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Mammals Target Protein of Rapamycin (mTOR) Expression in Adult Acute Myeloid Leukemia Patients

YASSER H. EL-NAHASS, M.D.*; EMAN A. EL-TONSY, M.D.**; MOHAMED A. EL-RAHEEM, M.D.**; NAHLA F. OSMAN, M.D.**; REHAM EL-ZAYAT, M.Sc.** and FATMA A. EL-REFAEY, M.D.*

The Departments of Clinical Pathology, NCI, Cairo University and Clinical Pathology, Faculty of Medicine, Menoufia University**, Egypt*

ABSTRACT

Background: Acute myeloid leukemia (AML) etiology comprise many deregulated mechanisms that results from mutations in key genes; one of which, the mammals target protein of rapamycin (mTOR), enhances proliferation of abnormal hematopoiesis and contribute to the pathogenesis of AML.

Aim: To evaluate and correlate mTOR expression to different hematological parameters, blast percent, cytogenetic risk groups, response to induction chemotherapy and survival in AML patients.

Patients and Methods: Fifty AML patients (34 males and 16 females) with a median age 42 years (16-73) were included. mTOR expression was assessed by real time quantitative PCR (RQ PCR) using normalized copy number in relation to normal healthy controls.

Results: mTOR expression did not show an association with age, gender, organ involvement or complications of therapy (p=0.799, 0.349 and 0.704, 0.905, respectively). However, high mTOR expression was associated with *FLT3*-ITD (p=0.017) and blasts percent in the bone marrow (BM) (p=0.045) while its low expression was associated with M3 phenotype and BM complete remission (CR) at day 28 of induction chemotherapy (p=0.017). In multivariate analysis, overall survival (OS) showed significant association with low mTOR expression in the CR group (p=0.019).

Conclusion: mTOR association with *FLT*-3ITD and BM CR at day 28 underscores its importance as a molecular risk factor in AML. mTOR signaling pathway needs intensive focus and is advised to be assessed prior to therapy in order to predict response to induction therapy in AML.

Key Words: *mTOR – AML – FLT-3ITD.*

INTRODUCTION

Acute myeloid leukemia (AML) is characterized by a deregulated proliferation of immature myeloid progenitors leading to the accumulation of leukemic blast cells in the bone marrow (BM) and inhibition of normal hematopoiesis. It has a low complete remission (CR) and short disease free survival (DFS) **[1]**. Deregulated mechanisms result from mutations of key genes that lead to aberrant and constitutive activation of cellular pathways. This aberrant activation enhances cell growth and mediate anti-apoptotic responses which results in deregulation of normal hematopoiesis and promote malignant transformation in AML **[2,3]**.

Mammals target protein of rapamycin (mTOR) is a downstream effector of phosphatidylinositol 3-kinase (PI3K)/protein kinase B (AKT) and liver kinase B1 (LKB1)/adenosine monophosphate-activated protein kinase (AMPK) pathway. Its function is to control cell growth, proliferation and survival through essential signaling pathway **[4]**. Deregulation of mTOR signaling pathway may enhance proliferation of abnormal hematopoiesis, and contribute to the development leukemogenesis **[5]**.

A major problem in treatment of AML is the inability to efficiently target and eliminate leukemia initiating cells (LICs). Most of LICs are quiescent and not sensitive to various chemotherapeutic drugs that target and kill rapidly dividing blast cells **[6]**. This fact explains the difficulty in treating AML with chemotherapy and the relapses seen, in a high percentage of patients, despite initially achieving CR with classical chemotherapy regimens. Aberrant activation of pro-survival signaling cascades in leukemia stem cells may also promote their survival providing a potential therapeutic target for the treatment of AML **[6,7]**.

The PI3' kinase/AKT/mTOR pathway is a key regulatory network of signaling cascades in mammalian cells. It plays a major role in regulating important cellular signaling pathways important for cell activity and function. Controlling activation of the mTOR kinase and its downstream effectors will lead to regulation of mRNA translation of genes that encode for prooncogenic proteins which promote malignant cell survival **[8]**.

This study was undertaken to evaluate mTOR expression and correlate it with various biological characteristics, Fms-like tyrosine kinase Internal tandem duplication (*FLT3*-ITD), response to induction chemotherapy and survival in AML patients.

PATIENTS AND METHODS

Between 2015 and 2016, 50 AML patients (34 males and 16 females) presenting to the Medical Oncology Department, NCI, Cairo University, prior to any treatment were included. The study was carried in the Clinical Pathology Department of NCI.

Patients:

Inclusion criteria were AML phenotype and age ≥16yrs. Exclusion criteria were acute biphenotyic leukemia. All patients were subjected to completehistory and physical examination with particular attention to age, gender, presenting symptoms, performance status, presence of fever defined as a temperature of 38°C for more than 1 hour (taken orally), or a single elevation above 38.5°C, signs of infections, bleeding manifestations, hepatomegaly, splenomegaly, lymphadenopathy and symptoms of CNS infiltration (headache, vomiting, blurring of vision). All patients gave written informed consent and the study was approved by the Institutional Review Board of the NCI, Cairo University.

Methods:

Patients were evaluated for mTOR expression by Real time Quantitative PCR (RQ-PCR). Leukemias were characterized according to their morphology [(French-American-British (FAB) classification)], karyotype, immunophenotyping and *FLT3* mutations (*FLT3*-ITD). Karyotype risk groups were defined according to the US Southwest Oncology Group criteria, which define the following chromosomal abnormalities as high-risk cytogenetic status: del(5q)/5, 7/del(7q), abn 3q, 9q, 11q, 20q, 21q, 17p, t(6;9) t(9;22) and complex karyotypes (>3 unrelated abnormalities) **[9]**. *FLT3* ITD was performed after extraction of genomic DNA as previously described **[10]**. CR at day 28 was defined as normocellular BM with <5% blasts and normal blood counts.

Laboratory investigations included Full Blood count; total leukocytic count (TLC), blasts %, hemoglobin (Hb) level and platelet count. Biochemical analysis includedserum urea, creatinine, uric acid, liver function tests [serum bilirubin, alkaline phosphatase, AST, ALT, serum albumin], serum LDH, fasting and 2 hours postprandialblood sugar.

BM aspirate smears were examined for assessment of cellularity and blasts percentage. Cytochemistry including myeloperoxidase (or Suddan Black) supplemented by Periodic acid shiff, acid phosphatase; specific esterase and non-specific esterase was done when necessary. All patients were classified according to the French-American-British [FAB] classification.

CSF was examined in patients with symptoms of CNS involvement, AML M4, M5 and patients with TLC >100x10^9/L.

Immunophenotyping on PBL or BM blasts was performed at diagnosis using 4 color flow cytometry (Coulter Epics, XL) for confirmation of myeloid lineage **[11]**. An acute leukemia panel was run for all patients including: Pan leucocytic marker (CD45), myeloid markers (MPO, CD33, CD13, and CD14), B-lymphoid markers (CD19, CD10, and CD22), T-lymphoid markers (CD3, CD4, CD5, CD7, and CD8), the stem cell marker CD34 and the lineage non specific marker (HLA-DR).

mTOR detection by RT-PCR:

RNA Extraction: Total RNA was extracted from 300ul PBL or BM sample using a column precipitation procedure (Qiagen, USA) according to manufacturer's instructions. Sample was stored at –70°c till used. RNA quality was assessed by gel electrophoreses on an ethidium bromide stained 1% agarose containing 2.2 mol/L formaldehyde.

Reverse Transcription: cDNA synthesis was performed by reverse transcriptase using Gene Amp Gold RNAPCR Reagent Kit (Applied

Biosystems, USA), 1ug RNA was used in 20uL volume including 1xRT-PCR buffer, 2.5mM Mg CL2, 1mM dNTPS blend, 10 U/20 ulRNase inhibitor, 10mM DTT, 1.25uM Random Hexamer and 15U/20ul Multiscribe Reverse Transcriptase enzyme. Cyclic conditions consisted of 25°C for 10min and 42°C for 1 hour.

PCR for detection of mTOR gene transcripts:

PCR was performed on Step One cycler (Applied Biosystem) with 5ul cDNA using QuantiFast Probe RT-PCR Plus kit (R and D systems, US Biological, USA) using 2 X QuantiFast mix 2 (12.5ul) (probe) containing Hot Start Taq Plus DNA polymerase, Quantifast Probe RT-PCR Plus Buffer, and dNTP mix (dATP, dCTP,dGTP and dTTP), ROX dye solution and adjusting reaction volume to 25ul with RNAse free water. For each sample housekeeping gene GAPDH and mTOR were amplified with their specific primers (R&D systems, US biological, USA). mTOR primer sequence was F 5'-CCATCCAATCTGATGCTGGA-3' and R 5'-GGTGTGGCATGTGGTTCTGT -3' and detected on VIC and FAM channels, respectively.

Cycling conditions: Initial denaturation and Hot start activation at 95°C for 3min was performed followed by 45 cycles of amplification consisting of denaturation at 95°C for 3 seconds, annealing/extension at 60oC for 30 seconds. Each run included a non template control (NTC).

Normalized copy number equation used ($2^{-\Delta\Delta CT}$) to evaluate mTOR expression based on DCT of sample calculated as mTOR (FAM)-GAPDH (VIC), then DDCT using the equation DCT sample-DCT normal healthy control.

Statistical methods:

Data was analyzed using SPSS win statistical package version 21 (SPSS Inc., Chicago, IL). Numerical data were expressed as mean and standard deviation (SD) 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. Comparison between two groups regarding quantitative variables was done using Mann-Whitney test (non-parametric *t*-test). Survival analysis was done using Kaplan-Meier method and Cox-regression method. Comparison between two survival curves was done using Log-rank test. All tests used were two-tailed. *p*-value ≤0.05 was considered significant.

RESULTS

Fifty AML patients (34 males and 16 females), with a median age of 42 (16-73) years, were included in the study. According to FAB classification, 12 (24%) were M1, 13 (26%) M2, 6 (12%) M3, 14 (28%) M4, 3 (6%) M5 and 2 (4%) M6. Clinical and Hematological characteristics of AML patients are presented in Table (1).

Table (1): Clinical and hematological characteristics of 50 adult AML patients.

Parameter	Mean ± SD	Median (range)	Parameter	No.	%
TLC $x10^9$/L	52.62±50.57	38.40 (1.20-187.4)	Normocellular BM	7	12.0
Hb: gm/dl	7.89±2.02	7.80 (3.0-13.10)	Hypocellular BM	3	6.0
PLT.$x10^9$/L	59.90±53.71	36.50 (6.9-204.0)	Hypercellular BM	40	80.0
PB Blasts: %	43.0±26.42	36.50 (1.0-97.0)	Splenomegaly	15	30.0
BM blasts: %	60.92±26.71	66.50 (12.0-99.0)	Hepatomegaly	24	48.0
			Lymphadenopathy	11	22.0

TLC: Total leukocytic count. Hb: Hemoglobin. PLT: Platelets. PB: Peripheral blood. BM: Bone marrow.

Cytogenetics: 11 (22%) patients were classified as favorable risk; 5 were t(15;17) positive, 6 (12%) core binding factor positive while 23 (46%) patients were in the intermediate and 16 (32%) patients were in the high risk category.

mTOR: Mean mTOR expression in the 50 AML patients was 9.91±12.98 with a median of 5.6 (0.2-64). Fig. (1) presents mTOR expression at the individual case level. *FLT3* ITD was positive in 19 patients (38%) and negative in 31 (62%).

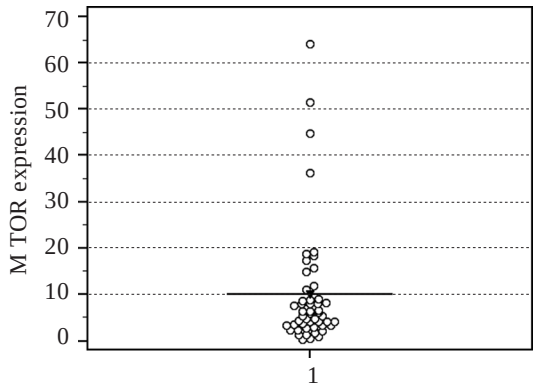

Fig. (1): mTOR expression in 50 adult acute myeloid leukemia patients.

mTOR expression was not associated with age or gender (Table 2). No association was either encountered with organomegaly or lymphadenopathy (Table 3). A higher mTOR expression was associated with *FLT3* ITD (p= 0.035) and poor risk cytogenetics patients versus favorable and intermediate (p=0.042) (Table 3).

Table (2): mTOR expression in relation to age and gender in 50 adults AML patients.

Parameter	No.	mTOR expression			p
		Mean ± SD	Range	Median	
Age: years					
16-40	21	9.52±13.55	0.42-64.0	5.20	
41-60	23	10.04±11.97	0.20-51.44	6.40	0.799
>60	6	10.81±16.84	1.58-44.83	3.78	
Gender:					
Male	34	10.24±14.65	0.20-64.0	6.03	0.349
Female	16	9.22±8.80	3.30-36.25	4.98	

Table (3): mTOR expression in 50 adult AML patients in relation to organomegaly and genetic/cytogenetic parameters.

Parameter	Present		Absent		p
	No.	m-TOR Expression	No.	m-TOR Expression	
Splenomegaly	15	7.87±8.23 6.40 (0.42-36.25)	35	10.79±14.57 4.30 (0.20-64.0)	0.649
Hepatomegaly	24	6.82±7.35 5.53 (0.20-36.25	26	12.77±16.21 5.86 (0.88-64.0)	0.332
Lymphadenopathy	11	5.36±4.16 5.20 (0.20-15.67)	39	11.20±14.32 6.39 (0.88-64.0)	0.276
FLT3-ITD	19	13.86±14.66 8.06 (1.58-51.44)	31	7.49±11.42 4.59 (0.20-64.0)	0.035
t(15;17)	5	3.45±1.96 3.30 (0.88-6.39)	45	10.63±13.49 6.20 (0.20-51.44)	0.073
Favorable	6	14.60±24.34 6.03 (0.42-64.0)	44	9.27±10.93 5.26 (0.20-51.44)	0.929
Intermediate	23	7.85±10.39 4.63 (0.20-51.44)	27	11.67±14.80 6.39 (0.42-64.0)	0.442
Poor	16	13.14±12.37 8.43 (1.30-44.83)	34	8.40±13.16 4.61 (0.20-64.0)	0.042*

A border line significantly lower mTOR expression was found in M3 vs. all FAB subgroups (3.54±1.77, median 3.40 (0.88-6.39) vs. 10.78±13.61, median 6.30 (0.20-64.0); p= 0.056). A weak significant positive correlation was encountered between m-TOR expression and BM blasts (r=0.285, p=0.045) but no correlation was encountered with TLC, Hb, Platelet count or PB blast %.

Response to therapy: At day 28, there was 19 responders (38%) and 31 non-responders (62%). m-TOR expression was significantly lower in the responders compared to non-responders (4.43±2.41, median 4.14 (0.42-8.86) vs. 13.28±15.52, median 7.96 (0.20-64.0), p=0.017*).

Survival: After a median follow-up of 1 year, median DFS was 5.6 months (4.2-10) and OS was 5.8 months (3.6-8.2) (Fig. 2). Patients who achieved CR had significantly longer survival than those who did not achieve CR (p=<0.001, Fig. 3). There was significant neg-

ative correlation between m-TOR expression and survival in those who achieved CR (r_S= –0.437, p=0.016) or did not achieve CR (rs =0.531, p=0.019) but only a trend for DFS in the CR remission group (r_S=–0.430, p=0.075) but not for the non-CR group (r_S=0.0207, p=0.396).

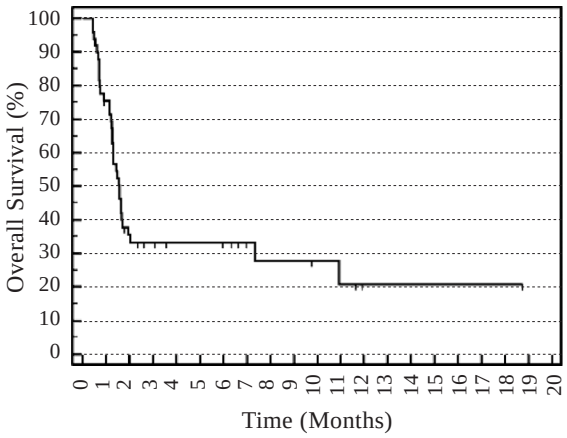

Fig. (2): OS of 50 adult AML patients.

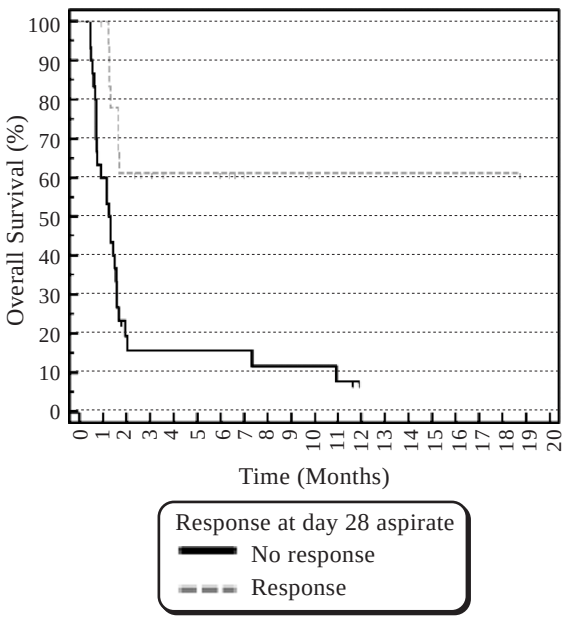

Fig. (3): OS according to CR at day 28 in 50 adult AML patients.

mTOR expression was comparable between the 34 patients who died (9.74±12.27, range 0.20-51.44, median 4.98) and the 16 alive (10.29±14.79, range 2.30-64.0, median 6.30) (p=0.045). There was no impact of mTOR expression level on OS (Fig. 4).

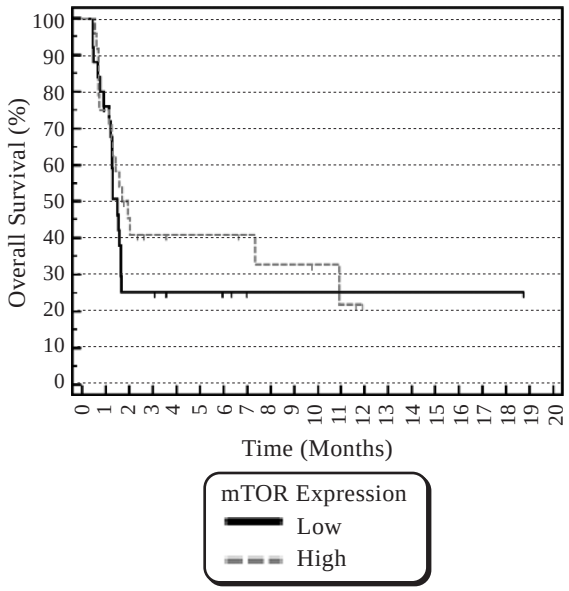

Fig. (4): OS according to mTOR expression level in 50 AML patients (p=0.327).

DISCUSSION

mTOR signaling pathway is crucial to cell survival and proliferation. Deregulation of this pathway is associated with poor prognosis, resistance to conventional chemotherapy and relapse in AML **[12,13]**.

In this study, mTOR expression was not associated with age, gender or organ involvement in AML patients. In concordance with our data, Gallay et al., found no association between age, gender, FAB classification or leukocytosis **[14]**.

We found that mTOR expression was significantly associated with achievement of CR at day 28 as its expression was significantly lower in patients who achieved CR compared to those who did not achieve CR at the end of induction therapy (4.14 vs. 7.96, p=0.017). This finding is pointing to an association between achievement of CR and lower mTOR expression and stated by several reports that claimed high mTOR expression as a mechanism of chemotherapy resistance **[12,13]**. A significant difference in mTOR expression was also found between APL and non-APL patients as mTOR expression was lower in M3 vs. other FAB subgroups adding to the better prognosis in APL. However; this association was not reported by others **[14]**.

Our data showed that *FLT3*-ITD positive patients displayed a significant higher mTOR

expression vs. wild type patients (8.06 vs 4.59). Previously, it was stated that FLT3 stimulates *PI3K/AKT/mTOR* pathway that promotes survival of *FLT3*-mutated AML cells, which means that *FLT-3* may be an upstream regulator of this pathway and can be taken as a target for the treatment of *FLT3* mutated AML patients **[15]**. In concordance with our data, several studies showed that mTOR up-regulation was due to FLT3, RAS or CKIT activation and patients displayed a shorter OS **[8,15,16]**. However, in dis-concordance with our results, no correlation was found between *FLT3* ITD and level of phosphorylation of its up-regulator AKT **[14]**.

In this study, 16 patients (32%) were in the high risk cytogenetics category. High mTOR expression was significantly associated with poor risk cytogenetics in this group. This finding was not found in favorable and intermediate risk categories. In one report, the level of phospho-Akt on Thr308, which leads to an up-regulated mTOR signaling, was significantly associated with high-risk cytogenetics and poor outcome **[14]**. In this study, high-risk cytogenetic samples were more sensitive to the Akt inhibitor, Akti-1/2, compared with intermediate-risk cytogenetic samples, suggesting that targeting Akt could be beneficial in this subgroup of AML patients with a poor prognosis on conventional chemotherapy. mTOR up-regulation was assessed by FCM and the ratio of mean fluorescence intensity on Threonine 308 was a continuum ranging from 0.3-2.87 in this study.

In our study, we could not find an association between mTOR expression and OS of the whole AML group, although proved by others **[14]**. The explanation of this controversy between results may be due to that, in their study, only patients with threonine 308 phosphorylation were associated with a shorter DFS and OS which was not investigated in our study **[14]**. As aberrant activation of the *PI3K/AKT/mTOR* pathway promotes malignant cell proliferation and survival; several studies have sought to examine the implications of constitutive activation of this pathway in tumori-genesis **[17,18]**. In contrast to others, Tamburini et al found that PI3K activation is an independent prognostic factor in AML and is associated with better OS **[19]**. The reasons for these different findings is not yet clear. One hypothesis for the lower relapse rate in PI3K+ve patients is that it may drive immature leukemic cells into S1 phase of the cell cycle increasing their susceptibility to S1 chemotherapies **[20]**. When analyzed by flow cytometry, only AKT thr 308 phosphorylation but not Ser473 has been found to correlate with high risk karyotype and shorter OS **[14]**.

There is now extensive evidence that deregulation of this pathway contributes to the tumorigenic potential, a more aggressive phenotype and poorer prognosis in several malignancies **[21,22]**. In addition, activation of this pathway has been associated with chemotherapy resistance **[23]** underscoring the importance of this signaling cascade as a therapeutic target for the treatment of AML.

mTOR expression did not impact OS of AML patients in our study. This may be due to numerous biological and molecular markers that may play a role in risk categorization of AML. In addition, many activators of mTOR pathway apart from *FLT3*ITD as *PTEN* deletion, AKt phosphorylation and mutation, cKIT and RAS has to be investigated, However, we found that high median mTOR expression was associated with failure to achieve CR at day 28 in addition to its positive correlation with BM blast percent. OS was significantly associated with low mTOR expression in the CR group at day 28 vs. a high mTOR expression in the group of patients who failed CR. Though, it is not clear if the better OS survival in the CR group was due to the lower mTOR expression or due to achievement of CR by itself, or absence of *FLT3* ITD in this group, yet a major goal for the development of new approaches for the treatment of AML is to enhance the anti-leukemic effects of standard chemotherapeutics and to design effective combinations targeting mTOR pathway in order to improve CR rates at day 28 and consequently survival.

There is emerging evidence that targeting of *PI3K/Akt/mTOR* by specific agents elicit much more potent responses against early leukemic precursors *in vitro*. Recent studies have shown that combinations of such agents with cytarabine result in enhanced anti-leukemic responses *in vitro*, raising the prospect and potential of use of these agents in combination regimens for the treatment of AML. For all these reasons, there has been a major interest in the development of pharmacologic inhibitors of the *PI3K/AKT/mTOR* pathway for hemato-

logical malignancies, which has further intensified after the detailed mapping and characterization of the pathway **[24,25,26]**.

In conclusion: mTOR is a tempting signaling pathway to be studied and targeted in AML. Its association with *FLT3* ITD and BM% CR after induction therapy underscores the importance of this pathway as another molecular risk factor to be added to various molecular genetic aberrations in AML.

Conflict of interest:

The authors declare no conflict of interest.

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