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Research Article

A new strategy to evaluate emerging tumour-associated antigens as biomarkers of acute lymphocytic leukaemia development

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Abstract
Acute lymphoblastic leukaemia (ALL) is the most common type of tumour in children aged from 1-14 years .Currently, diagnosis is made by bone marrow aspiration. For this reason, the present study aimed to find a new diagnostic method for acute lymphoblastic leukaemia based on the presence of specific biomarkers such as (α–enolase, P53, VDCI , and CIg) their ratios may reflect the tumor development status of patients. Eighty children with ALL were selected and compared with forty healthy children as control. The ages of ALL and normal cases ranged from 1 to 14 years. The serum levels of α–enolase, P53, VDAC1 and CIg were determined using ELISA method. The results showed a significant decrease in the levels of α–enolase, P53 and CIg in ALL patients (Mean ±SD 388.9062 ± 115.18294 pg/ml , 76.9207 ± 10.23092 pg/ml and 6.2747 ± 3.49786 µg/ml respectively) compared to the control cases (Mean ±SD 596.7733 ± 217.34848 pg/ml , 118.0352 ± 44.49135 pg/ml , 8.6873 ± 4.04248 µg/ml) respectively with $p<0.05$, while VDAC1 showed not significant decreased. The study concluded that changes in the selected tumour antigens reflected the physiological changes in the tumour lymphocytes, which can be adopted as new indicators to follow the tumour development and response to chemotherapy.

Keywords: Acute lymphocytic leukaemia, Alpha-enolase, Cancer, CIg, P53, VDACI

INTRODUCTION

Acute lymphoblastic leukaemia (ALL) is the most frequent kind of neoplasia (children 1–14 years old), with a peak incidence between 1 and 4 years old, and it accounts for approximately 25% of cancer diagnoses (Malard and Mohty, 2020). B-cells comprise 85% of the lineage, while T-cells comprise 15% in paediatric ALL patients. Currently, diagnoses are made via bone marrow aspiration. Still, most kids show significant psychological strain when it comes to this test because of the significant harm this method can cause (Rheingold *et al*., 2019).

A cancer biomarker is a biological molecule that indicates the existence of cancer, characterises cancer, and may even be the source of cancer (Sarhadi and Armengol, 2022). It may also show the body's reaction to the presence of cancer. A diagnostic biomarker is utilised to identify or validate a kind of cancer. Prognostic biomarkers are used to determine the chance of a patient's health result, both with and without therapy, and the risk of the disease progressing or reoccurring (Das *et al*., 2023). Proteins that are altered or mutated during tumour growth and creation can activate the immune system and cause the generation of antibodies directed against the tumour. Tumour diagnosis biomarkers may include autoantibodies and antigens seen in the serum of cancer patients (Monroy-Iglesias *et al*., 2022).

Alpha-enolase (α-enolase), also known as Enolase-1 (ENO1), is a glycolytic enzyme that acts as a plasminogen receptor on the surface of cells, particularly in malignancies and is one of the most significant antigens on the surface of cells, where it aids in the invasion, migration, metastasis, and proliferation of cancer cells. In addition, ENO1 stimulates other carcinogenic processes, such as the signalling of pathway activation, resistance to chemotherapy, and protein-protein interactions that control glycolysis (Cappello *et al*., 2018). Previous investigations have suggested that α-enolase could be used as a biomarker for early diagnosis or detection because it was found to be overexpressed in

many malignancies (Ji *et al.,* 2019; Qiao *et al*., 2019; Chen *et al*., 2020). According to previous studies (Zhang *et al*., 2020), α-enolase is a tumour-associated antigen that can cause the development of autoantibodies in malignant tumours. Acute leukaemia has also been linked to antibodies against α-enolase (Cui *et al*., 2005). ENO1 is a potential protein that can be used as a cancer biomarker and therapeutic target due to its overexpression at the cell level (Imaguel and Sanchez, 2021).

Tumour protein P53, or P53, is a protein that is made using instructions provided by the TP53 gene. This protein controls cell division by acting as a tumour suppressor, which prevents cells from proliferating – growing and dividing – too quickly or uncontrollably (Valentin *et al.,* 2018; Tchounwou and Kumar, 2022). The P53 protein is found in the nuclei of all cells in the body and directly binds to DNA there. This protein is essential in deciding whether a damaged cell will self-destruct (go through apoptosis) or have its DNA repaired when radiation, poisonous chemicals, or ultraviolet rays from sunshine harm it. Other genes are activated by P53 to repair damaged DNA if it is possible to do so. This protein also prevents the cell from replicating and tells it to enter programmed cell death if the DNA damage cannot be repaired. In this way, the tumour is suppressed by P53, which prevents cells with damaged or altered DNA from multiplying (Farooq *et al*., 2022).

The voltage-dependent anion channel 1 (VDAC1) is a mitochondrial protein located in the outer membranes of mitochondria and in the plasma membrane that regulates energy generation, cell development, and homeostasis. Moreover, VDAC1 mediates the regulation of apoptosis, the interaction between antiapoptotic proteins, and the release of apoptotic proteins from mitochondria (Fang, 2018; Karachitos *et al*., 2017). According to the finding that VDAC1 is overexpressed in several tumour types, it might be necessary to survive cancer cells. Reduction of VDAC1 expression in malignancies has been shown to dramatically slow tumour growth, indicating that VDAC1 could be an innovative therapeutic target (Arif *et al*., 2019). Targeted medications also inhibit the growth and spread of tumours by acting on VDAC1, a promising cancer therapy approach (Shoshan-Barmatz *et al*., 2020).

Cytoplasmic immunoglobulin (CIg) plays an important role in the differentiation and classification of ALL. Research has demonstrated that CIg, such as IgD, might affect leukaemic cells' behaviour in ALL patients, causing them to proliferate excessively and block apoptosis (Wu *et al*., 2023). Furthermore, the identification of cytoplasmic granules in blastoid cells has been highlighted as a useful morphological marker for distinguishing myeloid leukaemia from lymphoblastic leukaemia, aiding in accurate differential diagnosis of acute leukaemia (Sheikhpour *et al*., 2017).

The present study aimed to find a new diagnostic method to track ALL development based on specific non-invasive tumour-inducing biomarkers (α-enolase, P53, VDACI, and CIg), whose ratios may reflect the tumour's progression. This study is the first of its kind to utilise a selection of different globally known tumour antigens, their combination, and their adoption as indicators of the status of tumour lymphocytes in ALL patients.

MATERIALS AND METHODS

Study area

Samples were taken from patients with ALL under the supervision of a haematology specialist from the Al-Hadbaa Specialized Hospital in Mosul, Iraq, from March 2024 to July 2024.

Study subjects

Eighty blood samples were collected from paediatric patients who were diagnosed with ALL, and forty blood samples were collected from healthy children between one and fourteen years old. The experimental group included 50 male and 30 female paediatric ALL patients, and the control group included 20 male and 20 female healthy children. The patients' information was recorded in a special form, including age, gender, duration of illness, type of treatment, and family medical history. The ALL patients' samples were divided into three groups depending on the duration of the disease. Group 1 was less than one year, group 2 was one to two years, and group 3 was more than two years of age.

Serum collection

Three ml of venous blood taken from each child and was placed in a gel tube, left for 5 minutes at 37°C for coagulation, and then centrifuged for 5 minutes at 3000 rpm. The serum that had been isolated was put into Eppendorf tubes and stored at −20°C for examination.

Measuring the concentration of α-enolase, P53, VDAC1, and CIg by sandwich ELISA

The test depended on the interaction of the antigen present in the serum with the antibodies fixed on a polystyrene plate. This connection was detected using enzymes coupled with the antibodies that bind to the basic substance. The enzyme was measured from the approved colorimetric memory after adding the basic substance, which was read at a wavelength of 450 nm. The wavelength's intensity corresponded to the antigen's concentration in the sample.

Procedure

In small tubes, five dilutions of the standard were made, then 50 µl was drawn from each tube and trans-

ferred to an ELISA microplate, making 2 replicates for each dilution and placing them in the first 10 holes. Then, 40 µl of the dilution solution and 10 µl of the samples were added. After being incubated for 30 minutes at 37°C and washed 5 times, 50 µl of HRP conjugate reagent was added to each; then they were incubated and washed again, the same as in the previous step. To each well, 50 µL of the chromogen A solution and 50 µL of chromogen B solution were added and incubated at 37°C for 15 minutes. Finally, 50 µL of the stop solution was added to each well to terminate the reaction. The absorbance OD was measured at 450 nm using a microtiter plate reader. The kits used for measurement were supplied by the Chinese company Sunlong Biotech (Tabatabaei and Ahmed ,2022).

Ethical approval

Before beginning the clinical study, the ethical and scientific board of Mosul University in Mosul, Iraq, granted their consent according to the letter (476 on 21/1/2024) and the Iraqi Ministry of Health provided further approvals as per the letter dated (6428 on 12/2/2024). The study followed the ethical guidelines outlined in the Declarations of Helsinki. Before participating, each participant completed a consent form. All participants were told that their information would be kept confidential and would be only available to the researcher himself. Blood tests were free of charge.

Statistical analysis

A Duncan test with a probability of $P \leq 0.05$ was used to compare the means statistically. The SPSS 24.0 program and the SPSS 24 statistical analysis tool were used to perform the statistical analyses.

RESULTS

The study included the most important cancer biomarkers, which had a bearing on the illness. As displayed in Table 1, the results showed a significant decrease in the concentration of α-enolase, P53, and CIg in ALL patients (Mean ± SD 388.90 ± 115.18 pg/ml, 76.92 ± 10.23 pg/ml, and 6.27 ± 3.49 µg/ml, respectively) compared to the control group (Mean ± SD 596.77 ± 217.34 pg/ml, 118.03 ± 44.49 pg/ml, and 8.68 ± 4.04 µg/ml at 0.000*, 0.000*, and 0.014*, respectively) with $P \leq 0.05$, while the VDAC1 concentration did not show a significant relationship compared to the control.

The results also recorded that there were no statistically significant differences in α-enolase between each of the patient groups, as the *p*-value associated with the Duncan test was equal to 0.475, which is greater than 0.05, and the mean ± SD for the patient groups was equal to373.78 ± 86.28pg/ml, 407.87 ± 97.22 pg/ml,

Table 1. Comparison of alpha-enolase, P53, VDAC1, and CIg concentrations in acute lymphoblastic leukaemia patients and control group

	Groups	Number	Mean ± SD	Extreme value	p-value
α -enolase pg/ml	Patients	80	388.90 ± 115.18	967.60–300	0.000*
	Control	40	596.77 ± 217.34	988.51–300.58	
P53 pg/ml	Patients	80	76.92 ± 10.23	148.95–75	0.000*
	Control	40	118.03 ± 44.49	198.19–75	
VDAC1 pg/ml	Patients	80	163.62 ± 34.14	275.94–149	0.112
	Control	40	181.23 ± 61.61	323.42–149	
CIg µg/ml	Patients	80	6.27 ± 3.49	20.88–3.74	0.014*
	Control	40	8.68 ± 4.04	19.23–5.30	

*$P \leq 0.05$; SD:Standard deviation

Table 2. Duncan tests for pairs of comparisons and to test the significance level of the variable α-enolase

Variable	Groups	Number	Mean ± SD	Duncan test	
				Subset for alpha = 0.05	
				1	2
α-enolase pg/ml	Group 1 (less than 1 year)	30	373.78 ± 86.28	373.78	
	Group 2 (1 -2 years)	25	407.87 ± 97.22	407.87	
	Group 3 (More than 2 years)	25	390.36 ± 106.44	390.36	
	Control	40	596.77 ± 217.35		596.77
p-value				0.475	1.000

*$P \leq 0.05$; SD:Standard deviation

Table 3. Duncan test for pairs of comparisons and to test the significance level of the variable P53

Variable	Groups	N	Mean ± SD	Duncan test	
				Subset for alpha = 0.05	
				1	2
P53 pg/ml	Group 1 (less than 1 year)	30	76.73 ± 7.70	76.73	
	Group 2 (1 -2 years)	25	75.41 ± 1.78	75.41	
	Group 3 (More than 2 years)	25	78.70 ± 16.54	78.70	
	Control	40	118.04 ± 44.49		118.04
p-value				0.659	1.000

*$P \leq 0.05$; SD:Standard deviation

and 390.36 ± 106.44 pg/ml, respectively.

While the results showed a significant decrease between each of the three patient groups and the control group according to the Duncan test, this was because the control group did not share the same column with the remaining groups, as recorded in Table 2, and the mean ± SD value for the control group was equal to 596.77 ± 217.35 pg/ml.

P53 also showed no significant differences between the three pathological groups, as the *p*-value accompanying the Duncan test amounted to 0.659, which is greater than 0.05. The mean ± SD was equal to 76.73 ± 7.70 pg/ml for group 1, 75.41 ± 1.78 pg/ml for group 2, and 78.70 ± 16.54 pg/ml for group 3.

Each patient group also showed a significant decrease in concentration of P53 with the control group, in which the mean ± SD reached 118.04 ± 44.49 pg/ml according to Duncan's test, as recorded in Table 3.

As for VDAC1, no significant differences appeared between each of the three groups and the control group. The *p*-value accompanying the Duncan test amounted to 0.103, greater than 0.05. The mean ± SD was equal to 158.01 ± 24.10 pg/ml for group 1, 163.16 ± 30.60 pg/ml for group 2, 171.69 ± 46.95 pg/ml for group 3, and 181.23 ± 61.61 pg/ml for the control group, as shown in Table 4.

The CIg results showed no significant differences between group 1, group 2, and group 3. The mean ± SD were 6.39 ± 3.82 µg/ml, 6.82 ± 3.46 µg/ml, and 8.69 ± 4.04 µg/ml, respectively. The *p*-value accompanying the Duncan test amounted to 0.300, greater than 0.05. Group 3 showed a significant decrease compared to the control group as 5.573.12 ± µg/ml and 8.69 ± 4.04 µg/ml, respectively because they did not share the same column, as written in Table 5.

DISCUSSION

Acute lymphoblastic leukaemia (ALL) is the most prevalent malignancy in children (Ibrahim *et al.,*2023), resulting in the dysregulation of epigenetic modifiers, hematopoietic transcription factors, and cytokine receptors. In addition to specific somatic and genetic alterations, chromosomal abnormalities are the main characteristics

Table 4. Duncan test for pairs of comparisons and to test the significance level of the variable VDAC1

Variable	Groups	N	Mean ± SD	Duncan test
				Subset for alpha = 0.05
				1
VDAC1 pg/ml	Group 1 (less than 1 year)	30	158.01± 24.10	158.01
	Group 2 (1 -2 year)	25	163.16 ± 30.60	163.16
	Group 3 (More than 2 year)	25	171.69 ± 46.95	171.69
	Control	40	181.23 ± 61.61	181.23
p-value				0.103

*$P \leq 0.05$; SD:Standard deviation

Table 5. Duncan test for pairs of comparisons and tests the significance level of the variable CIg

Variable	Groups	Number	Mean ± SD	Duncan test	
				Subset for alpha = 0.05	
				1	2
CIg µg/ml	Group 1 (less than 1 year)	30	6.39 ± 3.82	6.39	6.39
	Group 2 (1 -2 year)	25	6.82 ± 3.46	6.83	6.83
	Group 3 (More than 2 year)	25	5.57± 3.12	5.57	
	Control	40	8.69 ± 4.04		8.69
p-value				0.300	0.056

*$p < 0.05$; SD:Standard deviation

that distinguish it. According to this viewpoint, the hunt for novel markers to serve as tools to track the advancement of the disease and identify the presence of tumours is crucial.

ENO1 is thought to be a developing cancer biomarker and therapeutic target because it has recently been shown to be a significant driver of tumour metabolism and progression (Cappello *et al*., 2018). It is an intriguing candidate due to a few important features, such as overexpression in cancerous cells relative to decreased expression in normal tissues, localisation to the cell surface where imaging and therapeutic applications may target it, and overexpression associated with diagnosis and clinical prognosis. Therefore, ENO1 may be a highly helpful biomarker to guide patient care, detect latent cancers, determine the course or recurrence of a disease, or evaluate the efficacy of therapy)Ji *et al*., 2019).

Anti-ENO1 autoantibody reactions are frequently linked to ENO1 overexpression (Zhang *et al*., 2020). Yu *et al.* (2022) noted that autoantigens linked to B-ALL may include VDAC1 and α-enolase. Consequently, autoantibodies against VDAC1 and α-enolase could be useful serological indicators for B-ALL in children. The presence of autoantibodies is one of the reasons for the decreased concentration of ENO1 antigen

Chemotherapy can also be considered a cause of a decreased concentration ENO1 in patients who are undergoing chemotherapy. The cornerstone of all treatments, L-asparaginase (ASNase), has been demonstrated to profoundly impact biochemical pathways, including those involving α-enolase as the presence of L-asparaginase can reduce the concentration of α-enolase (Maese and Rau, 2022; Ko *et al*., 2023).

This explains a decrease in the concentration of the ENO1in patient samples compared to the control. It might have caused the decline because it prompted the immune system to produce antibodies against it and neutralizing it. All patients were undergoing chemotherapy. Although there was no significant relationship between the three groups of patients, the concentration of the enzyme was lower in group 1 because they were in the early stages of the disease, in which the number of leukaemia cells in the peripheral blood was very small and the expression of antigens is at a minimum, leading to a decrease in the amount of cancer-related antigens in the peripheral blood. The enzyme's concentration began to rise somewhat in group 2; then it decreased in group 3 due to undergoing chemotherapy for long periods.

The tumour suppressor P53 is essential for maintaining genomic integrity and is involved in numerous basic functions. Cancer cells frequently use the inactivation of p53 activity to avoid apoptosis.Consequently, TP53 mutations are seen in many tumours such as breast and lung cancer, promoting oncogenesis by boosting cellular survival and proliferation (Hamed, 2022 ; Hameed and Hamed, 2023). Mutant p53 proteins are accumulated to extremely high concentrations within tumour cells; as a result, the accumulating p53-mut functions as an antigen, which prompts the immune system to recognise these antigens and produce antibodies that may ultimately eliminate p53-mut malignant cells (Blagih *et al*., 2020 ; Sobhani *et al*., 2021).

This explains the lower concentration of p53 in patient samples compared to the control. The decrease may be because it stimulated the immune system to form antibodies against it, which led to its neutralisation, and these antibodies were not present in the control samples. The decrease may be due to the accumulation of the p53 protein inside the cancer cell and its lack of secretion into the blood circulation. The samples used in the current study were serum samples, so the concentration of P53 was low.

In the early stages of cancer, VDAC1 may activate the immune system and is thought to be a cancer biomarker. Both VDAC1 and α-enolase can be used for diagnosis and immune monitoring in all patients. Chil-

dren with B-ALL also had antibodies against VDAC1 in their serum, suggesting that VDAC1 induced autoimmunity and raised VDAC1 antibodies (Yu *et al*., 2022 (. The result shows a non-significant decrease in the level of VDAC1 in group 1, whose level increased with the progression of the disease period due to chemotherapy.

In ALL, the level of CIg is a significant factor in understanding the disease. The results show no significant differences between group 1, group 2, and the control group. As for group 3, which includes patients who had ALL for more than two years, a significant decrease was observed compared to the control due to a long period of exposure to chemotherapy.

Prednisone, a chemotherapy drug used for ALL patients, has been shown to affect leukocyte cytochemical responses, which may be a sign of alterations in cytoplasmic elements such as immunoglobulins (Awang and Kamaludin, 2022; Erfanipour et al., 2023). This explains the lower concentration of CIg in patient samples compared to the control because all patients were undergoing chemotherapy

Conclusion

The antigens selected in this study reflected the changes that occur in the tumour lymphocyte through decreased levels of α-enolase, p53, and CIg in ALL patients compared to control cases, instead of through examining the BM to follow the body's response to chemotherapy in addition to monitoring whether the tumour develops.

Conflict of interest

The authors declare that they have no conflict of interest.

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