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The automated monocyte count is independently predictive of overall survival from diagnosis in chronic lymphocytic leukaemia and of survival following first-line chemotherapy

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ABSTRACT

We conducted an analysis of the effect of monocytosis at diagnosis of CLL on subsequent overall (OS) and treatment-free survival (TFS). Monocyte counts were performed using the Sysmex XE2100™ analyser. A monocyte count >0.91 × 10^9 L^{-1} at the time of diagnosis was associated with a shortened OS and TFS. Monocytosis at diagnosis was associated with lymphocyte count, deletions of chromosomes 17p and 11q, the extent of IgV_H somatic hypermutation and Binet stage. A multivariate analysis model which excluded somatic hypermutation found only monocyte count and age to be independently predictive of OS. The automated monocyte count is predictive of OS and TFS in newly diagnosed CLL.

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1. Introduction

Chronic lymphocytic leukaemia (CLL) is a clinically heterogeneous disorder which can vary from an indolent condition, with a prognosis similar to that of age-matched controls, to an aggressive disorder with a markedly shortened survival. Numerous methods for prognostication exist with some of the most robust including the assessment of clinical stage at diagnosis using the Binet or Rai systems, the detection of adverse cytogenetic abnormalities (deletions of 17p, 11q and the presence of trisomy 12), the extent of immunoglobulin V_H gene (IgV_H) somatic hypermutation and the strength of CD38 and zeta-associated protein of 70 kDa (ZAP70) expression by CLL-cells [1].

The survival of CLL-cells *in vivo* is critically dependent upon interactions with the microenvironments of the bone marrow and secondary lymphoid tissues and this dependency contributes to the poor survival of CLL-cells cultured *in vitro* where extrinsic signals important for the maintenance of CLL-cell viability are lacking. The addition of cultured stromal cells such as nurse-like cells, monocytes or macrophages to *ex vivo* tissue culture systems improves the viability of cultured CLL-cells [2].

Infiltration of tumour microenvironments by monocytes and macrophages has been identified in a variety of malignancies [3] with the extent of such infiltration being associated with an adverse prognosis in Hodgkin's lymphoma and other tumours [4]. In CLL the malignant B-cells are protected from apoptosis by monocytes in a process mediated by monocyte-derived CD14 which is present in elevated levels in the serum of patients with CLL when compared to normal controls [5]. This suggests that monocyte-derived survival signals may be important for the maintenance of the malignant clone in CLL.

We hypothesised that an elevated monocyte count at diagnosis would be associated with an adverse prognosis in CLL due to the possible tumour-promoting effects of these cells.

2. Methods

CLL was diagnosed according to National Cancer Institute (NCI) guidelines [6]. All patients had typical CLL with lymphocytes expressing CD5, CD19, CD23 and weak, clonally restricted, surface immunoglobulin. Data recorded for each patient included age, sex, date of diagnosis, date of first therapy, date of death and duration of follow-up. CLL-patients were identified from patients referred to a dedicated CLL-clinic within Hull and East Yorkshire Trust, the sole tertiary referral unit for a defined population of around 1.1 million people. Clinical staging was based on the Binet system. Treatment was initiated in patients with symptomatic disease and those with disease progression according to NCI criteria [6]. Patients were followed from diagnosis until last known follow-up or death. Mean follow-up was 48.5 months.

2.1. Ethics

Collection of blood samples and clinicopathological information from cases was undertaken with informed consent and with the relevant ethical review board approval in accordance with the tenets of the Declaration of Helsinki, and also

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following institutional review by the Hospital Trust Research and Development Committee.

2.2. Assessment of monocyte count

Peripheral blood samples were analysed on a Sysmex XE2100™ analyser, a multichannel analyser which generates a white cell differential count using a combination of fluorescent flow cytometry, impedance counting and chemical lysis of certain leucocyte subsets.

2.3. IgV_H gene mutation

RNA was extracted using the Qiagen RNeasy mini kit (Qiagen, Crawley, UK) and complementary DNA was synthesised using the Reverse-iT kit according to the manufacturer's instructions (ABgene, Epsom, UK). The IgV_H gene was amplified using PCR as previously described [7,8], and sequenced using BIG dye terminator sequencing method according to the manufacturer's instructions (Applied Biosystems, Warrington, UK). Sequences were compared to germline using IgBLAST (National Centre for Biotechnology Information, Bethesda, MD; http://ncbi.nih.gov/igblast/) and those with homology of 98% or higher were regarded as unmutated, and those with less than 98% homology were regarded as mutated [9].

2.4. Cytogenetic analysis, ZAP70 and CD38 expression

Fluorescent *in situ* hybridisation for deletions in chromosomes 13q, 11q and 17p as well as for trisomy 12 were performed according to published methodologies [10]. ZAP70 expression was assessed using flow cytometry following incubation of peripheral blood mononuclear cells with anti-CD19 and anti-ZAP70 monoclonal antibodies with reference to an isotypic control [11]. Cells were analysed by flow cytometry and at least 10,000 events were scored for each sample. The cut-off for ZAP70 positivity was 10%. CD38 expression was determined by triple colour immunophenotyping using anti-CD5, anti-CD38 and anti-CD19 monoclonal antibodies, as previously described [12]. The cut-off for CD38 positivity was 20%.

2.5. Statistics

Receiver operated curve (ROC) analysis was performed using Sigma Plot (version 11). All other analysis was performed using SPSS for Windows (version 17.0). OS and TFS were estimated using Kaplan–Meier analysis with comparison between curves made using Mantel–Cox log-rank analysis. Correlations between data were assessed using Pearson's correlation for continuous variables and Pearson Chi-square analysis for categorical variables. Multivariate analysis was undertaken using a Cox proportional hazard regression analysis. Statistical significance was defined as a p value less than or equal to 0.05.

3. Results

3.1. Validation of automated monocyte counts in CLL-patients

We utilised the automated Sysmex XE2100™ to assess monocyte counts in CLL-patients at diagnosis. This device has been demonstrated to produce white cell differentials which display a high level of concordance with cell counts obtained using flow cytometry [13], however, the alternative Coulter S Plus instruments have been reported as detecting a falsely high monocyte count in patients with CLL [14]. Therefore we performed 500-cell manual differential counts in 50 patients with CLL. The mean white cell count in these patients was $55.1 \times 10^9\,L^{-1}$ (range $3.8–260 \times 10^9\,L^{-1}$). There was a highly significant association between manual and automated neutrophil ($r = 0.94$, $p < 0.001$), lymphocyte ($r = 0.97$, $p < 0.001$) and monocyte counts ($r = 0.81$, $p < 0.001$). We concluded that the Sysmex XE2100™ produces an accurate differential white cell count in patients with an elevated white cell count due to CLL.

3.2. Patient characteristics

Our study investigated the prognostic power of monocyte count at presentation and following first-line chemotherapy in a single centre cohort of 191 CLL-patients. The characteristics of this patient group are shown in Table 1. The mean overall survival (OS) and treatment-free survival (TFS) for the whole cohort was 85 and 68.5 months, respectively. A shortened overall survival for the entire cohort was associated with age greater than 65 years ($p < 0.001$), an absolute lymphocyte count (ALC) of greater than $20 \times 10^9\,L^{-1}$ ($p = 0.006$), CD38 positivity ($p = 0.014$) and IgV_H unmutated disease ($p = 0.018$). A shortened TFS was associated with Binet stage B and C disease ($p < 0.001$), ALC of greater than $20 \times 10^9\,L^{-1}$ ($p < 0.001$), CD38 positivity ($p < 0.001$), IgV_H unmutated disease ($p = 0.004$) and deletions involving chromosome 11q or 17p ($p = 0.001$).

Table 1
Summary of clinical characteristics for the patient cohort.

Characteristics	Number
Sex	
Male	120
Female	71
Mean age at diagnosis in years	67.6
Binet stage (at diagnosis)	
A	155
B	18
C	18
Patients receiving at least one line of therapy	
Treated	69
Untreated	122
FISH	
Deletion 13q	33
Trisomy 12	9
Deletion 11q	7
Deletion 17p	5
Not tested	137
IgV_H mutation status	
Unmutated	42
Mutated	72
Not tested	77
ZAP-70	
Positive	20
Negative	86
Not tested	85
CD38 expression	
Positive	39
Negative	129
Not tested	23

3.3. Monocytosis at the time of diagnosis of CLL is associated with a shortened OS and TFS

ROC analysis was performed to establish a monocyte count that best defined subgroups of patients with differing outcomes. A threshold of $0.91 \times 10^9\,L^{-1}$ was found to be most discriminatory for predicting OS. Subsequently a monocyte count of greater than $0.91 \times 10^9\,L^{-1}$ is used to define monocytosis.

There was a shortened OS in patients with a monocytosis at diagnosis with a mean survival of 73.6 months compared with 89.2 months in patients with a monocyte count of less than $0.91 \times 10^9\,L^{-1}$ ($p = 0.028$, Fig. 1A). We hypothesised that a monocyte threshold greater than $0.91 \times 10^9\,L^{-1}$ might define a group with a worse prognosis in terms of overall survival from diagnosis, however, there was no significant difference in OS between patients with a monocyte count greater than $1 \times 10^9\,L^{-1}$ and those with a monocyte count of less than $1 \times 10^9\,L^{-1}$.

Monocytosis at diagnosis was predictive of a shortened TFS, with a mean of 55.7 months from diagnosis to treatment in patients with a monocyte count greater than $0.91 \times 10^9\,L^{-1}$ and a mean of 72.2 months from diagnosis to treatment in patients with a monocyte count less than $0.91 \times 10^9\,L^{-1}$ ($p = 0.013$, Fig. 1B).

3.4. Monocytosis at diagnosis of CLL is associated with high risk cytogenetics, ALC, the extent of IgV_H somatic hypermutation and Binet stage

Associations between monocytosis and recognised prognostic parameters in CLL were examined using the Pearson Chi-square

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