p53 Mutations in Mantle Cell Lymphoma Are Associated With Variant Cytology and Predict a Poor Prognosis

By Timothy C. Greiner, Michael J. Moynihan, Wing C. Chan, Debra M. Lytle, Alex Pedersen, James R. Anderson, and Dennis D. Weisenburger

Mutations of the p53 tumor suppressor gene have been described in several subtypes of non-Hodgkin’s lymphoma, but the incidence of p53 mutations in mantle cell lymphoma (MCL) is unknown. We hypothesized that cases of MCL with a variant or high-grade cytology would have a higher likelihood of p53 mutations than typical MCL. We were interested in the prognostic significance of p53 mutations in MCL. Therefore, a series of 53 well-characterized cases of MCL with DNA from 62 tissue samples was analyzed by the polymerase chain reaction with denaturing gradient gel electrophoresis for exons 5-8 of p53. Immunoperoxidase studies with the antibody DO-7 to p53 protein were also performed on frozen sections. We found mutations of the p53 gene in 8 of the 53 cases (15%) of MCL. Missense mutations predominated, and 50% of the mutations occurred at known p53 hotspot codons. Of 21 cases with variant cytology (ie, anaplastic or blastic), 6 (28.6%) had p53 mutations as compared with only 2 of 32 cases (6.3%) with typical MCL cytology (P = .05), and p53 mutations preceded the development of variant cytology in 2 patients. Overexpression of p53 protein was observed in 6 of the 8 cases (75%) with p53 mutations and in none of the 45 wild-type cases. The median survival of the cases with mutant p53 was only 1.3 years (all died), whereas the median survival of cases with germline p53 was 5.1 years (P = .023). These results suggest that mutations of p53 may be one mechanism involved in the development of variant forms of MCL and indicate that p53 mutations in MCL predict a poor prognosis.

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4302

MANTLE CELL LYMPHOMA (MCL) is a subtype of non-Hodgkin’s lymphoma (NHL) that is estimated to comprise 2.5% to 4% of NHLs in the United States. The neoplastic cells of MCL are thought to correspond to lymphocytes of normal primary follicles and the mantles around germinal centers of secondary follicles in lymph nodes, spleen, and other lymphoid tissues. The term MCL encompasses lymphomas that had been previously designated as lymphocytic lymphoma of intermediate differentiation, centrocytic lymphoma, and intermediate lymphocytic or mantle zone lymphoma, most of which correspond to diffuse small cleaved-cell lymphoma in the Working Formulation. Two architectural patterns are observed in MCL. The first pattern is a diffuse infiltrate that effaces the lymph node architecture, sometimes with characteristic histiocytes scattered among the lymphoma cells. The second pattern is a mantle zone pattern with the cells forming neoplastic nodules and infiltrating as wide mantles around residual benign germinal centers. The typical cytology of MCL is that of small- to intermediate-sized cells with irregular nuclear contours and coarse chromatin. The variant or high-grade cytological appearances described in MCL have considerable overlap with each other and include the blastic, anaplastic, and centrocytoid-centroblastic types.

Cytogenetic and molecular discoveries in MCL in recent years have helped establish MCL as a distinct clinicopathological entity. Karyotypic analysis led to the finding of a recurring nonrandom translocation between the bcl-1 locus and the Ig heavy chain in the t(11;14)(q13;q32). Southern blot analysis has shown a high percentage (50% to 70%) of bcl-1 rearrangements in MCL. The candidate oncogene is the PRAD1 gene located 3’ to most of the described bcl-1 breakpoints, and PRAD1 overexpression has been found to be highly associated with MCL. However, molecular studies to date have provided no putative mechanisms for the development of variant MCL, nor have molecular genetic markers with prognostic significance been identified.

Mutations in the p53 tumor suppressor gene have been described in several subtypes of lymphoid neoplasia and occur at an overall rate of about 14%. The frequency of mutations ranges from 5% to 40% including anaplastic large cell lymphoma (6%), peripheral T-cell lymphoma (8%), and chronic lymphocytic leukemia (CLL; 15%). Small noncleaved-cell lymphoma and adult T-cell leukemia/lymphoma (ATL) appear to have the highest de novo rates (30% to 40%) of p53 mutations in NHL, whereas a low mutation rate is observed in low-grade NHL. However, a high incidence of p53 mutations is present in high-grade lymphoid neoplasms that result from transformation or progression of a lower-grade NHL. These include CLL, in which 40% of cases that transform to diffuse large-cell NHL were found to have p53 mutations. In addition, at least 25% of diffuse large-cell lymphomas that arise from follicular lymphoma appear to harbor p53 mutations.

The major goal of our study was to determine the incidence of p53 mutations in MCL. First, we asked the question whether p53 mutations are present at a higher frequency in the variant or high-grade forms of MCL than in those with the typical cytology. Second, because p53 mutations are associated with a poor prognosis in Burkitt’s lymphoma (BL), acute lymphoblastic leukemia (ALL), and acute my-
Table 1. Immunophenotypic Characteristics and bcl-1 Rearrangements in the Two Subgroups of MCL

<table>
<thead>
<tr>
<th>Type</th>
<th>CD5*</th>
<th>CD10</th>
<th>CD23</th>
<th>bcl-1 RR</th>
</tr>
</thead>
<tbody>
<tr>
<td>Typical MCL</td>
<td>27/31(87.1%)</td>
<td>0/31(0%)</td>
<td>2/27(7.4%)</td>
<td>18/29(85.5%)</td>
</tr>
<tr>
<td>Variant MCL</td>
<td>17/21(81%)</td>
<td>4/21(19%)</td>
<td>1/21(4.8%)</td>
<td>9/20 (45%)</td>
</tr>
<tr>
<td>*P = 7.04</td>
<td>0.02</td>
<td>1.0</td>
<td>0.24</td>
<td></td>
</tr>
</tbody>
</table>

* Immunohistochemical studies performed on frozen tissue.
† Percentage positive; RR, detection of bcl-1 rearrangements by Southern hybridization using four probes (MTC, p94, A, D); ‡ the total in each group studied varied with the availability of frozen tissue or DNA.
¶ Fisher’s exact test statistics.

For oligo 4.06 Primer Analysis Software (National Biosciences Inc., Plymouth, MN). Primers with 4'-30'-nucleotide GC-clamps for one primer of each pair were synthesized on an ABI 380B synthesizer (ABI, Foster City, CA) by a solid-phase triester method.

Our modifications of the primers from Beck et al.26 (Table 2) include the use of a corrected sequence for the exon 7 forward strand, because the published forward primer lacked a thymidine 5'-triphosphate, 2.5 U Taq polymerase enzyme (Perkin Elmer-Cetus, Norwalk, CT), and 0.5 mM/L of each pair of primers. Reaction mixtures were incubated at 94°C for 9 minutes for denaturation followed by 45 cycles of 94°C for 1 minute and 15 seconds, 58°C for 1 minute and 15 seconds (60°C for exons 6), and 72°C for 30 seconds with 1 second of additional extension per each cycle. A terminal extension of 72°C for 10 minutes was performed after completion of the 45 cycles. A total of 10% of the reaction product (10 μL) was electrophoresed on a 2% 3:1 NuSieve agarose (FMCL, Rockland, ME) gel for determination of the success of amplification in producing the expected PCR product sizes.

Denaturing gradient gel electrophoresis (DGGE). DGGE was performed on the amplified DNA to screen for mutations and to isolate the mutated DNA for sequencing. Modifications of the electrophoresis times from Beck et al.26 for each exon were determined using stepwise-loading gels as described by Myers et al.46 To enhance the recovery of the DNA compared with precipitation techniques, the PCR products were dried with a Savant SpeedVac (Savant, Farmingdale, NY). A total of 40% of a 100-μL PCR product was electrophoresed at 160 V and 60°C in 8% polyacrylamide gels (37:1, acrylamide:bisacrylamide) poured with a model 150 Gradient Former (Life Technologies, Gaithersburg, MD).41 Denaturing conditions ranged from 35% to 65% through 60% to 80% (Table 2) in the gels analyzed on a model DGGE-4000 System (CBS Scientific Co, Inc, Del Mar, CA). After electrophoresis, the gels were stained with ethidium bromide (2 μg/mL) for 20 minutes and destained in distilled water for 10 minutes.41

Sequencing strategy. The PCR products that showed a shift in the migration of the sequence from the wild-type DNA were cut

| Table 2. Primer Sequences and Conditions for DGGE of p53 Mutations |
|--------------------|-----------------|----------------|
| p53 Exon | Primers† | Fragment Size | DGGE Conditions† |
| 5       | Forward 5′(GC)TTCCCTCTTCTGCAGTACTC-3′ | 282 | 60%-80% for 8 hours |
|         | Reverse 5′-CTGCGCAACAGCGGCTTCG-3′ | | |
| 6       | Forward 5′(GC)ACGACAGGGCGCTGG-3′ | 227 | 40%-70% for 6.5 hours |
|         | Reverse 5′-AGTTGCAACAACAGCCTAG-3′ | | |
| 7       | Forward 5′(GC)CCTCCTAGTGTCCTGAC-3′ | 174 | 35%-65% for 6.5 hours |
|         | Reverse 5′-CCAATGTTGCTCATGAC-3′ | | |
| 8       | Forward 5′-CCTATCTCGAGTTGAGTAT-3′ | 260 | 35%-65% for 8 hours |
|         | Reverse 5′(GC)AACTAGTCAAGGCTACA-3′ | | |

† (GC) is 5′-GCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGC-3′.
‡ The percentage of denaturing gradient range and electrophoresis run time at 160 V and 60°C. The best gradient for each PCR product was determined by optimizing the previously described conditions of Beck et al.26 in the authors’ laboratory.
†† Exon 8 reverse primer contains the primer sequence described by Gaidano et al.27
WT is wild-type p53. The wt sequences for exons 7 and 6 were aligned for the purposes of this figure: arrows, mutations as single bands above or below the wt bands; het, paired bands of heteroduplex formations between wt and mutated sequences (heteroduplex bands usually migrate slower than homoduplex bands); NA, bands above or below the wt bands; het, paired bands of hetero-
duplex formations between wt and mutated sequences (hetero-
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duplex bands usually migrate slower than homoduplex bands); NA, bands above or below the wt bands; het, paired bands of hetero-

from the DGGE gels, frozen at \(-70^\circ C\), thawed, and serially diluted in distilled H$_2$O to an estimated 10 pg/µl after comparison with a known quantity of OX174 HaeIII DNA (Life Technologies) used in the agarose gel. A reamplification of 10 to 100 pg of the first-round PCR product using the same primers (Table 2) without GC-clamps for the appropriate exon was performed. Our goal was to remove the GC clamp, which tends to interfere in sequencing reactions. The PCR product was centrifuged with a QiAQuick-Spin PCR Column Purification Kit (Quiagen, Chatsworth, CA) column to eliminate excess deoxynucleotide triphosphates and primers before sequencing. Sequencing was performed in both directions with the described exon primers without a GC clamp (Table 2), using the Dye-Labeled Dideoxy Terminator Kit (ABI) according to the manufacturer’s directions, purified with a Centri Sep PSR 00105 column (Princeton Separation Inc, Adelphia, NJ) and analyzed on an automated ABI 373A fluorescent sequencing system (ABI). DNA sequences were analyzed by Analysis 1.2 (ABI) and Sequence Navigator (ABI) software to compare with the wild-type p53 sequence.

Positive controls with previously published single nucleotide p53 mutations included six lymphoid cell lines (Exon 5-CEM; Exon 6-Raji, Jurkat; Exon 7-Raji; MC116; Namalwa; Ramos, CEM; see Fig 1) and a lymphoma sample with an exon-8 mutation (a gift of Dr M. Raffeld, National Cancer Institute, Bethesda, MD). Screening by DGGE identified mutational shifts (Fig 1) in each control sample, and sequencing showed the published mutations.

\textit{p53 Immunohistochemistry.} Overexpression of p53 protein was correlated with the p53 sequence results. Immunohistochemical studies using the avidin-biotin technique were performed on 6-µm frozen sections using the monoclonal antibody DO-7 (DAKO Corp, Carpinteria, CA) that reacts with an amino terminal epitope of the p53 protein.\textsuperscript{35} The antibody was applied at a concentration of 1:50 for 30 minutes, was developed with 3,3' diaminobenzidine tetrahydrochloride, and was counterstained with hematoxylin. A positive result was recorded when the percentage of nuclei staining exceeded 5% of the cells, a threshold determined after staining normal tissues and described previously in paraffin-embedded tissue.\textsuperscript{36}

\textit{Statistical analysis.} Comparisons of the distributions of categorical data characteristics between groups were made using Fisher’s ‘exact’ test for 2×2 tables. Survival was defined as the time to death from any cause and was estimated using the product-limit method of Kaplan and Meier.\textsuperscript{46} Distributions of time-to-event data were compared using the log-rank test.\textsuperscript{47}

\begin{table}[h]
\begin{center}
\begin{tabular}{cccccc}
\hline
\textbf{Lane} & \textbf{Exon} & \textbf{DNA} & \textbf{Codon} & \textbf{Mutation} & \textbf{Reference} \\
\hline
1 & Ex 7 & None & NA & NA & \\
2,3* & Ex 7 & Namalwa & 248 & CGG-CAG & Farrell et al\textsuperscript{43} \\
4,5* & Ex 7 & Ramos & 254 & ATC-GAC & Farrell et al\textsuperscript{43} \\
6 & Ex 7 & Placenta & NA & Wt & \\
7 & Ex 7 & Raji & 234 & TAC-CAC & Duthu et al\textsuperscript{44} \\
8 & Ex 7 & MC116 & 238 & TGT-TAT & Gaidano et al\textsuperscript{47} \\
9 & Ex 6 & SNC & NA & Wt & \\
10 & Ex 6 & Raji & 213 & CGA-CAA & Farrell et al\textsuperscript{43} \\
11 & Ex 6 & Jurkat & 196 & CGA-TGA & Cheng and Haas\textsuperscript{46} \\
12 & Ex 6 & Placenta & NA & Wt & \\
\hline
\end{tabular}
\end{center}
\caption{DGGE results in lymphoid cell lines with known p53 mutations.}
\end{table}

\textbf{RESULTS}

\textit{p53 Mutations in MCL.} A total of 32 cases of MCL showed typical cytology at diagnosis, whereas 17 cases (52%) showed variant cytology including 16 blastic and 1 anaplastic MCL. In 4 additional cases, the tumor transformed from typical cytology to blastic cytology in a subsequent biopsy specimen. Thus, 21 of our 53 cases (39.6%) showed variant MCL cytology at some time during the clinical course.

Of the 53 cases of MCL, 8 (15%) had p53 mutations (Table 3). The mutations were observed in exons 5 (1 case), 6 (1 case), 7 (3 cases), and 8 (3 cases), with 50% occurring at codons 213, 248, and 273. These codons are known to have a high frequency of p53 mutations and include CpG dinucleotides. Sequential biopsy specimens in 3 cases (cases no. 4, 5, and 6) showed the same mutation each time. The most frequent p53 abnormality was a missense mutation, which was observed in 6 of 8 cases. Approximately equal numbers of transitions (4) and transversions (3) were observed. In addition to the missense mutations, case no. 1 had a 2-bp deletion in codons 184-185. This deletion caused a frame shift and introduced a stop codon downstream at codon 207. Case no. 2 had a nonsense mutation at codon 213.

Figure 2 shows the DGGE screening results for mutations in exon 8 for cases of MCL. Each unique mutation migrates to a different position in the denaturing gel. Typically, four bands are observed in a heterozygous case, including one band for the wild-type sequence, one band for the mutant sequence, and two heteroduplex bands representing mixtures of wild-type and mutant sequences that migrate more slowly in the gel. The presence of paired heteroduplex bands facilitates the identification of mutated sequences, especially when the mutated cells comprise only 0.1% to 1.0% of the sample, which is the sensitivity cutoff level of DGGE in our laboratory. The presence of a strong mutant band out of proportion with the wild-type sequence band, such as those observed in cases no. 6b and 8 in Fig 2, suggests a deletion of the wild-type allele or, less likely, mutations in both alleles, shown in Fig 1 for Namalwa (lane 2).

To study the importance of p53 mutations and overex-
mutations in mantle cell lymphoma

expression of p53 protein in the development of variant forms of MCL, cases were classified as variant MCL if any biopsy specimen showed variant cytology. In the 32 cases with only typical lymphocytic cytology, 2 cases (6.3%) had p53 mutations. However, of the 21 cases with variant cytology, 6 cases (28.6%) had p53 mutations (P = .05); 5 of the variant cases had blastic morphology, and 1 case (case no. 1) was an anaplastic MCL. No correlation between CD10 expression and p53 mutation was identified because only 1 p53 mutant case was CD10⁺.

In 2 cases, the p53 mutation preceded the development of variant cytology. In case no. 4, approximately 1% of the tumor cells had a p53 mutation in the initial biopsy specimen (case no. 4a), but the mutation was more prominent in a subsequent biopsy specimen obtained 1 year later (case no. 4b). Figure 3 shows the pair of weak heteroduplex bands from the initial biopsy specimen (case no. 4a), which migrate to the same position as the bands from the subsequent biopsy specimen (case no. 4b). Morphologically, this tumor began with a mantle zone pattern and typical lymphocytic cytology (Fig 4A), but recurred with blastic cytology (Fig 4B) while retaining the mantle zone pattern. Case no. 5, which also changed from typical cytology to a variant form, also showed an architectural change from a mantle zone pattern to a diffuse pattern.

A previously described polymorphism at codon 213 (CGA → CGG) was observed in 3 of the 53 cases (5.5%). Figure 5 shows that the bands of the polymorphic sequence migrate to a different position in the gel than that of the mutation at the same codon 213 observed in case no. 2, which differs by only two nucleotides (CGA → TGA).

p53 Immunohistochemistry in MCL. Overexpression of the p53 protein was observed in 6 of the 8 cases with sequenced mutations using the antibody DO-7 (Table 3). None of the other 45 cases without a p53 mutation was positive with DO-7, which includes the 3 cases with the polymorphism at codon 213. Overexpression of p53 was present in the 2 mutated cases with stop codons (cases no. 1 and 2), because codons 207 and 213 are downstream from the DO-7 epitope.

Survival analysis. For the survival analysis, 15 patients were eliminated because of a lack of follow-up information (2 cases) or because they represented referrals at the time of relapse (13 cases). After these exclusions, 6 cases with p53 mutations and 32 cases with wild-type p53 remained for the survival analysis. The choice of chemotherapy provided no selection bias because a similar percentage received curative therapy in the p53 mutation group (4 of 6; 66.7%) and in the wild-type group (25 of 32; 74%; P = .16). Curative therapy was defined as a protocol including Adriamycin or an Adriamycin-like drug. There was a significant difference in the median survival of the MCL cases with p53 mutations as compared with that of those without mutations. The median survival of MCL cases with wild-type p53 was 5.1

![Fig 2. DGGE results for exon 8 in MCL. Case, MCL with exon 8 p53 mutations; WT, MCL with wild-type exon 8 DNA; Neg control, no DNA; Placenta, wild-type control; het, heteroduplex bands; arrows, mutant p53 bands; wt, wild-type p53 bands. Note that p53 mutation shifts are present in case no. 8, case no. 6a and 6b, and case no. 7. Occasionally, a gel artifact will produce the appearance of doublet bands (case no. 7), which has no significance.](image)

<table>
<thead>
<tr>
<th>Case No.</th>
<th>Pattern-Cytology</th>
<th>p53 Immuno-DO-7</th>
<th>Exon</th>
<th>Codon</th>
<th>DNA Changes</th>
<th>Amino Acid Change</th>
<th>Mutation Type</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>D-V</td>
<td>P</td>
<td>5</td>
<td>184-5</td>
<td>GATAGC→GAGCGA</td>
<td>AspSer→GluArg</td>
<td>Deletion 2 bp</td>
</tr>
<tr>
<td>2</td>
<td>D-T</td>
<td>P</td>
<td>6</td>
<td>213</td>
<td>CGA→TGA</td>
<td>Arg→Stop</td>
<td>Nonsense</td>
</tr>
<tr>
<td>3</td>
<td>D-V</td>
<td>N</td>
<td>7</td>
<td>234</td>
<td>TAC→TGC</td>
<td>Tyr→Cys</td>
<td>Missense</td>
</tr>
<tr>
<td>4a</td>
<td>MZ-T</td>
<td>N</td>
<td>7</td>
<td>248</td>
<td>CGG→CAG</td>
<td>Arg→Gin</td>
<td>Missense</td>
</tr>
<tr>
<td>4b</td>
<td>MZ-V</td>
<td>N</td>
<td>7</td>
<td>248</td>
<td>CGG→CAG</td>
<td>Arg→Gin</td>
<td>Missense</td>
</tr>
<tr>
<td>5a</td>
<td>MZ-T</td>
<td>ND</td>
<td>7</td>
<td>248</td>
<td>CGG→CAG</td>
<td>Arg→Gin</td>
<td>Missense</td>
</tr>
<tr>
<td>5b</td>
<td>D-V</td>
<td>P</td>
<td>7</td>
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<td>CGG→CAG</td>
<td>Arg→Gin</td>
<td>Missense</td>
</tr>
<tr>
<td>6a</td>
<td>D-T</td>
<td>ND</td>
<td>8</td>
<td>266</td>
<td>GGA→GTA</td>
<td>Gly→Val</td>
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<td>6b</td>
<td>D-T</td>
<td>P</td>
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<td>GGA→GTA</td>
<td>Gly→Val</td>
<td>Missense</td>
</tr>
<tr>
<td>7</td>
<td>D-V</td>
<td>P</td>
<td>8</td>
<td>273</td>
<td>CGT→CCT</td>
<td>Arg→Pro</td>
<td>Missense</td>
</tr>
<tr>
<td>8</td>
<td>D-V</td>
<td>P</td>
<td>8</td>
<td>278</td>
<td>CCT→ACT</td>
<td>Pro→Thr</td>
<td>Missense</td>
</tr>
</tbody>
</table>

Underlined nucleotides in case no. 1 were deleted, resulting in a stop codon at 207.

Abbreviations: N, negative p53 protein expression; P, positive p53 protein expression in greater than 5% of cells; ND, not done because no tissue was available; MZ, architectural pattern wherein tumor cells expand around germinal centers; D, diffuse pattern wherein the infiltrate effaces the lymph node architecture; T, typical lymphocytic cytology; V, variant cytology.
DGGE results for exon 7 in sequential biopsy specimens of MCL case no. 4: het, heteroduplex bands; arrows, mutant p53 band; wt, wild-type p53 band. Triplicate analyses of the biopsy specimen in case no. 4a with typical MCL from 1989 show heteroduplex bands. Case no. 4b is an analysis of the variant (blastic) form observed in 1990. The mutant p53 band (arrow) is easily identified in case no. 4b. The heteroduplex pair of bands observed in case no. 4b is also present in case no. 4a, indicating that a small population of cells with the mutant p53 sequence is also present in the earlier 1989 biopsy specimen. The mutant band is not observed in case no. 4a because the mutant sequence has been consumed in the heteroduplex bands. WT is an MCL case with wild-type DNA.

years, whereas all those with p53 mutations have died, with a median survival of only 1.3 years ($P = .023$; Fig 6). Multivariate analysis to determine if p53 mutations represent an independent prognostic factor apart from variant morphology could not be performed because of the small number of cases with mutations.

DISCUSSION

To assay mutations of the p53 gene in lymphoid neoplasms, most investigators have used modifications of the single-stranded conformation polymorphism protocol originally described by Orita et al. In contrast to single-stranded conformation polymorphism, DGGE uses double-strand PCR products in a computer-calculated and empirically determined denaturing gradient polyacrylamide gel constructed with increasing urea and formamide concentrations to isolate mutated sequences. Sheffield et al showed the analytical capabilities of GC-clamped DGGE to detect single base mutations in hemoglobin sequences with 100% sensitivity. Metzger et al first described the application of GC-clamped DGGE in detecting p53 mutations in ependymomas. This method was then applied by other investigators in rat and human tumors. Beck et al showed that a refined GC-clamped DGGE protocol can separate single base changes in p53 with high resolution and efficiency. A 40-nucleotide sequence of guanines and cytosines (GC-clamp), synthesized at the 5' end of one of the primers, provides the high-resolution capability. In view of these features and our experience with the DGGE technique, we chose DGGE as our mutation-screening assay.

In this study, we found p53 mutations in 15% of our cases of MCL. We also showed two other new significant findings. We found that (1) p53 mutations are highly associated with the variant cytology of MCL (28.6% of variant cases) and (2) p53 mutations appear to predict a poor prognosis. This report identifies p53 mutations in a large series of cases of MCL. Preliminary reports of smaller groups of cases at the recent US/Canadian Academy of Pathology meeting suggested findings similar to ours, ie, that p53 mutations and/or p53 overexpression may occur in MCL. Cazorla et al observed a p53 mutation in 1 of 14 cases (7%) of MCL, with the single mutation observed in one of four (25%) blastic variants. Finkelstein and associates reported p53 mutations in 2 of 18 cases (11%) of MCL and in 1 of 6 (16.7%) other lymphomas with bcl-1 rearrangements. In 23 cases of MCL with bcl-1 rearrangements, Louie et al observed p53 overexpression in 5 cases (16.6%), of which only
Fig 5. DGGE results for exon 6 in MCL. het, heteroduplex bands; arrow, mutant p53 bands; wt, wild-type p53 bands; p, polymorphism band; WT, MCL with wild-type exon 6 DNA; case no. 2, MCL with p53 mutation at codon 213 (CGA → TGA); POLY, 3 MCLs with paired biopsy specimens that have the codon 213 polymorphism (CGA → CGG); Jurkat, positive control cell line with a codon 196 mutation (CGA → TGA); Neg Control, no DNA.

2 had p53 mutations (10.1%). A median survival of 12 months was observed in the cases with p53 expression versus 63 months in the p53-negative cases. However, Louie et al could not show a correlation of p53 mutations with prognosis because only 2 cases had mutations. In addition, because no variant cases were identified in their series, the association of p53 mutations with variant cytology was not made. Finally, Zoldan et al observed p53 mutations in 1 of 10 cases (10%) of typical MCL and 1 of 4 (25%) MCL variants. These studies, taken in aggregate, support our data that show a low incidence of p53 mutations in MCL. Individually, these reports had insufficient cases to show a strong association of p53 mutations with variant MCL; however, in composite, they support our observation that a higher frequency of p53 mutations is observed in variant MCL.

The occurrence of variant MCL, which have larger nuclei and finer chromatin than typical MCL, is still an evolving concept, as reflected by the recent workshops on low-grade B-cell disorders conducted by the Society for Hematopathology in 1993 and on MCL conducted by the the European Lymphoma Task Force in 1994. An interesting finding in our study was the observation of variant cytology in 21 of 53 cases (39.6%) at some time during the clinical course. This is identical to the results of a recent study of the natural history of MCL published by Norton et al, who found that 39.4% of cases of MCL (26 of 66 cases) eventually developed transformation. The fact that transformation was found on rebiopsy or at autopsy in 24% (16 of 66) of these cases underscores an important point. Obtaining multiple biopsy specimens is necessary for the identification of the transformation to variant MCL and for studying the molecular pathogenesis of this process. Previous emphasis on diagnosing MCL as having only a lymphocytic cytology with infrequent transformation has a priori de-emphasized the importance of performing biopsies at relapse or evaluation at autopsy.

We asked the question of whether variant MCLs were associated with a specific type of p53 mutation. However, other than localization of 50% of mutations at codons with CpG dinucleotides, a spectrum of mutations was observed. To our knowledge, the deletion of two nucleotides in codons 184-185 has not been previously reported in hematopoietic neoplasms. However, a single nucleotide deletion at the first position in codon 185 has been observed in breast cancer.

The CGA → TGA nonsense mutation at codon 213 has been reported frequently in hematologic malignancies, including BL and BL cell lines, ATL, myelodysplastic syndrome (MDS) progressing to AML, and chronic myelogenous leukemia (CML) progressing to a blast crisis.

Our finding of frequent missense mutations parallels those of previous reports on other subtypes of NHL. The TAC → TGC mutation at codon 234 has been described in a BL cell line. The CGG → CAG mutation at codon 248 has been described in BL and in BL cell lines, transformed follicular NHL, CLL, immunoblastic NHL, ALL, T-cell line, CML in blast crisis, ATL, and MDS. Hsiao

Fig 6. Survival of patients with MCL. The solid line represents 32 patients with wild-type p53 sequences, and the dashed line represents 6 patients with p53 mutations.
et al.\textsuperscript{14} have shown that human T-cell acute leukemia cells with this CGG → CAG at mutation codon 248 have higher proliferation and metastasis rates as compared with those of wild-type p53 cells when implanted in SCID mice. The other mutations reported in Table 3 in our study have not been studied with this method. The CGT → CCT mutation at codon 273 has been reported in AML.\textsuperscript{73} The mutations at codons 266 and 278 have not been previously reported in lymphoid tumors.\textsuperscript{73}

In addition to the p53 mutations, a polymorphism at codon 213 was found at about the same rate as that reported by others.\textsuperscript{68,69} Because there is no change in the amino acid sequence in the protein (Arg → Arg), this polymorphism is thought to have no functional effect on the p53 protein.

The occurrence of p53 mutations has been suggested as one mechanism of clinical progression in NHL.\textsuperscript{76} In the transformation of a low-grade to a high-grade lymphoid process, such as the transformation of CLL\textsuperscript{27} or follicular lymphoma\textsuperscript{4,55} to large-cell lymphoma; and in the progression of chronic or smoldering ATL to the aggressive phase of ATL.\textsuperscript{72} Recently, mutations in p53 have been associated with high-grade MDS and progression to AML.\textsuperscript{67} A parallel observation has been described in CML progressing to blast crisis.\textsuperscript{68} In these settings, patients with p53 mutations have a poor prognosis.

We propose that a similar molecular mechanism involving p53 may be responsible for both the development of some cases of variant MCL and the poor prognosis in the mutant MCL. It is known that the p53 protein regulates the cyclin D1 – cyclin-dependent kinase complex in the cell cycle via the p21 (WAF1/Cip1) gene.\textsuperscript{77} Cyclin D1 participates in checkpoint control via phosphorylation of the retinoblastoma protein before the transition from G1 to S-phase.\textsuperscript{78-79} Recent "knockout" experiments in human cell lines with bcl-1 rearrangements have shown that cyclin D-1 is required for rapid G1 progression.\textsuperscript{79} As mentioned earlier, high levels of cyclin D1 mRNA and protein are present in MCL\textsuperscript{21-26} and are hypothesized to be oncogenic.\textsuperscript{78}\textsuperscript{80} Although elevated, the effect of cyclin D1 may be somewhat inhibited by the presence of wild-type p53. However, in cases with mutant p53, this inhibitory effect may be lost, thus further enhancing the growth-promotional effect of cyclin D1. The loss of p53 regulation of cyclin D1 may be one of the molecular mechanisms explaining the higher mitotic rate and proliferative fraction observed in blastoid variants of MCL.\textsuperscript{11,12} Other molecular lesions within cell cycle control may exist in the subset of variant MCLs without p53 mutations. The potential for finding other molecular abnormalities may be predicted by recent discoveries regarding the transformation of follicular lymphoma to diffuse large-cell NHL, wherein abnormalities of c-myc,\textsuperscript{80} p53,\textsuperscript{34,35} and bcl-6\textsuperscript{81,82} have been implicated.

Discrepancy between the results of p53 immunohistochemistry and mutational analysis has been observed in NHL.\textsuperscript{28,34,84} and, recently, in MCL.\textsuperscript{84} No mutations were identified in 3 of 5 MCL cases that were positive by p53 immunoperoxidase in the study by Louie et al.\textsuperscript{84} In our study, there was relatively good concordance between protein overexpression and detected mutations. Antibody DO-7 appears to be specific in identifying mutations in exons 5-8 in our study, because no case was positive in the presence of wild-type sequence. In our study, antibody DO-7 identified p53 overexpression in 6 of 8 cases (75%) with sequenced p53 mutations. We believe that the negative immunoperoxidase result on case no. 4a was secondary to the low number of mutant cells present (0.1% to 1%), as estimated from the DGGE of the first biopsy specimen (case no. 4a; see Fig 3). This hypothesis cannot be confirmed because no frozen tissue was available from the subsequent biopsy specimen (case no. 4b) with variant MCL.

Importantly, p53 mutations appear to be the first molecular marker to identify patients with MCL who will have a poor prognosis. The results of our survival analysis of p53 mutations in MCL parallel those of analyses previously described in small nonecleaved-cell lymphoma,\textsuperscript{31} CLL,\textsuperscript{29} MDS,\textsuperscript{67} CML,\textsuperscript{58} ATL,\textsuperscript{72} and ALL.\textsuperscript{26} Clearly, p53 mutations in most hematologic malignancies portend a poor prognosis,\textsuperscript{82} and our study indicates the importance of detection of p53 mutations in MCL. Other studies in MCL would be useful to confirm our results.

REFERENCES

14. Weisenburger DD, Sanger WG, Armitage JO, Portalio DT: Intermediate lymphocytic lymphoma: Immunophenotypic and cyto-


16. Vandenberghe E, De Wolf-Peeters C, van den Oord J, Wio-
darski I, Delabie J, Stul M, Thomas J, Michaux JL, Mecucci C, Casisman JJ, van den Bergh H: Translocation (11;14): A cyto-
genetic anomaly associated with B-cell lymphomas of non-follicle cen-

17. Mederios LJ, Van Krieken JH, Jaffe ES, Raffeld M: Association of bcl-1 rearrangements with lymphocytic lymphoma of inter-


19. Williams ME, Swedlow SH, Rosenberg CL, Arnold A: Char-
acterization of chromosome 11 translocation breakpoints at the bcl-
1 and PRADI loci in centrocyclic lymphoma. Cancer Res 52:5541, 1992 (suppl)

20. de Boer CJ, Loysen S, Kluin PM, Kluin-Nelemans HC, Schu-

homme O, Germain D, Magaud J-P: Rearrangement and over-
expression of the BCL-1/PRAD-1 gene in intermediate lymphocytic lymphomas and in t(11q13)-bearing leukemias. Blood 81:3063, 1993

22. Rosenberg CL, Wong E, Petty EM, Bale AE, Tsujimoto Y, Harris NL, Arnold A: PRAD1, a candidate BCL1 oncogene: Map-


cell lymphoma but not in other low-grade B-cell lymphomas, includ-


27. Gaidano G, Balerini P, Gong JZ, Inghirami G, Neri A, New-


29. El Rouby S, Thomas A, Costin D, Rosenberg CR, Potmesil M, Silber R, Newcomb EW: p53 gene mutation in B-cell chronic lymphocytic leukemia is associated with drug resistance and is inde-


33. Sakashita A, Hattori T, Miller CW, Suzushima H, Asou N, Takatsuki K, Koeffler HP: Mutations of the p53 gene in adult T-

34. Sander CA, Yano T, Clark HM, Harris C, Longo DL, Jaffe ES, Raffeld M: p53 mutation is associated with progression in follic-

35. LoCoco F, Gaidano G, Louise DC, Offit K, Chaganti RSK, Dalla-Favera R: p53 mutations are associated with histologic trans-
formation of follicular lymphoma. Blood 82:2289, 1993


37. Wattel E, Preudhomme C, Hequet B, Vanrumbeke M, Ques-


40. Myers RM, Maniatis T, Lerman LS: Detection and localization of single base changes by denaturating gradient gel electrophore-


42. Cheng J, Haas M: Frequent mutations in the p53 tumor sup-


odies on lung carcinomas. Mod Pathol 7:853, 1994

46. Kaplan GL, Meier P: Nonparametric estimation from incom-


48. Carbone D, Chiba I, Mitsudomi T, Matsuzaki T, Brambilla E: p53 immunolabeling in archival paraffin-embedded tissues: Optimal protocol based on microwave heating for eight antib-
odies on lung carcinomas. Mod Pathol 7:853, 1994

49. Mazars G-R, Jeanteur P, Lynch HT, Lenoir G, Theillet C: Detection and localiza-

50. Ichikawa A, Hotta T, Takagi N, Tsushita K, Kinoshita T,


53. Sheffield VC, Cox DR, Lerman LS, Myers RM: Attachment of a 40-base-pair G+C-rich sequence (GC-clamp) to genomic DNA fragments by the polymerase chain reaction results in improved detection of single-base changes. Proc Natl Acad Sci USA 86:232, 1989


68. Lukas J, Jadayel D, Bartikova J, Nucleva E, Dyer MJS, Strauss M, Bartek J: BCL-1/cyclin-D1 oncoprotein oscillates and subverts the G1 phase control in B-cell neoplasms carrying the t(11;14) translocation. Oncogene 9:2159, 1994


p53 mutations in mantle cell lymphoma are associated with variant cytology and predict a poor prognosis

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