Immunohistochemical Detection of p53, bcl-2, and Retinoblastoma Proteins in Follicular Lymphoma

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Mutations of p53 have been suggested to be involved in the histologic transformation of follicular lymphoma, but the role of the retinoblastoma (RB) gene, another tumor suppressor gene, in lymphomagenesis has not been established. To determine the roles of these tumor suppressor genes and their relationship with the anti-apoptotic bcl-2 gene in follicle center lymphoma, the immunohistochemical expression of p53, bcl-2, and RB proteins was correlated with cytologic grade in 50 cases of follicular lymphoma, and the results were compared to 23 cases of diffuse large B-cell lymphoma. The results showed that only 2 of 25 grade 1 follicular lymphoma were p53-positive compared to 16 of 25 grade 3 cases (P < .0001). A significantly lower number (13 of 25) of grade 3 follicular lymphomas expressed bcl-2 compared to grade 1 cases (23 of 25) (P < .004). Eight of 14 bcl-2-negative follicular lymphomas expressed p53, compared with 10 of 36 bcl-2-positive cases (P = .1). Twenty-four of 25 grade 3 follicular lymphomas showed 2+ to 3+ staining for RB protein compared to 9 of 21 grade 1 cases (P < .0002). Expression of p53 protein correlates significantly with higher cytologic grade in follicular lymphoma. Similar to earlier studies of breast cancer and lymphoma, there appears to be an inverse relationship between p53 and bcl-2 expression in follicular lymphoma. Inactivation of the retinoblastoma gene does not seem to be involved in the histogenesis of follicle center lymphoma or diffuse large B-cell lymphoma. (Key words: p53; bcl-2; Retinoblastoma protein; Apoptosis; Programmed cell death; Cell cycle; Lymphoma; Follicle center; Cytologic grade) Am J Clin Pathol 1996;105:538-543.

p53 is a tumor suppressor gene located on the short arm of chromosome 17, which encodes a nuclear phosphoprotein involved in the control of the entry of cells into S phase. Depending on the cell type, p53 can induce growth arrest or apoptosis (reviewed in reference 1). Mutations of p53 have been described in a large variety of malignancies and have been found primarily in aggressive neoplasms.1-7 Compared to the wild-type protein, the mutated p53 proteins often have a longer half-life, which results in their accumulation in the cell's nucleus and allows for their detectability by immunohistochemistry.8-10 Recent studies have suggested a role of p53 mutations in the histologic progression of follicular non-Hodgkin's lymphoma to diffuse large cell type.11,12 However, within the follicular lymphomas, it is unclear whether there is an association between immunohistochemical detection of p53 protein and higher cytologic grades.

Furthermore, although recent studies in breast cancer have suggested an inverse relationship between mutant p53 expression and the anti-apoptotic bcl-2 protein,13,14 similar studies in hematopoietic neoplasms have been limited to high grade B-cell lymphomas,15 or have compared across different histologic subtypes of non-Hodgkin's lymphoma.16 A review of earlier studies shows that the frequency of bcl-2 gene rearrangement or expression is not uniform among the follicular lymphomas, but appears to decrease with higher cytologic grade.17-20 Whether there is an inverse relationship between expression of the anti-apoptotic bcl-2 gene and the p53 tumor suppressor gene in follicular lymphomas has not been established.

In addition, although inactivation of the retinoblastoma (RB) gene, another tumor suppressor gene, has been associated with tumorigenesis in many tumor types,21,22 its role in lymphomagenesis has not been well characterized.

In this study, we correlated the immunohistochemical detection of p53, bcl-2, and RB proteins with cytologic grade in follicular center lymphoma. To evaluate the possibility that grade 3 follicular center lymphoma may...
be an intermediate stage between lower grade follicle center lymphomas and the higher grade diffuse large B-cell lymphoma, 23 cases of the latter were also subjected to the same analysis. Lastly, we examined the possibility of an interrelationship between \( bcl-2 \) and p53 expression and expression of the RB protein.

**MATERIALS AND METHODS**

**Materials**

We selected 25 cases of follicle center lymphoma, follicular, grade 3, and 25 cases of follicle center lymphoma, follicular, grade 1, which were seen in the Department of Pathology at the Massachusetts General Hospital between 1977 and 1994 and in which paraffin blocks were available. For comparison, we randomly retrieved 23 cases of diffuse large B-cell lymphoma seen in our department between 1990 and 1993. Routine histologic sections of each case were reviewed to confirm the diagnosis. Equivocal cases were reviewed with a senior hematopathologist (NLH), and final histologic subclassification was according to the Revised European-American Classification of lymphoid neoplasms, with cytologic grading of follicular lymphoma according to Mann and Berard.24

**Immunohistochemistry**

Paraffin sections of all cases were immunostained with monoclonal antibodies against \( bcl-2 \) (Dako, Carpinteria, CA; clone 124; 1:40 dilution) and p53 (Novocasta, Newcastle-upon-Tyne, UK; clone DO7, which recognizes both wild-type and mutant p53 proteins; 1:50 dilution) using the avidin-biotin-peroxidase technique.25 For detection of \( bcl-2 \) protein, the sections were developed with \( \text{H}_2\text{O}_2 \) and 3-amino-9-ethylcarbazole (Aldrich Chemical, Milwaukee, WI) and lightly counterstained in Gill's hematoxylin before microscopic examination. The reaction products for p53 protein were developed with \( \text{H}_2\text{O}_2 \) and 3-amino-9-ethylcarbazole (Aldrich Chemical, Milwaukee, WI) and lightly counterstained in Gill's hematoxylin before microscopic examination. In both grades of follicle center lymphoma, the

| Table 1. Immunohistochemical Detection of p53 in Follicular Lymphoma and Diffuse Large B-Cell Lymphoma |
|----------------------------------------|--------|--------|--------|
| Follicle center lymphoma, follicular grade 1 (\( n = 25 \)) | p53− | p53+ (rare) | p53++ |
| Follicle center lymphoma, follicular grade 3 (\( n = 25 \)) | 9 | 11 | 5 |
| Diffuse large B-cell lymphoma (\( n = 23 \)) | 9 | 4 | 10 |

considered to have lost RB function.26 When there was nuclear staining for RB protein, the following scale was used: tumors were scored as 1+ for RB protein expression if there was nuclear staining in less than one-third tumor cells, 2+ if one-third to two-thirds tumor cells showed nuclear staining, and 3+ if more than two-thirds tumor cells showed nuclear staining.

**Statistics**

Comparison of differences was by Fisher's exact test. \( P \) values at less than 0.05 were interpreted to be statistically significant.

**RESULTS**

**Follicle Center Lymphoma, Follicular (Follicular Lymphoma)**

**Immunohistochemical detection of p53.** In all, 18 of 50 follicular lymphomas, grades 1 and 3, showed nuclear staining for p53 protein. Sixteen of 25 (64%) grade 3 cases were p53-positive (14 cases with rare/1+ staining, 2 with 2+ staining) compared to 2 of 25 (8%; 1 with rare/1+ and 1 with 2+ staining) grade 1 cases (\( P < 0.0001 \)) (Table 1, Fig. 1).

\( bcl-2 \) protein. A significantly greater number of grade 1 cases expressed \( bcl-2 \) compared to grade 3 cases (23 of 25, 92% vs. 13 of 25, 52%; \( P < .004 \)) (Fig. 2).

**Retinoblastoma protein.** In four cases of grade 1 follicular lymphoma in which only B5-fixed tissue was available, staining was technically suboptimal. These cases were excluded from analysis. None of the studied lymphomas showed loss of RB function as measured by absent RB nuclear staining. RB protein expression was detected in a larger proportion of the tumor cells in grade 3 than in grade 1 follicular lymphoma: 24 of 25 (96%) grade 3 follicular lymphomas showed 2+ to 3+ nuclear staining with the RB-WL-1 antibody compared to only 9 of 21 (43%) grade 1 follicular lymphomas (\( P < 0.0002 \)) (Fig. 3). In both grades of follicle center lymphoma, the

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**FIG. 1.** Immunohistochemical detection of p53 protein with the DO7 antibody (Novocastra, Newcastle-upon-Tyne, UK). Images (A) and (B) show rare/1+ staining for p53 in a grade 1 follicle center lymphoma, follicular, and in a grade 3 follicle center lymphoma, follicular, respectively. 2+ positive staining for p53 is more frequently seen in grade 3 follicular lymphoma (C) and in diffuse large B-cell lymphoma (D) (counterstained with hematoxylin, ×160).

**FIG. 2.** Positive immunostaining for bcl-2 is shown in a grade 1 follicular lymphoma (A) and in a grade 3 follicular lymphoma (B). In contrast, image (C) illustrates staining of only rare tumor cells for bcl-2 protein (arrows) in a grade 3 follicular lymphoma which is interpreted to be negative (counterstained with hematoxylin, ×160).

**FIG. 3.** With the polyclonal antibody RB-WL-1 against retinoblastoma protein, a significantly higher proportion of grade 1 follicular lymphoma showed 1+ (A) staining compared to grade 3 follicular lymphoma, which more often showed 2+ (B) or 3+ staining (C). Note that the more intense staining is often seen in the larger centroblasts (counterstained with hematoxylin, ×160).
A greater intensity of staining was seen in the larger lymphoid cells (centroblasts).

**Association of p53 with bcl-2 and RB protein staining.**

Of the 14 bcl-2-negative follicular lymphomas, 8 showed accumulation of p53 protein (57%) compared to only 10 of 36 (28%) bcl-2-positive cases (Table 2). This difference approached but did not reach statistical significance (P = .1). With respect to RB status, 14 of 15 (93%) bcl-2-negative follicular lymphomas expressed 2+ to 3+ RB protein staining pattern compared to 19 of 31 (61%) bcl-2-positive follicular lymphomas (P <.05) (Table 2).

Of note, follicular lymphomas that stained negatively for p53 also tended to express less RB protein: only 16 of 28 p53-negative follicular lymphomas showed a 2+ to 3+ RB protein staining pattern compared to 17 of 18 p53-positive follicular lymphomas (P <.01) (Table 2).

**Diffuse Large B-Cell Lymphoma**

Of 23 diffuse large B-cell lymphomas, 14 showed positive staining for p53 (4 with rare/1+ nuclear staining, and 10 with 2+ staining pattern), 9 expressed bcl-2 protein, and 18 showed 2+ to 3+ RB protein staining (Table 1). Compared to grade 1 follicular lymphoma, a significantly higher proportion of diffuse large B-cell lymphomas expressed p53 protein (63% vs. 8%; P <.0003), and showed 2+ to 3+ staining for RB protein (78% vs. 43%; P <.04). Only 39% of diffuse large B-cell lymphomas expressed bcl-2 protein, in contrast to 92% of grade 1 follicular lymphomas (P <.0003).

There were no statistically significant differences between the diffuse large B-cell lymphomas and grade 3 follicular lymphomas with respect to p53, bcl-2, and RB protein expression. However, of the p53-positive cases, a greater number of grade 3 follicular lymphomas showed only rare positive cells, compared to diffuse large B-cell lymphoma, although this difference did not reach statistical significance (11 of 16 vs. 4 of 14; P <.07).

**DISCUSSION**

Previous studies have suggested a role of p53 mutations in the histogenesis of high grade non-Hodgkin's lymphoma and in the histologic progression of follicular lymphoma to diffuse large B-cell lymphoma. In follicular lymphoma, the reported frequency of immunohistochemical detection of p53 in various studies has ranged from 0% (in follicular small cleaved or mixed) to 33% (unspecified grades). Because the number of follicular lymphomas in those studies were small, ranging from 6 to 17, it has not been possible to characterize the role of p53 mutations in this particular histologic subtype of B-cell lymphoma. To our knowledge, the present study is the first to demonstrate a significant correlation between immunohistochemical detection of p53 protein and higher cytologic grade in follicular lymphoma. Although immunohistochemical detection of p53 protein is not entirely specific for mutations in this tumor suppressor gene, our results suggest that p53 mutations may play a role in the histogenesis of grade 3 follicle center lymphoma.

Furthermore, this higher frequency of p53 protein expression in grade 3 follicular lymphoma was accompanied by a significantly lower frequency of bcl-2 expression when compared to grade 1 cases. Only 10 of 36 (28%) bcl-2-positive follicular lymphomas expressed p53 compared to 8 of 14 (57%) bcl-2-negative cases. In an earlier study, Pezzella and colleagues reported expression of p53 protein in 2 of 3 bcl-2-negative follicular lymphomas in contrast to 2 of 14 (14%) bcl-2-positive follicular lymphomas. Thus, although the difference in the present study did not reach statistical significance, our findings, together with those reported by Pezzella and colleagues, would suggest that there may be an inverse relationship between p53 mutations and expression of the anti-apoptotic bcl-2 in follicle center lymphoma. In an earlier study in breast cancer cells, Haldar and coworkers demonstrated a similar inverse relationship between mutant p53 expression and bcl-2 protein. In addition, based on a series of experiments in which eukaryotic vectors carrying mutant p53 were transfected into several breast cancer cell lines, the same authors also showed that overexpression of a mutant p53 could induce down-regulation of bcl-2, either by repressing transcription initiation, or by altering mRNA stability and
processing. The present study does not address whether the same mechanisms prevail in lymphomas, but the results suggest that the link between the tumor suppressor gene p53 and the anti-apoptotic bcl-2 may be a common phenomenon shared by different cell lineages in the regulation of programmed cell death.

The finding of rare tumor cells with positive immunohistochemical staining for p53 has been described by other investigators. Although these cases have often been assumed to be p53-negative, the actual biologic significance of this finding is uncertain. In the present study, if we assumed that rare cells staining for p53 were the same as true negative staining, then we would expect to find the normal pattern in both grade 1 and grade 3 follicular lymphomas. In fact, we found a significantly higher proportion of grade 3 follicular lymphomas with rare cells staining for p53 compared to grade 1 cases (11 of 20 vs. 1 of 23; \( P < .0004 \)). Although we cannot explain the significance of rare tumor cells expressing p53, our result suggests that such a staining pattern may be biologically significant and raises the possibility that it may indicate an emerging clone of p53-mutated cells. In support of this hypothesis is the observation by Sander and colleagues of a sequential increase in the number of rare cells staining for p53 in sequential cases of follicular lymphoma. Additional studies with clinical follow-up may help determine the significance of this pattern of rare cells staining for p53 in follicular lymphoma.

Interestingly, only 4 of 14 (29%) p53-positive diffuse large B-cell lymphomas exhibited the pattern of rare cells staining compared to 11 of 16 (69%) p53-positive grade 3 follicular lymphomas, suggesting that the latter might be intermediate between grade 1 follicular lymphoma and diffuse large B-cell lymphoma in the proportion of tumor cells with p53 protein expression. In addition, with respect to the frequency of bcl-2 and p53 protein expression, the grade 3 follicular lymphomas in the present study also appear to be intermediate between grade 1 cases (predominantly p53 negative, bcl-2 positive) and diffuse large B-cell lymphoma (predominantly p53 positive, bcl-2 negative).

None of the lymphomas in the present study showed loss of RB protein function. In fact, our results showed a higher level of RB protein expression in grade 3 follicular lymphoma than in grade 1 cases. This finding may initially seem somewhat unexpected because as a tumor suppressor gene, inactivation of the retinoblastoma gene is expected to result in loss of protein expression and has been associated with tumorigenesis or malignant transformation. However, there is evidence that nuclear ac-

cumulation of RB protein is cell cycle-dependent and appears to be very low in G0 to middle G1, compared to that seen in G2/M and S phase. An earlier study by Cordon-Cardo and associates has demonstrated that in normal lymph nodes, strong RB protein expression is seen in the "lymphoblastic" cells within the germinal centers. Together with our observation of more intense RB protein staining in centroblasts compared to centrocytes, our results suggest that expression of the RB protein in follicular lymphomas may be a function of the tumor cell cycle and may be related to the degree of centroblastic differentiation. Our results indicate that inactivation of the RB gene does not appear to be involved in the histogenesis of follicular lymphomas of higher cytologic grade.

In the present study, because grade 3 follicular lymphomas tended to be p53 positive, we were unable to establish whether the apparent association between high level of RB protein expression and p53 staining was independent of cytologic grade. Neither were we able to determine if the apparent inverse relationship between high levels of RB protein staining and bcl-2 expression was in fact independent of p53 expression, because most bcl-2-negative follicular lymphomas in the study also tended to be positive for p53. However, it is possible to postulate that because expression of the RB protein is a function of the cell cycle, dysregulation of the control of cell entry into S phase following mutations of p53 may have an effect on the expression of the RB gene in follicular lymphoma.

In summary, we report a significant correlation between immunohistochemical detection of p53 and cytologic grade in follicular lymphoma, suggesting that p53 mutations may play a role in the histogenesis of grade 3 follicular lymphoma. In contrast to diffuse large B-cell lymphoma where many of the p53-positive cases exhibited a 2+ staining pattern, many p53-positive grade 3 follicular lymphomas showed only rare p53-positive cells, suggesting that the latter are intermediate between grade 1 follicular lymphoma and diffuse large B-cell lymphoma in the degree of p53 abnormality. The inverse relationship between p53 and bcl-2 protein expression is similar to that described in other tumor types and suggests an interaction of these two factors in the regulation of programmed cell death. Expression of the RB protein in follicular lymphomas seems to be related to the number of centroblasts, but inactivation of the RB gene does not appear to play a role in the histogenesis of either high grade follicular lymphoma or diffuse large B-cell lymphoma.

REFERENCES


