The Importance of Tyrosine Kinase-Like Orphan Receptor 1 (ROR-1) in Chronic Lymphocytic Leukemia

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ABSTRACT

Type 1 tyrosine kinase–like orphan receptor (ROR-1) is expressed on neoplastic B cell surface in chronic lymphocytic leukemia (CLL). In this study, we aimed to determine the prognostic significance of ROR-1 expression with relationship of clinical features and therapy requirements. Retrospectively, we analyzed the medical records of CLL patients who diagnosed at Hematology department between December 2016 and October 2019. The following panel 8 color combinations of monoclonal antibodies. While matutes scoring was accepted to indicate cell surface expression of 30% or greater, we practiced to determine its cut-off value with a ROC (receiver operating characteristic) curve. The optimal ROR-1 cut-off was evaluated 80.5%. White blood cell count, lymphocyte count and platelet count were found significantly higher in patients with ROR-1 level > 80.5% at diagnosis ($p< 0.05$). Furthermore, the majority of patients with ROR-1 level > 80.5% were presented with splenomegaly initially. Rates of therapy requirements was found similar in two groups. Higher higher level and uniform expression of ROR-1 may be used as a diagnostic marker rather than predictive or prognostic marker in CLL patients. Therefore, we suggest that ROR1 adding in routine antibody panels may be useful at diagnosis of CLL.

Keywords: Chronic lymphocytic leukemia, ROR-1, Flow cytometry; Prognosis

INTRODUCTION

Chronic lymphocytic leukemia (CLL) has a variable course.[1] While some patients have a mild clinical disease course that does not mostly require treatment, others may suffer an aggressive disease course. The heterogeneity of disease presentation has complicated with the estimation of survival among patients with CLL. Two well known clinical staging systems are currently used for the risk-stratification of CLL based on clinical and laboratory characteristics.[2,3] Rai and Binet staging systems are inexpensive and easy-to-use; furthermore, they also predict survival and treatment response. However, they do not include the known biological features of CLL cells.[1,4,5] For these reasons, other prognostic factors like chromosome abnormalities (17p deletion, 11q deletion, trisomy 12), elevated β2 microglobulin (β2M) and thymidine kinase, CD38 expression, immunoglobulin heavy chain variable gene (IGHV) mutation status and ZAP-70 expression have been studied and described as predictors of poor prognosis.[6-8]

ROR-1 and ROR-2 RTK (receptor tyrosine kinases) proteins were described first in neuroblastoma cell lines 20 years ago.

They were designated as neurotrophic tyrosine kinase receptor associated protein initially.[9] These two proteins share 58% similarity with the amino acid sequence in humans and are closely related to the Tyrosine kinase and MUSK receptor family.[9-10] 104 kDa proteins are produced by ROR-1 and ROR-2 encoding genes; however, ROR-1 is subjected to various posttranslational changes, and its molecular weight reaches 130 kDa through the glycosylation of its certain regions. Many studies have suggested that ROR-1 leads to an increased tumor survival, proliferation, and metastasis of various malignancies as the ultimate result of a variety of signal pathways, signal properties, and transcription activating factors.[11] ROR-1 overexpression has been reported in many cancers.[12-15] A uniform overexpression of ROR-1 is observed on the surface of leukemic cells, in CLL.[11,12,16] Interestingly, Baskar et al. reported that while ROR-1 protein is selectively on leukemic B cells, no expression on normal B cells or other tissues.[12,13] Moreover, it has been shown that ROR-1 is expressed uniformly on leukemic cells of B-CLL irrespective of any single biological feature.[17-19] In the current study, we aimed to assess the relationship of ROR-1 expression with clinical features and treatment status and to determine its prognostic significance.

PATIENTS AND METHODS

Patients and Samples

A retrospective review of the medical records of CLL patients diagnosed according to the National Cancer Institute-sponsored Working Group guidelines for CLL (NCI-WG) criteria was performed.[20] The study conducted between December 2016 and October 2019 in University of Health Sciences, Diskapi Yildirim Beyazit Training and Research Hospital, Department of Hematology. Complete blood count was examined and peripheral blood (PB) or bone marrow (BM) smears were obtained for morphological evaluation under May- Grunwald-Giemsa staining. PB or BM was withdrawn into 4-mL K3EDTA tubes (BD Vacutainer®, CA, USA) as an anticoagulant and processed within 2 hours after collection.

Disease features, hematological parameters including hemoglobin (Hb) level, platelet count, leukocyte count, lymphocyte count, presence of splenomegaly, disease stage (according to Rai staging), lactate dehydrogenase (LDH) levels and deletion 17p13/TP53 were determined at the time of diagnosis.

Flow Cytometry

For flow cytometric (FC) study, fresh PB and/or BM samples in 4 mL K3 EDTA tubes (BDVacutainer, CA) were studied rapidly. The cells were stained using monoclonal antibodies (Beckman Coulter (BC, USA) using a stain-lyse- and -then-wash method.

The following panel 8 color CD19/CD10/CD5/CD81/CD43/CD79b/CD20/CD3, CD19/CD103/CD22/CD11c/CD25/CD23/ROR1/CD3 and CD19/CD5/CD20/sIgκ/sIgλ combinations of monoclonal antibodies. Fluorescein Isothiocyanate (FITC), Phycoerythrin (PE), Phycoerythrin-Texas RedCy5.5 (Per-CPCy5.5), Allophycocyanin (APC), Allophycocyanin-H7 (APC-H7), V450, and BV500 was used in all cases.

Instrument alignment was confirmed daily using an alignment control bead (Flow-Check, BC, USA). The accuracy and precision of cell count were tested using international quality control spurchased from the United Kingdom National External Quality Assessment Scheme (UK NEQASLI, Sheffield,UK) (z scorerange - 2.0 to 2.0). Briefly, CD19+ B cells were selected from the data file using conventional gating strategies (forward and side scatter and the pattern of CD19 expression). Its Kauza (BC, USA) was used to determine a positive result with any antibody using a cut-off limit of 30% of lymphoid cells, as recommended by the British Committee for Standards in Haematology (BCSH) guideline.[21] Matutes scoring was accepted to indicate cell surface expression of 30% or greater.[22] However, as ROR-1 is a novel antibody, we chose to determine its cut-off value with a ROC (receiver operating characteristic) curve.

Ethical approval and informed consent

All procedures performed in studies involving human participants were in accordance with the ethical standards of the institutional and/or national

Table 1. Distribution of findings of the study population

Variables (n= 67)		n (%)	Mean ± S.D.	Median [Min-Max]
Gender	Female	34 (50.7)		
	Male	33 (49.3)		
Age (years)				64.12 (36-86)
Splenomegaly	Yes	46 (68.7)		
	No	21 (31.3)		
Treatment status	No treatment needed	58 (86.6)		
	Treated	9 (13.4)		
Deletion 17p	Negative	12 (75)		
	Positive	4 (25)		
LDH (U/L)			251.89 ± 158.33	
Hemoglobin (gr/dl)			13.24 ± 2.14	
Leukocyte (count/µl)			35932.99 ± 34278.33	
Thrombocyte (count/µl)			215044.78 ± 83630.40	
Lymphocyte (count/µl)			27473.28 ± 30879.01	
Rai Stage	0	7 (10.4)		
	1	39 (58.2)		
	2	15 (22.4)		
	3	2 (3)		
	4	4 (6)		
Follow-up duration (months)			12.7 (0.5-36.5)	

research committee and with the 1964 Helsinki Declaration and its later amendments or comparable ethical standards.This study was approved by the Local Ethics Committee and the approval number is 52/13.

Statistical Analysis

SPSS for Windows, version 24 (SPSS Inc., Chicago, IL, USA) was used for all statistical analyses. Risk factors were analyzed with respect to treatment need and overall survival (OS) during follow-up period. Descriptive statistics included the number of cases and percentages. Demographic, clinical, or laboratory variables were compared using Independent Sample-t" test (t-table value) for normally distributed variables and Mann Whitney-U test (Z table values) for non-normally distributed variables of two independent samples. The relationship between two qualitative variables was analyzed using "χ^2-cross tables". A ROC curve was used to determine an optimal ROR-1 cut-off level. A Logistic Regression model was constructed on the basis of treatment status.

RESULTS

Totally 67 patients, 34 female (50.7%) and 33 male (49.3%) were included the study. The mean age at diagnosis was 64.12±11.14 (years). Advanced stage (stage 3-4, according to Rai staging) disease was determined at 6 patients (9%). 46 (68.7%) patients had no splenomegaly at diagnosis; 9 (13.4%) patients required treatment during follow-up. Table 1 summarizes the demographic characteristics of the patients. The median duration of follow-up time was 12.7 (0.5-36.5) months.

The optimal ROR-1 cut-off was evaluated 80.5% with a sensitivity of 66.7% and specificity of 56.9%. Patients with ROR-1 > 80.5 had significantly higher white blood cell count, lymphocyte count and platelet count than those with ROR-1 < 80.5 at the time of diagnosis ($p< 0.05$) (Table 2). Additionally, patients with ROR-1 >80.5 had a greater frequency of splenomegaly at diagnosis. The two groups did not significantly differ with respect to having need for treatment.

Table 2. Comparison of some parameters according to ROR-1 groups

Variable (n= 67)	ROR-1		Statistical analysis*
	≤ 80.5	> 80.5	Possibility
Hemoglobin (gr/dl)	13.69±1.79	12.70±2.36	t= 1.428
			p= 0.159
Leukocyte (count/µl)	17770.0	33900.0	Z= -3.835
	[9800.0-92200.0]	[10700.0-203000.0]	p= 0.000
Lymphocyte (count/µl)	12500.0	25895.0	Z= -3.905
	[5900.0-79700.0]	[5300.0-182600.0]	p= 0.000
Thrombocyte (count/µl)	239676.47±90160.03	182700.00±67910.77	t= 2.970
			p= 0.004
LDH level (U/L)	198.0	225.5	Z= -1.325
	[132.0-837.0]	[146.0-1141.0]	p= 0.185
Rai Stage			
0	5 (%13.9)	2 (%6.5)	
1	23 (%63.9)	16 (%51.6)	χ^2= 3.790
2	6 (%16.6)	9 (%29.0)	p= 0.435
3	1 (%2.8)	1 (%3.2)	
4	1 (%2.8)	3 (%9.7)	
Splenomegaly			
No	29 (%80.6)	17 (%54.8)	χ^2= 5.119
Yes	7 (%19.4)	14 (%45.2)	p= 0.024
Treatment status			
Treated	3 (%8.3)	6 (%19.4)	χ^2= 1.740
No treatment needed	33 (%91.7)	25 (%)	p= 0.187
Follow-up duration (months)	16.3	12.3	Z= -1.9111
	[3.7-36.4]	[0.5-36.5]	p= 0.056

DISCUSSION

FC analyzing of cytoplasmic markers and cell surface markers on malignant cells is used for CLL diagnosis, routinely. However, due to the absence of a single specific marker to identify leukemic cells of CLL, applying multiple CD markers increases the rate of success during FC analyzing.[23] There is a constant effort to introduce reliable markers for CLL. Such markers should exhibit certain properties in order to be accepted for diagnosing CLL; while they should be constantly and regularly expressed on CLL cells, independently of the disease stage, they should be not expressed in any normal cell.[24,25] Each property of the diagnostic markers allows the distinction between malignant and healthy cells. Preliminary studies aimed to use ROR-1 as a diagnostic target have shown that the molecule has poorly expressed on normal blood cells.[26] Prior studies showed that very low levels of ROR-1 expression in normal blood cells. ROR-1 expression ratio is 3.7% of B-cells, 0.3% of T-cells, 1.4% of monocytes, 1.6% of granulocytes, and 1.9% of NK cells.[27] These findings suggest that ROR-1 is a suitable target marker in FC in early stages of CLL.[23,26] As for the specificity of ROR-1 expression in CLL, it has been recently clear that ROR-1 is not only expressed in CLL, but also in B cells of non-Hodgkin lymphoma (B-NHL) and B cell acute lymphoblastic leukemia.[14,27] Cells with positive CD19 expression like those in mantle cell lymphoma and follicular lymphoma also show a ROR-1 expression as high as 94.8%.[28] As a result of high expression of ROR-1 in other malignancies like B-NHL, ROR-1 does not seem to be sufficient alone to make an early diagnosis of CLL. However, according to the available antibody panel, a ROR-1 protein expression below 10% indicates the absence of CLL, which suggests that ROR-1

is complimentary to the performance of the CLL panel.[29]

The limitations of our study were its retrospective desing, small number of patient population and shorter duration of follow-up. In a large cohort of CLL patients formed in 2016, patients with higher level ROR-1 expression had a shorter median treatment-free survival and overall survival than those with a low level ROR-1 expression.[30] These data demonstrate that expression of ROR1 may promote leukemia-cell activation and survival and enhance disease progression in patients with CLL.[30] However, treatment-free survival and overall survival could not be examined in our study.

In our study, where we investigated the predictive value of ROR-1 expression level at diagnosis, we showed that ROR-1 expression > 80.5 at diagnosis was correlated to the presence of splenomegaly and lymphocytosis, whereas it was not a predictive parameter for the future treatment need among CLL patients.

In conclusion, higher level and uniform expression of ROR-1 may be used as a diagnostic marker rather than predictive or prognostic marker in CLL patients. Therefore, we suggest that ROR1 appling in routine antibody panels may be useful at diagnosis of CLL.

REFERENCES

1. Cramer P, Hallek M. Prognostic factors in chronic lymphocytic leukemia-what do we need to know? Nat Rev Clin Oncol 8: 38-47, 2011.
2. Binet JL, Auquier A, Dighiero G, et al. A new prognostic classification of chronic lymphocytic leukemia derived from a multivariate survival analysis. Cancer 48: 198-206, 1981.
3. Rai KR, Sawitsky A, Cronkite EP, et al. Clinical staging of chronic lymphocytic leukemia. Blood 1975;46: 219-234.
4. Kay NE, O'Brien SM, Pettitt AR, Stilgenbauer S. The role of prognostic factors in assessing 'high-risk' subgroups of patients with chronic lymphocytic leukemia. Leukemia 21: 1885-1891, 2007.
5. Letestu R, Levy V, Eclache V, et al. Prognosis of Binet stage a chronic lymphocyticleukemia patients: the strength of routine parameters. Blood 116: 4588-4590, 2010.
6. Dohner H, Stilgenbauer S, Benner A, et al. Genomic aberrations and survival in chronic lymphocytic leukemia. N Engl J Med 343: 1910-1916, 2000.
7. Rassenti LZ, Huynh L, Toy TL, et al. ZAP-70 compared with immunoglobulin heavy-chain gene mutation status as a predictor of disease progression in chronic lymphocytic leukemia. N Engl J Med 351: 893-901, 2004.
8. D'Arena G, Musto P, Cascavilla N, et al. CD38 expression correlates with adverse biological features and predicts poor clinical outcome in B-cell chronic lymphocytic leukemia. Leuk Lymphoma 42: 109-114, 2001.
9. Masiakowski P, Carroll RD. A novel family of cell surface receptors with tyrosine kinase-like domain, J Biol Chem 267: 26181-26190, 1992.
10. Forrester WC, Dell M, Perens E, Garriga G. A C. elegans Ror receptor tyrosine kinase regulates cell motility and asymmetric cell division. Nature 400: 881-885, 1999.
11. Hojjat-Farsangi M, Moshfegh A, Daneshmanesh AH, et al. The receptor tyrosine kinase ROR1-an oncofetal antigen for targeted cancer therapy. Semin Cancer Biol 29: 21-31, 2014.
12. Baskar S, Kwong KY, Hofer T, et al. Unique cell surface expression of receptor tyrosine kinase ROR1 in human B-cell chronic lymphocytic leukemia, Clin. Cancer Res 14: 396-404, 2008.
13. Daneshmanesh AH, Mikaelsson E, Jeddi-Tehrani M, et al. Ror1, a cell surface receptor tyrosine kinase is expressed in chronic lymphocytic leukemia and may serve as a putative target for therapy. Int J Cancer 123 :1190-1195, 2008.
14. Barna G, Mihalik R, Timár B, et al. ROR1 expression is not a unique marker of CLL. Hematol Oncol 29: 17-21, 2011.
15. Zhang S, Chen L, Cui B, et al. ROR1 is expressed in human breast cancer and associated with enhanced tumor-cell growth. PLoS One 7: e31127, 2012.
16. Hudecek M, Schmitt TM, Baskar S, et al. The B-cell tumor-associated antigen ROR1 can be targeted with T cells modified to express a ROR1-specific chimeric antigen receptor. Blood 116: 4532-4541, 2010.
17. Daneshmanesh AH, Porwit A, Hojjat-Farsangi M, et al. Orphan receptor tyrosine kinases ROR1 and ROR2 in hematological malignancies. Leuk. Lymphoma 54: 843-850, 2013.
18. Shabani M, Omran HA, Farsangi MH, et al. Comparative expression profile of orphan receptor tyrosine kinase ROR1 in Iranian patients with lymphoid and myeloid leukemias. Avicenna J Med Biotechnol 3: 119-125, 2011.
19. Zhang S, Chen L, Wang-Rodriguez J, et al. The onco-embryonic antigen ROR1 is expressed by a variety of human cancers. Am J Pathol 181: 1903-1910, 2012.
20. Cheson BD, Bennett JM, Grever M, et al. National Cancer Institute-sponsored Working Group guidelines for chronic lymphocytic leukemia: revised guidelines for diagnosis and treatment. Blood 87: 4990-4997, 1996.
21. Oscier D, Dearden C, Eren E, et al. British Committee for Standards in Haematology. Guidelines on the diagnosis, in¬vestigation and management of chronic lymphocytic leukaemia. Br J Haematol 159: 541-564, 2012.

22. Moreau EJ, Matutes E, A'Hern RP, et al. Improvement of the chronic lymphocytic leukemia scoring system with the monoclonal antibody SN8 (CD79b). Am J Clin Pathol 108: 378-382, 1997.

23. Craig FE, Foon KA. Flow cytometric immunophenotyping for hematologic neoplasms, Blood 111: 3941-3967, 2008.

24. Byrd JC. Introduction to a series of reviews on chronic lymphocytic leukemia, Blood 126: 427, 2015.

25. Fabbri G, Dalla-Favera R. The molecular pathogenesis of chronic lymphocytic leukaemia, Nat Rev Cancer 16: 145-162, 2016.

26. Uhrmacher S, Schmidt C, Erdfelder F, et al. Use of the receptor tyrosine kinase-like orphan receptor 1 (ROR1) as a diagnostic tool in chronic lymphocytic leukemia (CLL). Leuk Res 35: 1360-1366, 2011.

27. Shabani M, Asgarian-Omran H, Jeddi-Tehrani M, et al. Overexpression of orphan receptor tyrosine kinase Ror1 as a putative tumor-associated antigen in Iranian patients with acute lymphoblastic leukemia, Tumour Biol 28: 318-326, 2007

28. Daneshmanesh AH, Hojjat-Farsangi M, Khan AS, et al. Monoclonal antibodies against ROR1 induce apoptosis of chronic lymphocytic leukemia (CLL) cells. Leukemia 26: 1348-1355, 2012.

29. Rawstron AC, Villamor N, Ritgen M, et al. International standardized approach for flow cytometric residual disease monitoring in chronic lymphocytic leukaemia, Leukemia 21: 956-964, 2007.

30. Cui B, Ghia EM, Chen L, et al. High-level ROR1 associates with accelerated disease progression in chronic lymphocytic leukemia. Blood 128: 2931-2940, 2016.

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