

## Original Article

**Running Title:** MAP1LC3B, P62, and BECN1 Gene Expression and ALL

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### The Relationships between the Alteration of MAP1LC3B, , and BECN1 Gene Expression with Minimal Residual Disease in Acute Lymphoblastic Leukemia Patients

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#### Abstract

**Background:** Acute lymphoblastic leukemia (ALL) is known as a sort of malignancy in the blood lymphoid progenitors, specifically in B and T precursors of the lymphocyte. Autophagy is a protected hemostatic and catabolic process during evolution, through which lysosomes degrade the cytoplasmic components, such as redundant or dysfunctional organelles and misfolded proteins. We conducted the present study to investigate the link between gene expression changes of BECN1, MAP1LC3B, and P62 as the main regulators of remission and response to chemotherapy in ALL patients with minimal/measurable residual disease in ALL.

**Method:** In this case-control study, BECN1, MAP1LC3B, and P62 gene expression were assessed in 30 ALL patients at the diagnosis phase, 18 patients on day 15 of the therapy, and 11 controls employing qRT-PCR.

**Results:** The results revealed that BECN1 and MAP1LC3B gene expression levels were significantly lower in ALL patients whereas P62 gene expression levels were significantly higher than the controls ( $P < 0.05$ ). We found that the expression level of the BECN1 and P62 genes increased and decreased respectively in patients on day 15 of the therapy compared to newly diagnosed ALL patients. Nevertheless, neither BECN1 nor P62 genes were significantly different at the rate of 0.73-fold ( $P > 0.05$ ).

**Conclusion:** Our study demonstrated the relationship between autophagy-related markers, such as BECN1, MAP1LC3B, and P62 with pathogenesis in Iranian children with ALL. We found that BECN1 and MAP1LC3B genes significantly decreased in newly diagnosed ALL patients and may play a part in ALL pathogenesis.

**Keywords:** Precursor B-cell lymphoblastic leukemia, Autophagy, BECN1 protein, MAP1LC3B protein, Sqstm1 protein

## Introduction

Acute lymphoblastic leukemia (ALL) is a sort of malignancy in the blood consisting of about half of the neoplasms marked by clonal proliferation and stopped differentiation of lymphoid progenitors. It specifically involves B and T precursors of the lymphocyte.<sup>1,2</sup> There are two different kinds of acute leukemia with a different percentage; 80% goes for ALL, while acute myeloblastic leukemia accounts for 20% of the total percentage.<sup>1,3,4</sup> One common blood malignancy among children is ALL which is believed to be the cause of 25% of childhood cancers and 75-80% of childhood leukemia. The rate of an outbreak among children is 3 or 4 times more than that among adults.<sup>5</sup>

Cytogenetic abnormalities and gene mutations in the hematopoietic stem cells and lymphoid progenitors are the most prevalent causes of ALL.<sup>6</sup> Fever is one of the main symptoms of the disease;<sup>4</sup> there are also other symptoms, for instance, fatigue, anemia, bleeding, bone or articular pain, petechiae, and ecchymoses.<sup>6</sup> ALL is differentially diagnosed based on cytochemical staining properties and the immune phenotype of the leukemic cells. Recently, the Cooperative French-American-British (FAB) community has categorized ALL based on morphology of leukemic cells (subtype L1, L2, and L3); however, the accurate categorization is based on immunophenotyping.

Chemotherapy is the primary phase of treatment in the patients suffering from ALL. Therapy mainly aims to lead into full recovery in patients. Complete remission of patients could be achieved, but the amount of relapse would be still high, and they are at risk.<sup>3,6</sup>

The connection between autophagy and cancer has long been debated; numerous

new studies have shed light on various facets of this link.<sup>7</sup> However the precise function of autophagy in carcinogenesis remains unresolved.<sup>8</sup> Autophagy is a protected hemostatic and catabolic process during evolution, through which the lysosomes digest the cytoplasmic components like damaged organelles, misfolded, and unfolded proteins.<sup>1,9</sup>

Autophagy is divided into three different kinds, macro-autophagy, micro-autophagy, and chaperone-mediated autophagy.<sup>4,10,11</sup>

Macro-autophagy is known as a multi-stage mechanism which includes several steps, such as induction of Autophagy, autophagosomes nucleation, pro elongation, maturation of autophagosome, and lysosomal fusion and degradation.<sup>12</sup>

Various proteins are involved in the autophagy pathway, which are called autophagy-related proteins (ATG). Both microtubule-associated protein 1 light chain3 (MAP1LC3) and BECN1 genes play a crucial part in the autophagy of mammals.<sup>13</sup> MAP1LC3 is thought to be vital for Autophagy, as it is correlated with the autophagosome dynamic forming mechanism. MAP1LC3 has three isoforms (including MAP1LC3A, MAP1LC3B, and MAP1LC3C). Lately, it has been indicated that MAP1LC3B/LC3B expression detection is a simple and precise method for autophagy tracking.<sup>14</sup>

Beclin-1 is an essential element in the initiation and control of Autophagy; Beclin1 is a specific autophagy protein encoded by the BECN1 gene<sup>15</sup> and regulates the autophagosome formation.<sup>10,16</sup> Moreover, it is the main molecule in the relationship between autophagy and apoptosis through binding to anti-apoptotic targets, Bcl-2/BclxL for instance.<sup>17</sup> P62 is an adaptor protein that plays a role in controlling apoptosis and acts as a mediator protein

between autophagic proteins and their substrates.<sup>10</sup>

The current research aimed to examine the interaction between BECN1, MAP1LC3B, and P62 gene expression with minimal residual disease in acute lymphoblastic leukemia.

## **Materials and Methods**

### ***Subjects***

In this case-control study, 30 whole blood samples of patients with ALL were collected from Amir Oncology Hospital, University Hospitals, Shiraz, Iran. All the patients were diagnosed based on FAB classification guidelines. The diagnosis was made based on the morphological observation of Wright-Giemsa-stained smears of bone marrow aspirates and immune phenotyping analyses of the leukemic cells. In the current study, the control group comprised 11 healthy volunteers matched concerning age and sex. The informed consent of each patient was received in compliance with the principles of the Medical Ethics Committee of Shiraz University of Medical Sciences (IR.SUMS.REC.1397.819).

### ***Blood sampling***

The samples of peripheral blood (PB) were taken from the patients and collected on vacutainer tubes containing Na<sub>2</sub> EDTA for total RNA purification from the whole human blood.

### ***RNA isolation and real-time quantitative PCR***

The blood samples were initially analyzed, and the total RNA was extracted and purified using the Trizol method (Invitrogen, Carlsbad, CA, USA) in agreement with the manufacturer's Protocol stored at  $-20^{\circ}\text{C}$  until further use. Subsequently, the concentrations of the extracted RNA were determined using the NanoDrop instrument (Hellma, Denmark). Afterwards, 0.4  $\mu\text{g}$  of RNA was transcribed into cDNA to a final

volume of 10 $\mu\text{L}$  using the Prime Script First Strand cDNA Synthesis kit (Takara, Shiga, Japan).

The mRNA expression levels of beclin-1, MAP1LC3B, P62, and ( $\beta$ - Actin), as an internal control were measured employing quantitative one-step QuantiFast Probe QIAGEN's real-time PCR cycler (Rotor gene, Germany). In order to quantify BECN1, MAP1LC3B, and P62 genes expression, the components of real-time PCR mixture in a final reaction volume of 20 $\mu\text{L}$  were 1 $\mu\text{L}$  of cDNA, 10  $\mu\text{L}$  of SYBR Green PCR Master Mix (SYBR Premix Ex Taq<sup>TM</sup>II, Tli RNaseH Plus Yektatajhez, Iran), 8.2 $\mu\text{L}$  of deionized water, and 0.4 $\mu\text{L}$  of each forward and reverse primer. The stages of the amplification profile were as follows: initial denaturation at  $95^{\circ}\text{C}$  for 90 seconds followed by 40 cycles of subsequent denaturation at  $95^{\circ}\text{C}$  for 5 sec, annealing at  $55^{\circ}\text{C}$  for 30 sec and elongation at  $72^{\circ}\text{C}$  for 30 sec. The relative BECN1, MAP1LC3B, and P62 mRNA expression (fold changes) in our ALL patients were calculated utilizing the  $2^{-\Delta\Delta\text{CT}}$  method.

### ***Statistical analysis***

All the statistical analyses were conducted using the program Graph pad Prism 8.0.2 (Graph Pad Software Inc, San Diego, California, USA) and SPSS version 22.0 (SPSS IBM, Chicago, IL). N-fold changes were calculated using the equation  $2^{-\Delta\Delta\text{CT}}$ . BECN1 gene expression was compared between every two groups with the T-test. Pearson correlation (r) was utilized to analyze the correlation between BECN1, MAP1LC3B, and P62 expression with the clinical features of the patients. A p value  $< 0.05$  was considered significant while a p value of less than 0.01 and 0.001 was highly significant.

## Results

### *Patients' clinical features*

Table 1 represents the clinical and demographic data of our participants .

### *Correlation analysis of BECN1, MAP1LC3B and P62 expression levels in the studied group*

The expression of BECN1, MAP1LC3B, and P62 genes was assessed in 30 recently diagnosed ALL patients and 11 controls. The findings of this evaluation revealed that the expression level of BECN1 and MAP1LC3B genes was significantly lower in the ALL patients than the controls at the rate of 5/3 fold ( $P < 0.05$ ). Figures 1 and 2 depict the expression fold variations in BECN1 and MAP1LC3B mRNA in the ALL patients versus the controls. Meanwhile, P62 was found to be highly, significantly, and positively correlated in the ALL patients ( $P < 0.05$ ) (Figure 3).

### *BECN1, MAP1LC3B, and P62 gene expression levels in different studied groups*

A significantly lower mRNA expression of beclin-1 ( $P < 0.05$ ) and MAP1LC3B ( $P < 0.05$ ) was found in the ALL patients as compared to the control subjects while a significantly higher expression of P62 ( $P < 0.05$ ) was detected in the ALL groups compared to the controls (Figure 3). We also examined whether the patients' age or sex would affect these changes in mRNA expression levels. Pearson test was performed, showing that the expression of beclin-1, MAP1LC3B, and P62 was not affected by age or sex of the patients.

BECN1 gene expression level was found to be higher in the subjects with complete remission than the newly diagnosed ALL ones. We found that the expression level of BECN1 increased in patients on day 15 of the therapy compared to that in the newly diagnosed ALL patients, yet it was not significantly different at the rate of 0.73-fold

( $P > 0.05$ ). The findings are illustrated in figure 4.

P62 gene expression level was lower in the patients with complete remission than that in the newly diagnosed ALL patients. We found that the expression level of p62 decreased in the patients on day 15 of the therapy compared to that in the newly diagnosed ALL patients, but it was not significantly different at the rate of 0.73 fold ( $P > 0.05$ ). Figure 5 represents these findings.

No difference was observed concerning MAP1LC3B gene expression level between the subjects with complete remission and the newly diagnosed ALL patients. We found that the expression level of MAP1LC3B was not different in the patients on day 15 of the therapy compared to the newly diagnosed ALL patients. These results are shown in Figure 6.

## Discussion

In the present study, the expression of BECN1, MAP1LC3B, and P62 genes was evaluated in the pediatric ALL patients at diagnosis and following the treatment. We studied the expression of BECN1, MAP1LC3B, and P62 genes in the pediatric ALL patients employing real-time PCR. Our findings demonstrated that BECN1 (Figure 1) and MAP1LC3B (Figure 2) gene expression levels were significantly lower in the newly diagnosed ALL patients than those in the control group ( $P < 0.05$ ). Meanwhile, P62 gene expression levels were significantly higher than those in the controls ( $P < 0.05$ ) (Figure 3). Furthermore, based on our results, the expression level of the BECN1 (Figure 4) and P62 (Figure 5) genes increased and decreased respectively in the patients on day 15 of the therapy compared to the newly diagnosed ALL patients.

Autophagy is a current controversial issue that might play pivotal roles in both tumor suppression and induction. It is also expected that the assessment of Autophagy-dependent markers disclose various outcomes based on prognosis.<sup>7</sup> It is clear that the role of Autophagy-related proteins, as a prognostic factor, is now controversial although some researchers have documented no correlation between the expression of Autophagy-related proteins and prognosis.<sup>18</sup> Despite the fact that the mechanism of aberrance of BECN1 and MAP1LC3B expression is generally unclear in various types of cancers, our results were in accordance with those of the Egypt group's study, indicating that the expression level of BECN1 was lower in acute leukemia patients compared to normal controls.<sup>16</sup> Thus, the subsequent inhibition of autophagic capacity might be correlated with the development of acute lymphoblastic leukemia. This finding is in line with different reports, showing that Beclin-1 functions as a haplo insufficient tumor suppressor.<sup>19</sup> In contrast, the study of Keyvan et al. demonstrated the importance of Beclin-1 in acute leukemia patients. They did not find a remarkable difference in the expression of BECN1 between acute leukemia patients and control groups.<sup>20</sup> Moreover, Park et al. found that the overexpression of BECN1 in colon carcinoma patients was associated with worse prognostic effects.<sup>21</sup> Giatromanolaki et al. did not observe a significant difference in the expression of the MAP1LC3B gene with good prognosis in colon cancer.<sup>22</sup> Furthermore, another study reported that the expression of the MAP1LC3B gene in breast cancer could be an independent marker of poor prognosis.<sup>23,24</sup> Our results are in line with the findings of Li et al. who indicated that anti-tumor activity

of a drug was through knockdown of Beclin1 and increased P62 expression.<sup>25</sup>

The results of the current study shed light to the fact that BECN1, MAP1LC3B, and P62 might be a potential diagnostic biomarker in ALL patients. However, there were limitations for Beclin1, LC3B, and P62 protein measurement; therefore, further studies are needed to examine Beclin1, MAP1LC3B, and P62 protein expression using western blotting in ALL patients.

### **Conclusion**

Our study indicated that the expression level of autophagy-related markers, such as BECN1, MAP1LC3B, and P62 genes has a prognostic effect on Iranian children with acute lymphoblastic leukemia. We did not observe a significant relationship between the ALL patients at the diagnosis and following the treatment. Additionally, our study revealed a low expression of BECN1 and MAP1LC3B gene in the newly diagnosed ALL patients. Therefore, the inhibition of Autophagy could be used as a pathway to evaluate newly diagnosed status. In line with this, the reduced expression of BECN1 and MAP1LC3B genes may play a role in tumor genesis of the patients suffering from ALL.

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## Conflict of Interest

None declared.

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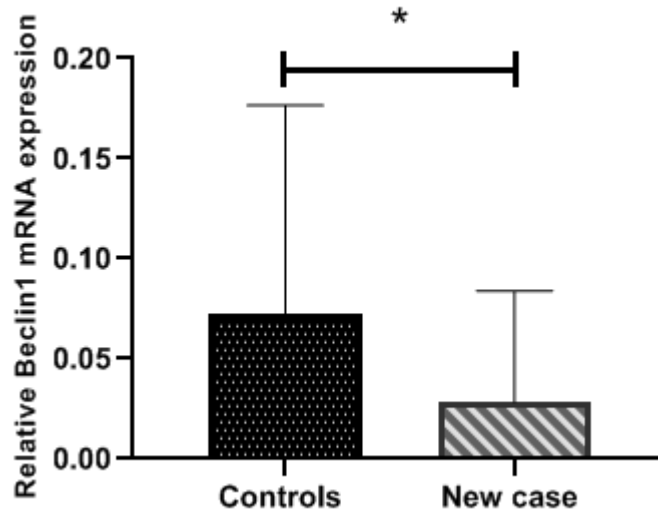
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Table 1. Clinical features of the ALL patients at diagnosis and complete remission stage

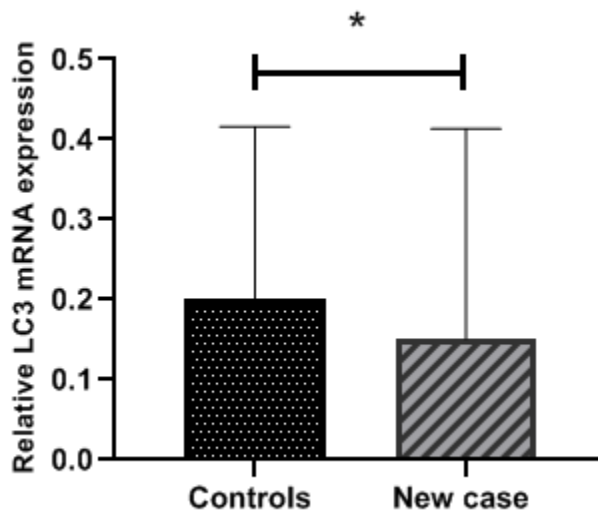
	<b>Newly diagnosed patients</b>	<b>Patients with complete Remission</b>
<b>Sex, male/female</b>	14/16	8/10
<b>Age, median</b>	6.52	6.5
<b>WBC (*10<sup>9</sup>/L), median(range)</b>	56.6	2.6 (0.4-16.9)
<b>PLT(*10<sup>9</sup>/L), median(range)</b>	65.8 (6-227)	87.2 (8-230)
<b>Hb (g/L), median(range)</b>	7.9 (4.4-11.2)	8.3 (7.2-10.4)
<b>BM Blast %, median</b>	85.17	< 5
<b>RBCs(*10<sup>6</sup>/L), median(range)</b>	3.08(2.98-3.18)	2.9 (2.81-2.99)

WBC= white blood cells, PLT= platelets, Hb= hemoglobin, BM= bone marrow



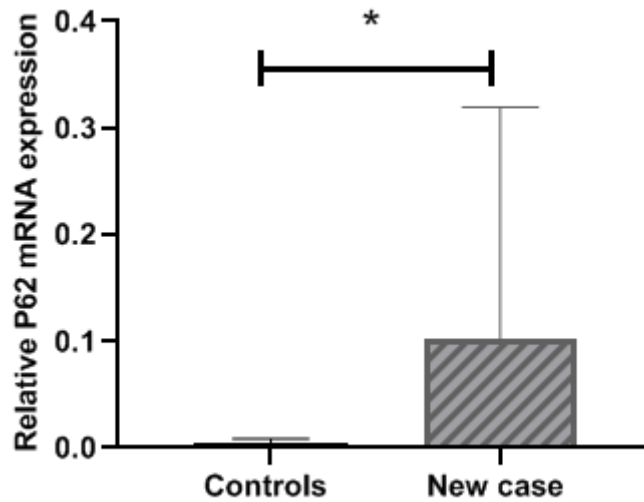


**Figure 1.** The expression of BECN1 mRNA in our newly diagnosed ALL patients compared to the controls. Fold changes of BECN1 mRNA expression were analyzed using equation  $2^{-\Delta\Delta CT}$ . \*Significant decrease in new case group compared to the controls (\* $P < 0.05$ ).

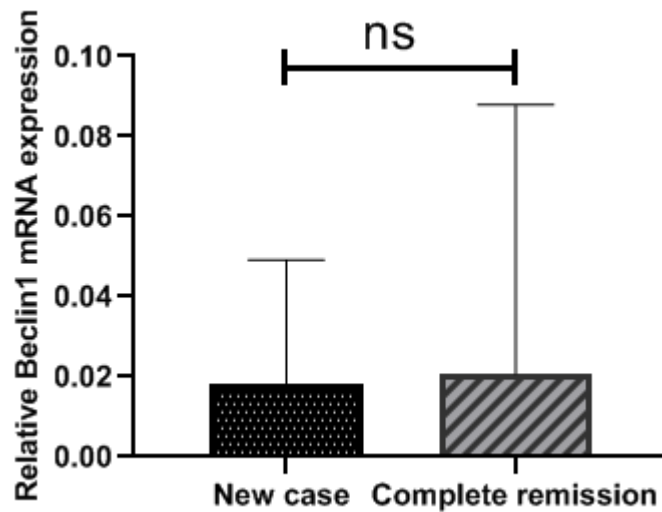


**Figure 2.** The expression of MAP1LC3BmRNA in the newly diagnosed ALL patients compared to the controls. Fold changes of BECN1 mRNA expression were analyzed with equation  $2^{-\Delta\Delta CT}$ .

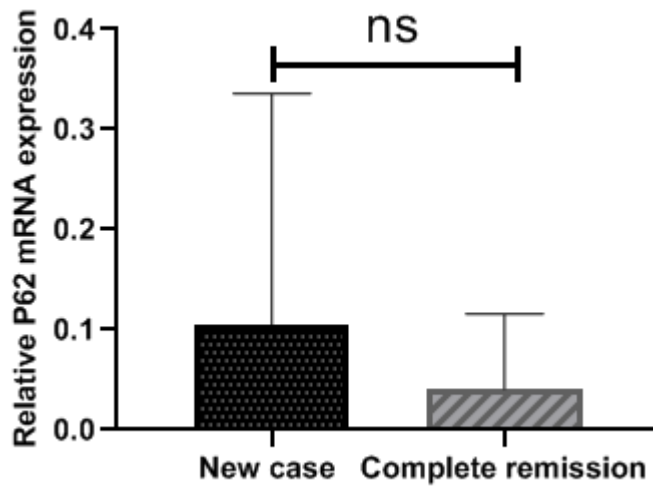
\*Significant decrease in new case group compared to the controls (\* $P < 0.05$ ).



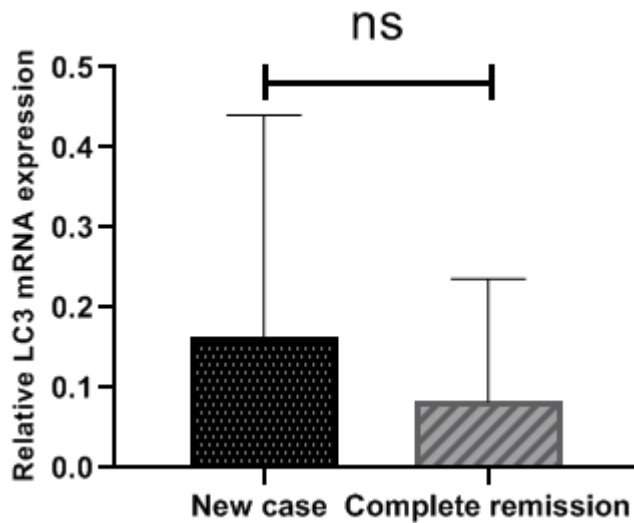
**Figure 3.** The expression of P62 mRNA in the newly diagnosed ALL patients compared to the controls. Fold changes of BECN1 mRNA expression were analyzed using equation  $2^{-\Delta\Delta CT}$ . \*Significant increase in new case group compared to the controls (\* $P < 0.05$ ).



**Figure 4.** The comparison of BECN1 mRNA expression in the patients with complete remission compared to the newly diagnosed ALL ones. The results are given as fold change of BECN1 mRNA expression ( $P > 0.05$ ). ns: Not significant.



**Figure 5.** The comparison of P62 mRNA expression between the patients with complete remission and the newly diagnosed ALL patients. The results are given as fold change of P62 mRNA expression ( $P>0.05$ ).  
 ns: Not significant.



**Figure 6.** The comparison of MAP1LC3B mRNA expression between the patients with complete remission and the newly diagnosed ALL patients. The results are given as fold change of MAP1LC3B mRNA expression ( $P > 0.05$ ).  
 ns: Not significant.