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# Diagnostic and Prognostic Significance of Histidine-Rich Glycoprotein in Acute Lymphoblastic Leukemia

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## Abstract

**Background:** Histidine-rich glycoprotein (HRG), a multifunctional plasma protein, has a regulatory role in homeostasis, angiogenesis, and immunity; which in turn could greatly affect tumor control and metastasis. **Objectives:** To assess the possible role of HRG in acute lymphoblastic leukemia (ALL) tumorgenesis and follow-up. **Design and Methods:** HRG was quantitatively measured in serum by ELISA and its expression was assessed by real-time PCR (qPCR) in 35 patients with ALL and compared to same 25 ALL patients after induction therapy and 30 age and sex matched healthy control subjects. **Results:** HRG-serum protein (at cutoff value 63.55 pg/ml) and HRG-RNA (at cutoff value 0.955) were positive in all ALL patients before therapy, but in only 76% after therapy for HRG-protein and 60% for HRG-RNA and they could not be detected in the control group; $P < 0.001$. Additionally, the serum HRG level showed a significant positive correlation with its expression level, bone marrow blast percentage, peripheral blood blast count, $P < 0.01$. Also its serum and expression levels were positively related to the poor risk Philadelphia chromosome; $P < 0.01$. **Conclusions:** HRG (protein and RNA) might be considered as a novel diagnostic and prognostic marker in ALL. HRG-serum protein level, detected by simple methodology of ELISA, has more significant advantages than its expression level, motivating its application in large clinical studies as a potential marker.

## Keywords

Acute Lymphoblastic Leukemia, Histidine-Rich Glycoprotein and Serum

Markers

## 1. Introduction

Acute lymphoblastic leukemia (ALL) is a highly heterogeneous disease comprising many entities for which distinct treatment strategies are pursued. Treatment of ALL remains one of the most challenging adult malignancies, especially with respect to therapy [1]. The inherent heterogeneity of ALL requires an accurate assessment of risk to aid treatment decisions. In the past, the classic prognostic factors were age, presenting white blood cell (WBC) counts, cytogenetic abnormalities and upfront response to induction therapy. One of the strongest adverse prognostic features is the presence of the Philadelphia chromosome t (9; 22) [2]. Although more than 80% of adult patients with Philadelphia chromosome (Ph)-negative ALL achieve complete remission (CR) with conventional induction therapy, their 5-year survival is only 30% - 40%. Leukemia relapse is the most common cause of treatment failure in ALL [3].

Histidine-rich glycoprotein (HRG) is, a ~75-kDa single polypeptide chain protein, synthesized by the liver and secreted from activated platelets [4]. It is a multidomain protein displaying two cystatin-like regions of the N-terminus and a histidine-rich region (HRR) flanked by proline-rich regions (PRR) closer to the C-terminus [5]. HRG could interact with many ligands, including heparin, phospholipids, plasminogen, fibrinogen, immunoglobulin G, C1q, heme, and $Zn^{2+}$ [6]. Through these interactions, HRG could function as an adaptor molecule and thereby modulates numerous important biologic processes, such as immunity, angiogenesis, cell adhesion, cell proliferation, and remodeling of the extra cellular matrix (ECM). Many of these functions are involved in tumor progression and antitumor response [7].

Some studies reported the proangiogenic effect of HRG through its high binding affinity to thrombospondin and interfering with TSP-CD36-mediated antiangiogenic signaling which inhibits angiogenesis induced by basic fibroblast growth factor [8]. While other studies suggested the antiangiogenic activity of HRG as it could inhibit endothelial cell adhesion and migration, block angiogenesis and induce apoptosis in endothelial cells [9]. Kärrlander and his colleagues [10] found that the quality of the vasculature is impaired by increasing expression of HRG in mouse malignant glioma cells. Meanwhile, Rolny *et al.* [11] reported that angiogenesis was improved in HRG-transduced tumors, including increased vessel perfusion and percentage of priest covered vessels. Moreover, the presence of HRG in the stroma of most tumor biopsies, indicates that its effects are likely dependent on their concentration in the tumor and type of tumor [12].

Previous studies evaluated HRG as a useful tool in several types of cancers, but rare of them were conducted in ALL, so the aim of the current study was to investigate HRG by two different approaches as ELISA assay and real-time PCR,

and evaluate its diagnostic and prognostic value in ALL patients.

## 2. Patients and Methods

### 2.1. Subjects

The current case-control study was conducted on 65 adult subjects (age ≥18 years) including 35 ALL patients, and 30 healthy control subjects. All patients were recruited from the Hematology and Clinical Oncology Unit, Internal Medicine Department, Faculty of Medicine, Ain Shams University, Cairo, Egypt in the period from July 2014 to March 2015. An informed consent was taken from all subjects participating in the present study according to declaration of Helsinki and was approved by the Research Ethics Committee of Ain Shams University, Cairo, Egypt.

Leukemia was diagnosed and classified according to the criteria of the French-American-British (FAB) Cooperative Group [13].

#### 2.1.1. The Subjects Enrolled in This Study Were Divided into the Following Groups

- ***Group-I* (*Malignant Group*):** Included 35 adult patients with newly diagnosed acute lymphoblastic leukemia (23 males and 12 females) with mean age of 38.8 ± 8.1 years, classified based on FAB classification into 23 patients having pre-B-ALL (8 with +ve Philadelphia chromosome), 5 patients as having B-ALL and 7 patients as having T-ALL (4 with +ve Philadelphia chromosome).
- ***Group-II* (*Follow-Up Group*):** Included 25 patients with ALL, they are the same individuals of group I after receiving induction chemotherapy protocol. Of them, unfortunately 10 have succumbed their illness during induction. The remaining 25 patients were segregated into chemotherapy responsive and chemorefractory patients in accordance with the complete remission criteria that will be detailed below.
- ***Group-III* (*Control Group*):** Included 30 healthy controls subjects (21 males and 9 females) with mean age of 40.5 ± 3.6 years with a complete normal demographic data.

#### 2.1.2. Inclusion Criteria

- Adults (age ≥ 18 years).
- Newly diagnosed ALL patients.

***Exclusion criteria*:**

- Age < 18 years.
- Relapsed or refractory ALL patients who have received prior chemotherapy protocols.

#### 2.1.3. Plan of Treatment in ALL Patients

- Patients were given eight induction-consolidation courses of alternating hyper-CVAD with high-dose methotrexate and cytarabine. Briefly, the treatment regimen was as follows. Odd courses (1, 3, 5, 7) were hyper-CVAD, while

Even courses (2, 4, 6, 8) included high-dose methotrexate and cytarabine: 200 mg/m$^2$ methotrexate. Addition of tyrosine kinase inhibitor namely imatinibmesylate; at a dose of 400 mg a day was done when the patient was proved to be pheladelphia positive ALL [14].

#### 2.1.4. Definition of Response

Response assessment was done at day 21 of course 1, if patient did not achieve complete remission, then he/she proceeded to course 2 and the response was assessed at day 21 of course. Response criteria were defined as no evidence of leukemic blasts in the BM (<5%), complete resolution of extramedullary manifestations, and recovery of peripheral cell counts [15].

***Follow up period***: Patients were followed up from the beginning of induction with course 1 to the end of course 2.

### 2.2. Methods

#### 2.2.1. Sample Collection

Blood samples (5 - 10 ml) were drawn from all subjects before any therapeutic intervention and after 3 weeks of completed induction therapy. Five-milliliters blood were collected into tubes without anticoagulant for serum samples; another 5 ml blood were collected into EDTA-anticoagulated tubes for RNA extraction and PCR protocol. Serum and RNA samples were separated and then stored at −80˚C until subsequent processing and measurements.

#### 2.2.2. Assay Procedures

HRG concentration was measured using enzyme-linked immunosorbent assay (ELISA) kit (Catalog No: E2267h; Wuhan EIAab Science Co., Ltd, China). The assay employs the quantitative sandwich enzyme immunoassay technique according to the steps described by the manufacturer.

#### 2.2.3. RNA Isolation and Real-Time Polymerase Chain Reaction (qPCR)

Aliquots of plasma from peripheral blood were processed using RNA extraction kits supplied by Ambion (nirvana $^{Tm}$Paris $^{Tm}$Kit). Extraction was carried on under complete sterile conditions in a level II Biosafety cabinet (Lobonco), steps were carried out according to the manufacture's instructions. Ethanol was added to the samples and they were passed through a filter cartridge containing a glass, fiber filter immobilizes the RNA. The filter was then washed few times and finally the RNA was eluted with a low ionic strength solution. The RNA purity and concentration were determined by spectrophotometric measurement of absorbance at 260 and 280 nm.

The reverse transcription reaction was carried out in 20 µL reaction mixture using 2 µg of RNA by using higher capacity RNA to c-DNA master mixsupplied by (Applied Bio-system, ABI). Real-time PCR was performed using a real-time PCR 7500 fast ABI thermal cycler (Applied Bio-system, USA), selecting the comparative CT as quantitation method. A final volume of 20 µL (10 µL of Quantifast SYBR Green PCR master mix, 1 µL of each primer, up to 8 µL diluted

c-DNA, $H_2O$ as required) was wanted. An initial denaturation at 95˚C for 10 minutes, then 40 cycles were done. Each cycle consisted of denaturation at 94˚C for 15 seconds, annealing at 60˚C for 25 seconds and elongation at 72˚C for 20 seconds. The following primer sequences were used for Histidine rich glycoprotein (HRG) (forward, 5′-GATCATCATCATCCCCACAAG-3′; reverse, 5′-GGGTCACAAGGTCCATAGTC-3′, GenBank: NM_000412.2). B-actin (forward, 5′-AGCGGG AAA TCG TGC GTG-3′; reverse, 5′-CAG GGT ACA TGG TGC C-3′) which was used as an endogenous reference. Bio-Rad software was used to calculate threshold cycle (Ct) values for the target gene and for the reference gene (B-actin). The expression values of the tumor samples are presented as a fold expression in relation to the control sample; the actual values were calculated using the $2^{-\Delta\Delta Ct}$ equation, where $\Delta\Delta Ct$ = [Ct Target – Ct B-actin] (tumor sample) – [Ct Target – Ct B-actin] (control sample).

### 2.2.4. Statistical Analysis

The analysis was done using the Statistical Package for the Social Sciences (SPSS software version 19, SPSS Inc., Chicago, IL). Statistical comparisons were made using parametric test, ANOVA (followed by Post Hoc test) or nonparametric Mann-Whitney U (to compare two groups) and Kruskal-Wallis tests (to compare three groups). Chi-square test was used to compare quantitative parameters between groups. Correlation between different variables was performed by Pearson's correlation coefficient. Statistical significance was set at a value of $p < 0.05$. The best cutoff value that maximizes sensitivity and specificity and differentiates acute lymphoblastic patients from controls was calculated by using the Receiver Operating Characteristic (ROC) curve, which was constructed by calculating the true positive fraction (sensitivity percent) and false positive fraction (100-specificity) of markers at several cut-off points. Positive predictive value was calculated as percent of truly positive patients while negative predictive value was calculated as percent of truly subjects that don't have the disease.

## 3. Results

The median percentage of bone marrow leukemic blasts in leukemic patients was 88.0% (range from 32% to 98%), while peripheral blood leukemic blasts it was 58.0% (range from 23% to 95%), Table 1. The leukemic patients before therapy showed higher HRG levels as detected by ELISA (657.9 ± 203.8 pg/ml; range 264.0 - 931.5 pg/ml) and by qPCR (5.51 ± 3.5; range 1.7 - 13.1) compared to control group (45.6 ± 8.0 pg/ml; range 37.0 - 59.0 pg/ml for HRG-serum protein and 0.41 ± 0.21; range 0.14 - 0.9 for HRG-RNA), $p < 0.01$. Moreover, HRG-serum protein (mean 185.4 ± 114.1 pg/ml; range 22.0 - 377.0 pg/ml) and HRG-RNA (mean 1.2 ± 0.97; range 0.13 - 0.34) in ALL patients after therapy had a significantly lower level than those patients before therapy ($P < 0.01$), and a significantly higher level than normal group for HRG-serum protein only ($P < 0.01$), Figure 1. The best cutoff value for HRG-serum protein levels in ALL detected by ROC curves, considering healthy subjects as control group, was 63.55

pg/ml with 90.0% sensitivity and 100.0% specificity, and for HRG-RNA was 0.955 with 83.3% sensitivity and 100% specificity ($p < 0.01$), Figure 2 and Table 2. Moreover, HRG positivity rates detected by both methods was 100% in ALL

Table 1. Clinicopathological parameters of ALL patients.

| Parameters | Mean ± SD | Median | Range |
|---|---|---|---|
| Age (years) | 38.3 ± 8.3 | 35.0 | 17.0 - 65.0 |
| TLC (1000/mm$^3$) | 8.3 ± 5.7 | 6.7 | 4.4 - 20.0 |
| HB % | 8.6 ± 2.3 | 7.0 | 6.0 - 14.7 |
| Platelet count (1000/mm$^3$) | 69.8 ± 66 | 45.0 | 6.0 - 21.1 |
| LDH (IU/L) | 714.7 ± 428.2 | 961.5 | 312.0 - 1561.0 |
| BM blast cells (%) | 78.7 ± 21.8 | 88.0 | 32.0 - 98.0 |
| Peripheral blast cells (%) | 87.7 ± 29.3 | 58.0 | 23.0 - 95.0 |

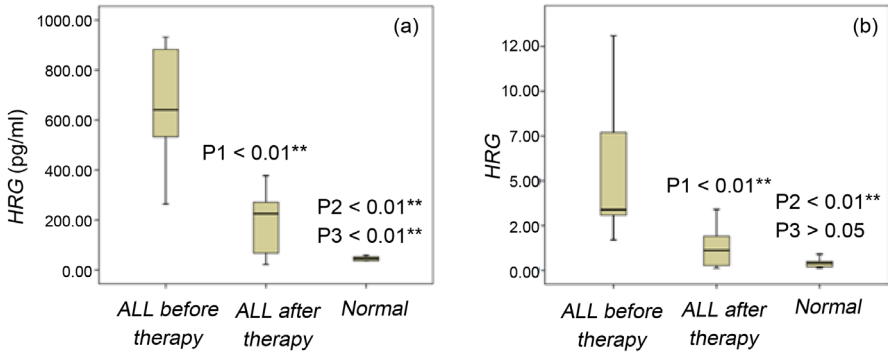


Figure 1. Serum HRG mean level detected by ELISA method (a) and qPCR (b) in ALL patients before and after therapy and control groups. (p1 = after versus before therapy, p2 = normal group versus ALL before therapy and p3 = normal group versus ALL after therapy **P < 0.01 is highly significant. P > 0.05 is non-significant).

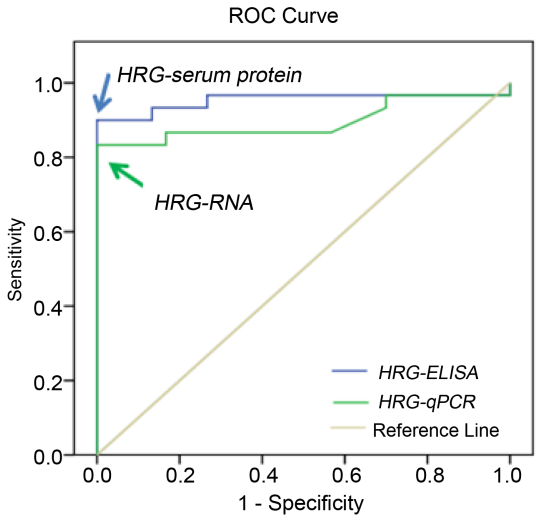


Figure 2. ROC curves for serum HRG-serum protein and HRG-RNA to discriminate between ALL patients and normal control group. The arrows denote best cutoff points of HRG-serum protein at 63.55 pg/ml and HRG-RNA at 0.955, P < 0.01.

patients before therapy and 0.0% in the normal group, while in follow-up group it was 76.0% for in HRG-serum protein and 60.0% for HRG-RNA ($P < 0.001$), Figure 3. No significant differences between ALL subtypes and HRG, however, the HRG protein level was significantly higher in AAL patients with +ve Philadelphia chromosome ($P < 0.01$) and both HRG protein and RNA protein were significantly high in ALL patients who died during the course of treatment compared to those had complete remission ($P < 0.01$), Table 3. Furthermore, HRG protein showed a significant negative correlation with platelets ($P < 0.01$), HB% ($P < 0.05$) and significant positive correlations with its expression level, LDH, BM blast cells and peripheral blast cells ($P < 0.01$), while HRG-RNA showed significant positive correlations only with LDH (<0.05) and peripheral blast cells ($P < 0.01$), Table 4.

## 4. Discussion

Histidine-rich glycoprotein (HRG) is a multifunctional plasma protein with two cystatin-like domains and a wide spectrum of targets and functions [16]. Extensive research studied the potential role of HRG in carcinogenesis, however, its effect on overall survival remains to be clearly determined. In the current study,

Table 2. Diagnostic performance of serum HRG-serum protein and HRG-RNA from patients with acute lymphoblastic leukemia and normal control group.

| Variable | Cut-off points | Sensitivity | Specificity | NPV | PPV | AUC |
|---|---|---|---|---|---|---|
| HRG-serum protein | 63.55 pg/ml | 90.0% | 100% | 83.3% | 100% | 0.953 |
| HRG-RNA | 0.955 | 83.3% | 100% | 75.0% | 100% | 0.896 |

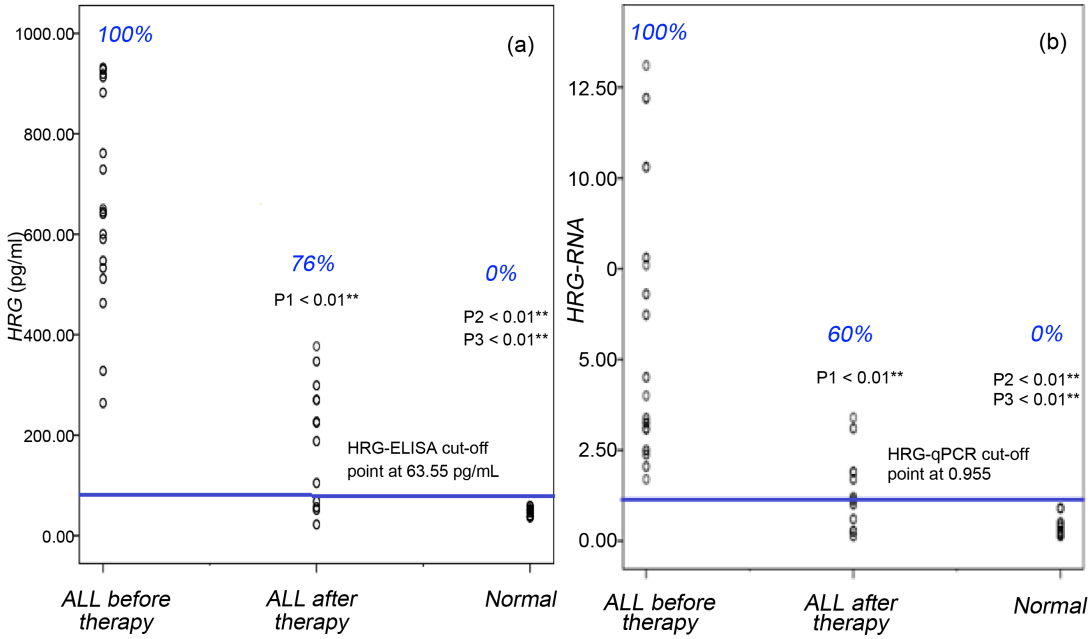


Figure 3. Positivity rate of patients of serum HRG-serum protein (a) and HRG-RNA (b) above the cutoff value in the different studied groups. (p1 = after versus before therapy, p2 = normal group versus ALL before therapy and p3 = normal group versus ALL after therapy **P < 0.01 is highly significant).

**Table 3.** Relation between mean rank of HRG serum level and RNA expression with clinicopathological factors in the ALL patients.

| *Clinicopathological factors* | *HRG serum level* | *HRG-RNA Expression* |
|---|---|---|
| Type of ALL;<br>Pre-B-ALL (23)<br>B-ALL (5)<br>T-ALL (7) | 19.24<br>18.5<br>15.2<br>$\chi 2$: 1.06<br>p: 0.5 | 17.74<br>20.5<br>17.55<br>$\chi 2$: 0.2<br>p: 0.8 |
| Chromosomal abnormalities;<br>Normal (23)<br>+ve Philadelphia chromosome (12) | 13.83<br>26.0<br>$\chi 2$: 11.1<br>*p: 0.001 | 16.28<br>21.29<br>$\chi 2$: 1.89<br>p: 0.16 |
| ALL patients<br>Complete remission (25)<br>Dead patients (10) | 13.48<br>29.30<br>$\chi 2$: 17.0<br>*p: 0.001 | 13.16<br>30.1<br>$\chi 2$: 19.5<br>*p: 0.001 |

P* Significant difference between types by applying non parametric Mann-Whitney U (to compare Chromosomal abnormalities) and Kruskal-Wallis tests (to compare Types of ALL).

**Table 4.** Pearson correlation between HRG-ELISA and HRG-qPCR and other parameters.

| Parameters | HRG-serum protein | HRG-RNA |
|---|---|---|
| Age | 0.08 | −0.19 |
| TLC | 0.489* | −0.08 |
| Platelet count | −0.56** | 0.02 |
| HB% | −0.38* | 0.34 |
| LDH | 0.6** | 0.47** |
| Peripheral Plast Cells | 0.87** | 0.42* |
| BM Plast Cells | 0.76** | 0.16 |
| HRG-RNA | 0.75** | ……. |

*P value < 0.05 is significant, **P value < 0.01 is highly significant.

we tried to detect HRG by two strategic approaches; ELISA and qPCR to investigate their values in ALL patients. We found that HRG-serum protein and HRG-RNA were significantly higher in ALL patients before therapy compared to control group. A significant reduction in HRG was observed in patients after therapy compared to at diagnosis, however, this level was still significantly higher than the normal group for only HRG-serum protein. According to ROC curve which discriminated ALL patients from healthy persons, none of the healthy control group expressed HRG detected by both methods above that calculated cutoff value giving up 100% specificity. Meanwhile, all ALL patients before thereby showed 100% sensitivity while after therapy, they were 76.0% positive for HRG-serum protein and 60.0% for HRG-RNA. Similar to our results, Matboli *et al*. [17] revealed that HRG tissue RNA and serum protein could be considered as promising novel markers for prediction of breast cancer prognosis with 71.7% sensitivity and 93.3% specificity for HRG tissue RNA and 86.7% sensitivity and 80% specificity for HRG serum protein. Moreover, Klenotic *et al*. [18] demon-

strated that HRG expression was increased by glioma cells in both subcutaneous and orthotopic brain tumor models resulted in an increase in tumor size and angiogenesis, possibly through interfering with the antiangiogenic activity of vasculostatin. On the other hand, HRG has been found to be reduced in the serum of alpha fetoprotein-negative hepatitis B virus-related hepatocellular carcinoma [19] and down regulated in endometrial carcinoma [20]. Moreover, Wu *et al*. [21] reported that fucosylated HRG levels were significantly higher in patients with stage III ovarian cancer compared to normal and benign donors but was not significantly higher in patients with stage I/II disease. HRG's antitumor activity has been ascribed due to its immune modulator functions and its effects on tumor vessels. The contradiction of the previous reports about pro- and antiangiogenic properties of HRG might be owing to its multi-domain structure and the activities of its proteolytically-released fragments, notably the histidine-proline rich region [22] and different experimental systems used [23]. Rolny *et al*. [11] assumed that HRG affects other cell types, such as macrophages, known to regulate angiogenesis and HRG might regulate tumor angiogenesis indirectly through tumor-associated macrophages.

HRG level was still high in follow-up ALL patients despite their complete remission (blast cells were around 5% and the rest of cells were normal), that had a statistically significant difference between HRG-serum protein levels after therapy and normal subjects. We previously explained it probably by persistence of residual tumor cells leading to relapse if no further additional consolidation chemotherapy was administered. This may highlight the molecular biology-based methods with a greater prognostic significance than conventional criteria for the detection of remission [24] [25]. Nagafuji *et al*. [26] reported that chemotherapy should be continued as follow up therapy in ALL even with good prognosis in order to prevent leukemia relapse. However, patients with less favorable prognosis should be treated more aggressively. Although allogeneic hematopoietic stem cell transplantation (HSCT) for patients with ALL in complete remision is much more intensive than multi-agent combined chemotherapy, it is associated with increased morbidity and mortality when compared with such chemotherapy. Minimal residual disease (MRD) status has been proven to be a strong prognostic factor for adult patients with Ph-negative ALL.

We also found a significant correlation between HRG-serum protein and its expression levels. Regarding their importance as a prognostic marker, significant positive correlations were excitsed between HRG-RNA and LDH, and peripheral blast cells. While HRG-serum protein showed more prognostic capacity through its additionally significant positive correlations with BM blast cells and significant negative correlations with platelets and HB%. Meanwhile, the HRG protein level was significantly higher in AAL patients with +ve Philadelphia chromosome. However, both HRG protein and RNA were significantly high in ALL patients who died during the course of treatment compared to those had complete remission. Contrary to our results, Roberts *et al*. [27] found that HRG was weak-associated with improved overall survival in a Phase III trial that compared

patients with pancreatic cancer. However, they explained this weak positivity of HRG as a prognostic marker due to its anti-angiogenic properties as the patients in trial phase were receiving anti-VEGF monoclonal antibody. Moreover, Zhang *et al*. [28] suggested the negative regulatory role HRG on hepatocellular carcinoma cell line through regulating cell proliferation via the Erk1/2 signaling pathway. Mantovani and Sica [29] revealed the antitumor activity of HRG as it is not only increased tumor infiltration by antigen-presenting DCs, cytolytic NK cells, and cytotoxic T-lymphocytes but also enhances their antigen presentation and tumor cell lysis potential, immune changes known to inhibit tumor growth. The possible, increasing levels of HRG in malignant cases might be due to the recognition of "malignant danger", in line with its presumed role as a "pattern recognition molecule" [16]. We could not find any literature evaluating serum HRG protein or its expression levels in ALL.

## 5. Conclusion

All these findings indicated that HRG might be a novel diagnostic biomarker in ALL patients, with high sensitivity and specificity. The more significant positive correlations of HRG-serum protein over HRG-RNA and its easy method of application, motivate its application in large clinical studies as a potential prognostic marker.

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## Conflict of Interest

The authors declare that they have no competing interests.

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## Abbreviations

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| ALL | Acute lymphoblastic leukemia |
| AUC | Area under ROC curve |
| BM | Bone marrow |
| ELISA | Enzyme-linked immunosorbent assay |
| HRG | Histidine-rich glycoprotein |
| LD | Lactate dehydrogenase |
| NPV | Negative predictive value |
| PPV | Positive predictive value |
| qPCR | Real-time polymerase chain reaction |
| ROC curve | Receiver operating characteristic curve. |
| TLC | Total leukocyte count |