



Neutrophil Extracellular Traps: A New Tool that Reflects the Progression of Tumor Status in Acute Lymphoblastic Leukemia Patients

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ABSTRACT

Acute lymphoblastic leukemia (ALL) is a malignant proliferation of immature lymphocytes known as lymphoblast that causes them to build up in several organs, including the bone marrow and blood. Cancer cells use Neutrophil Extracellular Traps (NETs) as a key component of their immune evasion strategies. The goal of the study is to estimate NETs levels in pediatric patients with ALL, as it is a promising immunological marker for tumor diagnosis and disease progression. A total of 80 children aged 1 to 14 years diagnosed with acute lymphoblastic leukemia were selected, alongside 40 healthy children served as the control group. The samples from ALL patients were split into three groups based on the duration of the disease. Serum levels of NETs were ascertained using the ELISA technique. The results showed a significant increase in the levels of NETs among ALL patients (Mean \pm SD 1125.400 \pm 739.165 ng/ml) compared to the control group (Mean \pm SD 622.363 \pm 127.983 ng/ml) with $P \leq 0.05$.

Additionally, no significant differences in NETs were observed among the three disease groups, as the p-value associated with Duncan test was 0.33. However, both the Less than 1 year and more than 2 years showed a significant increase in NETs levels compared with the control group, according to Duncan's test. The findings suggest that NETs were able to reverse the progression of the tumor during the treatment stages.

Keywords: Acute Lymphoblastic Leukemia, Biomarker, Cancer, Children, NETs.

INTRODUCTION

Acute lymphoblastic leukemias (ALL) are characterized by abnormal differentiation and growth of lymphoblast which suppress normal hematopoietic lineages and negatively affect immunity and blood cell generation (Hansen *et al.*, 2019). ALL can be either B-cells or T-cells precursor, and their rapid proliferation leads to their presence in the blood, marrow, and other organs (Saghir, 2023).

Innate immunity is the body's first line of protection against external intruders, serving as a vital guide for adaptive immunity in the event of recurrent infections. One of the first groups of responders sent to areas of inflammation are neutrophils, which are essential parts of the innate immune system.

Early on in an infection, local macrophages and activated endothelium cells draw circulating neutrophils and guide them to swiftly move from the bloodstream to affected tissues, where they bind to, phagocytose, and eliminate microorganisms (Borregaard, 2010).

Neutrophils also release neutrophil extracellular traps (NETs), a unique immune response, to efficiently collect and remove invading pathogens (Li *et al.*, 2020). This process involves the release of neutrophil DNA, which acts as a scaffold to attract and organize antimicrobial proteins, creating a mesh-like structure that is adept at stomping out infections. However, this complex mesh-like structure exhibits dual roles: it both harms the immune system and has defensive properties against infection (Vorobjeva and Chernyak, 2020).

NETs can have two different roles in the development of cancer, depending on the particular tumor microenvironment. By triggering cell death and preventing cancer cells from proliferating and spreading, neural endothelial cells have an anti-tumor effect in colorectal cancer. On the other hand, NETs have a pro-carcinogenic role in myeloproliferative neoplasms, breast cancer, and lung cancer by encouraging tumor proliferation and metastasis, sometimes exacerbating infection. NETs have an impact on the genesis and progression of disease through a variety of methods. On the one hand, releasing DNA fibers and related proteins creates a particular microenvironment that supports the growth and survival of tumor cells. However, the tissue damage and inflammation brought on by NETs act as catalysts for the growth, invasion, and metastasis of tumors. Moreover, it has been shown that direct contacts between NETs and cancer cells affect the adhesive qualities of the latter, promoting the spread of metastatic cells to distant organs (Yang *et al.*, 2020; Demkow, 2021; Yang *et al.*, 2020).

The study aimed to investigate the possibility of using NET as a promising new diagnostic tool to monitor tumor development during the treatment stages of acute lymphoblastic leukemia patients.

MATERIALS AND METHODS

Study area:

From March 2024 to August 2024, samples were collected from ALL patients at the Al-Hadbaa Specialized Hospital in Mosul/ Iraq. under the guidance of a hematology consultant. Before the clinical trial began, consent was obtained from the Iraqi Ministry of Health as well as the ethical and scientific board of Mosul University in Mosul, Iraq.

Study subjects:

80 samples of blood were drawn from pediatric acute lymphoblastic leukemia (ALL) patients and 40 healthy children's blood samples were obtained, aged between one and fourteen years. The control group consisted of twenty healthy boys and twenty girls, while the experimental group consisted of fifty male and twenty female ALL patients. Age, gender, period of disease, kind of therapy, and medical history in the family were among the general details of patient information entered into a unique form. Based on the duration of illness, three groups of ALL patient samples were created. Less than one year, one to two years, and more than two years were distinguished as groups 1 to 3.

Serum collection:

Each participant had 3 mL of venous blood drawn. The blood samples were placed in a gel tube and then centrifuged at 3,000 rpm for 15 minutes. After the serum was separated, assembled into Eppendorf tubes, and then stored at -20°C.

Determination of NETs concentration in serum by Sandwich ELISA

The principle of the test is based on the binding of the NETs present in the serum samples with its antibodies previously fixed on the surface of the polystyrene plate. Then, the biotin-labeled antibody is added, which in turn binds to the Ag-Ab complex. The HRP-Streptavidin Conjugate is added, as it depends on the strong binding between avidin and biotin to enhance the sensitivity and specificity of the test. After that, the substrate solution is added to show a color change whose intensity is proportional to the concentration of NETs. When the acid stop solution is added, the reaction was stopped, and the optical absorbance at 450 nm was measured.

Procedure

The measurement kit from the Korean company BT LAB Bioassay Technology Laboratory was used. Add 40µl of the standard solution to the first twelve wells. Then Add 40 µl of the sample was added to each sample well, followed by 10 µl of anti-NETs biotin-labeled antibody and 50 µl of streptavidin-HRP. The plate was thoroughly mixed, and the mixture was incubated at 37C° for 60 minutes. After incubation, five washes with the wash solution were performed on the plate and each well was soaked with 0.35 ml of the wash solution for 30 seconds for each washing stage. Add 50 µl of substrate solutions A and B and mix and incubate for 10 minutes at 37 C° in the dark.

After adding 50 µl of stop solution to each well, the optical density was determined by using a Rt-2100c microplate reader at a wavelength of 450nm within 10 min after adding the stop solution.

RESULTS AND DISCUSSION

The results revealed a significant increase in the concentration of neutrophil extracellular traps (NETs) in patients with acute ALL lymphoblastic leukemia compared to healthy controls. The mean \pm SD NETs concentration in ALL patients was 1125.400 ± 739.165 ng/mL compared to the control group, it was 622.363 ± 127.983 ng/mL at a significance level of 0.000 with a value of $P \leq 0.05$, as show in (Table 1).

Table 1: A comparison of NETs concentration between acute lymphoblastic leukemia patient and the control group

parameter	Groups	Number	Mean \pm SD	Extreme value	T	P-value
NETs ng/L	Patients	80	1125.400 ± 739.165	150 - 3361.52	2.614	0.000*
	Control	40	622.363 ± 127.983	150 - 886.34		

*P value <0.05

NETs concentration showed that there were no significant differences among the three patient groups, as the p-value accompanying the Duncan test amounted to 0.338 which is greater than 0.05.

However, Duncan's test indicated that the less than one year and more than two-year groups had significantly higher NETs levels compared to the control group, while the one - two group did not show a significant difference as in (Table 2) and Fig. (1).

Table 2: Duncan tests for pairs of comparisons and tests the significance level of the variable NETs

Variable	Groups	Number	Mean \pm SD.	Duncan test	
				1	2
NETs ng/ml	Controls	40	622.363 ± 127.983	622.363	
	1-2 years	25	1003.228 ± 659.529	1003.228	1003.228
	Less than 1 year	30	1148.608 ± 778.603		1148.608
	More than 2 years	25	1228.914 ± 787.992		1228.914
P-value				0.087	0.338

* P-value <0.05

Abbreviations: NETs: Neutrophil Extracellular Traps, SD: Standard deviation, T: t-

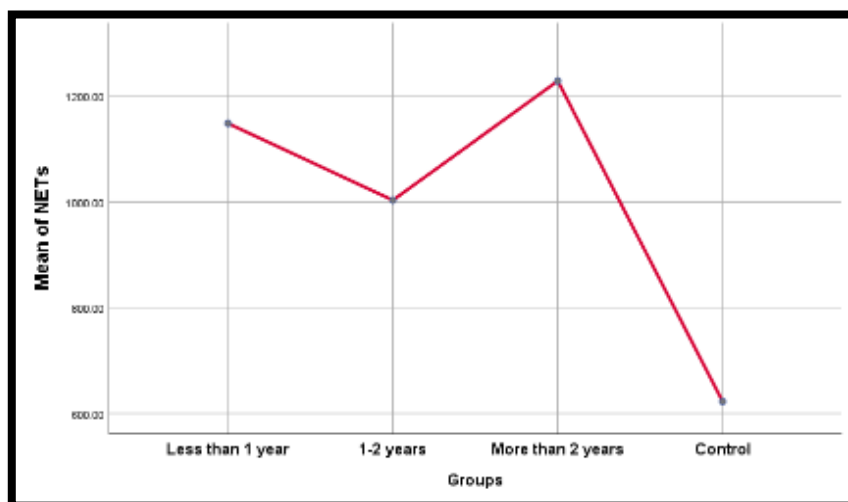


Fig. 1: shows the concentration of NETs in the ALL groups compared to the control.

Malignant progenitor cells proliferate excessively in acute leukemias, the most prevalent malignancies in children. Leukemia patients are vulnerable to infectious complications due to reduced blood cell formation, which is a leading cause of non-relapse mortality (Hansen *et al.*, 2019).

Inflammation is a hallmark of cancer and neutrophils exist as tumor-infiltrating inflammatory immune cells (Zhao and Jin, 2022). Neutrophils play a controversial role in tumor induction, promoting the proliferation, metastasis and invasion of cancer cells, and the antitumor effects of inhibiting proliferation and invasion through neutrophil extracellular traps (NETs). NETs have emerged as promising biomarkers for cancer diagnosis and prognosis and have been associated to various cancers, including lung, colorectal, ovarian, liver, breast, and stomach cancers (Li *et al.*, 2024; Hameed and Hamed, 2023; Hamed, 2022).

Several studies have indicated that NETs promote tumor progression and proliferation by inhibiting apoptosis and thus maintaining tumor progression and metastasis (Liu and Liu, 2019). The presence of a DNA receptor in NETs on cell membranes, which is essential for facilitating tumor metastasis as this receptor recognizes NET DNA outside the cell and activates pathways that mediate tumor metastasis (Yang *et al.*, 2020).

NETs components also play a role in stimulating cancer cells. The enzyme neutrophil elastase (NE), which is one of the enzymes that make up NETs, releases pro-tumor factors such as growth factor A, platelet-derived growth factor, and vascular endothelial growth factor. Endothelial growth factor, facilitates interaction with their receptors and tumor progression (Deryugina *et al.*, 2020).

Matrix metalloproteinase 9 (MMP-9) is a part of NET that stimulates tumorigenesis by impairing apoptosis, increasing angiogenesis, invasion, and metastasis (Al-Helaly and Younes, 2024; Mustafa *et al.*, 2022; Alaseem *et al.*, 2019). NET-associated Cathepsin G also contributes to stimulating tumor cell invasion (Guan *et al.*, 2021).

NETs play an important role in acute lymphoblastic leukemia (ALL) where their impact is multifaceted influencing immune modulation, treatment-related complications, and potential affecting prognosis (Huang *et al.*, 2022). NETs play a role in the immune defects associated with leukemia. In leukemias with ALL, NET formation is significantly impaired, contributing to the secondary immunodeficiency observed in patients (Ostafin *et al.*, 2021).

NETs play a significant role in promoting tumor growth and metastasis by creating a pro-inflammatory microenvironment. They can also damage endothelial cells, leading to increased vascular permeability and potentially contributing to leukemic cell dissemination (Mandrizzato *et al.*, 2022; Wang *et al.*, 2021). Additionally, NETs interact with various components of the tumor microenvironment, potentially facilitating cancer progression and metastasis by creating a suppressive environment for immune cells (Ma *et al.*, 2024; Zuo *et al.*, 2023).

NETs can impair T cell and NK cell activation, which may limit the effectiveness of immunotherapies, indicating a need for strategies that target NETs to enhance overall treatment efficacy (Ma *et al.*, 2024).

The high concentration of extracellular melanocytes observed in ALL as shown in (Table 1) may be attributed to the exploitation of NETs by cancer cells for immunological expression and invasion and providing a favorable environment for cell dissemination and disease progression.

Leukemic cells can induce neutrophils to release neutrophil extracellular traps upon activation, and this process is associated with immune evasion and metastasis of cancer cells (Tin *et al.*, 2024).

The (Table 2) and Fig. (1) show that the concentration of NETs increases with continuous chemotherapy. This elevation can be attributed to several reasons. First, chemotherapy increases the formation of neutrophil extracellular traps, which promote cancer cell death by inducing neutrophil death. For instance, studies involving colorectal cancer (Mousset and Albregues, 2024; Li *et al.*, 2024). Second, animal studies show that artificial neurons contribute to the inhibition of tumor growth, as their presence is associated with longer progression-free survival in patients undergoing specific chemotherapy regimens (Li *et al.*, 2023). Third, the formation of abnormal traps can also be affected by leukemia treatments. For example, in acute promyelocytic leukemia (APL), treatments can increase the formation of abnormal traps, leading to endothelial cell damage and complications such as bleeding. A similar mechanism may be relevant to acute lymphoblastic leukemia, where treatment-induced traps could contribute to adverse effects (Mandrizzato and Läubli, 2022). While studies on neuroendocrine involvement in acute lymphoblastic leukemia are limited, research in leukemia-associated conditions suggests that neuroendocrine cells might have diagnostic value, which could guide treatment strategies and improve patient management (Wang *et al.*, 2024).

CONCLUSIONS

The current study concluded that neutrophil extracellular traps (NETs) play a dual and controversial role in cancer biology. On one hand, NETs act as tumor promoters by enhancing the proliferation, invasion, and metastasis of cancer cells. On the other hand, they may exert anti-tumor effects by limiting these same processes. The current study highlights that elevated levels of NETs constitute a mechanism through which cancer cells, particularly in lymphocytic leukemia, evade immune surveillance. Therefore, NETs can be regarded as significant contemporary biomarkers for both the diagnosis and prognosis of tumor development and progression.

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مصادد العدلات الخارج خلوية: أداة جديدة تعكس تطور حالة الورم لدى مرضى ابيضاض الدم اللمفاوي الحاد

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الملخص

ابيضاض الدم اللمفاوي الحاد ALL هو تكاثر خبيث للخلايا اللمفاوية غير الناضجة المعروفة باسم الارومات اللمفاوية، مما يؤدي إلى تراكمها في العديد من الأعضاء، بما في ذلك نخاع العظم والدم. تستخدم الخلايا السرطانية مصادد العدلات خارج الخلية كعنصر أساسي في استراتيجياتها المناعية للتهرب. تهدف هذه الدراسة إلى تقدير مستوى مصادد العدلات الخارج خلوية لدى مرضى سرطان الدم اللمفاوي الحاد، حيث تُعد مؤشراً مناعياً واعداداً لتشخيص الورم وتطوره. تم اختيار ثمانين طفلاً مصاباً بسرطان الدم اللمفاوي الحاد، ومقارنتهم بأربعين طفلاً سليماً كمجموعة ضابطة. تراوحت أعمار المرضى والضوابط بين 1 و14 عاماً وقُسمت عينات مرضى سرطان الدم اللمفاوي الحاد إلى ثلاث مجموعات وفقاً لمدة المرض ومجموعة العلاج. تم تحديد مستويات مصادد العدلات الخارج خلوية في المصل باستخدام طريقة الاختبار المناعي ELISA المرتبط بالإنزيم. أظهرت النتائج زيادة كبيرة في مستويات مصادد العدلات الخارج خلوية لدى مرضى ابيضاض الدم اللمفاوي الحاد (متوسط \pm الانحراف المعياري 739.165 ± 1125.400 نانوغرام/مل) مقارنةً بمجموعة السيطرة (متوسط \pm الانحراف المعياري 127.983 ± 622.363 نانوغرام/مل) عند قيمة $p \leq 0.05$ كما أظهرت النتائج عدم وجود فروق كبيرة في تركيز مصادد العدلات الخارج خلوية بين مجموعات المرضى الثلاثة، حيث كانت قيمة p المرتبطة باختبار دنكن (0.338)، في حين أظهرت كل من مجموعة المرضى الذين مدة مرضهم أقل من سنة وأكثر من سنتين زيادة كبيرة في تركيز مصادد العدلات الخارج خلوية مقارنةً بالمجموعة الضابطة، وفقاً لاختبار دنكن. تمكنت مصادد العدلات الخارج خلوية من عكس تطور الورم خلال مراحل العلاج.

الكلمات الدالة: ابيضاض الدم اللمفاوي الحاد، العلامة الحيوية، السرطان، الأطفال، مصادد العدلات الخارج خلوية.