ISSN: 2230-9926

Available online at http://www.journalijdr.com

IJDR

International Journal of Development Research
Vol. 5, Issue, 07, pp. 5034-5038, July, 2015

International Journal of
DEVELOPMENT RESEARCH

Full Length Research Article

SEVERE IMMUNE THROMBOCYTOPENIA IN CHRONIC LYMPHOCYTIC LEUKEMIA (CLL)

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ARTICLE INFO

Article History:
Received 19th April, 2015
Received in revised form
07th May, 2015
Accepted 30th June, 2015
Published online 30th July, 2015

Key words:
Immune Thrombocytopenia,
Chronic lymphocytic leukemia,
Overall survival.

ABSTRACT

Severe immune thrombocytopenia (ITP) is a frequently associated hematologic condition in CLL with low frequency in the earlier stages of the disease. The aim of the study was to correlate of severe ITP (platelet count $<30x10^9$/L) evidence in the earlier CLL stages with biological features, phenotypic and cytogenetic abnormalities and disease outcome. Twelve of 175 (6.9%) diagnosed CLL patients were with severe ITP. The ITP occurrence was significantly associated with ZAP-70 positivity (58%, p=0.028). CD38 and P53 expressions was significantly higher (p=0.038 and p=0.013, respectively) than in CLL without ITP. Based on available FISH data, we found that among 12 cases with deletion of (11) (q22-23) region only one (9%) developed ITP. There was no statistical significance between ITP and cytogenetic deletion (13) (q14). The median overall survival of severe ITP patients was significantly shorter -68.6 months (p=0.016) than the other patients -111 months and overall survival dropped rapidly and was in stable rate after 12 months since the diagnosis. ITP cases had an increased risk of disease progression and mortality risk over 3 fold above the patients without ITP (p<0.001, p<0.05, respectively). Severe ITP patients showed shorter median free of treatment period-2.08 months, compared to CLL without ITP-45.10 months. Severe ITP is associated with a higher frequency of poor prognostic markers such CD38, ZAP-70 and P53 expressions and shortened free of treatment period and overall survival.

INTRODUCTION

Chronic lymphocytic leukaemia (CLL) is a heterogeneous disease with a highly variable clinical course. CLL is sometimes associated with autoimmune cytopenia: autoimmune hemolytic anemia (5-25%) (Mauro *et al*., 2000) and immune thrombocytopenia (1-12%) (Moreno *et al*., 2010; Zent, and Kay, 2010; Zent *et al*., 2009). Severe immune thrombocytopenia (ITP) is a frequently associated hematologic condition in patients with CLL with low frequency in the earlier stages of the disease (Dearden, 2008). In the earlier stages, mild thrombocytopenia is common in CLL patients. Later in the disease progression, the bone marrow will become more extensively infiltrated by the neoplastic lymphocytes, which results in more severe thrombocytopenia. Thrombocytopenia in patients with CLL dependents on a number of factors and could result from the disease progression, applied chemotherapy, splenomegaly or ITP development (Hodgson *et al*., 2011; Jiang *et al*., 2014). Pathogenesis of ITP in CLL patients is associated with a complex immunological character of the disease (Caligaris-Cappio, 1996; D'Arena *et al*., 2006; Shvidel *et al*., 2013). There is a CLL-associated immune dysregulation and leukemic cells are the dominant antigen-presenting cells that activate T-cells with auto-antigen. Auto-antigen presentation by leukemic cells could trigger normal B-lymphocytes to produce auto-antibodies via T-helper cell–mediated processes (Cooper *et al*., 2006; Hamblin, 2006; Ghia *et al*., 2007). Severe ITP complicates the course of CLL, compromise patients' quality of life, and in some cases can be fatal. For patients with CLL who develop severe thrombocytopenia, treatment options are limited and administration of platelet transfusions is common (Ding *et al*., 2007). Additionally, treatment of CLL patients with chemotherapy to treat the disease can be come off second-best due to severe

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thrombocytopenia (D'Arena *et al*., 2006). The clinical complications of severe thrombocytopenia include an increased tendency for bleeding, compromised hemostasis, and delayed administration of chemotherapy with the consequence of less optimal disease control (Fogarty, 2010).

Aim

The aim of the study was to correlate of severe ITP evidence in the earlier CLL stages with biological features, phenotypic and cytogenetic abnormalities and disease outcome.

MATERIAL AND METHODS

Design of the study

We studied 175 patients with B-cell CLL hospitalized in the Clinic of Hematology, University Hospital Alexandrovska, Sofia, Bulgaria for 3 year-period (informed consent obtained). All patients were diagnosed according to the revised criteria of the National Cancer Institute-sponsored Working Group (NCI-WG) on Chronic Lymphocytic Leukemia (CLL) and were classified according to the Rai staging system (Rai *et al*., 1975). Patients with an advanced Rai stage (3 or 4) were excluded. Twelve of B-cell CLL patients were with severe immune thrombocytopenia (ITP).In all cases, it appeared during the evolution of CLL.

Definition of severe ITP

The diagnosis of severe immune thrombocytopenia was based on the presence on unexplained fall in platelet count to $<30x10^9$/L and on more than two indirect parameters: evidence of normal bone marrow function (normal or increased megakaryocytes in bone marrow), no splenomegaly and no chemotherapy within the last month. The patients with thrombocytopenia due to bone marrow suppression (lymphocytes over 80% in bone marrow) were excluded from this analysis. Any other common cause of thrombocytopenia, such as pseudo-thrombocytopenia, disseminate intravascular coagulation, thrombotic thrombocytopenic purpura, HIV and HCV infections, acute infections, as well as heparin treatment, were ruled out by clinical and laboratory analysis. The CLL patients were separated into 2 groups: CLL group with platelet count more than $30x10^9$/l and CLL group (severe ITP group) with platelet count up to $30x10^9$/l.

Definition of total tumor mass score (TTM)

TTM is the sum of: (1) the square root of the number of peripheral blood lymphocytes per ml, (2) the diameter of the largest palpable lymph node in centimetres, and (3) the enlargement of the spleen below left costal margin in centimetres (Jaksić and Vitale, 1981).

Treatment regiment

The antileukemic treatment was obtained by chlorambucil + prednisone, CVP-regiment (cyclophosphamide + oncovin + prednisone), fludarabine, RFC-regiment (rituximab + fludarabine + cyclophosphamide) or alemtuzumab.

Definition of overall survival

Overall survival (OS) was calculated from the date of initial treatment to the date of death from any cause.

Definition of free of treatment period

Free of treatment period was defined as the time interval between the date of diagnosis and date of first CLL treatment.

Fluorescence flow cytometry

ZAP-70 and CD38expression were assessed by flow cytometric analysis on peripheral blood samples or bone marrow aspirates using a FAC Scan (Becton Dickinson Immunocytometry Systems, Mountain View, CA). Cytoplasmic ZAP-70 expression in more than 20% and surface CD38 expression on more than 30% of B-CLL cells were assessed as positive results. Mutated p53 protein expression was determined when the calculated intensity of the fluorescence (MIF) ratio was greater than 1.4.

Fluorescence in situ hybridization (FISH)

FISH was performed according to a guide of the manufacturer using a locus-specific probes for D13S25 (13q14) and ATM (11q22) labeled with Spectrum Orange (Vysis, USA) on peripheral blood samples in 36 and 35 patients, respectively. The presence of 1 copy per cell in more than 10% of the lymphocytes was considered indicative of a deletion. The presence of 2 copies per cell in more than 10% of the lymphocytes was considered a normal state.

Statistical analysis

Comparisons of quantitative variables among patient groups were made by one-way analysis of variance. A comparison of qualitative data was performed by means of the Chi-Square and T-test. All statistical tests were two-sided. Only probability values less than 0.05 were considered statistically significant. Univariable and multivariable Cox proportional hazards regression models were used to assess the risk for disease progression. Survival analysis was performed by the Kaplan–Meier method using IBM SPSS statistics for windows.

RESULTS

Biological parameters of B-CLL patients developing or not severe ITP

According to our definition, the diagnosis of severe ITP was confirmed in 12 (6.9 %) of 175 patients. Seventy fifth percentages of approved severe ITP-CLL were distributed in the 50- 70 age group with a male/female ratio of 2.0 (Table 1).

Table 1. Biological characteristics of 175 patients with B-CLL according to severe ITP occurrence

Parameters	CLL and ITP		CLL without ITP		P- value
	n	%	n	%	
Number	12	6.9	163	93.1	-
Age, years					
n, up to 50 years	0	0	19	11.7	NS
n, 50- 70 years	9	75	109	66.8	NS
n, over 70 years	3	15	35	21.5	NS
Gender					
Male	8	66.7	97	60	NS
Female	4	33.3	66	40	NS

NS- no significance

Phenotypic analysis of severe ITP patients

CLL patients with CD38 and ZAP-70 positivity were 81 of 175 (46.3%) and 51 of 175 (29.1%), respectively (Table 2). 75% of ITP-CLL cases were CD38 (+) compared to the other CLL patients -44.2% (p=0.038). The severe ITP occurrence was significantly associated with ZAP-70 positivity (7/12; 58.3%). In contrast ZAP-70 was found in 44 of 163 other B-CLL cases (27%, p=0.028). Among 175 B-CLL patients analyzed, 24 (13.7%) samples showed positive p53 mutated protein expression. ITP patients showed a higher incidence of p53 mutated protein expression than the other patients (41.7% *vs*. 11.7% respectively, p=0.013) (Table 2).

Severe ITP and survival

The median overall survival of severe ITP patients was significantly shorter -68.6 months (95% CI: 49.5-87.6 months) than the patients without severe ITP -111 months (95% CI: 104.7-117.4 months; p=0.016) and overall survival dropped rapidly and was in stable rate after 12 months since the diagnosis (Table 5, Fig. 1). Patients with severe ITP had shortened free of treatment period (median 2.08 months, CI: 0-5.99 months) than the other CLL cases (median 45.5 months, CI: 36.5-53.7 months), p=0.016 (Table 5). Accordingly, the risk rate of starting of initial CLL treatment in patients without severe ITP increased more slowly than the cases with ITP and reached considerably lower levels (Fig. 2) Mortality rate increased more slowly in B-CLL patients without an ITP development compared to ITP-CLL cases (Fig. 3).

Table 2. CD38, ZAP-70 and P53 mutated protein expression expressions in the B-CLL patients according to the platelet values

Platelets (x10^9/l)	CD38		ZAP-70		p53*	
	(-)	(+)	(-)	(+)	(-)	(+)
30 +; n (%)	91(55.8)	72 (44.2)	119 (73.0)	44 (27.0)	144 (88.3)	19 (11.7)
< 30; n (%)	3 (25.0)	9 (75.0)	5 (41.7)	7 (58.3)	7 (58.3)	5 (41.7)
Total; (n, %)	94 (53.7)	81(46.3)	124 (70.9)	51(29.1)	151(86.3)	24 (13.7)

*p53 mutated protein expression

Table 3. The frequency of del (11)(q22-23) and del (13)(q14) in the B-CLL patients according to the platelet values

Platelets (x10^9/l)	11(q22-23)		13(q14)	
	Normal	Del	Normal	Del
30 +; n (%)	22 (66.7)	11 (33.3)	11 (32.4)	23 (67.6)
< 30; n (%)	1 (50.0)	1 (50.0)	1 (50.0)	1 (50.0)
Total; (n, %)	23 (65.7)	12 (34.3)	12 (33.3)	24 (66.7)

Table 4. The relationship between ITP, total tumor mass (TTM) score and disease progression in B-CLL patients

Platelets (x10^9/l)	TTM score		Disease progression	
	Up to 9	Over 9	Without	With
30 +; n (%)	86 (52.8)	77 (47.2)	85 (52.1)	78 (47.9)
< 30; n (%)	3 (25.0)	9 (75.0)	3 (25.0)	9 (75.0)
Total; (n, %)	89 (50.9)	86 (49.1)	88 (50.3)	87 (49.7)

Table 5. Free of treatment period (in months) and overall survival (in months) in B-CLL patient with and without severe ITP

Platelets (x10^9/l)	Free of treatment period (in months)			Overall survival (in months)		
	N	$\overline{X}$	95% CI	N	$\overline{X}$	95% CI
30 +	163	45.10	36.50 - 53.70	163	111.02	104.66 -117.37
< 30	12	2.08	0 - 5.99	12	68.58	49.54 - 87.62

Fluorescence in situ hybridization analyses

Based on available FISH data, we found that among 12 patients with deletion of (11)(q22) region only one (9%) developed severe ITP with platelet count <30 x10^9/l. There was no statistical significance (p>0.05) between ITP development and unfavorable cytogenetic deletions (11q) (Table 3).There was no statistical significance (p>0.05) between severe ITP development and cytogenetic deletion (13)(q14). Among the 24 patients with del (13)(q14) only 1 (4%) showed a relation with severe ITP evidence (Table 3).

Severe ITP, total tumor mass (TTM) score and disease Progression

Three (25%) severe ITP cases presented a low (<9) TTM score, and the remaining cases (75%) had a high (>9) TTM score (Table 4). In contrast, only 47.2% of the other patients had a high TTM score. The statistical analysis showed no significant relationship (p>0.05) between the ITP and the disease progression (Table 4).

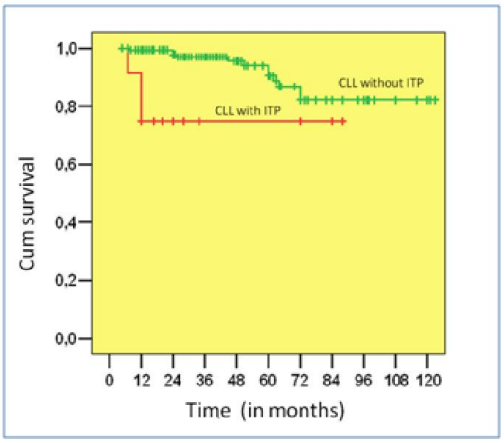

Fig.1. Overall survival of severe ITP-CLL patients vs. the other CLL patients

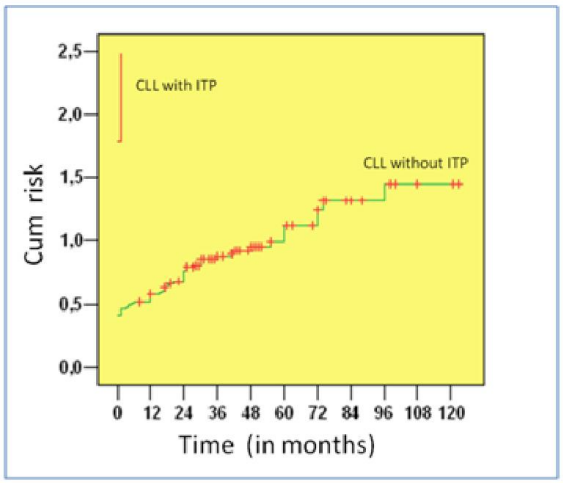

Fig.2. Risk rate of starting of initial CLL treatment in severe ITP-CLL patients vs. the other CLL patients

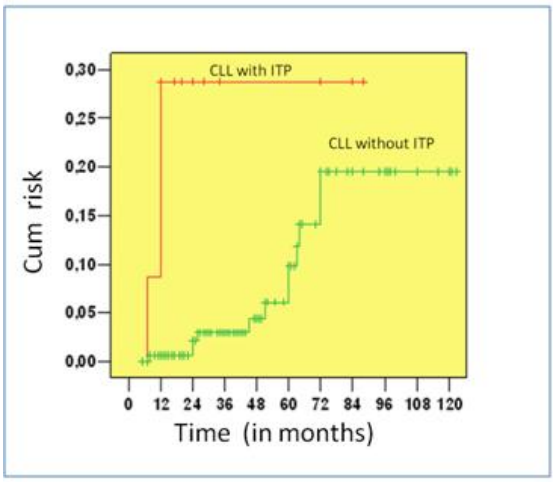

Fig.3. Mortality rate of severe ITP-CLL patients vs. the other CLL patients

DISCUSSION

The mechanisms underlying immune thrombocytopenia in chronic lymphocytic leukemia are still unclear. It has been estimated that ITP can complicate the course of CLL in approximately 2% of patients (Shvidel *et al*., 2013).The latest studies of autoimmune cytopenia in CLL revealed an incidence ranging between 4.5 and 7% ([a]Hodgson *et al*., 2011; Zent and Kay, 2010). In our small study, the prevalence was of 6.8% and in agreement with these studies. Several biological features of CLL have been associated with an increased risk of developing immune thrombocytopenia. In most studies, a correlation between advanced stage and older age and the risk of autoimmune events has been reported (Alzaki *et al*., 2014). Among clinical and biological variables, neither age, nor gender at CLL diagnosis was significantly associated with severe ITP development. There are a lot of scientific publications which confirm the relation of increased expressions of ZAP-70 and CD38 to autoimmunity development in CLL patients (Durig *et al*., 2002; Malavasi *et al*., 2011; Visco *et al*., 2010). Controversially, the other (Bosch *et al*., 2006) report an increased ITP risk in CLL patients with positive cells for ZAP70 expression, negative for CD38 and abnormal fluorescence in situ hybridization (FISH). We found that severe ITP occurrence in the earlier CLL stages was significantly associated with CD38 positivity (p=0.038) and ZAP-70 positivity (p=0.028). There was found a significant relationship between p53 protein expression and severe ITP occurrence (p=0.013).This finding implies that the presence of p53 expression may serve as a tool to identify patients with higher risk for severe ITP development. The development of new techniques, such as fluorescent in situ hybridization (FISH), has increased the detection of numerical and structural chromosome abnormalities in CLL patients. The most common cytogenetic abnormality is deletion 13q14 (51%), followed by deletion 11q22-q23 (17%-20%) (Kalil, and Cheson, 1999). Our data confirmed these frequencies - del 11q22-23 was found in 34.3% of total CLL patients and del 13q14- in 66.7 % of CLL patients. The severe ITP cases did not shown any statistical differences compared to the other CLL patients (p>0.05). Among the 24 patients with del(13)(q14) only 1 (4%) showed a relation with severe ITP.

The ITP evidence was higher in del 11q22-23 positive group- 1 of 12 CLL patients (8.3%) had chromosome 11q22-23 deletion. Cytogenetic 11q chromosome abnormalities have been related to poor outcome (Kalil, and Cheson, 1999). The poor disease prognosis has trend to be worse in severe ITP cases due to the hemorrhagic complications (Visco *et al*., 2008). The estimation of TTM score is an easy and reliable way to determine the volume of the malignant cell mass in CLL patients and is associated with intermediate prognosis (Jaksić and Vitale, 1981). Although obtained in a small number of patients, our study showed a high TTM score in 75% of ITP-CLL in contrast to the other CLL cases (47.2%, p=0.05).Possibly, higher TTM leads to an increased risk of developing of autoimmune events in CLL patients. The effect of immune thrombocytopenia on the clinical outcome and survival of patients with CLL is controversial ([a, b]Hodgson *et al*., 2011; Koehrer *et al*., 2010).

ITP has been associated with active disease without an impact on survival (Mauro *et al*., 2000; Visco *et al*., 2008). But there were not sufficient data about the association of severe ITP and disease outcome in earlier CLL stages without bone marrow failure. The performed analysis indicated that the 9/12 patients (75%) with severe ITP had increased disease progression rate, but there was no direct relationship between ITP development and disease progression (p>0.05). ITP was associated with an increased risk for starting an earlier CLL therapy. In our study, severe ITP evidence had have a negative impact on survival. Although numbers were small, patients with ITP had a significantly worse prognosis than other CLL patients, with median overall survival 68.6 months vs. 111 months of the other CLL cases (p=0.016).Accordingly, the mortality rate increases more rapidly in ITP-CLL cases compare to the other CLL patients.

Conclusion

This study indicates that in the earlier CLL stages, severe ITP is a rare event with a clear association with poor prognostic variables (CD38/ZAP-70 expression, high TTM score) and has an independent effect on overall survival and mortality rate.

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