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Aven and Survivin Expression in Egyptian Acute Leukemia and their Relation to Apoptosis

MAGDA ASSEM, M.D.[1]; THORRAYA ABEDEL-ALHAMEID, M.D.[2]; GIHAN A-BASSET, M.D.[1]; MAHMOUD M. KAMEL, M.D.[1]; AMANY M. HILAL, M.D.[2]; IMAN KHALIL, M.Sc.[3] and AYMAN METWALLY, Ph.D.[4]

The Departments of Clinical Pathology[1], Medical Oncology[2], National Cancer Institute, Cairo University and Faculty of Science, Cairo University[3] and Technology of Medical Laboratory, College of Applied Medical Science, Misr University for Science & Technology[4].

ABSTRACT

Background: One of the multiple resistance strategies exploited by cancer cells is the expression of inhibitors of apoptosis proteins (IAPs). Several anti apoptotic signals has been recently identified. Aven and survivin are broadly expressed and are conserved in mammalian species.

Objectives: The aim of this study was to assess survivin and aven expression levels with regard to the potential role of these genes as a prognostic marker in acute leukemia.

Patients and Methods: Sixty-four patients with acute leukemia 39 with acute myeloid leukemia (AML) and 25 with acute lymphatic leukemia (ALL) were used as the study group and 20 healthy volunteers were used as the control group. Reverse transcriptase (RT) PCR was used. Aven and survivin expression were detected at the messenger (mRNA) level. DNA fragmentation was carried out, before and 24 hours after the therapeutic dose, every day for 4 times during the initial induction of chemotherapy (everyday for ALL and every other day for AML).

Results: Survivin expression was found in 42 out of 64 (66%) patients with acute leukemia, more in AML (74%) than in ALL (52%), while aven expression was found in 26 out of 64 (40.6%) patients with acute leukemia equally expressed in AML (41%) and ALL (40%). Survivin was statistically related to CD7 expression (p=0.001) in AML only, while aven was statistically related to CD34 expression (p=0.014) in the whole acute leukemia cases. Aven was statistically correlated to alkaline phosphatase (p=0.036). Aven negative patients significantly respond well to chemotherapy (p=0.03). There was a significant dissociation between aven and survivin in AML (p=0.03) and near significant dissociation in ALL (p=0.07). Patients were categorized into 3 groups based on DNA fragmentation, whereby group I: DNA fragmentation found on day 1 or day 1+day 2, group II: DNA fragmentation found on days 1,2 and 3, group III where fragmentation was found on days 1+2+3+4 or/and day 5. Absence of aven expression significantly (p=0.007) contributed to DNA fragmentation as 24/35 (68.6%) of aven negative acute leukemia cases were in group III (the best group). Absence of survivin did not contribute as much. None of the double positive (survivin and aven) cases were in group III (the good DNA fragmentation group); this was statistically highly significant (p<0.001).

Conclusion: Aven seems to be more important as an inhibitor of apoptosis than survivin in acute leukemia. The presence of both aven & survivin confers a survival disadvantage & a significantly worse DNA fragmentation pattern, thus suggesting a synergistic inhibition of apoptosis when present together, although, they tend not to be expressed together. The highly significant relation between CD7 and survivin expression might suggest their involvement in a common signal transduction pathway.

Key Words: *Survivin – Aven – AML – ALL – DNA fragmentation.*

INTRODUCTION

The balance between cell death and cell viability is important in tissue homeostasis. Abnormalities in the control of programmed cell death (apoptosis) play an important role in tumorigenesis **[1]**. The evolutionarily conserved multi-step apoptosis cascade is regulated by proteins that promote or inhibit apoptotic cell death **[2]**. Inhibitors of apoptosis proteins (IAPs) are originally identified in malignant cells and during fetal development **[3]**.

In many instances IAP family proteins can suppress apoptosis across species barriers **[4]** implying that these proteins evidently target a common mechanism. Six human IAPs have been described so far: NAIP, CIAP1, CIAP2, XIAP, survivin and apollon **[5-10]**. Although there is some evidence that IAPs play an important role

in the chemoresistance of leukemia cell lines, little is known about their influence on this phenomenon in acute leukemia cells of human origin.

Survivin is an antiapoptotic gene, which is overexpressed in most human tumors and involved in mitotic checkpoint control. High levels of survivin expression have been associated with cancer progression, drug resistance, poor prognosis, and short patient survival **[11,12]** Recently, silencing of survivin gene by small interfering RNAs provides novel approaches for treatment of androgen-independent prostate cancer **[13]** childhood osteogenic sarcoma, as well as pancreatic cancer. To date several approaches have been taken to target and eliminate IAP function in an attempt to re-establish sensitivity, reduce toxicity, and improve efficacy of cancer treatment.

Aven, a novel apoptosis inhibitor identified, in the year 2000, **[14]** functions through binding Bcl-xL and Apaf-1. Aven is broadly expressed and is conserved in mammalian species. It suppresses apoptosis induced by Apaf-1 and caspase-9. Clinical relevance of aven was studied in acute leukemia in a Turkish population **[15]**.

The aim of the present study was to evaluate survivin and aven expression and to asses their prognostic relevance in Egyptian patients suffering from leukemia.

MATERIAL AND METHODS

This study included 64 patients with acute leukemia selected from the Pediatric and Medical Oncology departments of the National Cancer Institute (NCI) of Cairo University during the period between 2007 and 2008. These patients were classified as:

Group I: Twenty five patients with de novo ALL; they included 14 males and 11 females. Their ages ranged from 1 to 57 years.

Group II: Thirty nine patients with de novo AML; they included 26 males and 13 females. Their ages ranged between 4 and 60 years.

The study also included control group with 20 age and sex matched subjects. They were 9 males and 11 females. Their ages ranged from 3.5 and 55 years.

All the collected blood samples of the patients were subjected to assessment of the expression of survivin and aven in leukemic cells by RT-PCR. All patients of this study were treated according to the NCI ongoing induction, maintenance and consolidation regimens.

Methods:

1- *Sampling:*

From each patient 3 ml venous blood were obtained by a sterile venipuncture and were put in a sterile vacutainer containing EDTA as anticoagulant. This sample was divided as follows:

1- 1.0 ml for RNA extraction and PCR analysis by using QIA amp RNA extraction kit.

2- 1.0 ml for performing complete hemogram (Coulter- T 405).

3- 1.0 ml for DNA extraction and gel electrophoresis by using QIA amp DNA extraction kit.

2- *Detection of survivin and aven expression (RT-PCR):*

Using RT-PCR kits: Qiagen (Catalog number: 12110007).

Primers for survivin, aven and B actin amplification; (Operon primers):

- Primer-1 (sense strand primer) for survivin; the survivin mRNA Genbank accession number is NM001168. It has the following sequence: 5'-ACCAGGTGAGAAGTGAGGGA-3'.
- Primer-2 (Anti-sense strand primer) for survivin: 5'-AACAGTAGAGGAGCCAGGGA-3'.
- Primer-1 (sense strand primer) for aven: The aven mRNA Genbank accession number is AF283508. It has the following sequence: 5'-GATTTCAGTGTCCTCCTTAG-3'.
- Primer-2 (Anti-sense strand primer) for aven: 5'-CCTTGCCATCATCAGTTCTC-3'.

Primers for B actin amplification: The B actin mRNA Genbank accession number is NM 001101 Primer-1 (sense strand primer). It has the following sequence:5' TGACGGGGT-CACCCACACTG3'.

- Primer-2 (Anti-sense strand primer): It has the following sequence: 5' CTAGAAG-CATTTGCGGTGGA 3'.

Reverse Transcriptase (RT) PCR was carried out as follows:

1- One step RT PCR mater mix (Qiagen INC Catalog No 12110007) was used to amplify different amplicons using target specific primers. All reaction tubes were transferred to a thermal cycler (Eppendorf Mastercycler INC) and incubated at 55ºC for 30 minutes for RT step then, incubated at 95ºC for 15 minutes to inactivate the reverse transcriptase and to completely denature the template.

2- PCR amplification of survivin was performed for 32 cycles composed of denaturation at 94ºC for 60sec, annealing at 59ºC for 60sec and extension at 72ºC for 60sec with a final extension at 72ºC for 10 min.

3- PCR amplification of aven, was performed for 32 cycles composed of denaturation at 94ºC for 60sec., annealing at 60ºC for 60sec and extension at 72ºC for 60sec with a final extension at 72ºC for 10min.

4- PCR amplification of B-actin was performed for 32 cycles composed of denaturation at 94ºC for 60sec., annealing: 55ºC for 60sec. extension: 72ºC for 60sec, final extension 72ºC for 10 minutes. This was repeated for a total of 32 cycles.

5- Amplified material was stored at -20ºC until gel electrophoresis was performed. 2% agarose was performed using biotechnology grade agarose (Sigma, Inc) in Tris acetate EDTA buffer. The size of the amplified product was judged by the use of 100-1000bp Ladder, (GeneRuler™ Thermo Scientific) as DNA marker: 308bp for survivin, 252bp for aven and 600bp for B actin (Figs. 1-2).

3- *Detection of apoptosis by agarose gel electrophoresis* **[16]***:*

DNA fragmentation was detected on daily basis before chemotherapy and 24 hours after the therapeutic dose every day for 4 times during the initial induction of chemotherapy.

Before chemotherapy and 24 hours after the induction therapy peripheral blood samples were taken from each patient and detection of apoptosis by agarose gel electrophoresis was done (Fig. 3).

4- *Statistical analysis of the results:*

Data were analyzed using the SPSS Win Statistical Package, version 15. Numerical data were expressed as mean±standard deviation, median and range. Qualitative data were expressed as frequency and percentage. Chi-square test (Fisher's exact test) was used to examine the relation between qualitative variables. For quantitative data, comparison between two groups was done using the Mann-Whitney test (non-parametric test corresponding to student *t*-test for variables not normally distributed). Comparison between 3 groups was done using Kruskal-Wallis test (non-parametric ANOVA test). The post-Hoc comparison test "Schefe test" was used to compare pairs of groups on rank of the variables. Survival analysis was done using Kaplan-Meier method. Comparison between two survival curves was done using the Log-rank test. *p*-value ≤0.05 was considered significant, and less than 0.001 considered as highly significant.

RESULTS

Survivin expression in whole acute leukemia patients was 42 out of 64 (66%), higher in AML, as 29 out of 39 (74%) than in ALL, as 13 out of 25 (52%) cases expressed survivin. While aven expression was 26 out of 64 (40.6%) in whole acute leukemia patients equally expressed in AML, 16 out of 39 (41%) and in ALL, 10 out of 25 (40%) cases. Survivin and aven in control group were negative. Both survivin and aven expression in acute leukemia were highly statistically significant when compared to the control ($p<0.001$).

Survivin was statistically related to CD7 expression ($p=0.001$) in AML only (Table 1), while aven was statistically related to CD34 expression ($p=0.014$) as 43/64 (67.2%) AL patients have Concordant expression of aven and CD 34 and also, Aven was significantly associated with alkaline phosphatase ($p=0.036$) (Fig. 3). Survivin expression was not significantly related associated CD33 or CD34 and aven expression was not significantly associated with CD7 or CD33.

There was a significant dissociation between the expression of aven and survivin in AML ($p=0.03$), as 27/39 (69.2%) having one marker positive and the other was negative and 12/39 (30.8%) having concordant either positive or negative results, while in ALL there is a borderline significant dissociation between the expres-

sion of aven and survivin (p=0.07), as 17/25 (68%) having one marker positive and the other was negative and 8/25 (32%) having concordant either positive or negative (Table 2).

There was a significant good response to chemotherapy in aven negative patients (p=0.03) as 27/34 (79.4%) of patients who entered in complete remission (CR) were aven negative, while survivin dos not contribute so much (Table 3).

Patients were categorized into 3 groups based on DNA fragmentation where in group I DNA fragmentation was found in D1 or D1+D2, in group II found in D1+D2+D3, while in group III fragmentation was found in D1+D2+D3+D4 and/or D5. Apoptosis in group III was found in 15/22 (68.2 %) of ALL and in 16/34 (47.1%) of AML patients. Absence of aven was significantly associated with apoptosis as out of 35 aven negative AL cases, 24 (68.6%) were in group III (the best group) (p=0.007) but absence of survivin did not contribute as much. In fact 18 out of 31 (58%) of group III were survivin positive. None of the concordant both positive survivin and aven were in group III (the good 5 day fragmentation); this was statistically highly significant (p<0.001) relation (Table 4).

Table (1): Relation between the expression of Survivin and CD7 in acute leukemia:

Parameter	Survivin positive	Survivin negative	*p*-value
AML			
CD7 positive	26 (96.3%)	1 (3.7%)	0.001
CD7 negative	3 (25%)	9 (75%)	
ALL			0.85
CD7 positive	1 (25%)	3 (75%)	
CD7 negative	12 (57.1%)	9 (42.9%)	

Table (2): Relation between the expression of both survivin and aven in ALL and AML:

	Survivin		Total	*p*-value
	+ve	-ve		
AML				
Aven+	9	7		
Aven-	20	3	39	0.031
ALL				
Aven+	3	7		
Aven-	10	5	25	0.075
Total	42	22	64	

Table (3): Relation between the expression of aven and survivin and response to chemotherapy:

Parameter	CR	Refractory	*p*-value
Aven			
Positive	27 (79.4%)	7 (20.6%)	0.031
Negative	4 (44.4%)	5 (55.6%)	
Survivin			
Positive	21 (61.8%)	13 (38.2%)	0.5
Negative	6 (66.7%)	3 (33.3%)	

Table (4): Relation between the expression of aven and survivin and DNA fragmentation groups in acute leukemia:

DNA fragmentation / Marker expression	Group 1*	Group 2*	Group 3*	*p*-value
Aven				
Positive	9 (42.9%)	5 (23.8%)	7 (33.3%)	
Negative	3 (8.6%)	8 (22.9%)	24 (68.6%)	0.007
Survivin				
Positive	11 (29.7%)	8 (21.6%)	18 (48.6%)	
Negative	1 (5.3%)	5 (26.3%)	13 (68.4%)	0.1
Double positive**	9 (90%)	1 (10%)	0 (0%)	
Otherwise***	3 (6.5%)	12 (26.1%)	31 (67.4%)	<0.001

Group 1 : DNA fragmentation up to D2. i.e. (D1 or D1+D2).
*Group 2 : DNA fragmentation up to D3. i.e. (D1+D2+D3).
*Group 3 : DNA fragmentation up to (D1+D2+D3 D4 or D1+D2+D3+D4+D5).
**Double positive = Cases expressing both aven & survivin.
***Otherwise = Other combinations either both negative or expressing either.

After a follow-up period ranging from 1 and 21 months (median of 8 months) for all our acute leukemia patients, a mean overall survival was 18.41±18.30 months (95% confidence interval= 0-17.5%), and the median survival was 7.0±5.3 months. The Survival analysis carried out in all our acute leukemia patients in relation to the expression of the studied markers revealed a significantly higher overall survival in patients who were aven negative (p<0.001) (Fig. 4a).

A statistically significant higher overall survival was found in aven negative patients in both AML and ALL group (p=0.002 and 0.007 respectively) (Fig. 4b&c). On the other hand, there was no significant difference in ALL, AML or AL patients in relation with survivin expression. Survival analysis revealed a very high significantly shorter overall survival in all AL and AML patients who were positive for both aven and survivin (p<0.001 for both) (Fig. 5a&b). Unfortunately overall survival could not be done in ALL patients who expressed both aven and survivin due to the small number (only 3 patients). Survival analysis was carried out in all our acute leukemia patients regarding DNA fragmentation, there was highly significant overall survival in patients who have good DNA response (group 3) (p<0.001) (Fig. 5c).

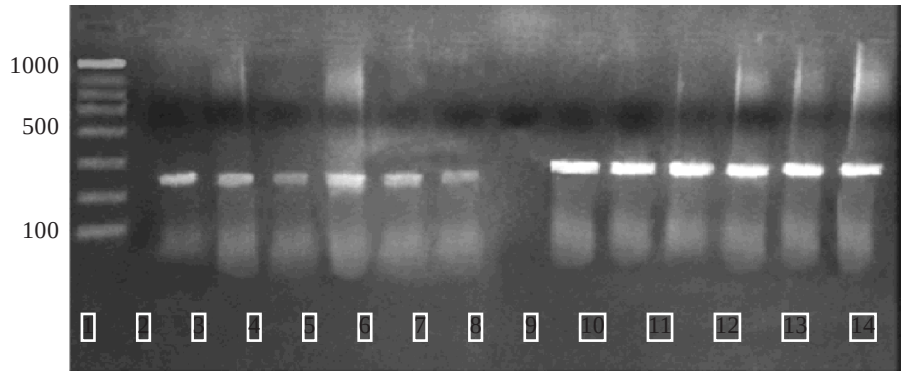

Fig. (1): Electropherogram of aven and survivin in acute leukemia patients.
Lane 1: Marker ladder (100-1000 bp). Lanes 2,3,4,5,6 and 7: Aven expression 252 bp.
Lane 8: Empty. Lanes 9,10,11,12,13 and 14: Survivin expression 305 bp.

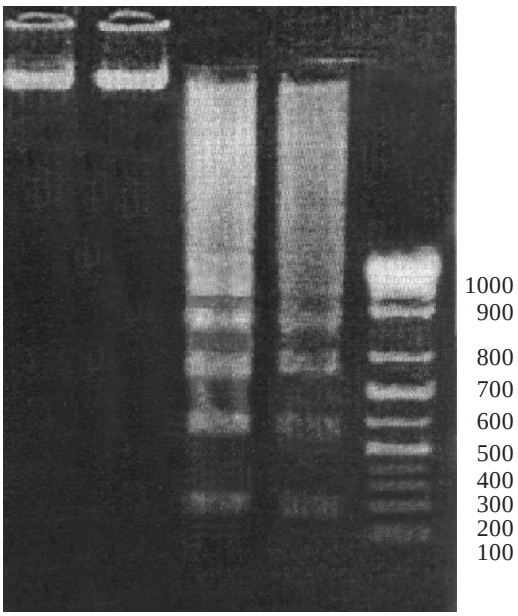

Fig. (2): DNA fragmentation at 200bp.
Lanes 1,2: Apoptosis by agarose gel electrophoresis before therapy.
Lanes 3,4: Apoptosis by agarose gel electrophoresis, 24 hours after therapy.
Lane 5: Marker ladder (100 -1000 bp).

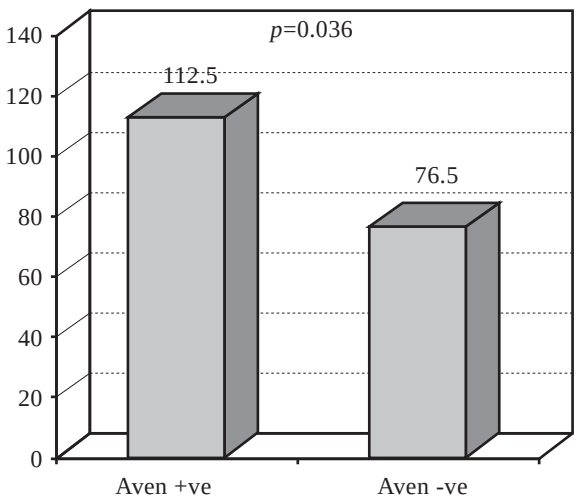

Fig. (3): Relation between aven and alkaline phosphatase in 64 cases of acute Leukemia.

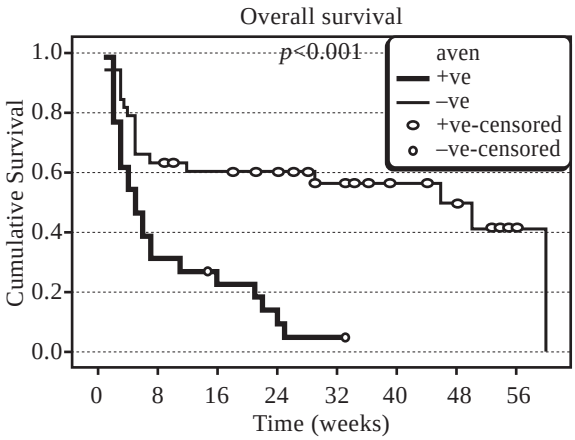

Fig. (4A): Overall survival in relation to Aven expression in acute leukemia.

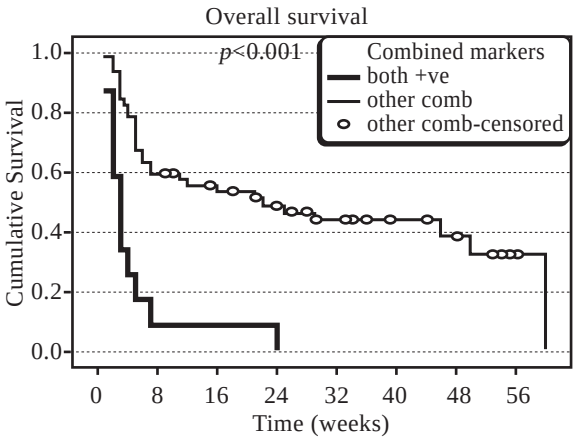

Fig. (5A): Overall survival in relation to both Aven and Survivin positive in AL.

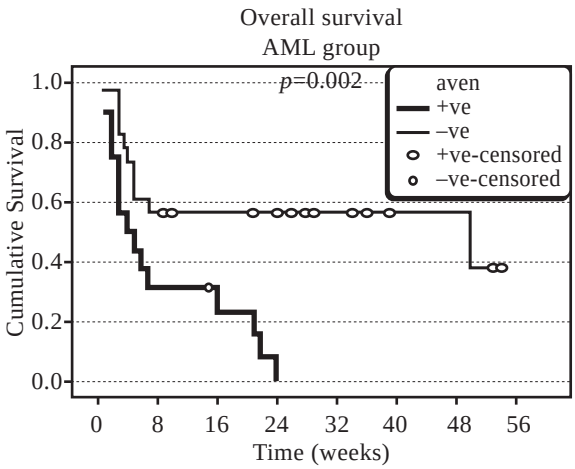

Fig. (4B): Overall survival in relation to Aven expression in AML.

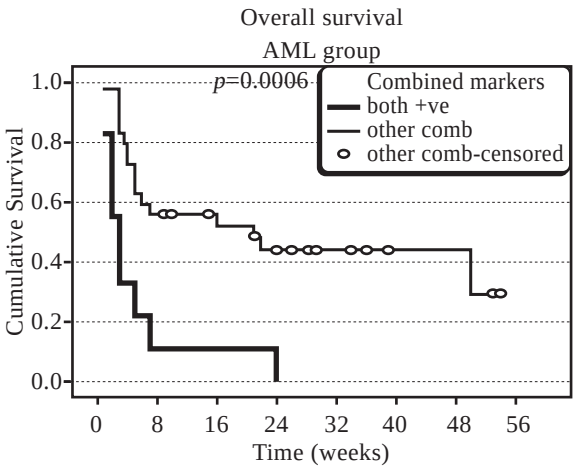

Fig. (5B): Overall survival in relation to both Aven and Survivin positive in AML.

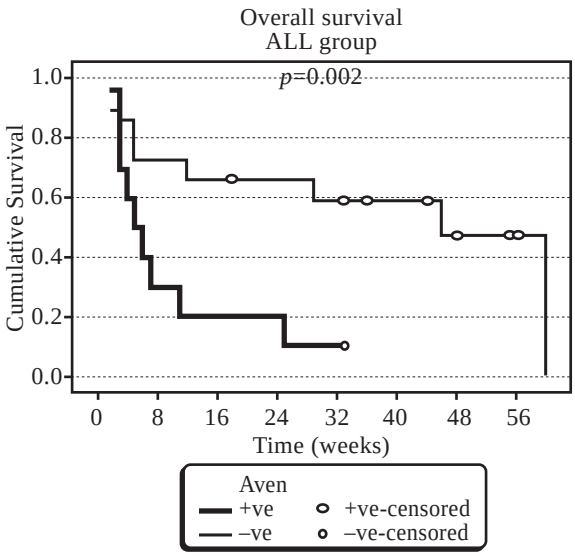

Fig. (4C): Overall survival in relation to Aven expression in ALL.

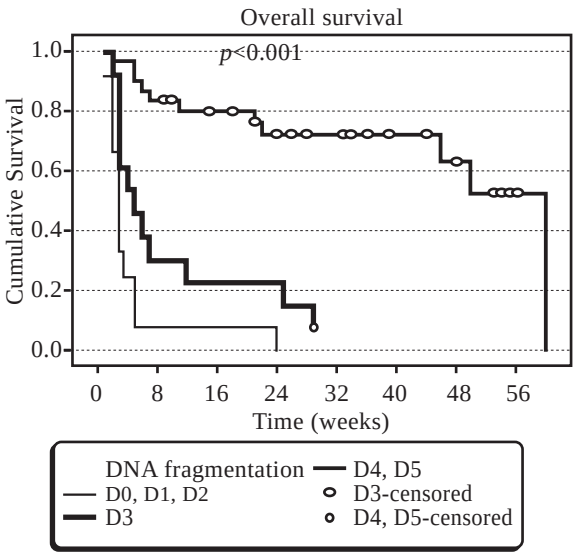

Fig. (5C): Overall survival in relation to DNA fragmentation in AL.

D0, D1, D2 (group I), D3 (group II), D4, D5 (group III)

DISCUSSION

According to the registry of the National Cancer Institute, Cairo University, acute leukemia is the 4th most common cancer among males and the 3rd most common cancer among females **[17]**. Acute lymphoblastic leukemia (ALL) constitutes about 80% of childhood cases, whilst acute myeloid leukemia (AML) constitutes about 80% of adult cases.

In the present study, we evaluated the expression of survivin and aven, status of CD7, CD34, CD33 cell surface markers expression as well as DNA fragmentation (before and during initial induction) in blast cells separated from peripheral blood for AML and ALL patients as well as 20 healthy volunteers. The results were correlated to clinical and hematological findings and response to therapy. The patients were followed up for 1-21 months.

Regarding survivin, 74.4% of the AML cases and 52% of the ALL cases were+ve. The expression of survivin was significantly associated with the expression of CD7 in AML (p=0.001), while in ALL; survivin did not show any significant association with CD7, CD33 or CD34, unlike the Turkish group **[15]** who found a significant association between the expression of CD7 and survivin in all acute leukemia (both AML and ALL) cases. It is known that CD7 mediated cell activation may be transduced via the lipid kinase phosphatidyl inositol 3-kinase (Pi3-kinase); at the same time the up-regulation of survivin occurs through the activation of Pi3-kinase. So, the activation is common for both survivin up regulation **[18-19]** and CD7 activation **[20]**. Thus the pathway CD7, Pi3AKT and survivin is most probably operational in Egyptian AML only.

In the present study we have evaluated the patients' response to therapy (cell apoptosis before & during initial induction of chemotherapy) using DNA fragmentation of peripheral blood blast cells.

We did not find association between survivin expression and DNA fragmentation (p=0.1). More studies are clearly needed on a larger number of samples to reach a final conclusion. Unlike survivin, the DNA fragmentation showed highly significant inverse relation with aven expression (p=0.007). This means that when aven is expressed, chemotherapy induced apoptosis is abolished. For our knowledge, this is the first study that correlates the expression of aven with the DNA fragmentation in acute leukemia.

In the current study, we found that aven is significantly correlated to the expression of CD34 (p=0.014); both markers are important indicators of poor prognosis. This finding is similar to that found in the Turkish population **[15]**.

In our acute leukemia cases, there is a tendency that these two inhibitors of apoptosis are not expressed together. Most of the cases (44/64) were either positive for surviving/negative for aven (30/64) or negative for surviving/positive for aven (14/64). The dissociation was significant in AML (p=0.03) and near significance in ALL (p=0.07); increasing the number of patients might bring it to the significant level. Up to our best knowledge, this is the first report to establish such a relationship.

In the present study, we found that aven significantly affect the response to chemotherapy (p=0.03) and the overall survival of the patients (p<0.001) while survivin did not significantly impact on the response to treatment or the OS in acute leukemia. Also, combined expression of both IAPs markers correlated significantly to survival (p<0.001). When10 out of 12 cases positive for both markers were analyzed for DNA fragmentation, all the 10 patients had reduced DNA fragmentation (p 0.001). This could be explained by a synergistic inhibitor of apoptosis effect when both markers are expressed. Up to our best knowledge, this is the only report on the relationship between the expression of aven, survivin and DNA fragmentation in acute leukemia.

In conclusion aven and survivin could be considered as two independent anti apoptotic pathways that could act separately to inhibit apoptosis in the leukemic cells. Aven seems to be more important as an inhibitor of apoptosis than survivin in acute leukemia. The presence of both aven and survivin confers a survival disadvantage and a significantly worse DNA fragmentation pattern, thus suggesting a synergistic inhibition of apoptosis when present together.

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