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ORIGINAL ARTICLE

The Prognostic Relevance of BAALC and ERG Expression Levels in Cytogenetically Normal Pediatric Acute Myeloid Leukemia

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Abstract Cytogenetic aberrations are important prognostic factors in acute myeloid leukemia (AML). About 45 % of de novo adult AML and 20 % of pediatric AML lack cytogenetic abnormalities, so identification of predictive molecular markers might improve therapy. Mutation status of *FLT3, NPM1* genes and gene expression levels of *ERG, BAALC* have been postulated as possible prognostic markers in pediatric AML with normal karyotype. Pretreatment blood samples from 47 cytogenetically normal AML patients were analysed for *BAALC* and *ERG* expression using real time RT-PCR. The patients were dichotomized at *BAALC* and *ERG* mean expression into low and high expression based on the median expression as cutoff. *BAALC* showed high expression in (24/47; 51.1 %) of patients and *ERG* high expression was detected in (22/47; 46.6 %). With follow-up for 1 year, patients with high *BAALC* and high *ERG* had inferior EFS ($P = 0.001$, $P = 0.017$ respectively), overall survival ($P = 0.001$, 0.08 respectively), and low rates of induction remission ($P = 0.001$, $P = 0.0017$ respectively) as compared to those with low expression. Also there was significant positive association between high expression of *BAALC; ERG* and FLT-ITD mutations ($P = 0.016$; $P = 0.007$ respectively). Multivariable analysis confirmed that high BAALC expression is an independent risk factor for EFS [HR for EFS 1.9(1.04–3.46) $P = 0.037$]; and OS [HR OS 1.55(1.7–3.36) $P = 0.03$]. In conclusion: Over expression of *BAALC* could predict adverse clinical outcome and may define important risk factor in cytogenetically normal pediatric AML.

Keywords *BAALC* · *ERG* · CN-AML · Prognosis

Introduction

AML is a heterogeneous illness composed of subtypes, with blast- intrinsic genomic aberrations serving prominent roles in disease classification and clinical management. AML constitutes 15–20 % of childhood leukemia and has an overall survival (OS) of 70 %. The prognosis has improved over the past couple of decades through optimization of treatment protocols, in which identification of disease subgroups that are based on genetic markers has turned out to be essential [1].

Approximately 45 % of adult AML patients and 20 % of pediatric AML patients are diagnosed with cytogenetically normal AML (CN-AML), which is currently classified and treated as a homogeneous intermediate-risk group. In order to refine risk stratification of pediatric CN-AML, it is important to study the underlying molecular aberrations [1].

Among the genetic markers that has been previously studied in CN-AML patients are Fms-related tyrosine kinase 3 gene (*FLT3) mutations* [2–5], the nucleophosmin

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gene (*NPM1) mutations* [6–15], *ERG* [16, 17] and *BAALC* [18–22] expression levels. Although studies concerning the prognostic relevance of *BAALC* [18] and *ERG* expression [16, 17] in adult AML patients with CN AML become fully clear; the studies regarding their expression and prognostic impact in pediatric AML are few and of controversial results. Therefore, this study was planned in order to assess the prognostic impact of the expression of *BAALC* and *ERG* in children with de novo CN-AML patients in parallel with well established genetic markers which include *NPM1 and FLT3*.

Patient Samples and Treatment Protocols

Patient samples and treatment protocols: This study included 49 consecutive children with denovo AML attending hematology/Oncology unit, Mansoura Cancer Institute; between January 2010 and 2013, after signing written consent. This cohort of pediatric patients included was with a normal karyotype. None of these 49 patients had AML- M3 or Down syndrome.

Children patients age range between 2 and 15 years (median age 7 years). The diagnosis of AML was based on the presence of blast cell $\geq$20 % in bone marrow (BM) smear. FAB subtypes were (2M0, 7 M1, 13 M2, 15 M4, 8 M5, 2 M6). The diagnosis and FAB subtype were confirmed by immunophenotyping using (Coulter Epics XL Flowcytometer PN 42372238 B, Coulter Corporation, Miami, Florida 33196, USA) to confirm diagnosis (Cyt. MPO, CD 13, CD 33, CD 117) as a primary panel for myeloid lineage, (CD14, CD36, CD11b) for M4 and M5, (CD61, glycophorin A) for M6 and (CD41, CD42) for M7. The patients were observed for 12 months or until death. History taking and clinical examination for organomegaly were done for all patients. All patients had normal karyotypes on conventional cytogenetic examination of at least 20 metaphases. Patients were characterized at the molecular level with regard to *FLT3ITD, NPM1* and were analyzed for *BAALC* and *ERG* expression. The study was performed in accordance to the Declaration of Helsinki, and parents and/or patients gave informed consent.

Therapy Protocol

The patients were treated according to standard protocols, most commonly 3 days of an anthracycline and 7 of ara-C (3 + 7) [23]. Bone marrow aspiration was done between 21 and 28 days after initiation of chemotherapy. Consolidation comprised of three to four courses of high dose cytosine arabinoside(3 g/m^2 every 12 h on days 1,3 and 5; total, 18 g/m^2) Following this, patients were followed up once every 3 months with clinical examination and complete counts for total period 12 months .

Cytogenetic and Molecular Genetic Analysis

Pretreatment samples from all patients were studied by G-banding analysis and fluorescence in situ hybridization (FISH). Conventional cytogenetic studies were performed using standard techniques, and chromosomal abnormalities were described according to the International System for Human Cytogenetic Nomenclature. To improve the accuracy of cytogenetic diagnosis, all specimens were also analyzed by FISH using a comprehensive DNA probe set allowing for the detection of the most relevant AML-associated genomic aberrations. Patients were classified as having normal cytogenetics on the basis of analysis of BM or PB metaphases; in most cases 20 metaphases were assessable.

RNA Extraction and Real-Time RT-PCR to Measure BAALC and ERG Expression Levels

Preparation of pretreatment blood samples and analysis of BAALC and ERG expression were performed as previously described 8–11. Briefly, total RNA extraction and isolation (QIAGEN) and complementary DNA was synthesized from total RNA. Quantitative real-time reverse-transcription-polymerase chain reaction (RT-PCR) amplification of BAALC, and ERG was performed using standard curves. BAALC and ERG expression levels are reported as copy numbers normalized to ABL1 copy numbers.

Nucleotide Sequence of the Primers and Probes Used for Detection of BAALC and ERG Expression Levels

BAALC (F)	5′-GCCCTCTGACCCAGAAACAG-3′
BAALC (R)	5′-CTTTTGCAGGCATTCTCTTAGCA-3′
BAALC Probe	FAM-5′-CTCTTTTAGCCTCTGTGGTCTGA AGGCCAT-3′- TMRA
ERG (F)	5′-AACGAGCGCAGAGTTATCGT-3′
ERG (R)	5′-GTGAGCCTCTGGAAGTCGTC-3′
ERG Probe	FAM-5′-GGAGTACAGACCATGTGCG GCAGTG- 3′-TMRA
GAPDH (F)	5′-GAAGGTGAAGGTCGGAGTC-3′
GAPDH (R)	5′-GAAGATGGTGATGGATTTC-3′
GAPDH Probe	FAM-5′-CAAGCTTCCCGTTC TCAGCC-3′-TMRA

Detect FLT3/ITD and NPM1 Mutations

DNA isolation and polymerase chain reaction Using QIA amp DNA blood mini kit (QIAGEN, USA) for DNA purification from whole blood and/or bone marrow aspiration.

To detect *FLT3* and *NPM1* genotype was determined as previously described [11]. Polymerase chain reaction (PCR) for exons 14 and 15 was performed on genomic DNA using published primer molecules for *FLT3* and exon 12 for *NPM1*. For Gene scan analysis of the *FLT3* mutant: wild-type ratio (*FLT3* ratio) PCR primer *FLT3* 14F was labeled with 6-FAM (TIB MOLBIOL, Berlin, Germany).

Fragment Analysis Post-PCR Products Using Gene Mapper Software of FLT3 and NPM1

Gene Mapper analysis software automatically analyzes the data collected by ABI prism 310 Genetic Analyzer to size and quantitate DNA fragment. Fluorescently labeled PCR products are electrophoresed through an acrylamide containing polymer, POP4 (PE Applied Biosystem, USA), which is then analyzed using an ABI prism 310 Genetic Analyzer. The associated gene mapper software version 4.1 is then able to convert the information into an correspond to intensity of fluorescence detected. Electropherogram show fluorescence intensity as a function of fragment size or migration time. Each electropherogram represents a single injection. The expected peak size for the wild *FLT3* PCR product is 330 bp. *FLT-ITD* fragment can be 18–108 bp larger than this. Only ITD positive cases are reported if the ITD represent at least 5 % of the peak area of *FLT3* WT fragment. As regards wild *NPM1*, its product expected peak size is 287 bp, while NPM1 mutant is usually 4 bp lager.

Statistical Analysis and Definition of Clinical Endpoints

The statistical analysis of data was done by using excel program and SPSS version 16 (statistical package for social

Table 1 Clinical and molecular characteristics at diagnosis according to BAALC and ERG expression status in CN-AML patients

Characteristic	High *BAALC* ($n = 24$)	Low *BAALC* ($n = 23$)	*P*	High *ERG* ($n = 22$)	Low *ERG* ($n = 25$)	*P*
Age, years						
Median	7.0	6.0	>0.05	8.0	9.0	>0.05
Range	(2.0–15)	(3.0–15)		(2.0–15)	(3.0–14)	
Sex, no. (%)						
Male	55	46	>0.05	52	53	>0.05
Female	45	54		48	47	
Hemoglobin, g/dl	7.0 (5.0–12.0)	8.0 (6.0–12)	>0.05	7.5 (6.0–12.0)	6.9 (5–11.5)	>0.05
Platelet count, $\times 10^9$/L						
Median	20	30	>0.05	32	25	>0.05
Range	(10–90)	(15–78)		(15–88)	(10–90)	
WBC count, $\times$ 10^9/L						
Median	95	97	>0.05	75	34.0	<0.01
Range	(12–190)	(20–150)		(12–190)	(20–140)	
Blood blasts, %						
Median	75	28	<0.01	45	52	>0.05
Range	(0–95)	(0–90)		(0–88)	(5–95)	
BM blasts, %						
Median	68	33	<0.01	27	18	>0.05
Range	(11–97)	(5–95)		(10–97)	(5–94)	
BAALC expression, no. (%)						
High				17(68.0)	6(24.0)	$P < 0.01$
Low				5 (32.0)	19(76.0)	
FLT3-ITD no. (%)						
Mutant	5 (20.8)	1 (4.3)	<0.01	6 (27.3)	0 (0)	<0.01
Wild	19 (79.2)	22 (95.7)		16 (72.7)	25 (100)	
NPM1 no. (%)						
Mutant	4 (16.7)	1 (4.3)	<0.01	4 (18.2)	1 (4.0)	<0.01
Wild	20 (83.3)	22 (95.7)		18 (81.8)	24 (96.0)	

science). Qualitative data were described in the form of numbers and percentages. Quantitative data were described in the form of mean (±) standard deviation (SD). Statistical analysis were done by comparison between groups using χ^2 test regarding qualitative data while quantitative nonparametric data comparison was performed using one way ANOVA and paired sample *t* test. The probability of being by chance (*P* value) was calculated for all parameters (*P* is significant if <or = 0.05 at confidence interval 95 %). The Event-free survival (EFS) and the OS were analyzed by Kaplan–Meier curve. OS is the time from diagnosis to last follow-up or death from any cause. EFS is the time from diagnosis until relapse.

Results

The patient's characteristics are shown in Table 1. *BAALC* showed high expression in (23/47, 49.9 %) of patients and *ERG* high expression was detected in (22/47, 46.6 %). The incidence of gene mutations of FLT3-ITD and NPM1 were detected in 6 out of 47 (12.6 %), and in 5 out of 47 (10.6 %) of pediatric CN-AML patients respectively. Interrelation between NPM1 and FLT3-ITD mutations and ERG, BAALC expression revealed that there is significant positive association between high expression of BAALC and ERG and mutant FLT3-ITD, and negative association with mutant NPM1 ($P < 0.01$ for all) (Table 1).

Study the *BAALC* and *ERG* expression in relation to induction remission response revealed that AML patients with high expression of *ERG a*nd BAALC are associated with inferior remission induction rate as compared with those with low expression ($P = 0.001$, $P = 0.0017$ respectively) (Table 2).

Table 2 *BAALC* and *ERG* expression in relation to induction response

	ND ($n = 4$)	Poor ($n = 13$)	Good ($n = 30$)	
BAALC				
Low expression ($n = 24$)	1	1	22	$X^2 = 19.5$
High expression ($n = 23$)	3	12	8	$P = 0.001$
ERG				
Low expression ($n = 25$)	1	3	21	$X^2 = 5.7$
High expression ($n = 22$)	3	10	9	$P = 0.0017$

Good response defined as <5 % BM blasts after induction therapy. Low and high expression defined as above and below the median value, respectively

Univariate analysis including many prognostic variables including WBCs counts (< = 50,000 vs. >50,000/cmm), *FLT3-ITD* mut vs wild type; NPM1 mut vs wild type; high expression of *BAALC* and *ERG* vs low expression of *BAALC* and *ERG*; and response to induction remission (poor vs., Good vs. non determined) revealed significant differences in all these parameters except *NPM1* regarding EFS and OS (Table 3; Figs. 1a, b, 2a, b).

The genetic markers that were significantly different in univariate analysis were tested in multivariate analysis in order to find the independent parameters regarding EFS and OS. The Cox regression analysis showed that high *BAALC* expression was the independent prognostic factor for EFS and OS in CN AML pediatric patients (Table 4).

Discussion

Chromosomal abnormalities provide a powerful tool to stratify AML patients into different prognostic risk groups. Patients lacking cytogenetic aberrations, accounting for approximately 45 % of newly diagnosed de novo AML cases, are contained in an intermediate risk group. For these patients the identification of novel molecular markers is necessary to overcome the limitations of current risk assessment and to design new risk- adapted treatment strategies [24].

Table 3 Univariate analysis for overall survival and Event free survival

Parameter	Number	Overall survival (OS) Log rank *P*	Event free survival (EFS) Log rank *P*
WBCs			
≤50,000/cmm	21	$X^2 = 3.4$, $P = 0.048$	$X^2 = 4.8$; P = 0.027
>50,000/cmm	26		
FLT3-ITD			
Wild	41	$X^2 = 63.8$, $P = 0.001$	$X^2 = 57.4$, $P = 0.001$
Mut	6		
NPM-1			
Wild	42	$X^2 = 0.001$, $P = 0.47$	$X^2 = 0.01$, $P = 0.9$
Mut	5		
BAALC			
Low	23	$X^2 = 28.8$, $P = 0.001$	$X^2 = 25.3$, $P = 0.001$
High	24		
ERG			
Low	25	$X^2 = 17.0$, $P = 0.008$	$X^2 = 15.6$, $P = 0.017$
High	22		
Response to induction			
Poor	13	$X^2 = 86.8$, $P = 0.001$	$X^2 = 81.9$, $P = .001$
Good	30		
ND	4		

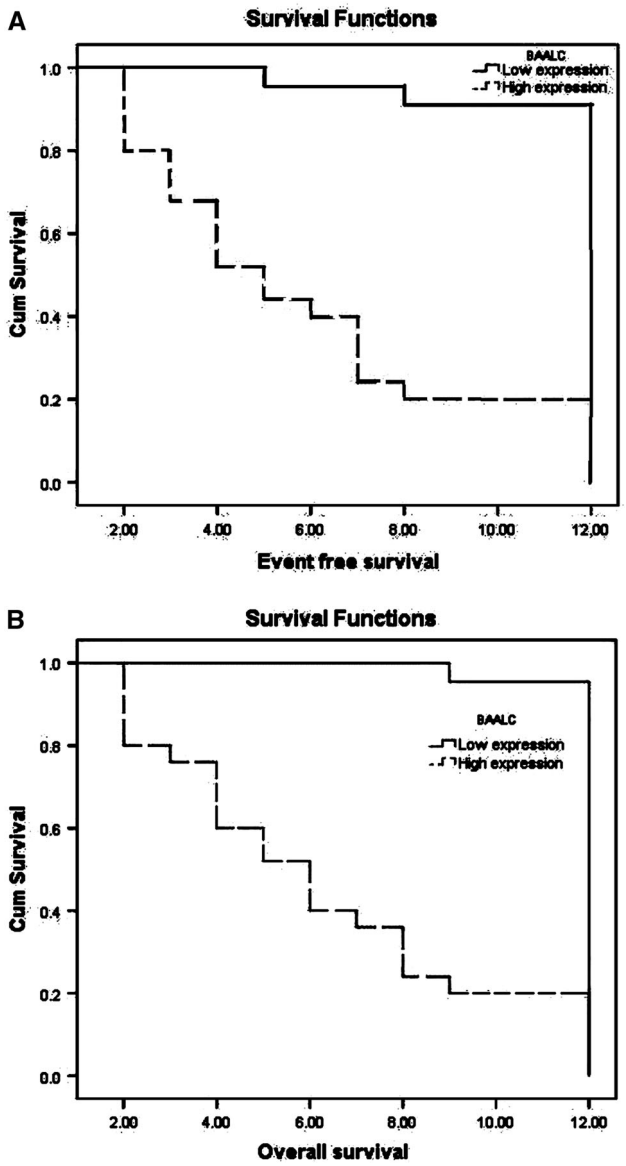

Fig. 1 **a** EFS for patients with high *BAALC* expression was significantly inferior than patients with low *BAALC* expression ($P = 0.001$). The event free survival was calculated by months. **b**)OS for patients with high *BAALC* expression was significantly inferior than patients with low *BAALC* expression ($P = 0.001$). The overall survival was calculated by months

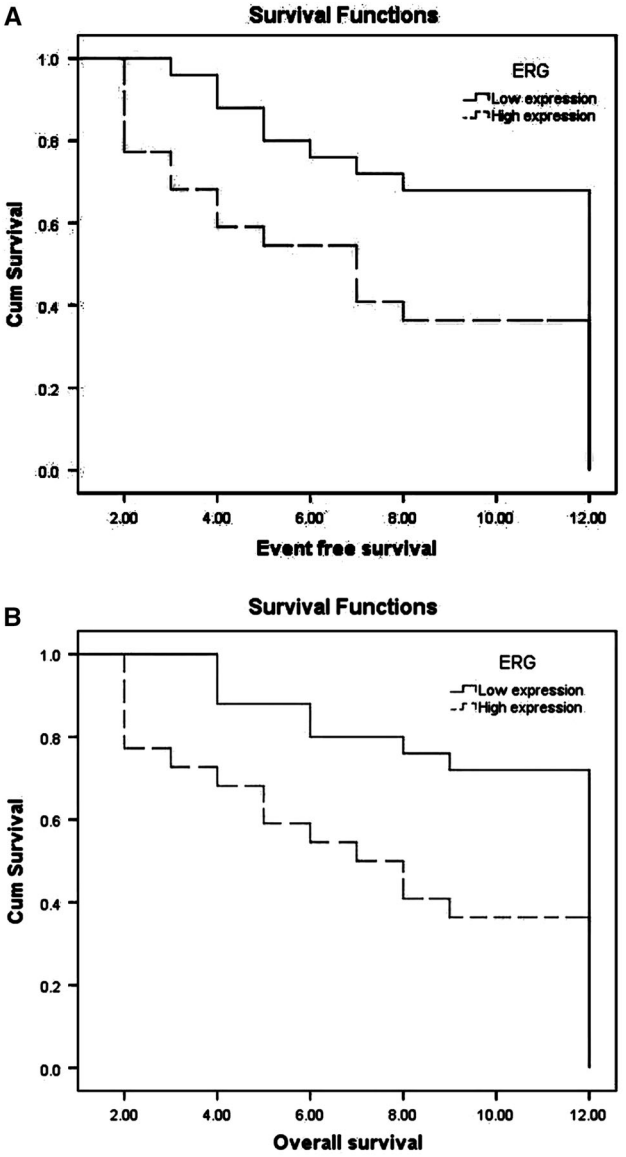

Fig. 2 **a** EFS for patients with high *ERG* expression was significantly inferior to patients with low *ERG* ($P = 0.017$). The event free survival was calculated by months. **b** Overall Survival for patients with high ERG expression was significantly inferior to patients with low *ERG* ($P = 0.008$). The overall survival was calculated by months

In the present study the high expression of BAALC and ERG were detected in 23 out of 47 (51.1 %) and in 22 out of 47 (46.8 %) of patients respectively. This finding is not in accordance with that reported by Eid et al. [25], they stated that high expression of *BAALC* and ERG were found in 70 and 33.3 % of patients respectively. In addition Eid et al. [25] stated that the high *BAALC* and *ERG* expression was correlated to both percentage of blast cells in the blood and BM, but did not associated with other clinical parameters of patients and high *ERG* expression was associated with higher white cell counts.

Our results indicated that the incidence of gene mutations of FLT3-ITD and NPM1 were detected in 6 out of 47 (12.6 %), and in 5 out of 47 (10.6 %) of pediatric CN-AML patients respectively. Significant association between high *BALLC* and *ERG* expressions and the presence of FLT3-ITD mutations were detected. Near findings were reported by Krstovski et al. [26] who detected *FLT3* mutations in 4/42 (9.5 %) and NPM1 mutations in 1/37 (2.7 %).

Table 4 Multivariate analysis of molecular and clinical factors

Factor	EFS		OS	
	Hazard ratio (95 % CI)	*P*	Hazard ratio (95 % CI)	*P*
FLT3-ITD	3.67 (1.43–9.42)	>0.05	2.73 (0.82–9.15)	>0.05
High *ERG* expression	1.28 (0.73–2.26)	>0.05	0.94 (0.48–1.84)	>0.05
High *BAALC* expression	1.90 (1.04–3.46)	0.037	1.55 (1.70–3.36)	0.03
WBCs > 50,000/cmm	1.02 (0.50–2.08)	>0.05	0.84 (0.33–2.12)	>0.05
Good response to induction therapy	0.38 (0.20–8.71)	>0.05	0.61 (0.28–1.32)	>0.05

In the current study high *BAALC* and *ERG* expressions were significantly associated with poor response to induction chemotherapy in CN-AML children. Similar finding was reported by Eid et al. [25]. Also, high BAALC and ERG expression had inferior EFS, and OS. These findings are in parallel with that reported by Eid et al. [25]. Univariate analysis revealed that High *ERG* and *BAALC* expression; showed a significant association with inferior EFS as well as shorter OS.

Based on in vitro culture studies observation Tanner et al. [27] suggested that the expression of *BAALC* is stage specific and postulate that BAALC represents a novel marker of an early progenitor cell common to the myeloid, lymphoid, and erythroid pathways. Moreover, Baladus et al. [28] stated that *BAALC* over expression is clearly nonrandom among AML French-American-British subtypes and AML cytogenetic groups, pointing to a role of *BAALC* in the leukemic phenotype. Its association with an adverse prognosis in AML is a further indication of it having a role in one or more processes that characterize these blasts.

In multivariate analysis, FLT3-ITD and high BAALC expression remained as prognostic factors for adverse EFS and OS. In Cox regression analysis high BAALC expression is independent prognostic markers for both EFS and OS. High ERG and BAALC expression showed a significant association with inferior EFS. These findings are parallel to that reported by Eid et al. [25] and Hovland et al. [24] Haferlach et al. [29]. On the other hand, high *BAALC* and *ERG e*xpression levels did not have independent prognostic significance in pediatric AML cohort or in the CN-AML subgroup specifically in the study done by Hermkens et al. [30]. This might be related to ethnic origin of the patients studied.

Expression levels of *ERG,* and *BAALC* were significantly associated with each other. Schwind et al. [31] believed that measuring *BAALC* and *ERG* expression at diagnosis seems to provide more prognostic information with regard to survival than NPM1 mutational status alone. Moreover, they stated that patients with high *BAALC* expression had worse outcome irrespective of their *NPM1* mutational status. Thus, the measurement and evaluation the pretreatment expression levels of *BAALC* and *ERG* in individual patients could be included as diagnostic testing for a more accurate risk stratification of older CN-AML patients.

In conclusion, we show that high BAALC expression is adverse prognostic marker for induction of remission achievement, EFS and OS in CN pediatric AML.

The number of patients enrolled was small, and during statistical analysis we found that there is a need to subclassify the AML patient's harbored mutations in FLT3-ITD and NPM1 to make association with degree of expression with BAALC and ERG. This led to decrease the statistical power of some analysis. So, we suggest extending the study to include larger group. Another limitation of the study is that the follow up period is short for AML, though effect is significant, however a longer follow up will show if these patients with low ERG will also relapse or die.

Conflict of interest The authors declare that there is no conflict of interest.

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