The impact of P53 and P21^{waf1} expression on the survival of patients with the germinal center phenotype of diffuse large B-cell lymphoma

Carlo Visco
Fabio Canal
Claudia Parolini
Annalisa Andreoli
Achille Ambrosetti
Mauro Krampera
Maurizio Lestani
Giovanni Pizzolo
Marco Chilosi

Immunohistochemically detected over-expression of *P53*-related protein (P53^{+++}) and absence of P21^{waf1} expression (P21^{-}) correspond to loss of function of the *P53*-gene in diffuse large B-cell lymphoma (DLBCL) patients. Using immunohistochemistry we examined 80 patients with DLBCL and found that 23% had the P53^{+++}/P21^{-} phenotype while 51% had a germinal center (GC) pattern. Both the P53^{+++}/P21^{-} phenotype and the non-GC pattern were associated with inferior outcome. Notably, the prognostic power of the P53^{+++}/P21^{-} phenotype was restricted to patients with a GC pattern, without effect on outcome of patients with a non-GC phenotype. Our results show that immunohistochemistry can parallel gene expression profiling in addressing clinical variability of DLBCL patients.

Key words: DLBCL, germinal center, prognosis, P53, P21.

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Frrom the Departments of Hematology, Ospedale S. Bortolo, Vicenza, Italy (CV); Clinical and Experimental Medicine, Section of Hematology, (MK, AAn, AAm, GP); Pathology, University of Verona, Verona, Italy (FC, CP, MC)

Correspondence:
Carlo Visco, Divisione di Ematologia, Ospedale S. Bortolo, Via Rodolfi 37, 36100 Vicenza, Italy.
E-mail: carlovisco@hotmail.com

Gene expression signatures can be used to predict the prognosis of patients with diffuse large B-cell lymphoma (DLBCL). Patients with a germinal center (GC) B-cell-like signature have a more favorable course than those with an activated B-cell like or post-germinal center profile (non-GC).[1,2] With the aim of translating these findings into readily available methods for clinical practice, there have been claims that immunohistochemistry for markers of GC or non-GC derivation (BCL-6, CD10, MUM-1, CD138), could be used to discriminate between GC-DLBCL and other tumors.[3,4] *P53* mutations have been associated with adverse outcome in DLBCL.[5] *P21^{waf1}* is a downstream effector of *P53* activation, and its induction determines cell cycle arrest. Mutations of *P53* result in the production of a protein with an abnormal structure and prolonged half-life, which is unable to induce P21^{waf1} expression and accumulates in the nucleus, resulting over-expressed by immunohistochemistry. Consequently, the pattern of P21^{waf1} cellular expression correlates with nuclear accumulation of *P53*-encoded protein and/or *P53* mutations.

We previously reported that point-mutations of *P53* were solely observed in 50 of 253 patients with the P53^{+++}/P21^{-} phenotype, defined by a cut-off of 50% for P53^{+} neoplastic cells.[6] Such a phenotype correlates strongly with a mutated or disrupted *P53* status, as also reported by others.[7–9]

Since a correlation between *P53* and *BCL-6* in the pathogenesis of tumors arising from the GC has been recently described,[10,11] we investigated the prevalence and the prognostic impact of the P53/P21^{waf1} pattern, identified by immunohistochemistry, among patients with GC or non-GC DLBCL in a series of homogeneously treated patients from a single center.

Design and Methods

Eligibility criteria and treatment

Eligibility criteria to enter this study included availability of complete data for the determination of the International Prognostic Index (IPI) at presentation; human immunodeficiency virus (HIV) negativity; treatment with anthracycline-based combination regimens; and a diagnostic specimen consisting of a lymph-node biopsy. Patients with primary mediastinal large B-cell lymphoma were excluded because of recently identified distinctive molecular features of this form of lymphoma.[12] Similarly, patients with DLBCL transformed from low-grade lymphomas, or with primary central nervous system, cutaneous or testicular lymphoma were excluded because of the peculiarity of their treatment and outcome.[13-15] This study was therefore restricted to a cohort of DLBCL patients with maximum clinical, pathological, and treatment uniformity.

Of 204 consecutive DLBCL patients who presented at Verona University between 1990 and 2003, 80 patients were eligible for this study. Clinical characteristics and outcome of these 80 patients did not differ from the remaining 124 DLBCL patients who presented in the same time period, except for extranodal presentation, which was significantly less represented among our cases (25% vs 45%, p=0.02), as a logical consequence of our eligibility criterion requiring lymph-node tissue for immunohistochemical analysis.

The patients had been treated with VACOP-B (57%), CHOP (29%, median 6 cycles), or intensified chemotherapy mega-CEOP (14%, median 6 cycles). Rituximab was routinely added to all of the regimens since 2002 and was administered to a total of ten patients.

Radiotherapy was administered after chemotherapy to 36 patients who presented with a bulky mass or who had a detectable residual mass after chemotherapy. All patients completed their planned treatment.

Pathology and immunohistochemical analysis

All diagnoses were made at our Institution; slides were reviewed in all cases and classified according to WHO criteria. The immunohistochemical evaluations were independently performed in all cases by two pathologists (*FC* and *MC*) on whole sections of the tissue, and the interpretation of results was reproducible in the vast majority of cases. Immunostaining was performed on formalin-fixed paraffin-embedded tissues, as previously described.[16] All samples were tested for BCL-2, CD10, BCL-6, MUM-1/IRF4, and CD138. Samples were considered positive when more than 30% of the tumor cells carried these markers. A semi-quantitative evaluation of P53 and P21 expression was performed by estimating the percentage of positive cells. A cut-off of 50% of positive tumor cells was used to define P53 over-expression ($P53^{+++}$), while P21 was considered positive when expressed in more than 10% of tumor cells. To determine the GC origin of the lymphoma, patients were subdivided following the indications given by Hans *et al.*, whose report provided immunohistochemical confirmation of the predictive value of cDNA microarray results.[4]

Results and Discussion

The characteristics of the patients at diagnosis are listed in Table 1. At present, after a median follow-up for survivors of 77 months (range 15-180), the 5-year overall survival and progression-free survival rates are 58% and 42%, respectively. Of our 80 patients, 46 (57%) stained positive for P53 to some extent, and 21 (26%) over-expressed the protein. All 21 patients with P53 over-expression, except two, stained negative for $P21^{waf1}$. Therefore 19 patients (23%) had a $P53^{+++}/P21^{-}$ phenotype. Of the remaining patients, 30 (38%) stained negative for both P53 and $P21^{waf1}$, 20 (26%) were $P53^{+}/P21^{+}$, 5 (6%) were $P53^{+}/P21^{-}$, and four (5%) were $P53^{-}/P21^{+}$. Forty-one patients were classified as having GC DLBCL, and 39 as having non-GC B-cell lymphoma. CD10 was positive in 28 patients, BCL-6 in 35, MUM-1/IRF4 in 31, and CD138 in two patients. Fifteen patients expressed both CD10 and BCL-6, 13 patients were $CD10^{+}/BCL\text{-}6^{-}$, 20 patients were $CD10^{-}/BCL\text{-}6^{+}$, while 32 patients were negative for both CD10 and BCL-6. Neither $P53^{+++}/P21^{-}$ phenotype nor GC pattern showed any correlation with the presence of any clinical or laboratory features by the $\chi 2$ test.

Survival analysis

The results of univariate analyses are shown in Table 1. The 19 patients with the $P53^{+++}/P21^{-}$ phenotype had a significantly lower 5-year overall survival compared with patients with other phenotypes (41% vs 56%, p=0.04). Likewise, progression-free survival was lower in the $p53^{+++}/p21^{-}$ patients than in patients with other phenotypes (27% vs 46%, p=0.02). Among these, patients with $P53/P21^{waf1}$ phenotypes other than $P53^{+++}/P21^{-}$ had similar progression-free survival. Patients with a GC phenotype had a better 5-year overall survival (72% vs 40%, p=0.006), and progression-free survival (57% vs 23%, p=0.01), as compared to those with a non-GC phenotype. Both the $P53/P21^{waf1}$ phenotype and the B-cell pattern maintained their predictive power in terms of progression-free and overall survival in each IPI risk group (*data not shown*).

Table 1. Clinico-pathological characteristics, and univariate analysis for progression-free survival (PFS) in 80 DLBCL patients.

	Patients	*5-years PFS (%)*	*p*
All patients	80	42	NA
Age			
60 or less	64	46	0.08
More than	16	17	
No. of extranodal sites			
<2	63	46	0.20
≥2	17	36	
AAS			
I-II	40	59	<0.0001
III-IV	40	22	
B-symptoms			
No	52	48	0.06
Yes	28	28	
Bulky			
No	45	46	0.36
Yes	35	35	
Hb			
≥ 10.5 g/dL or more	64	47	0.006
< 10.5 g/dL	16	15	
LDH			
Normal	35	52	0.03
High	45	32	
ECOG			
0-1	57	56	<0.0001
2 or more	22	5	
IPI			
Low	37	63	<0.0001
Low-int	14	36	
Int-high	16	18	
High	13	7	
P53/P21			
Others*	61	46	0.02
$P53^{+++}/P21^{-}$	19	27	
B-cell IHC pattern			
GC	41	57	0.01
Non-GC	39	23	

*ECOG: Eastern Cooperative Oncology Group; Hb: hemoglobin level; IHC: immunohistochemistry; GC: germinal center. Age-adjusted IPI was computed on 64 patients who were less than 60; NA indicates not applicable; *others includes $P53^{-}/P21^{-}$, $P53/P21^{+}$, $P53^{-}/P21^{+}$, $P53^{+}/P21^{-}$ phenotypes.*

We performed a multivariate analysis including all variables that had been found to be significant at univariate analysis. The Cox's proportional hazard model identified ECOG performance status (hazard ratio 0.29; p=0.008), $P53/P21^{waf1}$ phenotype (hazard ratio 0.43; p=0.01), and immunohistochemical B-cell pattern (hazard ratio 0.51; p=0.04) as independent variables influencing the progression-free survival of our patients. The 22 patients (28%) with none of these risk factors had a favorable 5-year progression-free survival of 87%. By contrast, the 22 patients with two or three adverse factors had a 5-year progression-free survival of 7%. The remaining intermediate group with only one risk factor had a 5-year progression-free survival of 30%.

Relations between $P53/P21^{waf1}$, and immunohistochemical B-cell pattern

No relation was found between P53 or $P21^{waf1}$ expression and the presence of a GC phenotype. Single expres-

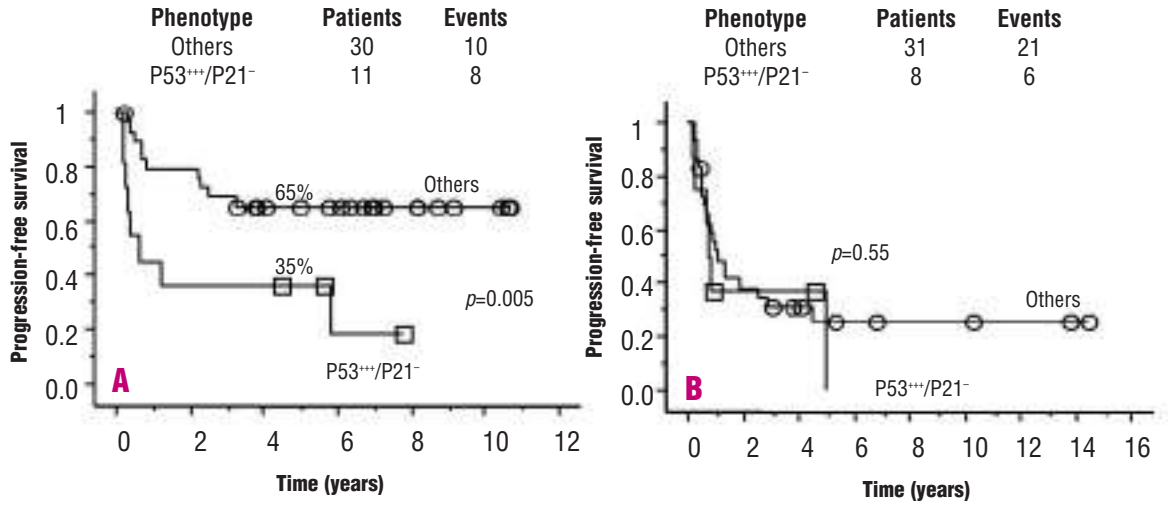

Figure 1. Progression-free survival of (A) GC, and (B) non-GC DLBCL patients according to P53/P21 phenotype.

sion of BCL-6, CD10, or BCL-2 was not associated with the presence of any particular P53/P21^{waf1} phenotype.

The presence of the P53^{+++}/P21^{-} phenotype significantly impaired the outcome of patients with GC-DLBCL, who had a 5-year progression-free survival of 35% compared with 65% for those with GC-DLBCL but a different P53/P21^{waf1} phenotype (p=0.005, Figure 1A). In contrast, the prognosis of patients with a non-GC phenotype was not affected, and remained poor, irrespective of the presence of the P53^{+++}/P21^{-} phenotype (5-year progression-free survival 30% vs 28%, p=0.55, Figure 1B). As regards overall survival among patients with GC-DLBCL, P53^{+++}/P21^{-} expression discriminated 11 patients with a 5-year overall survival of 40% from the remaining patients with different phenotypes who had a 5-year overall survival of 80% (p=0.02). Similarly to progression-free survival, the P53^{+++}/P21^{waf1} phenotype was not predictive of overall survival among patients with non-GC tumors (5-year overall survival 41% vs 37%, p=0.41).

Recent reports have indicated that *BCL-6* constitutive activation represses *P53* transcription by binding two specific DNA sites within the *P53* promoter region. *BCL-6* would immortalize B-cells only in the absence of *P53* function, and so disruption of the *P53* pathway may be crucial in the development of *BCL-6*- expressing B-cell lymphomas.[10,11] In our series we show that the prognostic power of the P53^{+++}/P21^{-} phenotype, which is highly suggestive of disrupted *P53* function,[6-9] is limited to GC-DLBCL, suggesting a central role of the *P53* pathway not only in the pathogenesis, but also in the clinical behavior of lymphomas of GC origin. However, we found no correlation between BCL-6 and P53 expression, assessed by immunohistochemistry. This is not surprising both because the relationship between gene expression and the synthesis of specific proteins is not straightforward, and because a wide spectrum of *P53* mutations or apoptotic loops implicated in regulating P53 expression could be responsible for by-passing the direct role of *BCL-6* on *P53* transcription. The specific prognostic impact of disrupted *P53* function on GC-DLBCL is not unexpected, since gains or losses in chromosomal regions (3p11-p12, 9p21) or t(14;18),[17] as well as single gene activation,[18] have recently been reported to provide prognostic information additional to that provided by gene expression-based models.[19,20] Such reports, with our observations, suggest that the genetic heterogeneity of DLBCL is even greater than so far determined and will probably lead to the definition of more subgroups. Since prohibitive expense and technical constraints have so far limited the clinical utility of gene expression profiling studies in DLBCL, a differential molecular diagnosis should concentrate on a manageably small set of readily measurable genes. Quantitative real-time polymerase chain reaction analysis would then parallel immunohistochemical assays for the products of specific genes, allowing results of gene microarrays to move toward clinicians, although the level of protein expression, rather than the level of gene expression (i.e. *P53, BCL-6*), might be a better predictor of patients' outcome.

CV was responsible for the conception and design of the project, collection of clinical samples, data handling and writing the manuscript. FC, CP, and ML contributed to collection and revision of pathological samples. AAn contributed to the collection of clinical samples. AAm, and MK critically revised the final version. MC and GP were responsible for the overall conception and design of the project. All authors approved the version submitted for publication. The authors declare they have no potential conflicts of interest. This work was partly supported by A.I.R.C. (Milan, grants to MC and GP).

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