Expression of recombination activating genes-1 and -2 immunoglobulin heavy chain gene rearrangements in acute myeloid leukemia: evaluation of biological and clinical significance in a series of 76 uniformly treated patients and review of the literature

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Background and Objectives. Early lymphoid differentiation is characterized by antigen receptor gene rearrangements; the rearrangement process is governed by two lymphoid-specific genes, RAG (recombinase activating gene)-1 and -2. The available data on the incidence and prognostic significance of clonal immunoglobulin heavy chain (IgH) gene rearrangements in acute myeloid leukemia (AML) are rather contradictory. The aim of this study was to evaluate the incidence and prognostic significance of RAG-1 and -2 mRNA transcripts and clonal IgH gene rearrangements in a cohort of uniformly treated AML patients; the available literature is also reviewed.

Design and Methods. The study was performed on 76 AML patients, newly diagnosed between August 1996 and November 1999. RAG-1/-2 gene expression was analyzed by a reverse transcriptase polymerase chain reaction technique and IgH gene rearrangements were detected with variable region (V\textsubscript{\alpha}) family-specific and consensus framework region (FWR)-2 and/or-3 primers. Statistical associations were explored between IgH monoclonality/ RAG mRNA expression and: (i) age, gender, FAB subtype, immunophenotype, cytogenetic risk groups; (ii) response variables (response/reapse incidence, survival).

Results. In total, 38/75 samples (50.6%) were RAG-1 and/or -2 positive; 30/76 samples (39.5%) carried clonal IgH genes, whereas 13/30 IgH-positive samples (43.3%) were RAG-1/2-negative. Significant associations were detected only for RAG-2 positivity and unfavorable karyotype and IgH monoclonality and FAB subtypes M4/MS; no association was identified with response outcome and survival.

Interpretation and Conclusions. Lymphoid-specific molecular markers are detected in a significant proportion of AML patients, regardless of differentiation status (assessed morphologically/ immunophenotypically); however, in our experience, they do not seem to constitute an adverse prognostic factor.

Key words: AML, immunoglobulin gene rearrangements, RAG.

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1996 and November 1999. Clinical and hematologic laboratory data at diagnosis before treatment were available in all 76 patients (Table 1). Patients were consecutively enrolled in two different treatment protocols according to age (> or < 60 years old); within each group, patients were treated uniformly.

Briefly, patients younger than 60 received 2 courses of induction therapy consisting of idarubicin 12 mg/m² i.v. and cytarabine 200 mg/m² in 24-hour continuous i.v. infusion (course 1: days 3+7, course 2: days 2+5). Patients achieving a CR were assigned or randomized to one of three post-remission therapies. Those with a histocompatible sibling donor were assigned to allogeneic hematopoietic cell transplantation (HCT). Those without matched donors were randomized to either intensive consolidation chemotherapy (two courses of cytarabine at 3 g/m² over 3 hours in an i.v. infusion twice daily over days 1, 3, 5) or autologous HCT with stem cells collected on recovery from a consolidation course as described above. Patients older than 60 were treated with two identical induction courses with oral idarubicin (20mg/m², days 1–3) and etoposide (160mg/m², days 1–5), followed by two identical courses administered as consolidation therapy;

patients were then given maintenance treatment with 6-mercaptopurine and methotrexate. Granulocyte colony-stimulating factor (G-CSF) was routinely administered to all patients, starting with the second induction course. In both age groups, AML-M3 cases received specific treatment containing all-trans retinoic acid.

FAB morphologic classification was based on morphology and cytochemistry. Flow cytometry was used to confirm FAB M0 cases. Cytogenetic analysis of bone marrow unstimulated mononuclear cells (obtained prior to induction treatment) was conducted by standard G-banding; karyotypes were interpreted using International System for Cytogenetic Nomenclature criteria (1995). Samples were considered normal diploid if no abnormalities were detected in a minimum of twenty mitotic cells examined. Three cytogenetic risk categories were defined: favorable [inv(16)/t16;16/del(16q), t(15;17), t(8:21) with/without secondary aberrations], intermediate (normal, +8), unfavorable (all other abnormalities).

RNA extraction and cDNA preparation

Total cellular RNA was isolated by the guanidinium isothiocyanate method. In vitro reverse transcription of 3 µg of total cellular RNA to cDNA was performed using Moloney Murine Leukaemia Virus reverse transcriptase (Gibco-BRL, Gaithersburg, MD, USA) and random hexamers as primers (Gibco-BRL, Gaithersburg, MD, USA). After an initial denaturation of 5 min at 65°C, the reaction mixture was incubated at 37°C for 60 min.
As a control for the presence of amplifiable RNA, 5 μL of the reverse transcription cDNA product were amplified by polymerase chain reaction (PCR) using primers specific for the retinoic acid receptor α (RARα) gene (Table 2). Amplification consisted of an initial denaturation step of 5 min at 94°C, followed by 35 cycles of denaturation at 94°C for 1 min, annealing at 53°C for 1 min and extension at 72°C for 1 min with a final extension step of 10 min at 72°C.

**PCR amplification of RAG-1 and RAG-2 transcripts**

Five microliters of the reverse transcription reaction product were amplified by PCR using 40 pmol each of primers specific for the RAG-1 (RAG-1A/RAG-1B)16 and RAG-2 (RAG-228/ RAG-229)17 genes (Table 2). PCR was carried out in a final volume of 100 μL with 40 pmol of each primer, 200 μM each of dNTP, 2.5 U Taq polymerase (Gibco-BRL, Gaithersburg, MD, USA) in PCR buffer (50 mM KCl, 10 mM Tris–HCl pH 8.0, 1.5 mM MgCl₂, 0.01% gelatin). Amplification consisted of an initial denaturation step of 5 min at 94°C, followed by 40 cycles of denaturation at 94°C for 1 min, annealing at 60°C for 1 min for RAG-1 and 55°C for 1 min for RAG-2 and extension at 72°C for 1 min with a final extension step of 10 min at 72°C.

**PCR amplification of immunoglobulin heavy (VH) chain variable region genes**

Five microliters of the reverse transcription reaction product were amplified by PCR using a mixture of oligonucleotides specific for each of the VH leader sequences of the VH1-618 gene families together with a mixture of oligonucleotides complementary to all possible JH gene segments J1-615 (Table 2). PCR was carried out in a final volume of 100 μL with 40 pmol of each equimolar mixture of oligonucleotide primers, 200 μM each of dNTP, 2.5 U Taq polymerase (Gibco-BRL, Gaithersburg, MD, USA) in PCR buffer (50 mM KCl, 10 mM Tris–HCl pH 8.0, 1.5 mM MgCl₂, 0.01% gelatin). Amplification consisted of an initial denaturation step of 5 min at 94°C, followed by 40 cycles of denaturation at 94°C for 1 min, annealing at 61°C for 1 min and extension at 72°C for 1 min with a final extension step of 10 min at 72°C. PCR products were analyzed on 6% polyacrylamide gels.

In the second round (nested PCR), 1 μL of amplified DNA (1st round product) was reamplified using as primers oligonucleotides representative of framework regions (FWR)-2 and -3 (FWR2/ FWR3)32 (Table 2) together with the mixture of J1-6 oligonucleotides. PCR was carried out as described above. Amplification consisted of an initial denaturation step of 5 min at 94°C, followed by 30 cycles of denaturation at 94°C for 1 min, annealing at 55°C for 1 min and extension at 72°C for 1 min with a final extension step of 10 min at 72°C. PCR products were analyzed on 8% polyacrylamide gels.

PCRsensitivity was performed with dilution experiments (RNA in RNA); the dilution was performed in 10-fold steps, from undiluted to 10^-5. We used a bone marrow sample of a patient with pre-B-ALL and almost 100% infiltration at diagnosis as the positive control and peripheral blood lymphocytes from healthy volunteers as the negative control. The first round PCR reached a sensitivity level of 10^-3/10^-4, while the second round of amplification reached a reproducible sensitivity level of 10^-4/10^-5.

Both in the reverse transcription reaction and in the ensuing amplification reactions, recommended measures to prevent cross-contamination of samples were followed.20 In addition, for each experiment, a control with no template was used to check for the presence of any contaminant. In positive controls, controls with all reagents including RNA albeit without the reverse transcriptase enzyme were used to rule out the possibility of cDNA contamination of the RNA samples.

**Statistical evaluation**

The categorical variables taken into account in this analysis are as follows: (i) age (two levels, <60 years and >60 years) (ii) gender; (iii) FAB subtype (seven levels, M0 through to M6) (iv) lymphoid-specific (B/T) surface markers (two levels, presence and absence); special emphasis was given to CD56 expression; (v) karyotype (three levels: favorable, intermediate and unfavorable risk groups); (vi) RAG-1, RAG-2, RAG-1/-2 mRNA (two levels, existence and non-existence); (vii) IgH monoclonality (two levels, presence and absence); (viii) response [two levels, complete remission (CR) and otherwise]; (ix) relapse (two levels, occurrence of relapse, no relapse). Survival time was measured with the related censoring information. The analysis of the relationships between categorical variables was split in two basic stages: i) in the first stage, the bivariate relationships between the existence of RAG (-1/-2) mRNA transcripts and IgH monoclonality with the remaining categorical variables were explored with the use of Fisher’s exact test at a significance level of 0.1 so as to allow for potential associations. The same test was used for the study of the relationships between response and relapse incidents with the remaining variables; ii) in the second stage, in cases showing significant bivariate relationships, a log-linear model was built to explore the existing associations, adjusting for other variables. Similarly, the analysis of the differences in survival time was implemented in two basic stages: i) in the first stage, the Kaplan–Meier procedure was conducted and a log-rank test was calculated to identify significant differences in survival.
time for all variables at a significance level of $p=0.1$; ii) in the second stage, a Cox's proportional hazards model for survival data was implemented to investigate the relationship between the prognostic variables detected in stage A and survival time. All analyses were implemented with the use of the statistical package SPSS version 10.

**Results**

**Detection of RAG-1 and RAG-2 transcripts and monoclonal Ig heavy variable region gene rearrangements**

RAG-1 mRNA transcripts were detected in 15/75 samples (20%) while RAG-2 was found in 34/75 samples (45.3%). In total, 38/75 samples (50.6%) were RAG-1 and/or -2 positive; 30/76 samples (39.5%) carried clonally rearranged IgH variable region gene sequences, whereas 13/30 IgH-positive samples (43.3%) were RAG-1/2 negative. Interestingly, the vast majority of IgH-positive samples (28/30) tested positive already from the first round of amplification (with a sensitivity level of $10^{-3}/10^{-4}$). RAG mRNA and IgH monoclonality data in specific karyotype and surface marker-positive AML subgroups are shown in Tables 3 and 4.

**Statistical analysis**

The analysis was based on measurements of 76 patients. When data were missing, the analysis was focused on cases with complete records. All variables were recorded at a percentage exceeding 95% except for expression of lymphoid markers (89.5%) and karyotype (92%). Table 5 shows the results of Fisher's exact tests between the existence of the particular genetic markers and all other categorical variables. It can be seen that monoclonally rearranged IgH genes were present at a statistically significant higher extent among patients with AML FAB-M4 and M5. Furthermore, RAG-2 mRNA transcripts were detected at a significantly higher rate in patients with unfavorable cytogenetics.

Concerning outcome, none of the genetic markers under study (IgH monoclonality, RAG mRNA transcripts) was significantly associated with response and relapse incidences. In order to focus on the relationship between all other variables and the response outcomes (response and relapse), all bivariate significance Fisher's exact tests were calculated. Significantly higher percentages of complete remission were recorded among patients aged less than 60 years and patients falling in the favorable cytogenetic subgroup. Log linear model analysis between response and each of the categorical variables, after adjusting for the remaining variables, showed that a higher complete remission rate was recorded among patients of younger age (<60) ($\chi^2=21.8$ at 1 d.f., $p<0.01$). Survival time was recorded for all 76 patients who entered the study. At the time of completion, 26 patients were still alive. With the use of the Kaplan–Meier procedure, survival time was calculated for each subgroup of patients and the log-rank test was used to explore survival differences between groups. None of the genetic markers under study was significantly associated with survival time (IgH monoclonality: $p =$...
approach (sensitivity level 10⁻⁵) and performed two
whole VH region
forms of PCR: RT-PCR, one- or two-round PCR,
sensitivities were adopted (Southern blot, various
several studies selected particular AML subtypes

0.88; RAG-1 mRNA expression: p = 0.79; RAG-2
mRNA expression: p = 0.98). On the contrary, a signifi-
cantly worse outcome was seen in patients of older age (p < 0.01), non-M2/M3 FAB subtypes (p < 0.01), and unfavorable cytogenetics (p < 0.02).

Mean and median survival times for each subgroup
are shown in Table 6. A Cox’s proportional odds
model investigating the simultaneous prognostic
effect of age, FAB subtype and karyotype on sur-

Table 6. Survival data: significant prognostic variables.

<table>
<thead>
<tr>
<th>Groups</th>
<th>Mean survival*</th>
<th>Median survival*</th>
</tr>
</thead>
<tbody>
<tr>
<td>Age: &lt; 60/&gt; 60</td>
<td>34.6/8.3</td>
<td>38.0/2.5</td>
</tr>
<tr>
<td>• FAB</td>
<td></td>
<td></td>
</tr>
<tr>
<td>M0/M1/M2/M3/M4/M5/M6</td>
<td>14.7/13.0/30.4/42.2</td>
<td>2.0/3.5/21.0/9.0</td>
</tr>
<tr>
<td></td>
<td>21.3/21.0/2.7</td>
<td>6.0/2.0</td>
</tr>
<tr>
<td>• Cytogenetics</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Fav/Interm/Unfav*</td>
<td>36.0/28.1/15.2</td>
<td>38.0/21.0/6.0</td>
</tr>
</tbody>
</table>

*In months; *Median survival time was not calculated due to high percentage of
censored data; **Fav: favorable; interm: intermediate; unfav: unfavorable.

Discussion

The precise frequency of immunoglobulin gene
rearrangements in AML is difficult to calculate from previously reported data7,10,11 not only because
several studies selected particular AML subtypes
but also, and perhaps more importantly, different
experimental protocols with markedly different
sensitivities were adopted (Southern blot, various
forms of PCR: RT-PCR, one- or two-round PCR,
whole VH region or FWR2 or FWR PCR).

To our knowledge, this is the largest study of IgH
rearrangements in uniformly treated AML patients.
Based on past experience both by our group and by
others,19,21,22 we adopted a sensitive RT-PCR
approach (sensitivity level 10⁻⁵) and performed two
round amplifications using primers derived from
VH family leader sequences (first round)18 and
FWR2 or FWR3 sequences (second round).19 Our
results indicate that immunoglobulin heavy chain
genes are rearranged in a significant proportion of
AML cases (30/76; 39.5%); similar results were
obtained in the studies by Kyoda et al.10 (14/35 cas-
es; 40%) and Yen et al.11 (11/39 cases; 28%). Of
course, one cannot rule out the possibility that by
adopting a sensitive RT-PCR protocol one may
detect monoclonal IgH rearrangements in minor
blast cell subpopulations; nevertheless, even if this
were true in some cases, it would point to the
propensity of the malignant clone to behave in an
illegitimate way and would be evidence for some

biological difference (with as yet undefined prog-
nostic significance) from cases lacking clonally
rearranged IgH genes. Importantly, we did not find
any case positive for monoclonal IgH rearrange-
ments among 20 healthy individuals; in the same
context, it is of interest that monoclonal IgH gene
rearrangements were detected in a previous study
by our group in only 6/23 patients with chronic
myeloid leukemia in chronic phase.23 Altogether,
the aforementioned results indicate that the re-
latively high incidence of clonally rearranged genes
in AML is not very likely to result from amplifica-
tion of minor clones or non-clonal reactive cells
and probably represents a bona fide feature of AML.
However, no associations have been detected
between IgH monoclonality and either specific
cytogenetic abnormalities (in analogy to what has
been reported for lymphoid-associated surface
marker expression in AML)5,6 or immunophenotyp-
ic patterns. The possible correlation with morphol-
ogy (FAB subtypes M4/MS) is intriguing given that,
exclusively among FAB subtypes, M4 and M5 have
also been described to express significantly more
frequently the lymphoid-associated markers CD4
(perhaps not surprisingly, as normal monocytes are
known to express CD4) and (perhaps) CD19.24

In the present study, RAG-1 mRNA transcripts
were detected in 15 cases and RAG-2 in 34. There
are several explanations for this discrepant finding:
(i) RAG genes appear to be expressed and act co-
ordinately to activate the recombinase;2 however,
exceptions to this rule are known to occur natu-
really;25 (ii) this rather discrepant result may be
attributed to technical reasons: generally, RAG-1
transcripts appear to be more difficult amplifica-
tion targets; (iii) a final explanation might be that
when the enzymatic recombination system func-
tions inappropriately some of its components
might be missing.26

Our analysis failed to detect any correlation
between RAG mRNA expression in AML blasts and
the presence of a clonally and completely
rearranged V(D)J gene complex (at least in the
sense that it could be amplified by the PCR proto-
cols applied here). Of interest, a similar result was
reported in the largest of the published series;6 in
that study, although RAG-1 mRNA expression was
detected in a significant proportion of AML cases,
it was not correlated with immunoglobulin gene
rearrangement. In general, RAG mRNA expression
was not associated with any biological feature of
the AML blasts. The possible association of RAG-2
mRNA expression with unfavorable cytogenetics is
of uncertain biological significance.

The recombinase normally recognizes highly con-
served recombination signal sequences (RSS) (the
heptamer CACAGTGG and the nonamer
ACAAAAACCC) that flank one or both sides of all
recombining gene segments.1 Interestingly, a com-

N. Stravoyianni et al.
puter-aided search detected typical RSSs in many of the genes participating in the most common chromosomal translocations in AML [t(16;21), inv(16), t(15;17), t(6;9)]. Taken together, this finding and the data presented herein concerning RAG mRNA expression allude to the possible role of the recombinase in mediating AML-specific chromosomal translocation events.

The prognostic significance of clonal antigen receptor gene rearrangements in AML remains unresolved, not only because the available data are limited but also because the two largest published series (35 and 39 patients, respectively) addressing this issue have reached conflicting results. Regarding the prognostic impact of RAG mRNA expression in AML, to our knowledge, the present analysis is the first to address this issue. Importantly, in our study, the presence of either monoclonally rearranged IgH genes or RAG mRNA transcripts was not found to affect remission and relapse incidence or duration of remission. Our series included almost twice as many cases as either of the aforementioned studies, had a longer follow-up and was the only one to discriminate patients for treatment according to age; furthermore, we adopted a significantly more sensitive approach [two-round, nested PCR vs. single-round, FWR3, PCR vs. Southern blot] for the detection of clonal IgH gene rearrangements.

Terminal deoxynucleotidyl transferase (TdT) was initially considered as a marker of immature lymphoid cells; however, many studies have conclusively demonstrated TdT expression in AML cases. The reported incidence of TdT expression in AML varies significantly (0–55%, average of combined adult data: 21%). In our study, with a 20% cut-off level for positivity, 9/76 cases (11.8%) were found to express TdT; on multivariate analysis, the expression of TdT was not correlated with any other variable. As in most studies (except, importantly, the study by Venditti et al.), no association was found between TdT positivity and immunoglobulin gene rearrangements; this is not biologically surprising given that, although TdT plays an important role in lymphoid ontogeny by inserting random nucleotides at the junctions of rearranging antigen receptor genes and thus increasing immune diversity, this enzyme is not essential for the gene rearrangement process.

In conclusion, the prognostic significance of lymphoid-specific genetic markers in AML remains unresolved and should be evaluated in further studies. However, an important parameter that must be taken into account in assessing the impact of a molecular marker is the methodology applied, which varies significantly between studies thus precluding direct comparison of the results. Finally, the results presented herein are in agreement with data from studies of hematopoietic development; thus, lymphoid-marker-positive AML might derive from an expansion of abnormal cells blocked at a particular stage of differentiation of a common lymphoid/myeloid precursor. However, one might argue that they fit an alternative model whereby primitive leukemic cells differentiate and acquire lineage markers depending upon the influence of specific transforming events.

Appendix

In addition to the authors, the following physicians and institutions participated in the study: D. Loukopoulos, G. Panagalis, I. Meletis, G. Vaiopoulos, I. Rombos, K. Konstantopoulos, N. Viniou, E. Variami, First Department of Medicine, University of Athens; N. Zoumbos, P. Matsouka, A. Kouraklis, M. Tiniakou, Hematology Department, University of Patras; P. Panayiotidis, First Department of Propaedeutic Medicine, University of Athens; T. Kalmantis, A. Kalokerakis, M. Filiotou, Second Department of Medicine, University of Athens; C. Poziopoulos, 401 Veterans Hospital, Athens; N. Anagnostopoulos, A. Galanopoulos, G. Gennimatas Hospital, Athens; J. Christakis, I. Korantzis, M. Papaioannou, Theagenion Hospital, Thessaloniki; G. Bourikas, C. Tsatalas, Hematology Department, University of Thrace; G. Eliopoulos, H. Papadaki, Hematology Department, University of Heraklion, Crete; P. Rousso, Third Department of Medicine, University of Athens; E. Stefanoudaki, Ag. Anargyri Hospital, Athens; G. Kokkinis, Simpsonogleneon Hospital, Athens; I. Papadopoulos, AHEPA Hospital, Thessaloniki.

References


