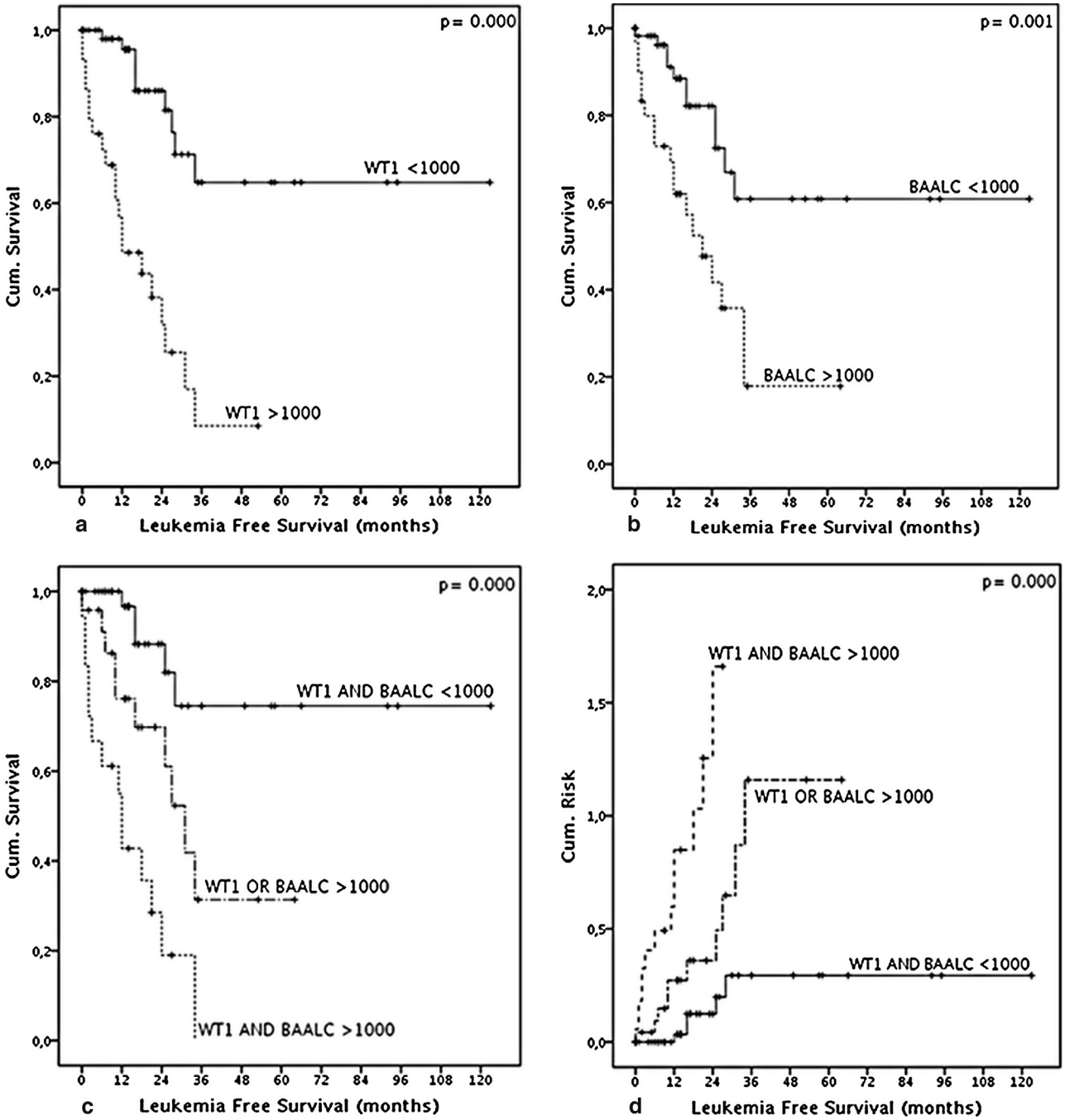

Fig. 1. Leukemia free survival according to WT1 and BAALC expression levels. (a and b) LFS according to WT1 (a) or BAALC (b) expression levels. (c and d) LFS (c) and cumulative leukemic evolution risk (d) according to molecular profile.

mutations. Gene expression was evaluated using TaqMan Gene Expression Assays (Opticon2-MJ Research) [29]. The combined evaluation of WT1 and BAALC gene expression at diagnosis was defined as "molecular profile".

Sixty-one patients showed a good risk karyotype, whereas 6 and 10 had intermediate and high risk karyotype, respectively. The cytogenetic study was not informative for 9 patients. On the basis of their IPSS score, 22 patients were classified as low risk and 27 and 28 as intermediate 1 and intermediate 2/high risk, respectively. According to the WHO classification, 27 patients had refractory anemia, 19 and 21 had refractory anemia with excess blasts 1 and 2, respectively, 3 patients had 5q-syndrome and 12 patients were diagnosed with refractory cytopenia with multi-lineage dysplasia (RCMD). Patients' features are summarized in Table 1.

Patients underwent different treatments including best supportive care, erythropoietin, hypomethylating agents and immunomodulating agents according to clinical guidelines and IPSS risk stratification.

2.2. Statistical analysis

Chi-square test and, when necessary, Fisher's exact test were used for univariate leukemic progression probability analysis. Two logistic regression models were built for multivariate analysis, including only variables reaching at least $p < 0.150$ at early univariate analysis. The first logistic-regression model included WT1 and BAALC expression levels as different covariates, the second model included combined WT1 and BAALC assessment (molecular profile).

LFS was calculated from diagnosis until last follow-up or documented leukemic progression as defined in the literature. Patients who died without documented leukemic evolution (i.e., death by any other cause) were censored at the time of death for LFS calculation. Overall survival (OS) was calculated from diagnosis until last follow-up or death by any cause.

LFS and OS survival curves were built with the Kaplan–Meier method; univariate survival analysis was performed by log-rank test. Two Cox proportional hazard model including only variables reaching at least $p < 0.150$ at early univariate analysis were built for multivariate survival analysis. The first proportional hazard model included WT1 and BAALC expression levels as different covariates, the second model included molecular profile.

A two-tailed p value <0.05 was considered statistically significant. All statistical analyses were performed using IBM SPSS v19.

3. Results

3.1. Leukemic evolution risk

After a median follow-up of 36 months (range 2–94), 29 patients progressed to AML. The probability of leukemic transformation was significantly affected by R-IPSS score, karyotype, IPSS score, WHO classification, WT1 expression levels, BAALC expression levels and molecular profile (Table 1).

Patients with high expression levels of both markers (defined as H/H profile) had the higher risk of leukemic transformation, whereas patients with low levels of both (defined as L/L profile) had the lowest. Patient with either elevated WT1 or BAALC had an intermediate risk of evolution, with no statistically significant difference between elevated WT1 only and elevated BAALC only (6/12 and 4/12 leukemic evolution, respectively, $p = 0.679$). Multivariate logistic-regression analysis showed that only WHO classification and WT1 levels at diagnosis were significant predictors of leukemic evolution ($p < 0.03$ and $p < 0.001$, respectively, Table 1); when analysis was performed including the molecular profile instead of single gene evaluation, this resulted the strongest predictor of leukemic evolution risk ($p < 0.001$, Table 1).

Molecular profile could enhance IPSS and R-IPSS based stratification. Among IPSS intermediate-1 and intermediate-2/high risk groups, patients with L/L profile had the lowest risk of leukemic evolution (3/16 and 0/6, respectively), whereas patients with H/H profile had the highest risk (2/4 and 9/11, respectively, $p < 0.05$ and $p < 0.03$ for intermediate-1 and intermediate-2/high risk, respectively).

Among intermediate and high/very high R-IPSS risk groups, L/L profile identified patients with the lower risk of leukemic evolution (2/15 and 1/7, respectively), while H/H profile conferred the greater risk of leukemic evolution (5/6 and 5/6, respectively, $p < 0.03$ and $p < 0.03$ for intermediate and high/very high risk, respectively).

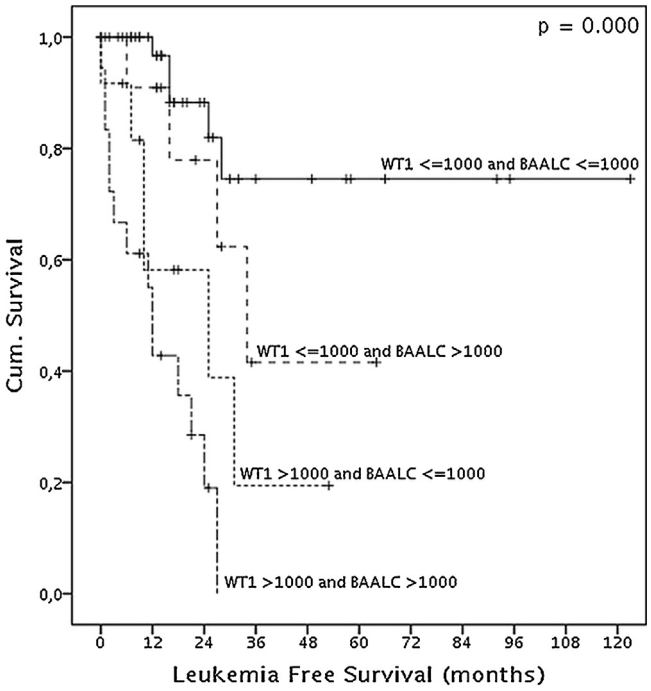

Fig. 2. Leukemia free survival according to molecular profile.

3.2. Leukemia-free survival (LFS)

Median LFS was 34 months (range 2–94). In univariate analysis, LFS duration was significantly affected by karyotype, R-IPSS and IPSS scores, WHO classification, WT1 expression levels (Fig. 1a), BAALC expression levels (Fig. 1b) and molecular profile (Figs. 1c and d and 2). Multivariate Cox Regression model showed that only WT1 and BAALC expression levels and R-IPSS risk group impacted on LFS duration ($p < 0.003$, $p < 0.03$, and $p < 0.01$, respectively, Table 1). When analysis was performed including the molecular profile, this resulted the strongest predictor of leukemia free survival duration ($p < 0.001$, Table 1).

Patients with L/L profile had a relatively favorable outcome (3-year LFS 74.5%, median not reached), whereas H/H profile patients had the worst prognosis (3-year LFS 0%, median 12 months, $p < 0.001$, Figs. 1c and d and 2).

Patients with either elevated WT1 or BAALC had a significantly worse prognosis than L/L profile patients ($p < 0.05$, Fig. 1c and d) but LFS duration was not significantly different between patient with only elevated WT1 and patient with only elevated BAALC ($p = 0.181$). However, a trend for worse LFS duration for WT1 elevated and normal BAALC appeared (Fig. 2).

Among patients with normal or elevated levels of BAALC, high levels of WT1 where associated with a significantly shorter LFS ($p < 0.005$ and <0.01, respectively, Fig. 3a and b).

Among patients with normal levels of WT1, patients with high levels of BAALC showed a trend for shorter LFS, without reaching statistical significance (Fig. 3c, $p = 0.181$). This also applied to patients expressing high level of WT1 (Fig. 3d, $p = 0.283$). This lack of significance is probably explained by the small sample size. This is supported by the observation that BAALC has a significant impact on LFS duration both in the univariate and multivariate analysis (Table 1§). Furthermore molecular profiling including BAALC can clearly identify a group of patients with "intermediate" outcome compared with a risk stratification based on WT1 alone (Fig. 1a, c and d).

Molecular profile could further enhance LFS prediction among various IPSS and R-IPSS groups. In particular, 3-year LFS in the

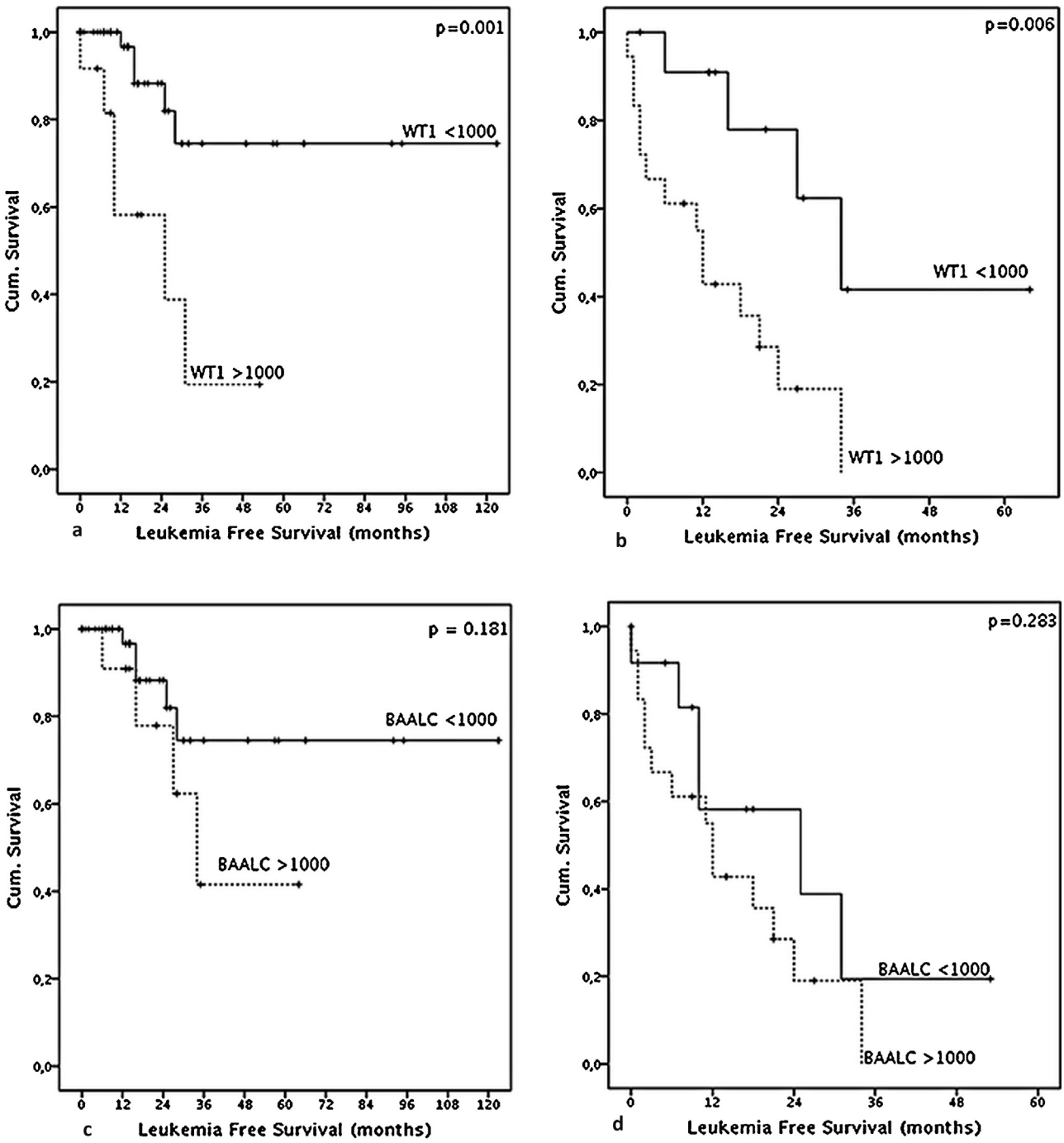

Fig. 3. Leukemia free survival according to different combinations of WT1 and BAALC expression levels. (a and b) LFS according to WT1 levels in patients with BAALC <1000 (a) or BAALC >1000 (b). (c and d) LFS according to BAALC levels in patients with WT1 <1000 (c) or WT1 >1000 (d).

intermediate-1 IPSS risk group was 66.5% for L/L profile patients (median not reached), compared with 0% for patients with H/H profile ($p < 0.05$, data not shown). No leukemic evolution was observed in IPSS intermediate 2/high risk group patients with L/L profile, whereas H/H profile patients had a very short LFS (median 12 months, 3-year LFS 0%, data not shown).

We tried to compare the prognostic value of both single gene expression and molecular profile among different R-IPSS groups but the small number of patients in each group prevented us from drawing any significant correlation. However, in IPSS intermediate 2/high risk group molecular profile seemed to predict outcome better than WT1 alone (leukemic evolution 0% vs 18%, 3yy LFS 100% vs 73%; 3yy OS 37.5% vs 29.6%, in L/L patients and low WT1 patients, respectively, data not shown).

3.3. Risk of death and overall survival

After a median follow-up of 32 months, 43 patients died. The main cause of death was leukemic evolution (31/43 deaths, 72%), while other causes were cardiovascular events and infections (data not shown).

The risk of death by any cause was significantly affected by leukemic evolution, diagnosis according to WHO classification, WT1 expression levels, BAALC expression levels and molecular

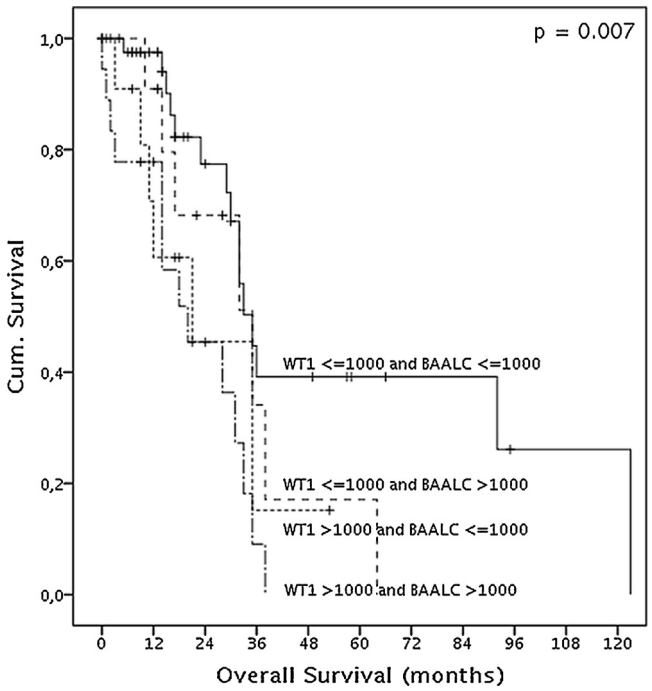

Fig. 4. Overall survival according to molecular profile.

profile. Multivariate analysis showed that leukemic evolution was the strongest independent predictor of death, in both models ($p < 0.001$).

Median OS was 32 months. In univariate analysis, OS was significantly influenced by diagnosis according to WHO classification, karyotype, R-IPSS score, leukemic evolution, WT1 levels of expression, BAALC levels of expression and molecular profile at diagnosis (Fig. 4).

Multivariate Cox-regression analysis revealed that IPSS score ($p < 0.02$), BAALC ($p < 0.01$), R-IPSS score ($p < 0.005$), and leukemic evolution ($p < 0.005$) were independent predictors of OS. When Cox regression model included molecular profile, leukemic evolution ($p < 0.03$), molecular profile ($p < 0.01$), R-IPSS score ($p < 0.005$) and IPSS score ($p < 0.003$) resulted independent predictors of OS. A detailed overview of death risk and OS analysis is provided in Table 2.

4. Discussion

Prognostic stratification is essential to establish the optimal therapeutic approach for MDS patients and several well-known scoring systems have been shown to predict survival [5–8]. Prognosis is mainly related to the risk of leukemic evolution, since treatment is poorly effective once progression has occurred. Accurate prediction of leukemic transformation risk is especially relevant in younger patients who are potentially eligible for hematopoietic stem cell transplantation (HSCT) [33]. In this setting new prognostic markers specifically related to leukemic evolution are strongly needed. Our study shows that a simple molecular profile performed at diagnosis (WT1 plus BAALC gene expression) might improve the prediction of leukemic evolution.

WT1, a tumor suppressor gene involved in the pathogenesis of Wilm's tumor, is overexpressed in most AML subjects. Although the molecular mechanism underlying WT1 overexpression and its role in leukemogenesis still remain unclear, several studies found a correlation between WT1 expression levels and outcome in AML patients [22]. The biological and clinical continuum between AML and MDS led investigators to evaluate the potential prognostic value of WT1 in MDS patients and several studies reported a negative impact of its overexpression [23,34–36]. Moreover, Cilloni et al. reported a significant correlation between WT1 expression and IPSS score [37].

The role of BAALC in physiologic and leukemic hematopoiesis has not been clearly defined but its involvement in the hematopoietic cell proliferation and differentiation has been hypothesized [38]. The prognostic role of BAALC gene expression has been investigated in hematological malignancies and its overexpression has been associated with poor outcome in AML [24–27]. Thol et al. evaluated the expression of several genes (BAALC, MN1, ERG, and EVI1) in MDS patients and found that overexpression of at least two of these genes had a negative prognostic value [28]. The prognostic value of WT1 and BAALC gene expression was retrospectively studied in a cohort of core-binding factor AML patients [39], but to the best of our knowledge, to date no data have been reported in MDS patients.

Despite the low number of patients, our study indicates that overexpression of either WT1 or BAALC may predict a higher risk of leukemic transformation. Although WT1 seems to exert a stronger prognostic impact when compared to BAALC, the prognostic value of the molecular analysis is higher when the combined expression of both genes is analyzed. In multivariate models molecular profile is indeed the best predictor of leukemic evolution risk and the strongest predictor of LFS duration. In comparison with WT1 based stratification the molecular profile allows to better define very low and very high risk patients (L/L and H/H profile patients, respectively) and to identify a new group of patients with intermediate risk of leukemic evolution. Moreover, the combined WT1 and BAALC expression retains a prognostic value regardless of IPSS risk group. In particular in patients assigned to intermediate risk groups ("Intermediate-1" according to IPSS and "Intermediate" according to R-IPSS) molecular profile may identify patients at greater risk of evolution to acute leukemia.

Finding the best therapeutic approach for intermediate risk patients still remains a challenge since there is currently no clear indication for hypomethylating agents or more aggressive treatments [40,41]. HSCT still represents the only curative approach for MDS but it is often difficult to establish which patients are eligible and what the optimal timing to perform the transplant is [42–45]. Our results seem to indicate that molecular profile may identify a subgroup of intermediate risk patients at higher risk of evolution to acute leukemia who might benefit from early and aggressive therapies. Recently developed HSCT risk score predictors could be integrated with molecular data and IPSS/R-IPSS scores in order to better select patients for whom an early BMT might be indicated [46].

Our study also suggests that the combined evaluation of WT1 and BAALC gene expression may also be of value for patients included in other risk groups. In particular low risk patients with H/H profile show a higher rate of leukemic evolution and, conversely, high risk patients with L/L profile display a lower risk of progression. However further prospective studies in larger cohorts of patients are needed to confirm our observations.

The biological background that may explain the effect of WT1 and BAALC expression on leukemic evolution is completely unknown. The hyper-expression probably represents the epiphenomenon of more complex underlying driver mutations that determine the aggressive biological behavior of the MDS clone.

In our series we did not analyze whether modifications of expression levels from baseline are somehow correlated to changes in leukemic evolution risk. It is possible that an increase in molecular expression may be associated with a higher risk of leukemic evolution.

Therapy tailored to the individual's risk of leukemic progression will probably represent the future goal in the management of MDS

Table 2
Factors affecting overall survival.

		Num. (%)	Dead (%)	p (univ.)	p (multiv.)	Median OS (months)	3-year OS (%)	p (univ.)	p (multiv.)
All Patients		86	43 (50)	-	-	32	27.3	-	-
Sex	Male	49 (57)	25 (51)	1.000	-	32	19.7	0.381	-
	Female	37 (43)	18 (48.6)		-	33	34.5		-
WHO Classification	MDS associated with isolated del(5q)	3 (3.5)	0 (0)	0.001	0.212§ 0.212*	NR	100	0.044	0.906§ 0.857*
	Refractory Anemia	27 (31.4)	8 (29.6)			33	38.5		
	Refractory Cytopenia with Multi-lineage Dysplasia	12 (14)	9 (75)			31	12.7		
	Refractory Anemia with Excess Blasts-1	19 (22.1)	11 (57.9)			29	27.3		
	Refractory Anemia with Excess Blasts-2	21 (24.4)	15 (71.4)			17	15.8		
	Other	4 (4.7)	0 (0)			NR	100		
Karyotype	Good Risk	61 (79.2)	26 (42.6)	0.082	0.329§ 0.329*	33	27.5	0.018	0.128§ 0.065*
	Intermediate	6 (7.8)	3 (50)			36	40		
	Poor Risk	10 (13)	8 (80)			14	12.5		
IPSS	Low-Risk	22 (25.6)	10 (45.5)	0.467	- -	35	37.1	0.149	0.018§ 0.002*
	Intermediate-1	27 (31.4)	11 (40.7)			33	28.1		
	Intermediate-2/High Risk	28 (32.6)	16 (57.1)			20	20.9		
R-IPSS	Very Low/Low Risk	24 (27.9)	11 (45.8)	0.067	0.315§ 0.315*	35	27.5	0.007	0.003§ 0.003*
	Intermediate	31(36)	11 (35.5)			36	47.4		
	High/Very High Risk	22 (25.6)	15 (68.2)			17	8		
WT1 Levels	< 1000	56 (65.1)	22 (39.3)	0.012	0.536§ -	35	37.8	0.002	0.161§ -
	>1000	30 (34.9)	21 (70)			21	11.6		
BAALC Levels	<1000	56 (65.1)	22 (39.3)	0.012	0.559§ -	35	33.3	0.016	0.008§ -
	>1000	30 (34.9)	21 (70)			28	18.2		
Molecular Profile	WT1 <1000 and BAALC <1000	44 (51.2)	15 (34.1)	0.013	- 0.760*	35	39.1	0.007	- 0.005
	WT1 <1000 and BAALC >1000	12 (13.9)	7 (58.3)			35	34.1		
	WT1 >1000 and BAALC <1000	12 (13.9)	7 (58.3)			21	15.2		
	WT1 >1000 and BAALC >1000	18 (20.9)	14 (77.8)			20	9.1		
Leukemic evolution	Leukemia-Free	29 (33.7)	17 (29.8)	0.000	0.000§ 0.000*	36	46.7	0.000	0.003§ 0.02*
	Progressed to leukemia	57 (66.3)	26 (89.7)			20	8.2		

patients. Several ongoing prospective studies will likely provide more information about the clinical application of molecular data in MDS. We acknowledge that our analysis does not include several important genes involved in MDS pathogenesis. However the present study indicates that a significant improvement of the prognostic scores in MDS can be achieved by using a relatively low-cost two gene expression analysis, that can be applied even in small centers.

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