Alterations of the p53 Tumor Suppressor Gene in Diffuse Large Cell Lymphomas With Translocations of the c-MYC and BCL-2 Proto-oncogenes

By M. Michèle Farrugia, Li-Juan Duan, Marciano D. Reis, Bo Y. Ngan, and Neil L. Berinstein

Diffuse large cell lymphomas are aggressive tumors of B-cell origin. In some cases they arise from low-grade follicular lymphomas carrying the t(14;18) translocation, an event that leads to the overexpression of the BCL-2 gene product. More frequently, however, they lack the t(14;18) translocation. Rearrangements of the c-MYC proto-oncogene and mutations of the p53 tumor suppressor gene have also been documented in these lymphomas. This study examines the extent to which alterations in the BCL-2, c-MYC, and p53 genes co-exist within individual lymphomas. Eight diffuse large cell lymphoma cell lines and 11 diffuse large cell lymphoma tumors were assessed for genetic alterations in these three genes. Our results indicate that there is a heterogeneity in the oncogene/suppressor gene profile among diffuse large cell lymphomas. Two cell lines and one tumor carried alterations in all three genes, one cell line carried alterations of c-MYC and p53, and one primary tumor and one cell line carried p53 mutations and the t(14;18). Single alterations of BCL-2 and p53 were also observed. Another cell line had no alterations in any of these genes. The heterogeneity indicates that varied mechanisms may be involved in the generation of diffuse large cell lymphomas.

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MATERIALS AND METHODS

Cell lines. The human diffuse large cell lymphoma cell lines used in these studies included: Karpas 422, obtained from Dr A. Karpas (Cambridge, UK);^4 OCI Ly1, Ly2, Ly3, Ly7, Ly8 C3, and Ly 17 obtained from Dr H. Messner (Toronto, Ontario, Canada);^5 and SU-DHL4. A Burkitt’s cell line, Raji, obtained from the American Type Culture Collection (Rockville, MD), was assessed as a t(14;18)-negative control. A lymphoblastoid cell line, 923, obtained from Dr L. Rubin (Toronto, Ontario, Canada), was used as a normal control on Southern and Northern blots. Cells were cultured in RPMI 1640 with 10% fetal calf serum (HyClone, Logan, UT), 2 mmol/L L-glutamine, 100 U/mL penicillin, 100 μg/mL streptomycin at 37°C, and 5% CO2. The rescue fusion cell line RF63c2 is a heterohybridoma made by fusing the K6H6 fusion partner with fresh lymphoma cells derived from the same patient from whom the OCI Ly8 C3 cell line was derived. 27

Fresh tumor samples. Fresh lymph node biopsies were divided into at least two parts. One part was examined by a pathologist for diagnostic purposes. The remaining part was used to prepare genomic DNA. Single-cell suspensions were obtained by passing the lymph node through a steel wire mesh. High molecular weight DNA was extracted using a GENEPURE nucleic acid purification system.

RNA isolation from cell lines. Cells were lysed in 10 mmol/L EDTA pH 8, 0.5 mol/L NaOAc, 0.5% sodium dodecyl sulfate, and 50 μg/mL Proteinase K (Pharmacia, Uppsala, Sweden), extracted, precipitated, resuspended in 10 mmol/L Tris, 1 mmol/L EDTA with 30 μg DNAase-free RNase for 2 hours, re-extracted, precipitated, and resuspended in sterile water as per standard techniques.

DNA isolation from fresh tumors. Fresh lymph node biopsies were divided into at least two parts. One part was examined by a pathologist for diagnostic purposes. The remaining part was used to prepare genomic DNA. Single-cell suspensions were obtained by passing the lymph node through a steel wire mesh. High molecular weight DNA was extracted using a GENEPURE nucleic acid purification system.

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p53, BCL-2, and c-MYC ALTERATIONS IN NHL

**Primer sequences.** JH: 5'-CACAAAGCTTACCTGAGAGGAGC- GCTGACCC: MBR1: 5'-CGGGATCTCTTTGACCCCTTAGGA- GTTGCTT: MBR2: 5'-CGGGATCTCTTTGACCCCTTAGGACC: MBR3: 5'-CGGGATCTCTTTGACCCCTTAGGACC: MBR4: 5'-CGGGATCTCTTTGACCCCTTAGGACC. 

The JH and P2 primers included a HindIII site and three protecting bases at their 3' ends. The MBR1, MBR2, MBR3, and P1 primers included an EcoRI site and three protecting bases at their 5' ends.

**RESULTS**

To define the profile of oncogene/tumor suppressor gene abnormalities in diffuse large cell lymphomas, we assessed the status of BCL-2, c-MYC, and p53 in eight diffuse large cell lymphoma cell lines. Subsequently, we surveyed primary lymphoma biopsies for similar alterations.

**Assessment of the BCL-2 and c-MYC oncogenes in large cell lymphoma cell lines.** Eight diffuse large cell lymphoma cell lines were assayed for the t(14;18) using a PCR assay. A JH consensus primer and primers flanking the major breakpoint region (MBR) or the minor cluster region (MCR) were used to amplify over the translocation breakpoint DNA. As shown in Fig 1, four of the eight cell lines were found to be t(14;18) positive after Southern blotting and probing with a BCL-2 cDNA probe. As expected, a Burkitt's lymphoma cell line, Raji, did not carry the t(14;18).

**Fig. 2.** Genomic Southern blot of diffuse large cell lymphoma cell lines probed with c-MYC. Peripheral blood lymphocytes (PBL) and a lymphoblastoid cell line, 923, were used as a negative control. The reactions were then extracted, precipitated, and resuspended in water. Half the product was ligated into the PBS+ phagemid (Stratagene, La Jolla, CA) with T4 DNA ligase (Bethesda Research Laboratories, Gaithersburg, MD). The plasmid was electroporated into XL1-Blue *Escherichia coli* (American Type Culture Collection) and clear colonies were screened by Southern blotting. Double-stranded templates were sequenced by the Sanger dideoxy method using the T7 sequencing kit (Pharmacia) and the universal (Stratagene) and M13 reverse sequencing (Amersham, Arlington Heights, IL) primers. Sequencing gels were exposed to Kodak X-OMAT film overnight and developed.

**Fig. 3.** Northern blot analysis of p53 mRNA expression. PolyA RNA was electrophoresed, blotted, and probed with an Actin probe as an internal control. Subsequently, membranes were re-probed with a p53 probe. Raji is a Burkitt's cell line, 923 is a lymphoblastoid cell line, C3 is Ly8 C3.
amplifying with the two 5′ primers (MBR2 and MBR3) but not the 3′ primer (MBR1).

We assessed the c-MYC gene for rearrangement in the eight diffuse large cell lymphoma cell lines by genomic Southern blotting. As assessed by three different restriction digests followed by probing with a c-MYC exon III probe, three of the eight diffuse large cell lymphoma cell lines carried c-MYC rearrangements (Fig 2). The Burkitt’s lymphoma cell line, Raji, as expected,33 also carried a c-MYC rearrangement.

Assessment for gross alterations of the p53 tumor suppressor gene in large cell lymphoma cell lines. We studied the p53 gene in the diffuse large cell lymphoma cell lines at three levels: gross DNA structure, mRNA expression, and sequence analysis. Gross DNA structure was assessed by Southern blotting followed by probing with a p53 cDNA probe. Restriction digests with three different enzymes (EcoRI, BamHI, and HindIII) showed no evidence of gross structural abnormalities of the p53 gene in any cell line studied (data not shown). Northern blotting of polyA mRNA and probing with a p53 cDNA probe showed that the Karpas 422 cell line expressed very little p53 mRNA (Fig 3). PCR amplification of the p53 gene from Karpas 422 cDNA indicated that the cell line did not totally lack p53 mRNA (data not shown). All other cell lines expressed relatively high levels of p53 mRNA.

Assessment of the sensitivity of direct sequencing for detecting p53 mutations. Determination of p53 sequence was accomplished by direct sequencing of genomic DNA. Advantages of direct sequencing include the elimination of PCR-related artifacts and the ability to assess all of the alleles within a cell. Before the evaluation of cell lines and patient samples for p53 mutations, we determined the sensitivity of our assay for two purposes: (1) to indicate whether or not we would detect the presence or absence of the wild-type allele at a point mutated site, and (2) to indicate whether we would be able to detect the presence of p53 mutations in mixed-cell populations containing malignant and nonmalignant cells, such as lymph node biopsies. To simulate both of these situations, we used a DNA preparation that contained p53 genes that differed at one nucleotide: genomic DNA containing wild-type p53 was mixed with genomic DNA containing point-mutated p53 in varying ratios. As can be seen in Fig 4A, even when the wild-type DNA was present in a ratio of one part wild-type to four parts mutant DNA, the wild-type sequence could be detected on
Assessment of p53 mutations in diffuse large cell lymphoma cell lines. Of the eight cell lines studied, five were found to have point mutations in the p53 gene (Table 1). All point mutations are predicted to result in amino acid changes. In four cases, only the mutated band was observed on the sequencing autoradiogram, implying that there were few normal cells in the biopsy, and that the wild-type allele in the malignant cells had been deleted. The rescue fusion was established from the same patient as the Ly8 C3 cell line and both were found to have the identical mutation. This is evidence that this mutation arose in vivo. Only the mutated band was observed in two diffuse large cell lymphoma cell lines. The presence or absence of the t(14;18) and c-MYC translocations were determined as previously described and the results are shown in Table 2. Limited amounts of biopsy DNA precluded Southern blot analysis of all samples for c-MYC rearrangements. Direct sequencing of the p53 gene was performed as for the cell lines. No point mutations were found in 10 follicular lymphoma samples sequenced. Point mutations were observed in two diffuse large cell lymphoma samples: in 1 of 10 primary biopsies from diffuse large cell lymphomas and in one sample assessed from tumor cells immortalized in a rescue fusion. In patient 1, the mutation was observed in exon 5 (Fig 4B). Only the mutated allele was identifiable, implying that there were few normal cells in the biopsy, and that the wild-type allele in the malignant cells had been deleted. The rescue fusion was established from the same patient as the Ly8 C3 cell line and both were found to have the identical mutation. This is evidence that this mutation arose in vivo. Only the mutated band was observed, indicating that the wild-type allele had been deleted. Both mutations were predicted to result in amino acid changes.

DISCUSSION

In this report, we show that the oncogene/tumor suppressor gene profile of diffuse large cell lymphomas is heterogeneous (Tables 1 and 2). We have documented examples of diffuse large cell lymphoma cell lines with alterations in both the BCL-2 and c-MYC proto-oncogenes, an observation consistent with the apparent functional synergy between BCL-2 and c-MYC. In addition, we show that mutations of the p53 tumor suppressor gene may accompany translocations of the BCL-2 and/or c-MYC genes. Spec-
Table 2. Summary of Clinical Status and Genetic Alterations in Primary Lymphoma Biopsies

<table>
<thead>
<tr>
<th>Patient</th>
<th>Histology</th>
<th>Stage</th>
<th>t(14,18)</th>
<th>c-MYC Rearrangements</th>
<th>p53 Mutations</th>
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<tbody>
<tr>
<td>6</td>
<td>DLC</td>
<td>IV A</td>
<td>+</td>
<td>wt/wt</td>
<td>aa266*</td>
</tr>
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<td>wt/wt</td>
<td>wt</td>
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Abbreviations: NA, not assessed; wt/wt, both alleles wildtype; wt/r, one allele rearranged.

* The p53 mutation observed in patient 1 at aa266 predicted an amino acid change of glycine to glutamine. Only one p53 allele was detected.

† The rescue fusion (RF) was established from the same patient as the Ly7 cell line as described in Materials and Methods. The amino acid change at position 282 predicts a change from arginine to proline. Only one p53 allele was detected.

specifically, we identified 2 cell lines in which BCL-2, c-MYC, and p53 were all altered (Ly8 C3, Karpas 422), 1 cell line in which c-MYC and p53 but not BCL-2 was altered (Ly7), and 1 cell line in which BCL-2 and p53 but not c-MYC were altered (Ly1). Two cell lines were found to have only a mutation of the p53 gene (Ly2, Ly17). Genetic abnormalities were also found in vivo. Of 11 diffuse large cell lymphomas assessed by analysis of tumor biopsy DNA or immortalized rescue fusion DNA, 1 was found to have a BCL-2 translocation and a p53 mutation (patient 1), and another was found to have BCL-2 and c-MYC translocations as well as a p53 mutation (RF).

The most frequent genetic abnormality in the eight diffuse large cell lymphoma cell lines was alteration of the p53 tumor suppressor gene, observed in 75% of our cell lines. Four of eight cell lines carried point mutations and allelic loss, another carried a point mutation on each allele, and one cell line had reduced p53 mRNA expression. The frequency of point mutations in tumor samples was lower; mutations were not observed in follicular lymphomas but were observed in 18% of diffuse large cell lymphomas. This discrepancy may be attributed to a number of factors. Firstly, we did not analyze p53 mRNA from tumor samples as we did from the cell lines. Secondly, the p53 mutations may have arisen in vitro. p53 mutations have been postulated to arise in cell lines; Farrell et al. reported that although 6 of 12 Burkitt’s lymphoma cell lines had mutations in conserved region 4, none of 6 primary Burkitt’s lymphoma biopsies sequenced in this region had mutations. Thirdly, the diffuse large cell lymphoma cell lines may represent a subgroup of very malignant tumors selected for by in vitro growth. Tweeddale et al. in their description of the OCL Ly cell lines, reported that only tumors from patients with advanced disease gave rise to cell lines, and that the ability of a tumor to give rise to a cell line correlated with poor survival of the patient. Fourthly, it is possible that our screening protocol was not sensitive enough to detect p53 mutations from lymph node biopsies containing mixtures of lymphoma cells and reactive host cells.

To eliminate the likelihood that our protocol would fail to detect p53 mutations from tumor biopsies, we chose to use direct PCR sequencing. This technique is more informative and sensitive than other protocols such as single-stranded conformation polymorphism (SSCP) analysis. If a mutation is detected by SSCP analysis, then the sample must subsequently be sequenced to both confirm and identify the mutation. Furthermore, SSCP analysis may not detect all p53 mutations. A potential problem of direct PCR sequencing of biopsy DNA was that contaminating host reactive cells in the lymph node biopsy might have obscured p53 mutations in the malignant cells. We minimized this problem in two ways. Firstly, lymph nodes used in this study were composed of at least 50% malignant cells. Previous investigators using SSCP and/or direct sequencing to screen for p53 mutations have successfully used cell samples (including lymph nodes) containing similar proportions of normal to neoplastic cells, or without enriching for tumor cells. Secondly, we showed that our sequencing protocol was sensitive enough to detect a mutant allele even if it was present in only one of five cells (Fig 4A), implying that our assay was at least twice as sensitive as it needed to be.

Further supporting the validity of our protocol and observations, Ichikawa et al. observed similar frequencies of p53 mutations in follicular and diffuse large cell lymphoma tumor samples. This group found p53 mutations in 1 of 11 primary follicular lymphomas, and in 8 of 34 intermediate-grade lymphomas, including diffuse large cell lymphomas, frequencies similar to those reported here for tumor samples: 0 of 10 and 2 of 11 for follicular and diffuse large cell lymphomas, respectively. In contrast, a previous report found no p53 mutations in 20 follicular and 14 diffuse large cell lymphomas. Methodology may account for this difference as these samples were assessed by SSCP, which may be less sensitive than sequencing.

Two types of p53 abnormalities were observed: reduced mRNA expression and point mutations. The Karpas 422 cell line had reduced p53 mRNA expression, although no gross rearrangements of the gene could be detected by Southern blotting. Similarly reduced levels of p53 mRNA have been observed in two lung cancer cell lines, but in these lines the gene was either homozygously deleted or rearranged. Similar to Karpas 422, the K562 chronic myelog-
enous leukemia line has reduced p53 mRNA but not genomic rearrangements.\textsuperscript{41} Several possible explanations exist; firstly, there may be mutations in the promoter or enhancer regions of the gene, decreasing its expression; secondly, a protein that transcriptionally activates or represses p53 may be altered so that p53 can no longer be expressed at normal levels; thirdly, the transcript may have been destabilized. Experiments to differentiate these possibilities may provide insight into the control of p53 mRNA expression.

The p53 mutations we observed are all predicted to result in amino acid changes and were always accompanied by loss of the wild-type allele, either by deletion or by a second mutation. Most tumors in which p53 mutations arise display a similar loss of heterozygosity.\textsuperscript{30,32,40,42,43} Two possible effects of the observed p53 mutations are (1) complete loss of p53 growth suppressor function and/or (2) gain of transforming activity. Either event may have contributed to cellular transformation and the development of the diffuse large cell lymphoma.

The low incidence of p53 mutations in follicular lymphomas suggests that there may be an association between p53 alterations and aggressive lymphomas. This hypothesis is supported by the higher incidence of p53 mutations in diffuse large cell lymphoma tumors, and the even higher incidence in diffuse large cell lymphoma cell lines, which may represent particularly aggressive lymphomas. In some cases, p53 mutations may be associated with the progression of a t(14;18)-positive follicular lymphoma to a t(14;18)-positive diffuse large cell lymphoma. This hypothesis is supported, but not proven, by the absence of p53 mutations in t(14;18)-positive follicular lymphomas and the presence of the t(14;18) and p53 mutations in diffuse large cell lymphoma cell lines (Ly 1, Ly8 C3, and Karpas 422) and two diffuse large cell lymphoma tumors (one of which gave rise to the Ly8 C3 cell line).

Our results show that multiple genetic events are involved in the development and progression of diffuse large cell lymphomas. In some cases, the BCL-2, c-MYC, and/or p53 genes may be altered, but it is likely that other proto-oncogenes and/or tumor suppressor genes are also involved.

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