

Remission DNA samples were available from 15 patients, nine of whom had a mutation at disease presentation. In each case the mutation was no longer detectable in remission, confirming that these were acquired leukemia-specific mutations and not germline polymorphisms. Relapse DNA samples were available from four patients (#3, 7, 10 and 17, Table 1). The two patients with mutations at diagnosis relapsed with the same mutation(s). Neither acquired a different mutation, and the two patients who were wild type at diagnosis did not acquire a mutation at relapse.

The results in our cohort of patients are comparable with those reported in the younger aged patients (Table 2) and emphasise the heterogeneity of mutations that can disrupt Notch-1 function. Of the 18 different mutations identified, only four have been previously described in the pediatric cohort or T-ALL cell lines.³ The HD domain is responsible for stable association of Notch-1 subunits⁶ and structural integrity in this region is required to prevent exposure of the site to unregulated proteolytic cleavage. Functional analysis using a Notch-sensitive luciferase reporter assay has shown that leucine to proline point mutations in the HD domain lead to increased activity.³ At least 10 of the 13 different HD domain mutations detected in our cohort are likely to result in an altered conformation, either through insertion of a proline residue, or loss or gain of amino acids. Conversely, the C-terminal PEST domain is an important negative regulatory site required for ICN degradation, and loss of this region also leads to increased activity in the Notch-sensitive reporter assay, probably through stabilization of ICN.³ All PEST domain mutations detected in our patients would lead to a similarly truncated protein. The probable functional consequence of the mutations identified in our study is supported by the loss of these mutations at remission and reappearance at relapse, which provides conclusive evidence that they are acquired and are specifically associated with the leukemic clone. The selective expansion of such clones indicates that the mutations are likely to provide some proliferative or survival advantage to the cell.

The presence of mutations in our cohort was not restricted to the young adult patients; 10 of the mutant-positive patients were over 30 years of age at presentation, and median age of mutant-positive patients was 35 years compared to 20 years for wild-type patients. This suggests that a similar pathogenic mechanism underlies the development of most cases of T-ALL irrespective of age, and the difference in curability in the two groups is not related to a differing frequency of Notch mutations. The median presenting white cell count was $58.3 \times 10^9/l$ and did not differ significantly between the two groups ($P=70$).

These results suggest that Notch mutations may be good candidate markers for minimal residual disease detection, particularly as they would not be susceptible to the antigenic

drift that desensitizes current monitoring techniques in T-ALL.⁷ Furthermore, γ -secretase inhibitors (GSI) have been shown to induce cell cycle arrest *in vitro* in T-ALL cell lines harboring Notch-1 mutations³ and therefore may offer a rational molecularly targeted approach for these patients who, in the older age group, have a poor outcome with current therapy. The experience gained from clinical trials using GSIs for Alzheimer's disease, where they inhibit the production of beta amyloid peptide from amyloid precursor protein, should expedite trials of these agents in T-ALL.

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Gain of chromosome 6p is an infrequent cause of increased PIM1 expression in B-cell non-Hodgkin's lymphomas

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Gains and losses of genomic material are changes that play central roles in tumor development and progression. Compar-

ative genomic hybridization (CGH) allows for the detection of over- and underrepresented sequences in tumor DNA as compared to normal DNA, and such genomic aberrations have been shown to have prognostic value in several studies of primary lymphomas. The serine/threonine kinase *PIM1* is a known oncogene that has previously been shown to be

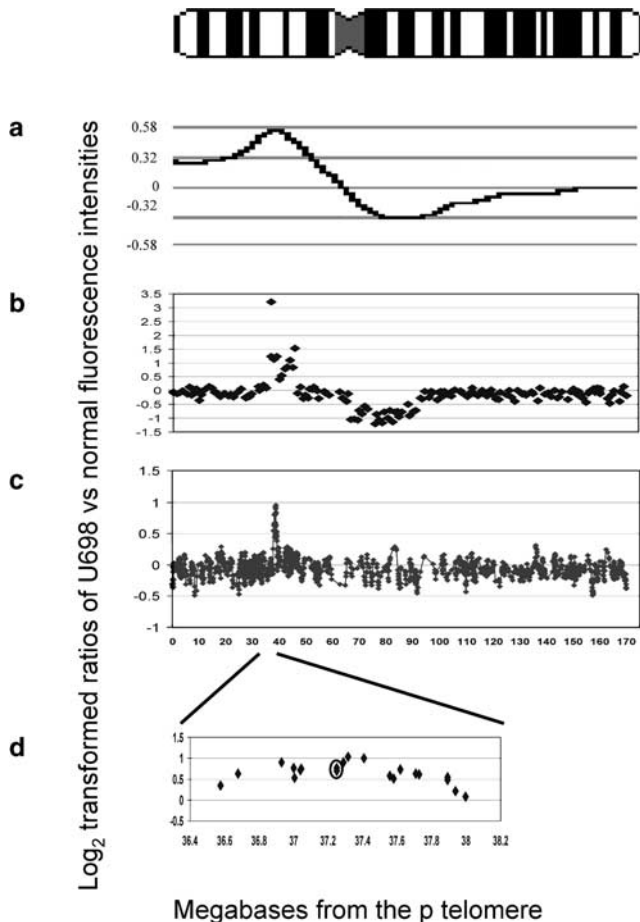


Figure 1 Gain of 6p21 in the lymphoma cell line U698. (a) CGH profile of chromosome 6 in U698. (b) BAC array CGH profile. (c) cDNA array CGH profile. (d) Detailed view of the cDNA array CGH profile of the 6p21 amplicon in U698. The two clones representing *PIM1* are encircled.

upregulated in prostate cancer. Overexpression of *PIM1* in cell lines protects against apoptosis induced by genotoxic agents. The *PIM1* gene is localized to chromosome 6p21.2. Parts of chromosome 6 or the whole 6p arm are gained in a subset of B-cell non-Hodgkin's lymphomas, and these gains are associated with a poor prognosis.¹

In order to identify genomic aberrations important for tumorigenesis of non-Hodgkin's lymphomas, we characterized in detail the gains and losses of genomic material in the mature B-cell lymphoma cell line U698, originally classified as a lymphoblastic lymphosarcoma.² The general procedures of DNA isolation, CGH hybridizations and gene copy number analysis by FISH has been described earlier.³ A local DNA gain mapping to region 6p21 was identified by conventional CGH analysis (Figure 1a). To identify genes in the amplified region, we performed array CGH analysis using a BAC-array with an average spacing of 1 Mbp between the probes (Figure 1b). This analysis narrowed the region of gain down to nine megabases. The most telomeric clone (RP3-431A14) was located on 6p21.31, whereas the most centromeric clone (RP11-546O15) was located on 6p21.1. A 10-fold gain was observed with the probe RP1-90K10. To further resolve any fine structure in the amplicon, DNA from U698 cells was hybridized to cDNA arrays with an average spacing of 150 kbp (Figure 1c). Although the dynamics and sensitivity of the array CGH analysis was

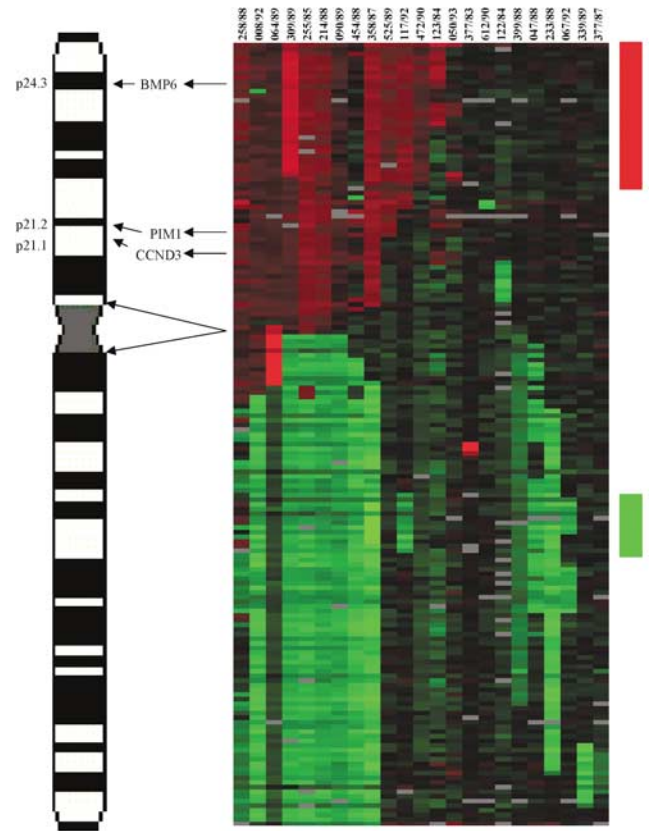


Figure 2 BAC array CGH profile of B-NHLs with chromosome 6 alterations. Genomic gains are shown in red whereas genomic losses are shown in green. The color intensities reflect the experimentally obtained log₂ transformed ratios. The most intense red and green colors reflect values above 1 and below -1, respectively. Regions with normal DNA copy number are shown in black.

much lower with cDNA arrays than with BAC arrays, the former analysis indicated that the amplicon was approximately 1.5 Mbp. *PIM1* was approximately in the center of this amplicon (Figure 1d).

Dual color interphase FISH studies showed that there were two copies of centromere 6 in both Reh and U698 cells. In contrast, U698 exhibited one normal signal and in addition a cluster of 15–20 signals from the *PIM1* probe (RP3-355M6), probably representing intrachromosomal amplification of the *PIM1* gene (Supplementary Figure 1). Translocations in lymphomas targeting *CCND3* has been described, and high *BMP6* expression has been shown to be of negative prognostic value in DLBCL.⁴ Considering these genes as possible targets for chromosomal alterations, we performed FISH with a probe for *CCND3* (RP5-973N23), located at 6p21 and *BMP6* (RP1-119C5) located at 6p24. Whereas there were only two signals from the *BMP6* probe, the *CCND3* probe generated four signals, indicating that this gene is also represented in a proportion of the amplicon in U698 cells.

Having established that *PIM1* is included in the high-level amplified region of 6p observed in U698, we investigated whether the increase in DNA content was reflected in high expression levels of the corresponding mRNA and protein. Northern blot analysis revealed that the basal mRNA levels of *PIM1* in U698 are higher than in other cell lines (Supplementary Figure 2). U698 cells also express high amounts of *PIM1* protein,

Table 1 B-NHL with chromosome 6 alterations: summary of chromosomal CGH findings, copy number data of *CCND3*, *PIM1* and *BMP6* obtained by FISH and the copy numbers of the same genes suggested by array CGH. The order of the tumors is the same as in Figure 2.

Case no.	Histology	Tumor fraction %B	Ploidy	CGH		Copy number determined by FISH				Copy number suggested by array CGH		
				Gain	Deletion	Centromere 6	<i>CCND3</i>	<i>PIM1</i>	<i>BMP6</i>	<i>CCND3</i>	<i>PIM1</i>	<i>BMP6</i>
258/88	dlbcl	44	1.07	6p		3	3	3	3	3	3	3
008/92	unclass	81	1	6p	6q	2	3	3	3	3	3	3
064/89	fcl II	69	1.26	6pter-q15		3	3	3	3	3	3	3
309/89	mcl	79	1.83	6p	6q	4	6	6	8	6	6	8
255/85	dlbcl	71	1.04	6p	6q	3	3	3	3	3	3	3
214/88	unclass	84	1.00	6p	6q	2	3	3	3	3	3	3
090/89	fcl III	52	1.21	6p	6q	2	3	3	3	3	3	3
454/88	dlbcl	92	1.02		6q	2	Inconcl.	Inconcl.	2	2	2	2
358/87	mcl	89	1.94	6p	6q	3	6	6	6	6	6	6
525/89	dlbcl	84	1.07	6p		2	2	3	3	2	3	3
117/92	dlbcl	98	1	6p21.3-ter		2	2	2	3	2	2	3
472/90	dlbcl	85	1.16	6p22-ter		2	2	2	3	2	2	3
123/84	mcl	94	1.04			ND	2	2	3	2	2	3
050/93	dlbcl	85	1.00			2	2	2	2	2	2	2
377/83	fcl I/II	78	1			2	ND	2	2	2	2	2
612/90	dlbcl	82	1.04			ND	ND	2	ND	2	2	2
122/84	dlbcl	86	1.23		6q	2	ND	2+5	2+5	2	2	2
399/88	fcl III	54	1		6cen-q24	ND	ND	2	ND	2	2	2
047/88	fcl I/II	72	1.00		6cen-q22	ND	ND	2	ND	2	2	2
233/88	fcl II	90	1.00		6q14-ter	ND	ND	2	ND	2	2	2
067/92	mcl	90	0.98			ND	ND	2	ND	2	2	2
339/89	sll	90	1.00			2	2	2	2	2	2	2
377/87	dlbcl	48	1.03			ND	ND	2	ND	2	2	2

dlbcl = diffuse large B-cell lymphoma; mcl = mantle cell lymphoma; fcl = follicle center lymphoma; ND = not determined; Inconcl. = inconclusive.

whereas the levels are undetectable in Reh cells (immunoblotting data not shown).

We now proceeded to define the core chromosome 6p amplicon in B-NHL to clarify whether *PIM1* could be the target of these gains. 93 B-cell lymphomas were subjected to analysis with array CGH as well as FISH with probes corresponding to *PIM1*, *CCND3*, *BMP6* and centromere 6. These tumors have previously been analyzed by chromosomal CGH, and in this study gain of chromosome 6p was found to have independent negative prognostic value.¹ Compared to the data obtained by chromosomal CGH, the BAC array CGH experiments revealed more copy number changes and could better define the boundaries of the gains and losses. The data of the array CGH experiments are available in Supplementary Table 1. The minimal consensus region for the gain on chromosome 6 was 6p22.1-ter (Figure 2). Loss of the whole arm of 6q was the most common change on 6q, and this was previously also detected by chromosomal CGH.¹ The minimal consensus region for the deletions at 6q was 6q16.2–q21 by BAC array CGH. In addition, we observed high-level gains at 6cen-q13 in tumor 64/89 (ratio = 2.9), 6q15 in 377/83 (ratio = 6.6) and 6p22.2-ter in 309/89 (ratio = 1.7) (Figure 2). The cDNA array CGH experiments did not reveal any high-level gains on chromosome 6 in this material. By FISH analysis, we found that 10 out of 92 successfully analyzed tumors exhibited increased copy number of *PIM1* relative to the DNA index. Nine of these tumors had also gain of 6p detected by chromosomal CGH analysis, whereas one tumor contained a clone of tumor cells with five copies of both *PIM1* and *BMP6* (122/84). Table 1 summarizes the chromosomal CGH data for chromosome 6, the copy number data of *CCND3*, *PIM1* and *BMP6* obtained by FISH, and the copy numbers of the same genes suggested by array CGH.

Given that gain of *PIM1* is a feature of a significant proportion of B-NHL, we wanted to clarify whether this gain is reflected in

increased expression levels that might influence on the tumor response to treatment. We therefore proceeded to analyze a set of diffuse large B-cell lymphomas,⁴ that was independent from the previously analyzed B-NHL.¹ By assessing the copy number of *PIM1* in 35 of the tumor samples from this study of which we had material available,⁴ we first examined whether increased copy number of *PIM1* in the tumor samples could account for the variation in the expression levels of the gene. Seven of the tumor samples had increased copy number of *PIM1*; three tumors had four copies while four had three. On average, these tumors exhibited 20% higher expression levels of *PIM1* compared to the ones without gain. This difference was, however, not significant (95% confidence interval 0.74–1.96, $P=0.41$).

We then proceeded to investigate the role of *PIM1* expression with respect to DLBCL subtype and prognosis. Analysis of the data revealed that expression higher than the median of *PIM1* is associated with the activated B-cell phenotype ($P<0.001$). Moreover, patients with high expression levels had significantly poorer outcome ($P=0.0054$) (Supplementary Figure 3).

Our data indicate that other genes than *PIM1* are the targets for the 6p gain frequently observed in malignant lymphomas, since the gene is not included in the core chromosome 6p amplicon. Moreover, the variation in *PIM1* expression seen in lymphomas cannot merely be explained by an increase of the copy number of the gene. Changes in transcriptional regulation could be a mechanism for *PIM1* upregulation. It has been shown that the NF-kappa B pathway is involved in *PIM1* regulation.⁵ This would fit well with the fact that high *PIM1* expression mainly is found in activated B-cell like DLBCL (ABC), which are characterized by constitutive activation of the NF-kappa B pathway.⁴ Recently, it was shown that that inhibitors of IkappaB kinase (IKK) are toxic for ABC DLBCL cell lines, but not for cell lines derived from the germinal center like DLBCLs. Interest-

ingly, treatment of the cells with such inhibitors was accompanied by changes in gene expression that included down-regulation of *PIM1*.⁶ This suggests that the high expression levels of *PIM1* seen in this tumor type, at least partly, are caused by transcriptional activation. However, other mutations of the genome than a mere increase in copy number could also lead to activation of the proto-oncogene, and *PIM1* has been shown to be involved in translocations as well as to be a subject for hypermutations in lymphomas. The highest number of *PIM1* copies detected by FISH in our material of 125 primary lymphomas was five, indicating that high-level gain of this locus must be a rare event in this tumor type. Previously, there have been sporadic reports on amplicon at 6p21 in primary lymphomas.^{7,8} Our findings in U698 where the high-level gain at this locus was accompanied by high expression levels of *PIM1*, suggest that *PIM1* can be the target of these rare amplifications, and thus in some cases play a role in the tumorigenesis of lymphomas.

The *PIM1* locus was not included in the consensus region of the 6p gain in our study, which is in line with the finding of others. Other candidate target genes for the gain are *BMP6* at 6p24 or *IRF4* at 6p25. High expression of both these genes has been associated to poor prognosis in DLBCL,⁴ and they are also included in the core 6p amplicon in our study.

It is now well established that certain gene expression features of the tumor in part cause the clinical heterogeneity of the lymphomas in terms of outcome, and the different tumor phenotypes have also been correlated to certain genotypes. The great variability of lymphomas regarding tumor genotype and phenotype as well as clinical behavior, is a therapeutic challenge. *PIM1* is promising as a therapeutic target, since it is overexpressed in tumors with poor response to therapy, and since silencing of this gene potentially could lead to higher sensitivity of the tumor cells to apoptosis inducing agents. However, since our data indicate that the transcriptional regulation to some extent overrides the gene dosage effect of this gene, the ideal targeting of *PIM1* probably also involves factors upstream in the regulation cascade. It will therefore be interesting to examine the effects of selective *PIM1* knock down combined with inhibitors of I κ B kinase in B-NHL.

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Reduced-intensity conditioning allogeneic transplantation is associated with a high incidence of extramedullary relapses in multiple myeloma patients

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Autologous stem cell transplantation (ASCT) has become the standard of care for young patients diagnosed with multiple

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myeloma (MM). Nevertheless, the median duration of response after ASCT does not exceed 3 years, and almost all patients ultimately relapse. By contrast, allogeneic transplantation has led to a long-term disease-free survival in a fraction of MM patients.¹ Unfortunately, allogeneic transplantation has been associated with a high transplant-related mortality (TRM),