Estrogen Receptor Methylation Is Associated with Improved Survival in Adult Acute Myeloid Leukemia[1]

Qing Li, Kenneth J. Kopecky, Avinash Mohan, Cheryl L. Willman, Frederick R. Appelbaum, James K. Weick, and Jean-Pierre J. Issa[2]

The Johns Hopkins Oncology Center, Baltimore, Maryland 21231 [Q. L., A. M., J-P. J. I.]; University of New Mexico School of Medicine, Albuquerque, New Mexico 87131 [C. L. W.]; Southwest Oncology Group Statistical Center, Seattle, Washington 98104; and Southwest Oncology Group Leukemia and Leukemia Programs, San Antonio, Texas 78245 [K. J. K., C. L. W., F. R. A., J. K. W.]

ABSTRACT

Estrogen receptor methylation (ERM) is a frequent molecular alteration in adult acute myeloid leukemia (AML). In this study, we sought to determine the clinical characteristics and prognostic significance of ERM in AML. ERM was determined for 268 patients who had leukemic blasts available for molecular analysis. ERM was measured by Southern blot analysis, and results were obtained for 261 patients (ages 17–69). ERM ranged from 0–99.1%, with a median of 25%. One hundred sixty patients (61%) had ERM values over 15% and were considered ERM+. In a subset of patients analyzed, ERM+ samples had markedly lower *ER* gene expression compared with ERM− samples. In multiple regression analyses of patient and disease characteristics at diagnosis, two factors had significant independent association with ERM: ERM decreased with increasing age ($P = 0.0001$) and was significantly lower in patients with French-American-British classification M4 or M5 ($P = 0.0019$). In regression analyses of outcome measures, ERM had no significant impact on complete remission rate after initial induction therapy. However, ERM+ patients had significantly better overall survival [OS; 18% at 6 years; 95% confidence interval (CI), 12–24% *versus* 9%; CI, 3–14% for ERM− patients; $P = 0.022$]. In multiple regression analyses, OS increased with increasing ERM ($P = 0.0044$). Similar results were seen for relapse-free survival (23% at 6 years; CI, 15–32% for ERM+ *versus* 10%; CI, 2–19% for ERM−), although the effect of ERM was not statistically significant ($P = 0.15$ in multiple regression analysis). Our results indicate that ERM at diagnosis may be a favorable prognostic factor for OS in adult AML.

INTRODUCTION

Cytosine methylation within the normally unmethylated promoters of selected genes has recently emerged as one of the most constant molecular changes in neoplasia, in general (1, 2), and leukemias, in particular (3). Such promoter methylation seems to be an alternate mechanism to coding region mutations in inactivating tumor-suppressor and other genes in neoplasia. The *P16* gene, for example, has recently been shown to be a tumor-suppressor gene for which promoter methylation and transcriptional inactivation are relatively common in multiple types of neoplasms (4–8). The *ER*[3] gene is another locus for which aberrant methylation has been described in multiple tumors, including hematopoietic malignancies (3).

The causes of aberrant methylation in cancer and the clinical determinants associated with such methylation have not been well defined. Observations in an animal model of lung cancer have suggested that, for the *ER* gene, methylation was partially dependent on the carcinogenic insult that induced tumor formation (9). In this model, spontaneous and radiation-induced tumors were much more likely to be methylated than tumors induced by the tobacco-derived carcinogen, NNK. These data suggested the possibility that ERM may be associated with specific clinical characteristics in human tumors, as well. In addition, the high frequency of promoter methylation at selected loci in cancer has raised the possibility that, similar to other molecular defects such as mutations, loss of heterozygosity, aneuploidy, or karyotypic abnormalities, this readily detectable DNA change could also be used as a prognostic factor and to supplement conventional clinical staging (3).

To explore these various possibilities, we have studied ERM in a well characterized and uniformly treated population of patients with AML. Previous data had indicated that ERM was very low (<10%) in normal peripheral blood or bone marrow-derived cells, but relatively common (70%) in patients with AML (10). We now show that ERM in AML is associated with specific clinical parameters, such as age and FAB classi-

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[2] To whom requests for reprints should be addressed, at The Johns Hopkins Oncology Center, 424 North Bond Street, Baltimore, MD 21231. Phone: (410) 955-8506; Fax: (410) 614-9884; E-mail: jpissa@welchlink.welch.jhu.edu.

[3] The abbreviations used are: ER, estrogen receptor; ERM, ER methylation; AML, acute myeloid leukemia; SWOG, Southwest Oncology Group; S8600, SWOG study 8600; CR, complete response; OS, overall survival; PH, proportional hazard; RFS, relapse-free survival; RT-PCR, reverse transcription-PCR; CI, confidence interval; FAB, French-American-British; GAPDH, glyceraldehyde-3-phosphate dehydrogenase.

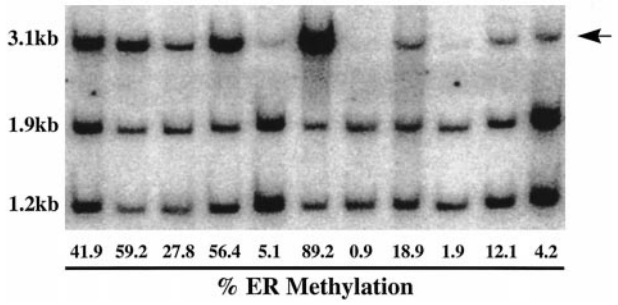

Fig. 1 Representative Southern blot of ERM in AML. DNA was digested with the restriction enzymes *Eco*RI and *Not*I (which is methylation-sensitive), electrophoresed on an agarose gel, transferred to a nylon membrane, and probed with a fragment of exon 1 of the *ER* gene. The band at 3.1 kb (*arrow*) indicates failure to digest with *Not*I, due to methylation of the *Not*I recognition site. The relative density of this band is indicated below each *lane* as percentage of ERM.

fication, and is an independent favorable prognostic factor for survival in this disease.

MATERIALS AND METHODS

Patients. S8600 compared high-dose *versus* standard dose 1-β-D-arabinofuranosylcytosine with daunorubicin in adult patients with previously untreated AML, including both *de novo* AML and secondary AML (11). S8600 was largely limited to patients <65-years-old, although a small number of older patients participated in an early pilot phase of the study. The results of S8600 have been reported (11). As part of the study protocol, pretreatment leukemic blasts derived from either peripheral blood or bone marrow were stored for future studies. For the present study, we selected all patients with centrally confirmed diagnoses of AML who had at least three vials of stored blasts available for analysis. Of 774 patients on S8600 with confirmed AML, 267 fulfilled this criterion. One additional patient who received the S8600 standard dose induction regimen on a different SWOG trial was also included in this study. In both trials, written informed consent in accordance with institutional and Department of Health and Human Services policies was obtained from all patients.

Measurement of ERM. DNA was extracted from mononuclear cell fractions using standard techniques. As in previous studies, Southern blots were used to determine the methylation state of the *ER* CpG island (10). This island contains a cluster of methylation-sensitive restriction enzymes. We used one of these enzymes, *Not*I, to study the methylation state of the island. *Not*I will digest DNA to completion if the two CG sites in its recognition sequence are unmethylated, but will not cut DNA if either of the two CG sites are methylated. Briefly, 5 μg of genomic DNA were digested with 50 units of *Eco*RI and 100 units of *Not*I for 16 h as specified by the manufacturer (New England Biolabs), run on a 1% agarose gel, transferred to a Zetaprobe nylon membrane (Bio-Rad), and probed with a 428-bp fragment of the first exon of the *ER* gene obtained by PCR amplification from normal genomic DNA using primers AACCTCGGGCTGTGCTCTTTTTCCAG (upper) and AGTAGCATCAGCGGGCTCGGAGACAC (lower). The Southern blots were then exposed on a phosphor screen for 2–3 days and developed using a Phosphorimager (Molecular Dynamics). For quantification of ERM, the relative density of the methylation band at 3.1 kb was measured using the ImageQuant software (Molecular Dynamics) and expressed as a percentage of the density of all bands (3.1 kb, 1.9 kb, and 1.2kb) in each lane. To rule out incomplete digestion with *Not*I, all of the blots were reprobed with a 5′ fragment of the c-*abl* gene, which contains two *Not*I sites in its 5′ CpG island. In all cases, an expected 5-kb band was the only band present, indicating complete digestion of the DNA with *Not*I.

RT-PCR. Total RNA was prepared using standard techniques. Total RNA (6 μg) was used to generate cDNA using random hexamers and M-MuLV reverse transcriptase enzyme, as recommended by the manufacturer (Boehringer Mannheim). About one-tenth of the cDNA was used as a template to amplify a 430-bp fragment specific to the *ER* gene (10). Another one-thirtieth of cDNA product was used to amplify a 306-bp fragment specific to the *GAPDH* gene transcript as a control for RNA integrity. The primers used are: CGGAGTCAACGGATTTGGTCGTAT (upper) and AGCCTTCTCCATGGTGGTGAAGAC (lower) for GAPDH. All amplifications were performed at least twice, and all included a positive control consisting of mRNA from the ER-positive breast cancer cell line ZR75 and a negative control consisting of mRNA from an ER-negative colon cancer cell line, RKO. Reverse transcription (-) controls, where the reverse transcriptase enzyme was omitted, were also used for each sample.

Statistical Analysis. Demographic, clinical, and outcome data for all patients were collected and evaluated according to standard procedures of the SWOG. Effects of patient and disease characteristics on ERM were examined using least squares regression analysis, two-sample *t* tests, and ANOVA. Preliminary examination indicated that the ERM values had a rather skewed distribution. Therefore, to more nearly satisfy the assumption of a normal (Gaussian) distribution under which least squares analysis is optimal, the ERM values were transformed to logits: logit(ERM) = log(ERM) − log(100 − ERM). Logits, which are often appropriate when the original data are proportions, proved to have a much more nearly Gaussian distribution. The effect of ERM on the probability of achieving CR was examined using Fisher's exact test and logistic regression analysis (12). OS was defined as the number of days from entry into the SWOG clinical trial until death from any cause, with observation censored at the date of last contact for patients last known to be alive. RFS was defined for patients who achieved CR as the number of days from establishment of CR until relapse or death from any cause, whichever occurred first, with observation censored at the date of last contact for patients last known to be alive without report of relapse. Distributions of OS and RFS were estimated by the method of Kaplan and Meier (13). The effects of ERM on OS and RFS were examined using PHs regression analysis (14). Because the skewness of the distribution of untransformed ERM values might inordinately increase the relative influence of the patients with extremely high ERM on the results of logistic or PHs regression analyses, parallel analyses were performed

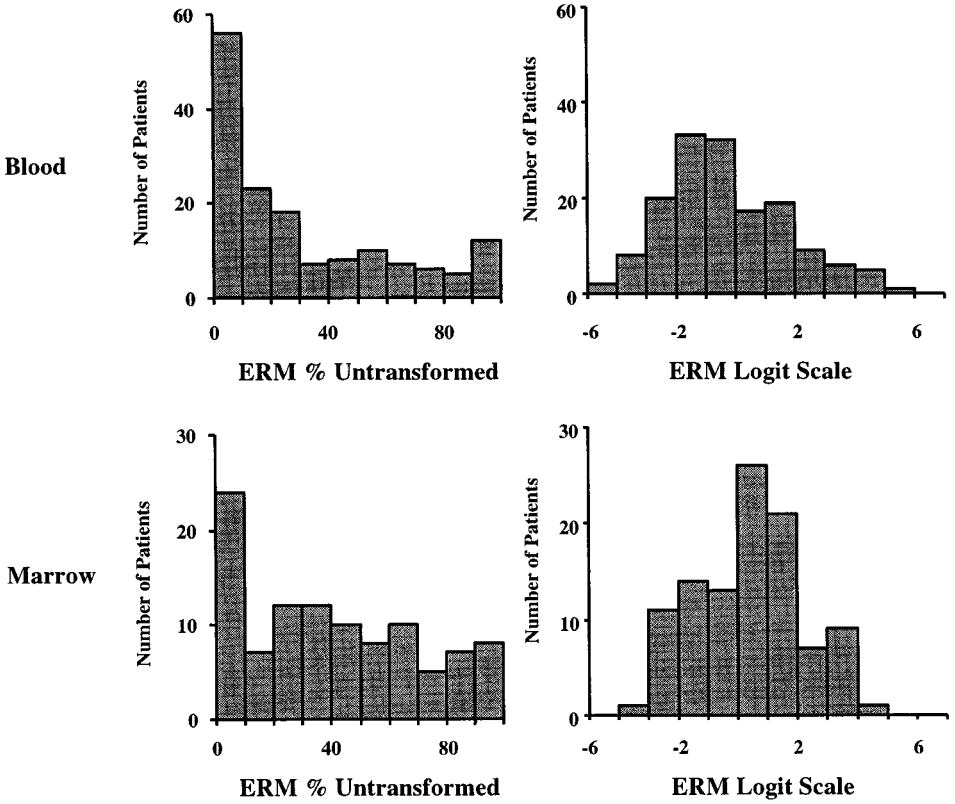

Fig. 2 Frequency distributions of untransformed and logit-transformed ERM values, by type of specimen.

using the untransformed and logit-transformed ERM values. Statistical significance was represented by two-tailed *P*s. OS and RFS results were based on data available on December 21, 1998.

RESULTS

ERM. Two hundred sixty-eight patients had leukemic blasts available for analysis. DNA was extracted from the leukemic blasts, and ERM was quantitatively determined using Southern blot analysis (examples in Fig. 1). The DNA from seven patients was degraded and unsuitable for ERM determination, leaving 261 cases for analysis. Of these 261 patients, blasts were obtained from the peripheral blood in 152 cases and from the bone marrow in 103 cases. The specimen type was unknown for six patients. As shown in Fig. 2, the distributions of ERM were somewhat skewed toward lower values, although they were approximately Gaussian after transformation to logits. In the entire group of 261 patients, ERM ranged from 0.3–99.1%, with a median of 24.7%. Because normal peripheral blood or bone marrow-derived cells have previously been shown to have up to 10% ERM (10), we arbitrarily chose 15% as a cutoff for calling a sample ERM+. In this analysis, 160 patients were ERM+ (61%), which is consistent with our previous findings. Patient characteristics are described in Table 1. Because S8600 was largely limited to younger patients, all but one were <65-years-old. Table 1 also summarizes the characteristics of 514 patients on S8600 with centrally confirmed diagnoses of AML who did not have ERM data, either because they had less than three vials of cryopreserved blasts (507 patients) or because their DNA was degraded (7 patients). The 261 patients with ERM data were similar in most respects to these 514 patients. They differed primarily in having relatively high WBC and peripheral blast counts, an artifact reflecting their selection on the basis of availability of stored cells: patients with low WBC and blast counts were less likely to have sufficient cells available for this study. FAB categories M6 and M0 were also somewhat less common among the 261 patients with ERM data.

ERM was significantly lower when based on blood (median, 18.8%; range, 0.3-99.1%) than marrow (median, 36.1%; range, 1.2- 97.2%; $P = 0.0077$ based on two-sample t test of logit-transformed values; see Fig. 2). Similarly, total WBC count [61.3 *versus* 30.7 (1000/μl)], peripheral blast percentage (68% *versus* 45%), and peripheral nonblast leukocyte count [16.2 *versus* 10.7 (1000/μl)] were higher in patients for whom blood specimens were submitted. These differences presumably arise because larger WBC counts increased the likelihood that a specimen of peripheral blood would be submitted. It is, therefore, possible that the lower ERM in samples based on blood is due to the larger number of nonblast (and possibly normal and unmethylated) leukocytes in these specimens. Alternatively, it is also possible that lower ERM in AML is associated with, or causes, a higher WBC count.

ERM and *ER* Gene Expression in AML. To determine whether differences in ERM status corresponded to potential

Table 1 Characteristics of 775 AML patients, by availability of ERM data and specimen type

	Patients with ERM data			Patients without ERM data[a] (n = 514)
	All with ERM data (n = 261)	Specimen type		
		Blood (n = 152)	Marrow (n = 103)	
Age (yr)				
Median	43	44	41	46
Range	17–69	17–64	18–69	15–72
Sex				
Male	150 (57%)	86 (57%)	59 (57%)	277 (54%)
Female	111 (43%)	66 (43%)	44 (43%)	237 (46%)
Race				
White	219 (84%)	127 (84%)	86 (83%)	439 (85%)
Black	28 (11%)	17 (11%)	11 (11%)	51 (10%)
Other	14 (5%)	8 (5%)	6 (6%)	24 (5%)
Histology (FAB)				
M1	58 (22%)	36 (24%)	22 (21%)	97 (19%)
M2	99 (38%)	54 (36%)	43 (42%)	196 (38%)
M3	17 (7%)	5 (3%)	12 (12%)	72 (14%)
M4	43 (16%)	25 (16%)	14 (14%)	54 (11%)
M5	34 (13%)	24 (16%)	10 (10%)	38 (7%)
M6	1 (<1%)	1 (1%)	0	20 (4%)
M7	1 (<1%)	1 (1%)	0	7 (1%)
M0	3 (1%)	3 (2%)	0	13 (3%)
Other	5 (2%)	3 (2%)	2 (2%)	17 (3%)
WBC (1000/μl)				
Median	48.6	61.3	30.7	9.3
Range	0.8–416	3.0–416	0.8–268	0.4–370
Peripheral blasts (%)				
Median	61	68	45	27
Range	0–99	0–99	0–98	0–99
Peripheral blasts (1000/μl)				
Median	20.0	26.5	9.2	2.0
Range	0.0–378	0.0–378	0.0–250	0.0–314
Hemoglobin (gm/dl)				
Median	9.4	9.6	9.1	9.2
Range	5.1–15.0	5.1–15.0	5.6–14.3	4.1–15.4
Platelets (1000/μl)				
Median	52	50	54	54
Range	3.0–700	7.0–700	3.0–333	2.0–347
Induction Ara-C dose				
200 mg/m^2	182 (70%)	109 (72%)	69 (67%)	347 (68%)
2.0 gm/m^2	62 (24%)	33 (22%)	28 (27%)	119 (23%)
3.0 gm/m^2	17 (7%)	10 (7%)	6 (6%)	48 (9%)
ERM (%)				
Median	24.7	18.8	36.1	
Range	0.3–99.1	0.3–99.1	1.2–97.2	
<15%	101 (39%)	71 (47%)	28 (27%)	
≥15%	160 (61%)	81 (53%)	75 (73%)	

[a] Includes 507 patients from S8600 without leukemic blasts available for study and 7 patients with degraded DNA.

functional differences among the AML samples, we measured ER expression by RT-PCR in a subset of the cases. Fifteen cases (seven ERM− and eight ERM+) were selected based on the availability of additional stored samples. RNA was degraded in one ERM− sample, leaving 14 cases for analysis. ER expression was very low or absent in all eight ERM+ cases, but present at significant levels in five of six ERM− cases (Fig. 3). These data suggest that ERM status may have functional significance in AML through differential expression of the ER gene.

ERM and Clinical Parameters. In simple linear regression analyses of the logit(ERM), which included an adjustment for the difference between blood and marrow specimens, ERM decreased significantly with increasing age (two-tailed $P = 0.0001$) and absolute nonblast leukocyte count ($P = 0.0005$) and was significantly heterogeneous among the six FAB categories [$P = 0.0006$ with M6, M7, M0, M0/M5, and Myeloid NOS (combined as a single category due to small numbers)]. The effect of FAB consisted primarily of lower ERM in the M4 and M5 categories, compared with the remaining categories ($P < 0.0001$). Marginally significant associations were seen with sex ($P = 0.079$, higher ERM among males), WBC count ($P = 0.043$, decreasing ERM with increasing WBCs), platelet count ($P = 0.079$, decreasing ERM with increasing count), and LRP expression ($P = 0.011$, decreasing ERM with increasing

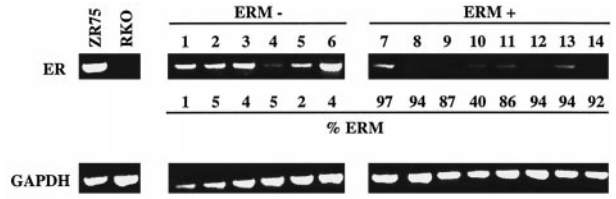

Fig. 3 Expression of *ER* in AML by ERM status. *ER* expression was determined by RT-PCR using primers that yield a fragment of 430 bp. Integrity of RNA was verified by RT-PCR using primers specific for the *GAPDH* gene (which yields a 306-bp fragment). All reactions were controlled using reverse transcription-negative samples where the reverse transcription enzyme was omitted (not shown). *ER* was expressed in ZR75 (positive control) and ERM- cases (AML1–6), but was very low or absent in RKO (negative control) and ERM+ cases (AML7–14). ERM values are indicated below each sample.

Table 2 ERM by specimen type, age, and FAB classification

Specimen type	Age	FAB	Number of patients	ERM (%) median	ERM (%) range	No. with ERM ≥15%
Blood	≤43	M4 or M5	27	12	1–94	13 (48%)
		Other	48	33	1–99	33 (69%)
	>43	M4 or M5	22	7	0–28	5 (23%)
		Other	55	23	1–97	30 (55%)
Marrow	≤43	M4 or M5	8	34	4–73	5 (63%)
		Other	47	55	4–97	41 (87%)
	>43	M4 or M5	16	23	2–81	9 (56%)
		Other	32	25	1–88	20 (63%)

Table 3 Treatment outcomes by ERM

ERM	CRs/ patients	CR	95% CI	Estimated OS at 5 yrs	95% CI	Estimated RFS at 5 yrs	95% CI
<15%	49/101	49%	39–58%	8%	3–13%	9%	1–17%
≥15%	95/160	59%	52–67%	18%	12–24%	23%	15–32%
Unknown	273/514	53%	49–58%	19%	15–22%	17%	13–22%

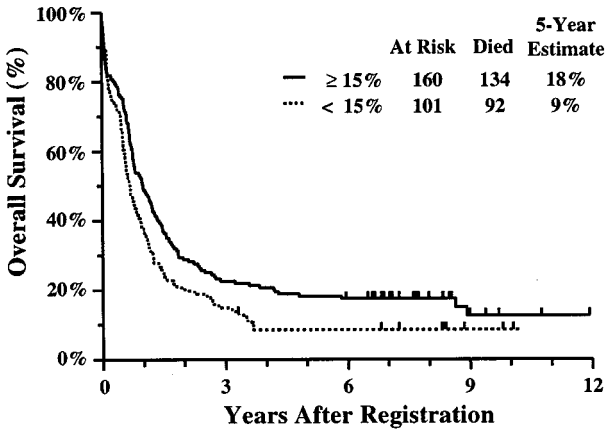

Fig. 4 OS by ERM status. The *solid line* represents ERM-positive (≥15%) cases, whereas the *dashed line* represents ERM-negative (<15%) cases. *Tickmarks* indicate censored observations.

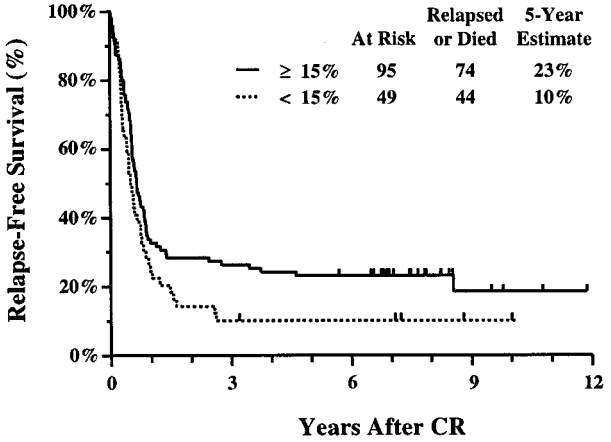

Fig. 5 RFS by ERM status. The *solid line* represents ERM-positive (≥15%) cases, whereas the *dashed line* represents ERM-negative (<15%) cases. *Tickmarks* indicate censored observations.

expression). ERM was not significantly associated with any of the other variables, including CD34 expression ($P = 0.13$) and multiple multidrug resistance markers such as expression of MRK16, MM4.17, and MRP, and functional efflux of $DiOC_2$ and Rhodamine (results not shown). In addition, there was no artifactual variation of ERM in relation to induction ara-C dose ($P = 0.60$).

In multiple regression analysis (also including adjustment for specimen type), only two factors retained clearly significant independent associations with ERM: logit(ERM) decreased with increasing age ($P = 0.0001$) and was significantly lower in patients with M4 or M5 AML ($P = 0.0001$). The difference between blood and marrow specimens remained marginally significant in this multiple regression model ($P = 0.026$). The effects of these variables on ERM are demonstrated in Table 2.

ERM and Response to Therapy. Of the 261 patients, 144 (55%) achieved CR. One hundred eight patients (41%) achieved CR after a single induction course. Of the remaining 153 patients, 87 received a second induction course under protocol, and 36 (41%) patients achieved CR. The CR rate of 55% was similar to that of the 514 patients without ERM data (Table 3). The CR rate was somewhat lower for ERM− patients (49 of 101 patients or 49%; CI, 39–58%) compared with ERM+ patients (95 of 160 patients or 59%; CI, 52–67%), but the difference was not statistically significant (two-tailed $P = 0.097$ by Fisher's exact test). In simple logistic regression analysis, the CR rate was not significantly associated with ERM treated as a quantitative variable ($P = 0.39$ with untransformed ERM and 0.23 with logit of ERM). Moreover, there was no significant interaction between specimen type and ERM in these analyses ($P = 0.80$ with untransformed ERM data; $P = 0.82$ with logit of ERM). In other words, there was no evidence that ERM measured in

peripheral blasts had a different association with CR rate, compared with ERM measured in marrow cells.

Simple logistic regression analyses of the other variables in Table 1 identified only one that was significantly associated with CR: FAB classification ($P = 0.0030$ for heterogeneity among six categories: M1–M5 and all others combined). The CR rates were particularly low for M1 (21 of 58 patients or 36%) and M3 (7 of 17 patients or 41%), intermediate for M5 (18 of 34 patients or 53%), and high for M2 (66 of 99 patients or 67%) and M4 (28 of 43 patients or 65%). The CR rate tended to decrease with increasing age (*e.g.*, from 62% among patients ≤30 years of age to 47% in patients ≥50 years of age), however this trend was not statistically significant in this data set ($P = 0.16$).

In multiple logistic regression analysis, after accounting for this effect of FAB classification, none of the other variables in Table 1 had a statistically significant independent association with CR. In particular, there was no significant effect of ERM, whether treated as a quantitative variable ($P = 0.22$ with untransformed ERM and 0.15 with logit of ERM) or dichotomized at 15% ($P = 0.088$). Because increasing age was associated with a large but nonsignificant effect on CR rate, multiple logistic regression analyses including both FAB and age were also performed; however, these analyses gave essentially the same results regarding the effect of ERM on response. Analyses of interactions produced no evidence that ERM had an effect on the CR rate, which varied according to age ($P = 0.45$) or ara-C induction dose ($P = 0.47$). Similar results were obtained in analyses using the logit of ERM.

ERM and OS. Of the 261 patients with ERM data, 226 have died. Except for one patient lost to follow-up after 40 months, the remaining 35 patients have been under follow-up between 6.0 and 11.9 years (median, 7.7 years). As shown in Table 3 and Fig. 4, OS was somewhat poorer for ERM− patients (9% at 5 years; 95% CI, 3–14%) compared with ERM+ patients (18%; CI, 12–24%). On the basis of PHs regression analyses, this difference was marginally significant (two-tailed $P = 0.022$). The estimated hazard ratio (ERM+ relative to ERM−) was 0.73 (CI, 0.56–0.95), indicating that the average mortality rate was an estimated 27% lower on average for ERM+ patients. Similarly, with ERM treated as a quantitative (continuous) variable, the trend in OS was marginally significant ($P = 0.024$). There was no significant interaction between specimen type and the effect of ERM ($P = 0.49$). In the parallel analysis based on logit(ERM), the association between OS and ERM was somewhat more significant ($P = 0.0042$) and, again, the effect of ERM did not differ significantly between the blood and marrow groups ($P = 0.51$). OS of the ERM+ patients was similar to that of the 514 patients from S8600 without ERM data (Table 3).

Of the other variables in Table 1, four were significantly associated with survival when tested separately in simple PHs regression analyses: OS decreased with increasing age ($P = 0.0002$) and absolute blast count ($P = 0.012$), varied significantly among six FAB categories (M1–M5 and other; $P = 0.0001$), and was significantly poorer for nonwhite patients ($P = 0.021$). In multiple PHs regression analyses, ERM and all four of these other variables retained independent statistical significance. As in the simple PHs regression analysis, the effect of ERM was rather more significant when based on logit(ERM; $P = 0.0044$) than on the untransformed values ($P = 0.016$). There was no significant evidence that the effect of ERM on OS varied according to age ($P = 0.36$ with untransformed ERM, 0.51 with logit of ERM) or ara-C induction dose ($P = 0.17$ and 0.13).

ERM and RFS. Of the 144 patients who achieved CR, 103 have relapsed and 15 others died without report of relapse. As shown in Table 2 and Fig. 5, RFS was somewhat poorer for ERM− patients (10% at 5 years; CI, 2–19%) compared with ERM+ patients (23%; CI, 15–32%). In simple PHs regression analyses, this difference was not statistically significant (two-tailed $P = 0.060$), with an estimated hazard ratio of 0.60 (CI, 0.48–1.01). Similarly, with ERM treated as a quantitative (continuous) variable, the trend in RFS was not significant ($P = 0.087$ based on untransformed ERM; $P = 0.085$ with logit of ERM). RFS of patients with ERM data was similar to that of those without ERM data.

Simple PHs regression analyses of RFS identified three other marginally significant prognostic factors: RFS decreased with increasing age ($P = 0.010$) and decreasing induction Ara-C dose ($P = 0.028$) and was poorer for nonwhites ($P = 0.032$). However, in multiple PHs regression analysis, only age ($P = 0.0042$) and race ($P = 0.012$ for whites *versus* nonwhites) retained independent prognostic significance for RFS. None of the other factors was significantly associated with RFS, including ERM ($P = 0.101$ based on untransformed ERM; $P = 0.15$ with logit of ERM). There was no significant indication that ERM had an effect on RFS, which varied according to age ($P = 0.23$ with untransformed ERM, 0.41 with logit of ERM), or according to ara-C induction dose ($P = 0.24$ and $P = 0.15$).

DISCUSSION

Our results confirm that ERM is frequently present in AML at diagnosis and is associated with repressed *ER* gene transcription *in vivo*. These data also suggest potentially important interactions between this molecular marker, clinical characteristics, and OS.

Among the clinical parameters that affect ERM in AML, the inverse correlation between age and ERM stands out as the most intriguing. Previous studies had suggested that aging is associated with an increase in ERM in normal appearing colonic epithelium (15) and other tissues.[4] Thus, we would have predicted that ERM should be more frequent in AML in the elderly. On the other hand, we were unable to detect a clear association between aging and ERM in normal hematopoietic cells (10), and it is possible that the relationship between aging, ERM and neoplasia is not conserved between epithelial cells and hematopoietic cells. One possible explanation for the present findings then, may reside in the triggering factors that lead to AML. As discussed earlier, we have previously shown that, in an animal

[4] J-P. J. Issa *et al.*, unpublished observations.

model of lung cancer, tumors induced by radiation exposure were associated with a higher rate of ERM than tumors induced by a tobacco-derived carcinogen (9). Furthermore, in glioblastoma multiforme, there is a strong correlation between methylation at the *ER* locus and methylation at *N33* (16), a distinct gene on a different chromosome (17), suggesting again that ERM may reflect a specific pathophysiological event that leads to hypermethylation at multiple loci. Thus, it is possible that the presence or absence of ERM in AML mark different pathways in the molecular pathophysiology of this disease. If this hypothesis is correct, the different rates of ERM among young and old patients with AML may, therefore, reflect differing exposures to carcinogenic insults. Future studies should clarify the nature and potential causes of this inverse association between age and ERM.

Perhaps most interesting is the independent association between ERM and a relatively favorable prognosis in AML. OS increased significantly with increasing ERM, even after adjustment for other significant prognostic factors, including age ($P = 0.0032$). Because both age and ERM were significantly associated with survival in this multiple regression analysis, ERM is not simply serving as a marker for age or age-related factors. Although the association of ERM with RFS was not statistically significant, the magnitude of the effect of ERM was similar for OS and RFS: the estimated hazard ratios (ERM+ relative to ERM−) were 0.71 (CI, 0.54–0.93) for OS and 0.67 (CI, 0.46–0.97) for RFS.

This, of course, is not the first time that a molecular abnormality has been associated with a good prognosis in acute leukemias. Indeed, several specific chromosomal translocations have previously been shown to be associated with a favorable prognosis in AML and acute lymphocytic leukemia (18, 19). Nevertheless, the mechanism of this association between ERM and survival remains obscure. We have shown that AML cases without ERM express relatively high levels of *ER*, and it is, therefore, possible that this expression itself is detrimental in AML. This possibility deserves further investigation because if *ER* expression is indeed responsible for this poorer outcome, than the use of ER antagonists such as Tamoxifen may be warranted in this subset of patients. Another possible explanation for the difference in outcome based on ERM status goes back to the hypothesis mentioned above that ERM might simply be a molecular marker for the carcinogenic event(s) that resulted in leukemia formation. Interestingly, a somewhat analogous situation has recently emerged in colorectal cancer, where tumors with mismatch repair deficiency appear to have more pronounced methylation defects, in association with a more favorable prognosis when compared with tumors proficient in mismatch repair (20). There are also other situations wherein, within a tumor type, different pathophysiological pathways are associated with differences in survival. For example, ovarian neoplasms in patients with germline BRCA1 mutations have a better prognosis than sporadic ovarian cancers (21).

There are two important limitations of our study that should be mentioned. First, because cytogenetics was not mandatory for patient registration on this clinical study initiated in 1986, we do not have reviewed high quality cytogenetic data on the majority of patients. Second, there is a relative lack of information regarding whether each patient developed AML after an antecedent hematological disorder and/or other factors linked to the development of secondary leukemia. This latter information may be particularly important because secondary leukemias tend to occur in older patients and are associated with a poor prognosis (22, 23). Although there is no *a priori* reason to suspect a particular link between ERM and specific chromosomal anomalies or secondary leukemias, this issue should be addressed in detail in future studies.

Whatever the mechanism of this association between ERM and better outcome in AML, our data has potentially important clinical implications. There are few clinical parameters of prognosis in AML that retain a predictive value in patients who achieve a CR (19, 24). These results, if confirmed in separate populations of patients, suggest the possibility that lack of ERM at diagnosis may mark a population of patients with such a poor outcome that alternate forms of treatment should be considered early in their management.

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