Expression of Heparanase Gene in Egyptian Acute Leukemia Patients
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ABSTRACT
Background: Heparanase is an endoglycosidase that degrades heparin sulfate, the main polysaccharide constituent of the extracellular matrix and basement membrane. Expression of the heparanase gene is associated with the invasive, angiogenic, and metastatic potential of diverse malignant tumors and cell lines.

Aim of the study: to investigate possible relation/correlation between Heparanase gene expression and quantitation in pediatric Acute leukemia patients and clinicopathologic variables as well as patients outcome in an attempt to determine it's prognostic value and the possibility of using it as a new target for treatment.

Patients and methods: Forty pediatric acute leukemia patients (20 acute myeloid leukemia (AML)&20 acute lymphoblastic leukemia(ALL) as well as 11 normal volunteers were analyzed for the expression and level of Heparanase gene using real time quantitative reverse transcriptase polymerase chain reaction (RTQ-PCR) to investigate a possible relation, association, or correlation with the clinical and laboratory features of patients at diagnosis, and patient outcome after treatment and follow up.

Results: Comparing the 3 groups as regards the Heparanase gene level there was high statistical significant difference (p<0.001) being maximum in AML and minimum in controls, with mean Relative quantitation (RQ) level 2336.2± 10405.2 in AML., median 8.0 and range (3.1-46543.0) , while mean RQ in ALL was 1.7±1.0 ,median 1.7 and range (0.1-3.1) and in controls mean was 0.8±0.3, median 0.8 and range (0.4-1.4).Comparison between each 2 groups as regards heparanase level was of high statistically significant difference, p value being (p<0.001) when comparing AML/ALL and AML/controls and (p=0.035) when comparing ALL/controls. Cut off value for heparanase gene was calculated using Roc curve and was found to be 1.413 with 80% sensitivity and 100% specificity. According to this cut off level, 20/20 (100%) AML cases were heparanase positive, 12/20(60%) (ALL) cases were heparanase positive and 8/20 ALL patients were negative, while all controls (100%) were negative. This was of high statistical significance (p<0.001). Comparing the overall survival (OS) of AML/ALL there was no statistically significant difference (p=0.2916), while comparing the disease free survival (DFS) of AML/ALL was of statistical significant difference (0.0312). Comparing the final status of the disease (complete remission(CR)/ progressive disease(PD) or death) as regards the heparanase gene level RQ , showed a high statistical significant difference (p<0.005) with the level being higher in patients with PD/death. There was no significant correlation between all group and heparanase gene level as regards age, TLC, hemoglobin, platelets and peripheral blood blasts (p=0.353,0.704,0.844,0.54 and 0.09) respectively, while there was significant negative correlation on comparing bone marrow blast% and heparanase gene level (r=-0.408 and p=0.09).

Conclusion: Heparanse gene is expressed in acute leukemia being higher in AML than ALL and controls. Patients with higher heparanase gene showed poorer outcome. These findings suggest that heparanase gene may be a novel significant therapeutic target for acute leukemia.

Key words: Heparanase gene, Acute leukemia, RTQ-PCR

INTRODUCTION
Acute leukemia is a heterogenous disease with numerous genetic abnormalities that define each subtype. Many of the known chromosomal translocations and mutations in leukemia target genes and pathways disrupt hematopoietic transcription factors and/or confer a proliferative and survival advantage to leukemia blasts (1).
Heparan sulfate proteoglycans (HSPGs) are ubiquitous macromolecules associated with the cell surface and extracellular matrix (ECM) of a wide range of tissues (2). The basic HSPG structure consists of a protein core to which several linear heparin sulfate (HS) chains are covalently O-linked. HS binds to and assembles extracellular matrix (ECM) proteins thus playing important roles in ECM integrity, barrier function, and cell-ECM interactions. The HS chains ensure that a wide variety of bioactive molecules bind to the cell surface and ECM and thereby function in the control of diverse normal and pathological processes (3). HSPGs not only provide a storage depot for heparin-binding molecules such as growth factors, chemokines and enzymes, but rather can decisively regulate their accessibility, function and mode of action (4). Recent discoveries indicated that HSPGs localized within the tumor microenvironment can be attacked by enzymes that alter proteoglycan structure resulting in dramatic effects on tumor growth and metastasis (5, 6).

Heparanase, an endoglycosidase, can specially cleave HS side chains from HSPGs and release a multitude of bioactive molecules. Then, the generated HS fragments and released bioactive mediators could facilitate tumor metastasis cooperatively. In addition, heparanase also exhibits non enzymatic activities, including cell adhesion and survival, upregulation of vascular endothelial growth factor (VEGF) and tissue factor, induction of signal transduction, and enhancement of certain HSPG from the tumor cell surface (7,8). A large body of evidence suggest that the expression of heparanase in the tumor closely relates with the potential for tumor invasion, angiogenesis and metastasis in most tumors examined (9,10).

The purpose of this study was to investigate possible relation/correlation between Heparanase gene expression and quantitation in pediatric Acute leukemia patients and clinicopathologic variables as well as patients outcome in an attempt to determine it’s prognostic value and the possibility of using it as a new target for treatment.

MATERIAL AND METHODS
Patients and samples from patients:
Peripheral blood (PB) samples and bone marrow (BM) aspiration samples from 40 Egyptian acute leukemia pediatric patients, were collected at diagnosis and centrifuged to obtain PB and BM mononuclear cells (MNCS).

Patients were recruited from 3 places, from the National Cancer Institute, the Cairo University Hospital and Beni Suef university hospital over the period from June 2012 and March 2013 after their agreement.

The diagnosis of Acute leukemia was made based on the morphologic findings from Giemsa stained smears of bone marrow (BM) aspirates, cytochemical stains criteria such as negativity for myeloperoxidase (MPO) and sudan black B (SBB) in cases of ALL or their positivity in cases of AML and positivity of acid phosphatase for (T-ALL) and immunophenotyping criteria as CD10, CD19, CD20, CD22 for B-ALL, CD2, CD3, CD5, CD7 for T-ALL, and positivity of CD13 and CD33 for AML cases.

These patients diagnoses were distributed as follows: 20 acute myelogenous leukemia (AML) including the following subtypes of the French-American-British (FAB) classification ( 4 AML-M1; 10 AML-M2; 3 AML-M3; 1 AML-M5, 2 AML-M7), 20 acute lymphoblastic leukemia (ALL) from B, T lineages or common ALL ( 14 B-lineage, 3 T-lineage and 3 common ALL). Eleven age & sex matched individuals were included as controls.

Heparanase gene was analyzed using real time quantitative reverse transcriptase polymerase chain reaction (RTQ-PCR) to investigate a possible relation, association, or correlation with the clinical and laboratory features of patients at diagnosis, such as: sex, age, lineage (B or T), hemoglobin (HB), TLC, platelets count and BM blast cell infiltration; and patient outcome after treatment and follow up.
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RNA isolation and real-time quantitative RT-PCR

Total RNA was extracted from bone marrow and peripheral blood mononuclear cells (MNCS) using a QIAamp RNA blood kit (Qiagen, Germany) according to the manufacturer’s instructions. Total RNA (1 µg) was used for a first strand complementary DNA (cDNA) synthesis using reverse transcription system (Promega, Madison, WI, USA) as described by the manufacturer and stored at -20°C till use. SYBR Green (Solis BioDyne, HOT FIREPOL EvaGreen) real-time PCRs for amplification of heparanase and the housekeeping gene GAPDH were performed using ABI PRISM 7000 Sequence Detection Software (Applied Biosystems). The sequence of primers used for detection of the Heparanase gene cDNA were

5′-GGTTCTAATGCTCAGTTGCTCCT-3′

and

5′-ACTCGACCCCCATTGTGCTCCT-3′

and for GAPDH were

5′-GCACCGTCAAGGCTGAGAAC-3′

and

5′-TGTTGAAGACGCCAGTGGA-3′.

All reactions were performed in triplicate using 20 µl samples containing 50 ng cDNA. The reaction protocol used involved heating for 10 minutes at 95°C, followed by 40 cycles of amplification (15 seconds at 94°C and 1 minute at 60°C).

The expression levels of Heparanase gene in tested samples were expressed in the form of CT (cycle threshold) level then normalized copy number (Relative quantitation) was calculated using the ΔCT equation. A negative control without template was included in each experiment.

Expression level of Heparanase gene was correlated with the clinical features of the studied patients at diagnosis including: age, sex, TLC, hemoglobin, platelets, lineage, etc.

Statistical Methods:

Data was analyzed using IBM SPSS advanced statistics version 20 (SPSS Inc., Chicago, IL). Numerical data of scores were expressed as mean and standard deviation or median and range as appropriate. Qualitative data were expressed as frequency and percentage. Chi-square test (Fisher’s exact test) was used to examine the relation between qualitative variables. For not normally distributed quantitative data, comparison between two groups was done using Mann-Whitney test (non-parametric t-test). Comparison between 3 groups was done using Kruskal-Wallis test (non-parametric ANOVA) then post-Hoc "Schefe test" on rank of variables was used for pair-wise comparison. Spearman-rho method was used to test correlation between numerical variables. Survival analysis was done using Kaplan-Meier method and comparison between two survival curves was done using log-rank test. Odds ratio (OR) with 95% confidence interval (CI) were used for risk estimation. The Receiver Operating Characteristic (ROC) curve was used for prediction of cut off values. Kappa test was used to evaluate agreement between two diagnostic methods. A p-value < 0.05 was considered significant.

RESULTS

The present study was conducted on 40 denovo pediatric acute leukemia patients (20 AML and 20 ALL), clinical characteristics of which are presented in table (1). Eleven normal age and sex matched normal volunteers were studied as a control group.

They were 23 male patients (10 AML & 13 ALL) and 17 female patients (10 AML & 7 ALL), while controls were 6 males and 5 females.

Concerning AML Patients:

They were 20 AML patients; 10 males (50%) and 10 females (50%), their ages ranged from (0.8-17 years) with mean 8.5 ± 5.0 years and median 7.5 years. The cases were: 4 cases M1(20%), 10 M2 (50%), 3 M3(15%), 1 M5(5%), 2 M7(10%). The mean total leucocytic count (TLC) at diagnosis was 36.1 ± 34.1x10³ with range (1.2-140x10³) and median 29x10³, mean hemoglobin was 7.1 gm/dl with range (3.3-13.4 gm/dl) and median 6.9 gm/dl, mean platelet count was 25.7x10³ with range (2-70x10³) and median 20x10³, mean peripheral blood blasts was 48.3% with range (10-84%) and median 50.5%, the mean bone marrow blasts was 52.8.8% with range (15-95%) and median 55%. 20/20 ALL patients (100%) had no
CSF involvement. 4/20 (20%) of AML patients had no organomegaly while 4/20(20%) had splenomegaly, 3/20(15%) had hepatomegaly, 9/20(45%) had hepatosplenomegaly and 6/20(30%) patients had no enlarged lymph nodes while 14/20(70%) had enlarged lymph nodes. After induction chemotherapy 18/20(90%) of AML patients entered in complete remission (CR) while 2/20(10%) showed no CR.

Concerning ALL Patients:
They were 20 ALL patients; 13 males (65%) and 7 females (35%), their ages ranged from (0.7 - 15 years) with mean 7.2 years and median 7.0 years. The cases were: 13 cases pre B (65%), 1 pro B (5%), 3 C ALL (15%), 3 TALL (15%). The mean total leucocytic count (TLC) at diagnosis was 82.1 x10³ with range (1.7-452x10³) and median 31.5x10³, mean hemoglobin was 8.2 gm/dl with range (4.3-14 gm/dl) and median 7.7 gm/dl, mean platelet count was 51.1 x10³ with range (7-152x10³) and median 39x10³, mean peripheral blood blasts was 36.6% with range (10-97%) and median 24.5%, the mean bone marrow blasts was 74.3±27.7% with range (8-98%) and median 87%. 17/20 ALL patients (85%) had no CSF involvement while 3/20(15%) had CSF involvement. 3/20 (15%) of ALL patients had no organomegaly, while 5/20(25%) had splenomegaly, 1/20(5%) had hepatomegaly, 10/20(50%) had hepatosplenomegaly and 1/20(5%) had mesenteric or splenic hilar lymph nodes. 3/20(15%) ALL patients had no enlarged lymph nodes while 17/20(85%) had enlarged lymph nodes. After induction chemotherapy 19/20(95%) of ALL patients entered in complete remission (CR) while 1/20(5%) showed no CR.

Comparative studies:
Comparing AML and ALL patients as regards their clinical and laboratory data showed no statistical significance for TLC, hemoglobin and peripheral blood blasts p value being(0.659,0.114 and 0.46) respectively, but was of statistically significant difference for platelets (p=0.046) and bone marrow blasts (p=0.006)

Comparing the 3 groups as regards the Heparanase gene level there was high statistical significant difference (p<0.001) being maximum in AML and minimum in controls, with mean Relative quantitation (RQ) level 2336.2 in AML ,median 8.0 and range (3.1-46543.0) , while mean RQ in ALL was 1.7 ,median 1.7 and range (0.1-3.1) and in controls mean was 0.8, median 0.8 and range (0.4-1.4).

Comparison between each 2 groups as regards heparanase level was of high statistically significant difference, p value being (p<0.001) when comparing AML/ALL and AML/controls and (p=0.035) when comparing ALL/controls.

Cut off value for heparanase gene was calculated using Roc curve and was found to be 1.413 with 80% sensitivity and 100% specificity. According to this cut off level, 20/20 (100%) AML cases were heparanase positive, 12/20(60%) ALL cases were heparanase positive and 8/20 ALL patients were negative, while all controls (100%) were negative. This was of high statistical significance (p<0.001).

Follow up of the whole patients group showed that post induction chemotherapy 37/40(92.5%) entered CR while 3/40 (7.5%) showed no CR, the event of these 37 patients after follow up of showed that 34/37 continued to be in CR while 3/37 relapsed. As for the final state of the disease (FSD) 33/40 (82.5%) of patients were in CR and 7/40(17.5%) had either progressive disease (PD) or died and for the state of the patient (SD); 33/40(82.5%) were alive free (AF) , 3/40(7.5%) were alive diseased (AD) and 4/40 were dead. The overall survival time of the whole group mean was 20.48 month with median 21.12 and range (0-34.9 month), while the disease free survival (DFS) time mean was19.28 month, median 19.34 month and range (1.61-34.28) month. Survival analysis of patients till the end of the study (34.9 month) was 89.73% (no median survival because more than half of the patients were still alive till the end of the study).

Comparing the OS of AML/ALL there was non statistically significant difference (p=0.2916), while comparing the DFS of AML/ALL it was of statistical significant difference (0.0312).

Comparing the OS and DFS of the whole group as regards heparanase gene
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expression showed non statistically significant difference with ($p=0.2968 \& 0.2739$) respectively, this may be because only 4 patients had different event (death). Also analyses of OS and DFS in each group and its relation to heparanase gene showed non statistical difference ($p=0.4142 \text{ and } 0$) respectively.

Comparing the final status of the disease (CR/PD or death) as regards the heparanase gene level (RQ), showed high statistical significant difference ($p<0.005$) with the level being higher in patients with PD/death. There were 7/40 (17.5%) patients in PD with heparanase gene level (RQ) mean 6662.40653±7436.4754, range (1.46-46543.02) and median 17.19 and 33/40(82.5%) patients in CR with heparanase gene level (RQ) mean 3.67663± 2.8543, range (9.381189-0.096973) and median 1.08.

**Correlation studies:**

There was no significant correlation between all group and heparanase gene level as regards age, TLC, hemoglobin, platelets and peripheral blood blasts ($p=0.353,0.704,0.844,0.54 \text{ and } 0.097$) respectively, while there was significant negative correlation on comparing bone marrow blast% and heparanase gene level ($r=-0.408 \text{ and } p=0.09$).

Correlation between heparanase +ve and heparanase –ve cases within AML and ALL groups as regards clinical and laboratory data including age, sex , TLC, hemoglobin, platelets, bone marrow and peripheral blood blasts, CSF affection, organomegaly and lymphadenopathy; and as regards the response to therapy (CR and no CR) there was non statistically significant difference.

![Figure (1): Mean of Age (Years) in Control (N=11), AML Patients (N=20) and ALL Patients (N=20).](image)

![Figure (2): Mean of TLC (X 1000 U/L) in Control (N=11), AML Patients (N=20) and ALL Patients (N=20).](image)
Figure (3): Mean of Haemoglobin (gm/dl) in Control (N=11), AML Patients (N=20) and ALL Patients (N=20).

Figure (4): Mean of Platelets (X 1000 U/L) in Control (N=11), AML Patients (N=20) and ALL Patients (N=20).

Figure (5): Mean of BM Blasts (%) in Control (N=11), AML Patients (N=20) and ALL Patients (N=20).

Figure (6): Mean of RBCs (X 10^6U/L) in Control (N=11), AML Patients (N=20) and ALL Patients (N=20).
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Figure (7): Median heparanase level (RQ) in Control, ALL and AML groups

Figure (8): Heparanase level in AML, ALL and control groups

Table (1): Relationship between patient outcome and heparanase gene expression

<table>
<thead>
<tr>
<th>Group</th>
<th>Overall survival (event) n=40</th>
<th>Disease free survival (event) n=37</th>
<th>p-value</th>
</tr>
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<tr>
<td>Acute leukemia</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Heparanase -ve</td>
<td>8(0)</td>
<td>8(0)</td>
<td>0.2968</td>
</tr>
<tr>
<td>Heparanase +ve</td>
<td>32(4)</td>
<td>29(4)</td>
<td>0.2739</td>
</tr>
<tr>
<td>AML</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Heparanase -ve</td>
<td>0(0)</td>
<td>0(0)</td>
<td>--------</td>
</tr>
<tr>
<td>Heparanase +ve</td>
<td>20(3)</td>
<td>18(4)</td>
<td>--------</td>
</tr>
<tr>
<td>ALL</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Heparanase -ve</td>
<td>8(0)</td>
<td>8(0)</td>
<td>0.4142</td>
</tr>
<tr>
<td>Heparanase +ve</td>
<td>12(1)</td>
<td>11(0)</td>
<td>0</td>
</tr>
</tbody>
</table>
Figure (9): Disease free survival and heparanase in acute myeloid leukemia group

Figure (10): Quantitative Real time PCR curve for heparanase gene (Target gene) and GAPDH (house keeping gene) in cases and controls

DISCUSSION

Acute Lymphoblastic Leukemia (ALL) is a heterogenous group of lymphoid malignancies that result from clonal proliferation and expansion of immature lymphoid cells in the bone marrow, blood and other organs\(^{(11)}\). In most patients, the cause of ALL is unknown. A higher incidence of ALL has been noted among monozygotic and dizygotic twins of ALL patients, reflecting possible genetic predisposition. Moreover, patients with trisomy 21, Klinefelter’s syndrome, Fanconi anemia, Bloom syndrome and ataxia telangiectasia have a higher risk of developing ALL\(^{(11)}\).

Recent data have indicated that in pediatric leukemia, most chromosome translocations and preleukemic clones arise during fetal hematopoiesis with secondary genetic events\(^{(1)}\).

Acute Myeloid Leukemia (AML) is infrequent, yet it is a highly malignant neoplasm responsible for a large number of cancer-related deaths. Known risk factors include age, antecedent hematological disease, previous chemotherapy and genetic disorders\(^{(12)}\).

In any event, leukemogenesis of ALL and AML is a multistep process that requires the susceptibility of hematopoietic progenitor cells to inductive agents at multiple stages\(^{(13,14)}\). Although the study of enzymes that cleave heparan sulfate is in its infancy, recent finding imply that heparanases play a critical role in promoting tumor growth and metastasis.

In humans, heparanase-1 (heparanase) appears to be the dominant, if not the only heparanase that can cleave extracellular heparan sulfate\(^{(15,16)}\). Although other heparanases are likely present in many cells, their activity appears to be localized to intracellular compartments\(^{(17,18)}\). Heparanase expression is rare in normal tissues, but becomes evident in many human tumors where it significantly
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increases both the angiogenic and metastatic potential of tumor cells (19). Elevated heparanase expression in humans has been correlated with advanced progression and metastasis of tumors of the breast (20), colon (21), ovary (22), bladder (23), pancreas (24) and acute myeloid leukemia (24).

The mechanism of heparanase function in tumors is under intense investigations and it appears that this enzyme may have multiple effects. For example, degradation of heparan sulfate likely facilitates tumor cell motility and budding of new blood vessels, an event that requires remodeling of the heparan sulfate-rich basement membrane.

Heparan sulfate proteoglycans within the tumor microenvironment can serve as a reservoir of heparin-binding growth factors and chemokines, and the activity of heparanase may act to release these factors for use by the tumor cells. Moreover it is important to note that active heparanase does not completely digest the heparan sulfate chains it attacks; rather it selectively cleaves the glycoside bonds of heparan sulfate chains at only a few specific sites, producing fragments that are only 10 to 20 sugar residues long (25,26). These fragments generated by heparanase or by enzymes that cleave heparan sulfate in a manner similar to that of heparanase have been shown to enhance the ability of heparan sulfate to potentiate the activity of bound growth factors (27,28). Thus, degradation of heparan sulfate surrounding the tumor may remove physical barriers and enhance signaling events that spark metastasis.

The present study was conducted on 40 denovo pediatric acute leukemia patients (20 AML and 20 ALL) together with eleven normal age and sex matched volunteers as a control group.

Heparanase gene was analyzed using real time quantitative reverse transcriptase polymerase chain reaction (RTQ-PCR) to investigate a possible relation, association, or correlation with the clinical and laboratory features of patients at diagnosis, such as; sex, age, lineage (B or T), hemoglobin (HB), TLC, platelets count and BM blast cell infiltration; and patient outcome after treatment and follow up. They were 23 male patients (10 AML &13 ALL) and 17 female patients (10 AML &7 ALL), while controls were 6 males and 5 females.

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Results repetition

Ostrovoyk et al. (29), investigated heparanase gene polymorphisms in patients acute myeloid leukemia (AML), myelodysplastic syndrome(MDS), acute lymphoblastic leukemia (ALL), chronic myeloid leukemia (CML), Hodgkin’s disease (HD) and multiple myeloma (MM).They found Significant correlation between rs11099592 and rs6535455 heparanase gene (HPSE) single nucleotide polymorphisms (SNPs) and ALL . Genotype frequency comparisons revealed a significant association with rs4693602 in MM patients and rs4364254 in AML patients. They examined heparanase gene mRNA expression by real-time RT-PCR and indicated a significant low HPSE gene expression level in ALL patients and a high expression level in MM and AML patients, compared to healthy controls. Moreover, statistically significant correlation was found between heparanase mRNA expression level and three HPSE gene SNPs (rs4693608, rs11099592 and rs4364254) among healthy individuals. They concluded that these data suggest that certain HPSE gene SNPs may contribute to basal heparanase gene expression and that alterations in this gene are an important determinant in the pathogenesis of ALL, AML and MM. This was in accordance to our work concerning AML, although we detected low levels of heparanase mRNA in ALL patients as well as normal control samples

Bitan et al. (30) evaluated heparanase expression in leukocytes isolated from peripheral blood of 71 patients with myeloid and lymphoid leukemias, or non-Hodgkin’s lymphoma. Analysis was performed at two levels: heparanase RNA was determined by reverse transcriptase polymerase chain reaction, and heparanase protein was evaluated by immunocytochemistry and flowcytometry. They found that in eight peripheral blood samples from normal donors, heparanase RNA was detected, and protein was found
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within the cytoplasm of granulocytes. In mononuclear cells derived from various leukemias, heparanase RNA was expressed in 14 of 15 acute myeloid leukemia (AML) samples. In contrast, cells derived from all 33 chronic lymphoblastic leukemia, all 7 non-Hodgkin’s lymphoma, 7 of 8 chronic myeloid leukemia, and 6 of 8 acute lymphoblastic leukemia patients showed no detectable expression of the heparanase RNA. They also revealed that heparanase protein was detected primarily within the cytoplasm of AML cells, indicating that the enzyme is produced and stored within the cytoplasm of myeloid cells, with limited expression on the cell surface. This was in agreement with our results concerning AML but we detected low levels of heparanase mRNA in ALL as well as in normal controls.

Thomas et al. (31) analyzed the activity, expression, and function of heparanase in myeloma patients. They analyzed heparanase activity in the plasma isolated from bone marrow biopsies of 100 patients and revealed 86 positive for heparanase activity and 14 negative. They further divided the bone marrow samples into three categories of heparanase activity, high activity (42 patients), low activity (44 patients), and negative (14 patients). In contrast to the bone marrow plasma, levels of heparanase activity in peripheral blood plasma of 29 myeloma patients were found to be either negative or low, they suggested that in multiple myeloma, heparanase functions in the local microenvironment of the bone marrow and its activity is not significantly elevated systemically. Immunohistochemistry was performed and revealed that patients with high levels of heparanase activity often have tumor cells with intense staining for the enzyme. Interestingly, they noted a marked heterogeneity among tumor cells, with clusters of heavily stained cells surrounded by cells with weak or negative staining for heparanase. They analyzed microvessel density and revealed a strikingly higher concentration of vessels in patients with high heparanase activity (78.96 vessels/mm²) as compared with patients negative for heparanase activity (25.03 vessels/mm²). When they implanted human myeloma cells transfected with the cDNA for heparanase in severe combined immunodeficient (SCID) mice, the resulting tumors exhibited a significantly higher microvessel density than did tumors established with control cells. Thus they concluded that expression of heparanase appears to play a direct role in enhancing microvessel density in myeloma cells.

Yan et al. (32) used a severe combined immunodeficient (SCID) mouse model, and demonstrated that enhanced expression of heparanase by myeloma cells dramatically up-regulated their spontaneous metastasis to bone. They noted that this occurs from primary tumors growing subcutaneously and also from primary tumors established in bone. Interestingly, they observed that tumors formed by subcutaneous injection of cells metastasize.

Not only to bone, but also to other sites including spleen, liver, and lung. In contrast, tumors formed by injection of cells directly into bone exhibit a restricted pattern of metastasis that includes dissemination of tumor to other bones but not to extramedullary sites. In addition, they stated that expression of heparanase by myeloma cells accelerated the initial growth of the primary tumor, increased whole-body tumor burden as compared with controls, and enhanced both the number and size of microvessels within the primary tumor.

Michael et al. (33) identified four putative estrogen response elements in the heparanase promoter region and found that transcription of a luciferase reporter gene driven by the heparanase promoter was significantly increased in estrogen-receptor positive MCF-7 human breast carcinoma cells after estrogen treatment. Estrogen-induced heparanase mRNA transcription in estrogen receptor-positive, but not in estrogen receptor-negative, breast cancer cells, confirmed the promoter study data. The estrogen effects on heparanase mRNA expression levels were abolished in the presence of the pure antiestrogen ICI
182,780, indicating that the classic estrogen receptor pathway is involved in transcriptional activation of heparanase. In vivo, exposure to estrogen augmented levels of heparanase protein in MCF-7 cells embedded in Matrigel plugs and correlated with increased plug vascularization. Collectively, our data suggest a new molecular pathway through which estrogen, independent of its proliferative effect, may induce heparanase overexpression and, thus, promote tumor-stromal interactions, critical for breast carcinoma development and progression.

Xu et al. \(^{(34)}\) assessed the role of HPA-1 by suppressing its expression using small interfering RNA (siRNA). They transfected the human heparanase-1 specific siRNA to the human gastric carcinoma cell line SGC7901 and assessed the effect of gene silencing by Real-Time PCR and Western Blot. They then evaluated the invasion potential of the cells using an in vitro cell invasion model system. They found that HPA-1 specific siRNA significantly suppressed expression of heparanase-1 in SGC7901 cells and that Invasion after RNAi treatment through membranes in a model system was significantly decreased. They concluded that HPA-1 facilitates gastric carcinoma invasion by up-regulating endoglycosidase activity in tumor cells.

Xiaotong et al. \(^{(35)}\) used a strategy of stable transfection with antisense to derive ovarian carcinoma cell lines that express different levels of heparanase and used these to demonstrate that invasion correlates with heparanase activity. They found that secreted heparanase activity was increased by reduction, hypoxia, and growth of cells under reduced oxygen (1%) augmented heparanase activity and invasion, both of which are inhibited by treatment with antiheparanase antibodies.

Liduan et al. \(^{(36)}\) designed, synthesized, and transfected three heparanase-specific small interfering RNA (siRNAs) into cultured gastric cancer cell line SGC-7901. They measured Heparanase expression by RT-PCR, real-time quantitative PCR and Western blot. Cell proliferation was detected by MTT colorimetry and colony formation assay. The in vitro invasion and metastasis of cancer cells were measured by cell adhesion assay, scratch assay and matrigel invasion assay. The angiogenesis capabilities of cancer cells were measured by tube formation of endothelial cells. They found that transfection of siRNA against 1496-1514 bp of encoding regions resulted in reduced expression of heparanase, which started at 24 hrs and lasted for 120 hrs post-transfection. The siRNA-mediated silencing of heparanase suppressed the cellular proliferation of SGC-7901 cells. In addition, the in vitro invasion and metastasis of cancer cells were attenuated after knock-down of heparanase. Moreover, transfection of heparanase-specific siRNA attenuated the in vitro angiogenesis of cancer cells in a dose-dependent manner.

Evgen et al. \(^{(37)}\) designed plasmid vectors to express hammerhead ribozymes or small interfering RNAs (siRNAs) directed against the human or mouse heparanase mRNAs. Human breast carcinoma (MDA-MB-435) and mouse lymphoma (Eb) and melanoma (B16-BL6) tumor cell lines, which have naturally high levels of endogenous heparanase or have been genetically engineered to over express heparanase, were transfected with anti-heparanase ribozyme or siRNA. Semiquantitative reverse transcription-polymerase chain reaction (RT-PCR) and measurements of enzymatic activity were used to confirm the efficient silencing of heparanase gene expression. Cells transfected with the anti-heparanase ribozyme and siRNA vectors were tested for invasiveness in vitro and metastatic dissemination in animal models of experimental and spontaneous metastasis. They noted that compared with cells transfected with control constructs, cells transfected with the anti-heparanase ribozyme or siRNA vectors had profoundly reduced invasion and adhesion in vitro, regardless of cell type, and expressed less heparanase. In vivo, tumors produced by cells transfected with the antiheparanase ribozyme and siRNA
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vectors were less vascularized and less metastatic than tumors produced by cells transfected with the control vectors. Mice injected with cells transfected with the anti-heparanase ribozyme and siRNA vectors lived longer than mice injected with control cells.

In conclusion, the current study provides characterization of heparanase gene expression and quantitation in acute leukemia and its effect in the patients outcome after treatment. We recommend the use of heparanase gene as a future therapeutic target for treatment of acute leukemias. Further studies are recommended to strengthen this hypothesis.

REFERENCES