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Original Article

Immunohistochemical algorithm alone is not enough for predicting the outcome of patients with diffuse large B-cell lymphoma treated with R-CHOP

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Abstract: Gene expression profiling (GEP), which can divide DLBCL into three groups, is impractical to perform routinely. Although algorithms based on immunohistochemistry (IHC) have been proposed as a surrogate for GEP analysis, the power of them has diminished since rituximab added to the chemotherapy. We assessed the prognostic value of four conventional algorithms and the genes in each and out of algorithm by IHC and fluorescence in situ hybridization in DLBCL patients receiving immunochemotherapy. The results showed that neither single protein within algorithms nor the IHC algorithms themselves had strong prognostic power. Using MYC aberrations (MA) either on the genetic or protein levels, we established a new algorithm called MA that could divide patients into distinct prognostic groups. Patients of MA had much shorter overall survival (OS) and progression-free survival (PFS) than non-MA (2-year OS: 56.9% vs. 98.7%; 2-year PFS: 26.8% vs. 86.9%; $P < 0.0001$ for both). In conclusions, using additional prognostic markers not associated with cell of origin may accurately predict outcomes of DLBCL. Studies with larger samples should be performed to confirm our algorithm and optimize the prognostic system of DLBCL.

Keywords: Diffuse large B-cell lymphoma, algorithms, immunohistochemistry, fluorescence in situ hybridization, MYC aberrations

Introduction

Diffuse large B-cell lymphoma (DLBCL) is the most common lymphoid malignancy in adults, accounting for 30%-40% of all cases of non-Hodgkin lymphoma (NHL) in Western countries [1]. The standard therapy for patients with DLBCL is rituximab combined with cyclophosphamide, doxorubicin, vincristine, and prednisone (R-CHOP), which has a 10-year disease-free survival (DFS) of approximately 42.6% [2]. Gene expression profile (GEP) studies have confirmed that DLBCL can be subdivided into subtypes depending on their gene signatures: germinal center B-cell (GCB) or GCB-like, activated B-cell (ABC) or ABC-like, and type 3 [3, 4]. The molecular and genetic distinction, such as MYC rearrangement concurrent with BCL2 rearrangement [5, 6], is also important, for patients in subgroups defined by these features respond differently and have different prognoses when treated with R-CHOP [7].

Because GEP is expensive and not readily available in routine practice, several algorithms have been proposed in recent years; these algorithms have been based on immunohistochemical (IHC) staining or tissue microarray analysis, which is a surrogate for GEP analysis [8]. GCB subtype tend to have a more favorable outcome than those with non-GCB subtype in patients treated with CHOP, irrespective of the International Prognostic Index (IPI) score [9]. However, since rituximab was added to CHOP as the standard of care, different factors have been shown to be important in determining a patient's prognosis when using different IHC algorithms for prediction [7, 10, 11].

The most commonly used algorithm, proposed by Hans et al [12] is based on the expression of three proteins (CD10, Bcl6 and MUM1/IRF4) which can classify DLBCL patients into two categories (GCB and non-GCB) with different prognoses. This algorithm, however, was created for

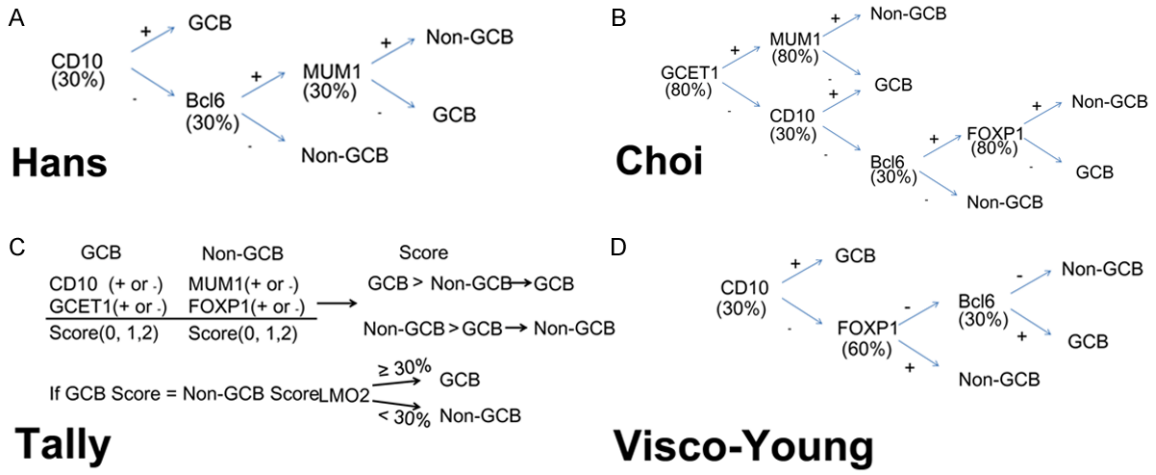

Figure 1. The four algorithms applied in this study were Hans (A), Choi (B), Tally (C), and Visco-Young (D). Abbreviation: GCB: germinal center B-cells.

use in patients to be treated only with CHOP. In addition, the predictions made by this algorithm had low concordance with those from GEP analysis (71% concordance for GCB, and 88% for non-GCB) [8]. The prognostic relevance of the Hans algorithm led to inconsistent results in subsequent studies performed in patient groups treated with R-CHOP [7, 13-18].

In 2009, Choi et al [15] reported a combination of five markers: GCET1, CD10, Bcl6, MUM1/IRF4, and FOXP1, which can achieve a concordance of about 90% with the GEP in patients treated with R-CHOP [15]. Compared with the Hans algorithm, the Choi algorithm integrated the analysis of two new molecules: FOXP1 and GCET1. Prediction was more accurate than that of the Hans algorithm and facilitated risk stratification of DLBCL patients.

In 2011, Meyer et al [19] reported another algorithm (called the "Tally" algorithm) that had a high concordance (93%) with GEP and was also based on the expression of five markers: CD10, GCET1, FOXP1, MUM1, and LMO2. This method includes an equal number of GCB (GCET1 and CD10) and non-GCB (FOXP1 and MUM1) antibodies. Classification is determined by the immunophenotype pair with more positive antigens. If an equal number of GCB and ABC antigens are positive, then LMO2 determines the immunophenotype (i.e., LMO2 ≥ 30% yields GCB).

Recently, a report from the International DLBCL R-CHOP Consortium introduced a new algorithm called "Visco-Young" ; it is based on the expression of CD10, FOXP1, and Bcl6, and it demonstrated a concordance of 92.6% with GEP and the ability to independently predict the rate of progression-free survival (PFS) and overall survival (OS) [8]. In multivariate analysis, both the IPI and the Consortium's algorithm were significant independent predictors of PFS and OS [8].

It is notable that most of these algorithms (**Figure 1**) have been developed and tested only in Western countries; few reports have confirmed the practicability of their use in Eastern or other nations. In this study, we analyzed the four algorithms to determine each one's power to predict the prognosis of Eastern DLBCL patients treated with R-CHOP-like therapies.

Material and methods

Ethics statement

All patients provided informed consent in accordance with requirements of the Declaration of Helsinki, and the research project was approved by the University and Institutional Review Boards.

Patients

We retrospectively studied 244 adult patients with de novo DLBCL who had been diagnosed between February 2006 and January 2014. All of the paraffin-embedded sections were reviewed by two hematopathologists (QXG and TXL), and the diagnoses were based on the

Table 1. Consistent and inconsistent numbers (percentages) of cases between pairs of algorithms

Algorithms	H-G	H-non-G	C-G	C-non-G	T-G	T-non-G	V-G	V-non-G
H-G	/	/	89 (36.5)	10 (4.1)	56 (23.0)	43 (17.6)	83 (34.0)	16 (6.6)
H-non-G	/	/	30 (12.3)	115 (47.1)	13 (5.3)	132 (54.1)	24 (9.8)	121 (49.6)
C-G	89 (36.5)	30 (12.3)	/	/	57 (23.4)	62 (25.4)	107 (43.9)	12 (4.9)
C-non-G	10 (4.1)	115 (47.1)	/	/	12 (4.9)	113 (46.3)	0 (0.0)	125 (51.2)
T-G	56 (23.0)	13 (5.3)	57 (23.4)	12 (4.9)	/	/	53 (21.7)	16 (6.6)
T-non-G	43 (17.6)	132 (54.1)	62 (25.4)	113 (46.3)	/	/	54 (22.1)	121 (49.6)
V-G	83 (34.0)	24 (9.8)	107 (43.9)	0 (0.0)	53 (21.7)	54 (22.1)	/	/
V-non-G	16 (6.6)	121 (49.6)	12 (4.9)	125 (51.2)	16 (6.6)	121 (49.6)	/	/

Abbreviations: GCB: germinal center B-cells; H-G: Hans GCB subtype; H-non-G: Hans non-GCB subtype; C-G: Choi GCB subtype; C-non-G: Choi non-GCB subtype; T-G: Tally GCB subtype; T-non-G: Tally non-GCB subtype; V-G: Visco Young GCB subtype; V-non-G: Visco-Young non-GCB subtype.

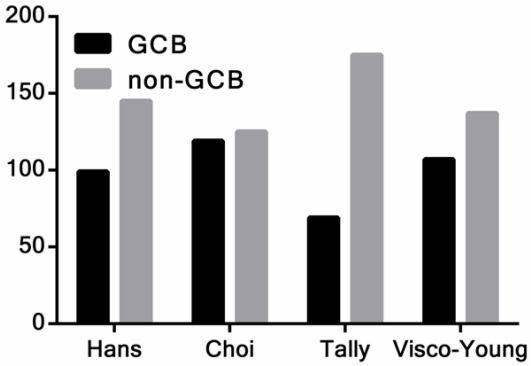

Figure 2. The distribution of GCB and non-GCB patients for each algorithm. There were significant differences among the algorithms ($P < 0.0001$). Abbreviation: GCB: germinal center B-cells.

World Health Organization classification criteria [20]. Among these 244 patients, 141 cases were treated with R-CHOP-like therapy, which was used for prognostic analysis.

IHC

IHC was performed on 4 μm formalin-fixed paraffin-embedded (FFPE) sections. The antibodies used were CD10, Bcl6, MUM1, FOXP1, GCET1, LMO2, Myc, and Bcl2. The cutoff scores for each antibody were described previously [8, 12, 15, 19].

Fluorescence in situ hybridization (FISH)

FISH analysis was performed using FFPE tissue sections according to the manufacturer's instructions with MYC dual-color, break-apart translocation probe (Vysis LSI) and IGH/BCL2 dual-color, dual-fusion translocation probe (Vysis LSI). The cut-off levels for the probes were established by evaluating the split signal distribution in samples of reactive lymphoid tissues, calculating the mean number of split signals plus three times the standard deviation. The cut-off levels were 14% and 5% for MYC break apart probe and IGH/BCL2 dual-color, dual-fusion translocation probe.

Statistical analysis

The OS and PFS distributions for each algorithm were estimated by the Kaplan-Meier method, with differences evaluated by the log-rank test. OS was defined as the time from initial diagnosis to death or last follow-up. PFS was defined as the time from initial diagnosis to disease progression, start of salvage treatment, additional (unplanned) treatments, relapse, or death from any cause, additional therapy, day of relapse, or day of death from any cause. Patients who were alive and progression-free at last follow-up were censored for this analysis. Chi-squared and Fisher exact tests were used to determine the level of consistency among algorithms and pairwise agreement between different proteins. The Spearman test was used to analyze correlations among variables. For all tests, a P value of 0.05 was considered statistically significant.

Results

Algorithms applied in this study

The published algorithms examined in this study were those of Hans [12], Choi [15], Tally [19], and Visco-Young [8].

According to the algorithms applied in this study, 244 cases of de novo DLBCL could be further investigated by IHC. For the Hans algorithm, 99 cases were classified as GCB and

Table 2. Concordance rates (x^2 values) and κ coefficients for the four IHC algorithms

Algorithm	Hans	Choi		Tally		Visco-Young	
Hans		x^2 = 112.789	κ = 0.671	x^2 = 65.724	κ = 0.500	x^2 = 108.180	κ = 0.664
Choi				x^2 = 44.090	κ = 0.387	x^2 = 200.178	κ = 0.901
Tally						x^2 = 42.445	κ = 0.394

$P < 0.001$ for all x^2 analyses. Abbreviation: IHC: immunohistochemistry.

Table 3. Baseline characteristics of the 141 patients in the R-CHOP-like group

Characteristic	No. (%) of patients
Age	
≤ 60	82 (58.2)
Male	92 (65.2)
Stage III-IV	75 (53.2)
Elevated LDH	59 (41.8)
PS ≥ 2	29 (20.6)
Extranodal sites ≥ 2	34 (24.1)
IPI score of 3-5	50 (35.4)
B symptoms	52 (36.9)
Treatment responses	
CR	82 (58.2)
PR	35 (24.8)
SD/PD	24 (17.0)

Abbreviations: PS: Eastern Cooperative Oncology Group performance status; IPI: International Prognostic Index; LDH: lactate dehydrogenase; CR: complete remission; PR: partial remission; SD/PD: stable disease/progression of disease.

145 cases as non-GCB. For the Choi, Tally, and Visco-Young algorithms, 119 and 125, 69 and 175, and 107 and 137 cases were classified as GCB and non-GCB types, respectively. Details of the results are shown in **Table 1** and **Figure 2**.

The consistency across all four algorithms was 63.52% (155/244). When the results of the algorithms were compared pairwise, however, the consistency was generally better. The Choi and Visco-Young algorithms showed the highest concordance rate (95.08%, X^2 = 200.178, κ = 0.901), while the Choi and Tally algorithms had the lowest concordance rate (69.67%, X^2 = 44.090, κ = 0.387). The details of the agreements among the algorithms were illustrated in **Table 2**.

Prognostic significance of original IHC algorithms

The baseline characteristics of the 141 patients in the R-CHOP-like group are listed in **Table 3**.

Table 4. Differences in survival for the four tested algorithms between GCB and non-GCB patient subgroups

Algorithm	Subtype	Numbers	*P* value (OS)	*P* value (PFS)
Hans	GCB	62	0.142	0.210
	Non-GCB	79		
Choi	GCB	71	0.705	0.808
	Non-GCB	70		
Tally	GCB	44	0.188	0.022
	Non-GCB	97		
Visco-Young	GCB	64	0.706	0.889
	Non-GCB	77		

Abbreviations: GCB: germinal center B-cells; OS: overall survival; PFS: progression-free survival.

None of the four algorithms showed significant differences in OS and PFS (except for Tally algorithm, P = 0.022 for PFS) between patients with GCB and non-GCB subtypes (**Table 4**, **Figure 3**).

Prognostic significance of single markers

Since the four algorithms showed poor prognostic significance, we analyzed single protein in each algorithm (**Table 5**). None of the proteins predicted significant differences in survival. In addition, pairwise agreement and correlation tests showed that LMO2 had a negative correlation with other GCB markers (data not show).

Furthermore, we observed a cohort of patients, treated with chemoimmunotherpy, whose disease had progressed and who had died mostly in the first two years. In order to determine whether these patients had special poor prognosis factors, we performed IHC and FISH with additional markers. MYC and BCL2, two factors receiving considerable attention currently, were analyzed in our cohort of patients. On the protein level, Myc expression showed significantly decreased survival (2-year OS, 53.4% vs. 96.6%, P < 0.0001; 2-year PFS, 27.5% vs. 81.9%, P < 0.0001). Bcl2 protein, however, pre-

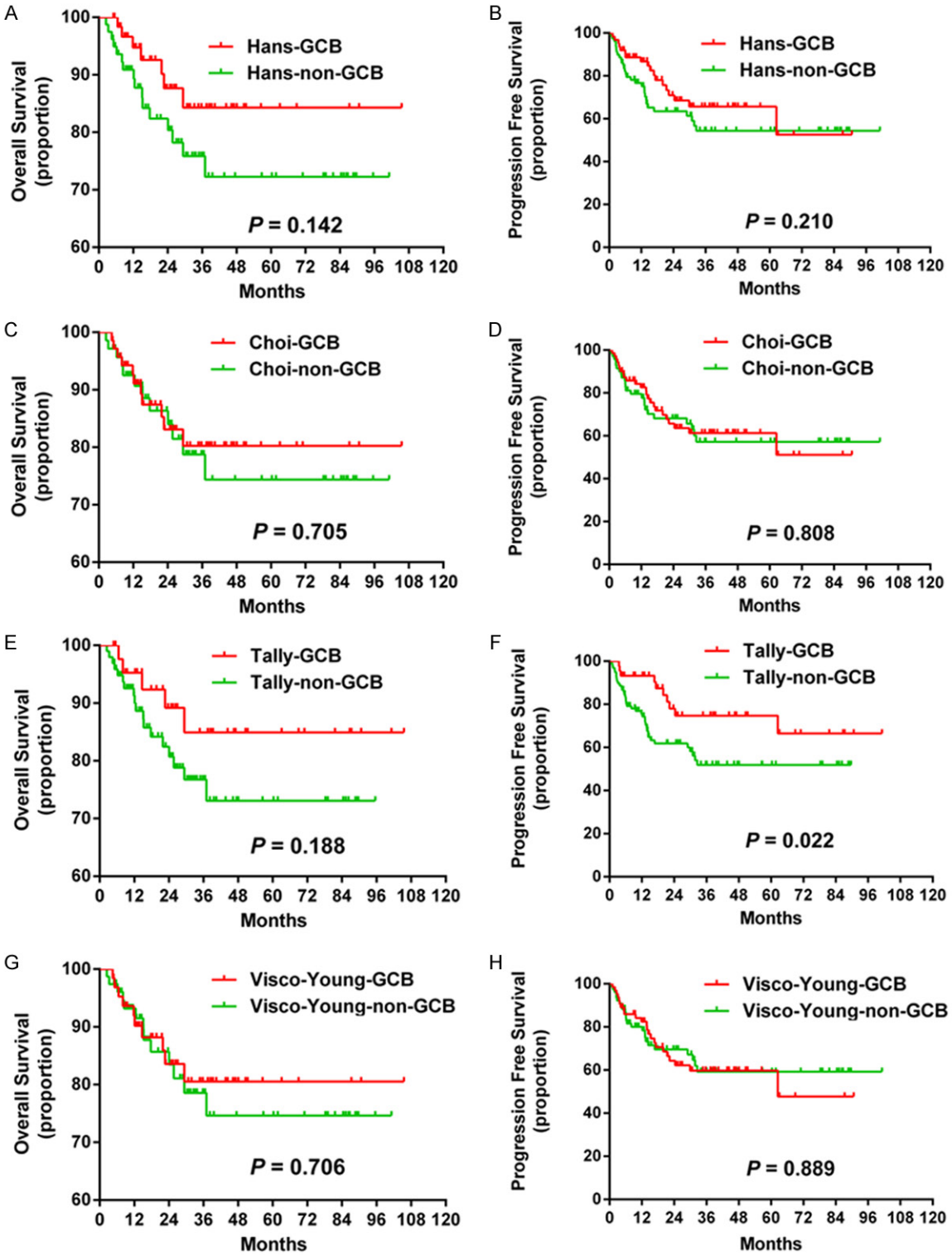

Figure 3. Survival curves calculated using the Hans, Choi, Tally, and Visco-Young algorithms. The Hans Choi and Visco-Young algorithms showed no differences in OS (A, C and G) or PFS (B, D and H). The Tally algorithm showed significant differences in PFS (F) but not OS (E). Abbreviations: GCB: germinal center B-cells; OS: overall survival; PFS: progression-free survival.

dicted significant differences in PFS (2-year PFS, 57.2% vs. 76.9%, P = 0.009) but not OS (2-year OS, 79.4% vs. 90.8%, P = 0.154). On the gene level, the results showed that MYC rear-

Table 5. Prognosis predicted by single protein expression or gene rearrangement

Variables	No. of patients	*P* value for OS	*P* value for PFS
CD10-30%	37 vs. 94	*P* = 0.456	*P* = 0.333
Bcl6-30%	98 vs. 43	*P* = 0.621	*P* = 0.263
MUM1-30%	86 vs. 55	*P* = 0.183	*P* = 0.315
GCET1-30%	41 vs. 100	*P* = 0.632	*P* = 0.175
GCET1-80%	17 vs. 124	*P* = 0.387	*P* = 0.885
FOXP1-30%	106 vs. 35	*P* = 0.282	*P* = 0.128
FOXP1-60%	84 vs. 57	*P* = 0.898	*P* = 0.559
FOXP1-80%	69 vs. 72	*P* = 0.710	*P* = 0.947
LMO2-30%	108 vs. 33	*P* = 0.587	*P* = 0.385
Myc-40%	42 vs. 99	*P* < 0.0001	*P* < 0.0001
Bcl2-50%	72 vs. 69	*P* = 0.154	*P* = 0.009
MYC rearrangement	15 vs. 126	*P* < 0.0001	*P* < 0.0001
BCL2 rearrangement	20 vs. 121	*P* = 0.392	*P* = 0.298

Abbreviations: OS: overall survival; PFS: progression-free survival.

rangement predicted decreased OS (2-year OS, 52.5% vs. 89.1%, *P* < 0.0001) and PFS (2-year PFS, 33.3% vs. 71.6%, *P* < 0.0001) (**Table 5**). However, BCL2 rearrangement showed no differences in survival for either OS ((2-year OS, 74.5% vs. 86.3%, *P* = 0.392) or PFS (2-year PFS, 48.5% vs. 69.4%, *P* = 0.298) (**Table 5**).

Prognostic significance of adding MYC aberrations to the original IHC algorithms

Since the four tested algorithms showed poor predictive ability and the single marker results showed only Myc protein and MYC rearrangement predicted significant outcomes for DLBCL patients. We decided to combine Myc protein and MYC rearrangement into an additional reference index, called "MYC aberrations" (MA). MA defined a single unfavorable group, with either Myc expression or MYC rearrangement. Cases without MA were reclassified using the original algorithms. Each new algorithm was designated by adding "-MA" to the original name (for example, the new Hans algorithm was called Hans-MA in order to distinguish it from the original).

All the four new algorithms showed significant differences in OS and PFS between MA and GCB or non-GCB (**Figure 4**). However, no differences of OS and PFS (except for the Tally-MA algorithm) were observed between the GCB and non-GCB groups.

Prognostic significance of MA

We then used MA as a single algorithm, without including the original algorithms. We defined three MA types. Type 1 was negative for both Myc protein and MYC rearrangement. Type 2 was as positive for either Myc protein or MYC rearrangement. Type 3 was positive for both Myc protein and MYC rearrangement. Survival analysis showed significant differences in OS and in PFS between Type 1 and Type 2 or 3 (P < 0.0001 for both), while no difference of OS and PFS was observed between Type 3 and Type 2 (**Figure 5**). We then combined the results of Type 3 and 2; Type 2/3 predicted extremely poor OS and PFS relative to the Type 1 (*P* < 0.0001 for both) (**Figure 5**).

Discussion

As a consequence of the work described above, we conclude that a new algorithm, based on MA, which showed a significant prognostic value in DLBCL patients treated with R-CHOP-like therapies. DLBCL is considered aggressive, and predicting the outcome of an individual patient is still difficult. This difficulty stems from the fact that DLBCL is a clinically and biologically heterogeneous group of lymphoma, with no clear histological criteria for subdivision [21]. Although new developments in chemoimmunotherapy, especially the anti-CD20 antibody rituximab, have improved the survival of patients with DLBCL, prognosis prediction is still difficult [22, 23]. Currently, GEP, the standard method to designate patients into molecular subsets, is not clinically practical. This fact has led to efforts to find robust, affordable, and reproducible techniques to approximate the information gained from GEP. IHC algorithms that are supposed to be useful surrogates for the classification of DLBCL subsets have been published, most of which use a combination of antibodies against GCB and ABC specific antigens [8, 12, 15, 19]. However, the prognostic power of these algorithms has weakened with the development of new drugs, including rituximab [7, 10, 11]. Based on previous reports [10, 11] and our data, we suggest none of the

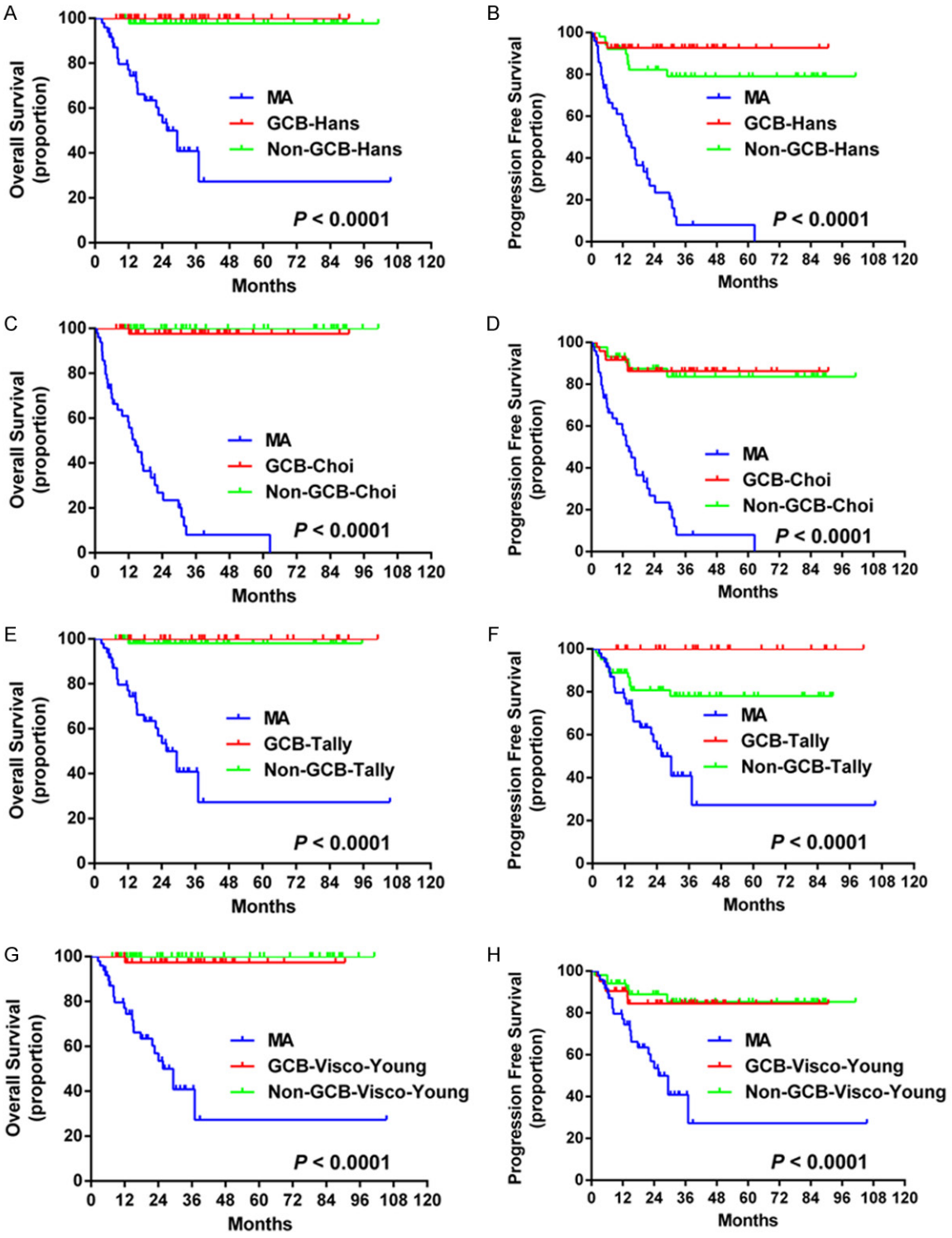

Figure 4. Survival curves for the Hans-MA, Choi-MA, Tally-MA, and Visco-Young-MA algorithms. The algorithms of Hans-MA, Choi-MA and Visco-Young-MA showed significant differences in OS (A, C, G) and PFS (B, D, H) between MA and GCB or non-GCB groups while no differences in OS and PFS were observed between the GCB and non-GCB groups. The Tally-MA algorithm showed significant differences in OS (E) between the MA and GCB or non-GCB groups and in PFS (F) among the three groups (GCB, non-GCB and MA). Abbreviations: GCB: germinal center B-cells; OS: overall survival; PFS: progression-free survival; MA: MYC aberrations.

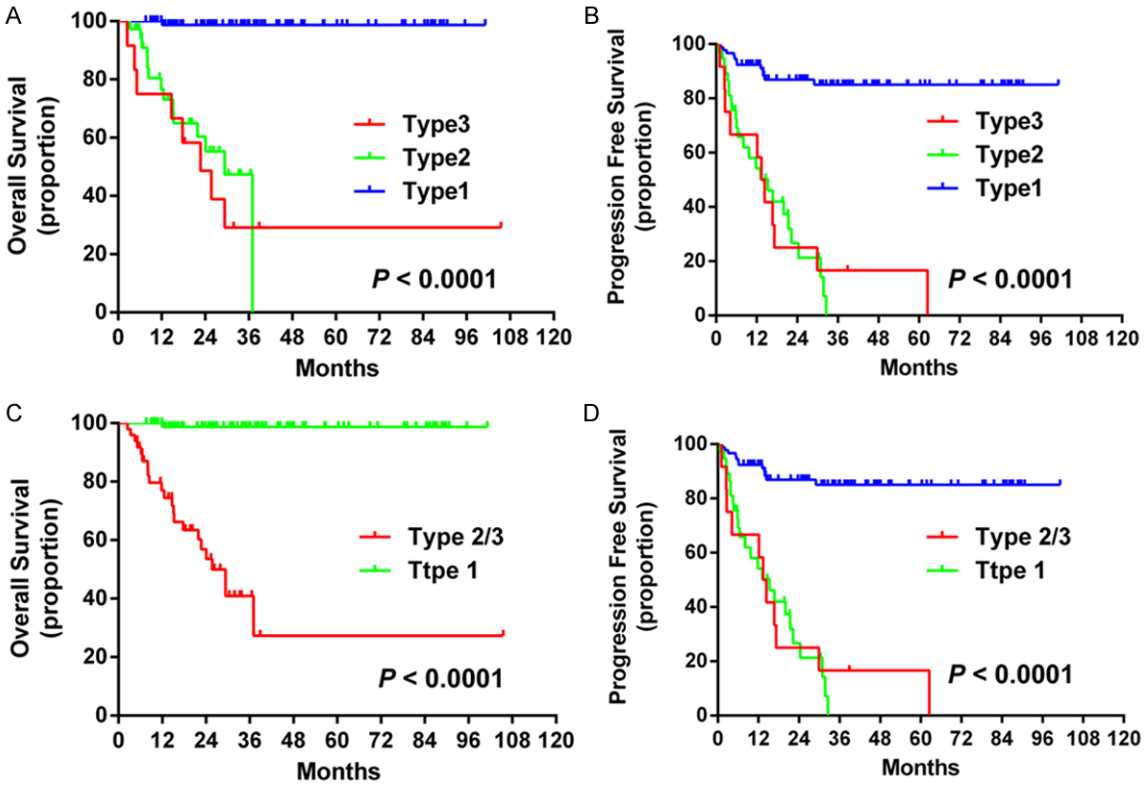

Figure 5. Survival curves for the MA algorithm. The MA algorithm showed significant differences in OS (A) and PFS (B) between patients with Type 1 and 2 or 3. No difference in OS or PFS was observed between those with Type 2 and Type 3 disease. Significant differences in OS (C) and PFS (D) were observed between Type 2/3 and Type 1 groups. Abbreviation: GCB: germinal center B-cells; OS: overall survival; PFS: progression-free survival; MA: MYC aberrations.

current algorithms alone dependably predict outcomes for DLBCL patients, especially for those patients receiving an R-CHOP-like therapy.

We then systematically analyzed the prognostic values by examining the predictive value of single markers in the IHC algorithms. It was previously reported that low CD10 expression (< 20% of cells) predicted poor OS in DLBCL patients [24]. However, we didn't find the correlation between OS/PFS and CD10 expression. Bcl6 is a marker associated with both GCB and ABC subtype [11], which suggests Bcl6 may not a dependable marker used to predict cell of origin [COO] alone. In our study, Bcl6 expression showed no impact on survival. MUM1, as a post- germinal center marker, was once reported to have a negative impact on OS in CHOP-treated patients [25]. Our patients who were treated with R-CHOP-like therapies, however, showed no survival difference on MUM1. We used the two different cut-off values of GCET1 recommended in the Choi and Tally algorithms. Although GCET1 is a marker restricted to GCB subtype [26], neither of the two cut-off values showed different effects on survival. FOXP1, an ABC subtype–associated transcription factor, also seemed to have less prognostic value than when used in the algorithms [27, 28], which was confirmed in our study. LMO2, the marker used in the Tally algorithm, was reported to predict an improvement of outcome with or without additional markers [29, 30]. In our study, we analyzed the value of LMO2 in the R-CHOP group. LMO2 expression predicted no effect on OS and PFS using a cut-off of 30%. However, in consistency and correlation analyses, LMO2 was found to have a negative relationship with other GCB markers.

Besides the markers included in each algorithm, we also analyzed the proteins beyond those encoded by these algorithms. Myc and Bcl2, for instance, play important roles in predicting prognosis in DLBCL [5, 31-33]. As re-

ported in most studies [31, 32], Myc expression conferred significantly inferior OS and PFS. Bcl2 expression, however, demonstrated no impact on OS but did contribute to decreased PFS. Since the Bcl2 protein had no consistent prognostic value in previous reports [34, 35], more inquiry is still needed to confirm the role of Bcl2 protein in DLBCL.

In addition, we performed FISH tests on MYC and BCL2, which are two classic genes rearranged in double-hit lymphoma [5, 6, 36, 37]. Like Myc expression, MYC rearrangement played a more robust role than BCL2 in predicting the outcome of DLBCL in the R-CHOP-like treatment group. Moreover, we paid great attention to the survival curves, which showed that each algorithm had an obvious survival overlap between GCB and non-GCB subtypes during the initial two years after diagnosis, which means these patients had extremely poor outcomes. A considerable number of these patients had MA (either Myc expression or MYC rearrangement). We therefore incorporated MA into the original algorithms and established a new algorithm (called MA). In accordance with our expectations, patients of MA had much shorter OS and PFS than non-MA. However, the non-MA patients (GCB or non-GCB), still showed no differences in survival (except for PFS of Tally-MA algorithm). One reason for this lack of difference is the heterogeneity of DLBCL, which makes prediction of DLBCL outcomes on the basis of IHC algorithms or prognostic markers alone inexact. The limited number of patients enrolled in our studies might be another factor. Moreover, besides R-CHOP, new targeted drugs have been developed [38, 39], which also blurs the survival boundary between GCB and non-GCB.

The MA algorithm could be used to classify DLBCL into three groups (MA, GCB and non-GCB), and our results showed that this division could be used to produce predictions that were better than any other algorithms. Since the final purpose of each algorithm was to predict the different outcome of patients and other algorithms had also used markers not associated with COO [19], we tried to abandon the original algorithms, which mainly depended on COO. Based on this, we established a new algorithm mainly depended on MYC aberrations, which could then classify the patients into three types. The results indeed showed much better power than any of the algorithms published before. Significant differences of OS were observed among patients with the three types of disease, while no difference of survival was seen between Type 2 (either Myc expression or MYC rearrangement) and Type 3 (both Myc expression and MYC rearrangement), mostly because of the limited cases. The Type 2/3 predicted extremely poor survival compared with the Type 1 group. Although not as accurate as GEP, this algorithm relies solely on MYC aberrations, and can be applied in routine practice, and had a better prognostic value than the conventional algorithms. In addition, it was much simpler than other algorithms, most of which applied many antibodies in a compulsory order. We believe that this MA algorithm will be useful in future research to predict outcomes for DLBCL patients. Since we enrolled a relative small group of patients, additional studies are needed to confirm our results and optimize our algorithm.

In summary, our data indicate that IHC algorithms alone are no long sufficient to predict outcomes for DLBCL patients. New prognostic markers may help to distinguish patients with poor survival from the total cohort. We suggested incorporating new markers into the prognostic systems of DLBCL and applying useful detection techniques to improve these systems' predictive ability.

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Disclosure of conflict of interest

None.

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