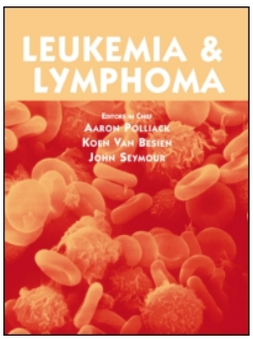

Leukemia & Lymphoma

ISSN: 1042-8194 (Print) 1029-2403 (Online) Journal homepage: http://www.tandfonline.com/loi/ilal20

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To cite this article: Robert Carr, Hilal Ozdag, Nilgun Tekin, Timothy Morris, Paulette Conget, Flavia Bruna, Botond Timar, Eva Gagyi, Ranjan Basak, Omkar Naik, Chirayu Auewarakul, Narongrit Srithana, Mark Pierre Dimamay, Filipinas Natividad, June-Key Chung, Nevin Belder, Isinsu Kuzu, Nader Omidvar, Diana Paez & Rose Ann Padua (2017) The effect of biological heterogeneity on R-CHOP treatment outcome in diffuse large B-cell lymphoma across five international regions, Leukemia & Lymphoma, 58:5, 1178-1183, DOI: 10.1080/10428194.2016.1231308

To link to this article: https://doi.org/10.1080/10428194.2016.1231308

LEUKEMIA & LYMPHOMA, 2017
VOL. 58, NO. 5, 1178–1183
http://dx.doi.org/10.1080/10428194.2016.1231308

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ORIGINAL ARTICLE: RESEARCH

The effect of biological heterogeneity on R-CHOP treatment outcome in diffuse large B-cell lymphoma across five international regions

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ABSTRACT

Addressing the global burden of cancer, understanding its diverse biology, and promoting appropriate prevention and treatment strategies around the world has become a priority for the United Nations and International Atomic Energy Agency (IAEA), the WHO, and International Agency for Research on Cancer (IARC). The IAEA sponsored an international prospective cohort study to better understand biology, treatment response, and outcomes of diffuse large B-cell lymphoma (DLBCL) in low and middle-income countries across five UN-defined geographical regions. We report an analysis of biological variation in DLBCL across seven ethnic and environmentally diverse populations. In this cohort of 136 patients treated to a common protocol, we demonstrate significant biological differences between countries, characterized by a validated prognostic gene expression score ($p < .0001$), but International Prognostic Index (IPI)-adjusted survivals in all participating countries were similar. We conclude that DLBCL treatment outcomes in these populations can be benchmarked to international standards, despite biological heterogeneity.

ARTICLE HISTORY
Received 21 February 2016
Revised 7 August 2016
Accepted 29 August 2016

KEYWORDS
DLBCL; gene expression; risk stratification; prognosis

Introduction

Cancer centers around the world may wish to compare their results against published studies from high-income Caucasian populations. Attempts to produce similar remission and survival rates across diverse populations may not be achievable if disease biology differs in low- or middle-income countries. Biological variation may arise from ethnic diversity which may influence host response, or the physical or microbiological environment which may influence causation and disease biology.

The International Atomic Energy Agency (IAEA) sponsored a prospective cohort study of diffuse large B-cell lymphoma (DLBCL) in countries from five United Nations defined geographical regions.[1] The study had two pre-defined aims: the first to investigate whether there was inter-country heterogeneity in speed of response, as assessed by positron emission tomography (PET), as a predictor of future survival; the second, to explore whether the biological characteristics of DLBCL differ and influence outcomes between countries. Analysis of the 327 patients with complete data for the PET monitoring component of the study has recently been reported.[2] We report here the analysis of between-country biological heterogeneity and relate this to survival.

Materials and methods

Patients and treatment

The protocol was developed by the IAEA with all international collaborators between 2006 and 2008. Adults >16 y with newly diagnosed DLBCL were recruited at major cancer centers in seven countries in Western

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Europe, Central Asia, South Asia, South East Asia, and South America, during 2008–2012. Treatment was with a common chemotherapy protocol: cyclophosphamide, adriamycin, vincristine, prednisolone with rituximab (R-CHOP), delivered at 21-d intervals. Omission of rituximab was permitted in a small number of patients who might otherwise have been excluded for financial reasons.[2]

Treatment response assessment

Treatment response was based on international criteria.[3] To accurately adjust outcomes for International Prognostic Index (IPI), pretreatment staging was based on CT and PET imaging in all centers, and final response status was based on central reading of all end-treatment PET scans at a final investigator meeting, as previously described.[2] Event-free survival (EFS) at the validated time-point of 24 months was used as the comparative outcome measure.[4] We do not report overall survival, as this is strongly influenced by the variable approaches between countries to salvaging those who relapse. The common study protocol and assessment of outcomes has been described in greater detail elsewhere.[2]

Measure of biological diversity

As the measure of biological variation, we used the 6-gene-expression score for predicting survival of patients with DLBCL, first published in 2004 by Losses et al.[5] This 'mortality score' is derived from expression levels of six informative genes (three predictive of better survival *LM02, BCL6, FN1,* and three of worse survival *CCND2, BCL2, SCYA3*) which together stratified patients into low-, medium- and high-risk groups. When considered as a continuous variable, the 6-gene score predicted overall survival for patients treated with CHOP [5] and both overall and progression-free survival in a larger cohort treated with R-CHOP.[6]

Molecular methods

RNA was extracted from formalin-fixed diagnostic tissue [6] and sent to a central laboratory (Biotechnology Institute, Ankara University, Turkey) for analysis. Analyses were all performed by authors NT and HO.

RNA, 1000 ng, was used to synthesize cDNA using ABI High-Capacity cDNA Reverse Transcription in 100 μl, and 2 μl (20 ng/μl) cDNA used for each quantitative polymerase chain reaction (QPCR) reaction. Standard curves were prepared from plasmids containing the cloned target genes. TaqMan QPCR assays of the six genes plus ABL were conducted on a Light Cycler 480 platform (Roche, Germany) using standard methodology. Expression ratios were calculated based on delta delta Ct[26] $R = 2(-\Delta\Delta CT)$. The 6-gene score was calculated as originally described [4,5] and included in analyses as a continuous variable.

Statistical methods

The analysis used a Cox model to stratify by country and initially included the 6-gene score, IPI and rituximab exposure as relevant predictive variables. However, as this model did not converge sufficiently due to few events per parameter, the final analysis investigated each variable separately in a Cox model stratified by country.

Research governance

The study was approved by the relevant Research Ethics Committee, or Institutional Review Board in each participating center. All patients were recruited into the study after gaining informed consent. Biological material and data were shared between countries only identified by a study code number and with all personal identifiers removed.

Role of the funding source

The IAEA provided funding which made the study possible, but had no role in the design, analysis, or interpretation of the data, nor the decision to publish.

Results

Complete molecular and clinical data were available for 136 patients. Sixty one were from high-income countries (Chile, Hungary, and South Korea), 40 from upper-middle income countries (Thailand and Turkey), 35 from lower-middle income countries (India and Philippines) (Table 1). There was good compliance with the common treatment protocol and 114/136 (84%) of all patients received rituximab (Table 1).

At a median follow up of 2 y and 6 months, 2-y EFS for the 136 patients was 74% (95% confidence interval 65–84%), similar to the 2-y EFS recently reported from a large UK R-CHOP trial, 75% (71–78%).[7] There was moderate variation between individual countries, from 85% 2-y EFS in Chile to 56% in Turkey (Figure 1).

The distribution of individual gene expression varied significantly between countries ($p < .0001$) (Figure 2(A)). When combined into the 6-gene score there similarly was significant between-country

Table 1. Patient characteristic and 2-y event free survival by country.

	Chile	Hungary	India	Philippines	South Korea	Thailand	Turkey	All cases
Patients *(n)*	27	27	22	13	7	24	16	136
Age in years *(median)*	61	56	52	51	48	56	54	55
IPI0–1	12 (44%)	18 (67%)	10 (45%)	4 (31%)	1 (14%)	9 (37%)	6 (37%)	44%
2	3 (11%)	2 (7%)	10 (45%)	3 (23%)	3 (43%)	6 (25%)	3 (19%)	22%
3	6 (22%)	5 (19%)	2 (9%)	4 (31%)	2 (29%)	6 (25%)	6 (38%)	23%
4–5	6 (22%)	2 (7%)	0 (0%)	2 (15%)	1 (14%)	3 (13%)	1 (6%)	11%
Treatment + Rituximab	26 (96%)	27 (100%)	14 (64%)	8 (62%)	7 (100%)	16 (67%)	16 (100%)	84%
2-y EFS %	85%	80%	67%	66%	71%	74%	56%	73%

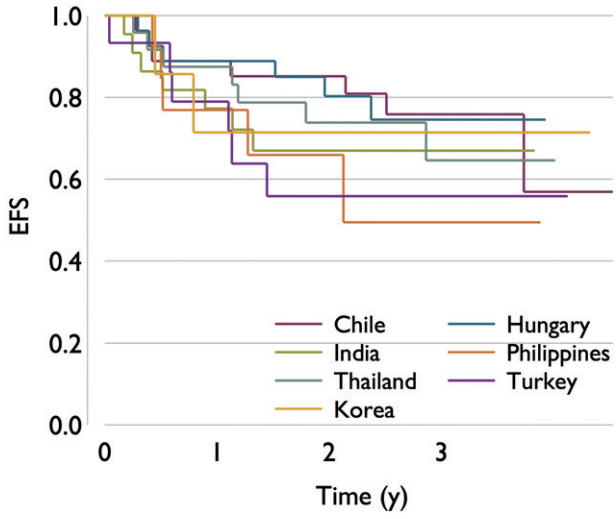

Figure 1. Composite of Kaplan–Meier plots showing event-free survival (EFS) for each country.

heterogeneity ($p < .0001$) (Figure 2(B)). The distribution of 6-gene scores within and between countries did not suggest any association with geographical region or country economic status.

Our interest was to investigate whether the highly significant between-country variation in the 6-gene prognostic score might explain the observed differences in EFSs. Multivariate analysis found only the IPI to be a significant predictor of outcome ($p = .009$), whereas neither the 6-gene score ($p = .21$), nor variation in use of rituximab between countries ($p = .08$) were significant explanatory factors.

To gain further insight into how adjustment for IPI or 6-gene score might individually influence the relative 2-y EFS between countries, Kaplan–Meier plots were generated from the Cox analysis, including country as a covariate rather than stratifying variable. Within each country, individual patient outcomes were normalized to remove the positive or negative influence of either the 6-gene score or IPI on EFS, then plotted as an adjusted survival curve. Composite figures displaying these modeled survival curves by country, without adjustment, adjusted for IPI, or adjusted for 6-gene score are shown (Figure 3).

Compared to the unadjusted 2-y EFS (Figure 3(A)), adjustment for IPI (Figure 3(B)) brings the survival curves closer together, with little difference at 2 y between Chile, Hungary, S. Korea, and Thailand. The survival curves for India, Philippines, and Turkey form a second cluster with comparatively inferior 2-y EFS. This figure reveals Turkey to have markedly improved 2-y survival after adjustment for IPI, reflecting the high proportion (75%) of stage IV and high IPI cases. India and Philippines have 2-y EFS approximately 15% lower than Chile, Hungary, and S. Korea, reflecting the lower use of rituximab (Table 1). By contrast, adjustment for the 6-gene score (Figure 3(C)) failed to bring the individual country survival curves closer together, thus demonstrating that variation in disease biology made no perceptible contribution to EFS differences between countries.

Discussion

The goal for oncologists in emerging economies is to achieve cancer outcomes comparable to the developed world.[8] The effects of late presentations with advanced disease, or reduced resources for expensive treatments are recognized challenges that may confound direct comparisons. The possible influence of variable biology across continents, even within the single histological entity of this commonest of lymphomas is currently unknown.

Global variation in the prevalence of different non-Hodgkin lymphoma (NHL) sub-types is well recognized.[9] The effect of environmental factors on DLBCL incidence, including levels of UV irradiation and infections have recently been reviewed and Epstein–Barr virus (EBV) associated with DLBCL is recognized as conferring poorer prognosis.[10–13] More recently, different inflammatory gene signatures within DLBCL tissue has been reported between Scandinavian and Egyptian cohorts.[14]

When designing this biological component of the IAEA DLBCL study, an important consideration was selection of biological markers with proven relationship to survival, and which could be assayed using

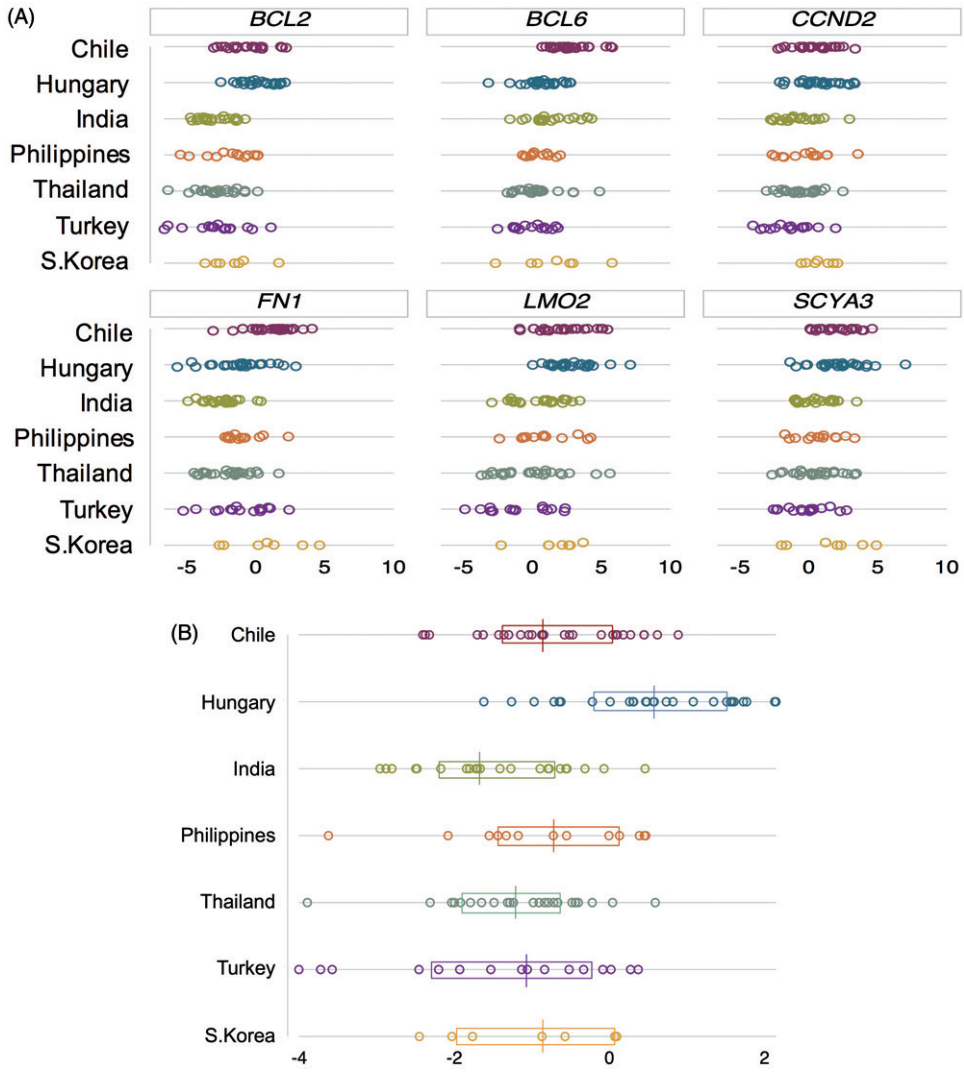

Figure 2. Heterogeneity of prognostic gene expression between countries. (A) Expression of the six individual prognostic genes for each study patient, by country. Heterogeneity between countries was significant ($p < .0001$). (B) Individual 6-gene scores, by country. Boxes represent median and inter-quartile range. Heterogeneity between countries was significant ($p < .0001$).

RNA from fixed tissue in a central laboratory. The 6-gene prognostic algorithm described by Lossos [5] had recently been published and had the merit of using standard methodology. Though initially devised for individual patient risk stratification, we used it as a global index of biological variation.

Though of lesser prognostic value in patients treated with immunochemotherapy,[15] the intention had been to also include classification of cases as activated B-cell (ABC) or germinal center B-cell (GCB) subtypes. However, this proved impracticable due to lack of resources for the necessary establishment of between center consistency in immuno-histocytochemistry, or central processing and reporting of all diagnostic biopsy sections. Recent reports have cast doubt on the robustness of immuno-histochemical (IHC) algorithms and highlighted the variability of classification between laboratories.[15,16]

The more recent 6-gene score study by Malumbres and colleagues [6] examined its relationship to outcomes of 132 patients treated with R-CHOP in three centers from North America, Canada, and Spain. The authors did not report any between-center heterogeneity in gene scores, but found in this population that, as in ours, IPI was the most significant predictor of individual progression-free survival. However, the cohort was more homogeneous than ours in terms of ethnicity and socio-economic background and, in contrast to ours, demonstrated identical unadjusted 2-y survival at the three centers.

No study, to our knowledge, has documented biological heterogeneity across a wide range of

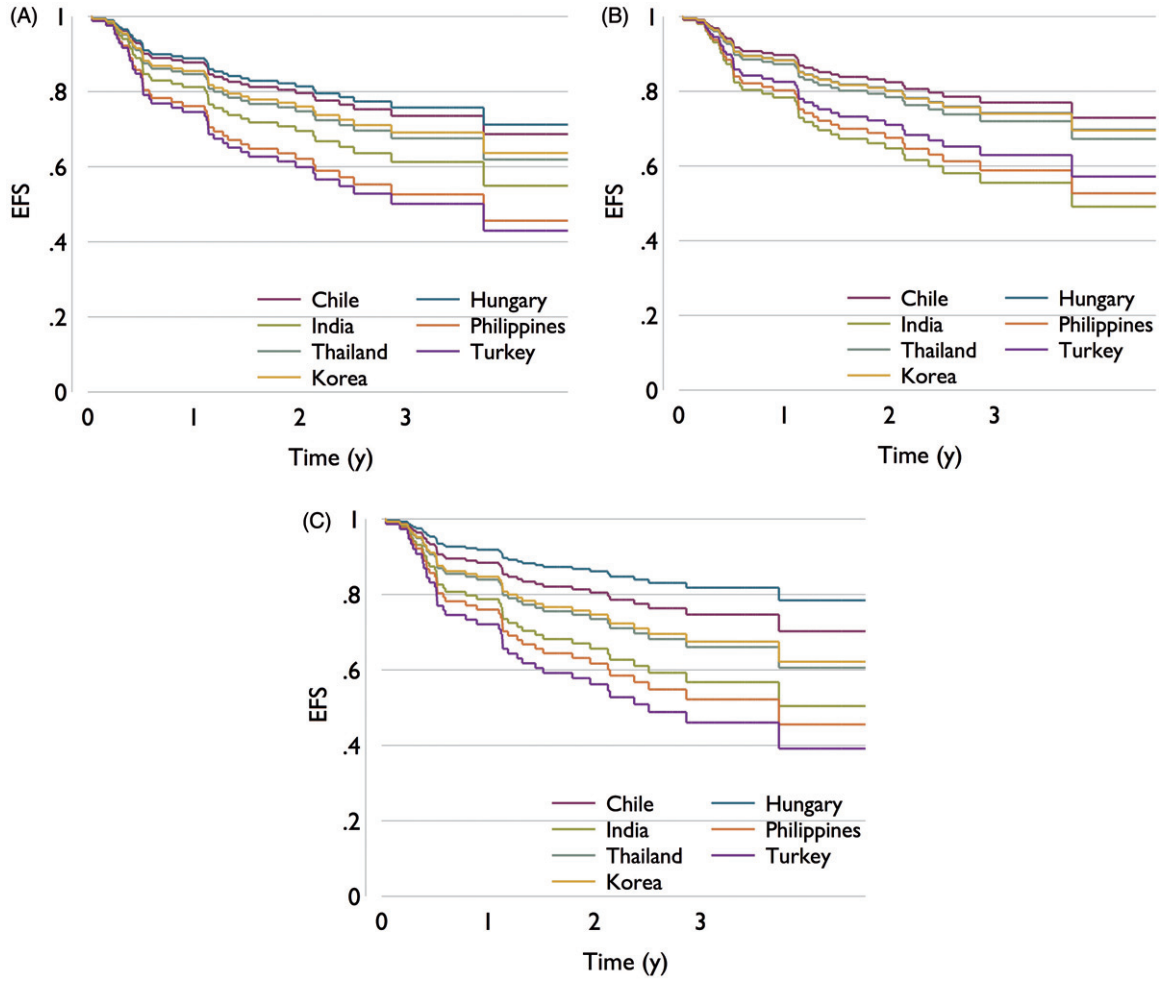

Figure 3. Kaplan–Meier EFS risk estimates for each country, generated from the Cox analysis. (A) Unadjusted survival risk estimates. (B) Risk estimates, normalized to adjust for IPI. (Note: Survival estimates for Hungary and S. Korea are superimposed in this figure). (C) Risk estimates, normalized to adjust for 6-gene scores.

socio-economic environments or ethnically diverse populations, and related this to outcomes.

Understanding the influence of variable disease characteristics between countries is an essential step in the global effort to improve lymphoma outcomes. Our study confirms the existence of significant biological heterogeneity between the populations investigated. It is to be hoped that in the future, more detailed analysis of genetic variation may be exploited to give insights into the causation of this common lymphoma.

For clinicians internationally, we have demonstrated that when adjusted for the IPI, event free survivals for DLBCL are comparable across seven socio-economic and ethnically diverse countries, despite significant differences in a validated prognostic gene-expression score. Where treatment includes rituximab, survivals are similar to recent European cohorts.

These observations provide necessary evidence that leading cancer centers around the world can benchmark their outcomes to those in high-income Western populations.

Acknowledgments

The study was made possible by financial and administrative support from the International Atomic Energy Agency.

Potential conflict of interest: Disclosure forms provided by the authors are available with the full text of this article online at http://dx.doi.org/10.1080/10428194.2016.1231308.

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