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All-Trans-Retinoid Acid (ATRA) Induced Upregulation of Autophagy Related Proteins (LC3-1, II and Beclin 1) in Acute Promyelocytic Leukemia

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Abstract: Treatment of Acute Promyelocytic Leukemia (APL) with All-Trans Retinoic Acid (ATRA) represents a paradigm in targeted cancer therapy as these drugs cause clinical remission by affecting the stability of the fusion oncoprotein promyelocytic leukemia (PML)/Retinoic Acid Receptor Alpha (RARA). Previous studies have implicated in the main mechanism involved in therapy induced PML/RARA degradation. Investigated the effect of ATRA on the autophagy responses LC3-1, II and Beclin 1 proteins and if ATRA-induced upregulation of autophagy and Beclin 1 in the acute promyelocytic leukemia cells or not. Study included 22 subjects diagnosed as Acute Promyelocytic Leukemia (APL) (M3), also included 20 healthy subjects as a control selected to match the study. Peripheral blood and BM examination, Immunophenotyping, Conventional cytogenetic, Western blotting for LC3-I and LC3-II formation expression and Enzyme Link Immunosorbent Assay (ELISA) kit for Beclin-1 (BECN1). Both beclin-1 and LC3-11 were significantly increased in patient than control group before induction therapy. Proteins induced autophagy LC3-11 was increased after induction therapy than before in APL cells and that indicate autophagic degradation. The present study indicate that the ATRA-induced up regulation of autophagy protein and Beclin-1 and LC3-1, 11 in the acute promyelocytic leukemia cells.

Key words: Leukemia, PML, RARA, APL, autophagy

INTRODUCTION

Leukemia, a malignant hematopoietic tumor, is a cancer of the blood or bone marrow which is characterized by the abnormal proliferation of white blood cells and is the 6th most common form of human cancer worldwide (Tallman and Altman, 2009).

Acute Myeloid Leukemia (AML) is characterized by an accumulation of abnormal hematopoietic progenitor cells which exhibit the morphology of a certain stage of myeloid differentiation in the bone marrow and the peripheral blood (Grignani *et al.*, 1998). The blocked differentiation restrains progenitor cells from maturing and subsequently undergoing apoptosis and it may also enhance their self-renewal capabilities. Unlike normal hematopoietic cells that require cell extrinsic signals to maintain metabolism and survival, leukemia cells often express constitutively active oncogenic kinases that promote these processes independent of extrinsic growth factors. When cells receive insufficient growth signals or

glucose metabolism decreases and the self-digestive process of autophagy is triggered to degrade bulk cytoplasm and organelles. Given its potential function in metabolism and cell survival, manipulation of autophagy may provide a critical means to eliminate certain type of leukemia cells (Bernardi and Pandolfi, 2007).

The clinical picture of APL is characterized by a rapidly increasing bleeding tendency which is due to pronounced coagulation disorders and severe thrombocytopenia. The microgranular variant (AML M3v) is a morphologically distinguishable special APL form which is mainly associated with increased leukocyte counts. The APL constitutes a hematological emergency which requires immediate diagnostic confirmation and specific therapeutic measures (Nasr *et al.*, 2008). Diagnosis requires the APL-specific chromosomal translocation t(15,17)(q22,q21) and/or the fusion gene PML/RARA (Nasr *et al.*, 2009).

The introduction of All-Trans-Retinoid Acid (ATRA) in APL therapy doubled the cure rate relative to

chemotherapy alone. Remission rates of 80-90% and rates of long-term survival exceeding 75%, were reached with the combination of ATRA and anthracycline-containing chemotherapy (Chomienne *et al.*, 1996). Yet, more than 10% of the patients treated in studies die in the course of induction therapy, mostly as a result of bleeding complications. All-*Trans*-Retinoic Acid (ATRA), a derivative of retinoic acid, reverses the differentiation block of promyelocytic blasts and induces the development of mature neutrophils, associated with a regression of the coagulation disorders within a few days. The extraordinarily high efficacy of ATRA is APL-specific (Lallemand-Breitenbach *et al.*, 2008).

Autophagy is a ubiquitous process in which “bad” cytosolic molecules damaged organelles or invaded pathogens are sequestered within double-membrane vesicles called autophagosomes that deliver their contents to lysosomes for degradation and/or recycling of the resulting macromolecules. Indeed, autophagosomes were found to specifically target damaged mitochondria. Peroxisomes and protein aggregates that lead to DNA damage and genomic instability, thereby maintaining intracellular organelle and protein homeostasis (Tatham *et al.*, 2008).

Autophagy may also provide nutrients through lysosome degradation of intracellular components of mitochondrial oxidation. Autophagy is more frequently compromised in solid cancer cells compared with their normal counterparts and acts as a protective mechanism in response to both extracellular and intracellular stress (Shen *et al.*, 2004). Heterozygosity for the autophagy-essential gene, Beclin1 led to greatly increased rates of tumorigenesis, possibly because of genomic instability when autophagy is reduced (Bence *et al.*, 2001).

The PML/RARA oncoprotein is known to be prone to aggregation, a feature that makes it a good substrate for autophagic degradation. The aggregation-prone characteristics of PML/RARA are further substantiated by showing that this protein chimera is considerably more effectively extracted by buffers containing the protein denaturation reagent UREA compared with buffers containing the detergents SDS or Triton X-100 (Pankiv *et al.*, 2007). Its ability to form protein aggregates is possibly attributable to the PML moiety of the fusion autophagy and proteasome-dependent degradation may thus cooperate in therapy-induced clearance of the APL-associated oncoprotein. In line with this finding, proteolytic crosstalk has been reported to clearly exist between the autophagy and ubiquitin-proteasome systems (Hu *et al.*, 2009).

Furthermore, proteolytic degradation of PML/RARA by caspases and lysosomal proteases has also been reported, suggesting the existence of multiple proteolytic pathways with a potential to target PML/RARA for degradation. However, because autophagy appears to become markedly induced in the presence of all-*trans* retinoic acid and arsenic trioxide, this degradation pathway may have a determinant role in therapy-induced PML/RARA clearance. Thus, both all-*trans* retinoic acid and arsenic trioxide induce clinical remission in APL patients by stimulating mTOR-dependent autophagy and causing concomitant autophagic degradation of the APL-associated oncoprotein PML/RARA (Nedelsky *et al.*, 2008). In patients with the characteristic morphological picture of an APL and severe coagulation disorders, the onset of therapy with ATRA is justified even before genetic analysis confirms the diagnosis. ATRA is administered continuously until complete remission is obtained, at maximum up to a period of 90 days. ATRA monotherapy achieves a complete remission in 80-90 (%) of the patients with newly diagnosed APL. However, long-term remissions are rare under ATRA monotherapy (Rowe and Tallman, 1997). The aim of this study was to investigate the effect of ATRA on the autophagic responses LC3-1, II and Beclin 1 proteins.

MATERIALS AND METHODS

This study was conducted in Mansoura Pediatric Hospital and Oncology center in Mansoura University hospital. It included that 22 patients (13 females and 9 males) diagnosed as Acute Promyelocytic Leukemia (APL) (M3). The study also included 20 healthy subjects (12 females and 8 males) as a control selected to match the study group in age and gender. The Ethics Committee of Mansoura, Faculty of Medicine approved the study protocol. Informed consent was taken before testing. All patients were subjected to the following.

Patient's history and physical examination and full laboratory investigation include the following:

Peripheral blood and BM findings: In the AML cases, clinical studies of the PB, BM cellularity, according to WHO classification 2008 (Swerdlow and Campo, 2008).

Immunophenotyping: We reviewed all available data, including Peripheral Blood (PB) smears, Bone Marrow (BM) aspirates, cytochemical staining immunophenotyping of cells was performed with flow cytometry against CD3, CD41a, CD14, CD34, CD33, CD20,

CD5, CD10, CD19, CD64, CD11c, CD13, CD117, CD56, CD2, CD7, HLA-DR, cytoplasmic CD22, cytoplasmic myeloperoxidase, cytoplasmic CD3 and cytoplasmic CD79a (coulter, San Jose, CA) (Dunphy *et al.*, 2004).

Conventional cytogenetics: Chromosomal analyses were performed by examining short-term cultures of BM specimens according to standard conventional cytogenetic protocols. At least 20 cells in metaphase were analyzed in each case. Clonal abnormalities were classified according to the 2009 International System for Human Cytogenetic Nomenclature guideline (Golomb *et al.*, 1976).

Fluorescence *in situ* hybridization: Fluorescence *in situ* Hybridization (FISH) analyses were performed to confirm translocation and the presence of t (15;17). These FISH analyses used pellets of cells remaining after conventional cytogenetic studies. Slides for FISH were prepared by using cells harvested for conventional cytogenetics and processing them for FISH according to the manufacturer's guidelines (Abbott V lysis, Des Plaines, IL, USA). (Gurrieri *et al.*, 2004). Analyses were performed on cells in either interphase or metaphasedual-fusion probe (Abbott V lysis) was used to identify t (15;17) and its variants (Rowley *et al.*, 1977).

Western blotting for LC3-I and LC3-II formation expression: First extracted in ice-cold Triton X-100 containing lysis buffer (50 mM NaCl, 10 mM Tris, 5 mM EDTA, 1% TritonX-100+protease and phosphatase inhibitor cocktails), the whole cell extract was harvested in lysis buffer. Cell lysates were then centrifuged (14000 rpm) for 10 min and the supernatants (soluble proteins) collected. The remaining protein pellets were washed with Phosphate Buffered Saline (PBS) before extraction with an SDS-containing buffer (2% SDS, 1 mM DTT, 50 mM Tris+protease and phosphatase inhibitor cocktails) to obtain SDS. The protein concentration was analyzed using a BCA assay (Thermo Fisher Scientific, Fremont, CA, USA). Protein extracts were loaded and resolved on 12% gels (Pierce, Rockford, IL) and subjected to sodium dodecyl sulfate-polyacrylamide gel

electrophoresis (SDS-PAGE). After the whole cell extract had undergone western blotting, the SDS-PAGE PVDF membrane was blocked with antibodies against Rabbit anti-LC3 (Cell Signaling, 1:5000), LC3 (Novus Biologicals, Littleton, CO, USA), PARP-1/2 (H-250) and then incubated with secondary antibodies, anti-rabbit IgG antibody (Jackson Immuno-research, West Grove, PA, USA). Actin or α -tubulin (Sigma-Aldrich) was used as an internal control (Klionsky *et al.*, 2008).

Enzyme-link immunosorbent assay (ELISA) kit for Beclin-1 (BECN1): Measurement the level of Beclin-1 (BECN1) by ELISA using USCNC life science InsUSA. Cut off 1.25 (ng L⁻¹) (Sinha and Levine, 2008).

Statistical analysis: Results were collected, tabulated and statistically analyzed using statistical package version 16 (SPSS Inc., 2007). Continuous data was expressed as were eventually appropriate statistical tests. For normally distributed parameters group, comparison was made by use of paired and unpaired Student's t-test. Mann-Whitney U-Test was used for non-normally distributed parameters. In comparing 3 groups, one way ANOVA for statistical significance, were analyzed by the Mann-Whitney U-test (2 groups). Statistical significance was defined as p \leq 0.05 normally distributed data, presented as Mean \pm SD.

RESULTS

Peripheral blood analysis of our study revealed anemia (Hb 9.36 \pm 629 g dL⁻¹) and leukocytosis (WBC 24.045 \pm 33.9 \times 10⁹ L⁻¹). All AML patients also exhibited thrombocytopenia platelet count (39 \times 10⁹ L⁻¹). The percentage of circulating blasts was variable and higher in AML patients (48%, p = 0.008). BM cellularity was nearly 100% and exhibited an increased number of blasts (60% in AML). Cytogenetic characteristics showed statistical significance (p = 00.1). Flow cytometric analysis showed a large population of myeloid blasts with the following immunophenotype; CD13, CD33, CD34, CD11c and myeloperoxidase (p = 0.001) (Table 1).

Table 1: Clinical and laboratory criteria in APL (M3) patients versus control before induction therapy

Variables	Mean \pm SD		p-value
	Patients	Control	
Age (years)	21 \pm 11.2	39 \pm 14.4	0.06
WBC count (\times 10 ⁹ L ⁻¹)	24.045 \pm 33.9	11.02 \pm 19.91	0.03
Plt count (\times 10 ⁹ L ⁻¹)	39.428 \pm 31.320	199.13 \pm 24.023	0.02
Hemoglobin (g dL ⁻¹)	9.36 \pm 629	12 \pm 312	0.01
PB blasts (%)	48.43 \pm 23.04	1.0.92 \pm 1.67	0.001
BM blasts (%)	60.85 \pm 24.88	1.64 \pm 0.25	0.07
Cytogenetic characteristics t (15-17)	10 (3-42)	(0) 0	0.001
Flow cytometric analysis: CD13, CD33, CD34, CD11c and myeloperoxidase	(20)	(1)	0.001

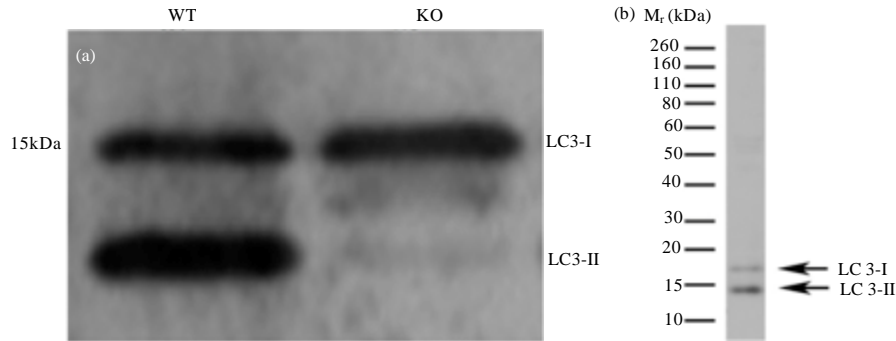


Fig. 1(a-b): Autophagic measurement by changes in LC3 localization: LC3-1 and LC3-11 (a) After treatment and (b) Before treatment

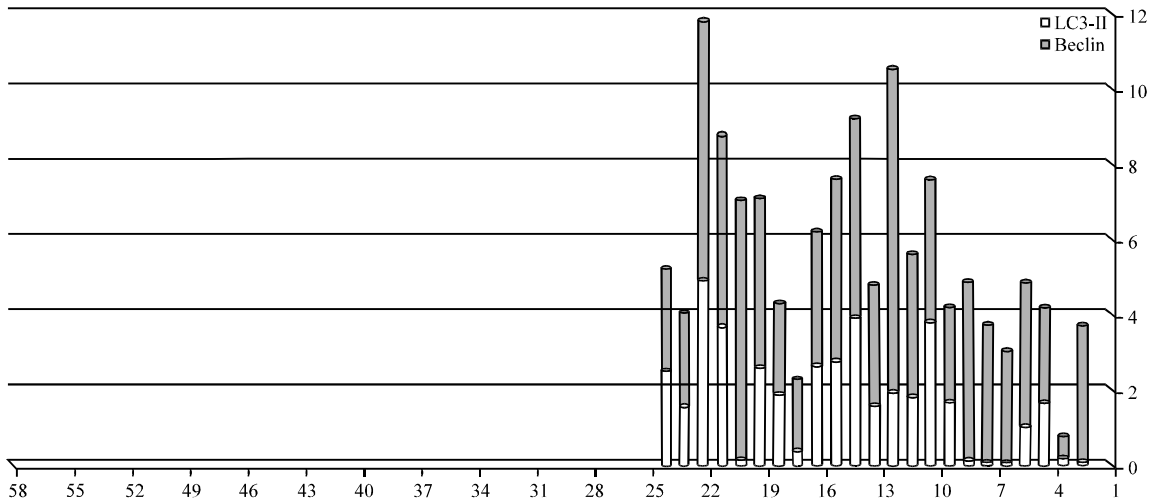


Fig. 2: Level of Beclin-1 and LC3-11 in APL (M3) patients group after ATRA induction and M3 patients after induction the level of LC3-11 and beclin -1 (indicator of autophagic activity with autophagosome formation)

Table 2: Levels of LC3-1,11 and beclin-1 in APL (M3) patients and control before treatment

	Mean±SD		Test of significance	
	Control N = 20	Patients N = 22	p	t
Serum Beclin(ng/l)	1.2±6.3	7.6±4.3	4.6	0.001*
LC3-I (kDa)	4.50±1.8	3.5±0.6	2.0	0.08
LC3-II (kDa)	7.4±3.9	13.9±1.7	5.6	0.002

There was significant increase in the level of Beclin-1 and LC3-II. On the other hand, non-significant difference with LC3-1 protein when compare with control before induction therapy (Table 2).

Autophagy can be measured by changes in LC3 localization; tracking the level of conversion of LC3-I to LC3-II provides an indicator of autophagic activity. In particular, the levels of LC3-II correlate with autophagosome formation due to its association with the autophagosome membrane. Western blotting (Fig. 1a, b)

found that, LC3 is detected as two bands, cytosolic LC3-I and LC3-II which is bound to autophagosome membrane. This makes the molecular weight of LC3-II greater than LC3-I. However, due to its hydrophobicity, LC3-II migrates faster in SDS-PAGE and therefore displays a lower apparent molecular weight (Fig. 1a, b).

Table 3 and Fig. 2 show the levels of beclin-1 and LC3-11 in the total cases of AML M3 before treatment compared to their levels in the same cases after treatment. There were significant increase in the levels of both

Table 3: Beclin-1 and LC3-11 level in APL (M3) patients group before and after induction therapy

Parameters	Before treatment	After treatment	Paired T-test	
			t	p
Serum Beclin(ng/ml)	7.6±4.3	25.49±8.76	11.04	0.001*
LC3-11 KD	13.9±1.7	22.10±18.20	0.18	0.059

markers in after treatment patients compared to before as following; beclin-1 (p = 0.001), LC3-11 (p<0.059). The levels of the markers in the AML M3 patients after treatment were significantly higher than in pre-treatment patients (p = 0.059).

DISCUSSION

Autophagy is a critical modulator of cancer cell fate in virtue of its ability to regulate cell survival, differentiation and proliferation was investigated. In the present study, we investigated the link between autophagy, cell death and differentiation in the context of ATRA-induced granulocytic differentiation of the Acute Promyelocytic Leukemia (APL). We provide, here, the first evidence showing that Beclin 1 increased after ATRA induction therapy in APL M3 and this data is in accordance with Trocoli *et al.* (2011) who demonstrating that that Beclin-1 is up regulated by ATRA in the myeloid leukemia and Beclin 1 displays a cytoprotective effect against numerous stressful situations. Such up regulations were also observed during the megakaryocytic differentiation of the chronic myelogenous leukemia, upporting the idea that autophagy and differentiation are interconnected processes (Bjorkoy *et al.*, 2005).

Our study found an increased level of LC3-11 after ATRA induction therapy (Table 3 and Fig. 2). Indicator of autophagic activity with autophagosome formation, consistent with the results of Nencioni *et al.* (2013) who reported that because ATRA and ATO-induced clinical remission is associated with degradation of PML/RARA, the ability of these drugs promote autophagy in NB4 cells. For these experiments we probed for the autophagy marker protein LC3 by Western blotting. Because LC3-II remains bound to autophagic membranes throughout the pathway, it is a useful marker to study autophagy and the LC3-II level is a good measure of autophagic activity. Suggesting that these drugs cause increased autophagic activity by using quantitative real-time polymerase chain reaction, he found that NB4 cells treated with ATRA or ATO had increased LC3 mRNA levels, indicating that these drugs also induce LC3 transcription. This newly synthesized LC3 is rapidly conjugated to LC3-II because there is no increase in the LC3-I levels (Lamark *et al.*, 2009).

Interestingly, Wang *et al.* (2008) revealed that Beclin 1 exerts an anti-apoptotic effect by impeding the binding of the pro-apoptotic protein Bad to Bcl-XL, during the cell differentiation induced by vitamin D3. Similarly, Vazquez and Colombo (2010) demonstrated that the anti-apoptotic effect *Coxiella burnetti* (a gram negative bacterium) exerts in infected host cells is prevented by Beclin 1 depletion and by the expression of a Beclin 1 mutant with defective Bcl-2 binding, suggesting that the interaction of Beclin 1 and Bcl-2 may modulate apoptosis (Klionsky *et al.*, 2008). These results support the idea that under some circumstances, the BH3-only domain of Beclin 1 may competitively disrupt the binding of pro-apoptotic proteins (e.g., Bad) to Bcl-2 or Bcl-XL, thus avoiding the induction of apoptosis. Paradoxically, the results of Ciechomska *et al.* (2009) showed that the binding of Beclin 1 to Bcl-2 does not modify the Bcl-2-mediated protection against apoptotic stimuli that initiates endoplasmic reticulum or mitochondria death signaling pathways. Thus, Beclin 1-regulated Bcl-2 anti-apoptotic function occurs only in certain circumstances (Youn *et al.*, 2013).

These findings open the question as to whether or not the accumulation of Beclin 1 during ATRA-induced neutrophil/granulocyte differentiation of APL cells exerts an anti-apoptotic function by displacing the BH3-only pro-apoptotic proteins from their binding to Bcl-2 and Bcl-XL. Further studies are also required to elucidate the molecular mechanisms underlying Beclin 1 upregulation by ATRA. Chang *et al.* (2009) reported that in contrast to the effects of normal Atg13 levels, increased expression of Atg13 inhibits autophagosome expansion and recruitment of Atg8/LC3, potentially by decreasing the stability of Atg1 and facilitating its inhibitory phosphorylation by TOR. Atg1-Atg13 complexes, thus, function at multiple levels to mediate and adjust nutrient-dependent autophagic signaling. Interestingly, in addition, autophagy is involved in a novel form of programmed necrosis that occurs in neutrophils exposed to the inflammatory cytokine, GM-CSF (Zhang *et al.*, 2009). Thus, the biological significance of Beclin 1 functions in normal neutrophil life span under both physiological and inflammatory conditions deserves to be investigated. In fact, to the best of our knowledge, there is no evidence in the literature showing that neutrophil function is abnormal in Atg-deficient mice (Liu *et al.*, 2014).

It was found that inhibiting the ATRA-mediated Beclin 1 upregulation by a specific RNA directed against BECN1 did not impair ATRA-induced autophagy or the differentiation of APL cells, whereas it did affect the viability of mature APL cells. These findings suggest that Beclin-1 had no crucial role in the induction of autophagy and differentiation in ATRA-treated APL cells. However, we cannot definitely rule out the role of Beclin 1 in these processes since extinction of Beclin 1 by siRNA was not complete in our experiments and this may be insufficient to prevent autophagy and differentiation induced by ATRA (Maycotte and Thorburn, 2011).

CONCLUSION

It was concluded that the ATRA-induced upregulation of autophagy protein and Beclin-1 and LC3-1, 11 in the acute promyelocytic leukemia cells. Most importantly, because PML/RARA proteolysis appears to be critical for APL remission, this data may provide new options for therapy-resistant patients because drugs known to induce autophagy already are used in the clinic.

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