Primary diffuse large B-cell lymphoma of bone

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Chapter 6

Nuclear factor-κB activation in primary lymphoma of bone

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Abstract

Primary lymphoma of bone is a rare type of extranodal diffuse large B-cell lymphoma with a relatively favorable outcome. Recent scientific interest has focused on elucidating the role of nuclear factor-κB pathway in lymphomagenesis and its possible value as a therapeutic target. In nodal B-cell non Hodgkin lymphomas, constitutive activation of nuclear factor-κB appears to be especially involved in tumor cell survival in the non-germinal center type of diffuse large B-cell lymphoma. In nuclear factor-κB activation, two major pathways are involved: the classical and the alternative pathway. We here investigate nuclear factor-κB activation via both pathways in primary lymphoma of bone, performing immunohistochemical staining procedures for nuclear factor-κB family members on tumor tissues of 50 cases. Nine cases (18%) showed nuclear positivity for p50, and one case showed nuclear co-expression of p52. Positivity for p50 was not restricted to either germinal center- or non-germinal center phenotype of the tumor, or related to an inferior prognosis or treatment resistance. P65 did not show significant nuclear expression. The immunohistochemical nuclear expression of p50 in 18% of the cases suggests constitutive activation of nuclear factor-κB via the classical pathway in a minority of primary lymphoma of bone patients. In contrast to other extranodal types of diffuse large B-cell lymphoma, there was a lack of nuclear co-expression of p65, which may suggest different pathway activation. Alternative pathway activation of nuclear factor-κB does not appear to be significantly involved, as only one case showed significant nuclear expression for p52. Finally, nuclear expression of p50 was not preferentially detected in non-germinal center type or germinal center type primary lymphoma of bone, or related to an inferior prognosis. Therefore, in contrast to nodal diffuse large B-cell lymphoma, the nuclear factor-κB pathway does not appear to be an attractive pathway for targeted therapy in primary lymphoma of bone.

Introduction

Primary non Hodgkin lymphoma of bone (PLB) is a rare neoplastic disorder, comprising 5% of extranodal lymphomas[1] and less than 2% of all non Hodgkin lymphomas.[2] PLB mainly presents as an extranodal subtype of diffuse large B cell lymphoma (DLBCL) and shows a non-aggressive radiological presentation.[3] Although extranodal DLBCL in general seems to represent a biologically very heterogeneous group of lymphomas with a varying prognosis, PLB shows morphological and clinical homogeneity with a relatively favorable clinical outcome. The 5-year overall survival rate is approximately 90%. [4;5] Complete remission is usually achieved with combined chemotherapy and radiotherapy regimens. PLB exhibits a germinal center B-cell (GCB) phenotype in at least half of the cases, but still a considerable amount of tumors show a non germinal center (GC)/activated B-cell (ABC) phenotype.[6] Age at presentation over 60 years and immunoblastic tumor cell morphology/non GC phenotype are considered the most important unfavorable predictive factors, although results concerning the prognostic value of the GC phenotype cannot be reproduced in all studies on PLB.[5;7-9] The most frequently encountered genomic aberrations include gain of 1q, amplification of 2p16.1 and loss of 14q32.33.[10] Considering the amplification of 2p16.1 in PLB, encoding for nuclear factor (NF)-κB family member c-Rel, we were interested in elucidating a potential role of constitutive activation of NF-κB in the pathogenesis of PLB.

NF-κB is a transcription factor involved in several cellular survival mechanisms. It has been implicated that NF-κB activity plays a role in the pathogenesis of different types of B-cell lymphomas, especially in nodal non-GC/ABC type DLBCL, although it is also encountered in a minority of GCB type DLBCL.[11] Constitutive activation of NF-κB is required for survival of activated ABC type DLBCL cells in vitro[12] and ABC type DLBCLs preferentially express known NF-κB target genes.[13] Furthermore, mutations in multiple genes involved in the NF-κB activation pathway can cause deregulation of NF-κB in DLBCL.[11] It is shown that a small molecule inhibitor of IκB kinase, an essential molecule in the activation of NF-κB, is selectively toxic to ABC type DLBCL cell lines.[14] These results suggest a role for the NF-κB pathway as a potential therapeutic target in DLBCL.

In this study, 50 cases of PLB were selected and examined for immunohistochemical expression of three NF-κB subunits (p65, p50 and p52) as surrogate read-outs for constitutive activation of the canonical pathway (p65 and p50) and alternative pathway (p52) of NF-κB. We evaluated the expression of NF-κB proteins in relation to the postulated cell of origin (GC versus non-GC B-cell/activated B-cell). Furthermore, we correlated the clinical parameters with the immunohistochemical results.

Materials and Methods

Fifty cases of primary lymphoma of bone were investigated, 27 were collected at the Leiden University Medical Center (Leiden, The Netherlands) and 23 at the Semmelweis University (Budapest, Hungary). According to the WHO classification[1] PLB was defined as a histologically proven non Hodgkin lymphoma arising within the medullary cavity of a bone, with or without regional lymph node involvement, but without evidence of other extranodal involvement, or as non Hodgkin lymphoma with multiple bone involvement without lymph node or visceral involvement. Staging procedures included Computed Tomography scan, iliac crest bone marrow biopsy and total blood count. The pathological diagnosis was established according to the WHO classification [15] using standard histological criteria and immunohistochemical staining procedures. All cases selected were reviewed independently by at least two pathologists. The samples were handled in a coded fashion, and all procedures were performed according to the ethical guidelines, 'Code for Proper Secondary Use of Human Tissue in The Netherlands' (Dutch Federation of Medical Scientific Societies).

Tissue microarray

Tissue microarrays were prepared using hematoxylin and eosin (H&E) stained sections from each paraffin-embedded, formalin-fixed block to select tumor rich areas. Accordingly, 3 representative 2-3 mm cores were obtained from each case and inserted in a grid pattern into a recipient paraffin block using a tissue arrayer device (TMA Master, 3D Histech Ltd, Budapest, Hungary).

Antibodies and Immunohistochemistry

Immunohistochemical staining was performed on 4 μm paraffin sections using standard procedures. After antigen retrieval by boiling for 10 minutes in 10 mmol/L citrate buffer (pH 6.0, tissue sections were incubated overnight with p50 (dilution 1:200, Abcam, Cambridge, UK), p52 (18D10) (dilution 1:100, Cell Signaling Technology, Beverly, MA, USA) and p65 (dilution 1:200, Neomarkers, Fremont, CA, USA). Sections were then incubated for 30 minutes with PowerVision Poly-horseradish peroxidase. Subsequently, a 10-minute incubation with diaminobenzidine (DAB) solution (Sigma-Aldrich, Zwijndrecht, The Netherlands) was performed. Finally, all slides were counterstained with Mayer's haematoxylin. Immunohistochemical stainings for BCL6, CD10 and IRF4/MUM1 were performed as previously described.[6]

Immunohistochemical Scoring

NF-κB expression was detected as nuclear and/or cytoplasmic staining of tumor cells for p50, p52 and/or p65. Nuclear staining was scored according to the estimated percentage of positive tumor cells; less than 10%, 10-20%, 20-30% or more than 30%, irrespective of cytoplasmic staining. In the largest cohort of DLBCL published thus far[10], cases were considered to be

positive for NF-κB activity when ≥30% of tumor cells showed nuclear staining. In our series, cases showed either virtually no nuclear staining (<10%, merely scattered positive tumor cells), or staining of 20% or more for p50 and p52. Because of this dichotomy, we considered cases of more than 20% positive tumor cells positive for nuclear staining. Tonsil sections were used as positive controls. As an extrinsic control group, paraffin sections of cases of nodal DLBCL and primary central nervous system DLBCL were also stained, since these groups have been previously reported to immunohistochemically express nuclear p50 and p52[11] and nuclear p65,[16] respectively.

Results

Patient characteristics

Patient characteristics are summarized in Table 1, subdivided by GC or non-GC phenotype as defined by diagnostically performed immunohistochemical staining procedures for CD10, BCL-6 and IRF4/MUM1, according to the Hans' algorithm. All stage IV patients had multifocal osseous disease without lymph node or visceral involvement. Forty-three patients received mulitagent chemotherapy, of which 9 were additionally treated with rituximab.

NF-κB expression

Nuclear immunohistochemical expression of p50 was detected in 9 cases (18%, see Figure 1), whereas almost all other cases just showed cytoplasmic expression throughout the tumor tissue. Nuclear expression of p50 was equally distributed between the GC- and non-GC type of PLB as determined by Hans' algorithm (see Table 2).

P52 showed nuclear expression of 20-30% of tumor cells in only one case. This case was a non-GC type PLB, and also showed nuclear p50 positivity in more than 30% of tumor cells. After a follow-up of ten years, this patient was still alive without recurrence of disease after polychemotherapy treatment.

Staining procedures for p65 did not show significant nuclear staining. Only scattered cells were considered positive (scored as less than 10% nuclear staining), while cytoplasmic staining in virtually all tumor cells was detected in 46 and 43 cases respectively.

None of the nuclear p50 positive cases presented with a recurrence or progression of disease, although for one of the positive cases, no follow-up data were available. Furthermore, exploring known prognostic factors in PLB, p50 positivity was evenly distributed between patients under and over 60 years of age and was not related to disease stage at time of presentation.

Discussion

The NF-κB comprises of a family of transcription factors that control genes that play critical roles in B-cell activation, proliferation and resistance to apoptosis. The NF-κB family has five members: p65 (RelA), p50 (NF-κB1), p52 (NF-κB2), RelB and c-Rel. In unstimulated human

Table 1: Patient characteristics.

	all 50	GC-type 25	non-GC-type 25
Sex			
male	32	16	16
female	18	9	9
Age (years)			
mean	49	50	47
range	18-78	18-78	20-72
Location			
femur	25	11	14
humerus	6	5	1
os ileum	6	3	3
vertebra	5	3	2
other	8	3	5
Stage			
I	33	17	16
II	4	0	4
III	0	0	0
IV	11	8	3
unknown	2	0	2
Treatment			
PCT + RT	25	13	12
PCT + RT	14	5	9
RT + resection	6	4	2
PCT + RT + resection	3	1	2
PCT + resection	1	1	0
unknown	1	1	0
Outcome			
complete response	37	19	18
partial response	3	0	3
progressive disease	7	4	3
unknown	3	2	1
Recurrence			
yes	2	1	1
no	45	22	23
unknown	3	2	1
Status			
A-	37	19	18
A+	0	0	0
D-	1	0	1
D+	9	4	5
unknown	3	2	1
Follow-up (months)			
mean	57	52	62
range	0-240	0-192	0-240

GC-type: germinal center phenotype, non-GC-type: non germinal center phenotype, PCT: polychemotherapy, RT: radiotherapy, A-: alive without evidence of disease, A+: alive with evidence of disease, D-: died of causes unrelated to disease, D+: died of causes related to disease

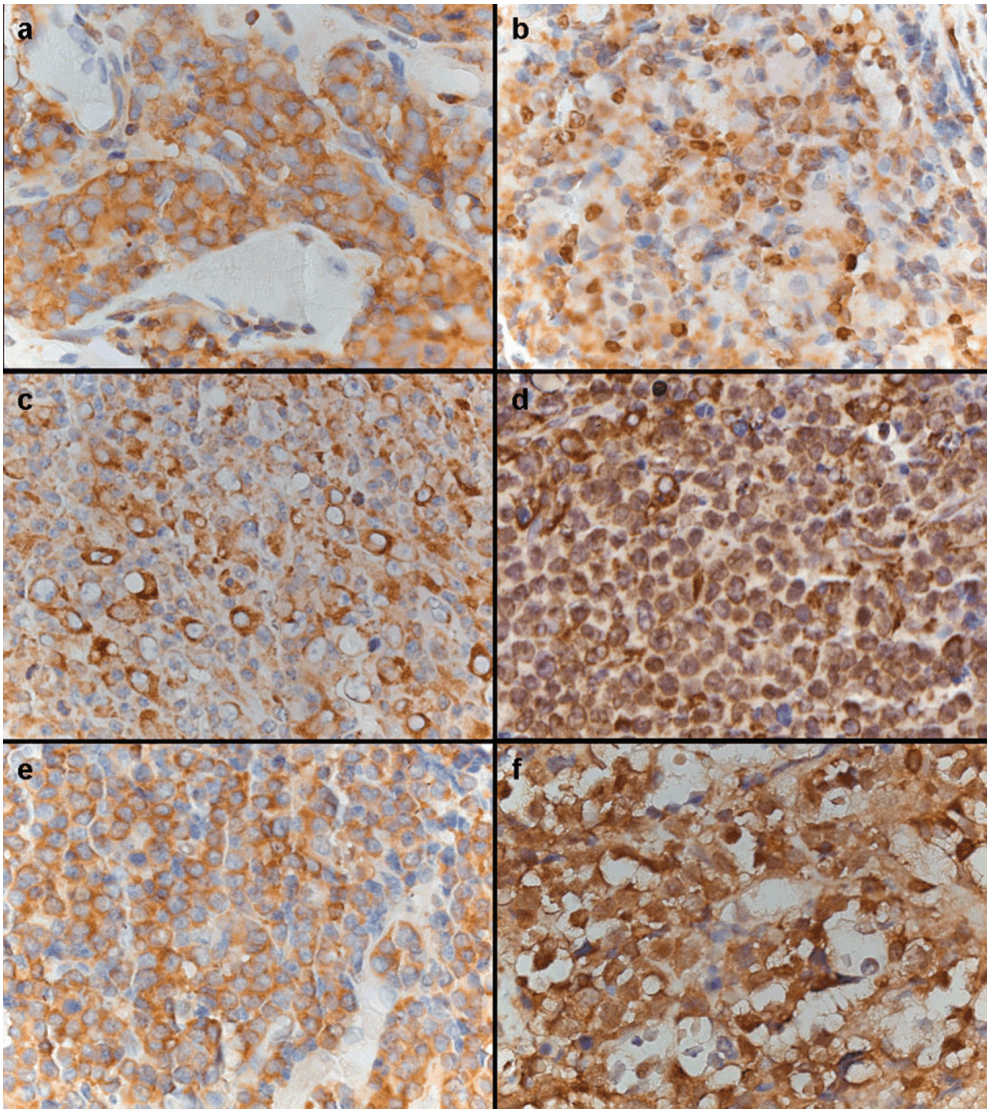

Figure 1: Immunohistochemical staining of nuclear factor-κB pathway members.
The majority of cases of primary lymphoma of bone merely showed cytoplasmic staining of the tumor cells for p50 (a) and p52 (c). P65 was only detected in the cytoplasm of tumor cells (e), while p50 and p52 showed nuclear staining in part of the tumor cells (b,d). P65 was not detected in the nucleus of tumor cells in primary lymphoma of bone (e). However, cases of primary central nervous system diffuse large B-cell lymphoma did show significant nuclear staining (f) and served as an external positive control.

Table 2: Nuclear immunohistochemical positivity for p50.

	All	GC-type	non-GC-type
<10%	41	21	20
10-20%	0	0	0
20-30%	2	0	2
>30%	7	4	3

GC-type: germinal center type, non-GC-type: non germinal center type.

cells, these NF-κB proteins and their precursor proteins reside in the cytoplasm in an inactive form bound to NF-κB inhibitory proteins (IκBs). The NF-κB pathway signals downstream after activation through different kinds of surface receptors, including the B-cell receptor. Two major signaling pathways account for the activation of NF-κB. In the canonical pathway, signal transduction events lead to activation of the IκB kinase complex resulting in phosphorylation and proteasomal degradation of IκB. Heterodimers and homodimers of p50, p65 and c-Rel can then be translocated to the nucleus to regulate gene transcription. The alternative pathway is characterized by IκB kinase complex activation through NF-κB induced kinase, eventually leading to processing of the p52 precursor subunit into active p52 translocating to the nucleus and forming a heterodimeric complex with RelB.[17;18] The clinical importance of the two different pathways of activation includes the potential of targeted therapy directed against NF-κB activation, as some specific inhibitors only target one of both activation pathways. [19]

This is the first study reporting on NF-κB activation in PLB. In our cohort, 18% of cases demonstrated nuclear positivity for p50, suggesting constitutive activation of the classical NF-κB pathway in a minority of PLB. Only one case showed significant nuclear positivity for p52, which implies that constitutive activation of NF-κB through the alternative pathway may not play a significant role in PLB. This is in contrast to previous reports showing nuclear staining for p52 in one-third (ABC-type) to one-fifth (GCB-type) of cases of nodal DLBCL.[11] We could not demonstrate co-localization of p65 with p50 in the nucleus, which is in line with recent results in nodal DLBCLs [20]. Interestingly, nuclear immunohistochemical staining for p65 was observed in primary central nervous system DLBCL and primary cutaneous DLBCL, two other variants of extranodal DLBCL.[16;21] It would be interesting to investigate whether the lack of nuclear staining for p65 indicates that another form of constitutive activation of NF-κB is involved in PLB, possibly by forming p50 homodimers in the nucleus or by co-localizing with C-Rel.

A considerable amount of nodal DLBCLs are known to show constitutive activation of NF-κB. In non-GC DLBCLs, gene set enrichment analysis showed high NF-κB transcriptional activity in >95% of cases, whereas this percentage was 47% in GC DLBCL. Immunohistochemical staining procedures in the same group showed nuclear localization of p50 and/or p52 in 61% and 30%, respectively.[11] Although this difference might be interpreted as a result of lower

sensitivity of immunohistochemical staining to detect aberrant NF-κB activity, it may also reflect the inability of gene set enrichment analysis to discriminate signals deriving from infiltrating reactive cells. Indeed, in our series, small infiltrating T-lymphocytes did also show nuclear staining for some of the NF-κB family members. Therefore, it seems reasonable to assume that immunohistochemistry may be a surrogate marker for determining constitutive activation of NF-κB.

Although constitutive activation of NF-κB in nodal DLBCL was not restricted to the non-GC type, a marked preference for non-GC tumors was observed [11]. In our cohort, nuclear expression of p50 was not confined to the tumors with a non-GC phenotype and was evenly distributed between GC- and non-GC like tumors. Activation of the NF-κB pathway as shown by upregulated gene expression of different NF-κB target genes is even considered as a specific gene array signature of the ABC/non-GC type DLBCL [12]. It therefore seems remarkable that in our series only 20% of non-GC type PLBs showed nuclear p50 staining. These results once more emphasize the heterogeneity of nodal and extranodal DLBCLs and of the different types of extranodal lymphomas.

It is hypothesized that NF-κB activation plays a role in chemotherapy resistance and a subsequent poor outcome in different types of lymphomas, by promoting cell proliferation, blocking apoptosis and potentially blocking differentiation and promoting metastases.[22;23] As PLB generally has an excellent prognosis, relating nuclear p50 staining to survival may not be relevant. Nevertheless, in our cohort, all patients with nuclear positivity for p50 are still alive without disease. This implies that p50 positivity in PLB is not a negative prognostic factor, and that constitutive activation of NF-κB is not likely to be significantly involved in (chemo) therapy-resistance in the minority of PLB cases that show progressive disease.

In conclusion, our results show that NF-κB activation through the classical pathway is seen in a minority of cases of PLB and is evenly distributed among cases with a non-GC or GC phenotype. Therefore, in contrast to nodal DLBCL, the NF-κB pathway does not appear to be an attractive pathway for targeted therapy in PLB.

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