



## ORIGINAL ARTICLE

# Expression of ROR1 Gene in Patients with Acute Lymphoblastic Leukemia

Hoda Enayati<sup>1</sup>, Hossein Ayatollahi<sup>2</sup>, Mohammad Reza Keramati<sup>2</sup>, Maryam Sheikhi<sup>2</sup>, Hassan Bagheri<sup>3</sup>, Seyyede Fatemeh Shams<sup>2</sup>, Mohammad Hadi Sadeghian<sup>2\*</sup>

<sup>1</sup>Mashhad University of Medical Sciences, Mashhad, Iran

<sup>2</sup>Cancer Molecular Pathology Research Center, Mashhad University of Medical Sciences, Mashhad, Iran

<sup>3</sup>BSc, Mashhad University of Medical Sciences, Mashhad, Iran

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### \*Corresponding author:

Mohammad Hadi Sadeghian,  
Cancer Molecular Pathology Research  
Center, Mashhad University of  
Medical Sciences, Mashhad, Iran  
Tel: +98 51 38012584  
Email: [sadeghianm100@yahoo.com](mailto:sadeghianm100@yahoo.com)

## ABSTRACT

**Background:** Acute lymphoblastic leukemia (ALL) results from genetic alterations in a single lymphoid progenitor cell. Expression of ROR1 is reported to be increased in ALL and mantle cell lymphoma. In this study the expression of ROR1 was assessed in newly diagnosed patients with ALL.

**Methods:** This study was carried out on 40 patients with newly diagnosed ALL and healthy individuals as control group. Quantification of ROR1 mRNA expression by Real Time quantitative PCR was performed. The expressions of ROR gene in patients were compared with the control group.

**Results:** ROR1 mRNA expression (Fold Changes) in patients with ALL was  $2.85 \pm 3.51$ . ROR1 mRNA expression in patients with ALL was significantly higher than normal individuals ( $P < 0.001$ ). There was not any relationship between the expression of ROR1 and cytogenetic abnormalities.

**Conclusion:** We assessed ROR1 gene expression on mRNA of 40 ALL patients at diagnosis. The results showed that ROR1 expression had significant increase in ALL patients compared with healthy controls.

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## Introduction

Acute lymphoblastic leukemia (ALL) results from multi-step genetic alterations in a single lymphoid progenitor cell. Its incidence peaks among the ages of 2 and 4 years.<sup>1</sup> ALL is the most common malignancy in children.<sup>2</sup> ALL accounts for 77% of childhood leukemia and acute myeloblastic leukemia (AML) for 11%.<sup>3</sup> ALL is categorized based on cytogenetic and molecular criteria into a few subgroups in terms of diagnosis, prognosis and treatment.<sup>4-6</sup>

There is no comprehensive information available in Iran about the prevalence of childhood leukemia; however literature review is suggestive of a high prevalence.<sup>7</sup> There are reports indicating that the prevalence of leukemia is around 16% in Iran, which is higher compared with other cancers such as skin, lung and breast.<sup>8</sup> Acquired genetic changes that are proposed to contribute to the

development of ALL include: dysregulation of genes encoding transcription factors and signaling molecules, resulting in a subversion of hematopoietic cell homeostasis. Cooperating lesions in genes encoding key regulators of lymphoid cell differentiation may also play an important role in leukemogenesis. Alterations of these genes would be predicted to block or delay normal B-progenitor cell differentiation, thus contributing to leukemogenesis.<sup>1</sup> Receptor tyrosine kinase-like orphan receptors (ROR 1, 2) are members of tyrosine kinase receptors which are located on chromosome 1 and 9, respectively. ROR1 and ROR2 were first identified on neuroblastoma cell line, and both are coding a 104 kDa protein.<sup>9</sup> The expression of this gene has well known role in embryogenesis and organ formation in the embryo.<sup>10</sup> Receptor tyrosine kinase-like orphan receptors (RORs) are made up of an extracellular domain including Immunoglobulin-like

motif, a cysteine-rich region domain, a kringle domain and an intracellular domain.<sup>11</sup> They were called orphan because their endogenous ligands are not yet discovered.<sup>12</sup> Two different variants are identified for ROR1.<sup>13</sup> One is called shortened or truncated-ROR1 (t-ROR1) which lacks all the extracellular domains.<sup>13</sup> The second variant lacks the intracellular and transmembrane domains. However, most of the studies have been focused on the extracellular domains.<sup>13</sup> Some studies indicate that the intra-cellular domain is devoid of biological activity, while others have considered its critical role in signal induced cell transformation.<sup>11</sup>

Many functions have been characterized for ROR family which include cell polarity and tumor-like behavior such as migration and invasion.<sup>14,15</sup> Increased expression of ROR1 has been reported in acute and chronic lymphoblastic leukemia<sup>14-17</sup> and mantle cell lymphoma.<sup>18</sup> Evidence of high expression of ROR2 is reported in osteosarcoma and renal cell carcinoma.<sup>15,19</sup> ROR1 has also been proposed as a diagnostic marker in chronic lymphoblastic leukemia (CLL).<sup>20</sup>

Fukuda and colleagues showed that ROR1 attaches to Wnt5a in Wnt signaling pathway and ROR1 as a co-receptor helps Wnt5a to activate NF- $\kappa$ B. Recently, it has been suggested that ROR1 attaches to STAT 3 and activates IL-6 in CLL patients.<sup>21</sup> Homozygous mutations in ROR2 is responsible for Robinow syndrome which is recognized with short extremities, defect in spine, and fetal face.<sup>22,23</sup> Furthermore, ROR1 may possibly be an oncofetal antigen for active immunotherapy. ROR1 gene is expressed in many malignancies and might act as a survival factor for tumoral cells.<sup>24,25</sup> In addition, monoclonal antibodies are used against tyrosine kinase receptors in treatment of CLL.<sup>26-29</sup> ROR1 with attachment to protooncogene TCL which is a known co-activator of AKT can accelerate leukemogenesis.<sup>30</sup>

In this study we aimed to evaluate the expression of ROR1 gene in patients with diagnosis of ALL and compare it with a control group.

## Materials and Methods

All patients diagnosed with ALL who referred to "cancer and molecular pathology research center" of Ghaem Hospital, Mashad during 2012-2013 were included in this study. This study was approved by the Ethics committee of Mashad University of Medical Sciences. Forty patients diagnosed with ALL (28 men and 12 women) were enrolled. The control group was selected from healthy people who were matched with the patients in terms of age and sex. All the patients were categorized based on the FAB-ALL classification.

(Nothing has been mentioned about obtaining the informed consent of the patients to perform the study here.)

## RNA Extraction and cDNA Synthesis

Immediately after sampling, RNA was extracted using RNX-Plus solution kit number RN7713C (Sinagen Company). To synthesize cDNA, 2  $\mu$ g of RNA were mixed with 4  $\mu$ l of 5x Reaction Buffer, 2  $\mu$ l dNTP composition,

1  $\mu$ l oligo(dt) primer, 1  $\mu$ l Reverse Transcriptase, 1  $\mu$ l RiboLock Rnase inhibitor and 9  $\mu$ l DEPC-water. The reaction mixture (20  $\mu$ l) was incubated in 42 °C for 60 minutes, and then the temperature rose to 70 °C for 5 minutes to stop the reaction. To evaluate the relative expression of ROR1, cDNA was multiplied using the RT-PCR method. Primer for ROR1 target gene and GAPDH gene (as an internal control gene) was designed by Pubmed blast software.

## ROR1 Expression Analysis

The SYBR Green real-time qPCR method was employed to express ROR1 gene. The relative expression of ROR1 was assessed by  $2^{-\Delta\Delta CT}$  method using GAPDH gene as internal control gene. The forward primer of ROR1 gene was AGCGTGCGATTCAAAGGATT and the reverse primer was 5-GACTGGTGCC GACGATGACT-3.

The RT-PCR reaction (20  $\mu$ l for each reaction) was prepared using 10  $\mu$ l SYBER Green Master Mix, 0.5  $\mu$ l forward primer 10 pmol, 0.5  $\mu$ l reverse primer 10 pmol, 2  $\mu$ l cDNA, and 7  $\mu$ l DDW. Finally, cDNA for ROR1 and GAPDH genes were multiplied using the following conditions: 95 °C for 30 seconds, 60 °C for 30 seconds, and 72 °C for 30 seconds (the second and third steps were repeated for 40 cycles). To ensure that the final product is specific, the melting curves were analyzed. To modify samples the GAPDH internal gene with forward primer of 5-TGCACCACCAACTGCTTAGC-3 and reverse primer of 5-GCATGGACTGTGGTCATGAG-3 were used.

## Statistical Analysis

Data were analyzed by SPSS version 21(version 21, SPSS Inc). To determine the difference in ROR1 between control and ALL group, T-test and Mann-Witheny U tests were used. Non-parametric Kroschal-Wallis test was used to compare ROR1 expression between various subgroups of ALL patients.  $P < 0.05$  was considered significant.

## Results

Forty patients were included in this study with a mean $\pm$ SD age of 8.4 $\pm$ 6.7 years. The mean ROR1 mRNA expression was 2.85 $\pm$ 3.51 and 0.45 $\pm$ 0.15 in the ALL and control groups, respectively ( $P < 0.001$ ) (Table 1).

Increased expression of ROR1 was observed in 55% of ALL patients. Based on these data, an arbitrary cut off level of 1 was defined with 100% specificity. The amount of ROR1 expression among subgroups of FAB-ALL was also evaluated. The results showed no significant difference for ROR1 expression among different FAB subtypes ( $P = 0.19$ ). Furthermore, there was no difference in terms of ROR1 gene expression among patients with t(12,21), t(9,22) and t(1,19) ( $P > 0.05$ ); 17 patients had these chromosomal translocations (Table 2).

## Discussion

We evaluated ROR1 gene expression on mRNA of 40 ALL patients at diagnosis. The results showed that ROR1 expression had significant increase in ALL patients compared with healthy controls. This result suggests that ROR1 may play an important role in ALL pathogenesis.

**Table 1:** The mean levels of *ROR1* mRNA expression in different subgroups of the ALL patients

	Total	Mean <i>ROR1</i> mRNA expression	P value
Sex			0.17
Male	28	3.34±3.87	
Female	12	1.70±2.18	
Age			0.86
<10 year	26	2.78±3.95	
>10 year	14	2.98±2.63	
Splenomegaly			0.65
Yes	15	2.57±2.31	
No	25	3.02±4.10	
Hepatomegaly			0.42
Yes	8	4.22±5.51	
No	32	2.51±2.84	
Lymphadenopathy			0.26
Yes	17	3.63±4.32	
No	23	2.27±2.73	
FAB subtype:			0.77
L1	28	2.72±2.76	
L2	12	3.16±4.99	
t(12;21)			0.38
yes	9	4.14±5.30	
No	31	2.47±2.81	
t(9;22)			0.93
yes	8	2.94±2.52	
No	32	2.83±3.75	
t(1;19)			0.89
yes	4	3.08±5.39	
No	36	2.82±3.35	

**Table 2:** Main laboratory characteristics at diagnosis of ALL patients divided according to *ROR1* expression status

	ROR1 overexpression		P value
	High	Low	
RBC (x10 <sup>12</sup> /L)	2.85	2.61	0.23
Hemoglobin (g/dL)	8.33	7.27	0.1
Hematocrit (L/L )	25.3	23.31	0.28
MCV (fL)	90.21	89.2	0.68
MCH (pg)	30.14	27.97	0.31
MCHC (g/dL)	30.07	30.17	0.93
PLT (x10 <sup>9</sup> /L)	54.91	57.44	0.86
WBC (x10 <sup>9</sup> /L)	22.68	23.66	0.92
L1 (FAB subtype)	17	11	0.26
L2 (FAB subtype)	5	7	

RBC: Red Blood Count, MCV: Mean corpuscular volume, MCH: Mean corpuscular hemoglobin, MCHC: Mean corpuscular hemoglobin concentration, PLT: Platelet, WBC: White Blood Count

Cancer as a life threatening disease has always attracted the attention of researchers for finding ways of early diagnosis and new treatments. Therefore, studying the changes in genetic materials of the cells through mutations and increased or decreased gene expression is an important and dynamic aspect of molecular studies.

Receptor tyrosine kinase-like orphan receptors (ROR1, 2) are members of tyrosine kinase receptors which are located on chromosome 1 and 9, respectively. RORs play an important role in expressing tumor-like behavior. Increased expression of this gene in fetal period is reported, but its expression would be decrease in adults. The increased expression of ROR1 gene has been reported

in a number of cancers.<sup>31</sup>

Expression of ROR1 on mRNA of patients with CLL and AML has been studied and compared with the results of the authors' previous study on ALL patients. RT-PCR was performed on bone marrow and peripheral blood of 84 CLL and 12 patients with AML. The ROR1 expression was found in 94% of CLL patients, but no significant difference for ROR1 expression was found in any CML patient. They concluded that ROR1 as a tumor-dependent antigen has increased expression in lymphoid leukemia (ALL and CLL), but not in myeloid leukemia (AML). It seems to be related to a step in lymphoid lineage differentiation.<sup>31</sup>

Daneshmanesh and colleagues evaluated ROR1 expression in lymphoid and myeloid malignancies in Sweden. Significant increase in ROR1 antigen was reported in CLL and Hairy Cell leukemia compared with follicular lymphoma, mantle cell lymphoma, marginal zone lymphoma, diffuse large B cell lymphoma, multiple myeloma, AML and ALL. The least expression of ROR1 antigen was observed in follicular lymphoma, but significant increases were observed in CLL, CML and hairy cell leukemia. Follow up showed that ROR positive cells remained unchanged for a long time in CLL patients who were not in advanced stages of the disease. ROR1 expression had increased in patients in advanced stage. Therefore a wide spectrum of ROR1 expression was observed which was very high (CLL, hairy cell leukemia), high (CML), medium (myeloma and large scattered B cell lymphoma) and low (follicular lymphoma).<sup>32,33</sup>

In our study we did not find any association between chromosomal translocations and ROR1 expression ( $P < 0.05$ ). However, another study which was performed by flow cytometry, ROR1 expression was reported in association with t(1,19).<sup>33</sup>

The increased ROR1 expression had been reported in many other cancers. Zhang and co-workers evaluated the ROR1 expression in biopsy samples of 45 patients with breast adenocarcinoma through microarray method. The increase or growth of tumoral cells demonstrated that substantial subgroups of human breast cancer express ROR1. Their results indicated that increase in ROR1 expression may be related to the degree of invasion of the disease.<sup>34</sup>

In another study, 23 (40%) patients with ALL had a high expression of ROR1. Furthermore, a similar expression was reported in 4 out of 12 (33%) leukemic cell lines. Stimulation of mononuclear cells of peripheral blood with Pokeweed mitogen and phorbol acetate myristate increased the expression of mRNA ROR1 in comparison with not stimulated cells.<sup>35</sup>

## Conclusion

We assessed ROR1 gene expression on mRNA of 40 ALL patients at diagnosis. The results showed that ROR1 expression had significant increase in ALL patients compared with healthy controls. It also seems to be the expression of ROR1 correlated with lineage and differentiation phases of leukemic cells with possible consequences for immunotherapy.

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**Conflict of Interest:** None declared.

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