Brief Definitive Report

Clonal Deleterious Mutations in the IκBα Gene in the Malignant Cells in Hodgkin’s Lymphoma

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Abstract

Members of the nuclear factor (NF)-κB family of transcription factors play a crucial role in cellular activation, immune responses, and oncogenesis. In most cells, they are kept inactive in the cytosol by complex formation with members of the inhibitor of NF-κB (IκB) family, whose degradation activates NF-κB in response to diverse stimuli. In Hodgkin’s lymphoma (HL), high constitutive nuclear activity of NF-κB is characteristic of the malignant Hodgkin and Reed-Sternberg (H/RS) cells, which occur at low number in a background of nonneoplastic inflammatory cells. In single H/RS cells micromanipulated from histological sections of HL, we detect clonal deleterious somatic mutations in the IκBα gene in two of three Epstein-Barr virus (EBV)-negative cases but not in two EBV-positive cases (in which a viral oncogene may account for NF-κB activation). There was no evidence for IκBα mutations in two non-HL entities or in normal germinal center B cells. This study establishes deleterious IκBα mutations as the first recurrent genetic defect found in H/RS cells, indicating a role of IκBα defects in the pathogenesis of HL and implying that IκBα is a tumor suppressor gene.

Key words: Hodgkin’s lymphoma • IκBα • nuclear factor κB • tumor suppressor gene • Reed-Sternberg cell

Introduction

Hodgkin’s lymphoma (HL) is the most common type of malignant lymphoma in the Western world. It is distinguished from other tumors by a peculiar histological presentation: the giant, often bi- or multinucleated malignant cells, termed Hodgkin and Reed-Sternberg (H/RS) cells, constitute <1% of all cells in the tumor tissue. They are surrounded by an excess of inflammatory cells, e.g., lymphocytes, macrophages, and eosinophils, which are believed to be drawn into the tumor by a deregulated immune reaction precipitated by the H/RS cells (1).

Investigations into the molecular biology of H/RS cells are hampered by their scarcity in the tumor tissue. A detailed analysis of their origin was made possible by the combination of micromanipulation of H/RS cells from frozen tissue sections with PCR amplification of genes from single cells (2, 3). Amplification of V(D)J gene rearrangements on the loci coding for IgH and IgL chains revealed the clonal nature of H/RS cells and their derivation from mature B cells. Deleterious (“crippling”) somatic mutations found in some of these rearrangements indicated that the B cell precursors of H/RS cells resided within the germinal center, and have acquired the capacity to survive loss of B cell receptor expression, an event that would otherwise invariably lead to death by apoptosis of a germinal center B cell (4). Consequently, resistance to apoptosis must play a crucial role in the pathogenesis of the malignant cells.

Despite the impressive progress made in the treatment of HL, the pathogenesis of the tumor and in particular the molecular events leading to transformation of the malignant cells remain to be elucidated. Even though studies on HL-derived tissue or cell lines suggested that p53 mutations or bcl-2 translocations might play a role in the pathogenesis of HL, neither of these ideas could be validated when primary H/RS cells were investigated (5, 6). In some cases of HL, the H/RS cells carry EBV and express EBV-encoded proteins such as the oncogenic latent membrane protein 1 (LM P-1 [7]). However, most cases of HL prevalent among young adults in Western countries are EBV-negative. The distinct epidemiology of these cases has led to various specu-
lating evidence indicates in addition a pivotal role of NF-

kappa B activity as their characteristic feature (8), suggesting a role for NF-
f-κB in the pathogenesis of HL. In line with this idea, inhibition of NF-κB activity in some of these cell lines led to increased susceptibility to apoptosis and impaired tumorigenicity in nude mice (9). NF-κB activity is mediated by the Rel/NF-κB family of transcription factors, members of which are expressed in virtually all mammalian cells. In most cells, NF-κB is retained in an inactive form in the cytoplasm by binding to members of the IκB family of proteins (for a review, see reference 10). A variety of stimuli leads to the degradation of IκB, which allows nuclear translocation of NF-κB and target gene activation. Most inducible NF-κB responses in the cell are mediated by the classical NF-κB heterodimer p50/p65 and are of a transient nature, an effect mainly due to the rapid resynthesis of IκBα, the prototypic member of the IκB family. Target genes activated by NF-κB include factors involved in apoptosis resistance, cell activation, and proliferation, as well as cyto- and chemokines involved in immunoregulation (10, 11). In fact, NF-κB has been likened to a master switch of immune responses, and accumulating evidence indicates in addition a pivotal role of NF-κB in several pathways of malignant transformation. In lympho-Id malignancies, for example, NF-κB deregulation may occur due to chromosomal translocations involving the nfkb2 or b3d loci (11, 12).

In view of this, the demonstration that constitutive activity of p65 is also a characteristic feature of the H/R S cells in primary cases of HL (9) is highly relevant for the pathogenesis of the tumor. In EBV-positive cases, this NF-κB activity may be caused by the expression of the viral oncogene LMP-1 on the H/R S cells. An intriguing alternative cause of constitutive NF-κB activity has been proposed on the basis of data from two HL-derived cell lines, which show defects in the expression of IκBα (13). However, these cell lines have been established from treated and relapsed patients at late stages of the disease (15), and it is unclear whether they are indeed derived from the rare H/R S cells. Likewise, a mutant IκBα fragment could be amplified from a minute fraction of a partially purified H/R S cell population from a relapsed case of HL (14). A gain, it is unclear whether the mutation is derived from the malignant cells. Even if this were the case, it is stated by the authors that this mutation cannot be present in all H/R S cells (14); its relevance for the NF-κB activity in the H/R S cell clone is thus doubtful. Therefore, it is an open question whether IκBα defects play any role in the pathogenetic process that leads to HL.

For many human malignancies, insight into the mechanisms of tumorigenesis has come from the recurrent detection of genetic defects that are common to all cells of the malignant clone and can already be found in primary tumors (rather than sporadic mutations scattered in the tumor that may arise due to its high mutability or upon treatment). It is the clonal nature of mutations that implicates their involvement in the process of malignant transformation—a central criterion for the identification of an oncogene or tumor suppressor gene. In this study, we have analyzed the role of IκBα defects in HL on the basis of this concept.

Materials and Methods

A analysis of HL-derived cell lines. IκBα protein was detected by Western blot with an antibody directed against the COOH terminus (C-21; Santa Cruz Biotechnology). The IκBα coding region was amplified from oligo(dt)-primed cDNA with primers P0S (5'-ACCGAGAAAGGACGGCCAG-3') and P2AS (5'-AGTCATGGTTCTTCATCGCC-3'), using the Expand PCR system (Boehringer Mannheim) supplemented with 2 mM MgCl2 and 2% DMSO. Cycling conditions were 5 min at 95°C; 40 cycles of 50 s at 95°C, 30 s at 63°C, and 90 s at 72°C; and a final 10 min at 72°C.

Primary cases. The primary cases were as follows: (1) mandibular lymph node biopsy from a 12-yr-old female patient, primary diagnosis of nodular sclerosing HL; (2) axillary lymph node biopsy from a 20-yr-old male patient, primary diagnosis of nodular sclerosing HL; (3) supravacular lymph node biopsy from a 35-yr-old male patient, primary diagnosis of nodular sclerosing HL; (4) cervical lymph node biopsy from a 29-yr-old male patient, relapsed mixed cellularity HL, primary diagnosis was 3 yr prior; and (5) abdominal lymph node biopsy from a 36-yr-old male patient, primary diagnosis of mixed cellularity HL.

A analysis of Primary cases. Total genomic DNA was isolated from sections of the lymph node using the QIampl kit. Single H/R S cells were micromanipulated from 5-μm histological sections stained for CD30, and stored in 20 μl of 1× Expand PCR buffer (Boehringer Mannheim) at -20°C. Thawed samples were incubated with 0.25 mg/ml proteinase K (Boehringer Mannheim) for 2 h at 50°C and then 8 min at 96°C. For amplification of exons 1 and 2, samples were adjusted to 50-μl reaction mixes supplemented with 2.5 mM MgCl2, 4% DMSO, 50 mM each of primers IκBα 1b (5'-TGTTCTCAGCTGGCTTGGAAATTCTC-3'), and IκBα 1c (5'-CATCGCTGTGGTCCCCGGGTTC-3'), and 16.6 nM of primers IκBα 2a (5'-CGAAGGCTGGGCTGATACAGG-3') and IκBα 2c (5'-GGATCTGGGTAGTACACGTGACA-3'). After incubation for 5 min at 95°C, 35 cycles of 50 s at 95°C, 30 s at 63°C, and 90 s at 72°C were followed by 10 min at 72°C. For amplification of exons 3-6, samples were supplemented with 2 mM MgCl2, 50 mM each of primers IκBα 3a (5'-AGTACAGGAGGAGGAGGAC-3'), IκBα 3d (5'-TGGGTCTGACTGGCTTGGAAATTCC-3'), and IκBα 4c (5'-ATACAGGAGGAGGAGGAC-3'). After incubation for 5 min at 95°C, 35 cycles of 50 s at 95°C, 30 s at 63°C, and 90 s at 72°C were followed by 10 min at 72°C. For amplification of exons 3-6, samples were supplemented with 2 mM MgCl2, 50 mM each of primers IκBα 3a (5'-AGTACAGGAGGAGGAGGAC-3'), IκBα 3d (5'-TGGGTCTGACTGGCTTGGAAATTCC-3'), and IκBα 4c (5'-ATACAGGAGGAGGAGGAC-3'). After incubation for 5 min at 95°C, 35 cycles of 50 s at 95°C, 30 s at 63°C, and 90 s at 72°C were followed by 10 min at 72°C.
(5'-GGAGGGTTGAAGGGAATGGCAC-3'), and lxkB6b (5'-CCCATCCTCCGCTTTGAGCAG-3') and lxkB6d were included, respectively. After 5 min at 95°C, 45 cycles of 50 s at 95°C, 30 s at 65°C, and 90 s at 72°C were followed by 10 min at 72°C. Amplification of products spanning several exons was performed accordingly. The same conditions were also used for amplification of the lxkB exons from total genomic DNA of cell populations and cell lines. Products were gel-purified and sequenced directly using the ABI BigDye system. For amplification of exons 1 and 4 from aliquoted DNA, single H/R S cells were incubated with 0.25 mg/ml proteinase K (Boehringer Mannheim) in 120 μl of a PCR buffer containing 1 ng/μl of 5S rRNA and incubated at 50°C for 14 h. After gentle mixing by 10 pipetting steps, 20-μl aliquots of the reactions were heated to 96°C for 8 min and subsequently adjusted to 50-μl Expand PCR reactions supplemented with 2 mM MgCl₂, 4% DMSO, 125 nM each of primers lxkB1s (5'-CTGAAGAAGGAGCGCTACTG-3'), lxkB1c, and lxkB4b, and 62.5 nM of primer lxkB5c. After incubation for 5 min at 95°C, 35 cycles of 50 s at 95°C, 30 s at 63°C, and 90 s at 72°C were followed by 10 min at 72°C. Seminested amplification of exon 1 (the Expand™ system was supplemented with 2 mM MgCl₂, 4% DMSO, and 125 nM each of primers lxkB1s and lxkB1c) and exon 4 (standard Taq DNA polymerase was used in 2 mM MgCl₂ and 125 nM each of primers lxkB4b2 and lxkB5c) was essentially as above. A analysis of Normal B Cell Populations. Tonsillar mononuclear cells were sequentially incubated with CD27-FITC (PharMingen) and anti-FITC microbeads (Miltenyi Biotec), then passed over a MACS column (Miltenyi Biotec). CD27⁺ cells were sequentially incubated with mouse anti-human IgD (Southern Biotechnology Associates) and anti-mouse IgG2a/b microbeads (Miltenyi Biotec), and IgD⁺ cells were isolated via an LS-MACS column (Miltenyi Biotec). Tonsillar mononuclear cells from the same patient were incubated consecutively with rat anti-human CD77 (Immunotech), mouse anti–rat IgM (Serotec), and anti-mouse IgG1 microbeads (Miltenyi Biotec), and CD77⁺ cells were isolated via an LS-MACS column. Total genomic DNA was isolated from both cell populations, and exons 1 and 2 of lxkBα were amplified as above using PfuTurbo™ polymerase (Stratagene). After 35 cycles, PCR products were purified, incubated with 200 μM dNTPs and Taq polymerase (GIBCO BRL) for 15 min at 72°C, and cloned into the pGEM-T Easy vector (Promega).

**Results and Discussion**

A panel of eight HL-derived cell lines was screened for lxkBα defects by Western blot, reverse transcription PCR amplification and sequencing of lxkB transcripts, and, if relevant, characterization of the exons of the lxkBα gene (Table I). The reported defects in lxkBα protein expression in two lines (L428, KM H-2 [13]) turned out to be due to deleterious mutations in one copy of the lxkBα gene and apparent loss of the other copy, in agreement with a recent study [14]. However, all other cell lines (including two not previously analyzed B lineage-derived lines) harbor wild-type lxkBα transcripts and express full-length lxkBα protein. Notably, the only cell line for which the derivation from H/R S cells is unequivocally proven (L1236 [17]), expresses wild-type lxkBα from both alleles of the gene, indicating that lxkBα mutations are not present in the H/R S cells in all cases of HL, if at all. As the limited number and ques-

| Table I. A analysis of the lxkBα Protein, Transcripts, and Gene in H L-derived Cell Lines |
|-----------------|-----------------|----------------------|-----------------|-----------------|-----------------|
| Cell line      | Origin/EBV status | Full-length lxkBα protein | Polymorphisms  | Aberrations  | M mutations in lxkBα gene  |
| L428           | B/−             | −                     | 175C, 399C, 1049C | 893C→U §       | 2278C→T $      |
| KM H-2         | B/−             | −                     | 175C, 399C, 1049C | Del. 509-641  | Del. 1497-1710 |
| L1236          | B/−             | +                     | 175Y, 399C, 1049U | −               | −               |
| DEV            | B/−             | +                     | 175Y, 399C, 1049C | −               | −               |
| L591           | B/+             | +                     | 175U, 399C, 1049U | −               | −               |
| L540           | T/−             | +                     | 175U, 399C, 1049Y | −               | −               |
| H DLM-2        | T/−             | +                     | 175C, 399C, 1049U | −               | −               |
| H D-M yZ       | M yeloid/-      | +                     | 175C, 399U, 1049C | −               | −               |

These sequence data are available from GenBank/EMBL/DDBJ under accession nos. A[J249290 and A]249291.

* The presumptive origin of the cell lines is indicated by the presence of rearrangements of the Ig or TCR gene loci, as well as the presence/absence of EBV in the cells. The sequence data for EBV in the cells is given (reference 15).

† The mutation generates a premature stop codon.

‡ The mutation is a silent mutation.

§ The mutation generates a stop codon.

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tionable origin of HL-derived cell lines allow no solid conclusions on this issue, we turned to the analysis of H/RS cells from tumor biopsies.

Five cases of HL were chosen for analysis. In two of them (cases 4 and 5), EBV-encoded small RNA (EBER) in situ hybridization detected EBV in the H/RS cells. To determine the germline sequence of the coding parts of the IkBα gene in the patients, DNA was extracted from whole sections of the infiltrated lymph nodes (i.e., primarily from the nonmalignant inflammatory cells in the tissue) and used for amplification and direct sequencing of the six IkBα exons. Three deviations from the published IkBα sequence (18) were consistently found in all five cases (738G, 2111T, 2734T), while at another six positions we detected frequent germline polymorphisms (Table II). For determination of the sequence of the IkBα gene in the malignant cells, individual H/RS cells micromanipulated from immunostained histological sections were used for seminested

| Case | Exon | Polymorphisms* | PCR-positive cells‡ | Products sequenced | Mutations in H/RS cells | Alleles amplified§ |
|------|------|----------------|--------------------|--------------------|--------------------------|-------------------|
| 1    | 1    | 212C           | 2/5                | 2 Deletion (254–255) | 2WT/ M                  |
|      | 2    | 1059C          | 2/5                | 2                  |                          |
|      | 3    | 1678G          | 5/8                | 5                  |                          |
|      | 4    | 2025C          | 6/8                | 6 Deletion (1994)   | 2WT, 1M, 3WT/ M         |
|      | 5    | –              | 6/8                | 6                  |                          |
|      | 6    | 2787T          | 4/8                | 1                  |                          |
|      |      |                |                    | 2921A              |                          |
| 2    | 1    | 212C           | 7/37               | 6                  |                          |
|      | 2    | 1059T          | 8/37               | 8 2 unique mutation§|                          |
|      | 3    | 1678G          | 5/14               | 5                  |                          |
|      | 4    | 2025C          | 5/14               | 5                  |                          |
|      | 5    | –              | 5/14               | 5 Deletion (2355–2356)| 5WT/ M                |
|      | 6    | 2787C          | 4/14               | 4                  |                          |
|      |      |                |                    | 2921G              |                          |
| 3    | 1    | 212Y           | 8/26               | 7 51C→T in 1 seq.  | 2C, 3T, 2Y               |
|      | 2    | 1059Y          | 5/26               | 5                  | 4C, 1T                   |
|      | 3    | 1678R          | 6/8                | 6                  | 2A, 4G                   |
|      | 4    | 2025Y          | 5/8                | 5                  | 4C, 1T                   |
|      | 5    | –              | 5/8                | 5                  | – ^                      |
|      | 6    | 2787Y          | 6/8                | 6                  | 4T, 1C, 1Y               |
|      |      |                |                    | 2921R              | 4A, 1G, 1R               |
| 4    | 1    | 212T           | 5/12               | 4 52C→T in 1 seq.  | 1T, 3Y                   |
|      | 2    | 1059C          | 6/12               | 4                  |                          |
|      | 3    | 1678G          | 5/10               | 5                  |                          |
|      | 4    | 2025C          | 7/10               | 6                  |                          |
|      | 5    | –              | 7/10               | 6                  |                          |
|      | 6    | 2787T          | 7/10               | 6                  |                          |
|      |      |                |                    | 2921A              |                          |
| 5    | 1    | 212Y           | 8/17               | 4                  | 1T, 3Y                   |
|      | 2    | 1059Y          | 7/17               | 7 6 unique mutations§| 4T, 3Y                   |
|      | 3    | 1678R          | 7/8                | 5                  | 1A, 1G, 3R               |
|      | 4    | 2025Y          | 5/8                | 5                  | 3C, 1T, 1Y               |
|      | 5    | –              | 7/8                | 5                  | – ^                      |
|      | 6    | 2787Y          | 5/8                | 4 3035G→A in 1 seq. | 1C, 3Y                   |
|      |      |                |                    | 2921R              | 1G, 3R                   |

* Nucleotides at polymorphic sites in the respective exons amplified from the whole tissue DNA. Y = C/T, R = A/G. Positions in the IkBα gene refer to reference 18.

**Indicates (in cases 1 and 2) amplification of wild-type (WT) or mutant (M) copies of the respective exon from individual cells, and (in cases 3 and 5) the number of sequences containing either of the two polymorphic nucleotides, or both.

† Products spanning exons 4 and 5 were amplified. Unmutated exon 5 on both alleles was concluded from the detection of both polymorphic nucleotides at position 2025 in unmethylated products.

These sequence data are available from GenBank/EMBL/DBJ under accession nos. AJ249283–AJ249289 and AJ249294–AJ249295. seq. = sequence.
PCR amplification. To avoid the detection of mutations introduced by Taq DNA polymerase, direct sequencing of the gel-purified PCR products was performed. In most cases \( \geq 4 \) PCR products of each exon were analyzed, since the single cell PCR approach may stochastically miss either of the two copies of the gene in individual reactions.

In case 1, mutations were detected in exons 1 and 4: the deletion of two and one nucleotides, respectively, leads to frameshifts (Table II). Both mutations were repeatedly detected in PCR products from H/R S cells (see also Table III), yet not in the germline sequences of the respective patient. We conclude that they represent clonal somatic mutations in the malignant cells. Notably, the PCR products from H/R S cells contain wild-type as well as mutant copies of the respective exons, indicating the presence of two distinct alleles of the IκB\( \alpha \) gene. To assign the two mutations to the two alleles, we separated the IκB\( \alpha \) loci of H/R S cells before coamplification of exons 1 and 4. Single H/R S cells were incubated with protease in buffer, which was then distributed to six tubes for PCR analysis. Most cells analyzed contain several copies of both wild-type and mutated exons 1 and 4 (Table III), presumably due to polyploidy, a characteristic feature of H/R S cells (19). Aliquots with evidence for the presence of more than one copy of either exon were disregarded. In most other cases, the mutant exon 1 was coamplified with wild-type exon 4, and vice versa, mutant exon 4 with wild-type exon 1. We conclude that the two mutations are located on the two different alleles of the gene, implying that no full-length IκB\( \alpha \) protein can be synthesized in the H/R S cells. The coamplification of wild-type exons 1 and 4 in two aliquots (ID, IIIIB) is apparently due to amplification of only the wild-type exons from tubes containing both wild-type and mutated copies.

In case 2, a deletion of two nucleotides, leading to a frameshift, was detected in all exon 5 products amplified from the H/R S cells (Table II). Again, both wild-type and mutant copies of the exon are evident in the H/R S cells, but no mutation was seen in the germline sequence. Thus, one of the two alleles of the IκB\( \alpha \) gene in the malignant cells is inactivated by a somatic mutation. For all other exons, the germline sequence was obtained, but an assignment of these wild-type products to either of the two alleles is not possible.

Analysis of PCR products spanning exons 3–5 and 5–6, respectively (allowing discrimination of the two alleles due to the mutation in exon 5), revealed no other point mutation or deletion in that region in either of the two alleles. Since exons 1 and 2 could not be included in this analysis (see Materials and Methods), it is unclear whether the entire coding region of the second IκB\( \alpha \) allele is unmutated. In the third EBV-negative case (case 3), as well as in the two EBV-positive cases (cases 4 and 5), no clonal mutations in any of the IκB\( \alpha \) exons were detected (Table II). For cases 3 and 5, we conclude that all coding exons of both alleles are unmutated, since germline polymorphisms allow their discrimination.

In several PCR products amplified from the H/R S cells of cases 2–5, in particular those spanning exons 1 and 2, unique nucleotide exchanges were detected. They were not seen in any of the other products of the respective exon of the respective case, and are thus apparently not due to mutations present in all malignant cells. These mutations might indicate enhanced mutability of the IκB\( \alpha \) gene, reflect the genomic instability of H/R S cells (20), or be derived from Taq DNA polymerase errors (although the latter seems unlikely). Be that as it may, the frequent occurrence of this type of mutation emphasizes an important issue: one can only invoke clonal genetic defects (i.e., those that can be assigned to all malignant cells) for all other exons, the germ-line sequence was obtained, but an assignment of these wild-type products to either of the two alleles is not possible.

Table III. Analysis of IκB\( \alpha \) Exons 1 and 4 Amplified from Genomic DNA from Single H/R S Cells of Case 1

|   | I   | II  | III | IV  | V   |
|---|-----|-----|-----|-----|-----|
| A | 1WT 4M ut | 1WT 4both | 1WT 4M ut |       |     |
| B | 1WT 4M ut | 1WT 4WT 1WT |       |     |     |
| C | 1M ut 4WT | 1WT 4both | 1WT 4both |       |     |
| D | 1WT 4WT | 1both | 1WT |       |     |
| E | 1both 4WT | 1both 4both | 1M ut 4WT |       |     |
| F | 1M ut 4WT | 1M ut 4WT 1WT 4M ut |       |     |

Five cells (I–V) out of eight analyzed in this experiment yielded PCR products for exons 1 and 4 in more than one of the six aliquots (A–F). Sequence analysis of the products revealed wild-type (WT), mutant (M), or mixed (both) sequences for exon 1 and 4, respectively. Negative control reactions yielded no products for either exon.

In conclusion, clonal deleterious somatic mutations in the IκB\( \alpha \) gene were detected in the H/R S cells in two of three EBV-negative and none of two EBV-positive cases of H.L. Ruminiscient of the mutations in H.L.-derived cell lines,
they lead to the synthesis of truncated IκBα proteins lacking a part of the ankyrin repeat and/or the COOH-terminal PEST domain (Fig. 1), which are required for interaction of IκB proteins with NF-κB and inhibition of its DNA binding, respectively (24–26). This suggests that loss or severe impairment of these functions was selected for during the pathogenesis of the tumor cell clone: it leads to constitutive nuclear activity of NF-κB. The severe phenotype of IκBα knockout mice indicates that none of the other IκB family members may fully take over IκBα function (27).

Loss of IκBα function presumably requires the inactivation of both copies of the gene. In the H/R S cells in most of the primary cases we investigated, two distinct alleles of the IκBα gene are clearly detectable, making proof of loss of function dependent on evidence for inactivation of both of them. Our single cell PCR approach, which is dictated by the peculiar histology of HL, does allow the detection of biallelic gene inactivation in the H/R S cells (in contrast to a previous one [14]), but it misses several ways of tumor suppressor gene inactivation, such as large deletions or chromosomal translocations. In light of this, we consider the detection of three inactivating mutations in the cases studied, and evidence for loss of IκBα function in one of these cases, to be a highly significant finding.

The data presented here establish deleterious IκBα mutations as the first recurrent genetic defect found in HL. It is intriguing that such mutations were only identified in EBV-negative cases (and lines)—a major subset of this disease for which no mechanism of transformation could be pinpointed to date. However, the limited capacity of the single cell PCR approach does not allow definite conclusions about whether IκBα inactivation is restricted to this subset, nor about its overall frequency in HL. Our attempts to analyze a larger panel of cases by specific detection of full-length IκBα protein in tissue sections failed, due to apparent cross-reactivity of the available antibody against the COOH terminus (C-21; Santa Cruz Biotechnology). Certainly, not IκBα inactivation but rather constitutive NF-κB (p50/p65) activation may be the unifying feature of H/R S cells (8, 9, 28), and it may apparently be brought about by different means. The finding that H/R S cells are derived from germinal center B cells that may survive the loss of B cell receptor expression warrants testing of the role of constitutive NF-κB activation in the rescue of such “crippled” B cells from apoptosis. Evidently, constitutive activation of NF-κB (p50/p65) in the context of a B cell provides an intriguing explanation for several of the distinctive clinical and pathological features of HL. It likely precipitates the secretion of a battery of cytokines and chemokines leading to massive attraction of inflammatory cells and profound disturbances in immunoregulation, contributes to the peculiar activated phenotype of H/R S cells, and, most importantly, confers apoptosis resistance and continuous proliferation as prerequisites of malignant transformation (10–12). The tumorigenic potential of members of the NF-κB family and of factors inducing NF-κB activity is well established. In contrast, ex vivo proof of a tumor suppressor function of an IκB family member has not been provided to date (but see reference 29). This study gives evidence for recurrent inactivation of the prototypic IκB family member, IκBα, in the most common lymphoma of the Western world. It will be interesting to see whether functional impairment of IκBα plays a role in the pathogenesis of other human malignancies.

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### Table IV. Analysis of IκBα Exons 1 and 2 in Normal B Cels

| PCR product | Cell population | N. of sequences | No. of Mutations | Mutation frequency |
|-------------|-----------------|-----------------|------------------|--------------------|
| VH3 rearr.  | Naive           | 17              | 0                | 0                  |
|             | GC              | 13              | 0                | 0                  |
| IκBα exon 1 | Naive           | 24              | 0                | 0                  |
|             | GC              | 25              | 1                | 0.009              |
| IκBα exon 2 | GC              | 22              | 0                | 0                  |

VH3 gene rearrangements and exons 1 and 2 of the IκBα gene were amplified from genomic DNA of naive (CD27+) and germinal center (GC, CD77+) B cells, cloned, and analyzed for somatic mutations. The expected frequency of mutations induced by Pfu DNA polymerase is 0.0035% (10⁻⁶ mutations bp/cycle) Pfu DNA polymerase error ≈ 35 cycles.
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Note added in proof. In a recent study of part of the IκBα gene in H/R/S cells, Emmerich et al. (Emmerich, F., M. Meiser, M. Hummel, G. Demel, H.D. Foss, F. Jundt, S. Mathias, D. Krappmann, C. Scheidereit, H. Stein, and B. Dörken. 1999. Blood. 94:3129–3134) report a clonal monoallelic nonsense mutation in 1 of 10 cases of HL.

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