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Prognostic Importance of *MN1* Transcript Levels, and Biologic Insights From *MN1*-Associated Gene and MicroRNA Expression Signatures in Cytogenetically Normal Acute Myeloid Leukemia: A Cancer and Leukemia Group B Study

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The Appendix is included in the full-text version of this article, available online at www.jco.org. It is not included in the PDF version (via Adobe® Reader®).

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A B S T R A C T

Purpose

To determine the prognostic importance of the meningioma 1 (*MN1*) gene expression levels in the context of other predictive molecular markers, and to derive *MN1* associated gene– and microRNA–expression profiles in cytogenetically normal acute myeloid leukemia (CN-AML).

Patients and Methods

MN1 expression was measured in 119 untreated primary CN-AML adults younger than 60 years by real-time reverse-transcriptase polymerase chain reaction. Patients were also tested for *FLT3*, *NPM1, CEBPA,* and *WT1* mutations, *MLL* partial tandem duplications, and *BAALC* and *ERG* expression. Gene- and microRNA-expression profiles were attained by performing genome-wide microarray assays. Patients were intensively treated on two first-line Cancer and Leukemia Group B clinical trials.

Results

Higher *MN1* expression associated with *NPM1* wild-type ($P < .001$), increased *BAALC* expression ($P = .004$), and less extramedullary involvement ($P = .01$). In multivariable analyses, higher *MN1* expression associated with a lower complete remission rate ($P = .005$) after adjustment for WBC; shorter disease-free survival ($P = .01$) after adjustment for *WT1* mutations, *FLT3* internal tandem duplications (*FLT3*-ITD), and high *ERG* expression; and shorter survival ($P = .04$) after adjustment for *WT1* and *NPM1* mutations, *FLT3*-ITD, and WBC. Gene- and microRNA-expression profiles suggested that high *MN1* expressers share features with high *BAALC* expressers and patients with wild-type *NPM1*. Higher *MN1* expression also appears to be associated with genes and microRNAs that are active in aberrant macrophage/monocytoid function and differentiation.

Conclusion

MN1 expression independently predicts outcome in CN-AML patients. The *MN1* gene- and microRNA-expression signatures suggest biologic features that could be exploited as therapeutic targets.

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INTRODUCTION

Nonrandom cytogenetic abnormalities are among the most important prognostic factors in acute myeloid leukemia (AML).[1-4] However, approximately 45% of adults younger than 60 years of age with primary AML have cytogenetically normal (CN) disease at diagnosis and thus lack informative chromosome markers for risk stratification.[1-4] Recently, this large cytogenetic group was shown to be composed of subsets differing for the presence of distinct submicroscopic genetic alterations.[5]

The meningioma 1 (*MN1*) gene is located at chromosome band 22q12 and encodes a protein that participates in a gene transcription regulator complex with the nuclear receptor RAR-RXR or the vitamin D receptor.[6,7] The involvement of this gene in human neoplasia was initially discovered in a case of meningioma carrying t(4;22)[8] and also found in myeloid malignancies with t(12;22).[9] High levels of *MN1* expression were recently associated with inv(16) AML,[10] and shown, in a mouse model, to cooperate with *CBFB-MYH11* gene fusion in the development of AML.[11] However, the mechanisms through which aberrant expression of *MN1* contributes to malignant transformation remain to be elucidated.[12,13]

Recently, Heuser et al[14] reported that overexpression of *MN1* predicted worse outcome in CN-AML patients. To date, however, these results have not been independently corroborated or tested in the context of several other established prognostic markers in CN-AML. Thus, to validate *MN1* expression's prognostic importance in CN-AML, we measured the *MN1* expression in diagnostic bone marrow (BM) samples from younger adult CN-AML patients that were also comprehensively characterized for other molecular markers associated with outcome. Furthermore, to gain insight into *MN1*-mediated leukemogenesis, we derived gene- and microRNA-expression signatures associated with changes in *MN1* expression levels.

PATIENTS AND METHODS

Patients, Treatment, Cytogenetic, and Molecular Analyses

One hundred nineteen adults younger than 60 years of age with untreated, primary CN-AML with material available for analyses were included. Patients were treated similarly on Cancer and Leukemia Group B (CALGB) protocols 9621 (n = 38) and 19808 (n = 81) with intensive induction chemotherapy and consolidation with autologous peripheral blood stem cell transplantation (SCT; Appendix, online only).[15,16] No differences in outcome (complete remission rate [CR], $P = .86$; disease-free survival [DFS], $P = .37$; overall survival [OS], $P = .33$) were observed between the patients studied for *MN1* expression and the remaining CN-AML patients not included (n = 121).

Pretreatment BM cytogenetic analyses were performed by CALGB-approved institutional cytogenetic laboratories on CALGB 8461, a prospective cytogenetic companion, and centrally reviewed.[17] *MN1* copy numbers normalized to *ABL* copy numbers were measured in BM samples by real-time reverse transcriptase polymerase chain reaction quantification (Appendix). The presence or absence of additional molecular markers such as *FLT3* internal tandem duplication (*FLT3*-ITD),[18,19] *FLT3* tyrosine kinase domain mutations (*FLT3*-TKD),[20,21] mutations in the *NPM1*,[22] *CEBPA*,[23] and *WT1*[24] genes, *MLL* partial tandem duplication (*MLL*-PTD),[25,26] and *ERG*[27,28] and *BAALC*[29,30] expression levels were assessed centrally. All patients gave informed consent for the research use of their specimens, in accordance with the Declaration of Helsinki.

Gene-Expression and MicroRNA-Expression Profiling

RNA samples from 75 of 81 patients studied for *MN1* expression enrolled on CALGB 19808 were analyzed for genome-wide gene expression using Affymetrix U133 plus 2.0 GeneChips (Affymetrix, Santa Clara, CA), as previously reported (Appendix).[10,31]

Of the 75 samples analyzed for genome-wide gene expression, 73 were also analyzed for genome-wide microRNA expression. Biotinylated first strand cDNA from total RNA extracted from pretreatment BM or blood mononuclear cell samples was synthesized using biotin-labeled random octamer primers and was hybridized onto microRNA microarray chips, as previously reported.[32] Images of the microRNA microarrays were acquired as previously reported.[33]

Statistical Methods

The main objective was to evaluate the impact of *MN1* expression on clinical outcome. We defined CR as BM cellularity ≥ 20% and fewer than 5% blasts, and recovery of leukocyte (≥ 1,500/μL) and platelet (> 100,000/μL) counts; relapse as ≥ 5% of BM, leukemic blasts, circulating blasts, or extramedullary leukemia; DFS as the interval from CR achievement until relapse or death, regardless of cause; OS as the date on study until death. Patients alive at last follow-up were censored for both DFS and OS. *MN1* expression values were calculated as the natural log transformation of the normalized *MN1* copy numbers; this continuous variable was used for all statistical analyses. Pretreatment CNS, spleen, liver, skin, nodes, gum, or mediastinal mass involvement constituted extramedullary disease.

The associations of *MN1* expression with baseline clinical, demographic, and molecular features, and achievement of CR were analyzed using one-way analysis of variance. Kaplan-Meier plots were generated for each time-to-event outcome measure (DFS and OS) using *MN1* expression quartiles. The corresponding tests for trend were calculated for each survival end point.[34] Comparisons between cases analyzed for *MN1* *v* those not analyzed were tested using the Fisher's exact test for CR rates and the log-rank test for the OS and DFS end points.

Multivariable logistic regression models were constructed to analyze factors related to the probability of achieving CR and multivariable Cox proportional hazards models were constructed to analyze factors important for the survival end points, OS and DFS. Factors examined for model inclusion were *MN1* expression, *FLT3*-ITD, *FLT3*-TKD, *NPM1* and *WT1* mutational status, age, hemoglobin, platelet count, WBC, percentages of BM and blood blasts, sex, race, and extramedullary involvement, and for survival end points only, *MLL*-PTD, *CEBPA* mutational status, and *ERG* and *BAALC* expression levels. For the multivariable Cox models, the proportional hazards assumption was checked for each variable individually. If the proportional hazards assumption was not met for a particular variable for a given end point, an artificial time-dependent covariate was included in the model for that end point. Variables considered for inclusion in the logistic and Cox multivariable models were those significant at $\alpha = .20$ from the univariable models . All models were constructed using a limited backwards selection procedure. Variables remaining in the final models were significant at $\alpha = .05$. For achievement of CR, estimated odds ratios (OR), and for survival end points, hazard ratios (HR) with their corresponding 95% CIs were obtained for each significant prognostic factor.

For microarray analyses, summary measures of gene and microRNA expression were computed, normalized, and filtered (Appendix).[35] Pearson correlation coefficients were computed between the resulting expression values of 24,183 Affymetrix probe sets and the natural log transformation of *MN1* expression, and between the resulting expression values of 305 microRNA probes and the natural log transformation of *MN1* expression values; significant Affymetrix probe sets ($P < .001$) and microRNA probes ($P < .005$) comprised the *MN1* gene- and microRNA-expression signatures, respectively. GenMAPP version 2.1 and MAPPFinder version 2.1[36] (Gladstone Institutes, the University of California, San Francisco, CA; http://www.genmapp.org/) were used to assess over-represented gene ontology (GO) terms within the identified gene-expression signature (Appendix).

All statistical analyses were performed by the CALGB Statistical Center.

RESULTS

Association of MN1 Expression With Molecular and Clinical Characteristics and Outcome

At diagnosis, higher *MN1* expression (*MN1*/*ABL* copy number range, 0.007 to 7.317) was associated with lower frequency of *NPM1* mutations ($P < .001$) and higher *BAALC* expression ($P = .004$) and less extramedullary disease ($P = .01$; Table 1; Fig 1). No other molecular or clinical characteristics were significantly associated with *MN1* expression.

The overall CR rate of the patients analyzed for *MN1* expression was 83%. Patients who failed to achieve CR had higher *MN1* levels ($P = .006$; Fig 2A). No interaction between *MN1* levels and induction treatment (ie, with or without PSC-833) was found for CR achievement. On multivariable analysis, patients with higher *MN1* expression were less likely to achieve CR ($P = .005$) after adjustment for WBC ($P = .005$; Table 2).

The median follow-up for patients with no event (ie, failure to achieve CR, relapse, or death) was 5.1 years (range, 2.7 to 9.9 years). Higher *MN1* expression was associated with shorter DFS ($P < .001$)

Table 1. Relationship of Clinical and Molecular Characteristics With *MN1* Expression Levels in Patients With Cytogenetically Normal Acute Myeloid Leukemia at Diagnosis (N = 119)

Characteristic	Summary Statistics		
	No.	%	P*
Median age, years	43		.64
Range	18-59		
Sex			.52
Female	62	52	
Male	57	48	
Race			.15
White	104	88	
Nonwhite	**14**	**12**	
Median hemoglobin, g/L	92		.21
Range	48-136		
Median platelet count, $\times 10^9$/L	55		.78
Range	8-395		
Median WBC, $\times 10^9$/L	27.3		.94
Range	1.4-273.0		
Median blood blasts, %	59		.78
Range	0-95		
Median bone marrow blasts, %	67		.20
Range	21-99		
Extramedullary involvement			.01
No	**85**	**72**	
Yes	33	28	
FLT3-ITD			.30
Negative	66	55	
Positive	53	45	
FLT3-TKD			.06
Negative	**109**	**92**	
Positive	10	8	
NPM1			< .001
Wild-type	**39**	**33**	
Mutated	80	67	
CEBPA			.15
Wild-type	97	83	
Mutated	**20**	**17**	
ERG expression			.86
Low	50	56	
High	39	44	
BAALC expression			.004
Low	46	50	
High	**46**	**50**	
WT1			.23
Wild-type	101	90	
Mutated	11	10	
MLL-PTD			.81
Negative	111	93	
Positive	8	7	

NOTE. Not all 119 patients were evaluated for all the molecular markers. For each molecular marker, the number of patients negative or positive, wild-type or mutated or low or high is reported in the Summary Statistics column.

Abbreviations: *FLT3*-ITD, internal tandem duplication of the *FLT3* gene; *FLT3*-TKD, tyrosine kinase domain mutation of the *FLT3* gene; *MLL*-PTD, partial tandem duplication of the *MLL* gene.

**P* values are from the one-way analysis of variance overall F-test, evaluating the presence of any linear relationship between the *MN1* expression and the variable tested. For tests with a *P* value < .20, the characteristic associated with higher *MN1* expression appears in bold.

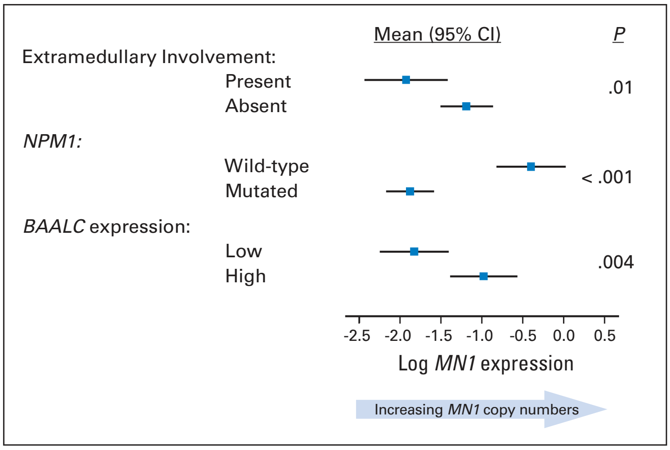

Fig 1. Clinical and molecular variables significantly associated with the meningioma 1 (*MN1*) gene expression. The direction of the correlation is shown by displaying the mean values and corresponding 95% CIs of *MN1* expression for each category of the clinical and molecular variables.

and OS ($P < .001$). An interaction between *MN1* levels and variations in the consolidation or maintenance treatments could not be evaluated because of sample size limitations. In multivariable models, higher *MN1* expression was associated with shorter DFS ($P = .01$) after adjusting for *WT1* mutations ($P = .01$), *FLT3*-ITD ($P = .02$), and high *ERG* expression ($P = .04$). Likewise, shorter OS ($P = .04$) was associated with higher *MN1* expression when controlling for *WT1* ($P < .001$) and *NPM1* mutations ($P = .04$), *FLT3*-ITD ($P = .01$), and WBC ($P < .001$; Table 2). Similar results were observed when the *FLT3*-ITD/*FLT3* wild-type allelic ratio (no *FLT3*-ITD *v* *FLT3*-ITD/*FLT3* wild-type < .7 *v* *FLT3*-ITD/*FLT3* wild-type ≥ .7) rather than presence compared with absence of *FLT3*-ITD, was utilized as a factor in the multivariable models.

To graphically display the relationship between *MN1* expression and clinical outcome, patients were divided into four groups corresponding to the quartile (Q) values of *MN1* expression (Figs 2B, 2C). The 5-year DFS and OS estimates were progressively lower from Q1 (ie, patients with the lowest 25% of *MN1* expression values) to Q4 (ie, patients with the highest 25% of *MN1* expression values; $P < .001$, test for trend for both DFS and OS). Patients in Q1 had remarkably favorable outcomes, with expected 5-year DFS and OS rates of 74% and 80%, respectively, compared with only 36% and 40%, respectively, for the remaining patients.

Biologic Insights

To gain insight into leukemogenic mechanisms associated with changes in *MN1* expression, we derived both gene- and microRNA-expression signatures using microarray assays. The *MN1*-associated gene-expression signature consisted of 555 probes (Appendix Table A1, online only; Fig 3). Expression of 261 probe sets positively correlated with *MN1* expression levels, and expression of 294 probe sets negatively correlated with *MN1* expression levels. The probe set for *MN1* had the highest positive coefficient of correlation ($r = .87$), corroborating the quantification of *MN1* expression obtained by real-time RT-PCR. Furthermore, we found *MN1* expression levels to be directly correlated with *BAALC* expression levels and with the expression of genes recently reported as associated with a *BAALC* expression signature,[30] specifically, *PROM1*, *CD34*, *FZD6*, *CRYGD*, *CD200*, and

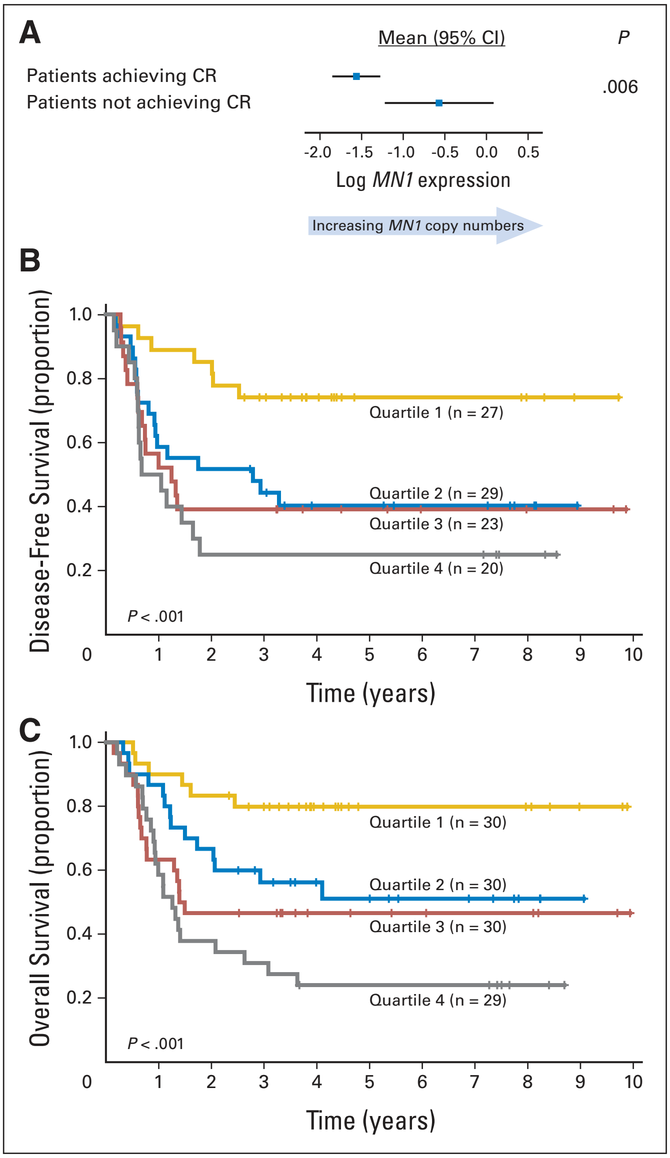

Fig 2. Outcome of cytogenetically normal acute myeloid leukemia (CN-AML) patients according to the meningioma 1 (*MN1*) gene expression levels. (A) Comparison of *MN1* expression in patients who achieved a complete remission (CR) compared with patients who did not achieve a CR. The direction of the correlation is shown by displaying the mean *MN1* expression and corresponding 95% CIs. (B) Disease-free survival of CN-AML patients according to quartile value of *MN1* expression levels. CR rates for each quartile are as follows: 90%, 97%, 77%, 69% for Q1, Q2, Q3, Q4, respectively. (C) Overall survival of CN-AML patients according to quartile value of *MN1* expression levels. For display purposes, *MN1* expression was treated as a categoric variable (patients were grouped according to the *MN1* copy quartiles from the lowest [quartile 1] to the highest [quartile 4] and Kaplan-Meier plots were generated). *P* values evaluate the trend in survival across *MN1* expression quartiles.

ABCB1 (*MDR1*). *MN1* expression levels were negatively associated with expression of *HOX* genes (ie, *HOXA2*, *HOXA3*, *HOXA4*, *HOXA5*, and *MEIS1*) that have also been reported to be expressed at lower levels in *NPM1* wild-type patients.[37] Thus, the microarray data were consistent with the association between higher *MN1* levels and high *BAALC* expresser and *NPM1* wild-type status observed at diagnosis in our patients (Table 1; Fig 1).

Using GO (www.geneontology.org), a project that groups together genes (referred to as members) participating in specific biologic processes (referred to as terms), we tested separately which terms were over-represented among the genes positively and negatively correlated with *MN1* expression levels. An over-represented term is one for which more members assigned to that term are found in the microarray signature than expected by chance. Thus, over-represented terms may provide insight into the biologic functions of the gene-expression signature associated with *MN1* expression changes. Sixteen GO terms were over-represented among the 261 gene probes positively correlated with *MN1* expression (Appendix Table A2, online only). Most of the 16 GO terms were related to the macrophage immune function of antigen processing and presentation.[38] Twenty-nine GO terms were over-represented among the 294 probe sets that negatively correlated with *MN1* expression (Appendix Table A2). Among those 29 GO terms, most were related to DNA, chromatin or chromosome organization, and tissue and organ development.

We derived an *MN1*-associated microRNA-expression signature comprising 15 microRNAs (Appendix Table A3, online only; Fig 4). Of the 15 microRNA probes, expression of 8 was positively and expression of 7 negatively correlated with *MN1* expression. Five of 8 microRNA probes positively associated with *MN1* expression corresponded to the *hsa-miR-126* family (including both *hsa-miR-126* and

Table 2. Multivariable Analyses for Clinical Outcome

Variables in Final Model by End Point	HR/OR	95% CI	*P*
CR*			
MN1 expression	0.54	0.35 to 0.83	.005
WBC	0.52	0.33 to 0.82	.005
DFS†			
MN1 expression	1.35	1.06 to 1.72	.01
WT1, mutated *v* wild-type	3.16	1.28 to 7.81	.01
FLT3-ITD, positive *v* negative	2.18	1.03 to 4.61	.02‡
ERG expression, high *v* low	1.99	1.03 to 3.84	.04
OS§			
MN1 expression	1.27	1.01 to 1.58	.04
WT1, mutated *v* wild-type	6.00	2.80 to 12.86	< .001‡
NPM1, wild-type *v* mutated	2.23	1.04 to 4.76	.04
FLT3-ITD, positive *v* negative	2.70	1.41 to 5.17	.01‡
WBC	1.75	1.34 to 2.28	< .001

NOTE. ORs < 1.0 mean lower CR rate for the higher values of the continuous variables. HRs > 1.0 indicate higher risk for an event for the higher values of the continuous variables and the first category listed for the categorical variables.

Abbreviations: CR, complete remission; DFS, disease-free survival; OS, overall survival; HR, hazard ratio; OR, odds ratio; *MN1*, natural log transformation of normalized *MN1* copy numbers; WBC, white blood count in 50 unit increments; *FLT3*-ITD, internal tandem duplication of the *FLT3* gene.

*Variables considered in the model based on univariable analyses were *MN1*, *FLT3*-ITD (positive *v* negative), age, hemoglobin, WBC (50 unit increments), and race.

†Variables considered in the model based on univariable analyses were *MN1*, *ERG* expression, *WT1* (mutated *v* wild-type), *FLT3*-ITD (positive *v* negative), WBC (50 unit increments), race, and *NPM1* (wild-type *v* mutated).

‡Does not meet the proportional hazards assumption. For DFS, the hazard ratio for *FLT3*-ITD is reported at 9 months (was not significant after this time point). for OS, the hazard ratio for *WT1* is reported at 9 months (not significant before this time point), *FLT3*-ITD is reported at 1 year (not significant after this time point).

§Variables considered in the model based on univariable analyses were *MN1*, *ERG* expression (high *v* low), *FLT3*-ITD (positive *v* negative), *WT1* (mutated *v* wild-type), *BAALC* expression (high *v* low), WBC (50 unit increments), age, hemoglobin, percentage of blood blasts, extramedullary involvement, and *NPM1* (wild-type *v* mutated).

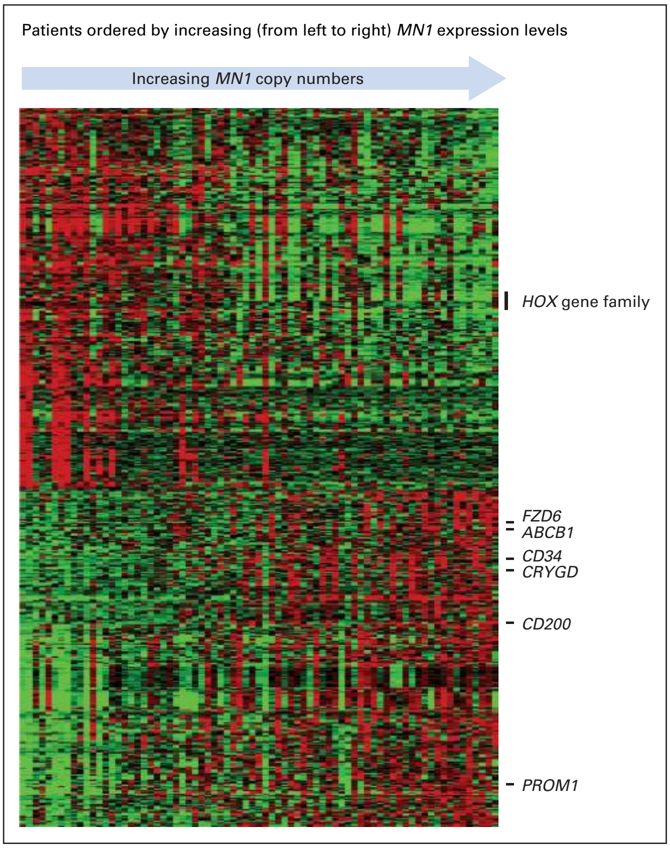

Fig 3. Heat map of gene probe sets that correlated significantly with the meningioma 1 (*MN1*) gene expression. Expression values of the probe sets are represented by color, with green indicating expression below and red expression above the median value for the given probe set. For display purposes, the expression values of the probe sets were centered so that each probe set has the same median expression value. Rows represent probe sets and columns represent patients. Patients are ordered according to *MN1* expression levels measured by real-time reverse transcriptase polymerase chain reaction.

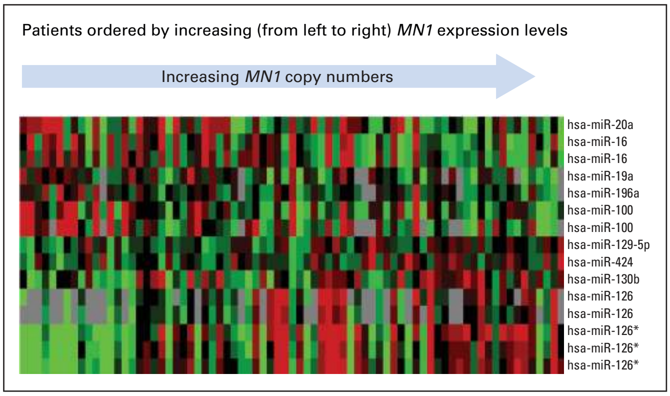

Fig 4. Heat map of microRNA probes that correlated significantly with the meningioma 1 (*MN1*) gene expression. Expression values of the probes are represented by color, with green indicating expression below and red expression above the median value for the given probe, and gray indicating a missing value. For display purposes, the expression values of the probes were centered so that each probe has the same median expression value. Rows represent probes and columns represent patients. Patients are ordered according to *MN1* expression levels measured by real-time reverse transcriptase polymerase chain reaction.

*hsa-miR-126**). This microRNA family was recently reported to enhance the proangiogenic activity of VEGF and regulate new blood vessel formation.[39,40] We also noted upregulation of *hsa-miR-424*, a regulator of monocyte and macrophage differentiation.[41] Among the microRNA probes negatively correlated with *MN1*, we found microRNAs involved in apoptosis (ie, *hsa-miR-16*)[42] or malignant transformation (ie, *hsa-miR-19a* and *hsa-miR-20a* members of the *miR-17-92* polycistron)[43,44] in addition to other microRNAs with unknown gene targets (ie, *hsa-miR-100* and *hsa-miR-196a*).

DISCUSSION

High levels of *MN1* expression were recently reported to negatively impact on outcome of CN-AML patients.[14] To our knowledge, these results have not yet been independently validated. Thus, we tested the prognostic value of *MN1* expression levels in younger CN-AML patients enrolled on similar CALGB first-line treatment protocols. We showed that the levels of *MN1* expression directly correlated with the risk of failing remission induction chemotherapy, relapse, and/or death, and predicted outcome independently of other clinical and molecular variables, thereby confirming the initial observation by Heuser et al.[14] All patients survived ≥ 30 days and were assessable for disease response after treatment. Therefore, the value of *MN1* expression to predict treatment-related mortality was not assessed.

The two studies presented several methodologic differences. We analyzed exclusively patients diagnosed with primary AML, whereas Heuser et al[14] also included patients with secondary AML. The patients in our study were similarly treated on two CALGB protocols that included consolidation treatment with autologous SCT (ASCT) or, in those few cases where ASCT was not possible, intensive consolidation chemotherapy. Patients who underwent allogeneic SCT in first CR were excluded. The German study included patients who underwent allogeneic SCT in addition to those receiving consolidation with ASCT or intensive chemotherapy.[14] In the German study,[14] *MN1* expression levels were measured using both BM and blood samples, and comparison of outcome was performed between higher and lower *MN1* expressers, dichotomized at the median value of *MN1* expression. In our study, only BM samples were analyzed, and we considered *MN1* expression as a continuous variable to avoid the need to adjust for different tissue types and eliminate the necessity of choosing arbitrary cutoff values to define groups of patients for comparison. Finally, Heuser et al[14] analyzed patients only for *FLT3*-ITD, *FLT3*-TKD, *MLL*-PTD, and *NPM1* mutations along with *MN1* expression. In addition to these molecular markers, we also analyzed *WT1* and *CEBPA* mutations, and *ERG* and *BAALC* expression levels. Despite these differences, the two studies were remarkably similar in their conclusions regarding the association of higher *MN1* levels with wild-type *NPM1* and poor outcome. However, while Heuser et al[14] showed that *MN1* expression was the only molecular marker that remained predictive of outcome in the final bivariable and multivariable models, we found that *MN1* expression provided prognostic information additional to that provided by *FLT3*-ITD and *WT1* mutations (for DFS and OS), high *ERG* expression (for DFS), and *NPM1* mutations (for OS).

Previous studies reported *MN1* as a fusion partner in the *MN1/ETV6* chimeric gene in t(12;22), and to be overexpressed in inv(16) AML.[11,12] *MN1* overexpression was shown to confer resistance to the differentiation activity of all-trans-retinoic acid (ATRA) in AML.[13]

Although murine models have in part recapitulated the ATRA-resistant phenotype of human *MN1*-associated AML, little is known about the mechanism through which aberrant expression of *MN1* drives myeloid leukemogenesis.[11,13] Thus, to gain insight into the functional significance of *MN1* expression in AML, we derived gene and microRNA profiles that correlated with *MN1* expression levels.

The gene-expression signature associated with *MN1* expression comprised 555 probes. Notably, *BAALC* was among genes that correlated most strongly with *MN1* expression. At diagnosis, high *BAALC* expressers indeed had higher levels of *MN1* expression (Table 1; Fig 1). Consistent with this finding, we observed similarities between a signature associated with *BAALC* expression that we recently reported[30] and the signature associated with *MN1* expression. Associated with higher *MN1* and *BAALC* expression were *PROM1*, *CD34*, *FZD6*, and *CRYGD* (genes expressed in noncommitted hematopoietic precursors), *CD200* (associated with poor outcome in AML), and *ABCB1* (involved in chemoresistance). Furthermore, in a comparative GO analysis (not shown), eight GO terms related to DNA, chromatin, and chromosome assembly, and organization were over-represented among the genes downregulated in both the *BAALC* and *MN1* gene-expression signatures. These findings suggest a potential functional interplay between *MN1* and *BAALC* in their contribution to myeloid leukemogenesis.

Despite the aforementioned similarities, the leukemogenic mechanisms associated with aberrant expression of *MN1* and *BAALC* are unlikely to be identical. Using GO analysis, we showed that genes involved in antigen processing and presentation were positively associated with the *MN1*, but not *BAALC*, gene-expression signature. Among those, there were genes encoding both MHC class I and class II proteins and CD74 that are central to the mechanisms of antigen processing and presentation for T-cell activation by macrophage and dendritic cells.[38] Interestingly, higher *MN1* expression was also associated with higher expression of *hsa-miR-424*, which is transactivated by SPI1 (PU.1) and upregulated during monocyte/macrophage differentiation.[41] We have recently published data suggesting that overexpression of certain microRNAs that potentially target genes encoding Toll-like receptors and *IL1B*, which also participate in macrophage and dendritic cell activation, are associated with worse prognosis.[33] Altogether, these data suggest that aberrant activation of mechanisms involved in both native and acquired immunologic response may play a role in sustaining myeloblast proliferation and survival.

Among the eight microRNA probes whose higher expression was associated with higher *MN1* expression, five corresponded to *miR-126* family members. *hsa-miR-126* and *hsa-miR-126** are generated from the splicing and processing of intron 7 of the *EGFL7* gene.[40,45] Consistent with these data, we observed that *MN1* expression positively correlated with expression of both *EGFL7* and *hsa-miR-126*. A leukemogenic role for *hsa-miR-126* has hitherto not been reported. However, two recent studies have shown that *hsa-miR-126* regulates vascular integrity and angiogenesis by repressing negative regulators of the VEGF pathways.[39,40] Whether aberrant activation of these mechanisms can contribute to leukemogenesis and impact the treatment response and outcome of CN-AML patients remains to be determined. Finally, since *hsa-miR-16* targets the antiapoptotic *BCL2* gene and is downregulated in cancer patients with poor outcome,[42] it is not surprising that lower *hsa-miR-16* expression was associated with higher *MN1* expression predicting treatment resistance and worse outcome. It was somewhat surprising, however, that lower expression levels of *hsa-miR-19a* and *hsa-miR-20,* both part of the *hsa-miR17-92* cluster, were associated with higher *MN1* levels as this cluster was previously reported to be overexpressed in aggressive neoplasms (ie, B-cell lymphoma and lung cancer) and function as an oncogene.[43,44]

In summary, we show that higher *MN1* expression is associated with wild-type *NPM1*, higher *BAALC* expression and worse outcome in CN-AML independent of other prognostic molecular markers. Patients with higher *MN1* expression appear to share biologic features with patients with higher *BAALC* expression, namely upregulation of genes involved in chemoresistance in noncommitted hematopoietic precursors, and/or those with wild-type *NPM1* (ie, lower expression of *HOX* genes). Aberrant *MN1* expression seemingly contributes to leukemogenesis by affecting mechanisms of monocytic/macrophage function and differentiation. Validation of these findings in preclinical models and larger clinical studies may lead to the designing of novel therapies targeting activation of these potentially leukemogenic mechanisms by *MN1* overexpression.

AUTHORS' DISCLOSURES OF POTENTIAL CONFLICTS OF INTEREST

The author(s) indicated no potential conflicts of interest.

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REFERENCES

1. Byrd JC, Mrózek K, Dodge RK, et al: Pretreatment cytogenetic abnormalities are predictive of induction success, cumulative incidence of relapse, and overall survival in adult patients with de novo acute myeloid leukemia: Results from Cancer and Leukemia Group B (CALGB 8461). Blood 100:4325-4336, 2002

2. Grimwade D, Walker H, Oliver F, et al: The importance of diagnostic cytogenetics on outcome in AML: Analysis of 1,612 patients entered into the MRC AML 10 trial. Blood 92:2322-2333, 1998

3. Slovak ML, Kopecky KJ, Cassileth PA, et al: Karyotypic analysis predicts outcome of preremission and postremission therapy in adult acute myeloid leukemia: A Southwest Oncology Group/

Eastern Cooperative Oncology Group study. Blood 96:4075-4083, 2000

4. Mrózek K, Heerema NA, Bloomfield CD: Cytogenetics in acute leukemia. Blood Rev 18:115-136, 2004

5. Mrózek K, Marcucci G, Paschka P, et al: Clinical relevance of mutations and gene-expression changes in adult acute myeloid leukemia with normal cytogenetics: Are we ready for a prognostically prioritized molecular classification? Blood 109:431-448, 2007

6. van Wely KHM, Molijn AC, Buijs A, et al: The MN1 oncoprotein synergizes with coactivators RAC3 and p300 in RAR-RXR-mediated transcription. Oncogene 22:699-709, 2003

7. Sutton ALM, Zhang X, Ellison TI, et al: The 1,25(OH)$_2$D$_3$-regulated transcription factor MN1 stimulates vitamin D receptor-mediated transcription and inhibits osteoblastic cell proliferation. Mol Endocrinol 19:2234-2244, 2005

8. Lekanne Deprez RH, Riegman PHJ, Groen NA, et al: Cloning and characterization of *MN1*, a gene from chromosome 22q11, which is disrupted by a balanced translocation in a meningioma. Oncogene 10:1521-1528, 1995

9. Buijs A, Sherr S, van Baal S, et al: Translocation (12;22)(p13;q11) in myeloproliferative disorders results in fusion of the ETS-like *TEL* gene on 12p13 to the *MN1* gene on 22q1. Oncogene 10:1511-1519, 1995

10. Valk PJM, Verhaak RGW, Beijen MA, et al: Prognostically useful gene-expression profiles in acute myeloid leukemia. N Engl J Med 350:1617-1628, 2004

11. Carella C, Bonten J, Sirma S, et al: MN1 overexpression is an important step in the development of inv(16) AML. Leukemia 21:1679-1690, 2007

12. Carella C, Bonten J, Rehg J, et al: MN1-TEL, the product of the t(12;22) in human myeloid leukemia, immortalizes murine myeloid cells and causes myeloid malignancy in mice. Leukemia 20:1582-1592, 2006

13. Heuser M, Argiropoulos B, Kuchenbauer F, et al: MN1 overexpression induces acute myeloid leukemia in mice and predicts ATRA resistance in patients with AML. Blood 110:1639-1647, 2007

14. Heuser M, Beutel G, Krauter J, et al: High meningioma 1 (*MN1*) expression as a predictor for poor outcome in acute myeloid leukemia with normal cytogenetics. Blood 108:3898-3905, 2006

15. Kolitz JE, George SL, Dodge RK, et al: Dose escalation studies of cytarabine, daunorubicin, and etoposide with and without multidrug resistance modulation with PSC-833 in untreated adults with acute myeloid leukemia younger than 60 years: Final induction results of Cancer and Leukemia Group B study 9621. J Clin Oncol 22:4290-4301, 2004

16. Kolitz JE, George SL, Marcucci G, et al: A randomized comparison of induction therapy for untreated acute myeloid leukemia (AML) in patients < 60 years using P-glycoprotein (Pgp) modulation with Valspodar (PSC833): Preliminary results of Cancer and Leukemia Group B study 19808. Blood 106:122a-123a, 2005 (abstr 407)

17. Mrózek K, Carroll AJ, Maharry K, et al: Central review of cytogenetics is necessary for cooperative group correlative and clinical studies of adult acute leukemia: The Cancer and Leukemia Group B experience. Int J Oncol 33:239-244, 2008

18. Whitman SP, Archer KJ, Feng L, et al: Absence of the wild-type allele predicts poor prognosis in adult *de novo* acute myeloid leukemia with normal cytogenetics and the internal tandem duplication of *FLT3*: A Cancer and Leukemia Group B study. Cancer Res 61:7233-7239, 2001

19. Thiede C, Steudel C, Mohr B, et al: Analysis of FLT3-activating mutations in 979 patients with acute myelogenous leukemia: Association with FAB subtypes and identification of subgroups with poor prognosis. Blood 99:4326-4335, 2002

20. Yamamoto Y, Kiyoi H, Nakano Y, et al: Activating mutation of D835 within the activation loop of FLT3 in human hematologic malignancies. Blood 97:2434-2439, 2001

21. Whitman SP, Ruppert AS, Radmacher MD, et al: *FLT3* D835/I836 mutations are associated with poor disease-free survival and a distinct gene-expression signature among younger adults with de novo cytogenetically normal acute myeloid leukemia lacking *FLT3* internal tandem duplications. Blood 111:1552-1559, 2008

22. Döhner K, Schlenk RF, Habdank M, et al: Mutant nucleophosmin (*NPM1*) predicts favorable prognosis in younger adults with acute myeloid leukemia and normal cytogenetics: Interaction with other gene mutations. Blood 106:3740-3746, 2005

23. Marcucci G, Maharry K, Radmacher MD, et al: Prognostic significance of, and gene and microRNA expression signatures associated with, *CEBPA* mutations in cytogenetically normal acute myeloid leukemia with high-risk molecular features: A Cancer and Leukemia Group B study. J Clin Oncol 26:5078-5087, 2008

24. Paschka P, Marcucci G, Ruppert AS, et al: Wilms' tumor 1 gene mutations independently predict poor outcome in adults with cytogenetically normal acute myeloid leukemia: A Cancer and Leukemia Group B study. J Clin Oncol 26:4595-4602, 2008

25. Caligiuri MA, Strout MP, Schichman SA, et al: Partial tandem duplication of *ALL1* as a recurrent molecular defect in acute myeloid leukemia with trisomy 11. Cancer Res 56:1418-1425, 1996

26. Whitman SP, Ruppert AS, Marcucci G, et al: Long-term disease-free survivors with cytogenetically normal acute myeloid leukemia and *MLL* partial tandem duplication: A Cancer and Leukemia Group B study. Blood 109:5164-5167, 2007

27. Marcucci G, Baldus CD, Ruppert AS, et al: Overexpression of the ETS-related gene, *ERG*, predicts a worse outcome in acute myeloid leukemia with normal karyotype: A Cancer and Leukemia Group B study. J Clin Oncol 23:9234-9242, 2005

28. Marcucci G, Maharry K, Whitman SP, et al: High expression levels of the *ETS*-related gene, *ERG*, predict adverse outcome and improve molecular risk-based classification of cytogenetically normal acute myeloid leukemia: A Cancer and Leukemia Group B study. J Clin Oncol 25:3337-3343, 2007

29. Baldus CD, Tanner SM, Ruppert AS, et al: *BAALC* expression predicts clinical outcome of de novo acute myeloid leukemia patients with normal cytogenetics: A Cancer and Leukemia Group B study. Blood 102:1613-1618, 2003

30. Langer C, Radmacher MD, Ruppert AS, et al: High *BAALC* expression associates with other molecular prognostic markers, poor outcome and a distinct gene-expression signature in cytogenetically normal patients younger than 60 years with acute myeloid leukemia: A Cancer and Leukemia Group B (CALGB) study. Blood 111:5371-5379, 2008

31. Radmacher MD, Marcucci G, Ruppert AS, et al: Independent confirmation of a prognostic gene-expression signature in adult acute myeloid leukemia with a normal karyotype: A Cancer and Leukemia Group B study. Blood 108:1677-1683, 2006

32. Calin GA, Ferracin M, Cimmino A, et al: A microRNA signature associated with prognosis and progression in chronic lymphocytic leukemia. N Engl J Med 353:1793-1801, 2005

33. Marcucci G, Radmacher MD, Maharry K, et al: MicroRNA expression in cytogenetically normal acute myeloid leukemia. N Engl J Med 358:1919-1928, 2008

34. Klein JP, Moeschberger ML: Survival Analysis: Techniques for Censored and Truncated Data. New York, NY, Springer-Verlag, 1997

35. Irizarry RA, Bolstad BM, Collin F, et al: Summaries of Affymetrix GeneChip probe level data. Nucleic Acids Res 31:e15, 2003

36. Dahlquist KD, Salomonis N, Vranizan K, et al: GenMAPP, a new tool for viewing and analyzing microarray data on biological pathways. Nat Genet 31:19-20, 2002

37. Verhaak RGW, Goudswaard CS, van Putten W, et al: Mutations in nucleophosmin (*NPM1*) in acute myeloid leukemia (AML): Association with other gene abnormalities and previously established gene expression signatures and their favorable prognostic significance. Blood 106:3747-3754, 2005

38. Vyas JM, Van der Veen AG, Ploegh HL: The known unknowns of antigen processing and presentation. Nat Rev Immunol 8:607-618, 2008

39. Fish JE, Santoro MM, Morton SU, et al: miR-126 regulates angiogenic signaling and vascular integrity. Dev Cell 15:272-284, 2008

40. Wang S, Aurora AB, Johnson BA, et al: The endothelial-specific microRNA miR-126 governs vascular integrity and angiogenesis. Dev Cell 15: 261-271, 2008

41. Rosa A, Ballarino M, Sorrentino A, et al: The interplay between the master transcription factor PU.1 and miR-424 regulates human monocyte/macrophage differentiation. Proc Natl Acad Sci U S A 104:19849-19854, 2007

42. Cimmino A, Calin GA, Fabbri M, et al: *miR-15* and *miR-16* induce apoptosis by targeting BCL2. Proc Natl Acad Sci U S A 102:13944-13949, 2005

43. O'Donnell KA, Wentzel EA, Zeller KI, et al: C-Myc-regulated microRNAs modulate E2F1 expression. Nature 435:839-843, 2005

44. Hayashita Y, Osada H, Tatematsu Y, et al: A polycistronic microRNA cluster, *miR-17-92*, is overexpressed in human lung cancers and enhances cell proliferation. Cancer Res 65:9628-9632, 2005

45. Musiyenko A, Bitko V, Barik S: Ectopic expression of miR-126*, an intronic product of the vascular endothelial EGF-like 7 gene, regulates prostein translation and invasiveness of prostate cancer LNCaP cells. J Mol Med 86:313-322, 2008

