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DNA Polymerase μ Gene Expression in B-Cell Non-Hodgkin's Lymphomas

An Analysis Utilizing *in Situ* Hybridization

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DNA polymerase μ (pol μ) is a novel error-prone DNA repair enzyme bearing significant structural homology with terminal deoxynucleotidyltransferase. Whereas other human error-prone DNA polymerases identified thus far show no preferential lymphoid tissue distribution, the highest levels of pol μ mRNA have been detected in peripheral lymphoid tissues, particularly germinal center B cells. Conceivably, up-regulation of the pol μ gene may be biologically significant in lymphomagenesis, especially in the development of B-cell non-Hodgkin's lymphomas (B-NHLs), because of enhanced error-prone DNA repair activities. To explore this possibility, we generated a digoxigenin-labeled riboprobe to pol μ mRNA and used the probe and *in situ* hybridization to examine the expression pattern of the pol μ gene in formalin-fixed, paraffin-embedded tissue sections of 37 B-NHLs. This included eight chronic lymphocytic leukemia/small lymphocytic lymphomas, six mantle cell lymphomas, seven follicular lymphomas, nine diffuse large B-cell lymphomas, three splenic marginal zone lymphomas, two Burkitt's lymphomas, and two precursor B-lymphoblastic lymphomas. We also correlated the pol μ mRNA expression levels with the tumor proliferation index, which was assessed in each case by image analysis of Ki-67 immunostained slides. Nineteen of 21 (90%) B-NHLs arising from postgerminal center B cells (follicular lymphomas, diffuse large B-cell lymphomas, splenic marginal zone lymphomas, and Burkitt's lymphomas) exhibited high expression of pol μ mRNA. In contrast, only 2 of 16 (13%) B-NHLs arising from pregerminal center B cells (chronic lymphocytic leukemia/small lymphocytic lymphomas, mantle cell lymphomas, and precursor B-lymphoblastic lymphomas) expressed significant levels of pol μ mRNA. Pol μ gene expression did not seem to correlate with the proliferation index, especially because a significant level of pol μ mRNA was not detected in either case of precursor B-lymphoblastic lymphomas. In conclusion, pol μ gene expression is highly associated with B-NHLs of postgerminal center B-cell derivation. Furthermore, the expression level is independent of the proliferation rate and thus is unrelated to the biological aggressiveness of the tumors. These findings, along with the error-prone nature of the enzyme, suggest that up-regulation of pol μ gene expression may be a contributing factor to the pathogenesis of a subset of B-NHLs through DNA repair-associated genomic instability. *(Am J Pathol 2002, 161:1349–1355)*

Numerous DNA polymerases that possess either replicative or reparative functions have been identified in mammalian cells. The integrity of genetic information depends not only on faithful replication and proofreading activity during DNA synthesis by replicative DNA polymerases, but also on cellular DNA repair enzymes capable of correcting mutations accumulated during DNA damage.[1] One well-characterized cellular DNA polymerase serving a specific role in DNA repair is DNA polymerase β (pol β), which is ubiquitously expressed among all eukaryotic cell types and is essential for base excision repair.[2] However, as in most reparative DNA polymerases, pol β acts in an error-prone manner, ie, in repairing DNA it introduces an enhanced mutational activity in the cells at a rate of 10^{-3} to 10^{-4} per bp per generation.[3] Such error-proneness intrinsic to mutator DNA polymerases, as exemplified by pol β, is thought to be evolutionarily essential for generating genetic heterogeneity.

Many mutator DNA polymerases other than pol β have also been identified within mammalian cells. Polymerase μ (pol μ) is a novel DNA repair enzyme recently isolated in parallel by Dominguez and colleagues[4] and Aoufouchi and colleagues.[5] Pol μ shares significant structural homology with terminal deoxynucleotidyltransferase (TdT), an enzyme with the unique function of randomly inserting nucleotides in the V-D and D-J junctions during the process of immunoglobulin and T-cell receptor gene rearrangement.[6] Unlike pol β and other error-prone DNA polymerases identified thus far, both TdT and pol μ ap-

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pear to be preferentially expressed in lymphoid tissues.[5,7] However, whereas TdT expression is restricted to primary lymphoid tissue, ie, thymus and bone marrow, the highest levels of pol μ mRNA have been detected in secondary lymphoid tissues such as lymph nodes and the spleen, although base levels have been detected in other tissue types as well.[4,5] In particular, the highest levels of pol μ mRNA have been detected within B lymphocytes of germinal center derivation, as demonstrated by both Northern blot analysis and *in situ* hybridization. These distinctive properties of pol μ, namely its error-proneness and preferential localization within peripheral lymphoid tissue B cells, suggest that it may play a role in B cell lymphomagenesis, perhaps by enhancing mutational activity and thereby contributing to genomic instability in these cells. To gain additional insight into this proposed biological role for pol μ, we investigated the pattern of pol μ mRNA expression in the principal clinicopathological categories of B-cell non-Hodgkin's lymphoma (B-NHL) using *in situ* hybridization and correlated the findings with our current understanding of B cell lymphomagenesis.

Materials and Methods

Specimens

Thirty-seven lymph node biopsy specimens containing B-NHL were selected from the files of the Immunopathology Laboratory of the Weill Cornell campus of the New York Presbyterian Hospital for inclusion in this study based on the following criteria: 1) the case was typical of a given Revised European American Classification of Lymphoid Neoplasms (REAL) nosological entity in terms of morphology and immunophenotype;[8] and 2) formalin-fixed, paraffin-embedded tissue blocks were available for the preparation of tissue sections for morphological evaluation, *in situ* hybridization studies, and determination of the proliferation index (PI). The 37 cases consisted of 8 chronic lymphocytic leukemia/small lymphocytic lymphomas (CLL/SLLs), 6 mantle cell lymphomas (MCLs), 7 grade I or II follicular lymphomas (FLs), 9 diffuse large B-cell lymphomas (DLBCLs), 3 splenic marginal zone lymphomas (SMZLs), 2 Burkitt's lymphomas (BLs), and 2 precursor B-lymphoblastic lymphomas (B-LBLs). Three human tonsils were also included as normal controls. Representative tissue samples were fixed in 10% formalin and embedded in paraffin, from which multiple 4-μm histological sections were prepared for the studies performed here.

RNA Isolation, Reverse Transcriptase-Polymerase Chain Reaction (PCR), and Cloning

Total RNA was extracted from 0.5 to 1.0 × 10^6 cells using the RNeasy total RNA kit (Qiagen Inc., Valencia, CA). Residual genomic DNA was eliminated by DNase I according to the manufacturer's protocol (Life Technologies, Inc., Rockville, MD). Total RNA (1.0 μg) was used for reverse transcription of the first strand cDNA using the SuperScript preamplification system with the oligo(dT) 12-18 primer (Life Technologies, Inc., NY). Pol μ cDNA was then amplified using novel primers flanking almost the entire sequence (~2.5 kbp) of pol μ (5′-CTGGAGGCTGTCGTCCCAATGCT; 3′-TGGTCTGGGAAATCCTCGCCTAA). Long-range PCR was performed using Elongase (Life Technologies, Inc.). Amplification was performed on an automated thermal cycler (GeneAmp PCR System 9700; PE Applied Biosystems, Foster City, CA) with an initial denaturation step at 94°C for 4 minutes, followed by 30 cycles at 94°C for 1 minute, 55°C for 1 minute, and 72°C for 1 minute and 30 seconds. The reaction was concluded by an extension step at 72°C for 7 minutes. The PCR products were resolved by 1% agarose gel electrophoresis, visualized by ethidium bromide staining, and subsequently purified using the QIAquick PCR purification kit (Qiagen, Inc., Valencia, CA). The purified PCR products were then cloned in the pCR4Blunt-TOPO vector using the Zero Blunt cloning kit (Invitrogen, Carlsbad, CA) following the manufacturer's instructions. The plasmids were then extracted and purified using the QIAPrep plasmid clean-up kit (Qiagen, Inc., Valencia, CA).

Riboprobe Preparation

Forward and reverse primers spanning a 227-bp nucleotide sequence specific to pol μ were designed, upstream to which a 22-bp nucleotide promoter for either T3 or T7 RNA polymerase was added, respectively (5′-TCAATTAACCCTCACTAAAGGGGTGGCTGGGCCAAGGAAG; 3′-GTAATACGACTCACTATAGGGCTGCAGCTGGCTCAGGGTT). The 227-bp sequence was amplified using 10 to 20 ng of purified plasmids containing the pol μ cDNA with the cycling condition of 94°C for 4 minutes, followed by 35 cycles at 94°C for 45 seconds, 55°C for 45 seconds, 72°C for 45 seconds, and a final extension at 72°C for 7 minutes. The PCR products were purified as described above and used as template to generate riboprobes. Both anti-sense and sense riboprobes were synthesized by *in vitro* transcription using T7 and T3 RNA polymerases (Roche, Mannheim, Germany), respectively, where digoxigenin 11-UTP (Roche) was incorporated following the manufacturer's recommendations.

In Situ *Hybridization*

Four-μm tissue sections prepared from cases of B-NHL and normal human tonsils (as controls; included with every run of *in situ* hybridization), were deparaffinized according to a standard protocol and digested with 10 to 50 μg/ml of proteinase K in 0.1 mol/L of Tris and 0.05 mol/L of ethylenediaminetetraacetic acid at 37°C for 30 minutes. The tissue sections were postfixed in 4% paraformaldehyde for 20 minutes. Prehybridization was performed in 50 to 100 μl of prehybridization buffer (2× standard saline citrate, 50% formamide, 250 μg/ml salmon sperm DNA, and 0.3% bovine serum albumin) at 42°C for 30 minutes. The tissue sections were then hybridized overnight at 42°C in 30 μl of hybridization buffer (2× standard saline citrate, 50% formamide, 10% dextran sulfate, 0.25% bovine serum albumin, 0.25% Ficoll,

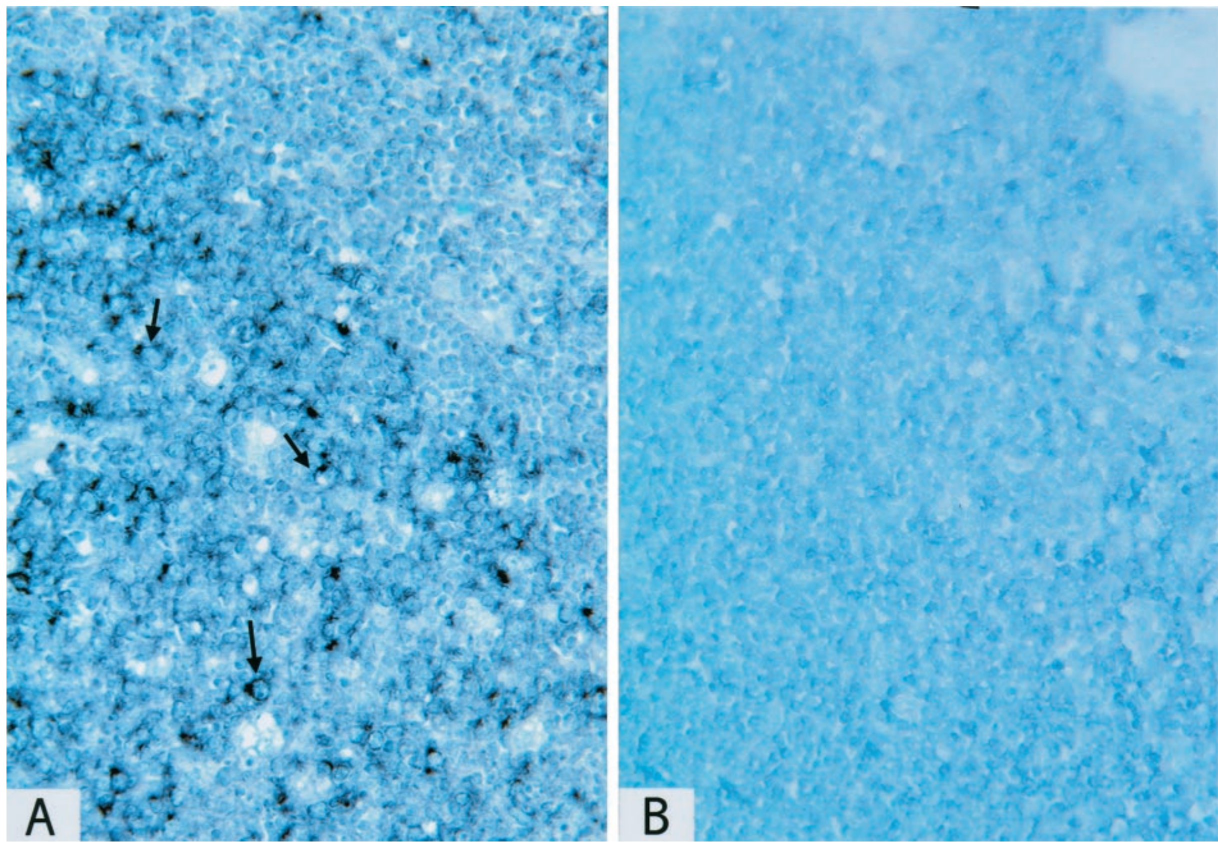

Figure 1. *In situ* hybridization of pol μ mRNA in tissue sections of human tonsil. Digoxigenin-labeled anti-sense (**A**) and sense (**B**) riboprobes were hybridized to human tonsil tissue sections, showing preferential expression within the germinal center using the anti-sense riboprobe as indicated by the dark purple-black staining. No comparable signal was detected using the sense riboprobe under similar conditions using a sequential tissue section.

0.25% polyvinyl pyrrolidone (PVP), 0.25 mol/L Tris, 2 mol/L sodium pyrophosphate, 0.5% sodium dodecyl sulfate, and 250 μg/ml salmon sperm DNA) and 1.5 μl of anti-sense riboprobe per section. Hybridization using sense riboprobes was performed under similar conditions as a negative control. The sections were first washed in 2× and then 1× standard saline citrate at 37°C for 10 minutes each. Detection was performed using anti-digoxigenin alkaline phosphatase (Roche) and 5-bromo-4-chloro-3-indolyl phosphate and nitro blue tetrazolium chloride (BCIP/NBT) as substrate (Invitrogen). The slides were counterstained in hematoxylin, coverslipped using Crystal Mount (Biomeda, Foster City, CA), and visualized under a light microscope. The intensity of the reaction was graded on a 0 to 4+ scale with 0 being negative and 4 being strongly reactive. The percentage of cells expressing pol μ mRNA was also assessed. A case was considered to be positive for pol μ mRNA expression when it demonstrated at least 2+ reactivity in at least 20% of the neoplastic cells.

PI Analysis

The PI was calculated in each B-NHL by image analysis of Ki-67 (Zymed, South San Francisco, CA) immunoperoxidase-stained and methylene-counterstained paraffin-embedded tissue sections using a CAS200 image analyzer and the quantitative nuclear antigen program (Becton Dickinson, Franklin Lakes, NJ) according to the manufacturer's recommendations.

Results

Tonsil tissue sections were included in every hybridization experiment. In each of these experiments intense pol μ mRNA expression was found within all tonsil germinal centers (Figure 1). Specifically, the highest expression was found within those germinal center cells possessing

Table 1. Polymerase μ mRNA and Proliferation Indices in B-NHLs

Diagnosis	No. + cases (%)	Mean PI	Range
B-LBL	0/2 (0%)	69.7	60.4–74.5
CLL/SLL	1/8 (13%)	18.5	5.0–43.7
MCL	1/6 (17%)	45.8	39.8–52.7
BL	2/2 (100%)	67.5	60.8–78.5
FL	6/7 (86%)	31.9	7.9–58.6
SMZL	3/3 (100%)	23.0	2.1–36.5
DLBCL	8/9 (89%)	50.9	21.7–66.7
Total	21/37 (57%)		

B-NHL, B-cell non-Hodgkin's lymphomas; No., number; PI, proliferation index; B-LBL, precursor B lymphoblastic lymphoma; CLL/SLL, chronic lymphocytic leukemia/small lymphocytic lymphoma; MCL, mantle cell lymphoma; BL, Burkitt's lymphoma; FL, follicular lymphoma (grade I or II); SMZL, splenic marginal zone lymphoma; DLBCL, diffuse large B cell lymphoma.

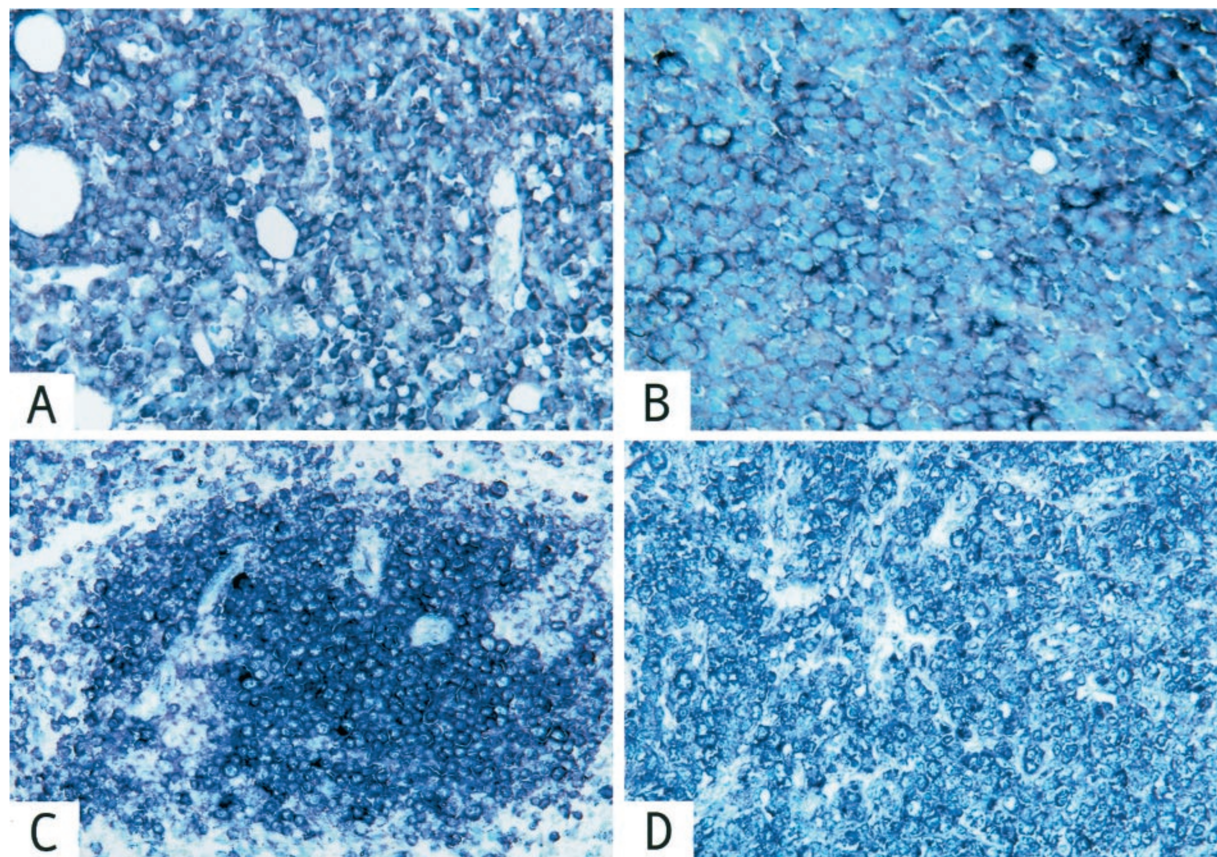

Figure 2. *In situ* hybridization of pol μ mRNA in tissue sections of BL (**A**), FL (**B**), SMZL (**C**), and DLBCL (**D**). These B-NHL subtypes displayed strong pol μ expression.

relatively abundant cytoplasm and vesicular nuclei containing multiple small nucleoli, ie, those exhibiting centroblastic morphology. The percentage of germinal center cells expressing pol μ mRNA, in which the intensity of expression was moderate to strong (3 to 4+), ranged from 30 to 50%. Low (0 to 1+) levels of pol μ hybridization signal were detected in a variable number of tumor cells in all cases of B-NHL. However, pol μ mRNA expression varied among the B-NHL categories, clearly differing between B-NHLs originating from pregerminal and postgerminal center B cells (Table 1). Specifically, 19 of 21 (90%) B-NHLs arising from postgerminal center B cells (FLs, DLBCLs, SMZLs, and BLs) expressed pol μ mRNA at high levels (Figure 2). Among these cases, both BLs and all three SMZLs expressed pol μ mRNA at intensity levels comparable to that of normal tonsils (3 to 4+), whereas most cases of FL (six of seven) and DLBCL (eight of nine) displayed somewhat weaker expression levels that typically fell in the range of 2 to 3+. In contrast, only 2 of 16 (13%) B-NHLs of pregerminal B cells (CLL/SLLs, MCLs, and B-LBLs) expressed significant levels of pol μ mRNA, including one of eight cases of CLL (2 to 3+ by 50 to 60% of the tumor cells) and one of six cases of MCL (2 to 3+ by 20 to 30% of the tumor cells) (Figure 3). Hybridization using sense riboprobes under the same experimental conditions did not produce comparable signals. Pol μ gene expression did not appear to correlate with the PI, especially because neither case of B-LBL significantly expressed pol μ mRNA. Also, strong expression was found in all three cases of SMZL, in which the PIs ranged from 2.1 to 36.5 (mean, 23.0).

Discussion

In this study, we have confirmed previously published findings that pol μ mRNA is expressed at high levels in germinal center cells.[4,5] In addition, we demonstrated the strong association of pol μ mRNA expression with B-NHLs of postgerminal center B cell derivation. Specifically, both cases of BL, all three cases of SMZL, and the vast majority of the FLs (six of seven) and DLBCLs (eight of nine) studied here expressed high levels of pol μ mRNA. The two pol μ-negative cases, one FL and the other DLBCL, did not display any obvious atypical clinical and pathological features. Conceivably, the lack of apparent pol μ mRNA expression in these cases may be attributable to the presence of splice variants, which actually represent the dominant species of pol μ mRNA (~90%) occurring naturally in B cells.[5] Most of the variations occur in exons 5 to 11, which are situated outside the region targeted by the riboprobe used in this study. It is possible that the alternate splice variants involved the region covered by the riboprobes in the negative cases.

In contrast, most neoplasms of pregerminal B cells did not show significant pol μ mRNA expression. Neither of

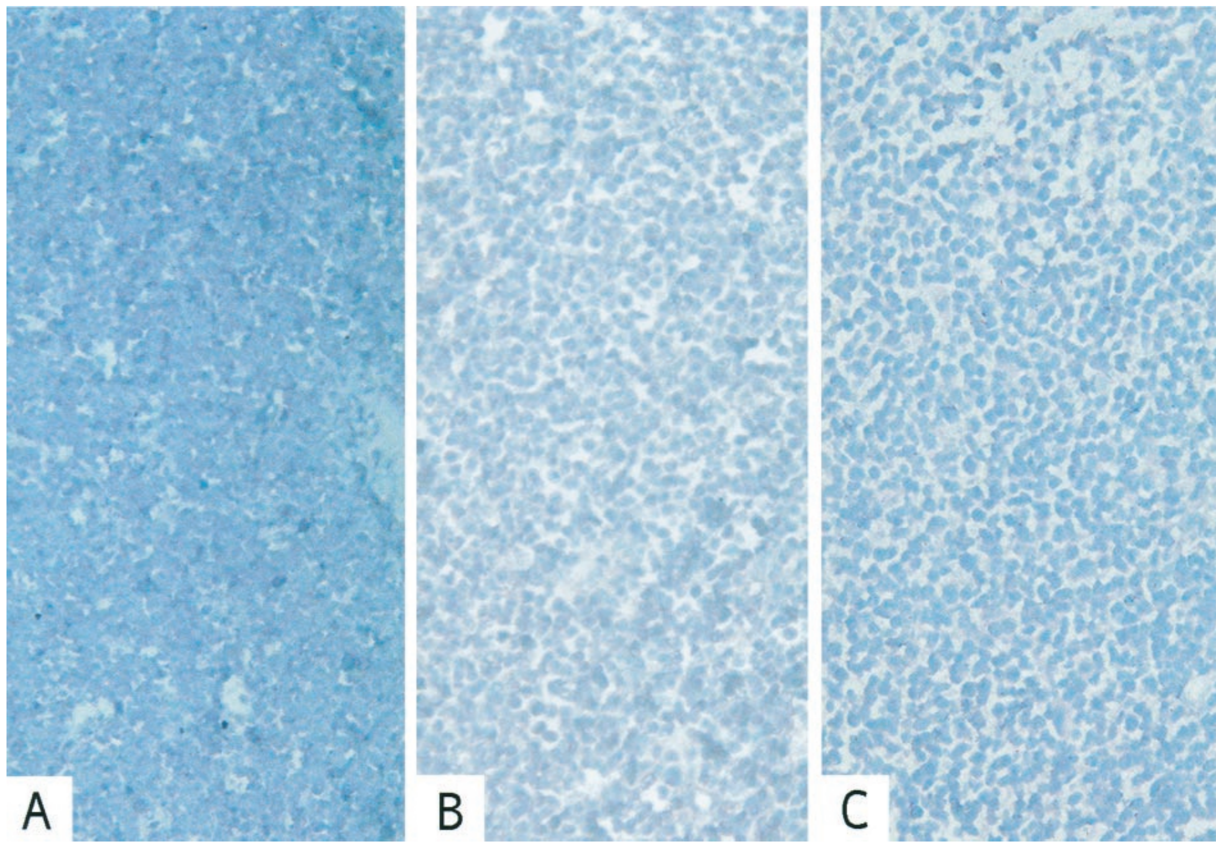

Figure 3. *In situ* hybridization of pol μ mRNA was performed under similar conditions in tissue sections of B-LBL (**A**), CLL/SLL (**B**), and MCL (**C**). Low to undetectable levels of pol μ expression were observed in these diagnostic categories.

the two cases of B-LBL and only one of eight and one of six cases of CLL/SLL and MCL, respectively, expressed pol μ mRNA. Perhaps exposure to the germinal center and thus somatic hypermutation had taken place in the positive case of CLL/SLL, but further studies are needed to distinguish pol μ expression levels between those CLL/SLL cases with and those without immunoglobulin variable region gene mutations. Of interest, the solitary positive case of MCL was also the only one that displayed overexpression of p53 protein as demonstrated by immunohistochemistry, suggesting that blastoid transformation had occurred in this case. Perhaps pol μ may somehow have been up-regulated in this case, contributing to the transformation, although this observation will also need to be confirmed with further studies.

The germinal center represents an exceedingly dynamic environment that creates intense genomic instability among B cells. The process of somatic hypermutation generates mutations in the variable region of the immunoglobulin heavy chain gene at the high rate of ~1/1000 bp per generation, thereby creating vast intraclonal diversity in the antigen-binding sites of immunoglobulins from only a few precursor cells.[9,10] Somatic hypermutation may also introduce genetic alterations outside the immunoglobulin loci; it has been recently shown that the gene encoding for BCL-6, a nuclear zinc-finger transcription factor expressed by both centroblasts and centrocytes, also undergoes somatic mutation in normal germinal center B cells.[11]

However, the precise mechanism accounting for somatic hypermutation is not well understood. Studies involving transgenic mouse models indicate that somatic hypermutation and transcription are intimately linked and likely involve DNA repair processes. This conclusion is based on several observations. First, the mutation domain spans a region ~1-kb downstream from the promoter site. In addition, it has been documented that transcriptional enhancers are necessary for mutation targeting, whereas mutation frequencies correlate with transcriptional levels. Lastly, insertion of a transcriptional promoter element upstream of an immunoglobulin constant region, which is normally spared from significant mutations, is also able to generate a new mutation domain downstream.[12–16]

Given these observations and the error-prone nature of mutator DNA polymerases, it is likely that these enzymes are recruited to create immunoglobulin diversification by introducing mutations.[17] Various mutator DNA polymerases have been proposed as candidates for the somatic hypermutase, including the ubiquitously expressed pol β.[18] However, knockout experiments involving mice deficient in pol β have excluded the role of pol β in hypermutation.[19] More recently, it has been shown that DNA polymerase zeta (pol ζ) plays a major role in immunoglobulin and BCL-6 somatic hypermutation.[20] Pol ζ is a translesion DNA polymerase, ie, it performs DNA synthesis bypassing lesions, with relatively high fidelity compared to error-prone polymerases. Pol ζ has been pro-

posed to perform somatic hypermutation in concert with an error-prone polymerase, in which pol ζ extends DNA past a lesion most effectively after the insertion of one or two mismatched nucleotides by an error-prone polymerase.

Regardless of the precise mechanism for somatic hypermutation, which remains speculative, the germinal center reaction represents a highly volatile environment generating intense genomic instability among B cells. It is highly likely that such an environment may lead to the genetic alterations responsible for malignant transformation in at least a subset of B-NHLs. There are several characteristics of pol μ that led us to hypothesize that this enzyme may represent an important molecular mechanism contributing to this genomic instability leading to B cell lymphomagenesis.

The human gene encoding for pol μ has been mapped within chromosomal band 7p13, one of the four known fragile sites in lymphocytes with a high incidence of molecular alterations such as deletions, insertions, and translocations.[4] Pol μ belongs to a family of mutator DNA polymerases named polX, which are low processive enzymes with no proofreading 3′ to 5′ exonuclease activity.[5] To date, there are only four eukaryotic members in this family, including TdT, pol β, and the newly identified pol μ and pol λ. Of these, TdT and pol μ are most closely related to each other, sharing an overall 41% amino acid identity, and exhibiting a preferential lymphoid tissue distribution. However, whereas TdT expression is restricted to primary lymphoid tissues, ie, thymus and bone marrow, the highest representation of pol μ has been detected in secondary lymphoid tissues, ie, lymph nodes and spleen, although base levels have been detected in other tissue types as well.[4,5] In particular, it has been demonstrated by both *in situ* hybridization and Northern blotting that pol μ is significantly expressed by germinal center B cells. In agreement with its structural similarity with TdT, pol μ also displays terminal TdT activity, although it does so more efficiently in the presence of a template.[4]

In short, pol μ is an error-prone DNA-dependent DNA polymerase showing preferential peripheral lymphoid tissue expression, especially within germinal center B cells. The close association with germinal center cells, the error-prone nature, and the strong expression by postgerminal center B-NHLs are attributes of pol μ that favor its role as an important underlying molecular mechanism leading to genomic instability in a subset of B-NHLs, ie, those originating from postgerminal center B cells. Our finding of close association of pol μ expression with B-NHLs of postgerminal center cell derivation supports this role.

Because the germinal center normally harbors highly proliferative B cells,[21,22] we assessed the PI in each case to determine whether or not pol μ mRNA expression is simply a function of cell-cycle status. We found that pol μ mRNA expression did not correlate with PI, because pol μ mRNA was not detected in considerable levels in either case of B-LBL, a tumor type typically associated with a high PI. In addition, all three cases of SMZL, which were associated with low to intermediate PIs, showed moderate to strong reactivity. Therefore, the expression of pol μ mRNA seemed to be independent of the proliferation status and thus the biological aggressiveness of the tumor cells. Thus, up-regulation of pol μ does not appear to occur because of cell proliferation but is the consequence of an as yet unknown mechanism, possibly involving one or several oncogenes and/or signal transduction pathways.

In conclusion, we established a close association between pol μ expression and B-NHLs of postgerminal center B cell derivation. This association suggests that certain stages of B cell differentiation may be particularly susceptible to the actions of pol μ, which may include enhancing genomic instability and promoting lymphomagenesis. Further studies analyzing the relationship between pol μ expression and mutational activity may lead to additional insight into the exact mechanism of action of pol μ and B cell lymphomagenesis.

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