Alterations of p53 and c-myc in the Clonal Evolution of Malignant Lymphoma

By Hong Chang, Sam Benchimol, Mark D. Minden, and Hans A. Messner

We derived the lymphoma cell lines OCI-Ly 13.1 and OCI-Ly 13.2 from a patient with non-Hodgkin’s lymphoma at the time of presentation and during chemotherapy-resistant relapse. These lines were of T-cell phenotype and contained the identical T-cell receptor β-chain rearrangement, indicating that both lines were members of the same malignant clone. The lines differed in their growth characteristics; OCI-Ly 13.1 grew slowly and required growth factors for colony formation, whereas OCI-Ly 13.2 grew rapidly and formed colonies without addition of growth factors. To test whether or not these biologic differences were associated with specific genetic changes, we evaluated the status of the c-myc and p53 genes of both cell lines. The p53 and c-myc genes of OCI-Ly 13.1 were in germline configuration and produced normal-sized transcripts. The p53 protein expressed in OCI-Ly 13.1 was recognized by the anti-p53 monoclonal antibody, PAb240, indicating a conformation typical of p53 proteins expressed by p53 alleles containing a missense mutation. However, sequencing studies of the entire p53 coding region did not reveal any point mutations. In contrast, the cell line OCI-Ly 13.2 contained structural abnormalities of both the c-myc and p53 genes. In addition, one of the p53 alleles was lost as determined by a cDNA probe for the p53 gene (17p 13.1) and the YN22.1 probe (17p 13.3). These changes resulted in the absence of p53 protein and mRNA in OCI-Ly 13.2 as detected by immunoprecipitation and Northern blot analysis, respectively. They may be a reflection of disease progression and may be associated with the altered behavior of the malignant cell population within the patient and in vitro.

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Table 1. Growth Characteristics of OCI-Ly 13.1 and OCI-Ly 13.2

<table>
<thead>
<tr>
<th>Parameters</th>
<th>OCI-Ly 13.1</th>
<th>OCI-Ly 13.2</th>
</tr>
</thead>
<tbody>
<tr>
<td>Colony formation without added growth factors</td>
<td>–</td>
<td>+</td>
</tr>
<tr>
<td>Colony formation in the presence of exogenous factors*</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>Feeder-cell activity</td>
<td>–</td>
<td>+</td>
</tr>
<tr>
<td>Growth-factor production</td>
<td>–</td>
<td>+</td>
</tr>
<tr>
<td>Doubling time in liquid-suspension culture</td>
<td>72 hrs</td>
<td>48 hrs</td>
</tr>
</tbody>
</table>

* Medium conditioned by phytohemagglutinin-stimulated leukocytes (PHA-LCM) or conditioned medium from OCI-Ly 13.2.

Fig 1. Lysate of OCI-Ly 13.1 reacted positively with both PAB1801 and PAB240. No immunoprecipitates for p53 were identified for OCI-Ly 13.2. The control cell line OCI-20 showed a positive reaction with PAB1801 but not with PAB240. None of the lysates reacted with the control antibody PAB419.
Fig 2. Total RNA from OCI-Ly 13.1 and OCI-Ly 13.2 was used to examine expression of p53 and c-myc. RNA from normal, human T cells stimulated with PHA was used as a positive control. The top panel indicates hybridization with the p53 probe. The same membrane was reprobed for c-myc (bottom panel) after stripping off the p53 probe.

5'-CCCTTGCCGTCCCAAGCAATGGA-3'). PCR was performed for 30 cycles using the same temperature changes as described earlier. The reaction mixture was treated with phenol/CHCl₃ and passed through a spin column of sephadex G-50 (Pharmacia, Uppsala, Sweden). The sample was precipitated with ethanol and redissolved in distilled water for direct sequencing.

Sequencing was performed by the dideoxynucleotide-chain termination method using specific p53 primers and DNA polymerase (Sequenase 2.0, US Biochemicals, Cleveland, OH) in the presence of 10% formamide. The sequencing reactions were fractionated on 6% polyacrylamide gels in TBE and 8 mmol/L urea. The primers used for sequencing PCR products of p53 cDNA were: 5'Sx1, 5'Sx4, 5Ax4 (antisense, exon 4), 5'-GGGTAGGAGCTGCTGGTG-GCA-3'), 5'Sx5(antisense, exon 5, 5'-CTCCCCTGCCCTCA-ACAAG-3'), 3Ax9(antisense, exon 9, 5'-GGGTGAATATTCTCATCCTAGTGGG-3'), and 3Sx3. The 5Sx4 primer was used to sequence PCR products derived from p53-genomic DNA.

RESULTS

TCR rearrangement. DNA prepared from OCI-Ly 13.1 and OCI-Ly 13.2 was examined by Southern blot hybridization using a probe for the TCR-β gene. Identical rearrangement patterns of the TCR-β gene were observed for both cell lines using either HindIII or BamHI restriction enzymes (data not shown). These results indicate that the two lines are members of the same malignant clone. To identify possible genetic changes responsible for the phenotypic differences between the cell lines, we examined the structure and expression of the c-myc and p53 genes, two genes that are associated with disease progression in malignant lymphomas.14,15,22

Protein analysis. To determine the status of the p53 gene expressed in both cell lines, p53 protein was immunoprecipitated using two different antibodies. PAbl801 recognizes all forms of human p53 protein, whereas PAb240 recognizes preferentially mutant p53 protein. Immunoprecipitation of p53 protein from 35S-methionine labeled OCI-Ly 13.1 and OCI-Ly 13.2 cell extracts showed that p53 was only present in OCI-Ly 13.1 but not in OCI-Ly 13.2 cells. Fig 1 shows the precipitation of p53 protein by PAbl801 and PAb240 in OCI-Ly 13.1 cell extracts; no p53 protein was precipitated by either antibody from lysates of OCI-Ly 13.2 cells. The B-cell lymphoma line denoted as OCI-Ly 20 was used as a control. It is known to express a p53 protein that is recognized by PAbl801 but not by PAb240. The reactivity of p53 protein with PAb240 expressed in OCI-Ly 13.1 suggests the presence of an aberrant conformation that may result from an underlying missense mutation.

Genetic analysis. To determine whether the absence of p53 protein in OCI-Ly 13.2 was caused by transcriptional regulation, RNA was isolated and analyzed by Northern blot hybridization. As shown in Fig 2, a normal sized 2.8-kb message was present in OCI-Ly 13.1, but not in OCI-Ly 13.2. To ensure the quality of mRNA in both lines, the same filter was washed and reprobed with the c-myc gene probe. Both lines were found to express c-myc mRNA (Fig 2). The amount of RNA in each lane was equal as judged by ethidium-bromide staining (data not shown).

<table>
<thead>
<tr>
<th>Restriction Enzymes</th>
<th>DNA Fragments (kb)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>OCI-Ly 13.1</td>
</tr>
<tr>
<td>EcoRl</td>
<td>5'</td>
</tr>
<tr>
<td></td>
<td>3'</td>
</tr>
<tr>
<td>BglII</td>
<td>5'</td>
</tr>
<tr>
<td></td>
<td>3'</td>
</tr>
<tr>
<td>BamHI</td>
<td>5'</td>
</tr>
<tr>
<td></td>
<td>3'</td>
</tr>
<tr>
<td>HindIII</td>
<td>5'</td>
</tr>
<tr>
<td></td>
<td>3'</td>
</tr>
</tbody>
</table>
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Fig 3. The p53 gene status of OCI-Ly 13.1 and OCI-Ly 13.2 was examined by Southern blot analysis using the restriction enzymes EcoRI and Bgl II (A), HindIII and BamHI (B). DNA of normal human skin fibroblasts was used as germline control. The p53 cDNA probe was used for hybridization. The size of the respective germline bands is indicated by line markers. Rearranged bands are identified by arrows.

Fig 4. (A) Depicts the normal p53 gene map with different restriction enzyme sites (E = EcoRI, B = BamHI, Bg = Bgl II, H = HindIII). (B) shows the predicted 4-kb insertion into intron 1 of the p53 gene based upon the results of Southern blot analysis. This is only a hypothetical map and will need to be confirmed by cloning and sequencing. The cDNA probe for the coding region of p53 is denoted as (a). The genomic DNA probe for exon 1 is shown as (b).
germline bands of 13 and 7.8 kb; the 13-kb fragment is often not seen, probably because of the small amount of exon present in the probe. DNA from OCI-Ly 13.2 digested with BamHI contained a germline band of 7.8-kb and a new band of 4 kb. Digestion of OCI-Ly 13.1 and OCI-Ly 13.2 DNA with HindIII and probed with the p53 cDNA showed bands of 7 and 2.5 kb similar to the germline pattern. To assess the status of exon 1, DNA was digested with EcoRI and BamHI and probed with a genomic fragment containing exon 1; germline sized bands of 3.5 and 13 kb, respectively, were observed (Fig 5).

The results of the Southern blot analysis are summarized in Table 2. Digestion of DNA with HindIII gave a germline pattern indicating that the HindIII site just 5' of exon 2 and the two HindIII sites 3' of exon 2 were intact. However, digestion of DNA with EcoRI, BglII and BamHI indicated that the gene had undergone a rearrangement. Based on the finding that the 5'-EcoRI and BglII fragments were 4 kb larger than expected, whereas the 3' fragments were of normal size, it is likely that a DNA rearrangement had occurred between the BglII site in intron 1 and the HindIII site just 5' of exon 2. The fact that both the new EcoRI and BglII fragments were 4 kb larger than expected and that the first exon was intact suggests that the rearrangement is caused by an insertion rather than a translocation or deletion (Fig 4B). Verification of this will require cloning of the p53 gene from OCI-Ly 13.2.

The above studies indicate that the p53 gene is altered in OCI-Ly 13.2. In addition, the results suggest that there has been loss of one copy of the p53 gene. In a cell carrying two copies of the p53 gene in which one of the copies had been rearranged, one would expect to see a germline pattern and evidence of rearrangement. In OCI-Ly 13.2 a normal p53 germline pattern was not seen. Allelic loss was confirmed by use of the probe pYNZ22.1 which detects a highly polymorphic region (17p 13.3) that is closely linked to the p53 gene on chromosome 17p. DNA of OCI-Ly 13.1 digested with BamHI resulted in 2 bands. In contrast, only the smaller band was present in BamHI-digested OCI-Ly 13.2 DNA (Fig 6).

**Southern blot analysis of myc.** When DNA from OCI-Ly 13.1 was digested with EcoRI and probed with c-myc, a 13-kb germline band was seen. In contrast, OCI-Ly 13.2 DNA contained two fragments of 13 and 15 kb that hybridized with the c-myc probe (Fig 7) indicating rearrangement of one of the c-myc alleles.

**Sequencing analysis.** The presence of PAb240 + p53 protein in OCI-Ly 13.1 cells suggested a putative mutation in the p53 gene. Therefore we sequenced the entire coding region of p53 cDNA from OCI-Ly 13.1. One nucleotide change (CCC-CGC) was identified at codon 72 that converts proline to arginine (Fig 8A). This has been shown to be a naturally occurring polymorphism in the human p53 coding sequence. Although this change alters the electrophoretic mobility of p53 protein, it is not believed to alter the biologic activity or the immunologic reactivity of wild-type p53 protein. No other differences were found in the p53 cDNA from OCI-Ly 13.1. As expected, the codon for arginine at position 72 was also found in the genomic DNA of...
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Fig 6. DNA of OCI-Ly 13.1 and OCI-Ly 13.2 was evaluated by Southern blot analysis using the pYNZ22.1 probe.

OCI-Ly 13.2 (Fig 8C). No primary tumor sample DNA was available to determine whether the changes present in OCI-Ly 13.2 were in the presentation or relapse samples. It is possible that the changes in OCI-Ly 13.2 arose during the establishment of the cell line, however, our experience with other cell lines indicates that this is unlikely.

DISCUSSION

The derivation of two cell lines from a lymphoma patient at diagnosis and during relapse provided the unique opportunity to examine genetic events that may be associated with progression of disease as shown by the clinical course of the patient. We focused on investigations of the p53 and c-MYC gene because a series of previous studies suggested their involvement in disease progression. The results of our studies are summarized in Table 3. Immunoprecipitation studies of OCI-Ly 13.1-derived lysates showed the presence of a PAb240-reactive p53 protein. This form of reactivity has been associated with mutant forms of p53. Sequence studies of the entire translated region of p53 only yielded a previously described polymorphism in codon 72. A mutation that could account for reactivity with PAb240 was not observed. The view that PAb240 reacts only with mutant, but not wild-type p53 protein was recently challenged. Rivas et al described reactivity of CD34+ normal BM cells with PAb240. Zhang et al reported several cases of acute myeloblastic leukemia that expressed PAb240 reactive p53. A sequence analysis of exons 5 through 9, the generally acknowledged hot spot for point mutations, did not contain any abnormalities. Similarly, examination of the remaining coding sequence by single-strand conformation polymorphism analysis did not show mutations. They also showed that normal activated lymphocytes contained PAb240-reactive protein. These findings and the results of our study indicate that wild-type p53 protein can adopt a conformation that is usually associated with mutant p53 proteins. Recently Halazonetis et al reached a similar conclusion using a different set of antibodies against mouse-p53 protein. In their case, wild-type p53 adopted a “mutant” conformation when complexed with DNA. The functional

Table 3. p53 and c-MYC Related Changes of OCI-Ly 13.1 and OCI-Ly 13.2

<table>
<thead>
<tr>
<th>Parameters</th>
<th>OCI-Ly 13.1</th>
<th>OCI-Ly 13.2</th>
</tr>
</thead>
<tbody>
<tr>
<td>p53 Protein</td>
<td></td>
<td></td>
</tr>
<tr>
<td>PAb1801 reactivity</td>
<td>+</td>
<td>—</td>
</tr>
<tr>
<td>PAb240 reactivity</td>
<td>+</td>
<td>—</td>
</tr>
<tr>
<td>p53 mRNA</td>
<td>+</td>
<td>—</td>
</tr>
<tr>
<td>p53 Gene structure</td>
<td>Germline</td>
<td>Rearrangement and loss of one p53 allele</td>
</tr>
<tr>
<td>pYNZ22.1</td>
<td>2 alleles</td>
<td>1 allele</td>
</tr>
<tr>
<td>c-MYC Gene structure</td>
<td>Germline</td>
<td>Rearranged</td>
</tr>
<tr>
<td>c-MYC mRNA</td>
<td>+</td>
<td>+</td>
</tr>
</tbody>
</table>

Fig 7. Southern blot analysis of the c-MYC gene status of OCI-Ly 13.1 and OCI-Ly 13.2. The rearranged band in OCI-Ly 13.2 is indicated by the arrow.
significance of PAb240-reactive p53 protein derived from a wild-type gene is not known. The cell line OCI-Ly 13.1 may facilitate studies to determine why in some cells, wild-type p53 adopts a PAb240-reactive conformation.

In contrast, OCI-Ly 13.2 neither contained detectable p53 protein by immunoprecipitation nor p53 mRNA by Northern blot analysis. The loss of p53 expression in OCI-Ly 13.2 was the result of two independent events. The changes included rearrangement of one p53 allele and loss of the second allele. The loss of one p53 allele at 17p13.1 was associated with a loss of a highly polymorphic locus at 17p13.3 as identified by the probe pYNZ22.1. Loss of one allele and rearrangement of p53 in the remaining allele have been observed in a number of tumors including Friend-virus induced murine erythroleukemia, osteosarcomas, and CML in blast crisis. Miller et al. found that approximately 20% of tumors from patients with osteosarcoma and 50% of osteosarcoma cell lines had a rearrangement of the p53 gene that had also lost the normal p53 allele. Similar to OCI-Ly 13.2, these osteosarcomas did not express p53 mRNA or protein. The present case further documents the association between alterations of p53 and progression of the malignant state.

In addition, we were able to observe changes in c-myc. OCI-Ly 13.1 showed c-myc in germline configuration. In contrast, OCI-Ly 13.2 presented with a rearrangement of one allele of c-myc. Although the significance of this rearrangement is not clear. We noted an association between mutant forms of p53 and c-myc rearrangement. A similar observation has also been made in Burkitt’s lymphoma cells where the majority of studied cases have both a rearranged c-myc and a mutated or deleted-p53 gene. The significance of this association is not clear. In addition to the alterations of myc and p53 other genetic changes may have occurred that differentiate OCI-Ly 13.1 from OCI-Ly 13.2. The mechanism resulting in the observed changes is unknown but may be related to an intrinsic genetic instability of the cells or as a result of the chemotherapy given to the patient at presentation. Both cell lines will be of value to investigate the role of myc: p53 or other genes that have undergone genetic modifications in the progression of malignant clones.

ACKNOWLEDGMENT

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