

# Livin Expression by Semi-quantitative Immuno-flourecent Staining in Hodgkin Lymphoma: A Promising Marker or a Leading Role in Pathogenesis?

Amin Ziaei<sup>1,2</sup>, Amir Pouya Tanhaei<sup>1,2\*</sup>, Safoura Mazrouei<sup>3</sup>, Mona Kharaji<sup>2</sup>, Kianoosh Keyhanian<sup>4</sup> and Mansoor Salehi<sup>1</sup>

<sup>1</sup>Medical School, Isfahan University of Medical Sciences, Genetics and Molecular Biology, Isfahan, Iran

<sup>2</sup>Medical School, Isfahan University of Medical Science, Medical Student Research Center, Isfahan, Iran

<sup>3</sup>Faculty of Medicine, Friedrich Schiller, University of Jena, Jena, Germany

<sup>4</sup>Ottawa Hospital Research Institute, Ontario, Canada

## Abstract

A novel human inhibitor of apoptosis protein family member, termed Livin, was demonstrated in the pathogenesis of different human malignancies, and also it was studied as a potential treatment target in malignancies. However, there is no report on the Livin expression profile in Hodgkin Lymphoma. In this study, we evaluated the Livin expression in 78 paraffin embedded blocks including 39 staged cases of Hodgkin Lymphoma and 39 control subjects (normal and reactive hyperplasia lymph nodes). Tissue Microarray-based Semi-quantitative Immuno-flourecent Staining was applied for protein expression profiling in both infiltrating non-neoplastic cells (morphologically typical Lymphocytes) and neoplastic cells (Hodgkin and Reed-Sternberg) of cases and control samples. Our results demonstrated that the mean ratio of Livin/GAPDH expression was significantly increased between infiltrating background cells in Hodgkin Lymphomas and control cases (0.54596 vs. 0.50827,  $P < 0.001$ ). Also, a significant difference was found in the mean ratio of Livin/GAPDH expression between neoplastic cells and major background cells in the tumor microenvironment (0.59024 vs. 0.54596,  $p < 0.001$ ). Furthermore, this study confirmed a significant increase of Livin expression from early-stage to advanced-stage of Hodgkin Lymphoma (0.52888 vs. 0.580146,  $P < 0.01$ ). These findings suggest that Livin may play a critical role in the pathogenesis of Hodgkin Lymphoma. It also can be a novel prognostic marker and a potential therapeutic target in this type of lymphoma.

**Keywords:** Hodgkin lymphoma; Reed-Sternberg cell; Inhibitor of Apoptosis Protein; Livin/BIRC7; Semi-quantitative Immunoflourecent Staining

## Introduction

Hodgkin Lymphoma (HL) is the most striking example of tight tumor-host relationship [1]. About 95% of HL belongs to the classical form of the disease [2] and based on the frequencies of cellular components and histo-pathological features, it is sub-typed to four entities: Nodular Sclerosis Classic Hodgkin Lymphoma (NSCHL), Mixed Cellularity Classic Hodgkin Lymphoma (MCCHL), Lymphocyte-Rich Classic Hodgkin Lymphoma (LRCHL), lymphocyte Depleted Classic Hodgkin Lymphoma (LDCHL) [3], while the remaining 5% represent Nodular Lymphocyte Predominant Hodgkin Lymphoma (NLPHL) [2,3]. This lymphoma is characterized by heterogeneous cellularity, including a majority of reactive and inflammatory non-neoplastic cells and a minority of specific neoplastic cells -the multinucleated Reed Stern-berg and its mono-nucleated variants, Hodgkin and Reed-Sternberg (HRS) cells. Unlike most other cancers, malignant cells in HL are outnumbered by background cells in the microenvironment of tumor [4,5], which accounts for only about 1% of all cells in the tumor tissue [1,2,4]. In addition, as for HL, major reactive cells in lymph node are lymphocytes (mostly T-cell) [6], macrophages/histiocytes, eosinophils, mast cells, plasma cells, fibroblast and neutrophils [6-9].

Growing attention has been focused on understanding the exact pathological aspect of HL so far, but its molecular biology is still poorly understood [5]. Although HL in general has a favorable prognosis but more molecular studies are required for the refractory and relapsed patients [9-12] to discover the unresolved molecular pathobiological issues of HL [2,13,14]. Recently, advances in molecular profiling of HRS cells have shown that this small number of malignant cells have a potential role in the HL pathogenesis through different pathways like providing a typical microenvironment by secretion of cytokines and chemokines (CCL5, CCL17, CCL22, IL5, TGF- $\beta$ , PDGF, IL2, IL6

[2,4,15], deregulating of transcription factor network [2], and escaping from apoptosis [13,15,16].

The numerous literature has been reported on the critical role of apoptosis in different pathological conditions like neurodegenerative disorders [17] and cancers, and particularly hematolymphoidneoplasias [16,18-22]. Interestingly, this is a solid notion that defective apoptosis rather than increased cell proliferation is the primary cause of malignant lymphopoiesis [21]. So, the impaired regulation of apoptosis is considered to be a prominent event in the development and progression of hematological malignancies [20,21]. The mechanisms of these defects, however, have not been fully elucidated. But Bcl2 and Inhibitor of apoptosis protein (IAP) gene families, which have been known as hallmarks of apoptosis regulation [20], are considered as the main culprits in the pathogenesis of these malignancies [18,23-27]. So far, as many as eight human IAP members have been identified, NAIP/BIRC1, cIAP-1/BIRC2, cIAP-2/BIRC3, XIAP/BIRC4, SURVIVIN/BIRC5, BRUCE/BIRC6, ILP-2/BIRC8 and our subjected novel one in this study, Livin/ML-IAP/BIRC7 [27,28].

Livin is a 39 kDa protein consisting of a single BIR domain and a RING finger motif. Structurally, Livin-BIR forms a globular

**\*Corresponding author:** Amir Pouya Tanhaei, Medical School, Isfahan University of Medical Science, Medical Student Research Center, Isfahan, Iran, Tel: +98-311-6244828; Fax: +98-311-6688597; E-mail: [a\\_p\\_t80@yahoo.com](mailto:a_p_t80@yahoo.com)

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conformation conserved by four  $\alpha$ -helices and a three-stranded anti-parallel  $\beta$ -sheets [29,30]. The Livin gene has two splice variants, Livin  $\alpha$  and Livin  $\beta$ , which are almost identical but with different tissue and cellular distributions [31,32]. Livin plays a critical role in apoptosis inhibition [33]. It has been reported that Livin directly interacts with executioner Caspase-3 and-7 in vitro and initiator Caspase-9 in vivo through its BIR domain [34]. BIR domain has a vital role in IAP function, and mutations in the BIR domain reduce both inhibition of Caspase-9 and general anti-apoptotic capacity of the cells [29,35,36]. In addition to apoptosis inhibition, Livin is also involved in the regulation of cell proliferation by arresting cell cycle at G0/G1 phase [32,37]. Regarding Livin function in human cells, numerous studies have reported that Livin up-regulation is a risk factor for cancer progression and unfavorable prognosis [38]. There are evidences showing that Livin expression significantly increases in series of cancers [39] including colon cancer [40], bladder cancer [32,41], lung cancer [26,32], neuroblastoma [17,42], pancreatic cancer [43], osteosarcoma, renal cell carcinoma [34], leukemia [38], and of course non-hodgkin lymphoma [16,32,44].

So far, there is no report regarding the Livin expression in HL, especially the Livin role in the deregulation of apoptosis in the reprogrammed-HRS cells and also no study has discussed the prognostic significance of Livin in HL as a promising and informative biomarker. In order to find possible differences in Livin expression in both malignant and non-neoplastic cells, we examined the Livin expression in 39 staged cases of HL and the same number of control samples using semi-quantitative immunofluorescent staining on Formalin-Fixed Paraffin-Embedded (FFPE) sections. Besides, we evaluated the Livin expression in early-stage cases (clinical stages of I and II) and advanced-stage ones (clinical stages of III and IV based on Ann Arbor staging classification for HL) [45,46], and Livin expression among different histologic subtypes of HL.

## Materials and Methods

### Patient and donor material

In this study, Livin expression was examined in 78 paraffin embeds blocks including 39 HL FFPE-tissue blocks and 30 and 9 FFPE-tissue blocks with diagnosed normal and reactive hyperplasia lymph nodes, respectively.

The lymph nodes were excised in the department of surgery, University Hospital of SeyedAlshohada and Al'zahra, Isfahan University of Medical Sciences, from 2006 to 2010.

Primary diagnosis of CHL was made based on histological examination through H&E staining and confirmed morphology diagnosis of RS cells by expert panel and also based on immunohistochemical examination we accepted RS cells (in order to consider as CHL cases) which is express CD15 (85 percent of cases) and CD30 (all cases except case of NLPHL), and lack global expression of pan-B (CD19, CD20, CD79a) and pan-T (CD3, CD7) antigens. Finally, eligible lymph node blocks for this study were confirmed again by hematopathologist with supplementary immunohistochemical staining (Data not shown). The histological examination included 39 HL lymph node blocks, and also, 18, 14, 4, 2 and 1 of NSCHL, MCCHL, LRCHL, LDCHL, and NLPHL respectively.

Clinical features of all patients including sex, age, B-symptom (fever, night sweats, weight loss), lymph node involvement site (cervical, auxiliary, mediastinum, paraaortic, perihilar, inguinal) and extra-lymphatic organ involvement (lung, liver, spleen, bone marrow)

were recorded and clinical staging based on Ann Arbor criteria was performed by an oncologist. Among 39-HL patients, 10, 9, 4 and 8 were diagnosed to be at stages of I, II, III and IV, respectively. Besides, in this study, we determined another staging style, which assumed stage I and II as early-stages and stage III and IV as advanced-stages [45,46]. Some limited cases did not have the required clinical information for staging, so they were considered in an undefined stage group. Informed consent was taken for all donor materials.

### Immunostaining

The samples were subjected to paraffin wax histology using a standard method. Histological sections (3  $\mu$ m) were cut using a Jung rotary microtome, floated out on a 50-50 volume mixture of absolute ethanol-distilled water at 48°C, and then mounted on glass microscope slides, which were previously coated with 3-aminopropyltriethoxysilane prior to overnight storage at 37°C.

The semi-quantitative method used in this study was the same method developed and applied in our previous studies [47-49]. We used two antibodies against Livin and GAPDH. Livin and GAPDH protein expression were measured using semi-quantitative immunofluorescent assay. For Livin detection, we used Livin primary antibody of Baculoviral IAP Repeat -containing (BIRC7/Livin) Rabbit anti-Human, IgG polyclonal antibody (Life Span Bio Sciences, Lot ID:LS-B456/10844) GAPDH primary antibody was Glyceraldehyde 3-Phosphate Dehydrogenase (GAPDH) mouse anti-Human, IgG monoclonal (2D4A7) antibody (Life Span Bio Sciences, Lot ID:LS-B520). Fluorochrome-conjugated secondary antibody against the species of the anti-livin and anti-GAPDH antibodies were Fluorescein (FITC) Affini Pure Goat anti-rabbit,IgG (H+L) and Texas Red Affini Pure Goat anti- mouse,IgG (H+L) respectively. These reagents were purchased from Jackson Immuno Research Laboratories (West Grove, PA, USA).

Slide preparation involved transferring sections to slides, removal of paraffin, re-hydration and also antigen retrieval treatment. As antigen retrieval treatment we used R&D Systems protocol to promote epitope availability and enhance immunoreactivity. Antigen Retrieval Reagent-Basic is an antigen retrieval system that utilizes heat-induced recovery of cell and tissue antigens. (Catalog NO. CTS013) Sections were de-paraffinized through Microwave oven (67°C for 45 min) and Xylene treatment (4 $\times$ 10 min). Then, they were gradually rehydrated in the following order: Absolut Alcohol (1 $\times$ 5 min), 95% Alcohol (1 $\times$ 5 min), 85% Alcohol (1 $\times$ 5 min) and finally 75% Alcohol (2 $\times$ 5 min), deionised water (5 min), Phosphate buffer saline (PBS) (2 $\times$ 5 min). After re-hydration, slides were allowed to dry at room temperature, and then with DAKO Pen, the rim of tissue section on the slides was marked. Next, 150  $\mu$ l of PBS was added to each slide and it was incubated for 15 minutes at 37°C in a humidified chamber before antibody treatment. Primary antibodies (Livin and GAPDH) were diluted to its optimal dilution (1/100) in diluents. After that, 50  $\mu$ l of the primary antibodies were added to the slides and they were incubated for 45 minutes at 37°C before rinsing slides with PBS. The following steps need to be done in darkness.

The secondary conjugated antibodies were diluted to its optimal dilution (1/50) in diluents. Then, 50  $\mu$ l of secondary antibody was applied to each slide, the slides were then incubated for 1 hour at 37°C, before washing in PBS (2 $\times$ 10 min). In this study, Livin was stained with FITC and GAPDH was stained with Texas Red conjugation. Finally, all slides covered with a cover slip, sealed with clear nail polish and kept in a cold and dark place.

### Image analysis

Prepared slides were examined using a LEICA fluorescence microscope (BZ00) with filter sets suitable for FITC & Texas Red dyes. Two images were taken from each microscope field, one with blue filter (350-450 nm) and the other with green filter (550-650 nm).

Furthermore, 20 to 30 images were taken for each sample from different sites of the tissue section, randomly. Images were captured using a cooled charge coupled device (CCD) camera (LEICA: DC 350F) interfaced with a PC computer. We analyzed these saved images using image-processing algorithms in MATLAB.7 software (<http://www.mathworks.com>).The whole boundary of desired cells in both green and red planes of our available images was selected and then the ratio of intensity between the mean of pixels in the green and red plane of selected cells was computed by following ratio formula:

$$\text{Ratio} = \frac{\text{Mean of pixels intensity for a cell in the green plane (indicated Livin expression)}}{\text{Mean of pixels intensity for the same cell in the red plane (Indicated GAPDH expression)}}$$

Unlike some previous studies, which selected and analyzed images randomly among all samples [17,48], samples were analyzed one to one in this study in order to acquire highly accurate and trustworthy results. Of all taken images for each sample, a 30-cell- collection was selected and then analyzed by MATLAB 7. The total cell count was around 2060 cells in all prepared HL and control sections.

We could not completely eliminate autofluorescence, so we excluded autofluorescence artifacts from the evaluation by selecting appropriate cells. The desired cells for analyzing were morphologically typical lymphocytes. Although the selection of HRS cells in immunofluorescence stained samples may be challenged by using conventional immunofluorescence as morphological details, we selected about 20 morphologically definite typical neoplastic HRS cells in HL samples and also confirmed it by two Hematopathologists independently.

### Statistical analysis

One-way analysis of variance (ANOVA) and student's t-test were run to analyze the mean level of Livin expression ratio between case and control groups and their subdivides. Furthermore,  $\chi^2$  and Fisher's exact tests were applied to analyze data in clinical features of this study (Table 1).

### Results

In this study, the expression of Livin in 39 staged HL was compared to 39 non-neoplastic lymphoid tissues (30 normal and 9 reactive hyperplasia lymph nodes). Livin protein expression was measured in all samples. To fulfill this purpose, multi-color FISH method was modified and applied for semi-quantitative Immuno-flourescent method [47]. This procedure had been developed to detect expression of more than one protein within the same cell, at the same time [48,49]. In fact, the quantitative evaluation of any subjected protein expression would be achieved only by considering the relative hybridization signal intensities from two different proteins: One with a variable expression and another with a constant expression termed housekeeping gene in neoplastic and normal lymphoid tissue. We calculated alteration in the expression of Livin protein in our samples based on the decrease or increase in Livin/GAPDH ratio. Any change in the ratio of green (conjugated hybrid with Livin) to red (conjugated hybrid with GAPDH) indicates variation of the expression of Livin (Figure 1).

To determine whether or not Livin up-regulation occurs

in neoplastic tissue, 1030 infiltrating cells were analyzed in the tumor environment (morphologically typical lymphocytes) and approximately the same number of background cells in normal and reactive environment of lymph node was considered. Mean Ratio (M.R) of Livin/GAPDH expression in all HL cases was  $0.5459 \pm 0.0612$  and in the control group, it was  $0.50 \pm 0.0267$ . So, Livin expression was significantly higher in HL than normal and reactive samples ( $P < 0.001$ , t-test) (Figure 2).

Surprisingly, there was no statistical difference between the Livin expression in normal (M.R of Livin/GAPDH expression =  $0.5085 \pm 0.0284$ ) and reactive hyperplastic lymph nodes (M.R of Livin/GAPDH expression =  $0.5068 \pm 0.0213$ ) ( $p > 0.05$ ) (Figure 3).

Furthermore, all diagnosed malignant cells (HRS) in the HL (Figure 1) were analyzed for Livin expression (M.R of Livin /GAPDH expression =  $0.5902 \pm 0.0135$ ) and compared with all infiltrating cells (M.R of Livin /GAPDH expression =  $0.5459 \pm 0.06126$ ) in HL, ( $P < 0.001$ , paired t-test) (Figure 4). The expression of Livin in malignant cells was significantly higher than reactive inflammatory cells either, ( $P < 0.001$ , paired t-test) (Figure 4).

Based on Ann Arbor classification, we examined Livin expression in 32 patients with definite staged HL. Of the total of 32 cases, 19 and 13 were at early-stage (I and II) and advanced-stage (III and IV), respectively. In the first group, the M.R of Livin /GAPDH expression was  $0.5288 \pm 0.0418$  and for the second one, it was  $0.5801 \pm 0.06941$ . The differences between stages in HL were statistically significant ( $P < 0.01$ , t-test) (Figure 5).

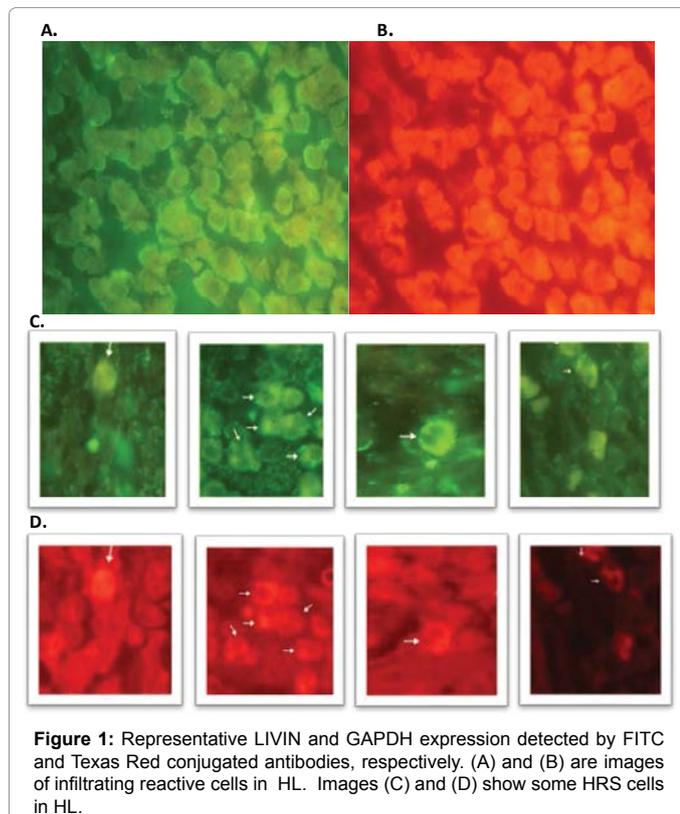
Based on the varying proportion of neoplastic and non-neoplastic cells, of 39 HL samples, 18, 14, 4, 2 and 1 cases were diagnosed with NHCHL, MCCHL, LRCHL, LDCHL and NPLHL histological patterns, respectively. There was no significant relevance between Livin expression in different entities of HL (Figure 6). Although the livin expression is the same for all histological types of Hodgkin lymphoma, it may be acceptable for NHCHL, MCCHL. However, there are a few number of the other types like LRCHL, LDCHL and NPLHL.

The ROC curve reveals a cut-off point for Mean Ratio (M.R) of Livin/GAPDH expression at 0.5231, maximized true positives and also true negatives (sensitivity 74.9%, specificity 74.4%), distinguishing HL from non neoplastic lymph node samples as a control group in difficult cases with Area under the curve (AUC) of 0.761 (Figure 7 and Table 2).

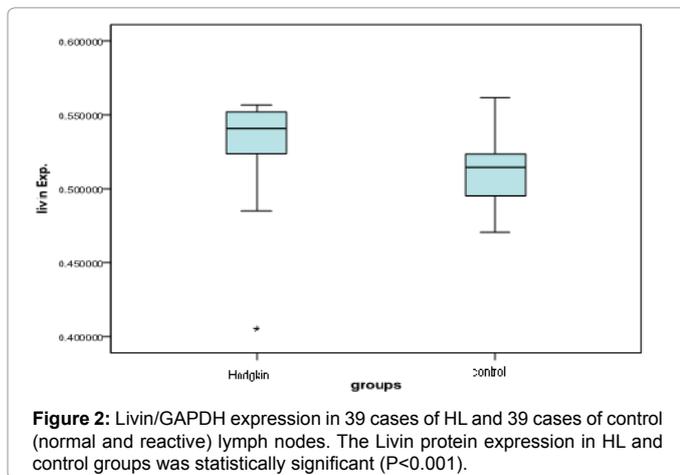
The ROC curve revealed that the Mean Ratio (M.R) of Livin/GAPDH expression could be an accurate marker in order to distinguish HL from non neoplastic lymph node samples as a control group. (AUC= 0.761 , P value <0.0001)

Medium age	33.8 ± 15.2						
Sex	Male=21			female=18			
B symptom	Positive=14			negative=17			
1-Fever	9 out of 14						
2-nightsweat	7 out of 14						
3-weight loss	8 out of 14						
Lymph node involvement	Cervical /auxiliary/mediastinal/paraortic/perihilar/ inguinal						
	Yes	26	5	9	3	3	4
No	5	25	22	29	28	28	
extra-lymphatic organ involvement	Lung	liver		spleen		bone marrow	Others
	3	2		3		0	4

Table 1: Clinical features of patients with HL.



**Figure 1:** Representative LIVIN and GAPDH expression detected by FITC and Texas Red conjugated antibodies, respectively. (A) and (B) are images of infiltrating reactive cells in HL. Images (C) and (D) show some HRS cells in HL.



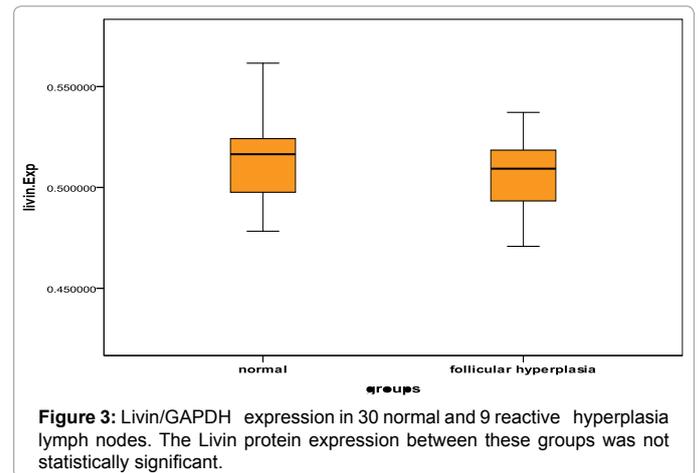
**Figure 2:** Livin/GAPDH expression in 39 cases of HL and 39 cases of control (normal and reactive) lymph nodes. The Livin protein expression in HL and control groups was statistically significant ( $P < 0.001$ ).

Our study, moreover, revealed that probably there are no correlation between the level of the Livin expression and other factors like age, gender, B symptom and lymphoid and extra-lymphoid tissues involvements. Clinical features of case groups are shown in (Table 1).

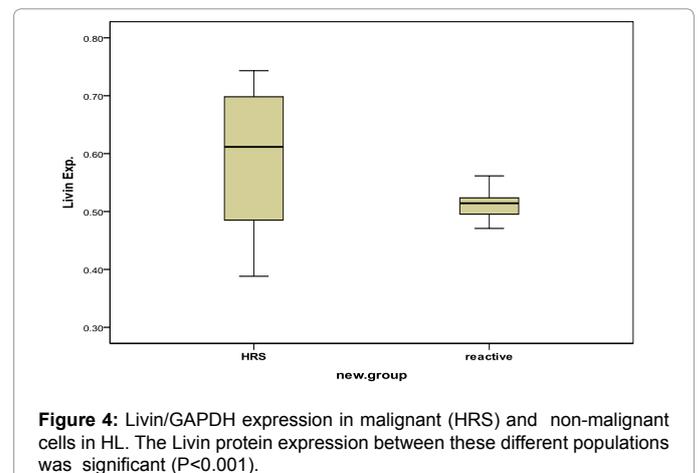
## Discussion

This study sheds new light on the prognostic significance of Livin expression previously described in human malignancies [21,39]. Livin was identified from malignant melanoma, and described as the melanoma IAP [28]. So far, it is reported that up-regulation and/or abnormal Livin expression has been detected during the process of cancer formation as well as progression [32] and also our reports show the critical role of Livin in HL. Numerous studies have validated that over expression of Livin is a potential prognostic and

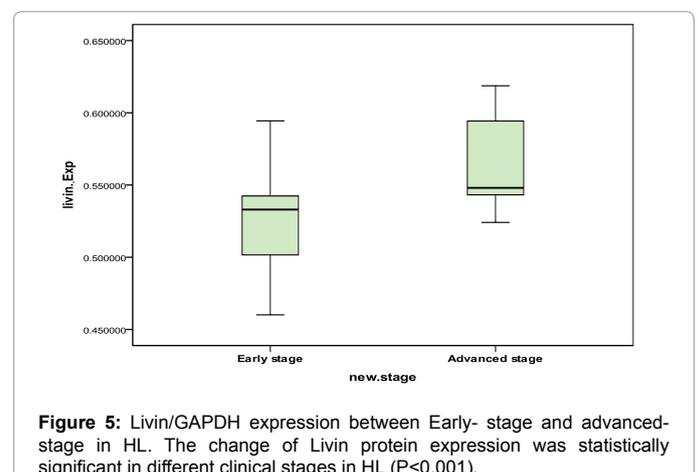
diagnostic biomarker [28]. While in some studies, this issue has not been confirmed. The prognostic importance of Livin has been shown in neuroblastoma, bladder cancer and NHL [28,41,42,44]. In 2005, Kim et al., have shown that the expression of Livin in neuroblastoma cells has a positive correlation with prognosis and outcome of the disease [42]. Also, Gazzaniga et al., in 2003, reported the role of over expression and prognostic significance of Livin in the progression of superficial bladder cancer, with median relapse-free time of Livin-positive patients being



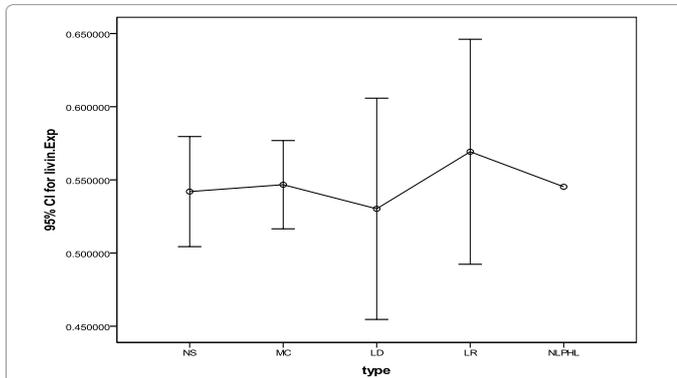
**Figure 3:** Livin/GAPDH expression in 30 normal and 9 reactive hyperplasia lymph nodes. The Livin protein expression between these groups was not statistically significant.



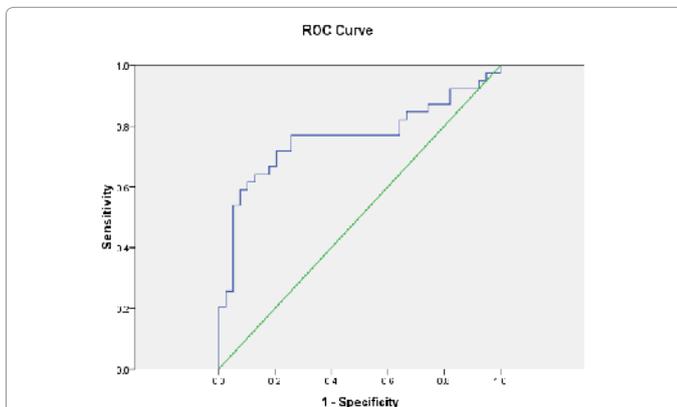
**Figure 4:** Livin/GAPDH expression in malignant (HRS) and non-malignant cells in HL. The Livin protein expression between these different populations was significant ( $P < 0.001$ ).



**Figure 5:** Livin/GAPDH expression between Early-stage and advanced-stage in HL. The change of Livin protein expression was statistically significant in different clinical stages in HL ( $P < 0.001$ ).



**Figure 6:** The M.R. of Livin/GAPDH expression among five histological entities of HL. No significant relevance is noticed between Livin expressions.



**Figure 7:** Diagnostic statistics = ROC curve of Livin/GAPDH expression for the discriminating CHL from non-neoplastic lymph node samples as control group (AUC = 0.761).

	Points	Sensitivity%	Specificity%	AUC
Optimal Cut-off point	0.5231	74.9	74.1	0.761 (95% CI 0.648 to 0.874)
Cut-off point with higher sensitivity	0.5032	82.1	35.9	0.761 (95% CI 0.648 to 0.874)
Cut-off point with higher specificity	0.5334	61.5	89.7	0.761 (95% CI 0.648 to 0.874)

AUC, areas under the curve; 95% CI, 95% confidence interval.

**Table 2:** The cut-off points to discriminate HL from non neoplastic lymph node according to MR of Livin/GAPDH expression.

obviously shorter than that of Livin-negative patients [28,41]. In 2010, clinical significance of Livin expression in NHL was evaluated by Gao et al. They found that the expressions of Livin mRNA and protein were positively correlated with clinical stage of NHL, B symptom and some other clinical features [44]. On the other hand, some studies on Lung cancer [50,51], colon cancer [40], nasopharyngeal carcinoma [28] and metastatic melanoma have not shown any correlation between Livin over expression and any clinical outcome, despite the up-regulation of Livin in these malignancies. Considering all these controversial results about the prognostic importance of Livin expression in different malignancies, we decided to examine the expression of Livin in different stages of HL.

The present study is the first description of evaluation and clinical significance of Livin expression in HL. We evaluated the

Livin expression, not only in the bulk of infiltrating background cells but also in malignant ones and then compared Livin expression in affected lymph nodes to non-neoplastic lymphoid tissues. We found that differences in the expression of Livin in both neoplastic and non-neoplastic cell population is statistically significant in comparison with cell population in the control lymph nodes. Surprisingly, we found that the expression of Livin in malignant cells is higher than the non-neoplastic background cells.

One way to explain our finding is to consider the role of nuclear factor- kappa B (NF-k B) on up-regulation of IAPs [52]. It is known that TF (NF-kB) is activated in the reprogrammed HRS cells [2]. This activation can be effective in up-regulation of IAPs in the malignant cells compared to inflammatory background cells. More comprehensive molecular profiling studies are required to underpin this issue.

Our findings also revealed possibility of significant difference in Livin expression between the early stage cases and advanced-stage HL samples. Therefore, we suggest Livin as a potential prognostic factor for staging of HL as well as Livin targeting treatment in HL patients.

Different studies have indicated that histological type of HL is a prognostic indicator for HL survival, with NSCHL (the most frequent type) having a more favorable prognosis [3,4]. In the present study, LDCHL samples (the most aggressive form of HL [4] had the lowest M.R of Livin/GAPDH expression while the LRCHL cases (with a rich background of normal lymphocytes) had the highest M.R. of Livin/GAPDH expression (Figure 6). This might be attributed to the low number of samples we studied. Hence, the authors recommend more studies with larger number of samples to evaluate the Livin expression in different types of HL.

Finally, it should be noted that a large body of evidence now supports the important function of Livin expression in the pathogenesis and progression of cancers. Livin has become an attractive target for cancer therapy by different strategies such as antisense oligonucleotides, protein function blocking and immunotherapy [28]. But, it is currently unknown whether pan-IAP inhibitors or a specific Livin inhibitors are the most useful therapeutically for the treatment of malignancies. Evaluation of all members of IAP family is therefore recommended to find a more vivid picture of their role in different stages of HL. We have extended our study to evaluate the NAIP expression profile in HL [49].

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