GTMB Vol 17, 1-13, 2018

APRIL Gene Expression in a Cohort of Egyptian Acute Myeloid Leukemia Patients: Clinical and Prognostic Significance Research Article

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Key words: *AML, APRIL, RTQ-PCR*

Abbreviations: Acute myeloid leukaemia (AML) APRIL (A Proliferation Inducing Ligand)

Received: 1 June 2017; electronically published: June 2018

Summary

Background: APRIL (A Proliferation Inducing Ligand) is a member of the tumor necrosis factor (TNF) family. It is essential for the survival of normal and malignant B lymphocytes. Increased expression of APRIL is noted in most of hematological malignancies and auto immune diseases.

Patients and methods: We investigated the expression level of APRIL mRNA in 50 de novo acute myeloid leukemia (AML) patients, together with 20 healthy controls using a Real-Time Quantitative Reverse-Transcriptase Polymerase Chain Reaction (RTQ-PCR) with a specific aim of determining its relation to clinical features and laboratory findings at diagnosis and its impact on the response to therapy.

Results: APRIL mRNA expression level was significantly higher in AML patients than in controls ($p<0.001$). APRIL expression level was significantly higher in patients who didn't achieve CR compared to those who achieved CR ($p<0.001$).

Patients who didn't achieve CR also had higher TLC, lower platelets and older age than CR patients. The difference was statistically significant (*$p<0.001$*, *$p=0.047$*, *$p=0.019$*) respectively. APRIL levels showed significant positive correlation with TLC *($r=0.743.p<0.001$)*, with age *($r=0.296$, $p=0.037$)*and a negative correlation with platelets count *($r= -0.443, p=0.001$)* and no correlation with gender, Hb level, BM blast, HSM or LNs enlargement .

Conclusion: Our study has shown that APRIL is overexpressed in AML patients, its level might serve as an indicator for disease progression. APRIL might be an indicator for poor prognosis and treatment resistance in AML patient; therefore, APRIL antagonists may represent a novel therapeutic approach for the treatment of AML.

I. Introduction

Acute myeloid leukaemia (AML) is a malignant clonal disorder of the haematopoietic stem cell in which both failure to differentiate and overproliferation in the stem cell compartment occurs; resulting in the accumulation of non-functional myeloid cells (myeloblasts) and loss of normal haematopoietic function ***(Estey 2012).***

AML is a heterogenous disease; there is no identifiable predisposing cause nor there definite predicator for prognosis. A number of factors such as age, performance status, chromosomal abnormalities or a history of prior chemotherapy have been associated with outcome ***(Byrd et al., 2002).*** Several studies tried to identify new diagnostic and prognostic markers in AML.

APRIL (a proliferation-inducing ligand), also known as TNFSF13, CD256, TRDL-1(TNF-related death ligand-1), TALL-2 or n zTNF2, is a member of the tumor necrosis family (TNF) ***(Kelly et al., 2000 & Dillon et al., 2006).*** April shares significant homology with another member of the TNF family; B cell-activating factor (BAFF) **(*Mackay et al., 2003).*** APRIL is a type II transmembrane protein that also exists in soluble form deriving from the intracellular cleavage of the full-length protein which is produced by several cells ***(Matsuda et al., 2015).*** It can bind with high affinity to two members of the TNF receptor superfamily, the B-cell maturation antigen (BCMA) and the transmembrane activator and calcium modulator and cyclophilin ligand-interactor (TACI) **(*Ingold et al., 2005).*** It can also bind to heparan-sulfate proteoglycans (HSPG) such as syndecan-1(CD138) ***(Shivakumar & Ansell 2006):***

II. PATIENTS AND METHODS

Patients and controls series:

Fifty Egyptian patients with Acute Myeloid Leukemia (AML) were included in this study. Patients and controls were recruited from the National Cancer Institute (NCI), Cairo University and BeniSuef University Hospital, from December 2014 to July 2015. Informed consent was taken from all contributors prior to their inclusion in the study. All work was performed in accordance with the ethical standards of the **Revised Helsinki Declaration of Bioethics (2008).**

Patients were 24(48%) males and 26(52%) females. Their age ranged between 19-68 years with a mean of 39±13.2 years. All the patients were newly diagnosed and did not receive any treatment.

Twenty age and sex matched healthy individuals with normal laboratory findings were included as a control group. They were 10(50%) males and 10(50%) females. Their age ranged between 24-56 years with a mean of 46±6.5 years.

Diagnosis of AML was based on (1) morphologic findings from Giemsa stained smears of bone marrow (BM) aspirates, (2) cytochemical stains criteria such as positivity for myeloperoxidase (MPO) and Sudan Black B (SBB) (3) immunophenotyping criteria as positivity of CD13 and CD33. A complete blood count and a differential count including blast cell percentage were done for all patients. Peripheral blood (PB) samples and bone marrow (BM) aspiration samples were collected at diagnosis from the 50 Egyptian acute myeloid leukemia patients, while peripheral blood samples were obtained from the control group.

APRIL gene was analyzed in patients and controls using Real-Time Quantitative Reverse Transcriptase Polymerase Chain Reaction (RTQ-PCR) to study mRNA expression levels.

RNA isolation and real-time quantitative RT-PCR for APRIL gene:

PB and BM mononuclear cells (MNCS) were isolated at diagnosis by Ficoll density gradient centrifugation. Total RNA was extracted from MNCs using a**QIAamp® RNA Blood Mini Kits (Catalog No. 52304) (Qiagen, Germany)** according to manufacturer's instructions. Complementary DNA (cDNA) was synthesized using (dt) 15-mer primer by Superscript III Reverse Transcriptase and stored at –20 ◦C till use.

The mRNA expression levels of APRIL gene and GAPDH (endogenous control) were measured by quantitative RT-PCR using an ABI PRISM 7000 Sequence Detector System (Applied Biosystems, Foster City, CA). The quantitative RT-PCR amplification was performed using the predeveloped Assays-on-demand Gene Expression Set for the APRIL (Assay ID: Hs00601664_g1; Catalog no. 4331182, Applied Biosystems)andTaqMan GAPDH control reagents (human;Catalog no. 402869, Applied Biosystems) with the following sequence, the forward primers (GAPDH-Fw: 5'-GTCCATGCCATCACTGCCAC-3') the reverse (GAPDH-Rv: 5'-ATGACCTTGCCCACAGCCTT -3') with the TaqMan Universal PCR Master Mix (Catalog no. 4440043,Applied Biosystems).

All reactions were performed in duplicate using 20 μl samples containing 50 ngcDNA. The reaction protocol used involved heating for 2 min at 50 ◦C and 10 min at 95◦C, followed by 40 cycles of amplification (15 s at 95 ◦C and 1 min at 60 ◦C). Analysis was performed using ABI PRISM 7000 Sequence Detection Software (Applied Biosystems) and **Applied Biosystem Step One ^{TM} Instrument (USA).**

The expression levels of APRIL gene in tested samples were expressed in the form of CT (cycle threshold) level **(Fig 1);** then normalized copy number (relative quantitation) was calculated using the ΔΔCT equation as follows: ΔΔCT = ΔCT of case - ΔCT of control, then the normalized copy number (relative quantitation) = $2^{-\Delta\Delta CT}$. A negative control without

template was included in each experiment.

Expression level of APRIL was correlated with the clinical and laboratory features of the studied patients at diagnosis including: age, sex, total leukocytic count (TLC), hemoglobin (Hb), platelet count, lineage, blast cell percentage and response to treatment.

Treatment of AML patients:

AML patients were treated according to Department of Oncology, National Cancer Institute (NCI), Cairo University. All patients received induction chemotherapy 7-3 protocol consisted of a course of 12mg/m² novantrone on day 1,3 and 5; Ara-C 100mg/m² continuously every 12 hours from day 1through 7.If the patient did not enter into remission, this protocol was repeated. If no or minimal response, patients were shifted to high dose chemotherapy. Patients who entered into remission received 4 courses of high dose Ara C as consolidation. This was Ara-C 2g/m² on 2 hours infusion every 12 hours day 1, 3 and 5.

Assessment of the response to induction chemotherapy:

By the end of induction therapy, complete remission (CR) status was defined by normalization of the neutrophil count (at least≥1.5/ul) and platelet counts(>100x103/cm³), and marrow examination that demonstrate at least 20% cellularity, less than 5% blasts and no Auer rods, as well as absence of extramedullary infiltration. Resistance to treatment is defined as more than 25% blasts in the BM, lack of regeneration of normal hematopoiesis, persistence of peripheral blood blasts and/or extramedullary leukemia after induction. Death during induction is defined as death during or after the first course of therapy with aplastic or hypocellular marrow.

Statistical methods:

Data was analyzed using IBM SPSS advanced statistics version 22 (SPSS Inc., Chicago, IL). Numerical data were expressed as mean and standard deviation or median and range as appropriate. Qualitative data were expressed as frequency and percentage. Chi-square test or 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). Spearman-rho method was used to test correlation between numerical variables. All tests were two-tailed. A p-value < 0.05 was considered significant.

III. RESULTS

This study was conducted on 50 denovo AML patients, clinical and laboratory characteristics of which are presented in **Table (1).**

Twenty age and sex matched normal volunteers were included in the study as a control group.

Expression level of APRIL gene mRNA in AML patients was of mean 4.86±2.4 (range 1.02-9.27) and median 5.07 while it was of mean 0.53±0.40 (range 0.007-1.13) and median 0.52 in controls.

Comparative studies between all AML patients and controls revealed statistically significant difference as regards APRIL gene expression levels, TLC, Hb and Platelet count with ***p-value < 0.001***, while there was no statistically significant difference as regards age and gender with p>0.05**(Table 2).**

Comparing APRIL gene level with different clinical variables was not significant statistically as regards gender, hepatomegaly, splenomegaly and lymphadenopathy with p-value (0.077, 0.602, 0.735 and 0.169 respectively).

Comparing APRIL gene level to patients outcome (CR/no CR) showed high statistically significant difference **(p<0.001)** the gene being higher in patients who didn't achieve CR.

Comparing patients′ response to treatment after 28 days of chemotherapy i.e those who achieved complete remission (CR) versus those who didn't achieve complete remission (no CR) as regards laboratory and clinical variables showed high significance for APRIL gene level ***(p<0.001)***,being higher in patients who didn't achieve CR, TLC ***(P<0.001),*** also higher in patients who didn't achieve CR, platelets count ***(p=0.047)***, were lower in patients who didn't achieve CR, and age ***(p=0.019)***, as patients who didn't achieve CR were older in age than those achieved CR. While there was no statistically significant difference as regards bone marrow blast percentage (p=0.072), gender (p=0.308), hepatomegaly (p=0.161), splenomegaly (p=0.101) and lymphadenopathy (p=0.650) although it was near significance in patients without hepatomegaly, splenomegaly and lymphadenopathy showing complete remission after 28 days of treatment **(Table 3).**

No p-value could be calculated for different FAB subgroups as regards the gene level and relation of gene level to their outcome after treatment because of the small number of cases within subgroups.

We regrouped the patients into 3 groups: group 1: M0+M1+M2, group 2: M4+M5+M6 and group 3: M3. There was statistically significant difference between group 1 and 2 as regards gene level **(p=0.003)** but there was no statistically significant difference between APRIL gene level in relation to outcome after treatment in group 1 and 2 (p=0.336) while no p-value in cases of M3 as all 4 cases entered in CR .

Correlation studies between APRIL gene levels and different clinical and laboratory data of patients showed

significant good positive correlation with TLC ***(r=0.743,p<0.001)***, significant weak positive correlation with age ***(r=0.296,p=0.037)***and a negative correlation with platelets count ***(r= -0.443,p=0.001)*** and no correlation with Hb level (r=0.053, p=0.691) and Bone marrow blast % (r=0.199, p=0.167) **(Table 4).**

Table 1: Clinical and laboratory Characteristics of AML Patients

Characteristics	Value
Acute Myeloid Leukemia (n)	50
Age in years Range mean±SD median	 19-68 39± 13.2 37 years
Gender Male (n %) female (n %)	 24(48%) 26(52%)
***FAB classification (n %)** M0 M1 M2 M3 M4 M5 M6	 3 (6%), 17 (34%), 18 (36%), 4 (8%), 6 (12%), 1 (2%) 1 (2%)
Total Leucocytic count (x /L) Mean±SD Range Median	 50.2 ± 48.3 2.5-327 46
Hemoglobin (gm/dl) Mean±SD Range Median	 7.6 ± 1.6 2.7- 11.6 7.8
Platelets (x /L) Mean±SD Range Median	 41.7 ± 34 6-172 34.5
Bone marrow blast (%) Mean±SD Range Median	 59.1 ± 27.6 1-97 67
Hepatomegaly **present (n %)** **absent (n %)**	 29/50 (58%) 21/50(42%)
Splenomegaly **Present (n %)** **Absent (n %)**	 33/50 (66%) 17/50(34%)
Lymphadenopathy **Present (n %)** **Absent (n%)**	 6/50 (12%) 44/50(88%)

Response to induction therapy (n %) CR No CR	. 16/50 (32%) 34/50 (68%)

*FAB: French American British system

Table (2): Comparison between AML patients & control groups regarding APRIL gene levels, clinical and laboratory data

P-Value	CONTROL N=20	AML N=50	
P < 0.001	0.5303±0.4014 0.0070-1.1300 0.5180	4.8569± 2.4071 1.0256- 9.2708 5.0793	**APRIL gene** Mean±SD Range Median
P = 0.2	46.3±6.5 24.0-56.0 45.5	39.0±13.2 19.0-68.0 37.0	**Age (years)** Mean±SD Range Median
P = 0.8	10(50%) 10(50%)	24(48%) 26(52%)	**Gender** Male (n %) female (n %)
P < 0.001	7.4±1.8 4.2-10.3 7.7	50.2±48.3 2.5-327.0 46.0	**Total Leucocytic count (x /L)** Mean±SD Range Median
P < 0.001	12.9±0.7 11.9-14.0 12.8	7.6±1.6 2.7-11.6 7.8	**Hemoglobin (gm/dl)** Mean±SD Range Median
P < 0.001	290±45 245-335 280	41.7±34.0 6.0-172.0 34.5	**Platelets (x /L)** Mean±SD Range Median

Bold values are statistically significant

*p-value ≤ 0.001 highly significant

p-value ≤ 0.05 significant

p-value> 0.05 non significant

Table (3): Relation of patients' response to APRIL gene and different clinical and laboratory variables

Parameter	AML with CR N=16	AML with no CR N=34	P-value
APRIL gene Mean±SD Range Median	2.0750±0.8264 1.0256-3.2750 2.0763	6.1661±1.6657 3.0997-9.2708 6.1196	***P < 0.001***
Age (years) Mean±SD Range Median	33.0±12.4 15.0-62.0 31.0	41.8±12.8 20.0-68.0 41.5	***P =0.019***
Total Leucocytic count (x /L) Mean±SD Range Median	16.9±13.0 2.5-48.0 15.8	65.9±51.0 16.0-327.0 54.7	***P < 0.001***
Hemoglobin (gm/dl) Mean±SD Range Median	7.5±2.0 3.6-11.0 8.0	7.7±1.4 2.7-11.6 7.8	P =0.832
Platelets (x /L) Mean±SD Range Median	47.8±37.8 6.0-172.0 37.5	28.6±19.0 7.0-75.0 26.5	***P=0.047***
Bone marrow blast (%) Mean±SD Range Median	46.8±34.1 1.0-97.0 59.0	64.9±22.3 8.0-92.0 72	P = 0.072
Hepatomegaly (n %)	7/29(24.1%)	22/29(75.9%)	P=0.161

Splenomegaly (n %)	8/33(24.2%)	25/33(75.8%)	P=0.101
Lymphadenopathy (n %)	1/6(16.7%)	5/6(83.3%)	P=0.650

Bold values are statistically significant

Table (4): Correlation between APRIL gene and clinical and laboratory variables

Variable	APRIL gene
Age Correlation Coefficient -r P-value	 **0.296** ***0.037****
TLC Correlation Coefficient-r P-value	 **0.743** ***<0.001****
Hb Correlation Coefficient-r P-value	 0.058 0.691
PLT Correlation Coefficient-r P-value	 **-0.443** ***0.001****
BM blast % Correlation Coefficient-r P-value	 0.199 0.167

Bold values are statistically significant

r< 0.5 weak correlation (underlined)

r = 0.5 ---fair correlation

r >0.5 ----0.75 good correlation

r> 0.75 ---- very good correlation

*p-value ≤ 0.001 highly significant

p-value ≤ 0.05 significant

p-value> 0.05 non significant

Figure (1): APRIL genes and GAPDH (endogenous control) in AML cases and controls

III. DISCUSSION

APRIL (A Proliferation Inducing Ligand) is a member of the tumor necrosis factor (TNF) superfamily which plays a key role in normal B-cell development, it promote peripheral B-cell survival, maturation, and differentiation and play important roles in the production of antibodies. Serum level of APRIL is increased in several autoimmune diseases ***(Chu et al., 2009, Zhang et al., 2015 & Polverino et al., 2016).***

Increased expression of APRIL is noted in most of hematological malignancies. IT is essential for the survival of normal and malignant B lymphocytes, and altered expression of APRIL or its receptors have been reported in various B-cell malignancies including B-cell non-Hodgkin's lymphoma, chronic lymphocytic leukemia, Hodgkin's lymphoma, multiple myeloma, and Waldenstrom's macroglobulinemia ***(Moreaux et al., 2009).***

From this context, this study was done to investigate the possible relationship between APRIL and AML. In this study, APRIL mRNA gene expression level was measured in 50 de novo AML patients, together with 20 normal age and sex matched healthy unrelated controls using Real-Time Quantitative Reverse Transcriptase Polymerase Chain Reaction (RTQ-PCR).

We found that APRIL mRNA expression level was significantly higher in AML patients than in controls **($p<0.001$).** Mean expression levels were 4.86±2.4 in AML patients versus 0.53±0.40 in controls.

Our results were in accordance with **Huang et al., (2010)** who found that APRIL mRNA expression level was significantly higher in AML patients than that in normal controls **($p < 0.05$),**but they didn't find a statistically significant difference when comparing APRIL expression levels in ALL and CML patients with controls ($p > 0.05$). So, they concluded that APRIL gene over expression exits in AML patients only. They also added that APRIL protein produced by AML cells probably plays an important role in abnormal proliferation and drug-resistance of AML cells.

In 2008; Bonci & colleagues found that APRIL mRNA and its protein, including the secreted form, are expressed in leukemic cells of patients with M0, M2 and M4 AML subtypes but not in normal hematopoietic progenitors. They added

that AML blasts express considerable levels of APRIL protein and that its expression results in increased resistance to drug-induced apoptosis.

Our findings were in agreement with **Bolkun et al., (2015)** who also found higher expression levels of APRIL in AML patients compared to healthy controls.

This could be explained by **Matthes et al., (2011)** findings who found that APRIL production is induced during hematopoiesis in myeloid cells by non-lineage-committing factors such as stem cell factor, thrombopoietin, IL-3, and FMS-like tyrosine kinase 3 ligand. They also found that APRIL production, both in the human and mouse systems, peaks in myeloid precursor cells, before dropping in fully mature granulocytes. So, myeloid precursor cells are producing high amounts of APRIL before getting fully mature and reaching the blood circulation.

This finding was supported by **Maia et al., (2011)** findings who stated that APRIL is expressed by cells of the BM microenvironment known to support leukemia, as well as by the leukemia cells themselves.

In the current study, when we compare results of APRIL expression level on patients outcome, after induction of chemotherapy; we found higher expression levels in patients who didn't achieve CR compared to patients who achieved CR , the difference was statistically significant **($p<0.001$)**

Our findings were in accordance with **Bolkun et al., (2015)** who found that the concentration of APRIL was significantly lower in AML patients with complete remission compared to non-respondent patients.

These findings could be explained by **Bonci and colleagues (2008)** who found that APRIL expression in normal hematopoietic progenitors increase resistance to chemotherapeutic drugs-induced apoptosis. On the other hand; blocking APRIL function by recombinant soluble APRIL receptors increased chemotherapeutic drugs-induced cell death in AML cells. These results indicate that APRIL acts in an autocrine fashion to protect AML cells from drug-induced death. These data suggest that APRIL activity is part of AML blast response to cytotoxic agents and may play an important role in protecting the cells from cell death induced by exogenous stimulation, possibly contributing to chemotherapy resistance.

The anti-apoptotic activity of APRIL has been demonstrated in B cell lymphoma, multiple myeloma and B-CLL cells, suggesting a general oncogenic role for APRIL in haematological malignancies ***(Bolkun et al., 2015).***

Previous researchers found that the anti-apoptotic protein Bcl-2 is induced by APRIL in B-cell lymphoma ***(Chiu et al., 2007).*** Also, high Bcl-2 expression is associated with poor chemotherapy response especially in AML patients ***(Tóthová et al., 2002).*** So, when **Bonci and colleagues (2008)** investigated the relationship between APRIL and Bcl-2 in AML by western blotting analysis; they found that exogenous expression of APRIL up-regulated Bcl-2 in CD34+ cells, whereas APRIL neutralization resulted in Bcl-2 downregulation in primary AML cells, suggesting that APRIL protects AML from chemotherapeutic drugs through the upregulation of Bcl-2. Thus, APRIL expression promotes the resistance to chemotherapy of normal myeloid progenitors, while APRIL neutralization considerably increases the cytotoxic activity of chemotherapeutic drugs against AML blasts. This ability to promote the resistance to chemotherapeutic drugs in AML suggests a general oncogenic role for APRIL in hematologic malignancies ***(Bonci et al., 2008)***.

In this study, when we compared patients′ response to therapy i.e those who achieved CR versus those who didn't achieve CR as regards laboratory and clinical variables, showed; high significance for APRIL gene level ***(p<0.001)***,being higher in patients who didn't achieve CR, TLC ***(P<0.001),*** also higher in patients who didn't achieve CR, platelets count ***(p=0.047)***, were lower in patients who didn't achieve CR, and age ***(p=0.019)***, as patients who didn't achieve CR were older in age than those achieved CR. While there was no statistically significant difference as regards BM blast percentage, gender, hepatomegaly, splenomegaly and lymphadenopathy (p>0.05) between the two groups of patients **(Table 3).**

As far as we know, no other study compared results of APRIL gene expression between CR and no CR patients, but our results could be explained by ; as APRIL expression level were higher in patients who didn't achieve CR (bad prognosis) , also higher TLC, lower platelets count and older patients age were of the bad prognostic signs in AML patients which indicates disease progression.

In the current study, when we correlated APRIL gene levels with different clinical and laboratory data of patients; we found a significant good positive correlation with TLC ***(r=0.743.p<0.001)***, significant weak positive correlation with age ***(r=0.296, p=0.037)***and a negative correlation with platelets count ***(r= -0.443,p=0.001)*** and no correlation with Hemoglobin level and BM blast % **(Table 4).**

In agreement with our study, **Huang and colleagues (2010)** only found a positive correlation between APRIL mRNA expression level and white blood cell count ($p < 0.05$) in AML patients but contrary to our findings; didn't found any other correlations.

Contrary to our findings, **Bolkun et al., (2015)** found that concentration of BAFF not APRIL was positively associated with WBC count, also found a positive correlation between the concentration of APRIL and the counts of blastic cells in a bone marrow smear.

We didn't find any statistically significant difference when comparing APRIL gene level with different clinical variables as gender, hepatomegaly, splenomegaly and lymphadenopathy (p>0.05).

Our findings were in accordance with **Huang and colleagues (2010)** who found that APRIL mRNA expression level are not related with extramedullary infiltration. The previous studies done on APRIL in AML **(Bonci et al., 2008, Matthes et al., 2011& Bolkun et al., 2015)** didn't report if there was a difference or not.

In conclusion, our findings suggest that APRIL (A Proliferation Inducing Ligand) is significantly associated with acute myeloid leukemia (AML) and that its level might serve as an indicator for disease progression. APRIL might be an indicator for poor prognosis and treatment resistance in AML patient; therefore, APRIL antagonists could be used in the treatment of AML.

Conflict of interests: The authors have no conflicts of interests to declare.

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