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Heyning, F.H.; Jansen, P.M.; Hogendoorn, P.C.W.; Szuhai, K.

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# Array-based comparative genomic hybridisation analysis reveals recurrent chromosomal alterations in primary diffuse large B cell lymphoma of bone

F H Heyning,<sup>1</sup> P M Jansen,<sup>2</sup> P C W Hogendoorn,<sup>2</sup> K Szuhai<sup>3</sup>

<sup>1</sup>Department of Internal Medicine, Medical Center Haaglanden, The Hague, The Netherlands

<sup>2</sup>Department of Pathology, Leiden University Medical Center, Leiden, The Netherlands

<sup>3</sup>Department of Molecular Cell Biology, Leiden University Medical Center, Leiden, The Netherlands

## Correspondence to

Dr Karoly Szuhai, Department of Molecular Cell Biology, Leiden University Medical Center, Einthovenweg 20, 2300 RC Leiden, The Netherlands; k.szuhai@lumc.nl

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## ABSTRACT

**Aims** Primary non-Hodgkin's lymphoma of bone (PLB) is a rare subtype of primary extranodal diffuse large B cell lymphoma. PLB has morphological homogeneity and a relatively favourable clinical behaviour. Recent studies report that array-based comparative genomic hybridisation (array-CGH) analysis can be used to classify lymphomas into clinically and biologically relevant phenotypes and possibly reveal differences in oncogenic mechanisms. Here the authors performed the first array-CGH study to detect illness related genomic alterations in nine, clinically well-staged primary lymphoma of bone cases.

**Methods** Nine frozen samples from primary lymphoma of bone patients were immunophenotyped and subsequently investigated using a well-established array-CGH platform. The array-CGH results were confirmed by fluorescence in situ hybridisation. Clinical data and follow-up were obtained for all nine patients.

**Results** Of the nine patients, eight reached complete remission, and one had progressive disease and died of primary lymphoma of bone. Frequent aberrations were: loss of 14q32 (n=7), trisomy 7 (n=6), gain of the long arm of chromosome 1 (n=5) and amplification of 2p16.1 (n=4). No statistically significant correlation between genetic abnormalities and clinical outcome was found.

**Conclusions** The authors found several recurrent genomic aberrations, including five cases with gain of 1q and four cases with 2p16.1 amplification. These findings are associated with a germinal centre-like phenotype and favourable treatment outcome, and differ from chromosomal aberrations found in other extranodal lymphomas. These findings further substantiate the notion that primary lymphoma of bone should be considered as a distinct entity not only on clinic-pathological grounds but also on the genomic level as well.

## INTRODUCTION

Primary non-Hodgkin's lymphoma of bone (PLB) is a rare neoplastic disorder, comprising 5% of extranodal non-Hodgkin's lymphomas (NHLs).<sup>1</sup> It is a subtype of primary extranodal diffuse large B cell lymphoma (DLBCL), which, as a whole, is the most heterogeneous group of lymphomas. PLB as an entity, however, has morphological and clinical homogeneity.<sup>2</sup> Characteristically, these lymphomas present in the long bones such as the humerus or the femur with pain or a palpable mass. During MR imaging, it might not present infrequently as a non-aggressive lesion.<sup>3</sup> Complete remission is usually achieved with a combination of chemotherapy and radiotherapy, with only a few patients relapsing during follow-up. Needless to say,

adequate staging including a CT-scan of the thorax and abdomen, and iliac crest bone marrow biopsy are essential in order to rule out disseminated DLBCL involving the bone instead of a primary bone presentation.

Studies on extranodal lymphoma are infrequent, even though the incidence of extranodal lymphoma in Western countries has increased in the last 40 years.<sup>4-5</sup> This discrepancy can be explained by the low frequency of primary involvement of any particular extranodal site. To overcome these small patient numbers, many authors have combined all extranodal cases to obtain enough statistical power for their research. However, it is questionable whether such a general distinction has any clinical relevance, since clinical outcome and tumour biology differ substantially between the various extranodal localisations.<sup>6-8</sup> Studies on PLB specifically are even rarer because, apart from the low patient numbers, the research on PLB is hindered due to limited availability of frozen tissue specimens and technological difficulties related to handling the tumour material of osseous origin.

Research in gene-expression profiling has led to the concept that most DLBCLs derive from germinal centre (GC) B cells or from their descendants, that is activated B cells or non-GC B cells. Recent studies have shown that the majority of PLBs are of the GC-like phenotype which is associated with a better prognosis than the non-GC phenotype.<sup>2,9-10</sup> Array-based comparative genomic hybridisation (array-CGH) enables us to detect the genomic copy number of alterations of cancers with high resolution. Recent studies report that this technique can be used to classify lymphomas into the clinically and biologically relevant phenotypes, GC-like and non-GC-like, and possibly reveal differences in oncogenic mechanisms.<sup>11</sup>

No studies using array-CGH analysis on PLB have been published so far. We investigated genomic alterations in nine well-documented cases of PLB using this technique and analysed the results in the context of data available from literature on studies of other distinct subtypes of extranodal DLBCL such as skin, brain and testis.<sup>12-13</sup>

## METHODS

### Tissue sample collection and selection

Ten frozen samples from PLB patients were collected from the tissue bank at the Leiden University Medical Center, and one sample was collected from the tissue bank at the University Medical Center Groningen, The Netherlands. Following quality control of DNA, we disregarded

two samples and performed an array-CGH analysis on nine of the 11 cases. All 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). Clinical data and follow-up were obtained on all nine patients. PLB was defined as a histologically proven non-Hodgkin's lymphoma arising within the medullary cavity of a bone, with or without regional lymph node involvement, but without evidence of other extranodal involvement.<sup>1</sup> Multiple bone lesions were acceptable as long there was no evidence of earlier lymphoma elsewhere. All patients were staged adequately with an MRI of the tumour site, CT-scan of the thorax and abdomen, and iliac crest bone marrow biopsy. The relevant clinical and follow-up data for the patients investigated are summarised in table 1.

### Histological classification and immunohistochemistry

The pathological diagnosis was established according to the WHO classification<sup>14</sup> using standard histological criteria and immunohistochemistry using antibodies directed against Vimentin, CD45, CD3, CD20, CD79a and CD99. Immunohistochemical staining was performed on 4 µm sections of formalin-fixed, paraffin-embedded tissues, using standard procedures as detailed elsewhere.<sup>2</sup> In addition to the diagnostic marker panel listed above, a set of markers relevant to a GC/non-GC phenotype was used: CD10, BCL6 and MUM-1.

### Array-CGH

Genomic DNA was isolated using high salt after SDS/proteinase K digestion; 500 ng was labelled with Cy3-dCTP (GE Healthcare, Diegem, Belgium) using the BioPrime DNA Labelling System (Invitrogen, Breda, The Netherlands). As a reference DNA, 500 ng of either male or female human genomic DNA (Promega, Leiden, The Netherlands) was labelled using Cy5-dCTP. Labelled samples were hybridised array slides containing ~3500 BACs clones spaced at ~1 Mb density over the full genome, a set of subtelomeric sequences for each chromosome arm and a few hundred probes selected for their involvement in oncogenesis, and were meticulously validated.<sup>15</sup> As all clones were part of the Human Genome Project, updated sequencing information is available from the ENSEMBL web page (<http://www.ensembl.org>). The clones were grown, amplified and spotted as described previously, and made available by the Wellcome Trust Sanger Institute (<http://www.sanger.ac.uk>). The array slides were produced in-house at Leiden University Medical Center according to Knijnenburg *et al.*<sup>16</sup>

Hybridisation and posthybridisation washing steps were performed on a HS400 TECAN automated hybridisation station

(Tecan, Giessen, The Netherlands) according to Knijnenburg *et al.*<sup>17</sup> and slides were then scanned with a GenePix Personal 4100A scanner at 5 µm resolution (Axon Instruments, Union City, California). The spot intensities were measured using GenePix Pro 4.1 software. With this software, spots in which the reference DNA intensity was below five times the mean of the background or presented more than 3% saturated pixels were excluded from further analysis. The test/reference ratios were normalised for the median of the ratios of all features. The triplicates of the features were averaged in a homemade routine developed in Microsoft Excel 2000, and spots outside the 20% CI of the average of the triplicate were excluded. Only those targets presenting at least two spots within 20% CI of their average were used. Any imbalances in the targets were determined based on log<sub>2</sub> ratios of the average of their replicates, and we considered sequences as amplified or deleted when outside the ±0.3 range.

Resulting data files were further analysed, and log<sub>2</sub> ratio values were analysed using R packages CGHCall and VAMP webtool.<sup>18 19</sup> Hemi- and homozygous loss were defined as one and two levels lower than normal respectively, and gain as one or two levels higher than normal. Gains with more than two levels were identified as amplified regions. Genomic locations (chromosome band and clone positions) were determined according to Ensembl Gene build (database version 54.36p) ([http://www.ensembl.org/Homo\\_sapiens](http://www.ensembl.org/Homo_sapiens)).

### Confirmatory interphase fluorescence in situ hybridisation (FISH) on formalin fixed paraffin embedded tissue (FFPE) samples

To confirm the array-CGH results, we performed interphase FISH on 4 µm thick FFPE tissue of the L2736 with small amplified regions of chromosomes 2p16.1 containing the *BCL11A* and *REL* gene loci. A panel of BAC probes was selected covering the region, and as a reference chromosome two centromer specific alphoid repeat probes were combined. The following BAC probes were used: RP11-416L21, RP11-498O5, RP11-493E12, RP11-373L24 and RP11-440P5 (the latter two were present on the BAC array-CGH platform detecting the amplification) for the alphoid repeat sequence D2Z2 plasmid clone. Probes were labelled using standard protocols as described earlier.<sup>20 21</sup>

Interphase FISH experiments was performed according to previously described protocols on formalin-fixed paraffin-embedded tissue slides.<sup>22</sup> Slides were embedded in Citifluor antifading solution containing with 4',6-diamino-2-phenylindole-dihydrochloride (DAPI)/citifluor (500 ng/ml) (Brunschwig Chemie, Amsterdam, The Netherlands). Image acquisition was performed using a DM-RA epifluorescence microscope (Leica Microsystems b.v. Rijswijk, The Netherlands) equipped with a Quantix camera (Roper Scientific, Fairfield, Iowa). Grey scale

**Table 1** Overview of the clinicopathological data and test results

Patient	Gender	Age (years)	Stage	Localisation	Treatment	Result	Follow-up
L2734	M	46	I	Humerus	CHOP-like+RT	CR	Disease-free
L2735	M	58	I	Humerus	CHOP-like+RT	CR	Disease-free
L2736	M	61	IV	Os ilium/vertebra	CHOP-like+RT	PR	Dead from disease
L2737	M	25	I	Femur	R-CHOP	CR	Disease-free
L2738	F	72	I	Femur	R-CHOP+RT	CR	Disease-free
L2739	F	32	I	Femur	CHOP-like+RT	CR	Disease-free
L2740	M	33	I	Tibia	CHOP-like+RT	CR	Disease-free
L2385	M	25	I	Tibia	R-CHOP+RT	CR	Disease-free
L2060	M	35	I	Scapula	CHOP+RT	CR	Disease-free

CR, complete remission; F, female; M, male; PR, progressive disease; RT, radiotherapy; Stage, Ann Arbor stage (Stage IV is multifocal).

images were collected with a 63× oil immersion objective by using appropriate filters to visualise the FITC, Cy3 and DAPI stainings. For further image processing, in-house-developed software (ColourProc) was used.<sup>23</sup>

## RESULTS

All patients presented with pain and/or a palpable mass, most often at a single location in one of the long bones. Multifocal bone involvement, scored as stage IV, was noted in one case, and none had iliac crest bone marrow involvement. The male:female ratio was 7:2. The mean age at presentation was 43, ranging from 25 years to 72 years. Clinical findings, including age, gender, primary location of tumour, treatment and outcome, are summarised in table 1. The minimum follow-up was 12 months. All patients were treated with CHOP (cyclophosphamide, doxorubicin, vincristin, prednisone) or CHOP-like chemotherapy; three were also treated with rituximab. Eight patients were also treated with involved field radiotherapy. Of the nine patients, eight reached complete remission; one had progressive disease within 3 months after completing R-CHOP and died of disease, after an initial response to chemotherapy.

### Immunohistochemical features

Applying Hans' algorithm, the GC phenotype was defined as CD10+BCL-6+, and the non-GC phenotype was defined as CD10-BCL-6-. In the case of CD10-BCL-6+, the phenotype was defined as GC if MUM-1 expression was negative and as non-GC if MUM-1 was positive.<sup>24</sup> Eight cases were of the GC phenotype, and one case was of the non-GC phenotype. The results are summarised in table 2.

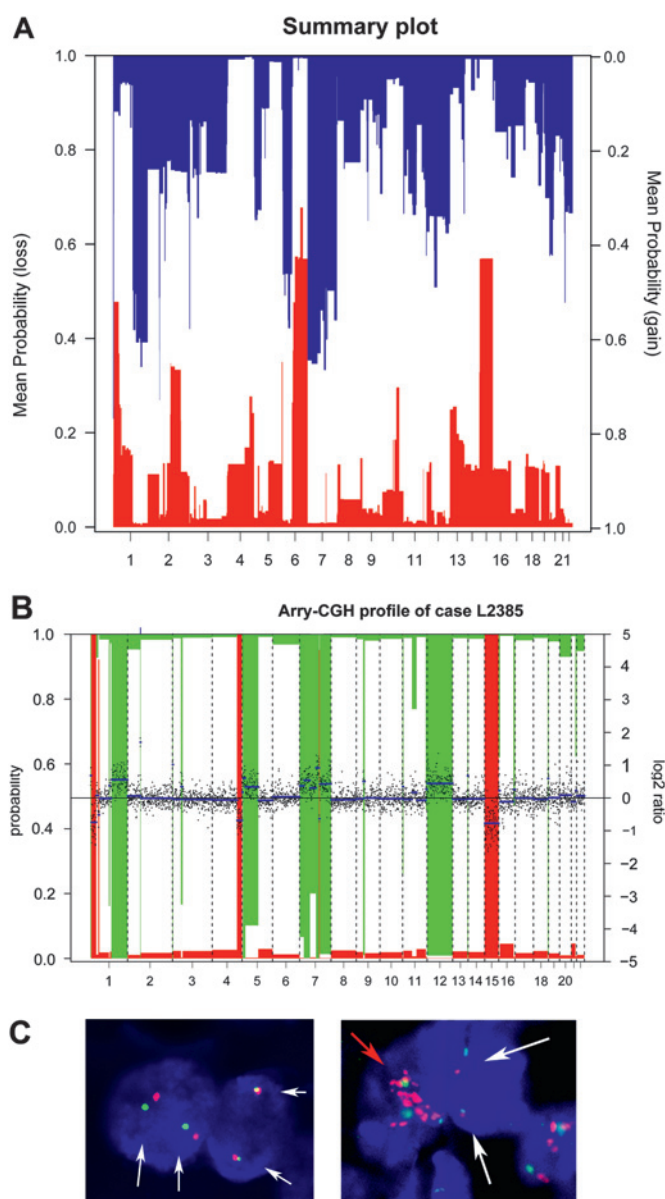
### Array-based CGH

Following quality control, frozen tumour biopsy samples of nine patients diagnosed as having PLB were analysed for copy-number alterations using array-CGH. The array-CGH profiles showed numerous chromosomal alterations in all analysed PLB samples. No common alteration was observed in all cases. The overall gain/loss frequency was plotted using an R script, CGHCall (figure 1A). The overall pattern of chromosomal alterations of PLB is characterised by gains of large genomic regions on chromosome 1q, 6p and 7, and losses of regions on chromosome 1p, 6q and 15. A high level of amplification was observed involving the 2p15–16.1 region in 4/9 cases (table 2). None of the analysed samples presented homozygous deletions. In order to delineate the smallest recurrent chromosomal regions with altered probes common to the set of array-CGH profiles in at least 35% of the analysed cases, we determined the minimal common regions (MRC) containing potentially relevant genes<sup>25</sup> and the VAMP

**Table 2** Overview of Hans' algorithm and selected array-based comparative genomic hybridisation results

Patient	CD10	BCL-6	MUM-1	Phenotype	Gain of 1q	Amplification of 2p16.1
L2734	–	+	–	GC	+	–
L2735	–	+	–	GC	+	+
L2736	+	+	–	GC	–	+
L2737	–	+	–	GC	–	+
L2738	–	–	+	Non-GC	–	–
L2739	+	+	–	GC	+	–
L2740	+	+	–	GC	+	–
L2385	–	+	–	GC	+	+
L2060	–	+	–	GC	–	–

GC, germinal-centre-like phenotype; Non-GC, non-germinal-centre-like phenotype.



**Figure 1** Overview of the array-based comparative genomic hybridisation (array-CGH) results and confirmatory fluorescence in situ hybridisation (FISH) experiments. (A) Array-CGH cumulative summary plot of all analysed PLB samples generated by default settings of the CGHCall R software. Probabilities for genomic losses displayed on the left y axis and gain probabilities are on the right y axis. The calculated probabilities for genomic loss or gain are represented by red and blue lines, respectively. (B) Representative array-CGH profile of case L2385. Normalised log<sub>2</sub> ratios are plotted with the scale on the right axis. Vertical bars indicate loss and gain probabilities. The probability scale is on the left axis; reversed ('1–') for the gains. Segments are plotted as horizontal lines. Segments with a bar extending beyond the middle axis (probability >0.5) are called gains or losses. Amplification locations are indicated by tick marks on the top axis. This case shows loss of 1p, gain of 1q, amplification of 2p15–16.1 (see blue tick mark on the top), gain of chromosome 7 and loss of chromosome 15 without involvement of chromosome 6. (C) Interphase FISH results using 2p16.1 locus-specific pore set (red) and chromosome 2 centromer-specific alphoid repeat sequence probe (green) in combination with DNA counterstaining agent: DAPI (blue). Left panel: two normal cells with two green and two red signals as indicated by white arrowheads. Right panel: interphase FISH proved the amplification of the 2p16.1 locus in case L2736. The red arrowhead indicates amplified signals in a tumor cell; white arrowheads point to a normal cell with two green and two red signals.



web tool.<sup>26</sup> An overview of the MCRs is given in table 3. Eight MCRs were identified, and four of the eight regions were full chromosome or full chromosome arms (gain: 1q, 6q and chromosome 7, loss: chromosome 15). The four MCRs with smaller genomic changes were: loss of chromosome 1p36.3–1p35.1 (~30 Mb region), high level of amplification of chromosome 2p16.1–2p15 (0.9 Mb), gain of chromosome 6p21.31 (3.7 Mb) and loss of 14q32.33 (1 Mb). Analysis of the co-occurrence frequency of the genomic alteration revealed that the deletion of 1p, 6q and 14q and the monosomy of chromosome 15 were mostly together, while no such similar association was seen for other regions. Clustering of the analysed samples, due to the low numbers of samples and homogeneous clinical group, were not informative.

Next, we matched the four MCRs with the Cancer Gene Census, a list of genes for which mutations have been casually identified in cancer.<sup>27</sup> In table 3B, the four identified MCRs with the 12 identified tumour suppressor genes and oncogenes are presented. The most highly recurrent MCR with loss of the *IGH* gene may represent a clonal immunoglobulin gene rearrangement rather than direct oncogenic involvement.

### Confirmatory interphase FISH

For two cases (L2735 and L2736) with small amplification of the 2p16.1 region containing the *BCL11A* and *REL* gene loci, 4 µm FFPE sections were cut and analysed by interphase FISH. A probe mixture was used containing five BAC clones covering the amplified region - including those two that were present on the BAC array and showed the amplification (RP11-373L24 and RP11-440P5)- labeled in red and mixed with a chromosome 2 specific alphoid repeat probe labeled in green. As a control, an FFPE section from skin was used and showed a distinct pattern of two centromeric signals in green with two 2p16.1 locus-specific signals in red (figure 1C left panel). The FISH showed a clear amplification pattern in case L2736 with a high level of amplification involving the 2p16.1 locus mingled with normal cells (figure 1C right panel; white arrows indicate the normal cells, and the red arrow points to a tumour cell with amplified red signals).

### Correlation with clinical data

Because of the very good clinical outcome of this group of patients, which results in few statistical events, no statistically

significant correlation between genetic abnormalities and prognosis could be made. The one patient with a dismal clinical course showed no specific array-CGH pattern. It did have the 2p16.1 amplification, but not a gain of 1q. The clinical parameters were unfavourable, with age at presentation over 60, multifocal disease and bulky tumour load. The one case with a non-GC phenotype did show an excellent response to chemotherapy. There was no specific array-CGH pattern in this non-GC phenotype either.

### DISCUSSION

Here we present the first array-CGH study on PLB. The sample size of the cohort was restricted due to the rarity of this tumour and, more specifically, the scarcity of fresh frozen tumour samples of PLB. As a result, few studies on PLB have been published because of these difficulties, which stresses the importance of the current investigation. We and others demonstrated in previous reports, using clinical and immunohistochemical data, that PLB has a favourable prognosis and is of GC-like origin in the majority of cases.<sup>2 10 28</sup> Our current cohort is representative of the typical clinical spectrum of PLB, with the majority of the tumours presenting in the long bones, and all but one patient achieving complete remission. Most prior studies on PLB excluded patients with multiple bone involvement—stage IV patients—which leads to an imperfect representation of the spectrum of this disease. The patient with progressive disease was the only patient in this cohort with multifocal disease. Moreover, his age at presentation was over 60. As we previously reported, these two parameters, both included in the IPI risk index, are the main adverse clinical prognostic factors in PLB.<sup>2 29</sup>

DLBCL is the most heterogeneous group of lymphomas. Over the years, various subtypes and classifications have been designed in an attempt to predict clinical course and prognosis for individual patients. Historically, DLBCL is divided into nodal and extranodal lymphoma. The data currently available in the literature on extranodal lymphoma are often obtained from studies performed on extranodal lymphoma in general. However, since clinical outcome varies substantially among all the specific sites of primary lymphoma, this generalisation might be clinically inappropriate. It is therefore important to study any particular primary site of lymphoma as a separate

**Table 3** Array-based comparative genomic hybridisation result overview in PLB

(A) Most recurrent chromosomal alterations in PLB (losses and gains are separated)							
Chromosome band	Start clone	End clone	Start position (bp)	End position (bp)	Size (bp)	CNA	No of cases
1p36.3–1p35.1	RP4-785P20	RP1-117N3	3214521	33379650	30165129	Loss	5
6q14.1–6q27	RP11-2506	RP5-1086L22	83405494	170509779	87104285	Loss	4
14q32.33	RP11-417P24	CTC-820M16	105267358	106278173	1010815	Loss	7
15q11.2–15q26.5	RP11-289D12	RP11-14C10	20363717	100036184	79672467	Loss	5
1q21.1–1q44.1	RP3-365I19	RP11-438H8	142642781	247249719	104606938	Gain	5
2p16.1–2p15	RP11-440P5	RP11-479F13	60501800	61422449	20349	Amplification	4
6p21.31	RP11-175A4	RP1-90K10	33521322	37077773	3556451	Gain	4
7p22–7q36.2	RP11-449P15	RP11-518I12	885103	157752947	156867844	Gain	6

(B) Candidate genes residing in frequently altered regions					
Chromosome	Chromosome band	Size (bp)	CNA	No of cases	Cancer gene census
1	1p36.3–1p35.1	30165129	Loss	5	LCK, MDS2, SDHB, PRDM16, PAX7
14	14q32.33	1010815	Loss	7	IGH
2	2p16.1–2p15	920349	Amplification	4	REL, BCL11A
6	6p21.31	3556451	Gain	4	HMG1A, SFRS3, FANCE

CNA, copy number alteration.

entity. Research in genomic scale gene-expression profiling has resulted in the definition of two tumour phenotypes in DLBCL, one GC-like, and one non-GC-like. A difference in response to multiagent chemotherapy is noted between these subgroups, with a favourable outcome for the GC phenotype. Unfortunately, these two phenotypes still show considerable clinical and morphological heterogeneity.

Recent studies show that array-CGH can be used for identifying these tumour phenotypes in malignant lymphoma as well.<sup>11</sup> More importantly, array-CGH can identify chromosomal aberrations within the same tumour phenotype, which is a subsequent step in making the group of (extranodal) DLBCL less heterogeneous.

For example, primary cutaneous large B cell lymphoma, leg-type, which has a moderately aggressive course, frequently shows a 9p21.3 deletion.<sup>13</sup> The prognosis of immune privileged associated DLBCL, such as testis and CNS DLBCL, is only slightly better. The typical aberration of these immune privileged lymphomas is deletion of 6q21–22.<sup>12</sup> Both subtypes of extranodal lymphoma are mostly of the non-GC origin, but differ considerably in clinical outcome. The dissimilar array CGH results between these subtypes of extranodal DLBCL suggest another tumour aetiology via different oncogenic mechanisms.

In this study, we found several recurrent aberrations, all of which have been described in DLBCL before. Intriguingly, several of these chromosomal changes are described in the literature as negative prognostic factors, which cannot be confirmed in this study focusing on PLB specifically. The data from our and other previous studies<sup>11–13</sup> suggest that for extranodal DLBCL, genotype-based classification (ie, array-CGH study) in combination with the site of involvement is a better class identifier. Loss of 14q32.33 was the most frequently observed event (seven cases). This deletion indicates a breakpoint in the IGH locus, which has been frequently described in B cell malignancies and, more specifically, in extranodal cutaneous DLBCL, such as large B cell lymphoma of the leg, and in primary cutaneous follicle centre lymphoma.<sup>30</sup> Trisomy 7 (six cases) is associated in the literature with progression of follicular lymphoma and with DLBCL, mostly as an adverse prognostic factor.<sup>31</sup> 2p16.1 amplification (four cases) and gain of 1q (five cases) have been associated with GC phenotype in the literature.<sup>8, 32, 33</sup> Our array-CGH results and immunohistochemical results confirm the previously described GC-like origin of PLB and are in accordance with its favourable prognosis (table 2). Of note, the one patient with non-GC phenotype did not have a gain of 1q or 2p16.1 amplification. It has been suggested that the REL proto-oncogene is the target gene of 2p16.1 amplification in DLBCL.<sup>34</sup> It encodes a transcription factor in the nuclear factor (NF) kappa-B family. Studies on the prognostic influence of 2p16.1 amplification in DLBCL are still controversial.<sup>35</sup> The one case in this study with 2p16.1 amplification, age over 60 at presentation, stage IV at presentation and an unfavourable outcome together with the favourable outcome of the case with non-GC phenotype, which had stage I at presentation, does suggest that clinical parameters in PLB have a strong influence on prognosis. Gain of the short arm of chromosome 6 and loss of the long arm of chromosome 6 are both adverse prognostic factors frequently found in DLBCL.<sup>12, 36</sup> In the future, array-CGH could be helpful in risk-stratification of extranodal DLBCL patients, which are all treated in a similar way at this moment. The goal would be to select those patients who need more intensive therapy than the standard regime of R-CHOP and radiotherapy on the one hand, and to protect the patients with a more favourable prognosis against too intensive a treatment on the other hand.

## Take-home messages

- ▶ Primary lymphoma of bone (PLB) has a homogeneous clinicopathological behavior.
- ▶ Array-CGH and expression profiling separates DLBCL to GC-like and non-GC-like types.
- ▶ GC-like DLBCL subtype is associated with better clinical outcome.
- ▶ Array-CGH profiling of PLB showed GC-like associated genetic profile (1q gain and 2p16.1 amplification).
- ▶ The genetic profile of PLB differs from other extranodal DLBCL.

## Conclusions

We found several recurrent genomic aberrations, including five cases with gain of 1q and four cases with 2p16.1 amplification, which are both associated with the GC phenotype. These findings concur with the relatively good prognosis of this rare type of extranodal lymphoma and differ from array-CGH results in other extranodal lymphomas.

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**Competing interests** None.

**Contributors** FH drafted the manuscript; KS carried out the array-CGH studies, participated in the design of the study and its coordination, and helped to draft the manuscript; PJ carried out the immunohistochemical studies; and PCWH coordinated the study.

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