Fine-Mapping Chromosomal Loss at 9p21: Correlation with Prognosis in Primary Cutaneous Diffuse Large B-Cell Lymphoma, Leg Type

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Primary cutaneous diffuse large B-cell lymphoma, leg type (PCLBCL, LT) is the most aggressive type of primary cutaneous B-cell lymphoma. In a recent study on 12 patients it was found that inactivation of CDKN2A by either deletion of 9p21.3 or promoter hypermethylation is correlated with a worse prognosis. In the present EORTC multicenter study, skin biopsies of 64 PCLBCL, LT patients were analyzed by multiplex ligation-dependent probe amplification to validate these previous results and to fine-map the losses in this region. Although no minimal common region of loss could be identified, most homozygous loss was observed in the *CDKN2A* gene (43 of 64; 67%) encoding p16 and p14ARF. Promoter hypermethylation of p16 and p14ARF was found in six and zero cases, respectively. Survival was markedly different between patients with versus without aberrations in the *CDKN2A* gene (5-year disease-specific survival 43 versus 70%; $P = 0.06$). In conclusion, our results confirm that deletion of chromosome 9p21.3 is found in a considerable proportion of PCLBCL, LT patients and that inactivation of the *CDKN2A* gene is associated with an unfavorable prognosis. In most patients the deletion involves a large area of at least several kilobase pairs instead of a small minimal common region.

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INTRODUCTION

Primary cutaneous diffuse large B-cell lymphoma, leg type (PCLBCL, LT) is the most aggressive type of primary cutaneous B-cell lymphoma (CBCL). It is generally characterized by rapidly growing tumors that present on the leg(s), but in a minority of patients skin lesions can also arise at other sites. Histologically it is defined as a tumor with a predominance or confluent sheets of large, atypical B cells (resembling centroblasts and immunoblasts), which generally express Bcl-2 and MUM-1. The disease has an intermediate prognosis with a 5-year survival rate of only 50% (Willemze *et al.*, 2005). As it is recognized that this disease represents a distinct type of CBCL, it will be included as a separate entity in the forthcoming WHO 2008 classification.

In a recent study by our group, using array-based comparative genomic hybridization (aCGH), it was found that inactivation of the CDKN2A region, encoding for the tumor suppressor genes p16 and p14ARF, by either deletion of chromosome 9p21.3 or promoter hypermethylation, is associated with a worse prognosis. However, these results were based on only 12 cases (Dijkman *et al.*, 2006).

Multiplex ligation-dependent probe amplification (MLPA) has recently been described as a new method for relative quantification of multiple different DNA sequences in a single reaction, requiring only small amounts of DNA (Schouten *et al.*, 2002). Moreover, the application of this

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Abbreviations: aCGH, array-based comparative genomic hybridization; CBCL, primary cutaneous B-cell lymphoma; DSS, disease-specific survival; FFPE, formalin-fixed, paraffin-embedded; MLPA, Multiplex ligation-dependent probe amplification; PCFCL, primary cutaneous follicle center lymphoma; PCLBCL, LT, primary cutaneous diffuse large B-cell lymphoma, leg type

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technique on DNA isolated from formalin-fixed, paraffin-embedded (FFPE) material has previously been reported to be reliable and less sensitive to DNA degradation (Natte *et al.*, 2005; Takata *et al.*, 2005; van Dijk *et al.*, 2005). Targeted MLPA probe panels are commercially available including a set of probes targeting the chromosomal region of 9p21 containing several known genes (CDKN2A, coding for p16 and p14ARF, CDKN2B, coding for p15 and MTAP).

In the present study, MLPA was used to confirm that deletion of CDKN2A is an unfavorable prognostic marker in a large patient group and to further fine-map the 9p21.3 region in order to determine a possible minimal common region.

RESULTS

Testing the reliability of the MLPA technique

We first investigated whether results generated by MLPA were in accordance with our previous aCGH results (Dijkman *et al.*, 2006). In addition, we aimed to obtain more information on the precise location of deletions as the MLPA technique has a much higher spatial resolution than the aCGH technique applied previously (Figure 1) (Dijkman *et al.*, 2006). To that end, we subjected available isolated DNA from 12 patients (9 PCLBCL, LT and 3 primary cutaneous follicle center lymphomas (PCFCL)) previously investigated by aCGH to MLPA. We were indeed able to confirm chromosomal aberrations in 9p21.3 as detected by aCGH in all patients, with homozygous loss in 4 of 12 patients and hemizygous loss in 2 of 12 patients using MLPA (Figure 2). Moreover, in 3 of the 6 patients that did not show loss in the aCGH analysis (3 PCLBCL, LT and 3 PCFCL), MLPA allowed detection of hemizygous loss (and in one patient even homozygous loss) of individual probes within the complete set (2 PCLBCL, LT and 1 PCFCL) (Figure 2). This demonstrates the higher sensitivity of the MLPA technique as compared to the aCGH platform previously used. However, we could not detect a minimal common region of deletion within the *CDKN2A* gene region.

As a considerable part of our samples was derived from FFPE material, it was felt important to test the claim that MLPA can be applied as reliably to DNA isolated from FFPE material as to DNA isolated from frozen material (van Dijk *et al.*, 2005). To accomplish this, we analyzed DNA from fresh-frozen samples and FFPE material taken simultaneously from the same tumor. This was performed in 2 patients from whom at 3 different time points biopsy material was collected (primary tumor, first skin relapse, and second skin relapse). We observed full concordance of the results as depicted for

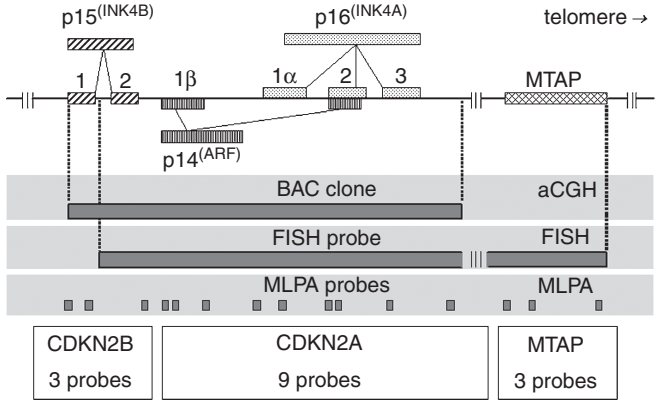

Figure 1. Schematical representation of 9p21.3 showing the spatial resolution of different techniques. MLPA can be used to fine-map the chromosomal aberrations as found by aCGH (BAC clone RP11-149I2 corresponding to 21899259-22000413 (according to Ensembl) on chr. 9) and FISH (for example LSI p16 probe from Vysis which, according to the manufacturer, at least includes the region from 21792942 (D9S1749) to 21995210 (D9S1752) on chromosome 9). Exact genomic positions of the MLPA probes can be requested at MRC Holland (info@mlpa.com).

MLPA results	PCLBCL, LT($n=9$)									PCFCL ($n=3$)		
	# 1	# 2	# 3	# 4	# 5	# 6	# 7	# 8	# 9	# 10	# 11	# 12
p15, promotor+exon 1	0.17	0.13	0.41	0.5	1.02	0.99	1.03	0.86	1.13	0.93	1.09	0.81
p15, end exon 1	0.13	0.13	0.41	0.45	1.04	0.88	1.05	0.98	1.02	0.99	1.09	0.95
Intron. p15/p14ARF	0.17	0.14	0.23	0.42	0.74	0.65	1.01	0.54	0.24	0.95	1.03	0.93
p14ARF, promotor	0.18	0.09	0.5	0.42	1.14	0.91	1.09	0.77	0.67	0.99	1.4	1.17
p14ARF, promotor near TSS	0.13	0.11	0.42	0.52	1.18	0.97	1.07	0.83	1.33	0.97	1.23	0.99
p14ARF, exon 1	0.16	0.08	0.25	0.32	0.93	0.73	0.9	0.73	0.63	1.04	1.17	1.01
Intron, p14ARF/p16	0.13	0.09	0.28	0.43	0.4	0.61	0.92	0.62	1	0.91	0.93	0.86
Intron, p14ARF/p16	0.12	0.06	0.37	0.42	0.41	0.74	0.86	0.67	1.05	0.73	0.81	0.67
p15, exon 1	0.19	0.09	0.57	0.48	0.59	1.02	1.32	0.82	1.15	1.19	2	1.45
p15, exon 1	0.17	0.07	0.5	0.41	0.57	0.91	1.13	0.82	1.35	1.04	1.23	0.97
p15, exon 2	0.13	0.09	0.27	0.46	0.45	0.76	0.99	0.58	1.36	1.09	0.95	0.68
p15, exon 3	0.13	0.07	0.43	0.38	0.47	0.8	0.92	0.77	1.13	0.97	1.03	0.93
MTAP, End	0.13	0.07	0.31	0.34	0.81	0.69	0.85	0.89	0.78	0.95	1.01	0.95
MTAP	0.19	0.1	0.4	0.38	0.82	0.76	1.06	0.97	0.7	1.02	1.13	1.02
MTAP, Start	0.19	0.09	1.16	1.09	1	1.08	1.03	1.09	0.66	1.21	1.41	1.05
aCGH results (log2 ratio)	−0.92	−0.94	−0.48	−0.50	−0.26	−0.24	−0.05	−0.15	−0.06	−0.04	−0.02	−0.07
SD	0.12	0	0.05	0.04	0.05	0.03	0.03	0.03	0.03	0.07	0.03	0.01
Follow-up	D+12	D+26	D+21	D+11	A-85	A-60	A-82	D+54	D-18	A-264	A-62	A-60

Figure 2. Array CGH versus MLPA results for PCLBCL, LT ($n=9$) and PCFCL patients ($n=3$). D+ = died of lymphoma, D− = died of other cause, A− = alive in complete remission. Black = homozygous loss, grey = hemizygous loss, white = no loss.

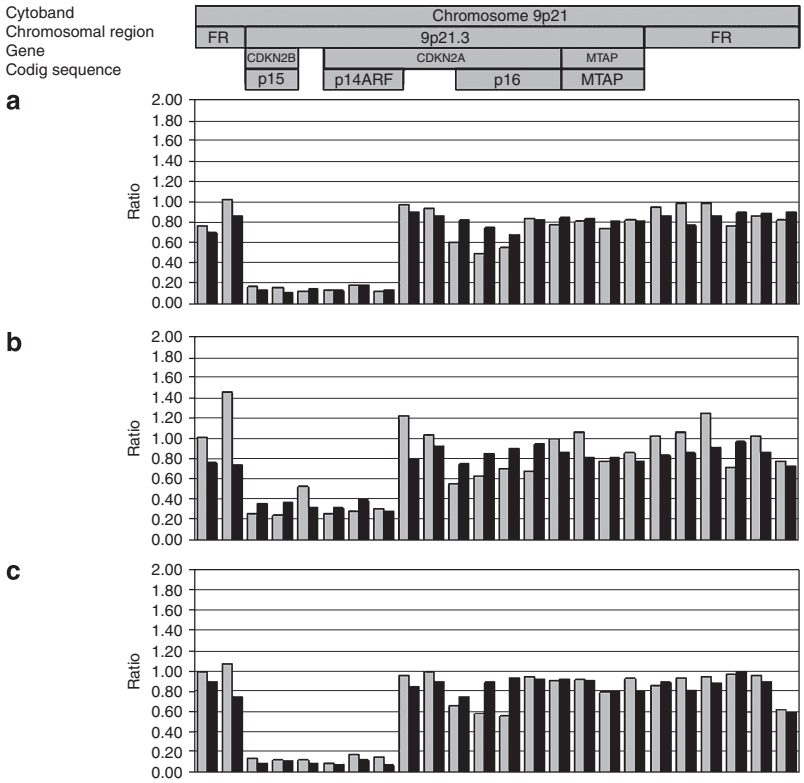

Figure 3. MLPA results for DNA from fresh-frozen (black bars) versus FFPE material (grey bars) from one patient. Skin biopsies obtained (**a**) at diagnosis, (**b**) at first skin relapse after 32 months, and (**c**) at second skin relapse after 39 months. FR: flanking region.

one patient in Figure 3, thereby demonstrating the applicability of this technique on partly degraded DNA. In addition, it was noted that the genetic lesions in the 9p21.3 region showed a stable pattern over time and did not alter with disease progression and treatment.

Allelic loss at the 9p21.3 locus

Having confirmed that MLPA can reliably detect genetic lesions in frozen as well as in FFPE material, we subjected the whole study group to MLPA. In total, tumor biopsies from 64 patients with a diagnosis of PCLBCL, LT were included in the final study group. This included the 9 patients previously analyzed by aCGH and in addition 55 new patients. The overall MLPA results for these patients are depicted in Figure 4. It was found that 45 patients (70%) showed homozygous loss of one or multiple probes within the 9p21.3 region. Hemizygous loss was found in 14 patients and 5 patients did not show any detectable loss in this region.

Most chromosomal aberrations were localized in the *CDKN2A* gene. Homozygous loss within this region was found in 43 of 64 cases (67%). Homozygous losses in the coding regions for p16 (exon 1α, 2, and 3) and p14ARF (exon 1β and 2), as well as both promoter regions, were found in 40 of 64 (63%) and 37 of 64 (58%) cases, respectively. Specific probes, most often lost were located in exon 1α coding for p16 and exon 2 coding for both p16 and p14ARF (both probes were lost in 31 of 64 cases; 48%). In most patients however, the deletion covered a large part of chromosome 9p21.3 instead of a smaller minimal common region (Figure 4).

Analysis of p16 and p14ARF promoter methylation status

From 20 of 21 patients without homozygous loss in CDKN2A, sufficient DNA was available to determine the promoter methylation status of p16 and p14ARF. In none of the samples methylation of the p14ARF promoter was found. However, methylation of the p16 promoter was detected in 6 of 20 samples. Five of these samples showed hemizygous loss within the p16 coding and promoter region in MLPA analysis and one sample had no loss within this region.

Correlation with survival

Survival analysis revealed a clear correlation between homozygous loss in chromosome 9p21.3 and reduced survival. Patients without homozygous loss in this region had an actuarial 5-year disease-specific survival (DSS) of 68%, whereas patients with loss of at least one probe in this region had a 5-year DSS of 39% ($P=0.06$). Although statistically not significant, these results are in line with the findings described by Dijkman *et al.* (2006). As most loss occurred in the CDKN2A region, we also performed survival analysis for losses specific to this region and in addition also for loss in areas coding specifically for p14ARF and p16 separately. Loss in the CDKN2A region was most strongly correlated with prognosis. Five-year DSS for patients with or without loss in this region was 38 versus 69% ($P=0.03$)

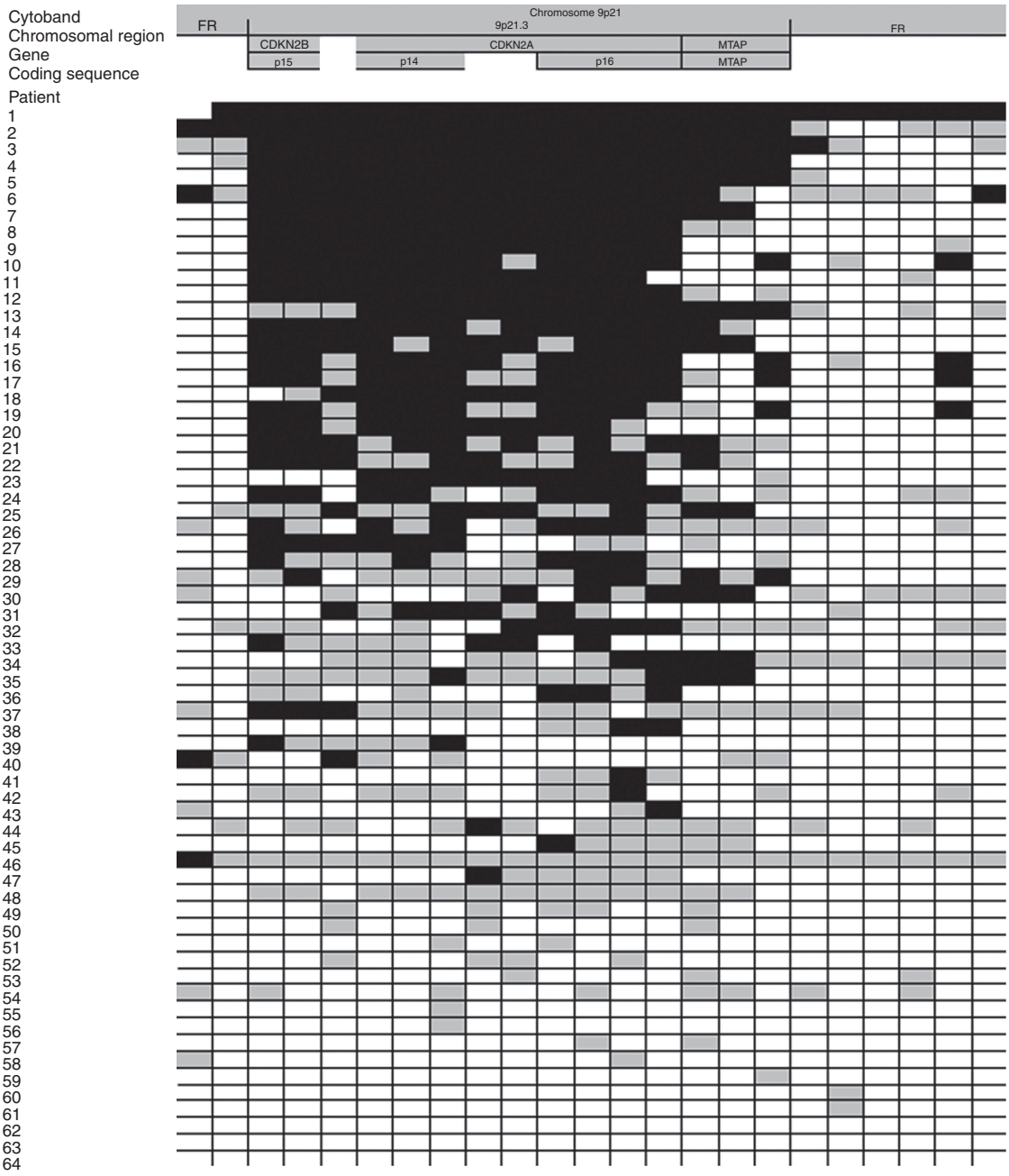

Figure 4. Total MLPA results for 64 PCLBCL, LT patients. Black = homozygous loss (ratio <0.4), grey = hemizygous loss (ratio between 0.4 and 0.7), white = no loss (ratio >0.7). FR: flanking region.

(Figure 5). Differences in 5-year DSS between patients with or without losses in the regions coding p14ARF or p16 were not or borderline significant, respectively.

Finally, we tested whether inclusion of the methylation data affected the survival analysis. In the complete study group a total of 48 patients can be considered to have an inactivated *CDKN2A* gene (43 patients with homozygous loss in the *CDKN2A* gene and 5 patients with a methylated p16 promoter combined with hemizygous loss in CDKN2A). Five-year DSS for patients with and those without inactivation of CDKN2A are 70 and 43%, respectively ($P = 0.059$) (Figure 6).

DISCUSSION

In this study we aimed to confirm recently reported data describing that loss of 9p21.3 and more specifically, inactivation of CDKN2A is commonly found and associated with inferior prognosis in PCLBCL, LT (Dijkman *et al.*, 2006). By using MLPA we were able to confirm loss of 9p21.3, including the *CDKN2A* gene, on a large group of patients with PCLBCL, LT. We observed full concordance with the results as obtained by aCGH, and, in addition, detected small areas of loss in three patients. So comparison between aCGH and MLPA confirms the higher sensitivity of the latter

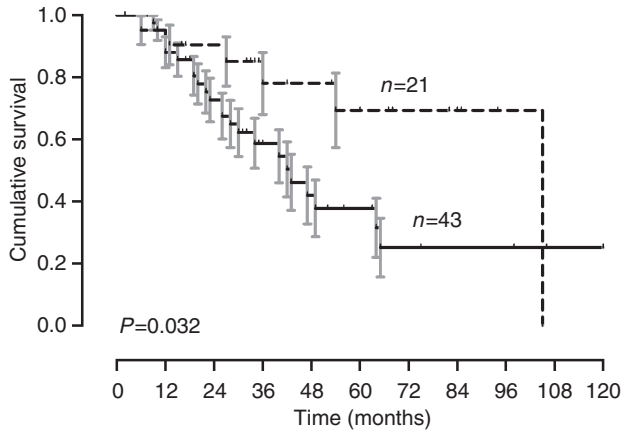

Figure 5. Disease-specific survival of 64 PCLBCL, LT patients according to chromosomal aberrations within CDKN2A. Solid line: patients with homozygous loss of one or multiple probes in CDKN2A ($n=43$), dashed line: patients with no or hemizygous loss in CDKN2A ($n=21$). Error bars indicate standard error.

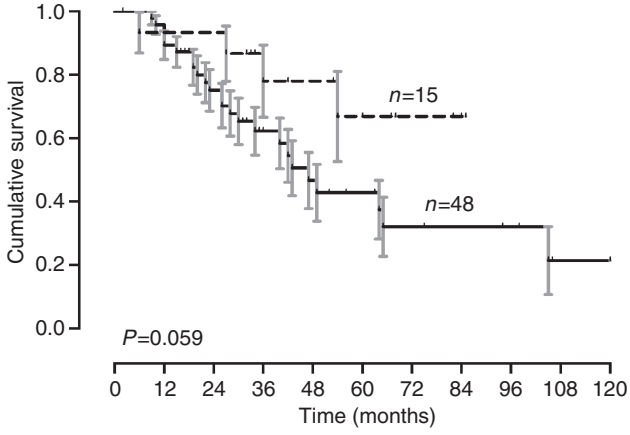

Figure 6. Disease-specific survival of 63 PCLBCL, LT patients according to CDKN2A status. Solid line: patients with inactivation of CDKN2A ($n=48$), dashed line: patients without inactivation of CDKN2A ($n=15$). In one patient no DNA was available for determining methylation status, this patient is not included in this analysis. Error bars indicate standard error.

technique and its ability to fine-map larger areas of loss as found by genome-wide analyses such as aCGH using BAC clones as in the previous study. It was found that in most patients the deletion covers a substantial part (up to several tens of thousands of base pairs) of this chromosomal region. Although no minimal common region of loss could be detected, most chromosomal aberrations converged on the *CDKN2A* gene.

An additional advantage of the MLPA technique is that it can be applied reliably on FFPE material of CBCL patients. Comparison between DNA derived from fresh-frozen and FFPE sections, obtained from the same tumor in two patients, showed identical results. Moreover, comparison of skin biopsy specimens obtained from consecutive tumors in these two patients, demonstrated identical chromosomal aberrations, indicating that these losses can display a stable pattern over time.

Loss or inactivation of the *CDKN2A* gene either by deletion or promoter hypermethylation has been extensively reported in hematological malignancies, including B-cell non-Hodgkin lymphomas (Dreyling *et al.*, 1998; Baur *et al.*, 1999; Sanchez-Beato *et al.*, 2001; Raschke *et al.*, 2005). CDKN2A codes for p16 and p14ARF, both of which are tumor suppressor genes and are negative regulators of cell-cycle progression. In our study group, inactivation of CDKN2A was mostly due to (homozygous) deletion. Promoter hypermethylation of p16 was found in a minority of cases, which is in accordance with the results of previous studies in CBCL (Gronbaek *et al.*, 2000; Belaud-Rotureau *et al.*, 2008). Promoter hypermethylation of p14 was never detected.

Besides confirming the loss in this chromosomal region we further wanted to validate the prognostic significance of the findings as reported previously. Although less striking than the results reported by Dijkman *et al.* (2006), loss or inactivation of CDKN2A, was still associated with reduced survival (Figures 5 and 6), which is also consistent with previous reports of others (Gronbaek *et al.*, 2000; Child *et al.*, 2002; Belaud-Rotureau *et al.*, 2008). Even though the results described herein show a clear, and borderline significant, correlation with reduced survival, loss of CDKN2A cannot be used as the sole tool to optimize management in individual patients.

In our study group there are several patients with deletions in 9p21.3, but have a favorable clinical course thus far. More importantly, 5 of 21 patients without inactivation of CDKN2A died of lymphoma 6–54 months (median 27 months) after diagnosis. Especially this latter group runs the risk of being undertreated when management would be solely based on CDKN2A status.

In conclusion, in a large part of PCLBCL, LT patients chromosomal loss is seen in 9p21.3. In most patients these losses are concentrated on the *CDKN2A* gene coding for p16 and p14ARF. Inactivation of this gene is caused by homozygous deletion or, less commonly, by promoter hypermethylation and is associated with a worse prognosis. However, caution is warranted before these results are incorporated into clinical decision making.

MATERIALS AND METHODS

Sample collection

Cases were collected from centers collaborating in the EORTC Cutaneous Lymphoma Group. Tumor DNA from pretreatment skin biopsies of 80 patients were initially submitted for the study. Patients with incomplete staging investigations (minimum requirements being routine laboratory screening, CT scans of chest and abdomen, and bone marrow biopsy) or follow-up of less than 12 months (unless caused by death due to lymphoma) were excluded from further analysis ($n=6$). In addition, of all submitted cases hematoxylin and eosin sections were reviewed for morphological reference and estimation of percentage tumor- and admixed-reactive cells. In case of doubt about the percentage of tumor cells, we reviewed stainings for CD3 and CD20. If these were not available, the case was excluded. Combined with information on the expression of Bcl-2, MUM-1, and FOXP1, a diagnosis of PCLBCL, LT was confirmed or discarded. Cases in which a diagnosis of PCLBCL, LT could not be confirmed and cases with more than 30% admixed reactive T cells

were excluded ($n = 5$). Finally, five cases could not be analyzed due to poor quality DNA. The final study group consisted of 64 patients with a diagnosis of PCLBCL, LT. The study group contained 25 men and 39 women (male–female ratio: 0.6), with a median age at diagnosis of 78 years (range 47–92 years) and a median duration of follow-up of 34 months (range 2–158 months). Clinical characteristics and treatment data are presented in Table 1. In addition, three patients with a diagnosis of PCFCL were included in the experiment validating the MLPA technique. A total of 12 of the above described patients (9 PCLBCL, LT and 3 PCFCL) were formerly analyzed with aCGH (Dijkman *et al.*, 2006). Genomic DNA was extracted from either fresh-frozen material, or FFPE sections, using local protocols.

This study was performed in accordance with the Dutch code and Leiden University Medical Center guidelines on leftover material. Patient informed consent was not required because this code considers human tissue left over from surgery or diagnostic procedures as discarded material. The study was conducted according to the Helsinki guidelines.

Table 1. Clinical and treatment characteristics of 64 patients with PCLBCL, LT

Total number of patients	64
Age (years) (median (range))	78 (47–92)
Sex	
Male	25
Female	39
Male:female ratio	0.6
Site of skin lesions (%)	
Head/neck	2 (3%)
Trunk	7 (11%)
Arm(s)	2 (3%)
Leg(s)	59 (92%)
Extent of skin lesions (%)	
Solitary	26 (41%)
Regional	30 (47%)
Multifocal	8 (13%)
Treatment (%)	
Radiotherapy	32 (50%)
Chemotherapy	15 (23%)
Chemotherapy and radiotherapy	9 (14%)
Surgery	3 (5%)
Surgery and radiotherapy	2 (3%)
Rituximab	1 (2%)
Other	2 (3%)
Result of treatment (%)	
Complete remission	54 (84%)
Partial remission	6 (9%)
No response	3 (5%)
Progressive disease	1 (2%)
Status at last follow-up (%)	
Alive and well	23 (36%)
Alive with disease	6 (9%)
Died of lymphoma	30 (47%)
Died of other cause	5 (8%)

Fine-mapping chromosomal loss at 9p21.3 using MLPA

A commercially available MLPA Kit (SALSA MLPA Kit P024B; MRC-Holland, Amsterdam, the Netherlands) targeting the 9p21 region was used according to the manufacturer's protocol. The P024B kit contains 23 probes of which 9 probes are specific for the CDKN2A region, 3 probes for the CDKN2B region, and 3 probes for the MTAP gene, whereas 8 probes hybridize to regions flanking these genes. For the experiments we used 60–80 ng of genomic DNA and normal control DNA (a DNA mix of 15 healthy donors) was always included in the same reaction. The principles of the MLPA technique are concisely described by Vorstman *et al.* (2006), whereas detailed methodology can be found in the paper by Schouten *et al.* (2002).

Briefly, genomic DNA diluted in 5 μl of Tris-EDTA 10 mM, was denatured at 95 °C for 5 minutes, mixed with the probe set and the MLPA buffer, and incubated for 16 hours at 60 °C. After probe hybridization, products were ligated for 15 minutes at 54 °C. The ligase enzyme was then inactivated by incubation for 5 minutes at 98 °C. The ligation products were subsequently amplified by PCR using universal FAM-labeled primers. All these reactions were carried out in a PTC-200 Thermal cycler with heated lid (MJ Research, Waltham, MA). The resulting products were separated according to size on an ABI Prism 3730 DNA analyzer (Applied Biosystems, Nieuwerkerk aan den IJssel, the Netherlands) by the inclusion of GeneScan ROX 500 as internal size standard (Applied Biosystems). Resulting fragment analysis chromatograms were sized to standard fragment lengths by GeneMapper v3.7 (Applied Biosystems).

Promoter hypermethylation analysis

As promoter hypermethylation can be involved in gene inactivation, we also evaluated the methylation status of the CpG islands, located in the promoter regions of p16 and p14ARF, in patients without homozygous loss of (parts of) the *CDKN2A* gene. Promoter methylation status was determined by performing melting curve analysis of bisulfite-converted and PCR-amplified tumor DNA, as described previously (Worm *et al.*, 2001). Tumor DNA was modified with sodium bisulfite by using the EZ Methylation Kit (Zymo Research Corporation, Orange, CA). PCR primers were designed to anneal to bisulfite-converted DNA as template which amplified a region of the p16 and p14ARF gene promoter CpG islands (Table 2). PCR amplification of bisulfite-treated DNA and subsequent melting curve analysis in the presence of SYBRGReen (MyiQ Real-time PCR Detection System; Bio-Rad Laboratories BV, Veenendaal, the Netherlands) allowed detection of methylation present in the sample DNA, by generating a peak with a higher melting temperature as compared to unmethylated DNA. Ratios for methylated versus unmethylated DNA in each sample were determined by dividing the total area under the melting temperature curve(s) by the area under the methylation specific peak. All samples showing a ratio above 0.3 were considered to contain methylated tumor DNA.

Table 2. PCR primer sequences, designed to anneal to bisulfite-converted DNA as template, for p16 and p14ARF gene promoter CpG islands

Gene	Primer sequence (5′–3′)	CpGs in amplicon	Position of amplicon relative to transcription start site	Amplicon size (bp)
p16	GATTTAATTTGGTAGTTAGGAAGGTTGT GGTTGGGAGTAGGGAGGTCG	10	−299, −159	140
p14ARF	GAGGGGAGTTAGGAATAAAATAAGG CTAAAACGCAACTCCAAACAAC	10	−413, 268	145

Data analysis and statistical methods

Analysis of MLPA results was carried out upon the transfer of GeneMapper results to Coffalyser software, a data analysis tool which was designed by MRC-Holland for normalization of MLPA fragment data files. With this program, DNA copy number ratios of test samples can be computed, by comparison and normalization to a control sample (for full description see: http://www.mlpa.com/coffalyser/). As sample DNA is compared against a normal control sample, a ratio of 0.5 would ideally indicate hemizygous loss and zero would indicate homozygous loss. However, considering the fact that our samples contained a maximum of 30% admixed reactive cells, ratios between 0.4 and 0.7 were considered as hemizygous loss, whereas ratios below 0.4 were considered as homozygous loss.

For analysis of clinical data and performing survival analyses, SPSS 14.0 (SPSS Inc., Chicago, IL) was used. DSS was calculated from the date of diagnosis until death from lymphoma (including therapy-related death) or last follow-up without event. Survival curves were estimated using the method of Kaplan and Meier and statistical comparison between curves was performed by log-rank testing.

CONFLICT OF INTEREST

The authors state no conflict of interest.

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