Original Article

Evaluation of Urokinase Plasminogen Activator Receptor, Soluble Urokinase Plasminogen Activator Receptor, and β1 Integrin in Patients with Hodgkin's Lymphoma

Abstract

Background: The objective of this study is to indicate the role of urokinase plasminogen activator receptor (uPAR), soluble uPAR (suPAR), and β1 integrin in tumor growth and invasion of lymph nodes from Hodgkin's lymphoma (HL) patients. **Materials and Methods:** In this study, 25 lymph nodes from HL patients were analyzed for the expression of β1 integrin and uPAR on mononuclear cells using two-color flow cytometry and immunohistochemical analysis. Moreover, the levels of suPAR in the serum samples of HL patients were measured and compared with 32 healthy controls. **Results:** Flowcytometry and immunohistochemical results indicated no significant association of uPAR expression with tumor size, different stages, or different histological subtypes of HL; however, an increased expression of β1 integrin was detected in the advanced stages of HL. Higher expression of β1 integrin was detected in nodular sclerosis compared to lymphocyte predominant. No significant difference was observed between the serum levels of suPAR in patients with different stages of HL and healthy controls. Moreover, the levels of suPAR were significantly higher in nodular sclerosis in comparison with other subtypes. **Conclusions:** This study showed that the levels of suPAR and β1 integrin varied between different histological subtypes of HL. Although uPAR may play only a minor role in the growth and metastasis of lymphoma, β1 integrin may be important in predicting prognosis and metastasis in HL.

Keywords: *Hodgkin's lymphoma, metastasis, soluble urokinase plasminogen activator receptor, urokinase plasminogen activator receptor, β1 integrin*

Shirin Kouhpayeh, Alireza Andalib, Marjan Gharagozloo, Mohammad Hossein Sanei[1], Nafiseh Esmaeil, Mohammad Reza Maracy[2]

From the Departments of Immunology and [1]Pathology, Isfahan Medical School, [2]Department of Biostatistics and Epidemiology, School of Health, Isfahan University of Medical Sciences, Isfahan, Iran

Introduction

Metastasis, "the spread of malignant cells from a primary tumor to a distant site," is a multistep event involving degradation or remodeling of the extracellular matrix. Basement membrane of vessels forms natural obstacles against the migration of tumor cells and metastasis. Tumor progression involves the disruption of anatomical barriers, regulated by different proteolytic systems. Among the proteases that play an active role in these processes are the serine proteases of the plasminogen activator (PA) system.[1] The urokinase PA (uPA) system consists of a proteinase (the uPA), its receptor (uPAR or CD87), and two major inhibitors, the PA inhibitor 1 (PAI 1) and PAI 2. The uPA converts plasminogen into plasmin, a potent protease that initiates several proteolytic cascades involved in the digestion of extracellular matrix. Therefore, it plays a crucial role in cell migration and tumor invasion.[2]

uPA activation is regulated by its specific cell surface receptor, uPAR, which is expressed on a variety of cell types including, vascular endothelial cells, smooth muscle cells, blood neutrophils (as intracellular reservoir), monocytes, and activated T cells.[3] uPAR is attached to the cell surface through glycosylphosphatidylinositol (GPI) anchor and it may shed from the cell surface to form suPAR by GPI-specific phospholipase D (GPI-PLD), thus forming a soluble molecule (suPAR)[4] or produced through alternative splicing.[5]

Recently, it has been documented that uPAR lacks transmembrane and intracytoplasmic domains, thus needs to form a complex with other transmembrane proteins such as integrins.[6] Integrins are heterodimeric transmembrane receptors consisting of noncovalently associated α and β subunits. They play an important role in mediating cell–cell and cell–matrix interactions

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Address for correspondence:
Dr. Alireza Andalib, Professor of Immunology, Department of Immunology, Isfahan Medical School, Isfahan University of Medical Sciences, Isfahan, Iran.
E-mail: andalib@med.mui.ac.ir

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and regulate biological activities such as migration and differentiation. Previous reports have indicated that uPAR can physically interact with multiple integrins including β1 and β2 integrins, with highest binding affinity to α3 β1 and α5 β1 molecules.[7] Such interactions may be important for cell adhesion, differentiation, and survival in different cell types. Both uPA and uPAR, as well as their soluble forms detectable in plasma and other body fluids, represent markers of cancer development and metastasis. Increased levels of uPAR and suPAR have been found in different types of solid tumors, such as breast cancer,[8] ovarian cancer,[9] colorectal cancer,[10] hepatocellular carcinoma,[11] and gastric cancer,[12] in comparison with benign or normal tissues. Moreover, it is speculated that evaluation of uPAR expression has a potential role in the diagnostic or prognostic workup of several hematological malignancies, particularly acute leukemia and multiple myeloma.[2]

Although uPAR plays an important role in several malignancies,[13] there are fewer reports on the role of uPAR in lymphoma. Lymphoid neoplasms are divided into non-Hodgkin's lymphoma (HL), HL, lymphoid leukemia, and plasma cell dyscrasia.[14] HL is a rare malignant tumor of the lymphatic system, characterized by the presence of Reed–Sternberg (RS) giant cells with B cell origin, which replaces the normal lymphoid structure.[15]

Taking into consideration that uPAR plays an important role in tumor metastasis and prognosis, and integrins are involved in uPAR signaling, in this study, the expression of uPAR and β1 integrin on lymph node mononuclear cells was evaluated in HL patients. Moreover, the serum level of suPAR was measured in patients and compared with normal healthy individuals.

Materials and Methods

Patients

A total of 43 patients who had undergone lymph node biopsy in Surgery Departments of Alzahra and Omid University Hospitals, Isfahan, Iran, were selected during November 2012–September 2013. All lymph nodes were examined by an experienced pathologist and only 25 histologically confirmed HL were included in this study as cases. Fourteen of the 18 remaining cases were diagnosed with reactive lymph nodes. In general, 23 reactive lymph nodes were considered control samples. The diagnoses were based on the WHO classification for lymphomas. All patients were comprehensively staged through Ann Arbor staging system.[16] The protocol for the present study was approved by the Ethics Committee of the Isfahan University of Medical Sciences (Isfahan, Iran). Control samples for suPAR evaluation were obtained from blood samples of 32 age- and sex-matched healthy volunteers referring to Isfahan Blood Transfusion Center (Isfahan, Iran) which were compared with the blood samples of HL patients. Informed consent was obtained from all the patients who participated in this study.

Preparation of mononuclear cells

Lymph node biopsies were homogenized by pressing the lymph node through a mesh with a sterile syringe plunger. Cells were suspended in a medium containing RPMI 1640 (Sigma, USA) supplemented with 10 mM glutamine, 10 mM HEPES, 2 g/L $NaHCO_3$, 100 U penicillin/mL, and 100 μg streptomycin. Lymph node mononuclear cells were separated from tissue homogenates by Ficoll-Hypaque separation (Bahar Afshan Lab, Tehran, Iran). The cells were washed twice with phosphate-buffered saline (PBS) (0.5% bovine serum albumin and 2 mM ethylenediaminetetraacetic acid), and the number of mononuclear cells was counted in the presence of 1% trypan blue. The number of mononuclear cells was adjusted to the appropriate concentration for flow cytometry analysis.

Flow cytometric analysis

Two-color flow cytometry analysis was performed using FACSCalibur flow cytometer (Becton Dickinson Labware, San Jose, CA, USA) and CellQuest software (Becton Dickinson and Company, USA). 1×10^6 cells were stained with saturating concentration of each monoclonal antibody. All antibodies were purchased from IQ products (Groningen, The Netherlands), unless noted otherwise.

After incubating for 30 min at +4°C in dark, the cells were washed and resuspended in 0.5 ml of PBS. Ten thousand events were counted in each sample by gating using forward scatter versus side scatter, and the gated cells were analyzed with fluorescein isothiocyanate (FITC)-conjugated anti-CD45 and phycoerythrin (PE)-conjugated anti-CD14 to ascertain that cells were lymphoid in origin. Events were acquired using FITC-conjugated anti-CD3, and PE-conjugated anti-CD19 antibodies that expressed on the lymphocyte subsets.

Surface expression of β1 integrin and uPAR was determined by FITC-conjugated anti-β1 integrin (Chemicon, Temecula, CA, USA) along with a PE-conjugated anti-uPAR (R and D system, USA). Isotype-matched mouse IgG1 (PE)/ IgG2a (FITC) (IQ Products, Groningen, The Netherlands) was used for background staining. Flow cytometric data were analyzed with CellQuest software and expressed as percentages of total lymphocytes [Figure 1].

ELISA assay

Soluble uPAR in preoperative sera obtained from lymphoma patients was measured by a quantitative [Figure 2] ELISA kit (Quantikine, R and D, USA) according to the manufacturer's instructions. The levels of suPAR in serum samples of lymphoma were expressed in pg/ml. The lower detection limit of the assay was <33 pg/mL suPAR.

Immunohistochemical staining

Immunostaining was performed on 4-μm sections taken from formalin-fixed paraffin-embedded lymph node of

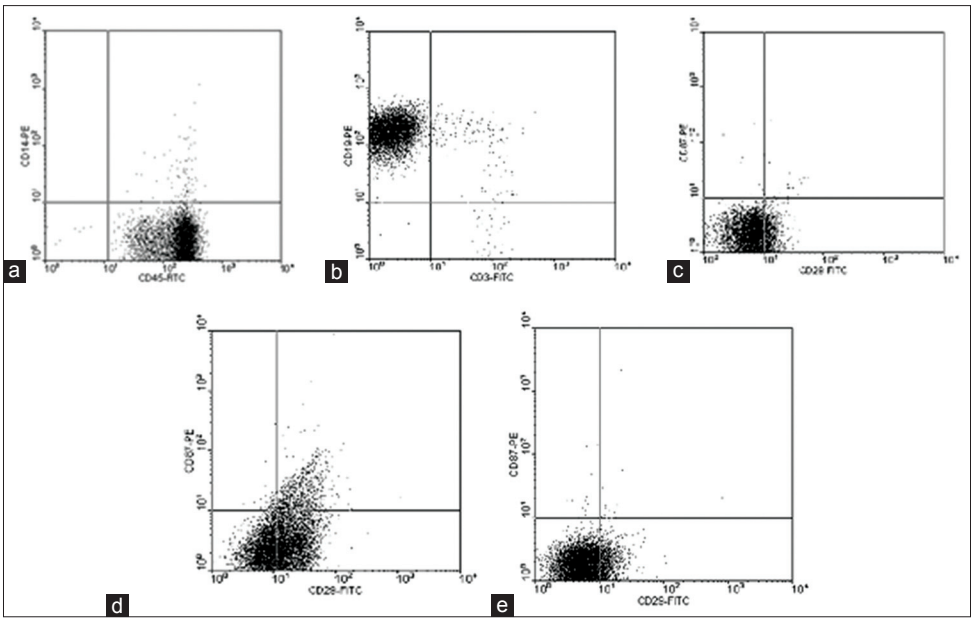

Figure 1: Flow cytometric representative dot plot results of urokinase plasminogen activator receptor and β1 integrin (markers) in Hodgkin's lymphoma. (a) Dual staining for CD14/45 to ensure urokinase plasminogen activator receptor, and β1 integrin expression was evaluated in the cells with lymphoid origin. (b) Lymphocyte predominance subtype with domination of B cell lineage marker (CD19). (c-e) Urokinase plasminogen activator receptor expression on B cell surface which is not different between the stages; while, the expression of β1 integrin on CD19+ population of B cells was higher in Stage IV (d) of Hodgkin's lymphoma samples compared to Stage I or II (c and e)

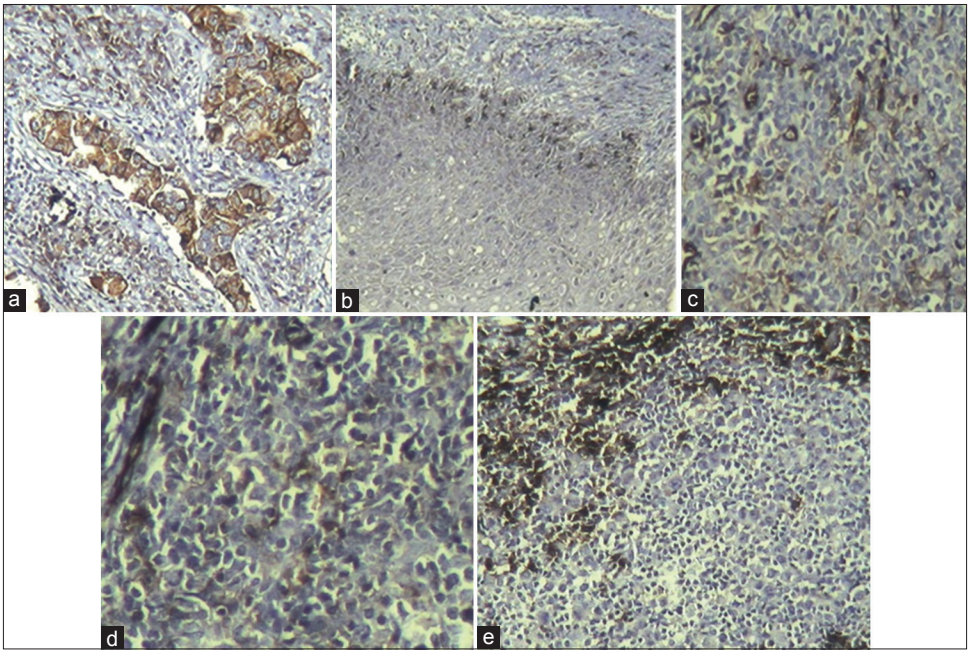

Figure 2: (a) Immunohistochemical staining of urokinase plasminogen activator receptor in invasive ductal carcinoma using anti-urokinase plasminogen activator receptor antibody, as positive control for urokinase plasminogen activator receptor expression. Cancer cells as well as fibroblasts are positive, and the staining illustrated in brown. (b) Immunohistochemical staining of β1 integrin using anti-β1 integrin antibody as positive control in skin sample, and the basal layer is shown as positive for β1 integrin expression. (c-e) Representative illustration images for comparison of β1 integrin expression between different stages of Hodgkin's lymphoma samples using anti-β1 integrin antibody. (c) Mixed cellularity, Stage II, shows moderate-to-faint staining of β1 integrin (d) lymphocyte predominance, Stage IV, exhibit a strong immunoreaction of β1 integrin (e) nodular scleroses, Stage III, moderate staining of β1 integrin. Reed–Stenberg cells could be seen in the images (×40)

HL patient's blocks. In short, paraffin-embedded sections were deparaffinized in xylene and rehydrated in ethanol. A microwave antigen retrieval technique using 0.01 M citrat (pH 6), 30 min, twice with intervening tris-buffered saline (TBS) (pH 7.4) wash was used. The sections were cooled to room temperature and sequentially rinsed with TBS, 3 times for 5 min. The sections were incubated with 3% hydrogen peroxide (diluted in methanol) for 20 min to

block endogenous peroxidase activity. Primary antibody against uPAR and β1 integrin (SantaCruz Biotechnology, CA, USA) was used at 1/100 and 1/200 dilution, respectively, overnight at 4°C. Slides were washed, and the Envision system (DAKO Cytomation, Denmark) was added to slides for 1 h at 37°C. Next, brown color was developed using 3, 3-diaminobenzidinetetra hydrochloride (Dako, Denmark) for 5 min, washed in distilled water, and counterstained with Mayer's hematoxylin for 1 min. Negative controls typically utilized the secondary antibody alone in the absence of labeling with the primary. Invasive ductal carcinoma and normal skin samples were used as positive controls for uPAR and B1 integrin expression, respectively [Figure 2].

Immunohistochemistry Evaluation

Pathologic score for β1 integrin was evaluated by the examination of staining intensity, in which sample was scored based on the intensity of signal (0, 1+, 2+, and 3+) and the percentage of positive cells (0 ≤10%, 1 = 10–25%, 2 = 25–50%, and 3 ≥50%). Expression of uPAR was based on the intensity of staining only. The degree of expression was graded as negative (no immunostaining), weak (definite but weak staining), or strong (intense staining). The level of significance was set at $P < 0.05$.

Statistical analysis

The two-tailed Mann–Whitney U-test and Kruskal–Wallis one-way analysis of variance test were used for statistical analysis with the level of significance set at $P < 0.05$. The analysis was performed using software package SPSS version 17.0 (SPSS Inc., Chicago, IL, USA). The results were presented as mean ± standard deviation.

Results

Lymph node size and the levels of urokinase plasminogen activator receptor, β1 Integrin, and soluble urokinase plasminogen activator receptor

Regarding the median diameter of lymph nodes, the patients were divided into two groups: Lymph nodes >1.5 and lymph nodes <1.5 cm. As shown in Table 1, the expression levels of uPAR and β1 integrin on gated lymph node B cells with CD3/19 markers were not significantly different between the two groups; however, higher serum levels of suPAR were detected in patients with larger lymph nodes than those patients with smaller lymph nodes [Table 1].

Table 1: Comparison of soluble urokinase plasminogen activator receptor, urokinase plasminogen activator receptor, and β1 integrin levels between two groups of lymph node size

Markers	D <1.5 cm (*n*=10)	D ≥1.5 cm (*n*=15)	*P*
uPAR	0.43±0.31	0.56±0.40	0.43
β1 integrin	10.82±7.11	7.73±7. 54	0.18
suPAR	665.9±165.8	689.27±310.07*	0.04

*Results were considered significant at $P<0.05$; Results were expressed as mean (%)±SD. D: Diameter, uPAR: Urokinase plasminogen activator receptor, suPAR: Soluble urokinase plasminogen activator receptor, SD: Standard deviation

Urokinase plasminogen activator receptor, β1 integrin, and soluble urokinase plasminogen activator receptor in different stages of Hodgkin's lymphoma

The Ann Arbor staging system was used for clinical and pathologic staging.[16] uPAR expression on B cell surface showed no difference between the four stages; however, the expression of β1 integrin on CD3/19 population of B cells was higher in Stage IV of HL samples compared to Stage I, II, or III, which was confirmed by IHC [Table 2 and Figure 1]. Serum levels of suPAR showed no significant differences between the disease stages.

Urokinase plasminogen activator receptor, β1 integrin, and soluble urokinase plasminogen activator receptor in different types of Hodgkin's lymphoma

Based on the WHO classification, 15 nodular scleroses (NS), 6 mixed cellularity (MC), and 4 lymphocyte predominance (LP) cases were diagnosed in 25 studied patients. Lymphocyte-depleted subtype was not included. In this study, surface expression of uPAR levels on CD14/45 and subsequently CD3/19 gated cells showed no difference among the stages of lymphoma [Table 3], although β1 integrin point up a significant difference between NS and LP patients with a higher level in LP cases. suPAR levels show significant differences between NS compared to MC and LP patients [Table 3]. Moreover, there was no marked difference between suPAR in HL patients and 32 healthy controls (787 ± 270.7 vs. 771.8 ± 235.5; $P = 0.8$).

Discussion

In circulating blood cells, uPAR is expressed particularly on monocytes and neutrophils, but not in resting T and B lymphocytes.[2,6] Some published studies showed that uPAR expression in leukemic cells with lymphoid origin was considerably weaker than those cells with myeloid origin.[17] In the present study, lymph node mononuclear cells from patients with HL showed a weak expression of uPAR with no significant association with tumor size or different stages (I–IV) of HL (LP, MC, and NS). Consistent with this finding, the latest research on leukemia by Atfy *et al*. demonstrated that uPAR expression in blast cells from acute lymphoblastic leukemia patients was considerably weaker than in acute myeloblastic leukemia cells.[17] Conversely, in different solid tumors and a few hematopoietic disorders with myeloid origin, higher expression of uPAR was detected in advanced stages compared to earlier stages, indicating the possible role of uPAR in the migration and invasion of solid tumor cells or hematopoietic malignancies with myeloid origin in contrast with lymphoid malignancies. Considering the

Table 2: Comparison of soluble urokinase plasminogen activator receptor, urokinase plasminogen activator receptor, and β1 integrin levels between different stages of Hodgkin's lymphoma

Markers	Stage I (*n*=7)	Stage II (*n*=8)	Stage III (*n*=7)	Stage IV (*n*=3)	*P*
uPAR	0.47±0.25	0.63±0.42	0.41±0.41	0.47±0.41	0.73
β1 integrin	2.70±1.07	6.75±5.41	14.44±6.36*	20.10±1.56*	0.001
suPAR	721.16±90.8	879.70±384.9	730.69±301.1	807.83±202.07	0.88

Results were considered significant at $P<0.05$; *is stands for a significance in comparison with Stages I and II. uPAR: Urokinase plasminogen activator receptor, suPAR: Soluble urokinase plasminogen activator receptor

Table 3: Comparison of soluble urokinase plasminogen activator receptor, urokinase plasminogen activator receptor, and β1 integrin in mixed cellularity and lymphocyte predominant subtypes with nodular sclerosis subtype of Hodgkin's lymphoma

Markers	MC (*n*=6)	NS (*n*=15)	LP (*n*=4)	*P*
uPAR (%)	0.49±0.35	0.62±0.47	0.39±0.24	0.25
β1 integrin (%)	9.22±7.99	4.90±4.84	16.62±1.74	0.06
suPAR (pg/ml)	700.72±84.81	1088.38±391.25*	645.75±275.1	0.03

*Significant in comparison with MC and LP subtypes. MC: Mixed cellularity, NS: Nodular sclerosis, LP: Lymphocyte predominant, uPAR: Urokinase plasminogen activator receptor, suPAR: Soluble urokinase plasminogen activator receptor

weak expression of uPAR on normal and malignant B lymphocytes, it can be concluded that uPAR may play only a minor role in tumor growth and metastasis of lymphoma.

As previously stated, interaction between specific cell surface molecules such as uPAR and integrins is crucial for tumor invasion and metastasis in some cancers.[18] Since uPAR and β1 integrin can form a functional complex to mediate signaling required for tumor cell proliferation and invasion,[6] in the present experiment, the expression of β1 integrin on lymph node mononuclear cells was evaluated in association with lymph node size and different stages of HL. The results revealed no significant association between lymph node size and β1 integrin expression, indicating no involvement of β1 integrin in tumor growth; however, the expression level of β1 integrin on lymph node mononuclear cells was significantly higher in more advanced stages (Stages III and IV) when compared to earlier stages (Stages I and II) of HL. Consistent with our finding, Arboleda *et al*. revealed the increased expression of β1 integrins to be positively correlated with increased metastasis in ovarian and breast cancer cells.[19] Using the Ann Arbor staging system, the stages of HL range from I to IV, of which Stage IV describing the most advanced stage where the disease has metastasized to other parts of the body.[16] A possible explanation for β1 integrin overexpression in advanced stages of HL is that it may participate in the spreading of lymphoma cells into the lymph nodes throughout the body in the late stages of the metastatic process. Moreover, the interactions between tumor cells and host stroma may regulate the expression of matrix metalloproteinases, which are abundantly expressed in malignant lymphomas.[20] Thus, it is speculated that contact between lymphoma cells and the vascular endothelium via adhesion molecules, for example, β1 integrin induces the expression of matrix metalloproteinases, which allow lymphoma cells to migrate through the extracellular matrix, reach the metastatic sites, and grow into secondary tumors.

In this study, the association of β1 integrin expression with different types of HL was evaluated. Results indicated that the expression levels of β1 integrin on lymph node mononuclear cells tended to increase in LP compared to NS subtype, although the difference was not statistically significant. Elucidation of β1 integrin expression discrepancy is based on the morphology and phenotype of the RS cells in different groups of HL. HL is currently divided into two broad groups: Classical HL with RS cells and nonclassical HL with the presence of lymphocytic and histiocytic (L and H) cells in uncommon LP subtype.[16,21] The L and H cells consistently expressed typical B-lineage markers, such as CD20 or the B-cell receptor, while RS cells differed markedly from L and H cells, in that B-lineage markers were found only rarely on RS cells.[21]

The interaction of B lymphocytes with the networks of follicular dendritic cells (FDC) is necessary for the selection of B cells in germinal center (GC), which is mediated by integrin α4 β1 (very late antigen-4) on the B cells and the vascular cell adhesion molecule-I expressed on FDC.[22] It has been shown that after immunoglobulin gene rearrangement, L and H cells with favorable mutations are selected in GC; however, instead of further differentiating into memory cells and plasma cells, the L and H cells manage to survive and lead to lymphoma disease.[23]

In the present study, the increased expression of β1 integrin in LP subtype might be responsible for the selection of L and H cells. In contrast to LP subtype, nodular sclerosis is composed of scattered RS cells.[16] The RS cells have clonally rearranged in GC, but the presence of unfavorable mutations in immunoglobulin gene inhibits BCR expression and results in the failure of clonal selection. Thus, the RS cells evade apoptotic cell death and lead to systemic

lymphoma disease.[24] Taken together, the overexpression of β1 integrin in LP subtype compared with NS might be due to the differences between the L and H and RS cells in clonal selection, reflecting a possible role of β1 integrin in the development of the L and H cells in GC.

Several studies have shown high levels of suPAR in the serum of patients with a variety of solid tumors and leukemia, which may have a role in predicting tumor prognosis and metastasis.[13] In contrast with previous studies, our data have not shown a significant difference between the serum levels of suPAR in patients with different stages of HL and healthy controls. As B lymphocytes are generally a poor reservoir of uPAR,[13] the release of suPAR into the bloodstream may not change considerably over the course of HL.

Results of the present study also revealed significantly higher serum levels of suPAR in patients with larger tumor (≥1.5 cm) compared to those with smaller tumor size (<1.5 cm). A possible explanation for this observation is that in contrast to low levels of uPAR expression on lymphocytes, high suPAR amounts may be produced mainly by tumor stromal cells, rather than by malignant B lymphocytes.

This study also revealed a marked difference in the serum level of suPAR between different types of HL, with the highest amount in NS in comparison with MC and LP. Nodular sclerosis subtype is characterized by dense collagen fibrosis in lymph nodes and myofibroblasts,[21] the primary collagen-producing cell in fibrotic lesions.[25] It has been previously reported by Eddy that reduced expression of full-length uPAR molecule by myofibroblasts compared with fibroblasts provides further evidence that lack of uPAR expression promotes fibrosis.[26] Thus, it can be concluded that higher amounts of suPAR as a result of uPAR shedding could be responsible for a more severe fibrosis in NS subtype.

Interestingly, an increased number of mast cells have also been observed in NS compared to other HL subtypes,[27] suggesting that these cells might play a pathogenic role in the fibrotic process as the bold characteristic. Mast cells are associated with fibrosis in various diseases, probably by producing fibrogenic cytokines, including platelet-derived growth factor, transforming growth factor-beta, and basic fibroblast growth factor.[28] Considering the increased levels of suPAR shown in this study, together with the high mast cell counts reported previously in NS subtype, it seems reasonable to conclude that mast cells may promote tissue fibrosis in NS by producing GPI-PLD. This enzyme could catalyze the conversion of uPAR to suPAR and result in increased levels of suPAR, which may be associated with lymph node fibrosis.

Conclusions

To the best of our knowledge, the results presented here demonstrate for the first time that β1 integrin could serve as a potential prognostic indicator for patients with advanced stage of HL. Regarding low uPAR expression on B-lymphoid lineage and lymphocytic hematopoietic disorder, we concluded that uPAR may play only a minor role in the growth and metastasis of HL. However, the current study was considered preliminary, and further investigation in more patients would be recommended to verify the role of β1 integrin and suPAR in the pathogenesis of HL.

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Conflicts of interest

There are no conflicts of interest.

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