Disrupted p53 Function as Predictor of Treatment Failure and Poor Prognosis in B- and T-Cell Non-Hodgkin’s Lymphoma

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ABSTRACT
Mutation of the p53 gene has been associated with treatment failure and poor outcome in various malignancies. It has been suggested that immunohistochemical analysis of p53 and p21Waf1, a downstream target, can be used to screen for p53 gene mutations. We determined the value of immunohistochemical screening for p53 gene mutations as a prognostic marker in a population-based group of B- and T-cell non-Hodgkin’s lymphomas (NHLs). On the basis of p53 gene mutation status and immunohistochemically detected p53 and p21Waf1 expression in 34 lymphomas, we established an immunophenotype (Δp53) correlating with p53 gene mutation. The immunohistochemical analysis was extended to encompass 199 lymphomas from a population-based registry and was correlated with clinical parameters. Δp53 showed 100% concordance with p53 gene mutation and was detected in 42 cases (21%). Multivariate analysis of advanced stage lymphomas showed that Δp53 was independently associated with treatment failure (relative risk, 3.8; P = 0.001). Δp53 predicted poor survival when analyzing all patients (P = 0.001), as well as B-cell (P = 0.04) and T-cell NHL (P = 0.000002). In multivariate analysis, Δp53 (relative risk, 2.2; P = 0.001) maintained prognostic significance. The impact on prognosis of Δp53 was highly significant in the low-intermediate-risk group (P = 0.00002). Comparing survival of the aggressive lymphoma patients in this group showed that the 8 Δp53 patients died within 1 year, whereas the median survival of the 28 non-Δp53 patients was 36 months. These results suggest that immunohistochemically assessed p53 status may predict treatment response and outcome in B- and T-cell NHL patients.

INTRODUCTION
NHL is becoming an increasing clinical problem because of a steady annual increase in incidence. The age-adjusted incidence increased 85% in the United States from 1973 to 1995 (1). Only four cancer types cause more person-years of life to be lost (1). It is, therefore, highly important to improve diagnosis and therapy of this disease. One important aspect of this is to identify patients with a poor prognosis to plan the treatment strategy accordingly, be it intensified standard treatment or an experimental regimen. Since the publication of the IPI (2), it has been widely used to categorize patients into risk groups. Although the IPI was constructed for aggressive NHL, it has been shown also to be valuable in other NHL subtypes (3–6). The IPI is based on patient characteristics related to patient constitution (age, performance status) and variables indirectly reflecting the tumors biology (LDH, extranodal disease, stage). To improve on the index, biological factors more directly relating to clinical characteristics of the individual tumor, such as chemo- or radio-sensitivity, will be needed. Efforts have been made to associate molecular factors related to such important tumor biology functions as proliferation (7), cell cycle progression (8), apoptosis (9), and invasion (10) with a predictive value in NHL.

The p53 tumor suppressor gene is one of the most frequently mutated genes in human cancer (11). It functions as an integrator of cellular responses to DNA damage (12, 13), and is vital in assuring genome stability (14). Mutation of the p53 gene is associated with poor prognosis in patients with aggressive B-cell NHL (15). Most p53 gene mutations in NHL are missense mutations stabilizing—possibly mediated by interaction with MDM2 (16)—the functionally defect protein (17–20). As a result, the mutated p53 protein is unable to induce downstream targets such as p21Waf1. Immunohistochemical detection of p53 and its downstream target p21Waf1 has been shown to predict presence of p53 gene mutations (18). This population-based study addresses the value of an immunophenotype indicative of p53 gene mutation (Δp53) in predicting outcome in B- and T-cell NHL. The optimal criteria for defining Δp53 was established by immunohistochemically analyzing 34 lymphomas with known p53 gene status from our previous molecular biological studies (21). We found that Δp53, identified by simple and cost-immunohistochemistry assays applicable in rou...
tine diagnostic laboratories, independently predicted poor survival in both B- and T-cell NHL.

PATIENTS AND METHODS

Patients and Tumors. Since January 1, 1983, the LYFO has registered clinical and pathoanatomical data on all newly diagnosed primary NHL patients ≥15 years of age in a population-based registry covering western Denmark (2.8 million inhabitants). We previously described the LYFO registry, including exclusion criteria, hematopathological analysis, staging, and follow-up procedures (22–24). Briefly, from the 3212 consecutive cases in the LYFO registry diagnosed between January 1, 1984, and December 31, 1994, we retrieved 206 previously untreated patients from Odense University Hospital by obtaining consecutive cases from each of a number of selected major histological entities. Of these 206 patients, 7 were excluded because no tissue from the time of initial diagnosis was available for analysis. For the present study, all specimens were reviewed and reclassified based on morphological examination of paraffin sections, as well as immunophenotyping using flow cytometry and immunohistochemistry on paraffin and frozen sections, according to the Revised European-American Lymphoma classification (25). For clinical grading, the scheme proposed by Hiddemann et al. (26) was used. Our final study population (n = 199) consisted of 72 indolent B-lymphomas [B-cell chronic lymphocytic lymphoma (14 cases), immunocytoma (17 cases), FCL grade I/II (21 cases), and extranodal marginal zone lymphoma (20 cases)], 74 aggressive B-lymphomas [FCL III (4 cases), FCL diffuse (4 cases), mantle cell lymphoma (15 cases), and DLCL (51 cases)], 37 aggressive T-lymphomas [peripheral T-cell lymphoma (21 unspecified, 6 angioimmunoblastic, 1 angiocentric, and 1 intestinal T-cell lymphoma) and anaplastic large T- or Null-cell lymphoma (8 cases)], and 16 very aggressive lymphomas [B-lymphoblastic lymphoma (5 cases), Burkitt’s lymphoma (3 cases), and T-lymphoblastic lymphoma (8 cases)]. The observation period analyzed was up to 5 years (until December 31, 1996) or death, whichever came first. No patients were lost to follow-up. The median follow-up period for patients alive at the end of the observation period was 60 months (range, 36–60).

The 72 indolent lymphomas were treated with combination chemotherapy in 25 cases (including anthracycline in 19 cases), single alkylating agents in 24 cases, radiotherapy and/or surgery in 21 cases, and no treatment in 2 cases. Forty-two patients treated with chemotherapy had advanced stage NHL, whereas 19 of 21 patients treated with radiotherapy and/or surgery had stage I or II disease. Treatment of the 74 aggressive B-lymphomas consisted of combination chemotherapy in 58 cases (anthracycline-containing in 46 cases, primarily cyclophosphamide, doxorubicin, vincristine, and prednisolone), single alkylating agents in 5 cases, and other approaches in 11 cases. The 37 aggressive T-lymphomas primarily received combination chemotherapy with (24 cases) or without (4 cases) anthracycline. Two patients received radiotherapy, four patients were treated with interferon or prednisone, and three patients had no initial therapy. Fifteen of 16 very aggressive lymphomas were treated with high-dose combination chemotherapy with central nervous system prophylaxis in 12 cases. One patient died before therapy was instigated.

Data allowing allocation of the patients to the IPI risk groups (2), which are based on age (>60 years), LDH (>1 × normal), Eastern Cooperative Oncology Group performance status (2–4), number of extranodal sites (>1), and stage (III or IV), were available for 193 patients.

We compared the clinical characteristics of our study population with those of the remaining patients (n = 3013) of the LYFO population from the study period for each histological entity separately, as well as the two populations overall. There were no significant differences in age, sex, stage, PS, B symptoms, LDH, number of extranodal sites involved, CR rates (27), and OS. Furthermore, the distribution of IPI risk group assignment was not significantly different between the study population and the remaining LYFO patients.

Laboratory Assays. Using sections from two compound paraffin blocks, one composed of numerous benign and malignant tissues of various histiogenesis and the other containing various lymphoma subtypes as well as benign lymphoid tissue, we established optimal conditions for immunostaining for p21Waf1 with the monoclonal antibodies 4D10 (Novocastra, Newcastle, United Kingdom), EA10 (Oncogene Research, Cambridge, MA), and 6B6 (PharMingen, San Diego, CA). The antibodies showed nearly identical reaction patterns, but because it had a superior signal-to-noise relation, EA10 was chosen for the study. We used the same material to optimize the conditions for the anti-p53 antibody DO-7 (DAKO, Glostrup, Denmark), which recognizes both wild-type and mutant p53 (28). Optimal dilution for EA10 and DO-7 was 1:25 and 1:100, respectively. Briefly, deparaffinized 4-μm sections were submitted to microwave oven antigen retrieval in T-EG buffer [10 mM Tris (Sigma Chemical Co., St. Louis, MO) and 0.5 mM EGTA (pH 9.0; Sigma Chemical Co.)] or 10 mM citrate buffer (DO-7; pH 6.0), followed by incubation overnight at 4°C with primary antibody. TechMate 1000 (DAKO) was used for automated staining using the LSAB+ kit (DAKO) with diaminobenzidine as chromogen and brief counterstaining in Mayer’s hematoxylin. Sections from the compound blocks served as positive and negative (omitted primary antibody) controls.

Brown nuclear coloration was considered positive immunoreaction. The reactivity was quantified using the image analysis software ImagePro Plus (Media Cybernetics, Silver Spring, MD). Briefly, in areas with highest expression of p53 and p21Waf1, respectively, the number of immunopositive cells was counted among the total number of cells. Typically 1000–3000 cells were counted. The quantification was done by a single observer, who had no previous knowledge of clinical outcome or other clinical variables.

Statistical Analysis. Univariate association was analyzed using Fisher’s exact test, Pearson’s χ² test, or McNemar’s test (29). OS was calculated from the time from definitive diagnosis to death from any cause in or to the end of the observation. RFS of patients with CR was calculated from the time from achievement of CR to relapse, death, or end of the follow-up period. Survival curves were calculated using the method of Kaplan and Meier (30) and compared using the log-rank test (31) stratified by clinical grade (indolent B-NHL, aggressive B-NHL, aggressive T-NHL, and very aggressive
Table 1  p53 gene mutations identified in 34 diffuse large B-cell lymphomas, and the corresponding expression of p53 and p21

<table>
<thead>
<tr>
<th>Patient no.*</th>
<th>Mutation</th>
<th>Immunohistochemistry</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Exon</td>
<td>Codon</td>
</tr>
<tr>
<td>1</td>
<td>5</td>
<td>151</td>
</tr>
<tr>
<td>2</td>
<td>5</td>
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<tr>
<td>3</td>
<td>6</td>
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<tr>
<td>4</td>
<td>6</td>
<td>194</td>
</tr>
<tr>
<td>5</td>
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<td>248</td>
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<tr>
<td>6</td>
<td>7</td>
<td>259</td>
</tr>
<tr>
<td>7</td>
<td>8</td>
<td>281</td>
</tr>
</tbody>
</table>

* The mutations have been described previously (21).

Table 2  Clinical characteristics of 199 patients with clinically indolent or aggressive lymphomas

<table>
<thead>
<tr>
<th>Variable</th>
<th>Non-Δp53 (n = 157)</th>
<th>Δp53 (n = 42)</th>
<th>P</th>
</tr>
</thead>
<tbody>
<tr>
<td>Indolent NHL</td>
<td>(n = 59)</td>
<td>(n = 13)</td>
<td>0.76</td>
</tr>
<tr>
<td>Age (&gt;60)</td>
<td>0.65</td>
<td></td>
<td></td>
</tr>
<tr>
<td>PS (2–4)</td>
<td>1.00</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Stage (II/IV)</td>
<td>0.83</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Extralateral sites (&gt;1)</td>
<td>0.60</td>
<td></td>
<td></td>
</tr>
<tr>
<td>LDH (≥1 × normal)</td>
<td>0.30</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Aggressive/very aggressive NHL</td>
<td>(n = 98)</td>
<td>(n = 29)</td>
<td></td>
</tr>
<tr>
<td>Age (&gt;60)</td>
<td>0.09</td>
<td></td>
<td></td>
</tr>
<tr>
<td>PS (2–4)</td>
<td>0.26</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Stage (II/IV)</td>
<td>0.25</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Extralateral sites (&gt;1)</td>
<td>0.38</td>
<td></td>
<td></td>
</tr>
<tr>
<td>LDH (≥1 × normal)</td>
<td>1.00</td>
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</tr>
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NHL, unless stated otherwise. Factors independently affecting CR, OS, and RFS were identified using a Cox proportional hazards regression model (Ref. 32; P ≤ 0.10 for entry into model, P ≤ 0.10 to remain in model) stratified by grade. All P values are two-sided and considered significant if <0.05. SPSS for Windows, version 8.0.0 (SPSS Ltd., Chicago, IL), was used for all calculations.

RESULTS

Association of p53 Gene Mutations with p53 and p21Warf1 Expression. The seven mutations found in the p53 gene in 7 of the 34 primary DLCLs analyzed were single nucleotide missense mutations (Table 1) and were described previously (21). The results of immunostaining of these seven tumors are shown in Table 1. On the basis of this, we wanted to establish which p53/p21Warf1 phenotype predicted p53 gene mutation status best. All mutated tumors would be included if a cutoff value for p53 of >5% was chosen. However, 8 of 27 of the remaining nonmutated tumors would also be included. A cutoff value of ≥20% excluded nonmutated tumors, but left out two of seven mutated tumors. To distinguish between mutated and nonmutated tumors with p53 expression ranging from 6–20%, p21Warf1 expression was very helpful. Mutated tumors, and only mutated tumors in this group, were p21Warf1-negative. An immunophenotype satisfying at least one of two criteria (p53 ≥20% or a combination of p53 >5% and negative p21Warf1), Δp53, showed both 100% specificity and 100% sensitivity in predicting p53 gene mutations in DLCL.

Characteristics of Patients with Δp53. We applied these criteria to all 199 patients and found Δp53 in 42 cases (21%), distributed as follows: chronic lymphocytic lymphoma, 1 case (7%); immunocytoma, 3 cases (17%); FCL, 8 cases (28%); extranodal marginal zone lymphoma, 5 cases (25%); mantle cell lymphoma, 1 case (7%); DLCL, 16 cases (31%); peripheral T-cell lymphoma, 4 cases (14%); anaplastic large cell lymphoma, 2 cases (25%); and very aggressive lymphomas, 2 cases (13%). There were no differences in proportions of Δp53 cases when comparing indolent B-NHL (18%), aggressive B-NHL (28%), aggressive T-NHL (16%), and very aggressive NHL (13%; P = 0.26) and when comparing B-cell (23%) with T/Null-cell (16%) NHL (P = 0.41). The IPI clinical pretreatment characteristics of Δp53 and non-Δp53 patients are compared in Table 2. There was no difference in proportions of Δp53 cases allocated to each IPI risk group (P = 0.66). Furthermore, for both indolent B-NHL (P = 0.25), aggressive B-NHL (P = 0.65), and aggressive T-NHL (P = 0.63) the proportions of patients treated with either combination chemotherapy, single alkylating agents, or other treatments were similar for Δp53 cases and non-Δp53 cases.

There was a significant difference in CR rate between Δp53 and non-Δp53 cases among patients with aggressive and very aggressive stage III or IV NHL receiving combination chemotherapy (n = 54). Of the non-Δp53 patients in this group, 64% (29 of 45) achieved CR, whereas CR was achieved in only 22% (2 of 9) of patients with Δp53 (P = 0.03). Also, in advanced stage indolent B-NHL treated with combination chemotherapy (n = 23), p53 was associated with failure to achieve CR because 0 of 5 patients with Δp53 obtained CR as opposed to 10 of 18 non-Δp53 patients (P = 0.05). Furthermore, analyzing all combination chemotherapy-treated stage III/IV
patients solely, Δp53 (RR = 3.8; \( P = 0.001 \)) and high PS (RR = 2.7; \( P = 0.007 \)) remained significantly associated with treatment failure in multivariate analysis with the IPI factors.

**Analysis of Survival.** Δp53 status was significantly associated with poor OS in our study population (\( P = 0.0001 \); Fig. 1). When considering the different clinical grades of NHL, differences emerge. The association of Δp53 with poor survival is highly significant in aggressive T-NHL (\( P = 0.000006; P = 0.0001 \) if stratified by peripheral T-cell lymphoma versus anaplastic large cell lymphoma), but also significant in lymphomas not included in this group (\( P = 0.03 \)). For both B-NHL (\( P = 0.04 \)) and T/Null-NHL (\( P = 0.000002 \)), p53 was associated with shorter survival. Multivariate analysis of OS of all patients with the IPI prognostic factors, treatment (combination chemotherapy versus single alkylating agent versus other treatments), and Δp53 in a Cox model showed that Δp53 maintained prognostic significance (Table 3). Furthermore, in Cox analysis excluding the stratum of aggressive T-NHL, where Δp53 is highly significant, Δp53 maintained independent prognostic significance (RR = 1.7; \( P = 0.05 \)).

The significance of Δp53 was not uniform among the IPI risk groups. Δp53 was unrelated to OS in the high-intermediate and high-risk IPI groups, whereas Δp53 significantly predicted poor survival in the low-intermediate-risk group (\( P = 0.00002; 13 \) of 65 patients had Δp53; Fig. 2). The very aggressive NHL group was not included in this analysis because none of the cases in this IPI risk group had the Δp53 phenotype. Looking only at aggressive B- and T-NHL in this risk group showed that all 8 patients with Δp53 died within 1 year, whereas the estimated 1-year survival rate for the 28 non-Δp53 patients was 64% and the median OS was 36 months (SE = 8.1 months). Comparison of OS of the 8 Δp53 patients from the low-intermediate-risk IPI group with all 21 patients—regardless of p53 status—in the high-intermediate-risk group showed that the 8 Δp53 patients had significant shorter survival (\( P = 0.04 \)), indicating that Δp53 status at least abolished the inherent difference between these two risk groups.

RFS of 64 patients with combination chemotherapy-treated indolent or aggressive NHL was shortened for patients with Δp53 (\( P = 0.02 \)). Multivariate Cox analysis of Δp53 and the IPI factors showed independent value of Δp53 in predicting RFS (RR = 3.2; \( P = 0.008 \)).

**DISCUSSION**

This study of a population-based group of NHL patients is the first to show that an immunohistochemical phenotype indicative of altered p53 function, Δp53, is independently associated with poor outcome in both B- and T-cell NHL. Δp53 predicts poor OS in indolent and aggressive NHL and, furthermore, is associated with treatment failure and shortened RFS.

Previous studies have also linked p53 with prognosis in selected NHL entities (20, 33, 34). However, only in aggressive B-cell NHL has an independent prognostic value of p53 gene mutation been demonstrated (15). The approach used was to identify p53 gene mutations in fresh tissue using a PCR-based single-strand confirmation polymorphism assay, followed by direct sequencing. Our approach of immunohistochemistry on paraffin-embedded formalin-fixed tissue has several advantages.
Immunohistochemistry is inexpensive, nonradioactive, non-labor-intensive, and readily applicable in routine diagnostic laboratories. Reproducibility of the immunostaining was ensured by using a semiautomated staining instrument. Furthermore, our method does not require fresh tissue, making it feasible in cases where only fixed tissue is available.

Studies correlating p53 gene mutation with p53 (and, in some reports, p21Waf1) expression in NHL show that a stronger correlation with p53 gene mutation is obtained with the DO-7 (20, 33–37) or DO-1 (18, 38) anti-p53 antibodies as compared with studies of other antibodies (39–41). p21Waf1 expression is helpful in predicting the functional status of p53, but it is not an absolute measure. Patient 2 (Table 1) expressed high levels of p21Waf1 (42) because this particular apoptosis-disruptive mechanism does not require fresh tissue, making it feasible in cases where only fixed tissue is available. The optimal cutoff values for p53 and p21Waf1 expression vary among laboratories (18, 20, 33–38). This reflects differences in fixation and processing of samples. It is, therefore, important for a laboratory introducing this immunohistochemical assay to establish which conditions are the most predictive in the particular laboratory setting, preferably by analyzing a sample of tumors with known p53 gene status.

An underlying assumption in our extrapolation from p53 gene mutation in DLCL to other NHL entities is that the types of mutations and their association with protein expression are comparable throughout the lymphoma entities. Previous studies justify this assumption (18, 20, 33–36, 38). A review of the 113 published p53 gene mutations with data on p53 expression using DO-7 or DO-1 antibodies available showed that among the 67 mutations identified in DLCL only 7 (10%) were not missense mutations or other mutation types with p53 expression. p53 status should be considered when planning and analyzing clinical trials. To significantly improve the treatment of patients with disrupted p53 function, it is imperative that alternative treatment strategies, not relying on p53-dependent apoptosis, are developed.

Table 3  Results of multivariate analysis, stratified by clinical grade, of OS and RFS

<table>
<thead>
<tr>
<th>Variable</th>
<th>RR</th>
<th>95% CI</th>
<th>P</th>
</tr>
</thead>
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<tr>
<td>OSa</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Age (≥60)</td>
<td>2.1</td>
<td>1.4–3.3</td>
<td>0.0004</td>
</tr>
<tr>
<td>Stage (III/IV)</td>
<td>1.8</td>
<td>1.2–2.8</td>
<td>0.004</td>
</tr>
<tr>
<td>PS (2–4)</td>
<td>2.9</td>
<td>1.9–4.4</td>
<td>&lt;0.0001</td>
</tr>
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<td>LDH high</td>
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<td></td>
<td></td>
</tr>
<tr>
<td>Extranodal sites (&gt;1)</td>
<td>0.15</td>
<td></td>
<td></td>
</tr>
<tr>
<td>p53 disrupted</td>
<td>2.2</td>
<td>1.4–3.4</td>
<td>0.001</td>
</tr>
<tr>
<td>Treatment</td>
<td>0.95</td>
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<td></td>
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<tr>
<td>RFSa</td>
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<td></td>
<td></td>
</tr>
<tr>
<td>Age (≥60)</td>
<td>0.53</td>
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</tr>
<tr>
<td>Stage (III/IV)</td>
<td>0.89</td>
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<td>PS (2–4)</td>
<td>3.3</td>
<td>1.5–7.1</td>
<td>0.002</td>
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<td>LDH high</td>
<td>0.47</td>
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<td>Extranodal sites (&gt;1)</td>
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<tr>
<td>p53 disrupted</td>
<td>3.5</td>
<td>1.1–11.5</td>
<td>0.04</td>
</tr>
</tbody>
</table>

a PS, as defined by Eastern Cooperative Oncology Group.
b CI, confidence interval.
c All patients (n = 193).
d Anthracyclin-treated aggressive lymphoma patients (n = 61).

Fig. 2  OS of the 65 indolent and aggressive lymphoma patients in the low-intermediate-risk IPI group according to p53 status. The 13 patients with altered p53 status had a significantly poorer outcome as compared with the 52 patients with a normal p53 phenotype (P = 0.00002). The number of patients at risk at time 0 and 30 and 60 months is shown for each group.

Disruption of p53-dependent apoptosis could contribute significantly to the poor prognosis of NHL with Δp53. Previous studies have shown that apoptosis induction, efficacy of anticancer agents of different classes, and radiotherapy are reduced in cell lines and tumors with p53 gene mutations (44–46), including Burkitt’s lymphoma cell lines (47, 48). An exception from this pattern is provided by antimitotic agents, the apoptosis-inducing capacity of which is not affected by p53 gene status (46, 49, 50). These results are in agreement with the finding in this and other studies (15, 35) of an association between p53 malfunction and treatment failure in NHL. This association is not absolute and, therefore, suggests that in cases with wild-type p53 and poor response to treatment apoptosis is circumvented by defects either of other components of the p53 pathway or of p53-independent apoptosis-inducing pathways. Furthermore, our finding of the adverse clinical impact of Δp53 being present primarily in the low-intermediate-risk IPI group and not in the higher risk groups, which is in agreement with a previous study (15), suggests that the tumors in the higher-risk groups have accumulated sufficient genetic changes to make p53 disruption less important.

These results must necessarily be confirmed on larger prospective series of NHL patients, making analysis of clinical impact of p53 status in individual NHL entities possible. Our finding, however, of Δp53 as an independent marker of poor prognosis and treatment failure, even abolishing the difference between the low-intermediate- and high-intermediate-risk groups in aggressive NHL, suggests that p53 status could be important to consider when treatment strategies for individual patients are selected. Likewise, p53 status should be considered when planning and analyzing clinical trials. To significantly improve the treatment of patients with disrupted p53 function, it is imperative that alternative treatment strategies, not relying on p53-dependent apoptosis, are developed.

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