Biomedical & Pharmacology Journal, December 2021. Vol. **14**(4), p. 2255-2260

Quantitative Determination of Serum Level of TLR4, TLR7 and TLR9 in Pediatric Acute Lymphoblastic Leukemia (ALL) Patients in Basrah, Iraq

Rasha Adnan Mustafa[1], Hanadi Abdulqader Jasim[1]* and Sadeq Khalaf Ali Al-Salait[2]

[1]Department of Microbiology, College of Medicine, University of Basrah, Iraq.
[2]Consultant Hematologist, Al-Zahraa Medical College, University of Basrah, Iraq.
*Corresponding Author E-mail: Hanadi.jasim@uobasrah.edu.iq

https://dx.doi.org/10.13005/bpj/2325

(Received: 28 October 2021; accepted: 18 December 2021)

Acute Lymphoblastic Leukemia (ALL) is one of most frequent malignancy detected in children, accounting for three quarters of all leukemia occurrences in children. Toll-Like Receptors (TLRs) have been shown to be expressed or up regulated in tumors (solid) and tumor cell lines, but their expression level or role in the etiology and progression of acute lymphoblastic leukemia in children is not studied widely. This study intended to explore the association of serum level of TLR4, TLR7, and TLR9 in children with acute lymphoblastic leukemia. A case control study was conducted on patients (pediatric) with ALL who have been admitted to Basrah Children Specialty Hospital, Basrah, Iraq. Three ml of serum samples were collected for the measurement of TLRs concentration by using Sandwich Enzyme-Linked Immuno Sorbent Assay (ELISA). The mean level of TLR4, TLR7 and TLR9 in patients were higher than the control group. However, the difference was statistically significant for TLR4 and TLR7 (P-value less than 0.005) but not for TLR9. The mean value of TLRs are higher in the newly diagnosed group than the relapse. The higher serum concentration of TLR4, TLR7 and TLR9 in patients, whether new or in relapse, compared to control group might be part of the immune-evasion mechanism developed by the malignant cells that plays a serious role in leukomogenicity and disease advancement.

Keywords: Acute Lymphoblastic Leukemia; Pediatric Patients; TLR4, TLR7, TLR9.

Leukemia is a broad category of hematological malignancies that includes numerous biologically different subgroups. It is a clonal neoplasm of hematopoietic cells caused by a variety of causes that create somatic mutations in pluripotent stem and progenitor cells (Kassahun et al., 2020). It is the most prevalent childhood cancer in most populations, accounting for 25% to 35% of all childhood cancers incidents (Pui et al., 2004). One third of all malignancies diagnosed in children are leukemia (Siegel et al., 2012).

ALL is four to five times more common in children aged 2 to 3 years than in newborns and it is also four to five times more common in children aged 10 years than older age (Howlader et al.,2013). It is hypothesized that activating recognition receptors, such as TLRs, that activate the innate immune system, may be able to restore

the antineoplastic immune response (Rakoff-Nahoum et al., 2009).

TLRs family in humans consists of ten members (TLRs 1–10). It is critical in the immune response to microbial infections because it recognizes particular pathogenic molecular components. TLRs, when triggered, create a signaling cascade that stimulates innate and adaptive immune responses directed towards the invading pathogen (Andreakos et al., 2004). Human TLRs are mostly expressed on antigen-presenting cells, predominantly found in immune-related cells including monocytes, macrophages, neutrophils, T cells, B cells, natural killer (NK) cells and dendritic cells (DC) (Harsini et al., 2014). The activation of TLRs causes the release of a number of pro-inflammatory cytokines and chemokines, that can promote tumorigenesis by promoting cell proliferation and migration and creating a favorable microenvironment for tumor cells. Aside from hematological cancers, overexpression of a number of TLRs has been related to tumor cell survival, proliferation, and metastasis in a range of cancers including colon, breast and lung cancers (Monlish, Bhatt and Schuettpelz, 2016). TLRs have been shown to be expressed or up regulated in tumors and tumor cell lines, but their expression level and function in the etiology and progression of acute leukemia in children is not studied widely (Fabricius et al., 2011).

This study attempted to explore the association of serum level of TLR4, TLR7, and TLR9 in children with acute lymphoblastic leukemia in order to obtain insight into their potential role in pathogenesis and prognosis.

MATERIALS AND METHODS

Study groups

A case-control study was conducted on pediatric patients with Acute Lymphoblastic Leukemia who have been admitted to Basrah Children Specialty Hospital. Over a period from September 2020 through June 2021, 60 patients (42 newly diagnosed and 20 relapsed), aged 6 months to 16 years, were enrolled. All children admitted to Basra Children Specialty Hospital fulfilled the following inclusion criteria: children aged (0-16) years-old, newly diagnosed cases with acute lymphoblastic leukemia and relapsed cases of known cases of ALL, who were treated with chemotherapy and achieved complete remission for any duration of time, then presented with re-appearance of blast cells in the peripheral blood and/or bone marrow in excess of 20% of all nucleated cells. The exclusion criteria included: Patients receiving chemotherapy, Patients taking steroid, patients with a History of recent blood transfusion (less than 1 week) and Patients with renal impairment**.** All pediatric patients were submitted to a questionnaire form which included; age, gender, home address and family history. The results of complete blood picture, peripheral blood morphology (blast percentage) and bone marrow blast percentage were reported. After obtaining informed consent from children's parents, blood samples were collected in vacuum gel tube, centrifuged and sera were stored in multiple tubes at -20°C. Forty-two pediatric patients were newly diagnosed with acute lymphoblastic leukemia (ALL) prior to any drug or treatment, 20 relapsed ALL cases and 60 apparently healthy children were included. All procedures were confirmed by the Research Ethical Committee of College of Medicine, University of Basrah according to code:7/39/3669.

Enzyme-Linked Immuno-Sorbent Assay (ELISA)

Three ml of serum samples were used to measure TLRs concentration by using Sandwich Enzyme-Linked Immuno-Sorbent Assay (ELISA). The level of TLR4, TLR7 and TLR9 was performed by specific commercial kits supplied by Human®, Germany. The procedure was performed and reagents prepared according to the manufacturer's instructions, which were included with the Kits. The kit was held at room temperature at 25°C for 20 minutes before use. The washing buffer was prepared in one step, and the 30ìl concentrated washing buffer was diluted with 750ìl deionized water. The standard solution was prepared by using 20ng/ml of standard stock solution prepared by adding 1ml of the standard dilution buffer into the standard tube. The tube was then kept at room temperature for 10 minutes and then mixed well. Serial dilutions were prepared, and then biotin-labeled antibody working solution was prepared prior to the experiment within 1 hour. Then 120 ml of biotin-detection antibody was diluted with 10ml of antibody dilution buffer and was mixed

thoroughly. HRP-Streptavidin conjugate (SABC) working solution was prepared within 30 minutes before the experiment. Also 120 ml SABC with SABC-buffer concentrate was diluted within 10ml of SABC buffer and then was mixed thoroughly. The plate was washed with wash buffer five times. After that 90il of TMB was added to each well with the substrate reagent, each well was filled with 50il Stop Solution and then mixed thoroughly. ELISA reader (Biotech®) with 450nm filter was used to measure absorbance and configure the curve.

Statistical analysis

Statistical Package of Social Science (SPSS) version 26 was used to analyze the data. Chi-square (X2) test was adopted to compare between percentages. Numeric data was described by Mean ± SD. The P-value is considered significant if d" 0.05 and highly significant if d" 0.001.

RESULTS

During the period of sample collection, the study was carried out on 122 samples divided into three groups: Group1 included newly diagnosed patients (n=40), group 2 relapsed cases (n=20) and group 3 healthy controls (n=60). The majority of these samples were in age group 2-5 years and 6-12 years (40.3%, 38.7% respectively), while a lower frequency appeared in both age groups < 2 years and >12years (11.3%, 9.7% respectively) without any statistically significant differences (P value =0.835). The distribution of pediatric acute lymphoblastic leukemia in our patients group according to sex revealed slightly more males (58%) than females (42%) with no statistically significant differences (P value= 0.103). Age and sex distribution of study sample is shown in table-1.

Hematological parameters of study groups were evaluated (Table 2). The mean value of Hb in newly diagnosed cases was (7.5590), which is markedly less than the value of relapse and control group, P-value (<0.001). Similarly, both neutrophil and platelet were found to be less in new and relapse than the control group (P-value <0.001).

In Table-3 It is clearly shown that the mean levels of both TLR4 and TLR7 are significantly higher in patients than the control group. While there is a slight elevation in TLR9 concentration in the patients than in the control group. However, the differences are statistically highly significant in all three parameters (P-value <0.001).

The study also revealed that the mean values of TLRs are higher in the newly diagnosed group than the relapse group (Table-4). The mean level of TLR4 appeared high in newly diagnosed

Table 1. The demographic distribution of study population

Age group	Case Group No (%)	Control Group No (%)	P-Value
< 2	7(11.3%)	6(10.0%)	0.835
2-5	25(40.3%)	22(36.7%)	
6-12	24(38.7%)	23(38.3%)	
>12	6(9.7%)	9(15.0%)	
Total	62(100%)	60(100%)	
Gender	Patients No (%)	Control group No (%)	P-Value
Male	36(58%)	35(58.3%)	0.103
Female	26(42%)	25(41.7%)	
Total	62(100%)	60(100%)	

Table 2. Mean level of hematological parameters in study population

Hematological parameters	New cases Mean[3] (No.= 42)	SD[4]	Relapsed patients Mean (No.= 20)	SD	Control group Mean (No.= 60)	SD	P value
Hb	7.5590	±2.17875	10.0170	±1.24276	11.5225	±0.79377	0.000
Neutrophil	1.46643	±1.734810	1.11905	±1.210888	3.73433	±1.091703	0.000
Platelet	74.8000	±102.61459	83.2055	±86.62986	387.2833	±78.14417	0.000
PB Blasts [1]	0.51	±0.323	0.29	±0.133	0	0	0.039
BM Blasts [2]	0.88	±0.139	0.76	±0.229	0	0	0.014

[1] Peripheral Blood, [2] Bone Marrow, [3]Mean level, [4]Standard Deviation.

patients than in relapsed ones with statistically highly significant difference (P-value <0.05).

DISCUSSION

When injury or a pathogen invasion occurs, the innate immune system gives an accurate protective mechanism, allowing the adaptive immune system to activate an antigen-specific response (El-Zayat et al., 2019). Notably, the immune response is controlled at several levels and one of the most important regulation mechanisms is the release of extracellular domains of immune receptors, including TLRs (Zunt et al., 2009; Kacerovsky et al., 2012; Ten et al.,2014). Numerous investigations have connected altered TLR signaling to hematological dysfunction and malignancy, indicating a role for this signaling in the etiology of these diseases (Monlish, Bhatt and Schuettpelz., 2016). In regards to the stimulus for increased TLR expression and signaling in hematopoietic neoplasms, the significance of this signaling to disease pathogenesis is not well explained in many cases, and more research is necessary to confirm the effects of enhancing or inhibiting TLR signaling on disease outcomes (i.e., complexity of cytopenias and initiation and/or progression of Leukemia) (Monlish, Bhatt and Schuettpelz, 2016).

To the best of our knowledge, measurements of the serum level of TLR4, TLR7 and TLR9 has not been reported in pediatrics with ALL in our region, so these results highlight this association.

We found in our study that the mean serum TLR4 level is higher in patients as in table-3 (5.76250±3.672829) than in the control group (0.20743±0.153867) with a very significant difference (P-value < 0.001). In addition, the mean serum level of TLR4 in newly diagnosed patients (6.69398 ±3.823063) was significantly higher than in the relapse group (3.80640±2.413277) (P-value <0.05).

In many types of cancer, such as breast cancer and hepatocellular carcinoma (Gonza et al., 2010) the serum TLR4 level was found to be highly expressed in human breast cancer tissues. Important data from preclinical investigations and clinical trials (Elkammah et al., 2020) indicate that patients with Hepatocellular carcinoma associated with hepatitis C exhibited higher blood levels of sTLR4. Ten et al., (2014) also found that the level of sTLR4 is higher in patients with inflammatory disorders than in controls. According to Hausen et al (2014) and Yiu et al. (2020) it is suggested that the host's immune responses to viruses play an essential role in cancer formation, and that tumor

Table 3. Mean Serum level of TLR types in ALL pediatric patients and control group measured by ELISA

TLR types	ALL Patients			Control group			
	No	Mean [1]	SD[2]	No	Mean[1]	SD[2]	P-Value
TLR4	62	5.76250	±3.672829	60	0.20743	± 0.153867	0.000
TLR7	62	7.753087	±3.7712491	60	0.334817	±0.1681693	0.000
TLR9	62	0.480131	±0.2370414	60	0.310133	±0.1797086	0.000

[1]Mean level, [2]Standard Deviation

Table 4. Mean Serum level of TLR types in both new cases and relapse of ALL pediatric patients measured by ELISA

TLR types	New cases			Relapse cases			
	No	Mean[1]	SD [2]	No	Mean[1]	SD [2]	P-Value
TLR4	42	6.69398	± 3.823063	20	3.80640	±2.413277	0.003
TLR7	42	7.930333	±3.6756367	20	7.366370	±4.0342202	0.582
TLR9	42	0.499526	±0.2074848	20	0.439400	±0.2914103	0.355

[1]Mean level, [2]Standard Deviation

growth may be due in part to a failure in the innate immune response. Wang et al (2015) also examined the clinical importance of serum sTLR4 in non-small cell lung cancer and discovered a substantial increase in sTLR4 in patients when compared to healthy controls. These increased levels of serum TLR4 as endogenous negative regulators may have a diagnostic and a prognostic benefit by counteracting tumor immune evasion mediated by TLR signaling to tumor cells leading to generation of pro-inflammatory interleukins 6 and 12. These variants result in cancer cells being resistant to attacking natural killer cells and evading immune surveillance (Huang et al., 2008).

With reference to TLR7, there is a study on urinary bladder cancer (UBC) in which the level of TLR7 was found to be lower in patients than in the control group (Al- Humairri et al., 2019). As a result, it is proposed that the risk of UBC is related to the decreased serum level of TLR7, and that its down-regulation may serve as an essential biomarker for the development of UBC (Al- Humairri et al., 2019). While in the present study, the TLR7 level was found to be higher in patients (7.753087±3.7712491) than in the control group (0.334817±0.1681693) with a highly significant difference (P-value<0.001). On the other hand, there was a slight elevation of serum TLR7 in new cases as mentioned in table-4 (7.930333±3.6756367) than in the relapsed cases (7.366370±4.0342202) with no statistically significant difference (P-value 0.582). We supposed that TLR7 is produced by the malignant cells to enable them to evade the immune response.

Regarding TLR9, this study revealed that serum level was also higher in patients (0.480131±0.2370414) than the control group (0.310133±0.1797086) with statistically significant difference (P-value<0.05). Also, in new cases serum TLR9 is higher (0.499526±0.2074848) than the relapsed cases (0.439400±0.2914103) but without significant difference (P-value = 0.355).

Abdullah's and Abdulwahid (2020) found that TLR9 has an elevated level in breast cancer patients. This finding was consistent with another study which revealed increased circulating levels of TLR9 in breast cancer patients compared to healthy control groups (Ivesaro et al., 2008; Klinman et al., 2004).

CONCLUSION

The serum levels of TLR4, TLR7 and TLR9 were found to be higher in patients, both new and in relapse, compared to control group. The difference was less remarkable between newly diagnosed and relapsed cases apart from TLR4. This supports the putative role of these TLRs, particularly TLR4, in pediatric ALL leukemogenicity, blast cells proliferation and survival.

ACKNOWLEDGMENT

We wish to thank the staff members of Basrah Children Hospital, the Children's parents and Dr. Asaad Yehya, the Statistician (University of Basrah) for their kind help.

Limitation of the study

We were unable to collect a larger number of samples due to the limited time for the study. Intracellular and surface membrane expression of these TLRs has not attempted due to limited access to required material and equipment.

Ethical consideration

The approval of Basra Health Authority was obtained prior to conducting this study at Basrah Children Specialty Hospital. Written consents were taken from the parents, or at least one of them, for patients' participation in this study for the purpose of enrolling their children in this study.

Conflict of interest

There is no conflict of interest.

REFERENCES

1. Kassahun W, Tesfaye G , Bimerew L , Fufa D , Adissu W, Yemane T. Prevalence of Leukemia and Associated Factors among Patients with Abnormal Hematological Parameters in Jimma Medical Center, Southwest Ethiopia: A Cross-Sectional Study. Volume Article ID 2014152, 7 pages (2020).
2. Pui, CH, Schrappe M, Ribeiro RC, Niemeyer CM. Childhood and adolescent lymphoid and myeloid leukemia. *Hematology Am Soc Hematol Educ Program*. **118** (2004).
3. Siegel R, Naishadham D and Jemal A.Cancer statistics, *CA Cancer J Clin,* **62**:10-29(2012).

4. Paul S, Kantarjian H, Jabbour EJ. Adult Acute Lymphoblastic Leukemia. *Mayo Clinic Proceeding,* **91**:1645–1666 (2016).
5. Howlader N, Noone AM, Krapcho M, Garshell J, Neyman N & Altekruse SF. SEER cancer statistics review, Bethesda, MD: National Cancer Institute. 1975–2010. 2013. Volume19,Total citations Cited by 1183(2014).
6. Rakoff-Nahoum S and Medzhitov R. Toll-like receptors and cancer. *Nat Rev Cancer,* **9**: 57-63(2009).
7. Andreakos E, Foxwell B, Feldmann M. Is targeting Tolll ike receptors and their signaling pathway a useful therapeutic approach to modulating cytokine-driven inflammation? *Immunol Rev;* **202**: 250-265(2004).
8. Harsini S, Beigy M, Akhavan-Sabbagh M and Rezaei N.Toll-like receptors in lymphoid malignancies: Double-edged sword. *Crit Rev Oncol Hematol,* **89**: 262-283(2014).
9. Monlish D, Bhatt S, Schuettpelz L.(The Role of Toll-Like Receptors in Hematopoietic Malignancies. Immunol. Sep 28;7:390. doi: 10.3389/fimmu.00390. PMID: 27733853; PMCID: PMC5039188(2016).
10. Fabricius D, Breckerbohm L, Vollmer A, Queudeville M, Eckhoff SM, Fulda S, Strauss G, Debatin KM, Jahrsdörfer B and Meyer LH. Acute lymphoblastic leukemia cells treated with CpG oligodeoxynucleotides, IL-4 and CD40 ligand facilitate enhanced anti-leukemic CTL responses. *Leukemia*, **25**: 1111-1121(2011).
11. El-Zayat S, Sibaii H& Fathia A. Toll-like receptors activation, signaling, and targeting: an overview. Mannaa Bulletin of the National Research Centre volume 43, Article number: 187(2019).
12. Wang Y, Song E, Bai B, Vanhoutte PM. Toll-like receptors mediating vascular malfunction: Lessons from receptor subtypes. *Pharmacol Ther.* **158**: 91-100. doi: 10.1016/j.pharmthera.12.005. Epub 2015 Dec 15. PMID: 26702901(2015).
13. Zunt S, Burton L, Goldblatt L, Dobbins E and Srinivasan M.Soluble forms of Toll-like receptor 4 are present in human saliva and modulate tumor necrosis factor-á secretion by macrophage-like cells. **156**(2):285–293(2009).
14. Kacerovsky M, Andrys C, Hornychova H, Pliskova L, Lancz K , Musilova I, Drahosova M, Bolehovska R, Tambor V, Jacobsson B. Amniotic fluid soluble Toll-like receptor 4 in pregnancies complicated by preterm prelabor rupture of the membranes. *J Matern Fetal Neonatal Med*. **25**: 1148–1155. doi: 10.3109/14767058.2011.62682 1(2012).
15. Ten Oever J, Koxm M, van de Veerdonk FL, Mothapo KM, Slavcovici A, Jansen TL, Tweehuysen L, Giamarellos Bourboulis EJ, Schneeberger PM, Wever PC et al. The discriminative capacity of soluble Toll like receptor (sTLR)2 and sTLR4 in inflammatory diseases. *BMC Immunol*, **15**: 55 (2014).
16. Gonza´lez-Reyes S, Marý´n L, Gonza´lez L, Gonza´lez LO, del Casar JM, et al. Study of TLR3, TLR4 and TLR9 in breast carcinomas and their association with metastasis. *BMC Cancer,* **10**: 665(2010).
17. Elkammah M, Gowily A, Okda T , Houssen M . Serum soluble Toll-like receptor 4 and the risk of hepatocellular carcinoma in hepatitis C virus patients. **24**(4): 216–220 DOI: https://doi.org/10.5114/wo. 102818(2020).
18. Hausen H, de Villiers EM. Cancer "causation" by infections—Individual contributions and synergistic networks. *Semin. Oncol*. **41**:860–875 (2014). [CrossRef] [PubMed]
19. Yiu S, Dorothea M, Hui KF, Chiang A. Lytic Induction Therapy against Epstein-Barr Virus-Associated Malignancies: *Past, Present, and Future. Cancers,* 12: 2142 (2020).
20. Huang B, Zhao J, Unkeless JC, Feng ZH, Xiong H. TLR signaling by tumor and immune cells: a double-edged sword. *Oncogene*. **27**(2): 218-224(2008).
21. Petricevic B, Vrbanec D, Jakic-Razumovic J, Brcic I, Rabic D et al. Expression of Toll-like receptor 4 and beta 1 integrin in breast cancer. *Med Oncol*, **9**: 486–94 (2012).
22. Al-Humairi R, Al-Musawi M and Ad'hiah A . Serum level and single-nucleotide polymorphisms of toll-like receptor-7 among urinary bladder cancer Iraqi patients. *Egyptian Journal of Medical Human Genetics,* **20**: 11 (2019).
23. Abdullah's H and Abdulwahid A. Expression of Serum IL-22, IL-23, and TLR9 as Tumor Markers in Untreated Breast Cancer Patients. *International Journal of Drug Delivery Technology,* **10**(03): 472-476 DOI:10.25258/ijddt.10.3.30(2020).
24. lvesaro JM, Merrell MA, Li L, Wakchoure S, Graves D, Brooks S, Rahko E, Jukkola-Vuorinen A, Vuopala KS, Harris KW, Selander KS . Toll-like receptor 9 mediates CpG oligonucleotide–induced cellular invasion. *Molecular Cancer Research*.; **6**(10): 1534-1543.32(2008).
25. Klinman DM. Immunotherapeutic uses of CpG oligodeo-xynucleotides. Nature Reviews Immunology. TLR9 concentrations were shown to be increased in serum breast cancer and ovarian cancer in a prior investigation; **4**(4): 249-259(2004).