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The evaluation of TLR1, TLR2, TLR4, TLR7, and TLR8 expression levels in the newly-diagnosed acute myeloid leukemia (AML) patients

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Abstract

Background: Acute myeloid leukemia (AML) is described by the clonal expansion of myeloid blasts with abnormal differentiation. Considering the role of Toll-like receptors (TLRs) in inflammation induction and the effect of chronic inflammation on cancer development, investigating the state of TLRs' expression in human malignancies has attracted scientists' attention.

Methods: In this study, 36 newly-diagnosed AML patients and 36 control samples were examined. The mRNA expression levels of TLR1/2/4/7/8 were measured in both groups using real-time PCR. The student's t-test was utilized to compare gene expression levels between the two populations and the one-way ANOVA test was used to compare data among multiple subtypes.

Results: All TLR gene expression levels were significantly up-regulated in patients compared to the control group ($p<0.05$). Positive correlations between different TLRs were observed as well. AML patients under the age of 55 showed significantly higher TLR1/2/4 expression in comparison with healthy individuals of the same age; a similar comparison in people above 55 also showed an elevated expression of TLR1/2/4/8. Male patients overexpressed almost all genes compared to healthy subjects; the levels of TLR1/2/4 were also higher in female patients. No difference was observed comparing blast percentages and FAB subtypes.

Conclusion: By considering the results of this experiment, it seems that TLRs up-regulation in AML patients may contribute to the pathogenesis and development of the disease; however, more investigations are required to elucidate the exact roles of these receptors in AML.

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1. Introduction

Acute myeloid leukemia (AML), the most frequent acute leukemia among adults (1), is described by the accumulation of myeloid blasts or other myeloid progenitors in the bone marrow (BM) (2, 3). With the short overall survival of patients, AML is considered to be an invasive hematological cancer. Upon receiving standard chemotherapy, while 60-80% of patients initially accomplish complete remission, more than 50% of AML patients undergo relapse, indicating the generally poor prognosis of this disease (4). Over recent years, researchers have

moved toward targeted therapies by focusing on detecting molecular mechanisms involved in cancer progression to improve AML prognosis. For instance, monoclonal antibodies, small molecule inhibitors involved in the obstruction of crucial signaling pathways, and cell cycle inhibitors are among the new drugs currently under investigation (5).

Toll-like receptors (TLRs), as parts of both innate and adaptive immune systems, play essential roles in the recognition of damage- and pathogen-associated molecular patterns that ultimately lead to anti-microbial and inflammatory responses (6). As yet, 10 TLRs have been discovered in humans; while TLR1/2/4/6/10 are located on the plasma membrane, TLR3/7/9 and slightly TLR4 are on the membranes of the cytosolic section (7). Noteworthy, it has been detected that TLRs are not only expressed on the surface of macrophages, monocytes, lymphocytes, and granulocytes but also on tumor cells. Indeed, aberrant expression of TLRs not only endows cancer cells an opportunity to escape from the immune system but also supports them through enhancing proliferation and angiogenesis (8). These receptors display varying degrees of involvement in hematological cancers (9). In addition, chronic inflammation is one of the noticeable reasons for leukemia progression that declares the association between TLRs and malignant clonal expansion, since TLR-induced inflammatory signaling transforms the crosstalk between hematopoietic stem cells in their niches and results in aberrant hematopoiesis (10).

The main TLRs under investigation in the field of inflammation and malignancies are TLR1/2/4/7/8 (11-13). For instance, Wei et al. evidenced the role of increased TLR1 and TLR2 in myelodysplastic syndrome (MDS) patients, suggesting that an activating variant of the TLR2 gene in CD34+ cells led to enhanced NF-κB activity and abnormal epigenetically-activated genes of the innate immunity. An anti-TLR2 antibody (OPN-305) was therefore shown to be beneficial in low-risk MDS patients as a second-line treatment in a phase I/II study (14). Similarly, TLR4 has recently been suggested to be effective in AML progression by regulating p53 and p21, and accordingly, TLR4 inhibition using a specific TLR4 blocker, TAK-242, could halt this process in AML cell lines, either as a single agent or in combination with arsenic trioxide (ATO) (15). On the other hand, there are studies that suggest TLR activation as an enhancer of immunogenicity in AML blasts. In this regard, Weigel et al. suggested that TLR7 activation could stimulate plasmacytoid dendritic cells in patients with recurrent hematologic malignancies and lead to improved conditions (16). Additionally, TLR9 triggering accompanied by STAT3 inhibition could break tumor tolerance and enhance the immune system in AML mouse models (17). Considering the contradicting studies mentioned above and despite various efforts in discovering AML etiology, the specific causes of this cancer remain unclear and little significant progress has been made in fully-effective therapy methods so far.

2. Patients and Methods

2.1. Patients

In this study, samples from 36 newly-diagnosed patients with AML (25 males and 11 females) collected at Taleqani Hospital (Tehran, Iran) were taken into account. The median age of the patients was 55, ranging from 19 to 80 years old. The initial control group contained 44 healthy subjects. However, since it has been stated by other experiments that infection and sepsis shock can be an influential factor to increase the expression of TLRs in leukocytes (18) and monocytes (19) of AML patients, we checked both patients and control groups for any signs of sepsis at the first stage of diagnosis by checking their clinical state and laboratory reports. The final control group contained 36 healthy individuals (15 males and 21 females) who had nearly the same age range as the patients.

This study was performed in agreement with the Helsinki protocol of 1975 and confirmed by the Ethics Committee of Shahid Beheshti University of Medical Sciences (Tehran, Iran) with the Ethics Committee code of IR.SBMU.RETECH.REC.1399.569. Once the consent form was signed by the patients, prior to any treatments, 5 ml of the BM from 24 patients and 5 ml of peripheral blood (PB) from the rest of the individuals were collected; meanwhile, peripheral blood (PB) samples were collected from all healthy subjects.

2.2. RNA extraction and cDNA synthesis

To isolate mononuclear cells from the samples, Ficoll-Hipague (INTRON South Korea) density gradient centrifugation was used. Next, TriZol (Yekta Tajhiz Azma) was added and total RNA extraction

was attained from each sample based on the manufacturer's instruction. The quantity and quality of the extracted RNA were assessed using Nanodrop (Thermo Fisher Scientific, USA). Besides, through the use of 1% agarose gel electrophoresis, the quality of the extracted RNA was observed. cDNAs were synthesized by reverse transcription (Thermo Fisher Scientific kit, USA) according to the manufacturer's guidelines using a master cycler Nexus Gradient.

2.3. Evaluation of mRNA expression level by quantitative real-time polymerase chain reaction (qRT-PCR)

SYBR Green I real-time PCR assay was carried out to evaluate the relative mRNA expression levels of TLR1, TLR2, TLR4, TLR7, and TLR8. Primer sequences are provided in **Table 1.** Accordingly, 20 µl total reaction volume was prepared which contained 2 µl cDNA (equal to 100 ng RNA), 0.5 µl of each primer (10 pM), 10 µl Universal Master Mix (Ampliqon, Denmark), and dH2O to accomplish the total volume. Thermal cycling was performed on a Rotor-Gene 6000 (Qiagen, USA) using a cycling program of 15 min at 95oC, followed by 40 cycles at 95oC for 15 s, 60oC for 15 s, and 72oC for 40 s. After each run, melting curves were assessed to verify the amplification's specificity. The mRNA expression level of each target gene was normalized to the housekeeping gene ABL and the expression of each mRNA was quantified according to the ΔΔCt formula.

2.4. Statistical analysis

SPSS software version 19 was used for the statistical evaluation of the results. All tests were carried out in triplicate and the results were statistically stated as mean ± SD. Regarding Kolmogorov-Smirnov test results, t-test and One-way ANOVA were used for comparing two groups and multi-groups of data respectively to assess the statistically significant results. Through the use of the Pearson correlation coefficient (PCC) test, linear correlations between two groups of data were determined; two-tailed P values less than 0.05 were referred to as significant. GraphPad Prism 8 was used to prepare the graphs and error bars were set based on the standard error of the mean (SEM).

3. Results

3.1. Patient characteristics

In this study, 36 newly-diagnosed patients with non-M3 acute myeloid leukemia (AML) (25 males and 11 females) were taken into account. The median age of the patients was 55, ranging from 19 to 80. Based on FAB classification, patients were categorized into three subgroups consisting of AML-M0 and M1 (N=15, 41/66%), AML-M2 (N=17, 47/22%), and AML-M4 and M5 (N=4, 11/11%) in accordance with the state of cell differentiation. By considering the immunophenotyping parameters, the patients' specimens varied from having 28 to 90% blasts (median 56/72%). Detailed demographic information of the patients is presented in **Table 2.**

3.2. Comparing the expression levels of various TLRs in patients versus the control group

Considering a plethora of studies indicating the effects of TLRs on different aspects of AML (20, 21), we decided to investigate the expression levels of TLR 1, 2, 4, 7, and 8 in patients and healthy individuals. As demonstrated in **Fig. 1**, by comparing the two groups, the expression levels of all TLR1/2/4/7/8 were significantly higher in the patient group with P values of 0.000, 0.000, 0.000, 0.008, and 0.000, respectively.

3.3. Correlations between different TLRs

In order to identify whether the expression levels of different TLRs were correlated, the Pearson's correlations of all these genes were studied in both AML patients and healthy subjects (**Supplementary Fig. 1**). Although with diverse values, all correlations were positive among TLR1/2/4/7/8 based on the results presented in **Table 3.** Noteworthy, all correlation amounts were statistically significant except for between TLR8 and TLR1/4 in healthy subjects. Looking in more detail, the strongest correlation among patients was observed between TLR1 and TLR2, followed by TLR2 and TLR4.

3.4. Comparing TLRs expression among different categories

The levels of expression of various TLRs were compared between categories to investigate their correlations with different characteristics of AML patients.

3.4.1. Comparing TLRs expression based on sample type

Since samples were taken from either the patients' bone marrow or peripheral blood, we compared the level of TLRs in each group. Our results showed no remarkable differences (**Fig. 2**), indicating that high TLR expression in AML patients may not be limited to the bone marrow and it can affect blood cells as well.

Table 1. Real-time PCR oligonucleotide primers.

Gene	Primers	Sequences (5'-3')	Tm (°C)	Accession Number	Amplicon size
TLR1	TLR1.F	CCACGTTCCTAAAGACCTATCCC	64	NM_003263.4	248 bp
	TLR1.R	CCAAGTGCTTGAGGTTCACAG	61		
TLR2	TLR2.F	ATCCTCCAATCAGGCTTCTCT	59	NM_001318796.2	118 bp
	TLR2.R	GGACAGGTCAAGGCTTTTTACA	60		
TLR4	TLR4.F	AGACCTGTCCCTGAACCCTAT	61	NM_003266.4	147 bp
	TLR4.R	CGATGGACTTCTAAACCAGCCA	62		
TLR7	TLR7.F	TCCTTGGGGCTAGATGGTTTC	61	NM_016562.4	80 bp
	TLR7.R	TCCACGATCCACATGGTTCTTTG	60		
TLR8	TLR8.F	ATGTTCCTTCAGTCGTCAATGC	60	NM_016610.4	143 bp
	TLR8.R	TTGCTGCACTCTGCAATAACT	57		
ABL	ABL.F	TGGAGATAACACTCTAAGCATAACTAAAG	59	NM_007313.3	124 bp
	ABL.R	GATGTAGTTGCTTGGGACCCA	60		

Table 2: Summary of patients' demographic data.

Sample size	36
Gender (Male/Female)	25/11
Age	19-80 (Median= 55)
Sample type (BM/PB)	24/12
Blast percentage	28-90 (Median= 56/72)
FAB classification (%)	
AML-M0, M1	15 (41.66)
AML-M2	17 (47.22)
AML-M4, M5	4 (11.11)
Blood Count	
WBC ($\times 10^9$/L)	66.6 (1.4-340)
Hb (g/dl)	9.2 (6.1-12.1)
PLT ($\times 10^9$/L)	86.1 (47-127)
Karyotype	All normal

FAB: French-American-British; WBC: White blood cell; Hb: Hemoglobin; PLT: Platelets; BM: Bone marrow; PB: Peripheral blood.

Table 3. TLRs' Pearson correlation coefficient (PCC) test results in patients and the control group (N= 36).

TLRs	Pearson's R	P value	α	State of correlation
TLR1 and TLR2	0.931**	0.000	0.01	Very strong
TLR1 and TLR2	0.855**	0.000	0.01	Very strong
TLR1 and TLR4	0.892**	0.000	0.01	Very strong
TLR1 and TLR4	0.639**	0.000	0.01	Strong
TLR1 and TLR7	0.570*	0.014	0.05	Moderate
TLR1 and TLR7	0.600*	0.000	0.01	Strong
TLR1 and TLR8	0.766**	0.000	0.01	Strong
TLR1 and TLR8	0.046	0.804	-	Very weak
TLR2 and TLR4	0.929**	0.000	0.01	Very strong
TLR2 and TLR4	0.643**	0.000	0.01	Strong
TLR2 and TLR7	0.542*	0.020	0.05	Moderate
TLR2 and TLR7	0.692*	0.000	0.01	Strong
TLR2 and TLR8	0.822**	0.000	0.01	Very strong
TLR2 and TLR8	0.329*	0.022	0.05	Weak
TLR4 and TLR7	0.596**	0.009	0.01	Strong
TLR4 and TLR7	0.635**	0.000	0.01	Strong
TLR4 and TLR8	0.860**	0.000	0.01	Very strong
TLR4 and TLR8	0.277	0.132	-	Weak
TLR7 and TLR8	0.616**	0.006	0.01	Strong
TLR7 and TLR8	0.389**	0.030	0.05	Weak

Data in blue presents the patients and data in black presents the healthy individuals. Concerning Pearson's R, 0.8-1: Very strong; 0.60-0.79: Strong; 0.40-0.59: Moderate; 0.20-0.39: Weak; and 0.00-0.19: Very weak.
Statistically significant values of *P < 0.05, **P < 0.01, and ***P < 0.001 are determined.

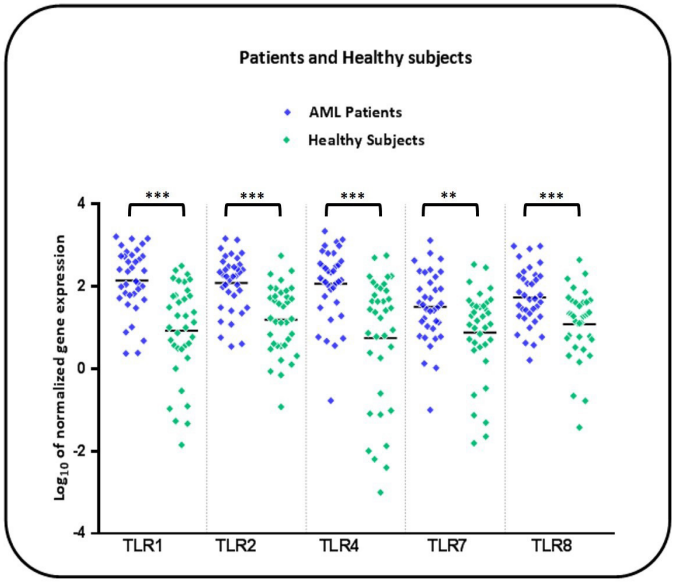

Figure 1. Comparing the expression levels of different TLRs between AML patients and healthy individuals showed that all genes were expressed noticeably higher in patients and presented significant differences. Statistically significant values of *P < 0.05, **P < 0.01, and ***P < 0.001 are determined compared to healthy subjects.

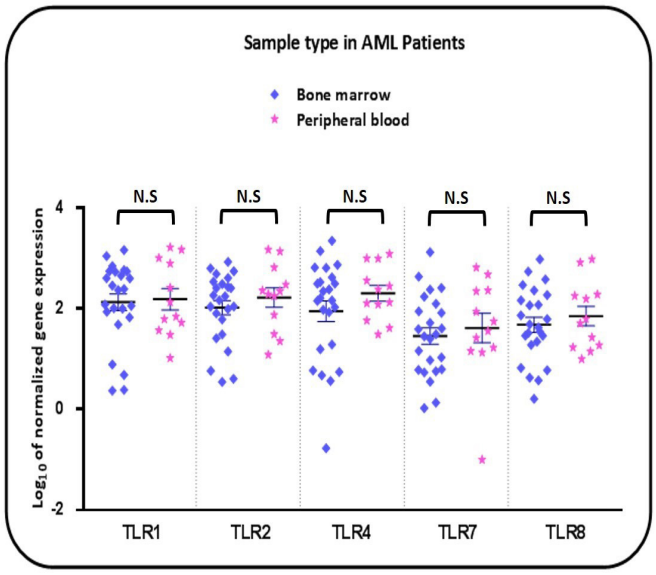

Figure 2. No significant differences (P > 0.05) between the expression levels of TLRs were detected when samples from the bone marrow (24 samples) and peripheral blood (12 samples) of AML patients were compared (N.S: Not significant).

3.4.2. Comparing TLRs expression based on gender and age

We also studied the expression levels comparing male and female patients which showed no significant difference **(Fig. 3A)**. However, comparing the expressions among male patients and healthy subjects resulted in noticeable differences in almost all genes except for TLR7 (P values of 0.0003, 0.0005, 0.002, and 0.0077 for TLR1/2/4/8 respectively) **(Fig. 3B).** Similarly, the same comparison among females showed different TLR1/2/4 levels (P values of 0.03, 0.047, and 0.043 respectively) **(Fig. 3C)**. Additionally, the differences in levels of expression were not statistically significant when patients older than 55 (based on the median age of all patients) and the younger age group were compared **(Fig. 4A)**. Conversely, comparing AML patients under the age of 55 with healthy individuals of the same age showed significant differences in TLR1/2/4 expression levels (P values of 0.011, 0.025, and 0.002, respectively). Similarly, significant differences in TLR1/2/4/8 expression levels were detected when patients and the healthy group above 55 years were compared (P values of 0.038, 0.000, 0.032, and 0.018 respectively). No significant contrast in TLR7 expression was observed between the patient group and the healthy subjects in either age group **(Fig. 4B, 4C).**

3.4.3. Comparing TLRs expression based on blast percentage and FAB subtype

TLR expression levels were compared within different sub-categories distinguished by the percentage of blasts and FAB subtypes. As patients were divided into three groups of having 20-50%, 50-75%, and 75-100% blasts, there was no significant difference among these groups for either of the TLRs **(Fig. 5A)**. Likewise, no remarkable difference was observed between FAB subtypes including three categories of AML-M0 and M1, AML-M2, and AML-M4 and M5 **(Fig. 5B).**

4. Discussion

A growing amount of evidence presents the fact that TLRs could be responsible for not only the immune system's proper function but also, they could surprisingly be beneficial to cancer cells, turning the immune system against one's body. Indeed, the discovery of these receptors in tumor cells has heralded a renaissance in the interconnection between innate immunity and tumor biology. In this context, some studies have highlighted the importance of TLRs' role in tumor development by indicating their involvement in cell proliferation, tumor infiltration, and providing a suppressed microenvironment (8). Regarding solid tumors, for instance, TLR4 signaling has become a great target in a variety of human malignancies (22), especially for suppressing ovarian and breast cancer invasion, since applying its specific inhibitor, TAK-242,

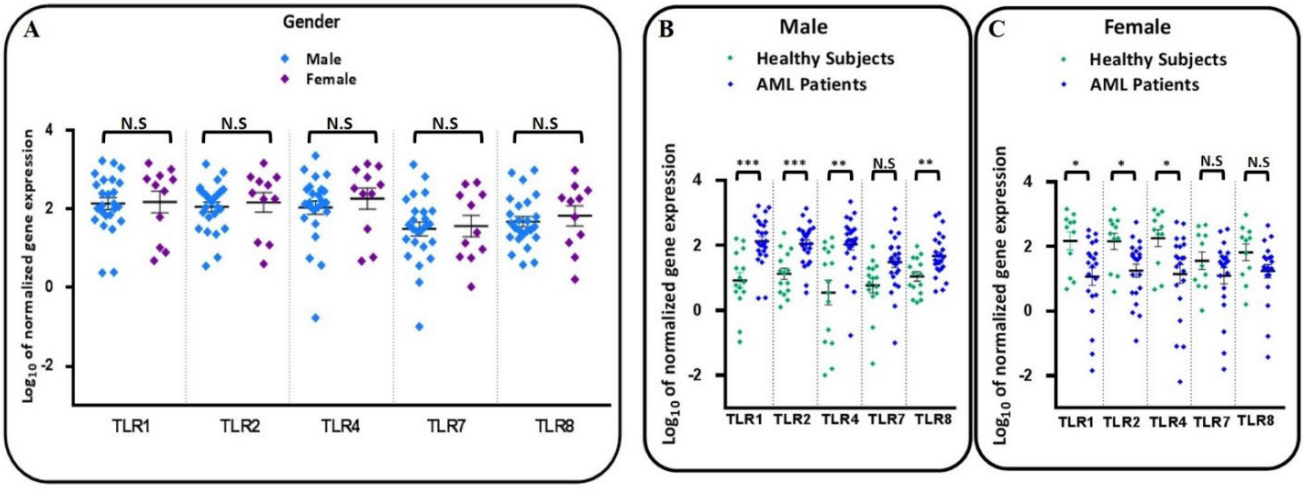

Figure 3. (A) No significant differences (P > 0.05) between the expression levels of TLRs were detected between male and female patients. When AML patients were compared with their healthy peers, however, most genes showed different mRNA levels among males (B) and females (C). Statistically significant values of *P < 0.05, **P < 0.01, and ***P < 0.001 are determined (N.S: Not significant).

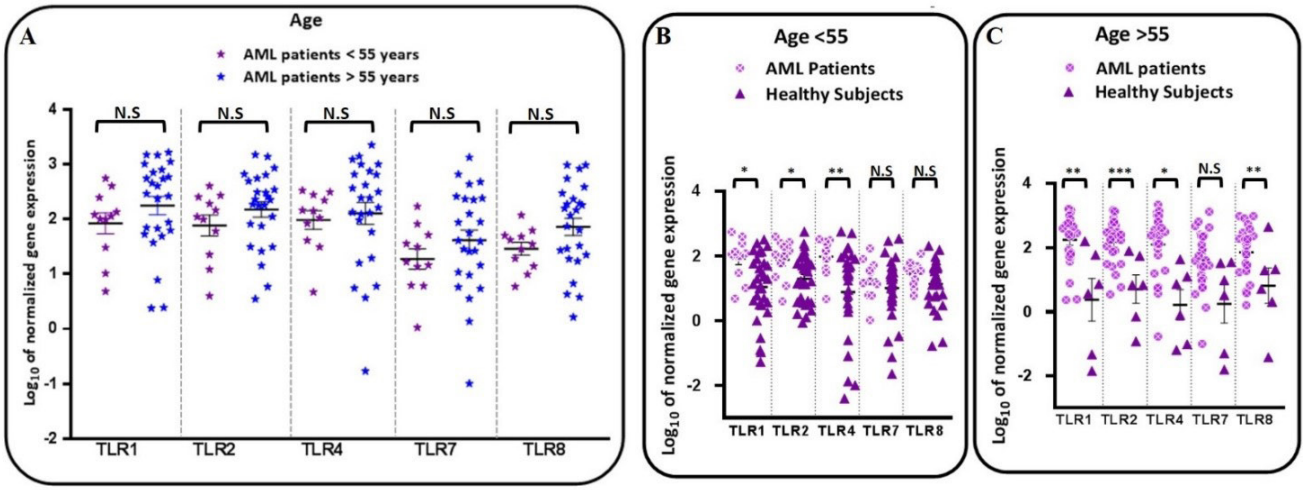

Figure 4. (A) No TLR showed a significant difference in expression among AML patients older and younger than 55 years of age. When AML patients under 55 years were compared with their healthy peers (B), TLR1/2/4 showed significant differences. The same comparison among people over 55 (C) evidenced significant differences in the expression of TLR1/2/4/8. Statistically significant values of *P < 0.05, **P < 0.01, and ***P < 0.001 are determined (N.S: Not significant).

was proposed as an effective targeted therapy either as a single agent (23, 24) or in combination with other chemotherapeutics (25). Similarly, another study has pointed to the role of TLR4 in local relapse and metastasis due to its activation by paclitaxel, regularly used as a chemotherapeutic drug against many cancers, resulting in inflammation and eventually metastasis (26). Likewise, in hematologic malignancies, overexpression of TLR2/4/9 has been reported as a mechanism to avoid the immune response in patients via both MEK/ERK-dependent and MyD88/TRAF6-dependent signaling pathways (27).

The idea behind the involvement of TLRs and their related signaling in the formation of human cancers has originated from a considerable number of studies reporting the abnormal expression of TLRs on tumor cells, where they may influence tumor growth and immune responses. According to a research conducted by Jego et al., a wide variety of TLRs are expressed on human myeloma cell lines and primary myeloma cells, of which TLR1, 7, and 9 are the most common (28). Correspondingly, our study showed that the mRNA expression levels of TLR1, TLR2, TLR4, TLR7, and TLR8 were significantly higher in drug-naïve AML patients

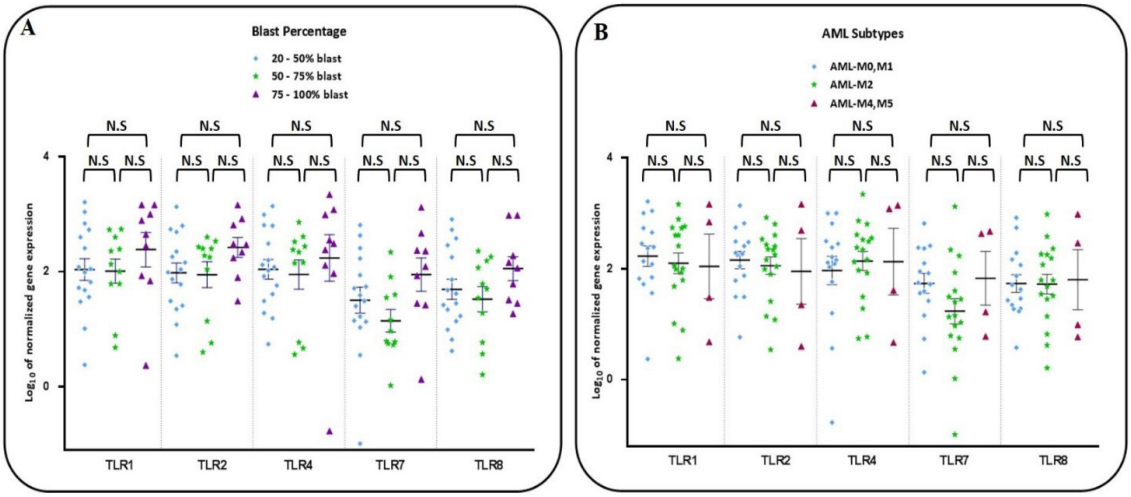

Figure 5. TLR expression levels were not significantly different (P > 0.05) when comparing either blast percentage (A) or FAB subtypes (B) (N.S: Not significant).

compared to the control group. Accordingly, Eriksson et al. discovered that stem cells highly expressed TLR1 in AML patients compared to the control group, revealing the necessity of TLR1 for LSC's (long-term stem cell) survival (29). Interestingly, an investigation conducted by Rybka et al. revealed that TLR2 and TLR4 mRNA expression levels considerably diminished in AML patients who experienced complete remission following the first step of chemotherapy compared to those resistant to therapy (21) which probably indicates that TLR2 and TLR4 may provide survival signaling in drug-resistant cancer cells. A recent study also showed that inhibiting the high activity of the TLR4 signaling pathway in AML cell lines using TAK-242 could enhance their response to ATO chemotherapy (15). Nonetheless, contrary to studies declaring high levels of TLRs in AML patients, Webb et.al announced that TLR4 expression levels were lower in AML patients compared to the control group, leading to insufficient immune response toward malignant leukemic cells (30). This discrepancy might be due to different target populations or sample sizes which calls for further research in this regard. Despite the differences observed in the effect of TLRs in numerous studies, it is noticeable that TLR1/2 and TLR7/8 show concurrent changes since they form heterodimers (31) or act simultaneously (32) in order to initiate immune responses.

In agreement with such studies, we found that a significant and very strong correlation existed between TLR1 and TLR2, as well as a strong correlation between TLR7 and TLR8 (Table 3). We also assessed the correlation between other TLRs, most of which showed strong to intermediate results. These correlations may be used to design targeted ligands or blockers for more effective therapeutic approaches (33), as Pam3CSK4 (TLR1/2) and R848 (TLR7/8) have previously been suggested to induce anti-leukemic effects (34, 35).

In order to explain the exact nature of TLRs' expression and their possible effect on cancer cells, various studies showed that multiple factors might be effective. For instance, Rybka et al. suggested that patients with myelomonocytic (M4) and monoblastic (M5) acute myeloblastic leukemia showed much higher mRNA levels of TLR2 and TLR4 than patients with other subtypes (21). We, however, could not find any difference in TLR expression among FAB subtypes in AML cases which could be due to the low number of M4 and M5 cases in our samples. As another possible effective factor, Renshaw et al. claimed that aging might negatively influence TLRs' level of expression which resulted in a more vulnerable state of the immune system against infections during old ages (36). On the contrary, in another study, Ramzi et al. declared no significant association between aging and TLR expression level (2). In the present study, we also could not detect any differences among AML patients above and below the median age (55 years). Our results showed that the differences in TLR1/2/4/8 levels of expression were significant when patients above 55 years were compared with their healthy peers; a similar result with TLR1/2/4 was observed in those under 55 compared to the control group.

Notably, we also studied gender as a potential contributing factor to mRNA levels of TLRs. While the results confirmed no statistically significant differences

among male and female patients, male patients had higher expression of TLR1/2/4/8 and female patients overexpressed TLR1/2/4 compared to healthy subjects of the same gender. Although we could not find any relevant studies on the correlation between TLRs and gender in hematologic malignancies, similar studies on monocytes in other diseases have shown no significant results as well (37, 38).

All in all, it seems that TLRs show different behaviors when it comes to cancer progression depending on TLR subtype, type of cancer, and tumor microenvironment. While TLR up-regulation in AML cases might induce differentiation, the chronic inflammation they trigger is closely linked to cancer development (21); therefore, the exact roles of TLRs in these cases remain vague. Our study supports the fact that the expression levels of TLR1/2/4/7/8 are significantly elevated in AML patients, suggesting their possible roles in cancer initiation and/or progression. However, a prolonged follow-up study involving drug treatment could further investigate the exact nature of these receptors by screening their expression through different stages of therapy and recurrence.

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Conflict of interests

The authors declare that there is no conflict of interest.

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