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QUANTITATION OF bcl-2 ONCOGENE IN CULTURED LYMPHOMA/LEUKEMIA CELL LINES AND IN PRIMARY LEUKEMIA B-CELLS BY A HIGHLY SENSITIVE RT-PCR METHOD

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ABSTRACT

Background. **The bcl-2 gene, isolated from the t(14;18) chromosomal translocation breakpoint, is able to prevent apoptotic death induced by various stimuli in different tissues. Therefore bcl-2 oncogene expression could be a key parameter for investigating the molecular mechanisms involved in the apoptosis of normal and neoplastic hematopoietic cells.**

Methods. **In order to evaluate bcl-2 expression in both follicular B-lymphomas carrying or not carrying the 14;18 translocation and in lymphatic leukemias, we optimized an internal standard-based method of reverse transcriptase-polymerase chain reaction (RT-PCR) for the rapid quantitation of bcl-2 mRNA cellular levels. A simple purification of the reverse transcription products resulted in very high PCR efficiency, so that radioactive labelling of the amplification products was avoided.**

Results. **bcl-2 mRNA levels proved to be higher in t(14;18) than in t(14;18) negative cell lines, and higher in primary leukemia pre-B cells than in early-B cells. Tested for sensitivity by identifying minimal residual t(14;18) B cells expressing the bcl-2/IgH gene, this RT-PCR method was able to detect bcl-2/IgH mRNA from just one t(14;18) positive cell out of ten million t(14;18) negative cells.**

Conclusions. **The RT-PCR method we optimized appears to be suitable for clinical use in both the leukemia/lymphoma characterization and in lymphomatous disease follow-up.**

Key words: bcl-2, lymphoma, leukemia, PCR, quantitative RT-PCR, minimal residual disease

Most hematologic neoplasias are related to tumor-specific chromosomal translocations.[1-4] The 14;18 translocation is consistently associated with human follicular B-cell lymphomas[3] and more rarely with acute lymphatic leukemias[5,6] (ALL). The *bcl-2* oncogene was originally identified in B-cell lymphomas as a result of its involvement in the t(14;18) that fuses the 3' untranslated region of *bcl-2* with the IgH locus, thus creating a *bcl-2*/IgH hybrid gene. The *bcl-2*/IgH fusion never implies disruption of the *bcl-2* open reading frame, nor are hybrid proteins produced.[7] Rather, higher levels of fused *bcl-2* mRNA and normal *bcl-2* protein are detected in all t(14;18) positive B-cells than in the matched t(14;18) negative counterparts expressing normal *bcl-2* mRNA.[8] Thanks to unknown mechanisms *bcl-2* is endowed with antiapoptotic properties and is thus responsible for the prolonged survival of t(14;18) B-cells both *in vitro* and *in vivo*.[9]

Studies of *bcl-2* gene expression in cell lines

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representing different stages of B-cell development indicate that *bcl-2* is physiologically regulated *in vivo* within the B-cell lineage;[8-11] nevertheless, extremely contrasting results have been reported so that the pattern of this regulation is far from being defined. Several observations also suggest that quantitative variations in *bcl-2* gene expression rather than a qualitative on/off switch of its activity control the lymphocyte lifespan and are responsible, if deregulated, for the emergence of lymphomas or leukemias. An accurate quantitation of the steady-state mRNA levels therefore appears to be essential for a better understanding of the molecular mechanisms involved in lymphocyte physiology and pathology.

Quantitative reverse transcription-polymerase chain reaction (Q-RT-PCR) is known to be the most sensitive method for evaluating cellular mRNA levels, giving unambiguous results from small starting samples.[12] We devised a highly sensitive internal standard-based RT-PCR method to quantitate *bcl-2* mRNA levels, and we determined *bcl-2* gene expression in both follicular lymphoma and other hematic neoplasia cell lines. Primary human B-cell leukemias were also tested in order to evaluate whether the different levels of *bcl-2* expression measured correlate with the maturation stage of the lymphatic cell giving rise to the neoplastic clone.

Moreover, the RT-PCR assay we optimized showed very high sensitivity in rapidly detecting minimal residual t(14;18) B-cells. Because it reveals only the *bcl-2*/IgH mRNA expressed, this method may represent the basis for detecting residual circulating B-lymphoma cells containing the *bcl-2*/IgH hybrid gene, seemingly predictive of lymphoma relapse.

Materials and Methods

Lymphoma/leukemia cell lines and primary leukemia B-cells

Two human follicular lymphoma B-cell lines with t(14;18)-related *bcl-2*/IgH fusion, i.e. DOHH2[13] and SU-DHL-4,[8] and the t(14;18) negative Raji[15] follicular lymphoma B-cell line were used for the experiments. Comparison with the Jurkat[14] T-cell line and the NB4[16] promyelocytic neoplastic cell line was also carried out. All cell lines were kindly provided by Dr. L. Capolongo (Farmitalia Carlo Erba Research Center, Nerviano, Italy) and maintained under standard conditions in RPMI 1640 medium (GIBCO, Gaithersburg, MD) with the addition of 10% fetal calf serum (FCS). Primary human leukemia cells were obtained after informed consent by venipuncture from two patients with pre-B lymphatic leukemia (immunophenotype TdT^+, $HLA\text{-}DR^+$, $CD19^+$, $CD20^+$, $CD22^+$, $CD34^+$, $CD\ 38^+$ and c-μ) and two patients with early-B lymphatic leukemia (immunophenotype TdT^+, $HLA\text{-}DR^+$, $CD10^+$, $CD19^+$, $CD20^+$, $CD22^+$, $CD34^+$ and $CD38^+$). Mononuclear cells were separated by Ficoll-Isopaque gradient centrifugation, thoroughly washed and cryopreserved in DMSO 7%. After thawing, cells were washed and, when there were more than 90% living lymphoid blasts, pelleted for RNA extraction. None of the samples showed the t(14;18) translocation.

RNA extraction and reverse transcription

Total cellular RNA was extracted from 10^6 cells either according to the guanidine thiocyanate method of Chomczynski and Sacchi[17] or using RNAzol B (Cinna Biotecx, Houston, USA) as reported in the manufacturer's instructions, and treated with RQ1 RNase-Free DNase (Promega, Madison, USA) according to Soubeyran *et al.*[18] DNase treatment of the RNA extracts before PCR excluded any possible contaminating genomic DNA amplification. After ethanol precipitation RNA was dissolved in 10 μL RNasin (1 U/μL), quantitated by spectrophotometry and analyzed on 1% agarose gel. Random hexamers were used as reverse transcription primers in order to obtain a total cDNA preparation. The reaction mixture (100 μL) contained 10 μL (about 1 μg) of total RNA, 100 U RNasin, 25 μg BSA, 1 mM dNTPs, 12.5 μM random hexamers (Genosys, The Woodlands, USA), 50 mM Tris-HCl, pH 8.3, 75 μM KCl, 3 mM $MgCl_2$ and 10% glycerol. After heating at 80°C for 5 min and rapid cooling, 1,000 U of Mo-MLV reverse transcriptase (Pro-

mega, Madison, USA) were added. Incubation was perfomed at 37°C for 60 min and stopped by heating at 95°C for 15 min. The cDNA solutions were then routinely purified by three phenol-chloroform extractions followed by ethanol precipitation, and concentrated in 10 μL H_20.

Quantitative polymerase chain reaction

Polymerase chain reaction (PCR) primers were designed by targeting different segments of the *bcl-2*/IgH cDNA obtained with the reverse transcription reaction. A segment of 137 bp located in the *bcl-2* coding region shared by both t(14;18) and untranslocated cells was targeted by primer 1 [5'-GGACAACATCGCCCTGTG-3', bases 551-568 in exon 1 of *bcl-2* cDNA, sense strand[7]] and primer 2 [5'-AGTCTTCAGAGACAGCCAGGA-3', bases 668-688 in exon 2 of *bcl-2* cDNA, antisense strand[7]]. A hybrid *bcl-2*/IgH segment of 312 bp, peculiar to DOHH2 cells, was targeted by primer 3 [5'-GGTGACCAGGGTCCCTTGGCCCCAG-3', bases 2973-2998 on the JH consensus sequence of the IgH locus, antisense strand[19]] and primer 4 [5'-GCAATTCCGCATTTAATTCATG-GTATTCAGGAT-3', bases 2866-2898 of the *bcl-2* cDNA, sense strand[7]]. A hybrid *bcl-2*/IgH segment of 198 bp, peculiar to SU-DHL-4 cells, was targeted by using primer 3 (see above) and primer 5 [5'-CGAAACCTGCTTTAAAAAAA-TA-CATGCATCTCACGC-3', bases 2544-2580 of the *bcl-2* gene cDNA, sense strand[7]]. Both β-actin and β_2m genes were chosen as internal standards, being expressed at constant per-cell levels (only data relative to β-actin are reported in this work). A 234 bp segment on the β-actin gene was targeted by primer 7 [5'-GCGGGAAATCGTGCGTGACATT-3', bases 2104-2127 of the β-actin genomic sequence, located in exon 3, sense strand[20]] and primer 8 [5'-GATGGAGTTG-AAGGTAGTTTCGTG-3', bases 2409-2432 of the β-actin genomic sequence, located in exon 4; antisense strand[20]]. A 120 bp segment of the β_2m gene was targeted by primer 9 [5'-ACCCCCACTGAAAAAGATGA-3': bases 1544-1563 of the β_2m genomic sequence, sense strand[21]] and primer 10 [5'-ATCTTCAAACCTCCATGATG-3': bases 2253-2262 and 3508-3517 of the β_2m genomic sequence, antisense strand[21]]. Samples obtained by omitting the Mo-MLV enzyme in the reverse transcription mixture were used as negative controls to detect possible residual DNA contamination. PCR was carried out according to the hot start procedure,[22] following the recommendations of Kwok and Higushi[23] concerning control of contamination by a previously amplified positive sample. Fifty μL of the PCR reaction mixture were as follows: 0-5 μL of cDNA solution, 1 μM primers, 200 μM dNTPs, 15 mM MgCl2, 50 mM KCl, 10 mM Tris-HCl pH 8.8, sterile water to 45 μL, 2.5 U of Taq polymerase (Perkin-Elmer-Cetus, Emeryville, USA). PCR was protracted for 25 cycles. Denaturation and extension were performed for 1 min at 92°C and 72°C, respectively, with all primers. Annealing was carried out for 1 min, at 56°C with primers 1 and 2 (*bcl-2* coding region), at 65°C with primers 3, 4, 5, 6 (*bcl-2*/IgH fusion segments) and 7, 8 (β-actin), and 48°C with primers 9 and 10 (β_2m).

Qualitative PCR assays were carried out first. Amplification products were analyzed on 2% agarose gel electrophoresis – using the ladder φ X174-Hinf I as molecular weight marker – and identified by sequencing with the *Cycle Sequencing Kit* (Perkin-Elmer, Norwalk, USA) according to the manufacturer's instructions. β-actin or β_2m standard genes were analogously amplified on the same total cDNA preparation. Quantitation of gene expression requires first finding the linear range between the amount of target or standard gene PCR products and the starting volumes of the relevant cDNA. Indeed PCR efficiency tends to decrease as the number of cycles increases until a *plateau* is reached, depending on both the starting amount of target copies and on a variety of other factors.[27] For quantitative RT-PCR, serial dilutions of cDNA were therefore amplified with a fixed number of PCR cycles, and amplification products were quantitated by densitometric analysis of the relevant ethidium bromide-stained bands obtained following agarose gel electrophoresis. Only for very small cell samples, or when low levels of total RNA were obtained was PCR carried out in the presence of 5 μCi of [α^{32}P]dATP (6,000 Ci/mmol) and radioactive products, analyzed

on PAGE, quantitated either by densitometric analysis of gel autoradiography or by radiometric counting of excised bands. Within the linear range of amplification, i.e. before PCR the *plateau* was reached, at least three values of PCR products relative to *bcl-2* mRNA were normalized to the starting cDNA volumes and referred to the values of PCR products relative to either the β-actin or the β_2m mRNAs used as internal standards.

Rapid detection of minimal bcl-2/IgH-expressing cells

PCR sensitivity was first compared to RT-PCR sensitivity in detecting minimal residual t(14;18) cells. Five hundred t(14;18) positive cells (DOHH2 and SU-DHL-4 cells) or 500 t(14;18) negative cells (Raji) were mixed with 500 μL of 5% Chelex 100 (Bio-Rad, Richmond, USA), a chelating resin that prevents nucleic acid breakdown,[25] boiled for 10 min, chilled on ice for 2 min and centrifuged at 1000 g for 10 min.[26] Extracts were incubated either in the presence or the absence of Mo-MLV reverse transcriptase and amplified by PCR as previously described. In order to enhance PCR sensitivity and to avoid aspecific amplification products a seminested PCR was carried out. The 312 bp hybrid PCR product obtained from DOHH2 cells (as previously described) was purified by ultrafiltration with a Millipore Ultrafree™ filter unit having a molecular weight cutoff of 30,000 (Waters-Millipore, Marlborough, USA), and again amplified for 25 cycles by using primer 3 (see above) and primer 6 [5'-CGTGGCCT-GTTTCAA-3', bases 3008-3022 of the *bcl-2* cDNA, sense strand[7]]; a 170 bp PCR product was expected.

Since RT-PCR analysis was to be employed in the detection of the minimal residual lymphomatous cells expressing the *bcl-2*/IgH gene, the t(14;18) positive DOHH2 cells were highly diluted (from $1:10^5$ to $1:10^7$) with the t(14;18) negative Raji cells used for total RNA extraction. Following DNase treatment the *bcl-2*/IgH fusion segment of cDNA peculiar to DOHH2 cells was amplified following the previously described seminested PCR protocol. In view of a further comparative assay between PCR and RT-PCR sensitivity, genomic DNA was also extracted using standard procedures[43] by the same cell dilutions and PCR was amplified with the same conditions employed for cDNA.

Results

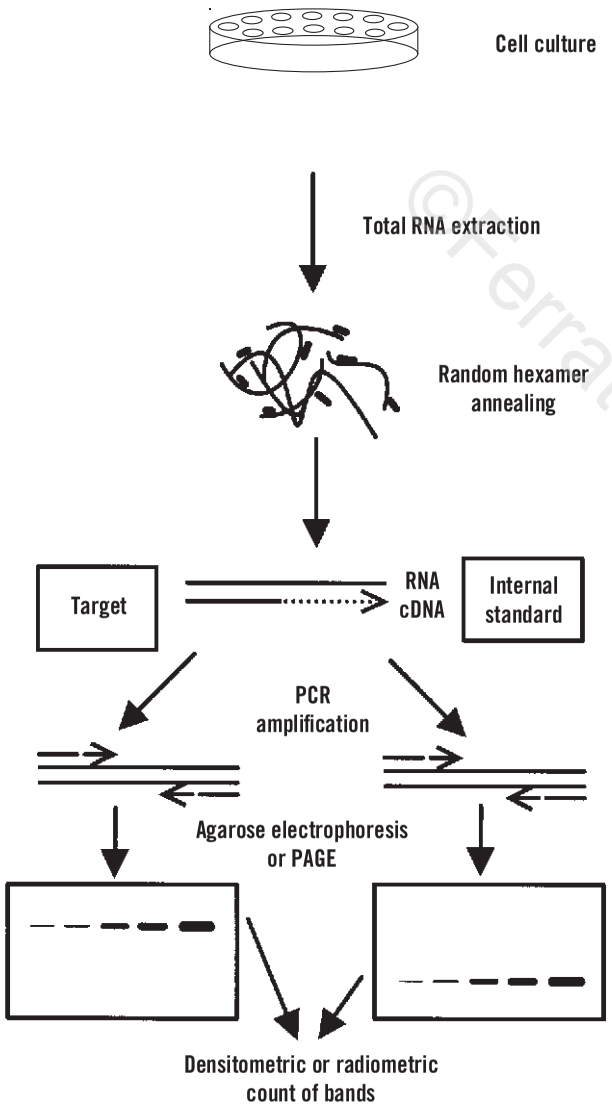
Identification of PCR products

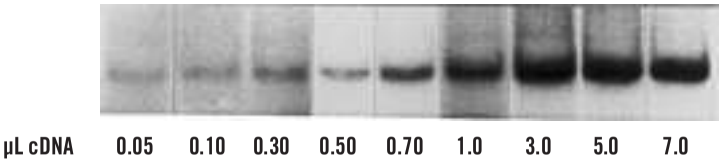
Preliminary qualitative PCR assays were carried out with cDNAs obtained from either t(14;18) or untranslocated cells (data not shown). With the primer pair designed for the segment located within the *bcl-2* coding region (1 and 2) shared by all cell lines, both the t(14;18) (DOHH2 and SU-DHL-4) and the untraslocated cells (NB4, Jurkat, RAJI and pre-B ALL) gave the expected 137 bp PCR product. With the primer pairs designed for the specific *bcl-2*/IgH fusion regions, only the two t(14;18) positive DOHH2 and SU-DHL-4 cell lines gave their peculiar *bcl-2*/IgH amplification products of 312 and 198 bp, respectively. Direct sequence analysis confirmed the specificity of all PCR products obtained.

Quantitative RT-PCR strategy

The scheme of the quantitative RT-PCR strategy we employed is reported in Figure 1A. Total RNA was rapidly extracted, DNase treated and reversely transcribed to cDNA using random hexamers. cDNA was amplified by PCR using the primer pairs designed for *bcl-2*, β-actin or β_2m, and the expected amplification products were quantitiated as described in the Materials and methods section.

Figure 1B shows the relationship between increasing amounts of DOHH2 cell cDNA and the PCR products obtained with primers 1 and 2, defining a 137 bp segment in the *bcl-2* mRNA. From densitometric quantitation of bands a linear region of amplification is followed by a *plateau* (Figure 1C). At least three values of *bcl-2* gene amplificates falling within the range of linearity were normalized to the starting cDNA volumes and referred to β-actin or β_2m. In general, for every gene segment targeted the linear PCR region fell in the range of twice the log concentration.





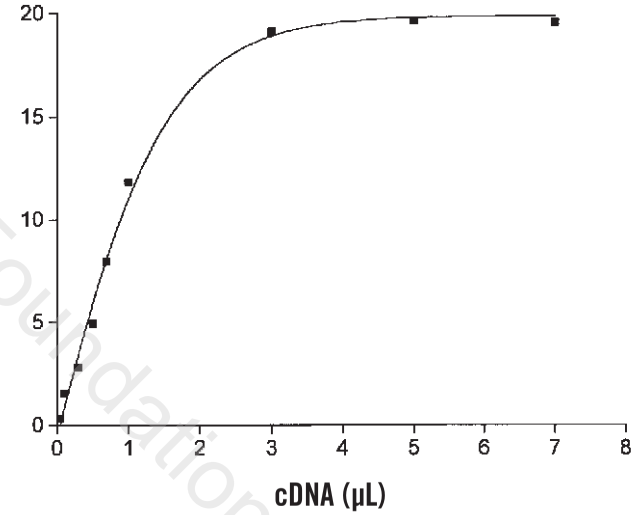


Figure 1. (A) Scheme of the quantitative RT-PCR assay. (B) PAGE analysis of PCR products. A 137 bp segment of the bcl-2 coding region was amplified with primers 1 and 2 in the presence of 32P-ATP and increasing amounts of DOHH2 cDNA, i. e., from 0.05 to 7 µl. The radioactive products were electrophoresed on polyacrylamide gel and autoradiographed for visualization. (C) Densitometric quantitation of bands indicates the relationship between the starting amounts of bcl-2 cDNA from DOHH2 cells and the relevant PCR amplification products.

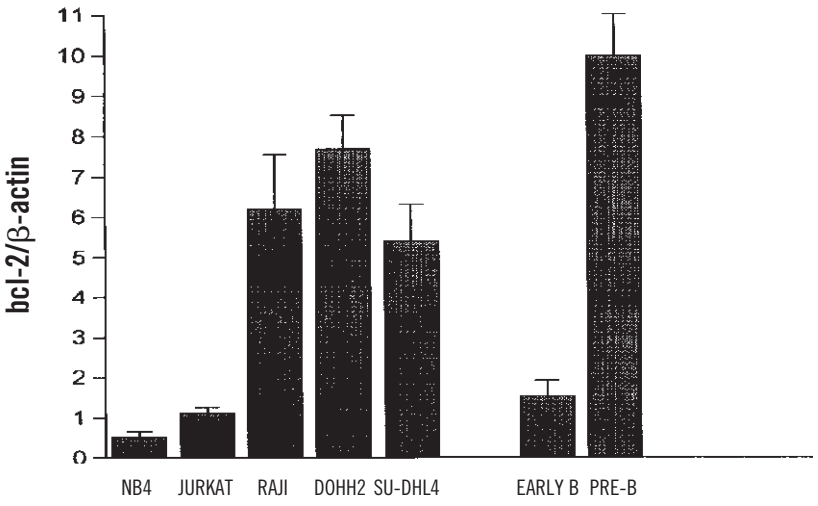


Figure 2. Cellular levels of bcl-2 mRNA in Raji, DOHH2, SU-DHL-4 B-cell lines, in the NB4 promyelocytic cell line and in the Jurkat T-cell line. Primary leukemia early B and pre-B cells were also tested. The relative yield of bcl-2 to ß-actin mRNA is obtained from values falling within the range of linearity of PCR amplification.

Levels of bcl-2 mRNA in follicular lymphoma B-cell lines and primary leukemia B-cells

Levels of *bcl-2* mRNA in human lymphoid cell lines and untranslocated primary leukemia B-cells are reported in Figure 2. It appears that both t(14;18) lymphoid B-cell lines (DOHH2, SU-DHL-4) and the t(14;18) negative pre-B Raji cell line present higher levels of *bcl-2* mRNA than the promyelocytic NB4 and Jurkat T cell lines. Furthermore, pre-B primary leukemias show higher levels of *bcl-2* mRNA than early-B primary leukemias.

Sensitivity of RT-PCR in the detection of rare lymphoma cells expressing hybrid bcl-2/IgH mRNA

The absolute sensitivity of our RT-PCR protocol in detecting chimeric *bcl-2*/IgH transcripts was evaluated by starting from the different amounts of cDNA obtained from 1 DOHH2 cell diluted in 10^7 Raji cells, corresponding to an initial ratio of about 10 pg of total DOHH2 RNA to 100 μg of total Raji RNA (Figure 3). Under these conditions PCR products were clearly detectable without radiolabelling, even when 1 fg of RNA (10^{-4} DOHH2 cells) was used. On the contrary, about a two log higher amount (100 fg) of RNA was generally required in order to clearly detect PCR products when cDNA purification through phenol-chloroform extraction and ethanol precipitation was omitted (not shown). No amplification product was detectable in the negative controls obtained using Raji cells without DOHH2 cells added, thus ruling out any possible contamination.

In order to assess the sensitivity of our RT-PCR method with respect to that of PCR, a comparative PCR analysis was carried out using two different protocols.

First, a crude mixture of total RNA and DNA obtained by Chelex from 500 t(14;18) DOHH2 cells was either subjected or not subjected to reverse transcription and then amplified. Untranslocated Raji cells were used as negative controls. Thirty-five PCR cycles carried out with primers 3 and 4 relative to the *bcl-2*/IgH fusion segment, were followed by 25 cycles of a seminested PCR with primers 3 and 6. The agarose gel in Figure 4 shows that the expected hybrid PCR product of 170 bp is detectable only in the samples of t(14;18) cells subjected to reverse transcription.

Second, the genomic DNA extracted from the same number of cells employed in the absolute sensitivity determination (Figure 3) was amplified with the identical amplification protocol used for this RT-PCR analysis. The agarose gel in Figure 5 shows that an amount of genomic DNA corresponding to 10^{-1} DOHH2 cells is required in order to detect amplification products. Comparison of Figures 3, 4 and 5 clearly indicates that the sensitivity of RT-PCR is much higher than that of PCR in detecting t(14;18) cells.

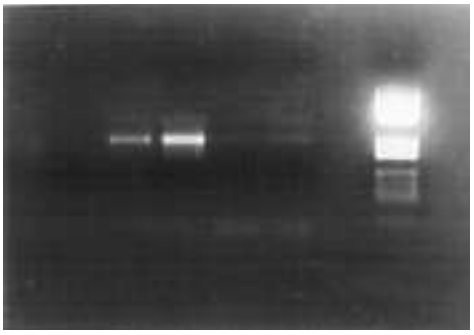


Figure 3. RT-PCR analysis carried out in one t(14;18) DOHH2 cell diluted with 10 million t(14;18) negative Raji cells. Serial dilution of cDNA was amplified. Agarose gel analysis of the 170 bp amplification products expected following the seminested PCR is reported. Products refer both to the fraction of DOHH2 cells and to the relative RNA/cDNA content. Lane 1. Ladder. Lane 2. 1 DOHH2 cell (10 pg RNA). Lane 3. 10-1 DOHH2 cells (1 pg RNA). Lane 4. 10-2 DOHH2 cells (100 fg RNA). Lane 5. 10-3 DOHH2 cells (10 fg RNA). Lane 6. 10-4 DOHH2 cells (1fg RNA). Lane 7. Negative control: 10-1 Raji cells, no DOHH2 cells. The bands of the amplification products refer to both the number of t(14;18) cells and to the relative RNA/cDNA amount present in the PCR mixture.

cator of the biological activity of the *bcl-2* pro

Figure 4. Comparison between PCR and RT-PCR sensitivity in the detection of rare t(14;18) cells carrying the bcl-2/IgH hybrid gene. Total DOHH2 or Raji cellular extracts were prepared by Chelex. Agarose gel analysis of the 170 bp amplification products expected following seminested PCR is reported. Lane 1. Negative control: extract omitted in the RT-PCR mixture. Lane 2. RT-PCR analysis of the extract obtained from 5×10^{-3} DOHH2 cells. Lane 3. RT-PCR analysis of the extract obtained from 5×10^{-2} DOHH2 cells. Lane 4. PCR analysis (reverse transcription omitted) of the extract obtained from 5×10^{-3} DOHH2 cells. Lane 5. PCR analysis (reverse transcription omitted) of the extract obtained from 5×10^{-2} DOHH2 cells. Lane 6. Negative control: RT-PCR analysis of the extract obtained from 5 Raji cells [no (14;18) translocated cells]. Lane 7. Ladder.

tein and of its role in cell survival.

The techniques adopted so far to study *bcl-2* gene expression, i.e. Northern blotting, S1 nuclease protection[8] and RNAase protection assay,[11] are limited by low sensitivity and the need for a considerable amount of sample RNA, and they often give rise to ambiguous results. After the introduction of PCR, the possibility of using this new molecular technique for quantitative aims was proposed. The first approach involved coamplification of a competitive synthetic template which is added to the reaction mixture and is amplified in the same tube and with the same primers used for amplification of the target gene.[27,29] An alternative strategy proposed consisted of coamplification of an endogenous reference gene as internal standard, which could be amplified either in the same reaction tube[21] or in separate tubes[24] using an additional pair of primers. To our knowledge, the first quantitative RT-PCR determination of *bcl-2* gene activity was that of Abe-Dohmae *et al.*,[30] who demonstrated high expression in the murine central nervous system during neurogenesis. This work follows a protocol that makes use of a competitive template, rendering the assay sensitive but laborious.

In the present study we developed an internal standard-based quantitative RT-PCR method suitable for the rapid quantitation of *bcl-2* mRNA levels from small cell samples. This method was inspired by the one proposed by Horikoshi *et al.*[24] for the study of low abundance

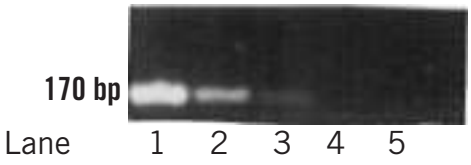


Figure 5. PCR analysis carried out on genomic DNA extracted from one t(14;18) DOHH2 cell diluted with 10 million t(14;18) negative Raji cells. Serial dilution of genomic DNA was amplified and the PCR conditions employed were the same as these for RT-PCR analysis (see Figure 4). Lane 1. 1 DOHH2 cell (10 pg genomic DNA). Lane 2. 10-1 DOHH2 cells (1 pg genomic DNA). Lane 3. 10-2 DOHH2 cells (100 fg genomic DNA). Lane 4. 10-3 DOHH2 cells (10 fg genomic DNA). Lane 5. 10^{-4} DOHH2 cells (1fg genomic DNA). DNase treatment of the RNA extracts before PCR excluded any possible contaminating genomic DNA amplification.

genes relevant to cancer drug activity. It takes advantage of the strategy by which an endogenous standard gene is amplified for normalization and avoids the problems deriving from a duplex amplification reaction in one tube involving two different primer pairs. The cDNA phenol-chloroform purification step we introduced into our method enhances PCR efficiency and sensitivity, so that, in most cases, radiolabelling of amplificates was not required. Besides the present work, we have used this method to assess the effects of antisense oligonucleotides on *bcl-2* gene expression[31] and, with slight modifications, on mdr1 gene[32] and urokinase receptor gene[33,34] expression in neoplastic cells.

Lately, many authors have been investigating differential *bcl-2* expression during B-cell maturation, but the results are contradictory. In a study carried out with human cell lines representative of the various stages of B-cell development, *bcl-2* mRNA levels were found to be high in pre-B cells and down-regulated in the course of maturation to undetectable levels in purified resting B-cells.[8] Furthermore, while *bcl-2* gene expression was detected in pre-B murine transformed cells, it was undetectable in plasmacytoma cell lines.[10] In an another study, four B-cell populations were identified in normal adult mice characterized by a several fold increase in *bcl-2* mRNA levels, with a positive correlation between normal B-cell longevity and *bcl-2* mRNA levels.[11] Although inconsistencies in results may arise from different ways of classifying lymphocytes, further studies are needed to clarify the trend in *bcl-2* mRNA levels in B-cell ontogeny.

Since human acute B-cell leukemias may derive from B-lymphocytes at different maturation stages, we thought it would be of interest to quantitate *bcl-2* expression in both follicular lymphoma cell lines carrying or not carrying the 14;18 translocation and in primary leukemic cell samples, with the aim of establishing whether the levels of expression could contribute to disease identification for possible clinical screening. B-cell lines with t(14;18)-associated *bcl-2*/IgH fusion and the pre-B untranslocated Raji cell line showed a very high expression of the *bcl-2* gene. Similarly, pre-B untranslocated primary leukemia cells revealed a dramatically higher *bcl-2* mRNA level with respect to the more immature untranslocated early-B primary leukemia cells. Mature B-cells are produced in large numbers and some of them are endowed with a very long life span. Homeostasis in a lymphocyte population is maintained by multiple factors. Our data, which indicate high levels of *bcl-2* mRNA in relatively more mature neoplastic B-cells, suggest that *bcl-2* expression may actually take part in the regulation of mature lymphocyte survival. What importance such an observation has in leukemogenesis remains to be determined through more extensive studies. Indeed acute lymphatic leukemias arising from phenotypically mature B-cells have a poor prognosis. The higher *bcl-2* expression paralleling B-cell maturation that we observed in agreement with Haury and coworkers,[11] if confirmed in neoplastic mature B-cells, seems to indicate that cell resistance to apoptosis could be an important factor in determining the aggressivity of the disease.

RT-PCR has been used for detecting a variety of translocation-derived chimeric transcripts in minimal residual lymphoma and leukemia disease.[43-48] We employed our RT-PCR protocol to detect cells expressing minimal *bcl-2*/IgH. Several methods, including PCR, have been optimized in order to quantify residual circulating B-cells carrying t(14;18) in follicular lymphoma patients;[35-39] however, the clinical validity of residual circulating B-cells with the t(14;18) translocation as a putative tumor marker in patients in complete clinical remission is far from being conclusively established. Some evidence supports the idea that the presence of residual t(14;18) B-cells with the *bcl-2* translocation is *per se* related to an increased risk of lymphoma relapse.[39] However, other evidence indicates that circulating t(14;18) B-cells are not clearly related to lymphoma relapse,[40] while the expression pattern of hybrid *bcl-2*/IgH mRNA seems to be more predictive.[18] The addition of a nested PCR step made our RT-PCR protocol a highly sensitive method for detecting *bcl-2*/IgH expression of minimal residual lymphoma B-cells, compared to other PCR or RT-PCR proto-

cols described so far.[18,37,38,41] With our technique, when the same *bcl-2*/IgH fusion segment of the t(14;18) DOHH-2 cells was targeted and the identical amplification protocols are used, the RT-PCR sensitivity proced to be on the order of three logs higher than that of PCR alone. The RT-PCR-based method we devised for the evaluation of *bcl-2* expression, since it is capable of detecting as little as one t(14;18) *bcl-2*/IgH mRNA-expressing cell diluted in 10^7 untranslocated Raji cells, may have clinical application as a tool in evaluating the risk of lymphoma relapse.

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