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Baseline differential blood count and prognosis in CD20-positive post-transplant lymphoproliferative disorder in the prospective PTLD-1 trial

Leukemia (2013) **27,** 2102–2105; doi:10.1038/leu.2013.110

Post-transplant lymphoproliferative disorder (PTLD) is a spectrum of lymphatic diseases associated with the use of potent immunosuppressive drugs after transplantation and ranges from polyclonal early lesions associated with primary Epstein-Barr virus (EBV) infection to monomorphic lymphoma.[1] The PTLD-1 trial, the largest prospective phase II trial in the field so far, has demonstrated the efficacy and safety of sequential therapy (rituximab followed by cyclophosphamide, doxorubicin, vincristin, prednisolone (CHOP) chemotherapy) with an overall response rate (ORR) of 90% and 6.6 years median overall survival (OS) in CD20-positive PTLD unresponsive to reduction of immunosuppression.[2] Because of the risk of treatment-related complications, such as infections in immunosuppressed transplant recipients, tailoring treatment to the individual patient is of particular importance in PTLD.[3] The European study groups on PTLD have already implemented risk stratification according to the response to rituximab (NCT00590447), with encouraging interim results.[4] However, stratification according to baseline parameters could potentially improve therapy even further.

With this in mind, we have noted with interest that a number of publications have demonstrated a significant prognostic effect of the differential blood count at initial diagnosis on OS in immunocompetent patients with diffuse large B-cell lymphoma (DLBCL): these included a poorer outcome in patients with an absolute lymphocyte count (ALC) $\leqslant$1000/μl in DLBCL treated with rituximab-cyclophosphamide, doxorubicin, vincristin, prednisolone (R-CHOP) immunochemotherapy,[5–8] inferior OS and progression-free survival (PFS) for patients with a baseline neutrophil/lymphocyte ratio (NLR) $\geqslant$3.5 in a cohort of 255 consecutive patients with DLBCL treated with R-CHOP at a single centre,[9] and significantly poorer treatment response, OS as well as PFS, for patients with a lymphocyte-to-monocyte ratio (LMR) $\leqslant$2.6 in 438 patients with DLBCL treated with R-CHOP.[10] In addition, Wilcox *et al.*[8] demonstrated a poor OS outcome in a cohort of 366 patients with DLBCL treated from 1993 to 2007 with CHOP or R-CHOP at a single institution not only for a low ALC $\leqslant$1000/μl but also for a high absolute monocyte count (AMC) $\geqslant$630/μl, and combined both in a model, the absolute monocyte and lymphocyte prognostic score (AMLPI), assigning one point each for either low lymphocytes or high monocytes. This model demonstrated a highly significant effect on OS and PFS—confirmed in a subgroup analysis of those patients receiving R-CHOP. The cohort of 70 patients treated in the PTLD-1 trial is the largest prospectively treated trial cohort in this disease entity so far. Because of uniform diagnostic criteria and treatment, it is ideally suited to examine the prognostic value of the baseline differential blood count in PTLD under sequential immunochemotherapy.

The current analysis is based on the published data set of the international, prospective, multicentre phase II PTLD-1 trial (NCT01458548, $n = 70$, data cut-off 1 June 2011):[2] solid organ transplant recipients with CD20-positive PTLD unresponsive to immunosuppression reduction received four weekly courses of 375 mg/m^2 rituximab followed by 4 weeks without treatment and four cycles of CHOP chemotherapy (cyclophosphamide 750 mg/m^2 IV day (d) 1, doxorubicin 50 mg/m^2 IV d1, vincristine 1.4 mg/m^2 IV d1 and prednisone 50 mg/m^2 Per OS (PO) d1–5) at 3-week intervals starting at day 50. In case of disease progression under rituximab treatment, patients proceeded to chemotherapy immediately (therefore, starting before day 50). Supportive treatment included granulocyte colony-stimulating factor support (mandatory) as well as antibiotic prophylaxis (cotrimoxazole and ciprofloxacin, recommended). Key exclusion criteria were central

Accepted article preview online 15 April 2013; advance online publication, 3 May 2013

Table 1. Baseline differential blood count as prognostic factors in univariate analysis in the PTLD-1 trial

	n/N (%)	*OS*		*TTP*	
		P	*HR (95% CI)*	P	*HR (95% CI)*
ALC $\leqslant$1000/μl	34/56 (60.7)	**0.027**	**2.809 (1.122–7.030)**	0.104	2.538 (0.827–7.792)
AMC $\geqslant$630/μl	14/56 (25)	0.873	0.928 (0.372–2.314)	0.450	0.618 (0.178–2.153)
AMC $\geqslant$495/μl	28/56 (50)	0.251	1.580 (0.723–3.452)	0.387	1.532 (0.583–4.026)
LMR $\leqslant$2.6	37/56 (66.1)	**0.034**	**2.891 (1.081–7.728)**	0.106	2.798 (0.803–9.747)
NLR $\geqslant$3.5	36/56 (64.2)	**0.006**	**4.479 (1.529–13.121)**	0.169	2.198 (0.716–6.751)
AMLPI	16,32,8 of 56	0.093	1.668 (0.917–3.034)	0.433	1.342 (0.643–2.799)
Adjusted AMLPI	10,30,16 of 56	**0.010**	**2.288 (1.222–4.282)**	0.052	2.153 (0.992–4.673)
Adjusted AMLPI >1	16/56 (28.6)	**0.016**	**2.642 (1.197–5.831)**	**0.027**	**2.949 (1.134–7.669)**

Abbreviations: ALC, absolute lymphocyte count; AMC, absolute monocyte count; AMLPI, absolute monocyte and lymphocyte prognostic score; CI, confidence interval; HR, hazard ratio; LMR, lymphocyte-monocyte ratio; *n/N*, number of patients with risk factor over number of patients analysed; NLR, neutrophil/lymphocyte ratio; OS overall survival; PTLD, post-transplant lymphoproliferative disorder; TTP, time to progression. AMLPI, one point each for low lymphocytes (ALC $\leqslant$1000/μl) and high monocytes (AMC $\geqslant$630/μl); adjusted AMLPI, one point each for low lymphocytes (ALC $\leqslant$1000/μl) and high monocytes (AMC $\geqslant$495/μl). Significant results ($P<0.05$) are highlighted in bold.

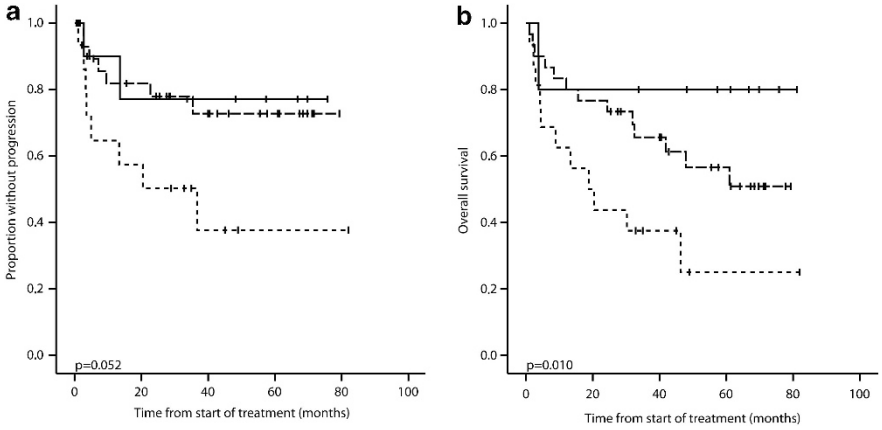

Figure 1. Kaplan–Meier estimates of TTP (**a**) and OS (**b**) in 56 PTLD patients treated with sequential immunochemotherapy in the PTLD-1 trial stratified by an adjusted absolute monocyte and lymphocyte prognostic index (adjusted AMLPI, one point each for low lymphocytes (ALC $\leqslant$1000/μl) and high monocytes (AMC $\geqslant$495/μl)). The solid line indicates a score of 0, the broken line indicates a score of 1 and the dashed line indicates a score of 2.

nervous system involvement, human immunodeficiency virus infection, severe organ dysfunction not related to PTLD and Eastern Cooperative Oncology Group (ECOG) performance status >2. Baseline differential blood counts were collected retrospectively from the recruiting centres. Baseline was defined as within 4 weeks before the first application of rituximab. Overall, baseline differential blood counts were available from 56/70 patients (80%) treated in the PTLD-1 trial from centres in Australia, France and Germany. Exploratory univariate analyses were performed by applying log-rank analyses to Kaplan–Meier statistics for time-to-event outcomes and χ^2-tests to categorical variables. Multivariable analyses were performed using Cox regression models (likelihood ratio, backward elimination). The level of significance in all cases was set at $P<0.05$. Statistical analysis was performed with IBM SPSS 20, Armonk, NY, USA.

In a univariate analysis of the differential blood count at diagnosis (Table 1), an ALC $\leqslant$1000 lymphocytes/μl ($P=0.027$), LMR $\leqslant$2.6 ($P=0.034$) and a baseline NLR $\geqslant$3.5 ($P=0.006$) were associated with inferior OS. However, neither ALC, NLR nor LMR had a significant effect on time to progression (TTP).

Median lymphocyte counts (830/μl) and monocyte counts (495/μl) were lower than those reported by Wilcox *et al.*[8] in immunocompetent lymphoma patients (1230 and 630/μl, respectively). Although ALC $\leqslant$1000/μl had a significant prognostic effect on OS (see above), AMC did not reach prognostic significance on OS or TTP as a dichotomized variable in the PTLD-1 cohort (neither Wilcox's cut-off of 630/μl nor a cut-off at the median, 495/μl, Table 1). The AMLPI had no significant effect on OS ($P=0.093$) or TTP ($P=0.433$). However, an adjusted AMLPI using a median monocyte cut-off ($\geqslant$495/μl) in analogy to the situation in immunocompetent patients was significant for OS ($P=0.010$) and borderline significant for TTP ($P=0.052$, Table 1). The group of patients with an adjusted AMLPI >1 in particular had significantly poorer OS ($P=0.016$) and TTP ($P=0.027$) than those with a score $\leqslant$1 (Figure 1).

We have previously published multivariate Cox regression analyses of factors affecting OS and TTP in the PTLD-1 trial and found that time-to-PTLD was a significant protective factor for both OS and TTP and EBV association for TTP only.[2] We performed backward log-rank Cox regressions with highly significant variables from the univariate analyses (NLR $\geqslant$3.5, and adjusted AMLPI for OS; adjusted AMLPI >1 for TTP) and the previously established parameters age, time-to-PTLD, EBV association, elevated serum LDH and ECOG $\geqslant$2. In these models, NLR $\geqslant$3.5 remained a significant predictor of OS ($P=0.019$, hazard ratio (HR) 3.701, 95% confidence interval (CI) 1.242–11.030) and an adjusted AMLPI >1 of TTP ($P=0.038$, HR 2.936, 95% CI 1.062–8.119) in multivariate analysis.

In PTLD, it is of particular importance to ascertain if inferior OS is related to poor treatment efficacy or treatment complications.

Furthermore, the differential blood count in an immunosuppressed patient is strongly influenced by their medication. We therefore investigated correlations between the ALC, NLR, LMR and adjusted AMLPI on the one hand and ORR, treatment-related mortality (TRM) and immunosuppressant drug classes (antimetabolites, mammalian target of rapamycin inhibitors, calcineurin inhibitors and steroids) on the other hand:

There were no significant correlations between TRM and either ALC $\leqslant$1000/μl, NLR $\geqslant$3.5, LMR $\leqslant$2.6 or the adjusted AMLPI. In immunocompetent DLBCL patients, a lower ORR for patients with ALC $\leqslant$1000/μl has been described;[5,7] however, in our PTLD cohort, there was no significant correlation between ALC $\leqslant$1000/μl, NLR $\geqslant$3.5, LMR $\leqslant$2.6 or the adjusted AMLPI with ORR to either rituximab or sequential immunochemotherapy. Regarding immunosuppression, steroids were significantly more common in patients with a NLR $\geqslant$3.5 (27/35 vs 10/20, $P = 0.039$). We could not identify significant correlations between ALC $\leqslant$1000/μl, LMR $\leqslant$2.6 or adjusted AMLPI and immunosuppression.

In summary, in 56/70 patients treated in the PTLD-1 trial, ALC $\leqslant$1000/μl, NLR $\geqslant$3.5, LMR $\leqslant$2.6 and an adjusted AMLPI were significantly associated with inferior OS in univariate analysis. NLR $\geqslant$3.5 was an independent significant predictor of OS in multivariate analysis. Only an adjusted AMLPI >1 had a significant effect on TTP in univariate and multivariate analysis. None of the examined counts or ratios had an effect on the risk of TRM or ORR. Of note, a NLR $\geqslant$3.5 was significantly correlated with steroid-containing immunosuppression.

In this journal, Wilcox *et al.* have recently discussed the theoretical basis for using peripheral myeloid and lymphoid lineage cells as biomarkers for tumour microenvironment and host immunity in immunocompetent patients with DLBCL.[8] We were surprised to make similar prognostic observations in PTLD, despite the effects of immunosuppressive drugs not only on leukocyte counts but also on leukocyte, particularly lymphocyte, function. Our observations suggest that the baseline differential blood count integrates information about tumour microenvironment, host immunity and the level of immunosuppression. The significant effect of NLR, ALC and LMR on OS, but not TTP, could suggest a contribution of additional, non-tumour-related factors. Such factors might include infection as well as cardiovascular disease. PTLD patients (heart- and kidney-transplant recipients in particular) are at high risk of cardiovascular events, and high NLR has been linked to poorer OS in patients with coronary artery disease.[11]

This retrospective analysis of a subgroup of patients from the largest phase II trial in PTLD so far demonstrates a potential independent prognostic role of the baseline differential blood count, a ubiquitously available and cheap investigation in the setting of post-transplant lymphoma. However, because of the limitations of multiple testing in a small cohort, prospective validation in an independent cohort will be required before incorporation into stratification protocols.

CONFLICT OF INTEREST

The German and the French PTLD Study Groups were supported by Roche, AMGEN and Chugai to conduct the PTLD-1 trial with an unrestricted grant. SC received payment for lectures, research grants and consultancy fees from Roche. GS received payment for consultancy, honoraria and/or advisory board membership from Roche and/or Genentech and is an advisory board member for Celgene, Janssen-Cilag, Genzyme, Calistoga/Gilead and Mundipharma. FM received payment for consultancy, honoraria and/or advisory board membership from Roche and/or Genentech and is an advisory board member for Celgene, Spectrum and Mundipharma. CT received honoraria from Roche. VL is on the advisory board for Janssen-Cilag and Pharmacyclics, and received travel support from Roche and Honoraria from Janssen-Cilag and Mundipharma. RUT received payment for lectures and consultancy from CSL Behring, Mundipharma and/or Roche, grant support from AMGEN, CSL Behring, Mundipharma and Roche, and travel support from AMGEN, CLS Behring and Roche. The remaining authors declare no conflict of interest.

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Distribution of genomic breakpoints in chronic myeloid leukemia: analysis of 308 patients

Leukemia (2013) **27**, 2105–2107; doi:10.1038/leu.2013.116

Translocation breakpoints of several types of leukemia have been sequenced in order to identify factors that lead to DNA breakage (for example, topoisomerase 2 in *MLL* and *PML-RARA* leukemia).[1,2] Several studies in chronic myeloid leukemia (CML) have shown a possible association of breakpoints with ALU interspersed repeat elements (IREs),[3–6] but remained inconclusive due to their small sample size. We sequenced 308 *BCR–ABL1* fusion genes and performed a bioinformatic analysis with greater statistical power to identify breakpoint motifs.

The research was approved by the ethics committees of the participating institutions. All CML patients in this study expressed *BCR–ABL1* mRNA with breakpoints in the major breakpoint cluster region of *BCR*. The *BCR–ABL1* genomic breakpoint was identified using one of three methods: long range,[7] short range[8] or inverse PCR.[9] These methods rely on the fact that *BCR* breakpoints are tightly clustered, so that using a limited number of *BCR* primers it should be possible to amplify across the *BCR–ABL1* junction. The success rates of the three methods were around 90%, 99% and 65%, respectively. The use of several methods with a high overall success rate avoids skewing of the data due to the limitations of a specific technique (for example, restriction enzyme sites). The data of 32 patients and five common CML cell lines were previously published.[7,10]

In *BCR* there were 129 breakpoints between exon 13 and the end of exon 14 (giving rise to e13a2 mRNA), and 179 breakpoints between exon 14 and the end of exon 15 (e14a2 mRNA) (Figure 1a). Ten breakpoints were in *BCR* exons: in these cases, the partial exon was spliced out of the mRNA. In *ABL1* 256 breakpoints were located in the large intron between the first alternative exon 1b and the second alternative exon 1a. Thirty-eight breakpoints were located in the smaller intron between exon 1a and exon 2 (Figure 1b). Two breakpoints were in the short intron between exon 2 and exon 3, resulting in the expression of e13a3 *BCR–ABL1* mRNA. Twelve breakpoints were in a region of $\sim$10 kb upstream of *ABL1* extending from the last exon of the *EXOSC2* gene. No breakpoints were found in *ABL1* exons. A modified Anderson–Darling test showed non-uniformity of distribution of *BCR* breakpoints ($P<10^{-5}$), but not *ABL1* breakpoints ($P>0.05$).[11]

MEME software was used to search for sequence motifs that might be over-represented in the vicinity of breakpoints (10 bp up- and downstream of *BCR* breaks, and 100 bp around *ABL1* breaks).[12] Where sequence neighborhoods overlapped for multiple breakpoints, the contiguous intervals were combined resulting in 34 distinct sequences in *BCR*, and 195 sequences in *ABL1*. The *de novo* search in *ABL1* returned only motifs resulting from the presence of IREs, while no motifs were over-represented in *BCR*, despite the significantly non-uniform distribution of breakpoints in *BCR*. The *ABL1* gene is rich in ALU and other IREs, which together make up $\sim$50% of the entire *ABL1* breakpoint region, and it has been proposed that these elements are somehow involved in the genesis of the *BCR–ABL1* fusion. ALU and L1 are the two most frequent types of IRE in the *ABL1* breakpoint region, accounting for more than half of all repeats. In the relevant portion of *BCR* there is a single ALU repeat contiguous with an L1 repeat, and two small L2 repeats. We calculated the interval between each *ABL1* breakpoint and the nearest ALU or L1 repeat and obtained a quantile–quantile (QQ) plot by comparing this distribution of intervals with a reference distribution (all 203 384 intervals between every nucleotide in the 203 kb reference sequence and its nearest repeat). This was also done for 1000 sets of 308 uniformly selected 'breaks' in place of the actual breakpoints to determine an approximate envelope of QQ plots under uniformity. The distribution of intervals for the CML patients lay within this envelope (Supplementary Figures), indicating that *BCR–ABL1* breakpoints are no more frequent than would be expected in and around these IREs.

The *de novo* search for motifs resulted in the discovery in *ABL1* of a large palindrome with 243 bp of perfect reverse complementarity separated by $\sim$2 kb (Figure 1b). It is difficult to assign statistical significance to this observation, but within the whole 141 Mb of chromosome 9 there are only eight such palindromes with arm lengths of $\geqslant$200 bp and loop lengths of $<$2 kb. In *BCR*, the nearest palindrome was downstream of exon 16, with an arm length of 100 bp and a loop length of $\sim$3.6 kb. In chromosome 22, there are 23 palindromes of comparable size and separation. Palindromic sequences can result in non-B DNA conformation and have been associated with pathogenic recombination in humans.[13] It is possible that secondary structures in DNA are involved in initiating an interaction between chromosomes 9 and 22, as postulated for a 76 kb duplicon in 9q34 and 22q11.2.[14]

We searched for 50 sequence motifs previously reported in association with DNA breakpoints (Supplementary Data). For every breakpoint, we determined the shortest distance between the breakpoint and each of the 50 motifs, upstream and downstream and on both genomic strands. These were compared (via QQ plots) with similar distances for cyclic permutations of the motifs, so as to reduce the effects of polynucleotide composition. None of the motifs was significantly associated with *BCR–ABL1* breakpoints.

Analysis of the breakpoint sequences showed an increase in the frequency of microhomology, a hallmark of non-homologous end-

Accepted article preview online 16 April 2013; advance online publication, 7 May 2013