Abstract

CD26/dipeptidyl peptidase IV (DPPIV) is a multifunctional membrane protein and it is strongly upregulated in activated B-cells. We aimed to evaluate CD26 expression in mature B cell neoplasms, and its prognostic role in B cell chronic lymphocytic leukemia (B-CLL). CD26 expression was evaluated by flow cytometry in various B cell neoplasms. CD26 expression was high in MM and HCL, variable in B-CLL and in CD5neg B-CLPDs. Kaplan–Meier curves revealed a significantly shorter progression free survival (PFS), and lymphocytic doubling time (LDT) in the CD26 high expression group (p=0.014, 0.024 respectively). High CD26, CD38 and/or ZAP70 showed significantly shorter PFS, (p=0.020, 0.022 respectively) and LDT (p=0.024, 0.024 respectively) when compared to both low expression CD26, CD38 and/or ZAP70. CD26 expression may identify subsets of B-CLL patients with an unfavorable clinical outcome, thus suggesting its potential role as a marker in a future routine cytofluorimetric panel for B-CLLs.

Keywords: B-cell mature lymphoid tumours; B-cell chronic lymphocytic leukaemia; CD26; CD38; ZAP-70; Expression; Progression free survival, Lymphocytic doubling time, Prognosis

Abbreviations

B-CLL: B Cell Chronic Lymphocytic Leukemias; B-CLPD: B Cell Chronic Lymphoproliferative Disorders; DPPIV: Dipeptidyl Peptidase IV; FITC: Fluorescein Isothiocyanate; HCL: Hairy Cell Leukemias; LDT: Lymphocytic Doubling Time; mAbs: Monoclonal Antibodies; MM: Multiple Myeloma; OS: Overall Survival; PBS: Phosphate Buffered Saline; PE: Phycoerythrin; PerCp: Peridinin-Chlorophyll Proteins; PFS: Progression Free Survival; RT: Room Temperature; Sig: Surface Immunoglobulin; ZAP-70: Zeta-Chain-Associated Protein Kinase.

Introduction

CD26 or Dipeptidyl peptidase IV (CD26/DPPIV) is a unique multifunctional 110 kDa membrane-bound glycoprotein, belongs to the serine protease family, acts as receptor, binding and proteolytic molecule. The crystal structure indicates that tetramerization of DP IV is a key mechanism to regulate its interaction with other components. A dipeptide mimicking inhibitor complexed to the active site discloses key determinants for substrate recognition. It is expressed on a variety of tissues including T lymphocytes, endothelial and epithelial cells. CD26 plays an important role in immune regulation, signal transduction, and apoptosis [1]. Studies have suggested that CD26 plays a regulatory role in the neoplastic transformation and progression of various types of tumours, and it may also play a role in tumour migration and metastasis as a result of its ability to bind extracellular matrix proteins [1,2]. It is thought that CD26 plays an important role in hematological malignancies, mainly in aggressive subtypes of T-cell non-Hodgkin lymphomas in which it is highly expressed by neoplastic cells [3,4]. Although CD26 expression is very low in B-cells, it is greatly up regulated following activation [5].

The B-cell neoplasms are a biologically heterogenous group of malignant diseases characterized by accumulation of mature B lymphocytes in the bone marrow, peripheral blood and lymphoid tissue [6]. B cell chronic lymphocytic leukemia (B-CLL) is an accumulative disease of slowly proliferating lymphocytes that develops in the aging population. Whereas some patients with B-CLL have an indolent course and die after many years from unrelated causes, others progress very rapidly within a few years from this currently incurable leukemia [7]. Thus, it is more important than ever to develop sensitive stratification parameters to identify patients with poor prognosis.

Subjects and Methods

Subjects

The present study involved 100 newly diagnosed patients with mature B-CLPD who were evaluated at the time of diagnosis. On the basis of the WHO classification of neoplastic diseases of hematopoietic and lymphoid tissues [8], there were 58 cases of B-CLL, 7 cases of hairy cell leukaemia (HCL), 23 cases of CD5neg B-CLPD and 12 cases of multiple myeloma (MM). All patients were diagnosed and followed up between 2009 and 2013 in Mansoura Oncology Center, Mansoura, Egypt. In addition to 10 control subjects of matched age and sex were enrolled in the study. Informed consent was provided by all subjects.

The study was based on 58 (40 males, 18 females) consecutive, previously untreated CLL, 7 HCL (5 males, 2 females), 23 CD5 negative LPD (15 males, 8 females) and 12 multiple myeloma (8 males, 4 females). Mean age of CLL, HCL, CD5 negative LPD and multiple...
myeloma groups was 60.47, 68.14, 59.04, 60.92 years respectively. B-CLL cases were classified at diagnosis according to Binet and Rai classifications. Patients required treatment, received chemotherapy following the Working Group for CLL criteria [9].

**Immunophenotype analysis**

Bone marrow aspirates or peripheral blood samples were collected on EDTA tubes and immediately transported to the flowcytometry laboratory. For surface antigen staining, the received samples were lysed using homemade lysing solution (8 g Ammonium chloride, 1 g EDTA, and 0.1 g dihydrogen potassium phosphate in 1 l-10x), washed with phosphate buffered saline (PBS) until complete RBCs lysis and resuspended in appropriate amount of PBS. The cells were stained with different fluorescently labeled monoclonal antibodies (mAbs) according to manufacturer recommendations (Dakoctymation, Denmark, and Beckman Coulter, France). One hundred microliters of cell suspension were mixed with 10 μl of the fluorescently labeled mAb and incubated in the dark at room temperature (RT) for 30 min. Washing with PBS containing 2% bovine serum albumin was done twice and the pellet was resuspended in PBS and analyzed immediately on flowcytometer. For detection of cytoplasmic and nuclear antigens, IntraPrep Permealization Kit was used (Beckman Coulter, France). Fifty microliters of EDTA PB/BMA sample were mixed with 100 μl of IntraPrep reagent 1 (fixative), incubated for 15 min at RT protected from light, and washed with PBS. 100 μl of IntraPrep reagent 2 (permealization) were mixed with the cells and incubated for 5 min at RT without vortexing or shaking. The tube was shook carefully and manually for 2-3 seconds and then 20-20 μl of the mAb were added, vortexed, and incubated for 20 minutes in case of cytoplasmic antigens and for 1 h in case of nuclear antigens at RT protected from light. Then, the mixture was washed and resuspended in PBS and analyzed on the flowcytometer immediately. The mAbs were used in different combinations of fluorochromes; namely fluorescein isothiocyanate (FITC), phycocerythrin (PE) and peridinin-chlorophyll proteins (PerCP). Different combination of mAb against the following antigens were used: CD45-PerCP, CD10-FITC, CD5-FITC, CD19-PerCP, CD20-PE, CD23-FITC, CD38-PE, CD 26-PE and FMC7 FITC (Becton Dickinson, Mountain View, CA) CD103-FITC (ImmuNo Quality Products, Groningen, The Netherlands); and CD79b-FITC (DAKO, Glostrup, Denmark). FITC-conjugated F (ab)2 fragments of rabbit anti-human Ig (DAKO, Glostrup, Denmark) were used for the SIg analysis. For ZAP-70, peripheral blood mononuclear cells were stained with CD3-PerCP and CD19-PE for 15 minutes at 4°C (Becton Dickinson), and washed and fixed/permeabilized using a Fix & Perm kit (Caltag Laboratories, Burlingame, CA) according to the manufacturer’s instructions. They were then washed and stained with an Alexa-Fluor 488-conjugated monoclonal antibody against ZAP-70. The immunophenotyping was performed on EPICS-XL flowcytometer (Coulter, Miami, FL). The cells were analyzed with the most appropriate fluorochromes in different combinations of fluorochromes; namely fluorescein isothiocyanate (FITC), phycoerythrin (PE) and peridinin-chlorophyll proteins (PerCP). The best cut-off point for CD26 expression was sought by constructing receiver operating characteristic (ROC) curves. Kaplan-Meier test was used for survival analysis and the statistical significance of differences among curves was determined by Log-Rank test. The Cox proportional hazards regression model was chosen to assess the independent effect of covariables on progression free survival. N.B: p is significant if ≤ 0.05 at confidence interval 95.

**Results**

**CD26 expression**

CD26 was positive in 50, 85.7, 56.5, and 75%, in CLL, HCL, CD5 negative CLPD and MM respectively (Figure 1). The proportion of CD19-positive neoplastic B-CLL cells expressing CD26 ranged from 2 to 97%, with a median value of 10%; (Figure 2). Fluorescence intensity isothiocyanate (FITC), phycoerythrin (PE) and peridinin-chlorophyll proteins (PerCP). Different combination of mAb against the following antigens were used: CD45-PerCP, CD10-FITC, CD5-FITC, CD19-PerCP, CD20-PE, CD23-FITC, CD38-PE, CD 26-PE and FMC7 FITC (Becton Dickinson, Mountain View, CA) CD103-FITC (ImmuNo Quality Products, Groningen, The Netherlands); and CD79b-FITC (DAKO, Glostrup, Denmark). FITC-conjugated F (ab)2 fragments of rabbit anti-human Ig (DAKO, Glostrup, Denmark) were used for the SIg analysis. For ZAP-70, peripheral blood mononuclear cells were stained with CD3-PerCP and CD19-PE for 15 minutes at 4°C (Becton Dickinson), and washed and fixed/permeabilized using a Fix & Perm kit (Caltag Laboratories, Burlingame, CA) according to the manufacturer’s instructions. They were then washed and stained with an Alexa-Fluor 488-conjugated monoclonal antibody against ZAP-70. The immunophenotyping was performed on EPICS-XL flowcytometer (Coulter, Miami, FL). The cells were analyzed with the most appropriate fluorochromes in different combinations of fluorochromes; namely fluorescein isothiocyanate (FITC), phycoerythrin (PE) and peridinin-chlorophyll proteins (PerCP). The best cut-off point for CD26 expression was sought by constructing receiver operating characteristic (ROC) curves. Kaplan-Meier test was used for survival analysis and the statistical significance of differences among curves was determined by Log-Rank test. The Cox proportional hazards regression model was chosen to assess the independent effect of covariables on progression free survival. N.B: p is significant if ≤ 0.05 at confidence interval 95.

**CD26 Cut off**

The best cut-off point for CD26 expression discriminating the patients who were diagnosed as B-cell neoplasms from controls was entry until objective disease progression, first line treatment according to NCI Working Group guidelines, death or last follow-up. Overall survival (OS) was measured from diagnosis to last follow-up or death. Lymphocyte doubling time was measured from diagnosis to time of lymphocyte doubling. Factors contributing to lymphocytosis or lymphadenopathy other than CLL (e.g. infection) were excluded. All patients underwent first treatment at the time of documentation of progressive and symptomatic disease according to National Cancer Institute–Working Group guidelines [13].

**Statistical methods**

All of the statistical calculations were made using excel program and SPSS (statistical package for social science) program (SPSS, Inc, Chicago, IL) version 16. Qualitative data were presented as frequency and percentage. Chi square test was used to compare groups. Quantitative data were presented as mean and standard deviation. For comparison between two groups; student t-test and Mann-whitney test (for non-parametric data) were used. The associations between variables were tested by Spearman’s correlation test. The best cut-off point for CD26 expression was sought by constructing receiver operating characteristic (ROC) curves. Kaplan-Meier test was used for survival analysis and the statistical significance of differences among curves was determined by Log-Rank test. The Cox proportional hazards regression model was chosen to assess the independent effect of covariables on progression free survival. N.B: p is significant if ≤ 0.05 at confidence interval 95.

**Definition of end points**

Progression free survival (PFS) is defined as the time from study

---

**Figure 1:** CD26 expression in the analyzed B-CLPD. Horizontal line demonstrate CD26 cutoff at 10%.
sought by constructing receiver operating characteristic (ROC) curves. The area under the ROC curve (AUC)=0.842, 95% CI=0.770-0.913, p<0.0001, Cut off=9.5, sensitivity 85.6% and specificity 100.0%).

In the B-CLL cases, according to the obtained cut-off value, 29 (50.0) cases had high CD26 expression (CD26high), their values ranged from 10 to 97 with a mean 27.52; median 16.0 and 29 (50.0) cases had low CD26 expression (CD26low), their values ranged from 2 to 9 with a mean 7.62 and median 8.00.

**Relationship of CD26 with markers of clinical characteristics**

No significant differences regarding age, sex, lymphadenopathy, hepatomegaly Binet, Rai staging, and hemoglobin in high versus low CD26 expression in CLL patients (p>0.05). High CD26 expression showed significantly higher incidence of hepatomegaly, CD38high and ZAP70high (p=0.027, <0.001, <0.001 respectively), significantly higher total leucocytic count and LDH (p=0.033, <0.001 respectively), lower platelets count (p=0.040). Absolute lymphocytic count was higher in high CD26 expression group, although did not reach significant level (p=0.081) (Table 1).

**CD26, CD38 and ZAP70**

CD 38 was positive in 55.2%, 0%, 56.5%, and 100% in CLL, HCL, CD5 negative LPD and MM respectively. Correlation analyses were made between CD26 reactivity and the expression of the other tested membrane antigens of relevance. High levels of CD38 (>30% CD19-positive cells) or ZAP-70 expression (>20% CD19-positive cells) were found in respectively 32 (55.2%) and 33 (56.9%) of the 58 B-CLL patients. Spearman’s test revealed a significant correlation between CD26 and CD38 reactivity’s and between CD26 and ZAP-70 expressions (rS: 0.795, p<0.001; rS: 0.855; p<0.001 respectively). When applying Friedman correlation in B-CLL group; CD26, CD38 and ZAP70 values were highly correlated p<0.001. CD26 and CD38 reactivities had different patterns in other B-cell neoplasms. There was a statistically significant Spearman’s rank correlation coefficient in MM patients (rS: 0.671, p=0.017), but no significant correlations were found in the patients with CD5neg B-CLPD (r=-0.344, p=0.108) and HCL (r=0.679, p=0.094).
Relevance of CD26 expression as prognostic factor in B-CLL

ROC analysis was used to explore the power of CD26 expression to distinguish patients who developed progressive disease from those with stable disease, and confirmed 10.5% as the best cut-off value (area under the curve 0.829, p<0.0001); all the subsequent data were therefore analyzed using a 10% threshold. When testing the prognostic relevance of CD26 expression in this study, B-CLL patients with high CD26 expression had significantly shorter progression free survival (PFS) (77.8 versus 38.4 months, p=0.014). In addition, 79.8% of the CD26 expression had significantly shorter progression free survival relevance of CD26 expression in this study, B-CLL patients with high (area under the curve 0.829, p<0.0001); all the subsequent data were with stable disease, and confirmed 10.5% as the best cut-off value to distinguish patients who developed progressive disease from those doubling after 12 months (Figure 5).

Relevance of CD26–CD38, CD26–ZAP-70 expression as prognostic factors in B-CLL.

The relevance of CD26 as a prognostic marker of progressive disease was compared with that of CD38 and ZAP-70 expressions. Twenty four cases were CD26low/CD38low and 27 were CD26high/CD38 high cases, while the remaining 7 cases were discordant for the two markers. PFS was significantly shorter in the CD26 high/CD38high than in the CD26low/CD38low cases (37.94, 76.49 months respectively, p=0.020), but when comparing the PFS of these cases and that of the discordant cases, no significant difference was found (p=0.151) (Figure 6). Lymphocytic doubling time (LDT) was significantly shorter in the CD26 high/CD38high than in the CD26low/CD38low cases (p=0.024) (Figure 7).

When we assessed the predictive power of ZAP-70 expression, there was no statistical significant difference in terms of PFS between the cases classified as ZAP-70low and ZAP-70high (log rank 0.711, p=0.399, not shown). There were 25 ZAP-70low/CD26low cases and 29 ZAP-70high/CD26low cases; 4 showed discordant markers. The combined expression of CD26 and ZAP-70 was analyzed in relation to PFS. The estimated mean PFS in the three groups were different, 76.64 months for ZAP-70low/CD26low, 38.37 months for ZAP-70high/CD26low patients and 62.0 months for discordant cases. After 3 years, respectively 93.3, 79.8 and 75.0% of the cases had stable disease. No significant difference was found in PFS between the three groups (p=0.146). However, there was significant difference between ZAP-70low/CD26low versus ZAP-70high/CD26high regarding PFS (p=0.022) (Figure 8). LDT was significantly shorter in the CD26high/ZAP70high than in the CD26low/ZAP70low cases (p=0.024) (Figure 9).

CD26, CD38, ZAP-70, Rai, absolute lymphocytic count and LDH were included in a proportional hazard regression model of Cox to test their strength as independent prognostic factors for PFS. CD26 seemed to be the sole unfavorable independent prognosticator for PFS among the biologic risk factors. According to these analyses, CD26 expression in CLL was associated with a hazard ratio (HR) for progressive disease of 1.091 (95% confidence interval (CI), 1.037–1.148; p=0.022) (Table 2).

Discussion

B-Cell neoplasms are a heterogeneous group of malignant clonal proliferations of lymphocytes [14]. B-CLL represents a B-CLPD with a high biological and clinical heterogeneity. This disease is related to
the accumulation of monoclonal B-cells with the morphology of small apparently mature lymphocytes, which is mainly due to inhibition of apoptosis rather than enhanced cell proliferation [15]. CD26 plays an important role in immune regulation, signal transduction, and apoptosis. Furthermore, CD26 appears to play an important role in tumor progression [1]. Previous studies found that CD26 status may be altered in certain malignancies and that the presence of CD26 is associated with aggressive tumours [1,2]. The aim of this study was to evaluate the expression of CD26 in patients with different forms of B-cell tumors, and its prognostic impact on B-CLL patients.

Our data show that CD26 expression is highly expressed in HCL, and heterogeneously expressed in MM, CLL and CD5neg B-CLPD, which are consistent with that reported by others [16]. CD26 was positive in 50, 85.7, 56.5, 75%, in CLL, HCL, CD5 negative LPD and MM respectively. This was confirmed by other studies [16-18]. Previous studies have reported that HCL and B-CLL can express high levels of ADA and CD26. Suggesting that their dramatic response to 2-deoxycoformycin and 2-chlorodeoxyadenosine, known inhibitors of ADA, relates to their surface expression of the ADA–CD26 complex [18,19]. Nishida et al. reported that CD26 is intensely expressed in activated osteoclasts in MM, but not in MM cells themselves, the direct interaction between MM cells and OCs results in the up-regulation of CD26 expression in MM cells [17].

The best cut-off point for CD26 expression discriminating the patients who were diagnosed as CLL, LPD or MB-cell neoplasms from control subjects was confirmed by the cut off obtained by others [16,20].

The proportion of B-CLL patients displaying high CD26 expression was equal to those displaying CD26 low expression. This was consistent with Cro et al. who reported that 58% of CLL cases had CD26low and 42% had CD26high, [16] whereas Bouvis et al. reported that 12% of CLL cases had CD26low and 88% had CD26high [21]. The observed differences may be due to differences in sample size.

CD26 expression, in the present study, could be correlated with some clinical and laboratory markers. Patients with high CD26 expression had significantly higher incidence of splenomegaly, higher total leucocytic count, LDH and tendency to higher peripheral mature lymphocytes. In addition to significantly lower hemoglobin concentration and platelet count. This is in part contradictory to a number of published studies [16,20,22]. These differences may be attributed to difference in sample size, ethnic, racial and environmental factors.

The determination of, CD38 and ZAP-70 are increasingly utilized as prognostic factors for CLL [23-26]. Cut-off points of 30% and 20% were used to define positivity for CD38 and ZAP70 respectively [26,12].

Interestingly, all our HCL cases were negative for CD38, which is considered as a germinal center-associated marker. This is approved by Forconi et al. Who found that the expression of CD38 markers...
confirmed the absence in HCL [27].

Our study found that CD26 reactivity closely correlates with CD38 expression in B-CLL and MM, but their reactivity patterns are different in HCL and CD3 negative CLPD. This was in agreement with some investigators [16], and disagreement with others [22]. While this may be due to the size of our cohort, it may also reflect clinical differences between the cohorts. In addition to differences in the ongoing antigen mediated activation of the neoplastic cells.

The B-CLL cases in the present study showed a statistically significant correlation between CD26/CD38, CD26/ZAP70 and CD26/CD38/ZAP70 expressions. These data correlated with a previous publication [16]. However, others found a borderline significance for ZAP-70 (P=0.07) [22].

We evaluated CD26 prognostic role in terms of OS, PFS and LDT. The optimal cut-off point yielding the best separation of B-CLL patients into two subgroups with different prognoses was fixed at 10% of positive cells. This cut off was confirmed by other study [16]. B-CLL patients with high CD26 expression had significantly shorter PFS and LDT. OS showed no significant differences between high and low CD26 groups. These data correlated with previous publications [16,22]. On the other hand, others did not find any association between CD26 and survival [20].

The combined evaluation of CD26/CD38 and CD26/ZAP70 reactivities allowed us to refine the prognostic role of the combined evaluation of the molecules in comparison with their individual reactivity. The presence of both CD26 and/or CD38, ZAP-70 (concordant positive) is associated with B-CLL with a shorter PFS, while the absence of both CD26 and CD38, ZAP-70 (concordant negative) grouped patients were associated with a longer PFS, and the discordant expression of CD26 and/or CD38, ZAP-70 was associated with an intermediate prognosis. The potential usefulness of an additional prognostic marker is supported by the results of others showing that the combined analysis of ZAP-70 and CD26 or CD38 and CD26 may be more informative than their individual evaluation [16].

CD26 shares many biological functions with CD38 [5], and may be involved in the sIgM-mediated signal competence that, in vitro, seems to unite the three important prognostic markers in B-CLL: VH-gene mutational status, CD38 and ZAP-70 [28]. Moreover CD26 expression, and particularly its DPPIV enzyme activity, induces constitutive p38 phosphorylation [29]. Which, in B-cell tumours, contributes to tumour growth [30]. In CLL B cells expressing ZAP-70, it is possible that activation (phosphorylation) of ZAP-70 via CD26 signalling may trigger downstream events with enhanced survival signals and cell growth, resulting in a more aggressive phenotype [31-33].

Our data show that the combined analysis of CD26, CD38 and ZAP-70 identifies subgroups of patients with a different prognosis, better than their individual evaluation. And our results suggest that the simultaneous estimate of CD26, ZAP-70, and CD38 might distinguish different B-CLL patient subgroups at least in terms of chemotherapy requirement.

These markers have a number of advantages: they can be easily evaluated by flow cytometry, which is widely used technique, and their combined evaluation in B-CLL cases may confirm the prognosis if one predictive factor is borderline. The determination of these markers and their reactivity could become a future routine panel for prognostic stratification.

Multivariate analysis showed that CD26 is an independent prognostic factor for PFS among the biologic risk factors.

In conclusion, the results of this study demonstrate the heterogeneous pattern of CD26 expression in B-cell neoplasms. CD26 expression is an unfavorable prognostic parameter in patients with B-CLL. CD26 correlates with the expression of CD38 and ZAP70 in B-CLL. We suggest its use as a part of a panel of molecules (i.e. CD38 and ZAP70) as a new flow cytometric prognostic model.

<table>
<thead>
<tr>
<th>Covariates</th>
<th>p</th>
<th>HR</th>
<th>95% CI</th>
</tr>
</thead>
<tbody>
<tr>
<td>Rai</td>
<td>0.365</td>
<td>1.704</td>
<td>0.538</td>
</tr>
<tr>
<td>Absolute lymphocytic count</td>
<td>0.405</td>
<td>1.005</td>
<td>0.993</td>
</tr>
<tr>
<td>LDH</td>
<td>0.097</td>
<td>1.034</td>
<td>0.987</td>
</tr>
<tr>
<td>CD26</td>
<td>0.001</td>
<td>1.091</td>
<td>1.037</td>
</tr>
<tr>
<td>CD38</td>
<td>0.344</td>
<td>1.237</td>
<td>0.012</td>
</tr>
<tr>
<td>ZAP70</td>
<td>0.285</td>
<td>2.666</td>
<td>0.236</td>
</tr>
</tbody>
</table>

HR: hazard ratio

Table 2: Progression-Free Survival as dependent parameter studied with other covariates (multivariate analysis).

References


