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Point mutations of the *BCL-6* gene: clinical and prognostic correlation in B-diffuse large cell lymphoma

U Vitolo[1], B Botto[1], D Capello[2], D Vivenza[2], V Zagonel[3], A Gloghini[4], D Novero[5], G Parvis[6], R Calvi[1], C Ariatti[2], I Milan[3], M Bertini[1], C Boccomini[1], R Freilone[1], P Pregno[1], L Orsucci[1], G Palestro[5], G Saglio[6], A Carbone[4], E Gallo[1] and G Gaidano[2]

[1]*UOA Ematologia, Dipartimento di Oncologia, Azienda Ospedaliera S Giovanni Battista, Torino, Italy;* [5]*Anatomia Patologica, Dipartimento di Oncologia, Azienda Ospedaliera S Giovanni Battista and Università, Torino;* [2]*Divisione di Medicina Interna, Dipartimento di Scienze Mediche, Università del Piemonte Orientale Amedeo Avogadro, Novara, Italy;* [3]*Divisione di Oncologia Medica B Centro di Riferimento Oncologico-Instituto Nazionale Tumori, Aviano, Italy;* [4]*Divisione di Anatomia Patologica, Centro di Riferimento Oncologico-Istituto Nazionale Tumori, Aviano, Italy* [6]*Divisione di Medicina Interna ed Ematologia, Dipartimento di Scienze Cliniche e Biologiche, Azienda Ospedaliera S Luigi e Università di Torino, Orbassano-Torino, Italy*

Although point mutations of the 5′ noncoding regions of the *BCL-6* proto-oncogene are frequently detected in B-diffuse large cell lymphoma (B-DLCL), a thorough analysis of the clinical correlation of these mutations has not been performed to date. In this study, *BCL-6* mutations were examined by DNA direct sequencing in 103 patients with B-DLCL. *BCL-6* mutations were found in 53/103 patients, including 38/76 treated with standard chemotherapy and 15/27 treated with autologous stem cell transplantation (ASCT) up front. The presence of *BCL-6* mutations was correlated with clinical features at diagnosis and outcome. Mutated patients had a significantly higher LDH level (66% *vs* 38%, $P < 0.05$), and bulky disease (51% *vs* 32%, $P = 0.05$). In the whole series of patients *BCL-6* mutations did not affect CR and OS. Patients with *BCL-6* mutations tended to have a prolonged 5-years DFS and FFS compared to those without mutations (DFS 82% *vs* 63%, FFS 63% *vs* 49%). Among B-DLCL treated with standard chemotherapy, mutated patients showed a significantly improved 5-year DFS (85% *vs* 61%, $P < 0.05$) and, notably, the only four relapses observed among mutated patients occurred in less than 8 months. The multivariate regression analysis ($P < 0.01$) with DFS as endpoint confirmed the independent prognostic value of *BCL-6* mutations. There was a trend for 5-year failure-free survival to be better for patients with *BCL-6* mutations (63% *vs* 43%, $P = 0.09$). In the 27 patients treated with ASCT, *BCL-6* mutations did not correlate with outcome. These results suggest that *BCL-6* mutations may predict a higher chance of being free of disease in B-DLCL treated with standard chemotherapy. Larger series of patients need to be analyzed to evaluate the clinical relevance of *BCL-6* mutations properly.
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Introduction

In recent years, molecular studies have revealed that the pathogenesis of B-DLCL is a heterogeneous process involving multiple independent molecular pathways. One such pathway involves *BCL-6*, a proto-oncogene mapping to chromosomal band 3q27 and encoding a POZ/zinc finger transcriptional repressor, which is required for germinal center formation and function.[1,2] Two major types of genetic alterations may affect the *BCL-6* gene in B-DLCL. The first type of *BCL-6* alteration, occurring in approximately 30% B-DLCL, is represented by chromosomal alterations that lead to substitution of the gene promoter with heterologous sequences derived from the partner chromosome.[3–7] The second type of genetic alteration affecting the *BCL-6* gene is represented by point mutations of the 5′ noncoding regions of the gene.[8] Mutations of the *BCL-6* gene are somatic in nature, may be biallelic and occur independent of cytogenetic alterations of band 3q27. The sequences affected by these mutations lie in the proximity of the *BCL-6* promoter and overlap with the major cluster of chromosomal breakpoints.[8–11] Mutations of the *BCL-6* gene are regarded as a marker of B cell transit through the germinal center (GC) because, in normal lymphoid tissues, they occur in approximately 30% to 50% of GC and memory B cells, whereas they are absent in pre-GC and virgin B cells.[8–12] A large survey of B cell neoplasm has documented that *BCL-6* mutations occur in approximately half of B-DLCL, including systemic nodal B-DLCL, primary extra nodal B-DLCL, $CD5^+$ B-DLCL, $CD30^+$ B-DLCL and primary splenic B-DLCL, whereas they are exceptional in primary mediastinal B-DLCL with sclerosis.[13]

In advanced stage B-DLCL, current standard treatment may allow long-term survival in only 40% of the patients. The prognosis of advanced B-DLCL has possibly further improved with the introduction of more intensive regimens.[14–16] Moreover well-defined clinical prognostic features, such as the International Prognostic Index (IPI),[17] may stratify patients in different risk groups, in order to tailor the therapy. However, despite advances in treatment and more defined clinical indicators, approximately half of the patients with B-DLCL fail the therapy and die of their disease.

The term B-DLCL is therefore likely to include more than one disease entity and patients with B-DLCL have highly variable clinical behavior, outcome and natural history.[18] Because the molecular pathogenesis of B-DLCL is heterogeneous, it has been proposed that the tumor genotype may affect the clinical behavior and the outcome of the disease. The identification of new prognostic markers may help to further stratify patients into different risk groups. In this respect, several studies have reported the clinical relevance of some genetic lesions of B-DLCL, including gross rearrangements of *BCL-6* and *BCL-2* or alterations of tumor suppressor genes involved in RB1 and p53 pathways.[3,4,7,19–23] Because some *BCL-6* gene mutations have been suggested to deregulate the *BCL-6* expression,[24] the presence of *BCL-6* mutations may identify B-DLCL patients with possibly different biological behavior and clinical outcome. To date, however, the clinical relevance of *BCL-6* mutations in B-DLCL patients has not been analyzed thoroughly.

The aim of the present study was to perform a detailed analysis of the clinical presentation and outcome in a cohort of *de novo* B-DLCL patients.

Correspondence: U Vitolo, UOA Ematologia, Azienda Ospedaliera S Giovanni Battista, Cso Bramante 88 10188 Torino, Italy; Fax: 39 11 675571; e-mail: uvitolo@molinette.piemonte.it
B Botto and D Capello contributed equally to this work.
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Patients and methods

General characteristics of the patients

This retrospective study included patients with previously untreated diffuse large cell lymphoma with B phenotype (B-DLCL), who had been diagnosed and treated at four Italian institutions from 1986 to 1997. Criteria for inclusion into the study were a revised histopatologic diagnosis of B-DLCL according to the Real classification,[18] available DNA, adequate and uniform staging procedures, no age limits, any stage of disease and only treatment with doxorubicin-containing regimens already reported in the literature. One hundred and three patients entered into this study. Of these, 64 patients have been studied previously for molecular characterization of *BCL-6* mutations.[13] They were followed up until 31 August 1999 or until death. The median follow-up duration from initiation of treatment for censored patients was 63 months. None of the patients had a history of previous low-grade lymphoma nor did the pathological material examined show areas of low-grade histology mixed with the B-DLCL pattern. Discordant B-DLCL cases were excluded from this study. Therefore all 103 patients were classified as *de novo* B-DLCL. Diagnosis was based on histopathology, immunophenotypic analysis of cell surface markers, and immunogenotypic analysis of immunoglobulin (Ig) gene rearrangement.[25] The histopathologic definition of B-DLCL was according to the REAL classification.[18] Based on previous data that showed that *BCL-6* point mutations are extremely rare in primary mediastinal B-DLCL, this subset of B-DLCL patients were excluded from this study.[13] Patients positive for human immunodeficiency virus were not included in the study.

Staging

Staging included routine blood chemistry tests; blood cell counts and differential; ECG; chest X-ray; computed tomography (CT) of chest, abdomen and pelvis; and bilateral bone marrow biopsy in all patients. Gastrointestinal series with endoscopy, lumbar puncture with CSF examination and brain CT scan were performed only when there was clinical concern of gastrointestinal tract or CNS involvement. Disease stage was assessed according to the Ann Arbor criteria.[26] The extent of extranodal involvement was documented radiographically or pathologically. Bulky disease was assessed as a mass >10 cm in one diameter or more than one-third of the chest diameter in the mediastinum. Bone marrow involvement, but not spleen or Waldeyer lymphatic tissue, was scored as an extranodal site. Patients were scored according to the International Prognostic Index (IPI).[17]

Treatment

All patients included in the study were treated with an anthracycline-containing regimen. However, chemotherapy programs were different depending on the stage of disease, time of diagnosis and prognostic factors. Eleven patients with localized stage of disease without adverse prognostic features were treated with brief chemotherapy, ACOPB (adriamycin, cyclophosphamide, vincristine, prednisone, bleomycin)[27] or three courses of CHOP (cyclophosphamide, adriamycin, vincristine, prednisone),[28] followed by locoregional radiotherapy at a dose of 36 Gy. Fifty-five patients with advanced stage or localized stage with adverse prognostic features were treated with CHOP regimens (41 patients) or a third generation chemotherapy scheme such as MACOPB (methotrexate, adriamycin, cyclophosphamide, vincristine, prednisone, bleomycin) (seven patients)[29] or VACOPB (etoposide, adriamycin, cyclophosphamide, vincristine, prednisone, bleomycin) (seven patients).[30] Ten elderly patients, over 65 years, received PVEBEC (prednisone, vinblastine, epirubicin, bleomycin, etoposide, cyclophosphamide),[31] or VMP (etoposide, prednimustine, mitoxantrone) chemotherapy.[32] Twenty-seven patients, with advanced stage disease and adverse prognostic features at diagnosis were treated with a reduced course of standard chemotherapy (MACOPB or CHOP) followed by an intensification chemotherapy with peripheral blood stem cell harvest and high-dose chemotherapy BEAM (carmustine, etoposide, Ara-C, melphalan) with autologous stem cell transplantation.[14]

Assessment of response

Response to treatment was evaluated 1 month after the end of the therapeutic program. Restaging tests included blood chemistries and CT scans of chest, abdomen and pelvis in all patients and repetition of bone marrow biopsy if abnormal at diagnosis. Response criteria were defined according to the recent International Working Group recommendations.[33] Complete remission (CR) was defined as the absence of any detectable clinical and radiographic disease with disappearance of all disease-related symptoms. Complete remission unconfirmed (CRu) was considered as persistent clinical or radiographic lymphnode mass that has regressed by more than 75% of the initial tumor volume and no signs or symptoms of active disease. If the radiological abnormalities were subsequently stable for at least 3 months, the patients were judged to have a CR. Patients with a 50% or greater reduction in tumor volume were considered to be in partial remission (PR). Failure was defined as anything less than a PR, progressive disease or treatment-related death.

Analysis of BCL-6 mutations and rearrangements

Genomic DNA was performed as previously reported.[34,35] Mutational analysis of the *BCL-6* gene was performed by DNA direct sequencing of a unique PCR product encompassing 739 nucleotides (positions +404 to +1142) of the *BCL-6* 5′ noncoding regions.[13,36] The choice of this fragment was based on the fact that $>95\%$ of *BCL-6* mutations detected in B-DLCL fall within this region.[13] The PCR amplimer was amplified by primers E1.21B (5′-CTCTTGCCAAATGCTTTG-3′) and E1.26 (5′-CACGATACTTCATCTCATC-3′) and directly sequenced with forward or reverse primers using a commercially available kit (ThermoSequenase, Amersham Life Sciences, Amersham, UK). [α-^{33}P]-labeled terminator dideoxynucleotides (Amersham Life Science) were included in the sequencing mixture. For each DNA fragment analyzed, sequencing of both strands was performed on independent PCR reactions. All the oligonucleotides used in this study were synthesized by the solid phase triester method.[13] Pilot experiments performed in our laboratory have shown that our mutation analysis technique allows the detection of *BCL-6* mutations occurring in $\geqslant 5\%$ of the tumor cell population.

Gross rearrangements of the *BCL-6* gene were investigated by Southern blot analysis using probes (Sac4.0 and Sac0.8)

and restriction enzymes (*Bam*HI and *Xba*I) that, in combination, explore the cluster of chromosomal breakpoints detected in NHL.[13] Only cases showing abnormally migrating bands with two restriction enzymes and/or two probes were scored as rearranged.[13]

Analysis of BCL-2 rearrangement

Molecular analysis of *BCL-2* rearrangement was performed as previously reported.[6]

Statistical methods

All the patients started on treatment were considered assessable. Survival includes all patients, with event defined as death of the patient due to any cause. Overall survival (OS) duration was measured from the beginning of treatment to the date of death or last follow-up alive. Failure-free survival (FFS) includes all patients, and was calculated from the beginning of treatment to the date of relapse, progression or death from any cause. Disease-free survival (DFS) applies only to the patients who achieved a CR or CRu: the duration was calculated from the time of CR or CRu assessment to the date of relapse or last follow-up free of disease. All curves were plotted according to the method of Kaplan and Meier[37] and statistical differences among curves were evaluated by the log rank test. Correlation between *BCL-6* point mutations and clinical characteristics were analyzed by Fisher's exact test. Means were compared by two sample *t*-tests. A multivariate regression analysis according to Cox's[38] proportional regression model was used to perform multivariate analyses of factors affecting OS, DFS and FFS. All calculations were done through the BMDP program (1985) developed at the Health Science Computing facility, UCLA (NIH) Special Research Resources.

Results

Patient characteristics

The clinical characteristics of the 103 patients are listed in Table 1. The median age was 55 years (range 16 to 86 years). There were 55 males and 48 females. All case displayed a monoclonal B cell population as determined by immunophenotypic and/or immunogenotypic analysis. *BCL-6* point mutations were found in 53 patients (51%), whereas they were absent in 50 (49%) cases. Rearrangements of *BCL-6* were studied in 81 patients: 23 (28%) showed a gross rearrangement of *BCL-6* and 58 (72%) did not. Of the 81 patients studied for both *BCL-6* rearrangement and *BCL-6* mutations, 13 showed both mutations and rearrangement, 33 displayed only mutations, 10 only rearrangement and 25 did not show any of the genetic lesions examined. CR, OS, DFS and FFS rates were not different between patients with or without *BCL-6* rearrangement.

BCL-2 gene rearrangements were evaluated in 88 patients: 12 (14%) showed a rearrangement and 76 (86%) did not. *BCL-2* rearrangement was present in 3/46 patients with *BCL-6* mutations compared to 9/42 without *BCL-6* mutations (P = NS).

Table 1 Clinical characteristics of all 103 DLCL patients

Clinical characteristics	*Patients (%)*
BCL6	
Unmutated	50 (49)
Mutated	53 (51)
Sex	
Male	55 (53)
Female	48 (47)
Age	
Median	55 years
Range	16–86
Stage	
I	10 (10)
II	24 (23)
III	20 (19)
IV	49 (48)
Sites	
Nodal	40 (39)
Extranodal	12 (12)
Nodal + extranodal	51 (49)
Constitutional B symptoms	39 (38)
Performance status	
0–1	71 (69)
>1	32 (31)
LDH[a]	
Normal	44 (46)
Above normal	52 (54)
Bulky	
No	60 (58)
Yes	43 (42)
No. extranodal sites	
0–1	71 (69)
>1	32 (31)
Bone marrow involvement	21 (20)
BCL6 rearrangement[b]	
Negative	58 (72)
Positive	23 (28)
IPI	
Low/low-intermediate	61 (59)
Intermediate-high/high	42 (41)
Standard chemotherapy	76 (77)
High-dose chemotherapy and ASCT	27 (23)
Response	
CR	69 (67)
CRu	4 (4)
PR	12 (12)
NR	18 (17)

[a]LDH level was not determined in seven patients.
[b]BCL6 rearrangement was not tested in 22 patients.
ASCT, autologous stem cell transplantation.

Correlation of BCL-6 point mutations with clinical features at diagnosis

The relationship between clinical features at diagnosis and *BCL-6* point mutations status was analyzed. The results of these correlations are shown in Table 2. Compared to patients without *BCL-6* mutations, those with mutations had a significantly higher LDH level: LDH above normal in 33 mutated patients (66%) *vs* 19 unmutated ones (41%), $P < 0.01$. A higher frequency of bulky disease was also observed in mutated patients: 27 mutated patients (51%) *vs* 16 (32%) unmutated ones, $P < 0.05$. Twenty-five patients (47%) with *BCL-6* mutations were at intermediate/high or high risk according to IPI compared to 17 (34%) of those without mutations, however the difference was not statistically significant. All other clinical features were well balanced between the two groups. The frequency of extranodal involve-

Table 2 Clinical characteristics of all 103 DLCL patients according to BCL6 mutations

Clinical characteristics	*BCL6 unmutated 50 patients (49%)*	*BCL6 mutated 53 patients (51%)*	*P*
Sex			
Male	29 (58)	26 (49)	NS
Female	21 (42)	27 (51)	
Age			
Mean	54 years	54 years	NS
Range	19–83	16–86	
Stage			
I	7 (14)	3 (6)	
II	11 (22)	13 (25)	NS
III	7 (14)	13 (25)	
IV	25 (50)	24 (44)	
Sites			
Nodal	24 (48)	16 (30)	NS
Extranodal	4 (8)	8 (15)	
Nodal + extranodal	22 (44)	29 (55)	
Constitutional B symptoms	17 (34)	22 (42)	NS
Performance status			
0–1	37 (74)	34 (64)	NS
>1	13 (26)	19 (36)	
LDH[a]			
Normal	27 (59)	17 (34)	0.01
Above normal	19 (41)	33 (66)	
Bulky			
No	34 (68)	26 (49)	0.05
Yes	16 (32)	27 (51)	
No. extranodal sites			
0–1	38 (76)	33 (63)	NS
>1	12 (24)	20 (37)	
Bone marrow involvement	12 (24)	9 (17)	NS
BCL6 rearrangement[b]			
Negative	25 (71)	33 (71)	NS
Positive	10 (29)	13 (29)	
IPI			
Low/low-intermediate	33 (66)	28 (53)	NS
Intermediate-high/high	17 (34)	25 (47)	
Response			
CR	35 (70)	34 (64)	
CRu	0	4 (8)	NS
PR	7 (14)	5 (9)	
NR	8 (16)	10 (19)	

[a]LDH level was not determined in seven patients.
[b]BCL6 rearrangement was not tested in 22 patients.

ment by lymphoma was not different between patients with or without *BCL-6* point mutations.

Analysis of BCL-6 mutations and outcome

In the whole series of patients, complete response (CR + CRu) was observed in 73 patients (71%) and with a median follow-up of 63 months the predicted OS, DFS and FFS rates at 5 years were: 62%, 74% and 56%, respectively. Complete response (CR + CRu) was achieved in 38 (72%) of 53 patients with *BCL-6* mutations: CR 34/38 and CRu 4/38. Thirty-five (70%) of 50 patients without *BCL-6* mutations showed a CR, no CRu was observed. Overall, the complete response rate was not different between mutated and unmutated patients. OS, DFS and FFS rates were analyzed in patients with or without *BCL-6* mutations. Five-year OS rate was not different between the two groups (63% *vs* 59%). Patients with *BCL6* mutations tended to have a prolonged 5-year DFS compared to those without mutations (82% *vs* 63%) (Figure 1a). Only seven (5 CR and 2 CRu) of 38 complete responders with *BCL-6* mutations relapsed compared to 12 of 35 CRs without mutations. All relapses in mutated patients occurred early within 1 year off therapy, whereas patients without mutations relapsed continuously up to 6 years. The 5-year FFS rate was higher in patients with BCL-6 mutations (63% *vs* 49%), however the difference was not statistically significant (Figure 1b).

Our series includes patients treated with two different treatment approaches: standard chemotherapy and high-dose chemotherapy (HDC) with autologous stem cell transplantation (ASCT) up front. Patients treated with HDC and ASCT up front had a more prolonged 5-year FFS rates than those treated with standard chemotherapy (67% *vs* 53% $P < 0.05$). Thus in order to evaluate the prognostic impact of the presence of *BCL-6* mutations on the outcome properly and to avoid a therapeutic bias, these two groups of patients were analyzed separately.

Among seventy-six patients treated with standard chemotherapy, 38 (50%) harbored *BCL-6* point mutations. Clinical characteristics according to the presence or the absence of BCL-6 mutations are shown in Table 3. As also observed for the whole series of patients, those with BCL-6 mutations had a significantly higher LDH level: LDH above normal in 22 mutated patients (63%) *vs* 10 unmutated ones (29%), $P < 0.01$. A higher incidence of bulky disease was also observed in mutated patients, however the difference was not statistically significant ($P = 0.09$).

The correlation between *BCL-6* mutations and the outcome was evaluated in this group of 76 patients treated with standard chemotherapy. CR rate was superimposable between patients with or without *BCL-6* mutations: 30/38 (71%) *vs* 25/38 (66%) patients. OS rate was slightly higher in those with *BCL-6* mutations, although the difference was not statistically

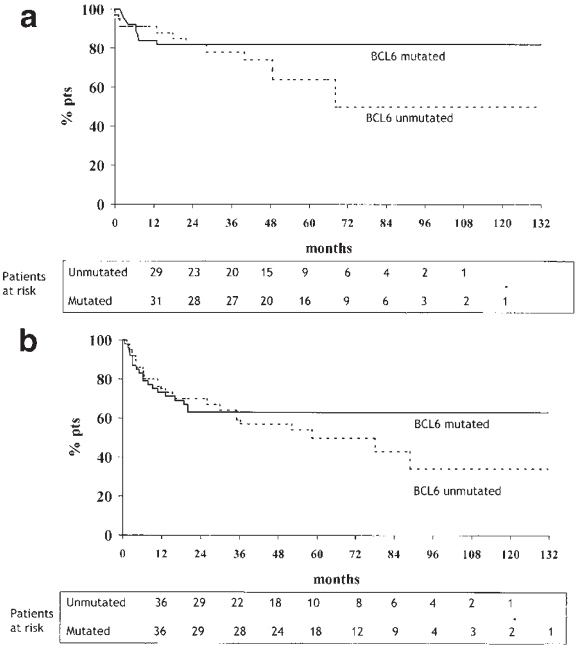

Figure 1 Disease-free survival (DFS) and failure-free survival (FFS) according to *BCL-6* mutations status in the whole series of 103 patients with B-DLCL. DFS (a) at 5 years was 82% for *BCL-6*-mutated patients (solid line) *vs* 63% for *BCL-6*-unmutated patients (dashed line) $P = 0.08$. FFS (b) at 5 years was 63% for *BCL-6*-mutated patients (solid line) *vs* 49% for *BCL-6*-unmutated patients (dashed line) $P =$ NS.

Table 3 Clinical characteristics of 76 DLCL patients treated with standard chemotherapy according to BCL6 mutations

Clinical characteristics	*BCL6 unmutated 38 patients (50%)*	*BCL6 mutated 38 patients (50%)*	*P*
Sex			
Male	22 (58)	18 (47)	NS
Female	16 (42)	20 (53)	
Age Mean			
Range	59 years 22–83	59 years 16–86	NS
Stage			
I	7 (18)	3 (8)	
II	9 (24)	11 (29)	NS
III	3 (8)	8 (21)	
IV	19 (50)	16 (42)	
Sites			
Nodal	16 (42)	10 (26)	NS
Extranodal	4 (11)	8 (21)	
Nodal + extranodal	18 (47)	20 (53)	
Constitutional B symptoms	8 (21)	13 (34)	NS
Performance status			
0–1	29 (76)	29 (76)	NS
>1	9 (24)	9 (24)	
LDH[a]			
Normal	24 (71)	13 (37)	<0.01
Above normal	10 (29)	22 (63)	
Bulky			
No	28 (74)	21 (55)	0.09
Yes	10 (26)	17 (45)	
No. extranodal sites			
0–1	29 (76)	24 (64)	NS
>1	9 (24)	14 (36)	
Bone marrow involvement	8 (21)	5 (13)	NS
BCL6 rearrangement[b]			
Negative	18 (72)	22 (66)	NS
Positive	7 (28)	11 (33)	
IPI			
Low/low-intermediate	26 (68)	22 (58)	NS
Intermediate-high/high	12 (32)	16 (42)	
Response			
CR	25 (66)	27 (63)	
CRu	0	3 (8)	NS
PR	7 (18)	4 (11)	
NR	6 (16)	7 (18)	

[a]LDH level was not determined in seven patients.
[b]BCL6 rearrangement was not tested in 18 patients.

significant (5-year OS: 65% *vs* 56%). Conversely, patients with *BCL-6* mutations showed a significantly higher 5-year DFS rate compared to those without this genetic lesion: 85% *vs* 61% ($P < 0.05$) (Figure 2a). In particular, among B-DLCL who achieved CR, relapse occurred in 4/27 cases with *BCL-6* mutations compared to 10/25 cases without *BCL-6* mutations. The four relapses in patients with *BCL-6* mutation occurred early in less than 8 months, compared with the continuous pattern of relapse, up to 69 months, observed in those without mutations. There was a trend for patients with *BCL-6* mutations to have a better FFS (5-year FFS 63% *vs* 43% $P = 0.09$) (Figure 2b). Of the 38 mutated patients, 14 failed and the last failure was observed at 20 months, whereas of the 38 unmutated patients, 21 failed with events observed continuously up to 88 months.

At a significant level of $P < 0.05$, the multivariate regression analysis with DFS as an end point identified three prognostic factors for B-DLCL treated with standard chemotherapy: performance status, *BCL-6* mutations and number of extranodal

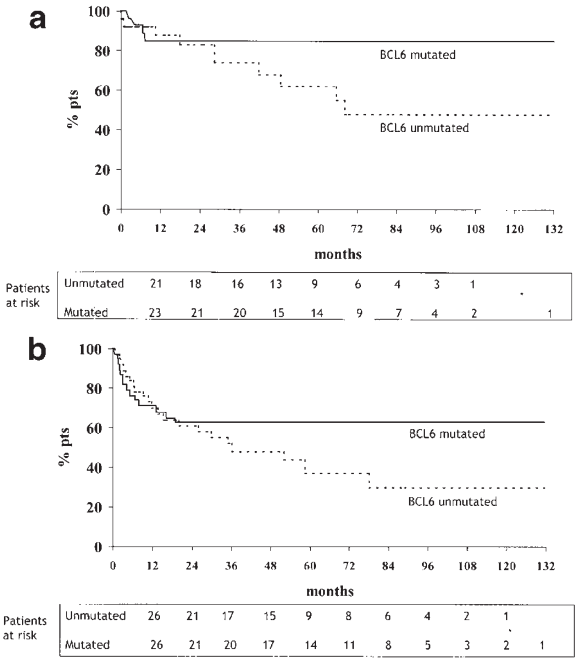

Figure 2 Disease-free survival (DFS) and failure-free survival (FFS) according to *BCL-6* mutations status in 76 patients treated with standard chemotherapy. DFS (a) at 5 years was 85% for *BCL-6*-mutated patients (solid line) *vs* 61% for *BCL-6* unmutated patients (dashed line) $P = 0.05$. FFS (b) at 5 years was 63% for *BCL-6*-mutated patients (solid line) *vs* 43% for *BCL-6*-unmutated patients (dashed line) $P = 0.09$.

sites. This analysis confirmed the independent prognostic value of the presence of *BCL-6* mutations for predicting a prolonged DFS (Table 4).

In the 27 patients treated with HDC and ASCT, *BCL-6* mutations were found in 15 patients (56%). In this group of patients there were no correlations between clinical features at diagnosis and BCL-6 mutations status. However, the majority of them had bulky disease (16 patients) or elevated LDH level (20 patients) at diagnosis. The presence of *BCL-6* mutations did not correlate with different CR, OS, DFS or FFS rates.

Discussion

This study aimed at defining the clinical relevance of point mutations of the 5′ noncoding regions of the *BCL-6* proto-

Table 4 Multivariate regression analysis for disease-free survival in 55 patients treated with standard chemotherapy

Clinical characteristics	X^2	*P*
Performance status	15.274	0.002
BCL6 point mutation	13.302	0.001
Number of extranodal sites	6.771	0.009
Age	2.14	0.14
Sex	0.06	0.81
Stage	2.16	0.14
Symptoms	0.67	0.41
LDH	2.14	0.14
Bulky	2.19	0.13
Bone marrow involvement	1.91	0.16

oncogene in B-DLCL. In our series, mutations of *BCL-6* are detected in approximately 50% B-DLCL, in agreement with previously reported data, and appear to be independent of the concomitant occurrence of a gross rearrangement of the proto-oncogene. Notably, the presence of *BCL-6* mutations in B-DLCL at diagnosis correlates with well-known unfavorable clinical characteristics, namely LDH level and bulky disease. Our series included two groups of patients, treated with two different therapeutic approaches, both of them containing anthracyclines: standard chemotherapy and high-dose chemotherapy (HDC) with autologous stem cell transplantation (ASCT) up front. With respect to outcome, the prognostic relevance of BCL-6 mutations was assessed both in the whole series of patients and in separate subsets of patients treated with either standard chemotherapy or with ASCT. This strategy seemed appropriate, since several studies have reported that ASCT may be superior to standard chemotherapy in achieving a cure for B-DLCL[14–16,39,40] and since patients treated with HDC and ASCT included in this study had a more favorable outcome than those treated with standard chemotherapy. Although our separate analysis according to therapy may help to avoid a potential bias in the assessment of the prognostic relevance of BCL-6 mutations, it does not allow any comparison between the two treatment groups of patients. In fact, treatment was not randomized and patients treated with HDC and ASCT were selected as younger, with a better performance status.

In the whole population of patients, the presence of *BCL-6* mutations did not show statistically significant correlation with outcome. Conversely, in the group of 76 patients treated with standard chemotherapy, *BCL-6* mutations appear to predict prolonged DFS despite their association with some poor clinical features at diagnosis, such as a higher LDH level or a higher incidence of bulky disease. Patients with *BCL-6* mutations treated with standard chemotherapy had a markedly lower incidence of relapses: 85% of them were still alive and free of disease at 5 years compared with only 61% of unmutated patients. Also, the timing of relapse appears to be different in B-DLCL with or without *BCL-6* mutations. The few relapses observed in the mutated group occurred early in less than 8 months, compared to the continuous pattern of relapses reported in the unmutated cases. There was a trend for patients with *BCL-6* mutations to have a better FFS (5-year FFS 63% *vs* 43%). Although the difference between FFS curves was not statistically significant, mutated patients showed a clear plateau compared to unmutated patients who continued to fail up to 88 months. Unmutated patients do not represent cases carrying t(14;18), because *BCL-2* rearrangement was found in a small proportion of patients regardless of the presence of *BCL-6* mutations.

In our study, the prognostic value of *BCL-6* mutations was found to be independent of other well-known prognostic factors of relapse, including performance status and number of involved extranodal sites as assessed by multivariate regression analysis with DFS as endpoint. Thus, patients with *BCL-6* mutations, treated with standard chemotherapy, appear to have a good chance of cure once CR has been achieved.

The different DFS rate of patients with or without *BCL-6* mutations was not influenced by the standard chemotherapy regimen used (data not shown). This is not surprising, considering that B-DLCL outcome is similar regardless of the second or third generation chemotherapy regimens adopted, as reported by large randomized trials.[41] The lack of statistically significant differences in overall survival rates between mutated and unmutated patients may be due to the impact of salvage therapy, mainly HDC and ASCT, used in the patients who failed or relapsed after first-line treatment.

The apparent lack of prognostic evidence of BCL-6 mutations in the subset of patients treated at the onset with HDC and ASCT may be due to the small sample size and prompts future studies, on a large panel of patients, aimed at defining the clinical relevance of BCL6-mutations in this subset of patients.

The reasons for the apparent clinical differences between B-DLCL with and those without *BCL-6* mutations are currently speculative. Although a pathogenetic role for *BCL-6* mutations has not been formally established, indirect observations suggest that mutations may carry functional consequences.[24] In particular, it is remarkable that *BCL-6* mutations cluster in highly conserved genomic regions, suggesting that some mutations may affect regulatory domains of *BCL-6* and thus deregulate the physiologic expression of the gene.[24] In this respect, the presence of *BCL-6* mutations, and consequently altered regulation of *BCL-6* protein, may render the tumor clone more sensitive to full eradication by chemotherapy or, alternatively, less prone to relapse.

Recently, investigations exploiting DNA microarray technology has subdivided B-DLCL into two molecularly distinct subgroup with different gene expression profiles: germinal center (GC) B cell-like B-DLCL and activated B cell-like B-DLCL.[42,43] GC B cell-like B-DLCL displays many known markers of GC differentiation, such as elevated *BCL-6* m-RNA levels and, interestingly, these patients did significantly better than those with activated B cell-like B-DLCL. Whether *BCL-6* mutations may lead to higher *BCL-6* mRNA levels, and thus to better prognosis, remains to be defined.

Although well-known clinical indicators, as those outlined by the IPI, may define the prognosis of B-DLCL, these patients continue to have different clinical features and outcome. Thus, it is commonly agreed that patients will certainly benefit from tailoring therapy to more precise subgroups of tumors. In particular, patients at high risk of relapse need an aggressive treatment early in the course of their disease, while those in good prognosis subgroups may be spared further therapy once CR has been achieved. On this basis, the results presented in this study may suggest a prognostic value of *BCL-6* mutations, at least in patients treated with standard chemotherapy. This finding requires confirmation by investigations aimed at defining the potential prognostic value of *BCL-6* mutations in larger series of patients.

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