The Expression of p53 Protein in Non-Hodgkin’s Lymphomas Is Not Always Dependent on p53 Gene Mutations

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p53 overexpression has been found to be a fairly common feature in high grade lymphomas in the majority of tumoral cells. The results vary from series to series, from 25% to 33% of cases. To assess whether immunohistochemical positivity for p53 correlated with the presence of structural gene abnormalities, DNA from 16 non-Hodgkin’s lymphomas with high and low p53 values was amplified and sequenced to determine the existence of point mutations in the highly conserved regions of the p53 gene. In the group of 8 cases containing high levels of protein, 3 cases showed missense point mutations at the codons mapping between exons 5 through 8. Of the 8 cases of tumors containing undetectable or low levels of p53 protein, 1 case presented a nonsense point mutation giving a stop codon. No missense mutations were detected in this group. The finding of p53 mutations in 4 of 16 cases confirms the presence of p53 gene mutations in high grade lymphomas distributed over different histologic groups. These include Burkitt’s lymphoma, together with centroblastic, immunoblastic, and large cell lymphoma of mucosa origin. Nevertheless, the absence of mutations in 5 of the 8 cases that overexpressed p53 suggests that the nuclear or cytoplasmic stabilization of p53 protein could also depend on other factors. The absence of detectable levels of p53 protein cannot discount the existence of p53 mutations, as is shown by a case of Burkitt’s lymphoma in which a nonsense mutation was detected. The impact of this range of p53 alterations on clinical course and treatment response of the patients deserves to be explored, in an attempt to differentiate the specific consequences of each one.

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P53 IS A NUCLEAR phosphoprotein encoded by a gene mapped at the short arm of chromosome 17 (17p13 band). It is involved in the negative regulation of cellular growth. Its precise function is not known, although there is evidence indicating that p53 is a potent transcriptional activator with a DNA binding region. Recent data suggests that p53 may be involved in DNA repair and apoptosis.13 Previous findings have shown that the wild type (WT) p53 protein has a suppressor activity, enabling it to inhibit in vitro transformation.4 Point mutations in p53 gene can cause the loss of its suppressor function, thus transforming it into a dominant oncogene.5,6

Deletions and mutations in the p53 tumor suppressor gene are the most common genetic alteration found in human tumors so far and have been described in a wide range of human tumors,7,8 including hematologic malignancies.9-12 The majority of p53 mutations occur as missense changes grouped in the highly conserved regions of the coding sequence (exon 5 through 8). These mutations have been associated with increased nuclear or cytoplasmic expression in human lung, breast, esophageal, ovarian, and colon carcinoma.13-16 Higher levels of p53 protein seem to result from a longer half-life of the mutated protein, arising from conformational changes that stabilize the protein for 4 to 8 hours.4,20 However, owing to its short half-life of 20 minutes, WT p53 protein does not accumulate to amounts that are detectable by immunohistochemical techniques.21

p53 overexpression has been found to be a fairly common feature in high grade lymphomas. Results in different series fluctuate between 25% and 33% of cases.22-26 Additionally, a pattern of focal expression in a small percentage of tumoral cells has also been identified in most high grade lymphomas and in a significant group of low grade lymphomas.22

Other investigators have described p53 inactivation independent of mutation. This is due to its association with DNA tumor viral proteins including the simian virus-40 large T antigen, papillomavirus E6 protein in cervical carcinomas,27,28 and adenovirus E1b protein. It has also been associated with cellular genes such as MDM2 protein in some human sarcomas.29 Inactivation by nuclear exclusion has also been proposed.16

To assess whether immunohistochemical positivity for p53 correlated with the presence of structural abnormalities of the gene, 16 cases of high grade non-Hodgkin’s lymphoma (NHL) in which immunohistochemistry analysis was performed (8 with diffuse positivity and 8 negative or only focal positivity for p53) were studied to determine the existence of point mutations within the most frequently altered regions of the gene, from exon 5 through 8.

MATERIALS AND METHODS

Tissue Samples

Tumor specimens from 16 high grade NHL cases and reactive tonsils from routine tonsillectomy specimens were obtained from the frozen tissue bank of the Pathology Department of the Virgen de la Salud Hospital. Representative samples of tumor were snap-frozen in liquid nitrogen and stored at or below –70°C. Tissue immediately adjacent to that stored was fixed for histopathologic diagnosis. Formalin-fixed sections of each specimen were stained with hematoxylin and examined histologically and then classified according to the Kiel classification.30

Immunohistochemical Analysis

Immunohistochemical analysis was performed in 16 cases on frozen sections or cytospins, using the monoclonal antibody...
Molecular Technique

Molecular analysis was performed in all 16 cases to locate p53 mutations within exon 5 through 8. Hematoxylin and eosin sections were reviewed to confirm the presence of neoplastic cells. The fraction of malignant cells, calculated using PanB(CD19) and PanT(CD3) antibodies, was at least 40%. DNA from tumoral tissue (frozen samples) was extracted following standard protocol by digestion with proteinase K, purification with phenol/chloroform, and precipitation by ethanol. Normal DNA from the same patients was obtained from bone marrow (BM)-smears without tumoral infiltration, according to protocol described by Fey et al.

Oligonucleotide primers. Primers used for polymerase chain reaction (PCR) were derived from the p53 genomic sequence described by Chumakov PM, available from the EMBL Data Library (Heidelberg, Germany) n X54156 (unpublished), excepting the primers p5-5' and p5-3' that were derived from published sequences. The sequence of the primers were as follows: for primer p5-5', 5' TCCTTCCTCTTCCTACAG 3'; for exon 6: p6-5': 5' ACAGGGCTGGTGCCCAAGGT 3', and p6-3': 5' AGTGACACACGCTGGAGCCG 3'; for exon 7: p7-5': 5' TCTTACTGTTGGCCTCTCAGTGT 3', and p7-3': 5' AGTGCC-CCTGACCTGAGGTCT 3'; for exon 8: p8-5': 5' GGGACAGGGTGAGACCTGATTTTCCTT 3', and p8-3': 5' ATCTGAAGGCCATTACGTGACACCCTTGG 3'.

PCR. Genomic DNA (500 ng) was amplified in vitro for exons 5 to 8, using the primers described above for 30 cycles in a 100 µL reaction containing 1.5 mmol/L C1Mg2+, 50 mmol/L KCl, 10 mmol/L Tris-Cl (pH 8.3), 0.2 mmol/L dNTPs, 50 pm of each primer, and 1.5 U of Taq polymerase. Reactions were overlaid with mineral oil. PCR conditions used were as follows: 94°C for 1 minute 30 seconds, 58°C (for exons 6, 7, and 8), 54°C (for exon 5) for 1 minute 30 seconds, and 72°C for 1 minute 30 seconds.

Nucleotide sequencing. Nucleotide sequences of amplified PCR products were determined by asymmetric PCR. One microtitre of DNA from the double strand PCR reaction was reamplified for 35 cycles using one primer at 1/50 dilution under the same conditions of PCR described above, except for the annealing temperature, which used 57°C for the exons 6, 7, and 8 and 53°C for exon 5. Single strand DNA products were purified by Sephadex G-50 and sequenced using the Sequenase kit (USB Dideoxi, Cleveland, OH).

RESULTS

Immunohistochemical Analysis

Immunohistochemical analysis was performed on 16 cases of NHL. p53 values that ranged from 0% to 52% were found. The highest p53 values were found in 3 cases of Burkitt’s lymphoma (Table 1). The percentage of p53 positive cells in cases lacking p53 mutations was variable (Figs 1A and B). Positive cells may correspond to proliferating cells, as has been described in mitogen-stimulated lymphocytes (Sanchez-Beato, submitted). In cases in which mutation was found, the percentage of positive cells was also variable. In case MT14, this percentage was higher (Fig 1C) than that in cases LL48 and LL47 (data not shown). p53 values were not related to the percentage of tumoral cells. To determine the possible relation between p53 positive cells and proliferation index, ki67 was assayed. The data shows that p53 values are lower or equal to ki67 values, because the values of ki67 (32%) and p53 (34%) in case MT14 are statistically equal (Table 1). Lack of staining was observed in case BK13 (Fig 1D), which has a nonsense mutation. Staining was nuclear in most cases, with the exception of BK10 in which cytoplasmic staining was also found.

Molecular Analysis

Molecular analysis was performed on all 16 cases with tumors. In the group of 8 cases containing high levels of protein detected by immunohistochemistry, 3 cases showed missense point mutations (Fig 2), whereas the other 5 contained exclusively WT sequence. Among the 8 cases with tumors containing undetectable or low levels of p53 protein, 1 case contained a nonsense point mutation giving a stop codon (Fig 2B). The remaining 7 cases with tumors contained WT sequences in the exons that were examined. The missense mutations were found at the codons mapping between exons 5 through 8. Two mutations were at codon 273 (Fig 2C), thus changing CGT to CAT and producing a conversion of Arg to His. One mutation was at codon 158 on exon 5, thus changing CGC to CAC, while also converting Arg to His (Fig 2A). The nonsense mutation in case BK13 was found at codon 196 in exon 6 that changed Arg to a chain-terminating codon (Fig 2B).

The intensity of the mutant band varied from case to case. In 2 cases (BK13, LL47) WT sequence was not detectable at the mutation site, suggesting deletion of the WT allele (BK13, Fig 2A; and LL47, data not shown). The other 2 cases (MT14 and LL48) show WT and mutant bands of similar intensity (Figs 2B and 2C). In case LL48, where tumoral cells make up 40% of the tumor, the WT band is probably caused by contamination of normal tissue. Nevertheless, in case MT14, where 80% of the cells are tumoral, the normal allele is probably present in the tumor.

To exclude the possibility that base changes detected in tumor samples could be polymorphism or germine mutations, genomic DNA from normal BM cells from the same patients was sequenced, and no nucleotide change was de-
MUTATION-INDEPENDENT p53 EXPRESSION IN LYMPHOMA

Table 1. P53 Gene Accumulation and Gene Mutation in 16 Cases of NHL

<table>
<thead>
<tr>
<th>Case</th>
<th>Diagnosis</th>
<th>p53 (1801) Expression (%)</th>
<th>p53 Mutations (exons 5–8)</th>
<th>Nucleotide Substitution</th>
<th>Amino Acid Substitution</th>
<th>Ki67 (%)</th>
<th>% Tumoral Cells</th>
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<tbody>
<tr>
<td>BK13</td>
<td>Burkitt</td>
<td>0</td>
<td>Codon 196</td>
<td>CGA → TGA</td>
<td>Arg → stop codon</td>
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<td>90</td>
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<td>LL66</td>
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<td>1</td>
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<td></td>
<td>44</td>
<td>75</td>
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<tr>
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<td>CB MALT</td>
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<td>WT</td>
<td></td>
<td></td>
<td>40</td>
<td>50</td>
</tr>
<tr>
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<td>CB MALT</td>
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<td>WT</td>
<td></td>
<td></td>
<td>20</td>
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</tr>
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<td></td>
<td>47</td>
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<td>IB-B</td>
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<tr>
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<td>34</td>
<td>Codon 196</td>
<td>CGC → CAC</td>
<td>Arg → His</td>
<td>32</td>
<td>80</td>
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p53 and Ki67 values are determined by the CAS system. Abbreviation: ND, not done.

DISCUSSION

p53 mutations have been described in a very wide variety of human tumors including hematopoietic malignancies such as acute myeloblastic leukemia (AML),36,37 plastic transformation of chronic myeloid leukemia,10 and acute lymphoblastic leukemia.11 In cases of NHL, p53 mutations have been described in Burkitt’s lymphoma,9,38 and Burkitt’s cell lines,9,39 chronic lymphocytic leukemia,9 and adult T-cell leukemia lymphoma.12,40 The presence of p53 mutations in 4 of our series of 16 cases confirms the presence of p53 gene mutations in high grade NHL. These mutations were found distributed in different histologic groups of high grade NHL, including Burkitt’s lymphoma, centrolast (CB), immunoblastic (IB), and large cell lymphoma of mucosa-associated lymphoid tissue (MALT) origin, in a similar frequency (20%) to that found in previous studies.41 p53 mutations were found in only 1 of 5 cases of Burkitt’s lymphoma that were studied. The frequency of p53 mutations in 33% of Burkitt’s lymphomas in previous studies,7 contrasts with the described presence of p53 mutations in 63%-83% of the Burkitt’s lymphoma cell lines studied9,30 thus far, suggesting that p53 mutations could be given a selective growth advantage for the in vitro establishment of cell lines.42

The presence of p53 mutations and/or p53 overexpression in some high grade lymphomas is consistent with the hypothesis that the p53 gene could play a role in the later stages of lymphomagenesis or in the progression of the disease, as has been pointed out by Ichikawa et al.41 Initial stages could be characterized by clonal B- or T-cell expansions that are occasionally associated with chromosomal translocation such as the (14;18) characteristic of follicular centrolast-centrocytic (CB-CC) lymphoma or the (8;14) frequently found in Burkitt’s lymphoma. Subsequent p53 alterations could contribute to transformation into more aggressive tumors, such as those found in colorectal cancer,43 those which have been proposed in brain tumor progression,44 or those arising in metastasis from human papillomavirus-positive cervical carcinoma.45

All 4 cases with mutations showed G to A transition. This is the type of base change most frequently found in lymphomas.7 Codons 196 and 273, where three mutations were found, correspond to CpG dinucleotide, a frequent site of mutations. All three basic changes (at codons 158, 196, and 273) have already been identified in lymphomas and other types of tumors.7,9 The results may underestimate the frequency of p53 mutations, because analysis was performed exclusively in these highly conserved regions of the p53 gene. Although the existence of mutations in other exons, introns, or promoter regions could not be excluded, it seems unlikely because conserved regions (exons 5 through 8) contain 98% of the mutation previously identified in human tumors.7,8,46 A potential cause of underestimating the real frequency of mutations is that direct sequencing could not detect mutations present in small tumor populations. To avoid this, we have only included cases in which tumoral cells represent at least 40% of the tissue.

Immunostaining with 1801 p53 MoAb, which recognizes an N-terminal epitope present in WT and mutant p53,47 was used to provide useful preliminary screening for detection of p53 mutations in tumors, because a relationship between the presence of p53 mutations and high levels of p53 protein expression has been described in lung cancer,13,48 breast cancer,14,16 ovarian carcinoma,18 and colorectal cancer cell lines.19 The detection of p53 protein through immunohistochemical techniques seems to depend on the stabilization of nuclear or cytoplasmic p53 as a conse-

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sequence of conformational changes secondary to mutations.\textsuperscript{5,20} This has led to the proposal that p53 detection may be synonymous with p53 mutation. Nevertheless, the findings of this series, where no mutation was detected in 5 or 8 cases with high p53 levels, do suggest that the nuclear or cytoplasmic stabilization of p53 protein could also depend on other factors, such as adhesion to other molecules (MDM2), as has been described in soft-tissue sarcomas.\textsuperscript{29} The presence of p53 independent of mutation has been described in human normal BM marrow blasts,\textsuperscript{49} in AML,\textsuperscript{50} and in the normal cells of a member of the family of a cancer patient.\textsuperscript{31} Recent findings suggest that p53 expression may be a stress response to a wide variety of DNA attacks. p53 protein is able to induce growth arrest in cells with DNA damage.\textsuperscript{1,2} Immunohistochemical findings have even shown p53 expression in reactive lymphocytes and epithelial basal cells in toxoplasmic lymphadenitis and reactive tonsils, as well in phytohemagglutinin-stimulated lymphocytes (Sanchez-Beato, submitted).

The combined analysis of p53 and ki67 expression shows that p53 values were lower or equal to ki67 results in all cases; this relation was independent of the presence of mutations. As well as confirming the existence of a relation between growth fraction and p53 expression, as has been already described (Sanchez-Beato, submitted), this result suggests that in lymphomas with low growth fraction, p53 values cannot be used as a selection criteria in the search for p53 mutations. This is due to the fact that mutations would

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**Fig 1.** Immunohistochemical detection of p53 protein is shown. (A) Case LL66: CB lymphoma shows scattered p53 positive cells in a case with no mutation in 5 to 8 exons. (B) Case BK15: Generalized p53 nuclear staining in a Burkitt's lymphoma lacking p53 mutation in exons 5 to 8 is shown. (C) Case BK13: Absence of p53 staining in a Burkitt's lymphoma with a nonsense mutation is shown. (D) Case MT14: Intense p53 staining in a case of MALT CB lymphoma with a mutation in codon 158 is shown.
in any case produce scarcely detectable p53 protein. The absence of p53 expression cannot rule out the existence of p53 mutations, as shown by case number BK 13, in which a nonsense mutation in codon 196 gives rise to a truncated protein in which C-terminal domain is lost.

To conclude, we found several different types of p53 disregulation in cases of NHLs. These include missense mutations with overexpression of the protein, nonsense mutation without accompanying mutations in the conserved regions. The impact of these p53 alterations on clinical course and treatment response of the patients deserves to be explored in an attempt to differentiate the specific consequences of each.

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