Development of a Real-Time Reverse Transcription Polymerase Chain Reaction Assay for c-myc Expression That Allows the Identification of a Subset of c-myc+ Diffuse Large B-Cell Lymphoma

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SUMMARY: Absence of a reliable method for determining the level of c-myc expression has impeded the analysis of its biological and clinical relevance in tumors. We have standardized the conditions for a real-time reverse transcription polymerase chain reaction analysis for c-myc expression, including the selection of an endogenous reference (18S rRNA), the adequate number of measurements for each sample (2 cDNA in triplicate), and suitable controls for determining inter- and intrarun variability (standard curve and calibrator). Subsequently, in a series of 56 non-Hodgkin's lymphomas, we analyzed the expression of c-myc mRNA, using real-time reverse transcription polymerase chain reaction, and of other functionally related proteins (bcl-6, p27, cyclin D3, and p53). As expected, all eight Burkitt's lymphoma cases analyzed had high levels of c-myc mRNA expression compared with that observed in reactive lymphoid tissue. There was a wider range of expression in diffuse large B-cell lymphoma, with 30% (15 of 48) of cases overexpressing c-myc. This overexpression was largely independent of c-myc translocations (4 of 5), as demonstrated by fluorescence in situ hybridization. In this large B-cell lymphoma series, a high level of c-myc expression predicted lower survival probability, irrespectively of the International Prognostic Index risk group classification. A slightly increased frequency of p53 inactivation was observed in the cases with c-myc overexpression, which suggests a growth advantage in lymphomas with concurrent deregulation of c-myc and p53. In addition, a moderate increase in bcl-6 protein expression was observed in the c-myc–positive cases, suggesting the existence of a complex interrelationship between these two genes. These findings suggest that c-myc may play a relevant role in the pathogenesis of a subset of large B-cell lymphoma and suggest the existence of additional regulatory mechanisms of c-myc expression to c-myc rearrangements. (*Lab Invest 2003, 83:143–152*).

C-myc is a multifunctional gene involved in important cellular processes, such as cell proliferation, differentiation, and apoptosis. c-myc targets include critical genes involved in growth, the cell cycle, signaling, and adhesion (eg, cyclin D2, cyclin D3, DP1, p27^{KIP1}, p21^{CIP1}, AKT, Cul-1, TGFR-2, CDC25C, APl2, *Bcl*II) (Coller et al, 2000; Eischen et al, 2001; Guo et al, 2000; Nasi et al, 2001; Neiman et al, 2001; Nesbit et al, 2000; O'Hagan et al, 2000; Schuhmacher et al, 2001).

Although c-myc gene was initially described as being involved in t(8;14) in Burkitt's lymphoma (BL), overexpression of c-myc has been found in other solid tumors such as breast, prostate, and gastrointestinal carcinomas and in melanoma. In these neoplasias, c-myc expression has been shown to be dependent on amplifications of the gene or to be derived from deregulation of upstream genes in the c-myc pathway, such as BRCA1, PTEN, and β-catenin/APC (Nesbit et al, 1999). In addition, N-myc has been implicated in the pathogenesis of neuroblastomas (Fulda et al, 1999).

c-myc activation is of particular relevance in leukemia and lymphoma. Virtually all BLs show translocation of the c-myc gene with the heavy- or light-chain immunoglobulin (Ig) locus, and most of them also contain somatic mutations (Cesarman et al, 1987; Dalla-Favera et al, 1982; Khaira et al, 1998). Diffuse large B-cell lymphomas (DLBCLs) display c-myc translocations in 1 to 15% of cases (Cigudosa et al, 1999; Kramer et al, 1998; Vitolo et al, 1998), amplifications in 15% (Rao et al, 1998), and somatic mutations in 32% of tumors (Pasqualucci et al, 2001). Translocations and mutations have been linked to c-myc activation in BL and DLBCL because these

structural alterations were accompanied by an altered pattern of c-myc transcription, specifically the removal of the block on transcriptional elongation (Bradley et al, 1993; Cesarman et al, 1987). Furthermore, regulatory elements of promoters and enhancers of Ig and c-myc genes have been shown to contribute to this deregulation (Gerbitz et al, 1999; Ji et al, 1994; Kanda et al, 2000; Madisen and Groudine, 1994).

Results of expression analyses using Northern blot (Hernandez et al, 1999), in situ hybridization (Greil et al, 1991), and immunohistochemistry (IHC) (Chang et al, 2000; Pagnano et al, 2001) coincide in showing that c-myc overexpression is a common observation in BL and DLBCL. Despite this, there is striking variability in the reported c-myc protein cellular localization and intensity of c-myc expression in reactive and tumoral lymphoid tissue (Chang et al, 2000; Jack et al, 1986; Loke et al, 1988; Pagnano et al, 2001). Despite the significant role that c-myc seems to play in lymphomagenesis, the difficulties for demonstrating c-myc expression at the protein or RNA level has impeded our gaining a deeper understanding of the mechanism regulating c-myc expression and the identification of c-myc targets in lymphoma tumoral specimens. Thus, under these circumstances, recent analyses using real-time RT-PCR of c-myc mRNA expression have demonstrated in breast cancer and other tumors that the level of expression of c-myc is clinically and biologically relevant and that the results are highly reproducible with this technique (Bieche et al, 2001; Bieche et al, 1999; Christoph et al, 1999; Latil et al, 2000).

Interest in c-myc has been fueled by recent results showing that its expression in B cells could be dependent on bcl-6 and blimp-1 genes. bcl-6 protein is a significant transcriptional factor involved in lymphomagenesis and has been proposed as being involved in the transcriptional activation of c-myc promoter through the repression of a c-myc transcriptional repressor, blimp-1 (Lin et al, 1997; Shaffer et al, 2000). Other important targets of c-myc are p27 and cyclin D3, molecules whose interaction plays a critical role in G1/S transition (Bouchard et al, 1999; Sanchez-Beato et al, 1999), and whose abnormal expression has been found to be associated with adverse clinical outcome in DLBCL (Saez et al, 1999). In nonlymphoid tumors, c-myc expression has been described as promoting apoptosis, which explains the high frequency of p53 mutation and apoptosis inactivation in neoplasms characterized by overexpressed c-myc (Teitz et al, 2000; Wagner et al, 1994).

Here we have quantified the level of expression of c-myc mRNA and other hypothetical c-myc targets (bcl-6, p27, cyclin D3) to establish whether the findings in a series of DLBCL corroborate the previous observations in experimental models. At the same time, we have analyzed the status of p53 gene to test whether aggressive B-cell lymphoma with c-myc overexpression experiences a pressure to inactivate the p53 pathway. The results confirm the role of c-myc in DLBCL and suggest the existence of different mechanisms of c-myc deregulation.

Results

Standardization of Real-Time Reverse Transcription Polymerase Chain Reaction for c-myc mRNA Measurement

For each experimental sample, the amount of the target (c-myc) and endogenous reference (18S rRNA) was determined from the standard curve. This standard curve was composed of five points obtained from 5-fold serial dilutions (1/5, 1/25, 1/125, 1/625, 1/3125) of cDNA from the Raji cell line, synthesized from 500 ng of total RNA. We considered only experiments in which the linear relationship between C_t (threshold cycle) and the log of the amount of standard curve for c-myc and 18S rRNA were higher than 0.98 (correlation coefficient). The c-myc amount was then divided by the amount of 18S rRNA to obtain a normalized c-myc value.

18S rRNA was included as an endogenous control gene to correct for variations in the degree of RNA degradation and efficiencies of reverse transcription. Before performing this study, we confirmed that expression of 18S rRNA was steady in a series of BL and DLBCL, including nodal and extranodal localizations, and immunoblastic, centroblastic, and plasmablastic cytologies. To demonstrate the suitability of the 18S rRNA gene as control, we used similar amounts of each sample, adjusting the quantity of each c-DNA by electrophoresis in agarose gels and absorbance at 260 nm and then studied the expression of this gene by real-time reverse transcription polymerase chain reaction (RT-PCR).

To test inter-run variability, we included a calibrator sample (single tonsil cDNA) in each PCR. We evaluated the variability of the technique testing the c-myc expression of this calibrator in every experiment. Thus, the standard deviation (SD) was 0.029, and the coefficient of variation was 13.096%. In addition, for testing intrarun variability in each sample, two cDNAs from each sample and two serial dilutions of each cDNA were analyzed. All samples were measured in triplicate. The measurements were considered to be acceptable when the SD of C_t triplicates was lower than 0.38 and when that of serial dilutions was lower than 0.06.

To determine the cutoff point of altered c-myc gene expression at the RNA level, we scored the mean of expression of reactive lymphoid tissue (0.23). The relative c-myc gene expression in each sample was normalized to this cutoff value to generate the final relative expression levels. Final results, expressed as n-fold differences in c-myc gene expression relative to the cutoff value, termed ^{r}c-myc (relative c-myc expression), were determined as follows: ^{r}c-myc = c-myc sample/c-myc cutoff expression.

c-myc Expression in Reactive Lymphoid Tissues

c-myc gene expression at the RNA level in lymphoid reactive tissue was determined for six tonsils. The values ranged between 0.20 and 0.36, with a mean of 0.23. These values were clearly lower than those

observed in the BL series analyzed here (ranging from 0.44 to 2.22), in a similar ratio to that previously reported in the comparison of reactive tonsils and BL (Hernandez et al, 1999).

c-myc Expression in the LBCL Series

A summary of the results is included in Table 1. Eight cases of BL were studied. The expression level (measured as ^{r}c-myc, in relation to the level of expression observed in reactive lymphoid tissue) ranged from 1.47 to 9.50, with a mean of 3.80 (Table 1; Fig. 1).

c-myc mRNA expression was analyzed in 48 cases of DLBCL, including 34 nodal DLBCLs and 14 extranodal DLBCLs. ^{r}c-myc levels had a range from 0.00 to 8.40, with a mean of 1.14. Low ^{r}c-myc levels (lower than reactive lymphoid tissue) were observed in 73% (35 of 48) of cases (26 nodal DLBCLs and 9 extranodal DLBCLs), and high levels were noted in 27% (13 of 48) of cases (8 nodal DLBCLs and 5 extranodal DLBCLs; Table 1; Fig. 1).

Levels of ^{r}c-myc expression from BL were significantly higher than those observed in DLBCL (χ^2 = 14.221, p = 0.003, Kruskal-Wallis test). No significant differences between means of ^{r}c-myc values were found when comparing nodal DLBCL and extranodal DLBCL cases ($p > 0.05$; Kruskal-Wallis test).

Fluorescence in Situ Hybridization

It was possible to analyze the presence of rearrangements and amplifications of c-myc gene by fluorescence in situ hybridization (FISH) in eight DLBCL and five BL paraffin-embedded samples (Fig. 2). One of five DLBCL cases with high c-myc expression and one of three DLBCLs with low c-myc expression showed c-myc rearrangements. Four of five BLs analyzed also showed c-myc rearrangements. All of these rearrangements were identified as the translocation t(8;14). We found no amplification of c-myc gene in any case.

Relationship between ^{r}c-myc mRNA Expression and MIB1, p53, p27, Cyclin D3, and bcl-6 Protein Expression in DLBCL Cases

To determine the possible relationship between ^{r}c-myc levels and the proliferative activity of these tumors, we examined MIB1 immunohistochemistry expression (Table 1). We found that the percentage of positive cells varied from 55 to 100 (mean: 88.02) in all DLBCL cases available for study. The MIB1 means of the different groups defined by ^{r}c-myc values were nearly identical (86.88, and 90.50 for low and high ^{r}c-myc levels, respectively; $p > 0.05$, Kruskal-Wallis test). No significant correlation was found between ^{r}c-myc and MIB1 expression (r: 0.082, $p > 0.05$, Spearman correlation).

bcl-6 is a transcription factor that has been proposed as playing a role in the regulation of c-myc expression (Shaffer et al, 2001). We examined immunohistochemically the expression levels of bcl-6 in DLBCL cases. bcl-6 reactivity could be evaluated in

41 cases. Fewer than 20% of positive cells were found in 7 cases, 10 showed an intermediate level of expression, and 26 cases had more than 80% positive cells (Table 1). The mean was 67.12, in agreement with previously reported results (Artiga et al, 2002). Mean levels of bcl-6 expression show a moderate increase in DLBCL cases with high c-myc mRNA expression (χ^2 = 5.159, p = 0.023, Kruskal-Wallis test; r = 0.324, $p <$ 0.05, Spearman correlation). The relationship between bcl-6 and c-myc expression was not observed in the BL group ($p > 0.05$, Spearman correlation), but the small size of the group (eight cases) precludes the possibility of reaching a conclusion concerning this relationship.

c-myc overexpression induces cell-cycle progression and apoptosis simultaneously, explaining the increased parallel inactivation of p53 (Wagner et al, 1994) seen in tumoral cell lines and the presence of caspase 8 deletion observed in neuroblastoma and other tumors (Teitz et al, 2000). We tried to establish whether there was a relationship between c-myc expression levels and inactivation of p53 in all of the series. Three cases of 54 showed p53 missense or nonsense mutations or an anomalous p53+/p21− phenotype (seven cases; Table 1) (Villuendas et al, 1997). We found that cases with high c-myc expression had a higher probability of being p53-inactivated, but this was not statistically significant.

p27 and cyclin D3 are two important c-myc targets for G1/S transition (Bouchard et al, 1999; Schuhmacher et al, 2001). In addition, it has been reported that cyclin D3 may contribute to p27 inactivation in DLBCL by sequestration into complexes containing CDK4, where p27 is rendered inactive but stabilized and detectable by immunohistochemical techniques (Bouchard et al, 1999; Sanchez-Beato et al, 1999). Confirming these results (data not shown), the expression level of p27 and cyclin D3 in this series showed a strong positive correlation in DLBCL cases (r = 0.416, p = 0.006, Spearman test). In these cases, there was no clear correlation between c-myc mRNA levels and p27 protein expression (r = 0.071, $p > 0.05$, Spearman test) or cyclin D3 (r = 0.072, $p > 0.05$, Spearman test), although c-myc–positive cases had a tendency to overexpress p27 (χ^2 = 2.811, p = 0.094, Pearson test).

Relationship between c-myc mRNA Levels and Clinical Outcome in DLBCL

In the DLBCL, the Kaplan-Meier plot and long rank test indicated that patients with high ^{r}c-myc level had lower overall survival (OS) and failure-free survival (FFS; p = 0.0008 for OS and p = 0.009 for FFS; Fig. 3). In addition, a univariate Cox analysis confirmed that high ^{r}c-myc levels were a very good predictor of shorter OS and FFS in the series of patients with DLBCL (p = 0.027, relative risk = 2.668 for OS; p = 0.016, relative risk = 2.940 for FFS).

The International Prognostic Index (IPI) is a recognized predictor of OS in high-grade lymphomas. In our DLBCL series, the risk defined by the IPI accurately distinguished different survival probabilities (p =

Table 1. Summary of the Results of c-myc Molecular Alterations (FISH); c-myc mRNA Expression (real-time RT-PCR); MIB1, bcl-6 Protein Expression (IHC); p53 Inactivation; and Clinical Outcome.

| Case | Tumoral cells (%) | Diagnosis | c-myc gen | | c-myc (RNAm) | | MIB1 | bcl6 | p53 status | | OS | |
			Amplif	Rearrang	Value	SD			gen	IHC	Time (months)	Status
1	70%	DLBCL			0		87	37	ND	Active	8	Alive
2	70%	DLBCL			0		95	90	Wt	Active	69	Alive
3	70%	DLBCL			0		55	37	Wt	Active	73	Alive
4	80–100%	DLBCL			0		74	0	Wt	Active	53	Alive
5	80–100%	DLBCL			0		95	n.a.	Wt	Active	9	Dead
6	80–100%	DLBCL			0.19	0.024	81	85	Wt	Active	19	Dead
7	70%	DLBCL			0.26	0.085	90	75	Wt	Active	82	Dead
8	80–100%	DLBCL	−	−	0.30	0.000	96	85	Wt	Active	22	Dead
9	80–100%	DLBCL			0.34	0.120	n.a.	n.a.	Mt, aa213, R-R	Active	n.a.	n.a.
10	80–100%	DLBCL	−	+	0.34	0.073	90	90	Wt	Active	3	Dead
11	80–100%	DLBCL			0.36	0.090	84	56	Wt	Active	n.a.	n.a.
12	80–100%	DLBCL			0.41	0.030	98	95	Wt	Active	96	Dead
13	80–100%	DLBCL			0.45	0.030	100	60	Wt	Active	n.a.	n.a.
14	80–100%	DLBCL			0.47	0.060	100	95	ND	Active	83	Alive
15	80–100%	DLBCL			0.47	0.060	81	83	Wt	Active	58	Alive
16	80–100%	DLBCL	−	−	0.50	0.060	86	80	Wt	Active	30	Dead
17	60%	DLBCL			0.51	0.024	85	57	Wt	Active	11	Dead
18	60%	DLBCL			0.51	0.059	88	10	Wt	Active	94	Alive
19	80–100%	DLBCL			0.58	0.160	92	n.a.	Wt	n.a.	33	Alive
20	80–100%	DLBCL			0.61	0.201	98	3	Wt	Active	17	Dead
21	80–100%	DLBCL			0.64	0.060	95	80	Wt	Active	24	Alive
22	80–100%	DLBCL			0.68	0.177	82	84	Wt	Active	87	Alive
23	80–100%	DLBCL			0.68	0.197	81	56	Wt	Active	110	Alive
24	60%	DLBCL			0.71	0.242	85	87	Wt	Active	64	Alive
25	80–100%	DLBCL			0.72	0.149	82	n.a.	Wt	Active	4	Dead
26	80–100%	DLBCL			0.75	0.171	90	n.a.	Wt	Active	9	Dead
27	70%	DLBCL			0.79	0.090	85	9	Wt	Active	82	Alive
28	60%	DLBCL			0.79	0.090	90	90	Mt, aa239, N-D	Inactive	n.a.	n.a.
29	80–100%	DLBCL			0.83	0.335	n.a.	n.a.	Wt	Active	n.a.	n.a.
30	80–100%	DLBCL			0.87	0.149	80	5	Wt	Active	4	Dead
31	80–100%	DLBCL			0.88	0.030	97	64	Wt	Active	3	Dead
32	80–100%	DLBCL			0.93	0.354	73	95	Wt	Inactive	48	Alive
33	80–100%	DLBCL			0.96	0.671	82	100	Wt	Active	77	Alive
34	70%	DLBCL			0.98	0.000	85	2	Wt	Active	n.a.	n.a.
35	70%	DLBCL			1.00	0.233	85	10	Wt	Active	80	Alive
36	80–100%	DLBCL			1.01	0.098	98	39	Wt	Active	3	Dead
37	80–100%	DLBCL			1.26	0.090	80	83	Wt	Active	3	Dead
38	70%	DLBCL			1.32	0.060	78	48	Wt	Inactive	3	Dead
39	60%	BL	−	+	1.47	0.090	100	82	Wt	Active	81	Alive
40	80–100%	DLBCL			1.65	0.030	95	84	Wt	Active	29	Dead
41	80–100%	BL			1.88	0.124	n.a.	n.a.	Wt	Active	1	Dead
42	80–100%	DLBCL	−	−	1.92	1.471	82	98	Wt	Active	n.a.	n.a.
43	80–100%	DLBCL	−	−	1.92	0.359	98	95	Wt	Active	28	Dead
44	80–100%	DLBCL			1.97	0.592	90	98	Mt, aa234, Y-Stop	Inactive	17	Dead
45	80–100%	DLBCL	−	−	2.12	0.209	83	80	Wt	n.a.	72	Alive
46	80–100%	BL	−	+	2.28	0.610	100	98	Wt	Inactive	1	Dead
47	70%	DLBCL			2.41	0.508	78	n.a.	Wt	Active	3	Dead
48	80–100%	DLBCL	−	−	2.95	0.359	95	100	Wt	Inactive	34	Alive
49	80–100%	BL	−	+	3.08	0.060	100	100	Wt	Active	10	Alive
50	70%	DLBCL			3.10	1.552	96	80	Mt, aa213, R-R	Active	1	Dead
51	80–100%	BL	−	−	3.12	1.552	100	80	Wt	Active	25	Dead
52	80–100%	BL			4.47	0.164	100	47	Wt	Active	60	Alive
53	80–100%	BL			4.66	2.652	100	95	Wt	n.a.	11	Alive
54	80–100%	DLBCL	−	+	6.88	0.816	98	90	Mt, aa248, R-Q	Inactive	58	Alive
55	80–100%	DLBCL			8.40	2.850	87	91	Wt	Active	9	Dead
56	80–100%	BL	−	+	9.51	3.423	100	31	Wt	Active	71	Alive

n.a., not available.
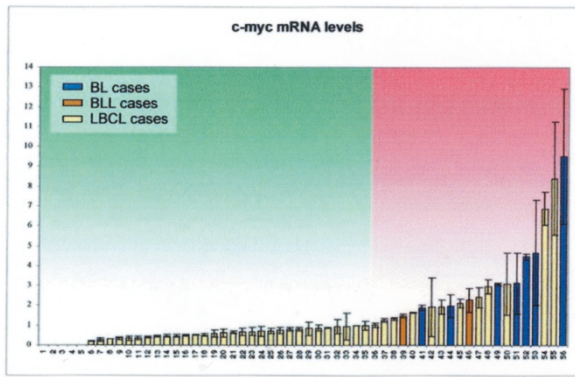
Figure 1.
The histogram shows the ⌐c-myc mRNA expression levels in diffuse large B-cell lymphomas (DLBCL) cases (*yellow bars*) and BL (*blue bars*). Eight DLBCL cases were in the range of c-myc expression of Burkitt's lymphoma (BL). Red background represents area of positive c-myc expression, using the mean of c-myc expression in reactive lymphoid tissue as a threshold. BL-like cases were considered for practical purposes as BL.
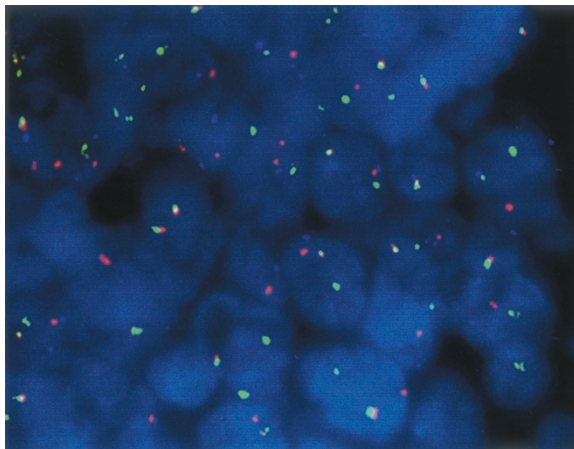

Figure 2.
Detection of t(8;14) by fluorescence in situ hybridization in a DLBCL sample. Green signals correspond to Ig heavy chain probes, red signals correspond to c-myc probes, and blue signals identified centromere of chromosome 8.

copy numbers in bladder cancer (Bieche et al, 1999; Christoph et al, 1999).

Analysis of c-myc expression using real-time PCR, as performed in this study, benefits from the use of multiple controls, which guarantee the reproducibility of the measurements. We previously found that the endogenous control provided by the 18S rRNA level was independent of growth fraction, morphology, and tumoral localization in this series of DLBCL and BL. In addition, the use of different dilutions of the replicates allowed the identification of variations in the expression level dependent on reverse transcription efficiency and polymerase potential inhibition.

Most of our results confirm previous reports using Northern blot, IHC, and in situ hybridization (Chang et al, 2000; Greil et al, 1991; Hernandez et al, 1999; Pagnano et al, 2001), which showed that a subset of DLBCLs expressed a high level of c-myc, stimulating interest in reproducible immunohistochemical techniques, which could facilitate the analysis of c-myc expression in the future. Although different groups have striven to demonstrate c-myc expression by IHC, the results obtained differed in the expression pattern in normal tissues, subcellular localization, percentage of DLBCL cases with increased expression, and even the intensity of expression in BL (Chang et al, 2000; Jack et al, 1986; Loke et al, 1988; Pagnano et al, 2001). Our own experience with IHC for demonstrating c-myc expression is that results vary greatly depending on the chosen antibody and on other unknown factors. This prompted us to look for alternative tools for measuring c-myc expression, such as real-time RT-PCR.

The results described here raise a number of questions about the clinical significance, mechanisms of c-myc deregulation, and relation with other cell cycle and apoptosis-regulator genes. Thus, considering patients with DLBCL, previous results obtained with IHC techniques showed that a high level of c-myc expression was associated with unfavorable IPI (Chang et al, 2000) and a relatively worse response to chemotherapy (Pagnano et al, 2001). We may now observe that deregulation of c-myc mRNA expression is associated with an adverse clinical course irrespective of the IPI scores. Although posttranslational changes (ie, somatic mutations in Thr 58 and Ser 62 [Gregory and Hann, 2000]) may contribute to the deregulation of the expression level of c-myc, the data shown here indicate that c-myc deregulation seems to play a significant role in the pathogenesis of a subset of LBCL cases.

Our observations underline the need to take into account mechanisms of c-myc deregulation other than translocations, which were absent in the majority of the DLBCL series and, specifically, in a subset of the cases with a high level of c-myc expression. Additional mechanisms of c-myc deregulation include exon 1–intron 1 somatic mutations and activation of upstream genes (Bradley et al, 1993; Cesarman et al, 1987; Gerbitz et al, 1999; Kanda et al, 2000; Pasqualucci et al, 2001; Wittekindt et al, 2000). It has been claimed that bcl-6 gene regulates c-myc transcription through
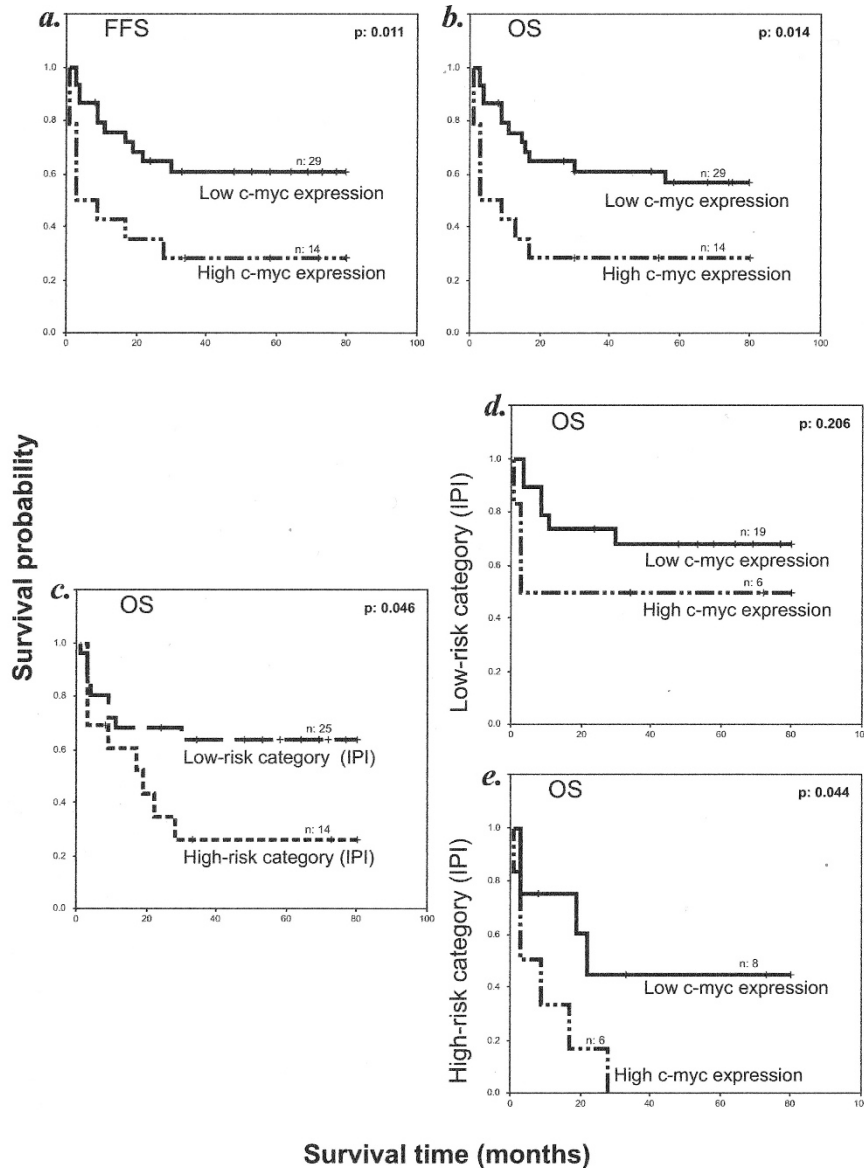
0.043, long rank test for OS; Fig. 3). To determine whether the effect of c-myc in the survival probability was independent of the IPI, we compared the survival probability in the groups of high- and low-risk patients. c-myc expression was an adverse prognostic factor in both groups (Fig. 3), although this was significant only in the group of cases in the high-risk category ($p = 0.044$, long rank test for OS).

Discussion

c-myc mRNA expression in this series of DLBCL is variable, over a range that includes a level of expression similar to that observed in BL. Reliable determination of the c-myc expression has been made possible by the use of real-time RT-PCR, as has been described in previous reports, where c-myc deregulation, as determined by real-time RT-PCR, was associated with larger tumor size and higher histological grade in breast cancer and increased c-myc gene

Figure 3.
Survival plots for DLBCL patients, according to c-myc expression. Patients with a high c-myc expression level had significantly shorter overall survival (OS; a) and failure-free survival (FFS; b). International Prognostic Index (IPI) distinguished two categories of patients, in terms of OS (c). High c-myc expression is associated with shorter survival in the low-risk group (d) and in the high-risk group (e), as defined by IPI, although the impact of c-myc is only significant in the latter group of patients.

the repression of a c-myc transcriptional repressor, blimp-1 (Lin et al, 1997; Shaffer et al, 2000). In this series of cases, we observed a weak relationship between the expression levels of c-myc and bcl-6 that could suggest a link between them. Nevertheless, the nature of the relationship between these two factors seems to be more complex than expected and demands additional experimental analysis (Shaffer et al, 2001).

c-myc is a multifunctional gene, and its pleiotropic effect is dependent on the cellular context. In addition, tumoral cells accumulate alterations that allow themselves to avoid c-myc–induced apoptosis (Felsher et al, 2000). In animal models, it has been shown that loss of ARF and/or p53 reduces apoptosis in B cells that express Eµ-myc (Eischen et al, 1999; Schmitt et al, 1999). We found that expression of c-myc was

associated with an increased frequency of p53 inactivation in DLBCL cases, thus complementing the similar observations made in other tumor types (Teitz et al, 2000) and confirming preliminary IHC results on DLBCL (Chang et al, 2000). Furthermore, our results prompted us to analyze the potential effects of c-myc deregulation on cell proliferation and on p27 and cyclin D3 (Bouchard et al, 1999; Schuhmacher et al, 2001). Cases with high levels of c-myc expression showed a weak increase in the proliferative index, although this difference was not significant. It is well accepted that p27 and cyclin D3 in particular are targets of c-myc in lymphoid tissue. However, the analysis of our data showed only a relatively weak relation. We interpreted this as indicating the multiplicity of genes in pathways converging at p27. First,

c-myc controls p27 expression at the transcriptional and posttranscriptional level through mechanisms including transcriptional repression, degradation, and stabilization of complexes that contain cyclin D3 and p27 (Bouchard et al, 1999; O'Hagan et al, 2000; Perez-Roger et al, 1999; Sanchez-Beato et al, 2001; Schuhmacher et al, 2001; Yang et al, 2001). The balance between these signals is also tissue- and cell type–dependent and may significantly rely on p53 mutations or other critical cellular events. In addition, the variability in p27 expression in cases with similar c-myc expression suggests the existence of complex multiple upregulatory genes that may well control p27 expression, such as bcl-6 (Shaffer et al, 2000).

In conclusion, real-time RT-PCR for c-myc allows the reproducible estimation of c-myc levels that, when analyzed in a series of DLBCLs, confirms the clinical and biological significance of c-myc expression and suggests the existence of a variety of mechanisms for deregulating this gene.

Materials and Methods

Tissue Samples, Patients, and Clinical Variables

Fifty-six samples of tumor specimens from 48 DLB-CLs and 8 BLs were selected from the CNIO Tumor Bank Network, CNIO (Madrid, Spain), on the basis of the availability of paraffin-embedded and frozen tissue for molecular studies, and the existence of an adequate clinical follow-up. We also selected six routine reactive tonsils, as representative of reactive lymphoid tissue. Diagnostic pretreatment samples were analyzed in all cases. Tumors were diagnosed according to the World Health Organization classification (Jaffe et al, 2001).

All patients were treated with chemotherapy regimens that included anthracyclins. Follow-up data were available in 49 of 56 cases. Periods of clinical follow-up of patients who were alive at the time of the study ranged from 12 to 110 months (mean: 62.68 months). Age, clinical stage, performance status, lactate dehydrogenase (LDH), and the number of involved extranodal sites were used as outcome criteria, as set out in the IPI (The International Non-Hodgkin's Lymphoma Prognostic Factors Project, 1993). Patients with 0 to 2 unfavorable variables were considered as being low risk, and those with 3 to 5 variables were considered as being high risk. Treatment outcome was measured using FFS and OS. FFS was defined as the time from diagnosis to the first occurrence of progression, relapse after response, or death from any cause. OS was measured from diagnosis to death from any cause. To help in the validation of the kinetic quantitative RT-PCR method, we also analyzed the Raji cell line, obtained from the American Tissue Type Culture Collection (Rockville, Maryland).

Real-Time RT-PCR

RNA Extraction and c-DNA Synthesis. Total RNA was extracted from frozen sections by the standard Trizol protocol, using TRIzol reagent (GIBCO BRL, Paisley, Scotland). Electrophoresis on agarose gels and absorbance measurement at 260/280 nm determined the quality of the RNA samples. Before RNA extraction, frozen sections of the specimens were analyzed to ensure that they were representative of the tumor. The percentage of tumoral cells was >60% in all cases.

Reverse transcription of RNA was done in a final volume of 20 μl containing 1$\times$ RT-PCR buffer, 40 units of RNAsin (a RNase inhibitor), 10 units of AMV reverse transcriptase, 50 ng of random hexamers (Promega, Madison, Wisconsin), and 500 ng of total RNA. The samples were denatured at 65°C for 5 minutes and incubated at 25°C for 7 minutes and 42°C for 60 minutes. Afterward, they were heated at 70°C for 15 minutes and cooled at 4°C to inactivate reverse transcriptase.

Real-Time RT-PCR. c-myc mRNA expression was measured by real-time quantitative RT-PCR using TaqMan technology. The principle of these methods has been described in detail elsewhere (Gibson et al, 1996; Heid et al, 1996). All PCR reactions were performed with the TaqMan Universal PCR Master Mix (PE Applied Biosystems), c-myc and 18S rRNA primers and probes supplied by Applied Biosystems as predeveloped assay reagents in a 25-μl final reaction mixture, according to the manufacturer's instructions. The thermal cycling conditions were composed of an initial denaturation step at 50°C for 2 minutes and 95°C for 10 minutes followed by 40 cycles consisting of 15 seconds of denaturation at 95°C and 1 minute at 60°C for hybridization of the probe and primers.

Immunostaining Techniques

The bcl-6 protein was detected with monoclonal antibody (MAb) for P6-B6p, a recombinant protein corresponding to amino acids 3 to 484 from DAKO (Glostrup, Denmark; 1/10). p27 protein was detected with a MAb from Transduction Laboratories (Lexington, Kentucky), K25020 (1/1000), generated against the full-length mouse KIP1 protein. cyclin D3 was detected with MAb DCS-22 from Novocastra (Newcastle upon Tyne, UK; 1/10). The proliferation index was evaluated using nuclear antigen Ki67 expression, detected with the MIB1 antibody from Immunotech (Marseille, France). p53 protein was detected with MAb DO7 (Novocastra; 1/50 dilution), generated against recombinant p53 protein, which recognizes both wild-type and mutant forms of p53 protein. p21^{WAF1} protein was detected by WAF1 (Ab-1; Oncogene Science, Cambridge, Massachusetts) mouse MAb (1/25 dilution) raised against recombinant human p21^{WAF1} protein.

Immunostaining techniques were performed in paraffin-embedded tissue sections. For antigen retrieval, before incubation with the antibodies, the slides were heated in a pressure cooker for 3 minutes in 0.01 M sodium citrate solution. In the case of bcl-6 protein detection, the slides were also subsequently digested with proteinase K for 10 minutes at room temperature.

After incubation with the primary antibodies, immunodetection was performed with biotinylated anti-mouse immunoglobulins, followed by peroxidase-labeled streptavidin (LSAB-DAKO) and diaminobenzidine chromogen as substrate. All immunostaining was performed using the Techmate 500 (DAKO) automatic immunostaining device.

Incubations omitting the specific antibodies or with unrelated antibodies were carried out to provide controls of the technique. Expression in small lymphocytes was used as an internal control of immunostaining of bcl-6 and p27. Internal controls for cyclin D3 were provided by the degree of staining of endothelial cells and macrophages. Cases that lacked internal controls were excluded from the series. This technique for demonstrating bcl-6 protein expression had previously been validated and found to match the results of bcl-6 mRNA expression as measured by real-time RT-PCR (Artiga et al, 2002).

Quantitative Studies

All scoring and interpretations of immunohistochemical results were carried out without knowledge of the clinical variables or results of the molecular analysis.

High-magnification fields were chosen for the evaluation of all of the proteins studied, focusing on tumoral areas and counting up to 300 cells. Scoring of p53 and p21 expression was performed semiquantitatively. We subdivided cases into three different groups according to the number of positive cells (negative [−], positive [+], and highly positive [++]). Expression of bcl-6, p27, cyclin D3, and MIB1 was scored quantitatively as the percentage of positive cells. All immunoreactive cells were considered to be positive. A manual cell-counting procedure was used so that all nontumoral subpopulations could be excluded on the basis of their cell morphology.

The functionality of p53 protein was evaluated taking into consideration the expression of itself and its effector p21. Cases with p53−/p21− or p53++/p21− were considered to have an inactive p53 protein (Villuendas et al, 1997).

The mean values of p27 and cyclin D3 in our series (27% and 44%, respectively) were chosen as the threshold of reactivity for those proteins. These are approximately equivalent to the level of expression of p27 and cyclin D3 within the germinal centers and have been previously reported as being reproducible (Saez et al, 1999; Sanchez-Beato et al, 1999). bcl-6 expression was considered as a continuous variable.

Mutational Analysis of p53 Gene

Exons 5 to 8 of the p53 gene were amplified from genomic DNA, extracted from tissue samples, using previously described primers and conditions in 54 of the 56 cases in this series (Sanchez-Beato et al, 2001; Villuendas et al, 1997). PCR products were purified using the Microcon PCR system (Millipore Corporation, Bedford, Massachusetts). Direct sequencing of purified PCR products was performed with an auto-mated DNA Sequencer ABI PRISM 3700 Genetic Analyzer (Applied Biosystems, Weiterstadt, Germany). Partial results of p53 sequencing (23 of 56 cases) in this series have been published previously (Sanchez-Beato et al, 2001; Villuendas et al, 1997).

FISH

FISH analysis was performed on paraffin-embedded samples. The fluorescently labeled DNA probes were purchased from Vysis (Downers Grove, Illinois). The LSI Ig heavy chain/myc CEP 8, tricolor probe is designed to detect the juxtaposition of the Ig heavy chain locus and myc gene region sequences and also to detect any amplification of the c-myc gene. The CEP 8 probe was used as the control to determine the copy number for chromosome 8. The probe extends approximately 400 kb upstream of c-myc and approximately 350 kb 3' beyond the gene.

After being deparaffinized and rehydrated, the slides were treated with EDTA (1 mM, pH 8) for 2 to 3 minutes, then with a pepsin solution (0.1 mM HCl, 0.05 mg/ml pepsin), and finally dehydrated and denatured in the presence of the probe on a plate at 75°C for 2 minutes. Finally, the slides were counterstained with DAPI in an antifade solution.

We established the cutoff values to avoid false-positive cases by scoring 800 nuclei from four different tonsil samples. The cutoff value was set at 18%. Cases with fusion signals greater than that value were considered positive for the translocation. At least 200 interphase nuclei were analyzed for each case. Cell images were captured using a CCD camera (Photometrics Sensys camera) connected to a computer running the Chromofluor Image Analysis system (Cytovision; Applied Imaging Ltd., New Castle, United Kingdom)

Statistical Analysis

Relationships between variables were investigated using the Pearson test for categorical variables, the Kruskal-Wallis test for single-ranked data, or the Spearman correlation for double-ranked data. The prognostic value of c-myc expression was evaluated by standard survival analysis, using the Kaplan-Meier method, long rank test, and Cox regression. All statistical analyses were performed using SPSS 10.0 for Windows (SPSS Inc., Chicago, Illinois).

Acknowledgements

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