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

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Abstract

Background: Several anti apoptotic signals have been recently identified. Aven and Survivin are broadly expressed and are conserved in mammalian species.

Patients and Methods: 39 AML and 25 ALL were tested. Aven and Survivin expression were detected by RT-PCR. DNA fragmentation was carried out daily after treatment.

.**Results:** Survivin was expressed (P=0.06) more in AML (74%) than in ALL (52%). While, Aven was equally expressed in both leukemias. Patients were categorized into 3 groups based on DNA fragmentation. Absence of Aven significantly ($p<0.001$) contributed to DNA fragmentation,but Survivin did not contribute as much. None of the concordant both positive Survivin and Aven were in group III (the good 5 day fragmentation, ($P< 0.001$). Survivin was statistically related to CD7 expression ($P<0.001$) in AML only. There was a significant dissociation between Aven and Survivin in AML ($p=0.03$) and near significant dissociation in ALL ($p=0.07$).

Conclusion: Aven seems to be more important as an inhibitor of apoptosis than survivin in acute leukemia. The presence of both confers a survival disadvantage and a significantly worse DNA fragmentation pattern suggesting a synergistic inhibition of apoptosis. 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 and DNA fragmentation.

Introduction

Both leukaemogenesis and resistance to chemotherapy can be attributed to blocks of apoptosis. Inhibitors of apoptosis proteins (IAPs) are originally identified in malignant cells & during fetal development (**1**).In many instances IAP family proteins can suppress apoptosis across species barriers (**2**) implying that these proteins evidently target a common mechanism. Six human IAPs have been described so far: NAIP, CIAP1, CIAP2, XIAP, survivin and apollon. (**3**-**8**). 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 (**9**&**10**). Recently, silencing of survivin gene by small interfering RNAs provide novel approaches for treatment of androgen-independent prostate cancer (**11**), 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 (**12**) 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 (**13**).

The aim of the present study is to evaluate survivin & aven expression in acute leukemia, and asses their prognostic relevance in acute leukemia patients.

Material and Methods

This study included 64 patients with acute leukemia selected from pediatric and medical oncology department of the National Cancer Institute of Cairo University in the period between 2006 and 2007. These patients were classified as: -

Group I:

Twenty five patients with de novo ALL, 14 males and 11 females. Their ages ranged from 1 to 57years.

Group II: Thirty nine patients with de novo AML, 26 males and 13 females. Their ages ranged between 4 to 60 years. All the collected blood samples of the patients were subjected to assessment of the expression of SURVIVIN & AVEN in leukemic cells by RT-PCR. All patients of this study were treated according to the institute ongoing induction 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 vaccutainer 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 haemogram (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, all primers were used for each gene (Operon primers)**.** ***Primer-1 (sense strand primer) for Survivin*** The survivin mRNA Genbank accession number is NM001168. It had the following sequence: 5'-ACCAGGTGAGAAGTGAGGGA-3'

Survivin sense strand, scale: 10 nmol. ***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 had the following sequence:

5'-GATTTCAGTGTCCTCCTTAG-3' *Aven* sense strand, scale: 10 nmol.***Primer-2 (Anti-sense strand primer) for Aven*** 5'-CCTTGCCATCATCAGTTCTC-3'*Aven* anti-sense strand, scale:10 nmol. ***Primers for B actin amplification.*** The *B* actin mRNA Genbank accession number is NM_001101 ***Primer-1 (sense strand primer).*** It had the following sequence: 5' TGACGGGGTCACCCACACTG3'.*B* actin sense strand, scale: 10 nmol.

Primer-2 (Anti-sense strand primer):5' CTAGAAGCATTTGCGGTGGA 3'

Reverse Transcriptase RT-PCR was carried,

1- All reaction tubes were transferred to a thermal cycler and incubated at 55ºC for 30 minutes for c-DNA synthesis then, incubated at 95ºC for 15 minutes to inactivate the reverse transcriptase and to completely denaturate the template.

2- For PCR amplification of Survivin , denaturation: 94ºC for 60 sec,annealing: 59ºC for 60 sec, polymerization: 72ºC for 60 sec,final extension 72ºC for 10 min.

This was repeated for a total of 32 cycles.

3-For PCR amplification of Aven ,denaturation: 94ºC for 60 sec.annealing: 60ºC for 60 sec,polymerization: 72ºC for 60 sec ,final extension 72ºC for 10 min.

This was repeated for a total of 32 cycles.

4- Another set of PCR master mix was used for amplification of *B*- actin, denaturation: 94ºC for 60 sec.annealing: 55ºC for 60 sec. polymerization: 72ºC for 60 sec.final extension 72ºC for 10 min.

5- Amplified material was stored at -20ºC until gel electrophoresis was performed.

The size of the amplified product was read with the use of a DNA marker of different molecular weight molecular weight (308bp for Survivin, 252 bp for Aven and 600bp for B actin), figure (1-2).

4-Detection of apoptosis by agarose gel electrophoresis: ***(14)***

DNA fragmentation was carried out on daily basis before chemotherapy and 24 hours after the therapeutic dose every day for 5 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 figure (3).

5- STATISTICAL ANALYSIS OF THE RESULTS:

Data was analyzed using 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 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). Then by 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 Log-rank test. P-value $\leq$ 0.05 was considered significant and less than 0.001 considered as highly significant.

RESULTS

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 leukemias, the most common malignancy. Survivin expression in acute leukemia 42 out of 64 (66%), in AML 29 out of 39 (74%) while in ALL 13 out of 25 (52%), Aven expression was (40.6%) in acute leukemia equally expressed in AML 16 out of 39 (41%) while in ALL 10 out of 25 (40%).(table,1).

Patients were categorized into 3 groups based on DNA fragmentation were by group I DNA fragmentation found in (D 1 or D1+D2), group II found in (D1+D2+D3), group III where fragmentation was(D1+D2+D3+D4or/andD5). Apoptosis in group III was found in 13 out of 22 (59%) ALL and 15 out of 34 (44.1%) AML patients.

Out of 35 Aven negative AL, 24 (68.6%) were in group III (the best group) (P = 0.007) table (2). absence of Survivin did not contribute as much. In fact 18 out of 31 (58%) of group III were Survivin positive (P= 0.1) table (3). 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).

Survivin was statistically related to CD7 expression (P<0.001) in AML only table (5), while Aven was statistically related to CD34 expression (P<0.014) in both ALL and AML table (6). However Survivin expression was not significant related with CD33 in AL and Aven expression was not significant with neither CD7 nor CD33.Aven was statistically correlated to alkaline phosphatase (P< 0.036).

There was a clear dissociation of expression of Aven and Survivin in acute leukemia 44/64(68.7%), that is to say these two markers tend not to be expressed together. This dissociation is statistically significant (P=0.03) in AML and near significant (p=0.07) in ALL table (7).

After afollow up period ranged between 1-21 months for all our acute leukemic patients, the mean over all survival was 18.41± 18.30 months (95% confidence interval).the median survival was 7.0±5.3 months. The Survival analysis

carried out in all our acute leukemic patients regarding the expression of the studied markers revealed a significantly higher overall survival in patients who were Aven negative (P <0.001) figure (4).

A statistical significant higher over all survival was found in each AML & ALL group and Aven negative patients (P 0.002, 0.007) respectively figure (5-6). On the other hand there was no significant difference neither in (ALL, AML nor AL) patients in relation with Survivin expression. Survival analysis revealed a very higher significant short over all survival in all AL and AML patients who were positive for both Aven and Survivin (P <0.001) for both figure (7-8). Unfortunately over all survival can not be done in ALL patients who expressed both Aven and survivan 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 over all survival in patients who have good DNA response (group 3) (P < 0.001) figure (9).

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. 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 as well as the 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 up to1- 21 months.

Regarding the survivin, 74.4% of the AML cases were +ve while 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 significance when correlated with CD7, CD33&CD34.Unlike the turkish group (**13**) where they found a significant correlation between the expression of both 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 (**15-16**) and CD7 activation (**17**).Thus the pathway CD7,Pi3AKT&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, where we collected blood samples from the 56 patients at days 1,2,3,4 and 5, the DNA was extracted and agarose gel electrophoresis was done.

We did not find significance between survivin expression and DNA fragmentation where the P value was border line (P= 0.1). More studies are clearly needed on a

larger number of samples to reach a final conclusion. Unlike survivin the DNA fragmentation with Aven was highly significant relation (P=0.007) where, there was an inverse relation between the expression of Aven and DNA fragmentation.

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. Aven was correlated, CD7, CD33, CD34. When aven was correlated to CD34 we found that aven was significantly correlated to the expression of CD34 (P =0.014), where both markers are important indicators of poor prognosis. This finding is similer to that found by (**13**) in the Turkish population.

In acute leukemia, there is a tendency that these two inhibiters of apoptosis are not expressed together. Most of the cases 44/64 were either positive for survivin & negative for aven (30/64) or negative for survivin & positive for aven (14/64). In AML 27/39 were having either marker aven negative and survivin positive, 9/39(23%) were concordant both positive, 3/39(7.6%) were concordant both negative. There was a significant dissociation (P=0.03) between the expression of aven and survivin in AML, while in ALL there is a borderline significant dissociation (P = 0.07%) between the expression of aven and survivin, however, 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 , patients were followed up for 1-21 months and when correlated to their aven status, we found that aven significantly affects the overall survival of the patients (P<0.001).However, survivin did not correlate significantly to 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 subjected 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 .This is the only report on the relationship between the expression of Aven , survivin and DNA fragmentation in acute leukemia.

From the present study we can conclude that:

1 The association between aven, CD34 & alkaline phosphatase which are strong negative prognostic factors in acute leukemia suggests that aven is an independent candidate parameter to determine the poor prognosis in acute leukemia.

2-Aven seems to be more important as an inhibitor of apoptosis than survivin in acute leukemia.

3- Aven and survivin could be considered as two independent anti apoptotic pathways that could act separately to inhibit apoptosis in the leukemic patients.

4- 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.

5- The association between higher expression of survivin and CD7 which is a strong negative prognostic factor in acute leukemia suggests that survivin is a marker for poor prognosis in acute leukemia & the pathway CD7 Pi3AKT and survivin is operational in AML.

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Table (1): comparison between Survivin & Aven expression in acute leukemic patients:

Item	AML (no 39)	ALL (no 25)	P Value
Survivin +	29 (74.4 %)	13 (52%)	0.06
Aven +	16 (41%)	10 (40%)	0.935

Table (2): Comparison between Aven expression and DNA fragmentation groups in acute leukemia:

P value	**Aven negative**	**Aven positive**	**Group**
0.001	3 (8.6%)	9 (42.9%)	Group 1
	8 (22.9%)	5 (23.8%)	Group2
	24 (68.6%)	7 (33.3)	Group 3

Table (3): Comparison between Survivin expression and DNA fragmentation groups in acute leukemia:

P value	**survivin negative**	**survivin positive**	**Group**
0.1	1 (5.3%)	11 (29.7%)	Group 1
	5 (26.3%)	8 (21.6%)	Group2
	13 (68.4%)	18 (48.6%)	Group 3

Table (4): Comparison between concordant double expression of both Survivin & Aven cases and all otherwise cases and DNA fragmentation in acute leukemia:

P value	****otherwise**	**Both* positive**	**Group**
0.000	3 (6.5%)	9 (90%)	Group 1
	12 (26.1%)	1 (10%)	Group2
	31 (67.4%)	0 (0%)	Group 3

*Both positive= cases expressing both aven & survivin.

**otherwise= other combinations either both negative or expressing either.

Table (5): Relation between the expression of Survivin and CD7 in acute leukemia

Items	Survivin Positive	Survivin Negative	P value
AML			
CD7 Positive	24	1	0.001
CD7 Negative	2	9	
ALL			NS
CD7 Positive	1	3	
CD7 Negative	12	9	

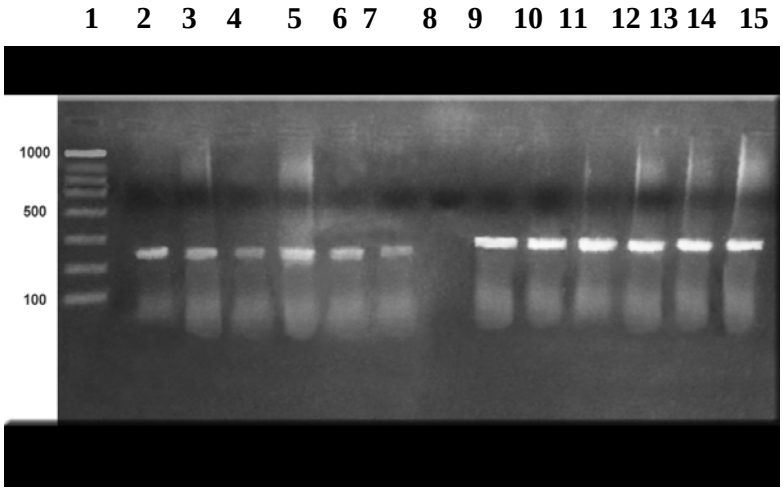
Table (6): Relation between the expression of Aven and CD34 in acute leukemia (ALL&AML)

P Value	Aven negative	Aven positive	
0.014	13 (43.3%)	17 (56.7%)	CD34 positive
	23 (74.2%)	8 (25.8%)	CD34 negative

Table (7) : Relation between the expression of both (Survivin and Aven) in ALL and AML.

	SURVIVIN +ve	SURVIVIN -ve	Total	P value
AML Aven + Aven -	9 20	7 3	39	0.031
ALL Aven + Aven -	3 10	7 5	25	0.072
Total	42	22	64	

Figure (2): shows PCR product of Survivin (305 bp) and Aven (252bp):

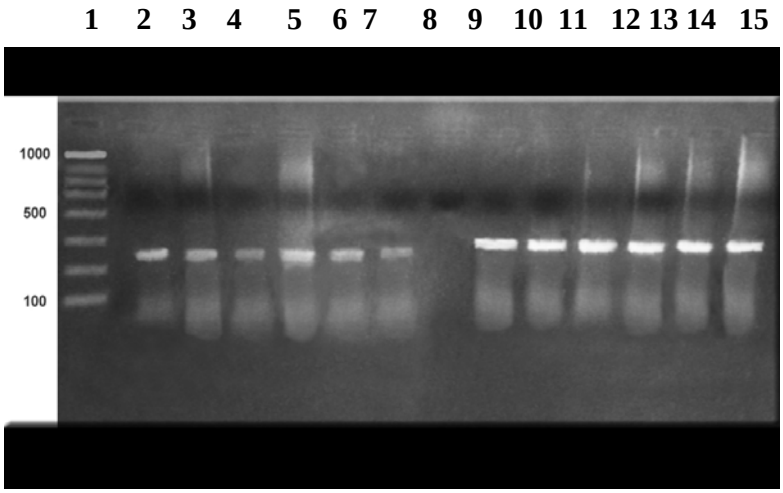


Lanes 2,3,4,5,6,7: show Aven expression of AL patients.

Lanes 9,10,11,12,13,14,15: show Survivin expression.

Lane 1: Marker ladder (100-1000).

Figure (2): shows PCR product of Survivin (305 bp) and Aven



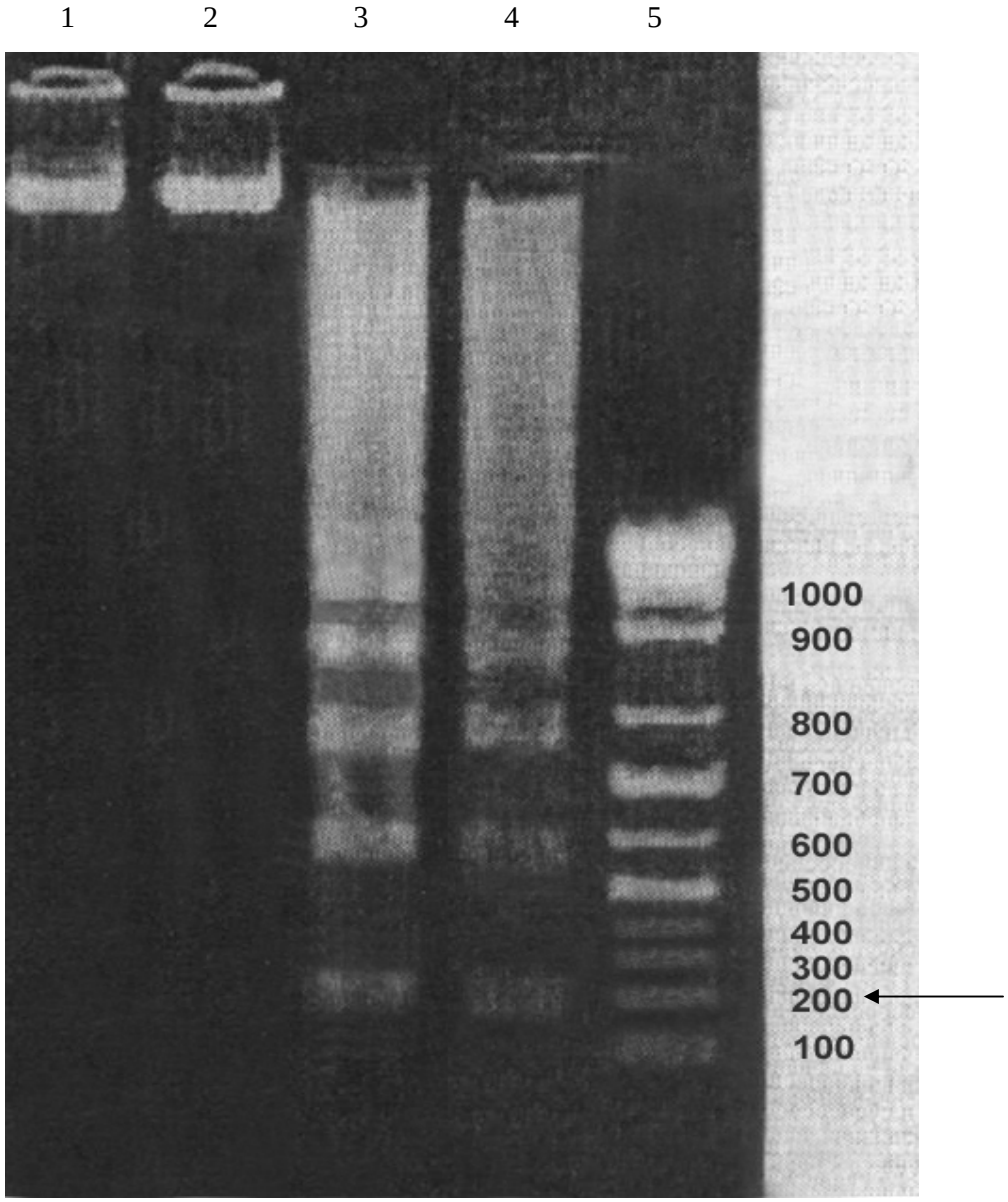
(252bp):

Lanes 2,3,4,5,6,7: show Aven expression of AL patients.

Lanes 9,10,11,12,13,14,15: show Survivin expression.

Lane 1: Marker ladder (100-1000).

DNA fragmentation at 200bp Figure (3):



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).

Figure (4): overall survival and Aven in acute leukemia.

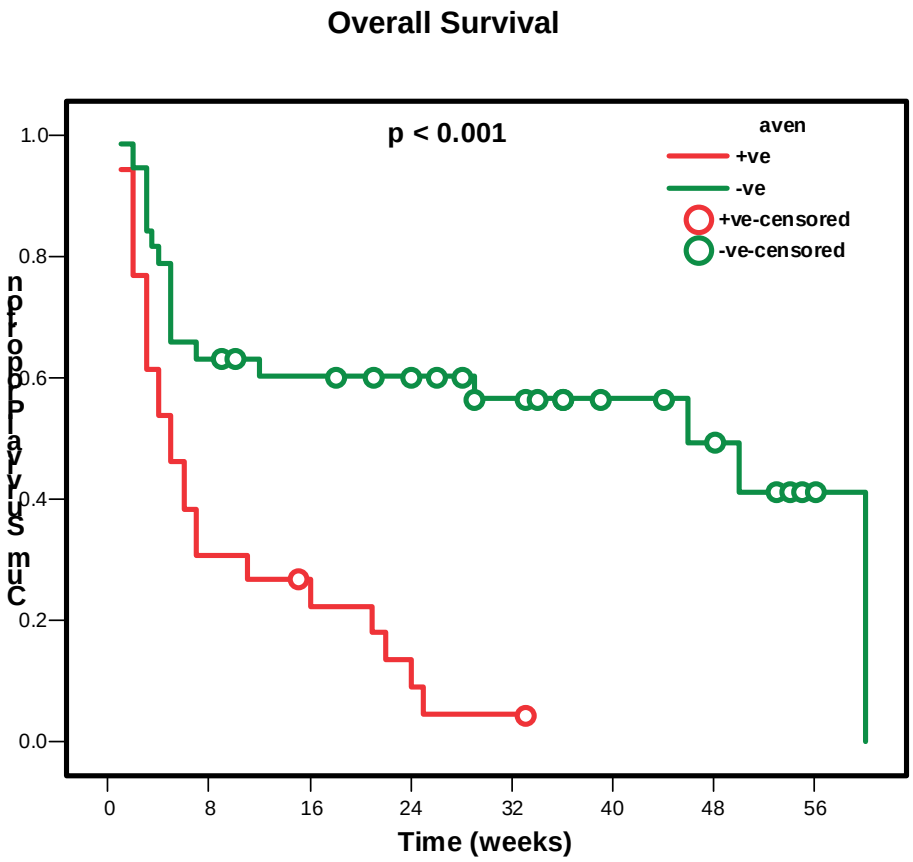


Figure (5): overall survival and Aven expression in AML.

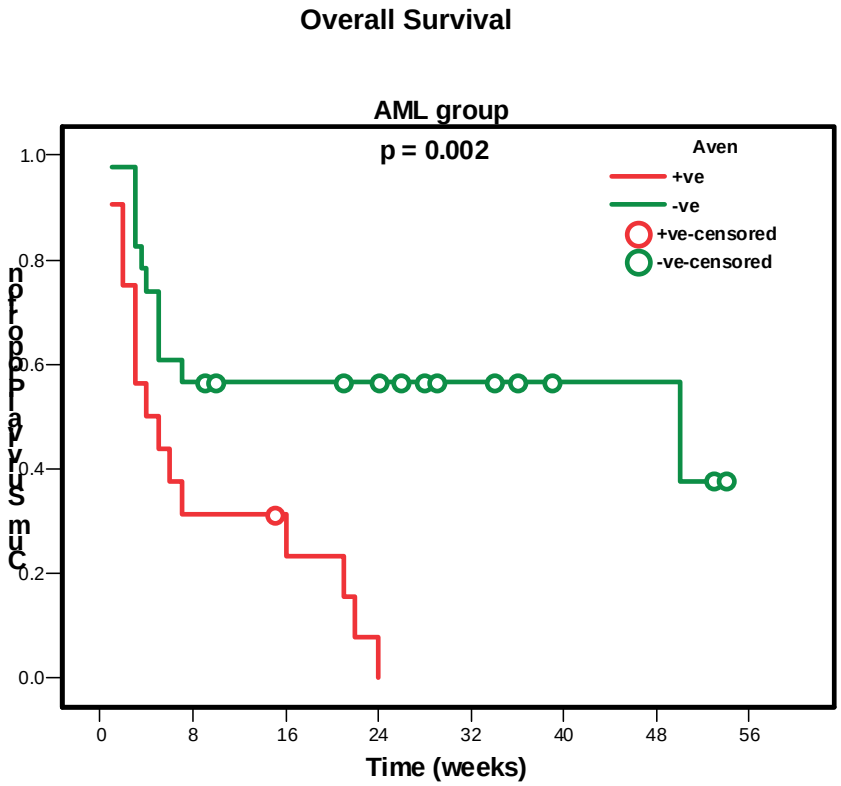


Figure (6) overall survival and Aven expression in ALL.

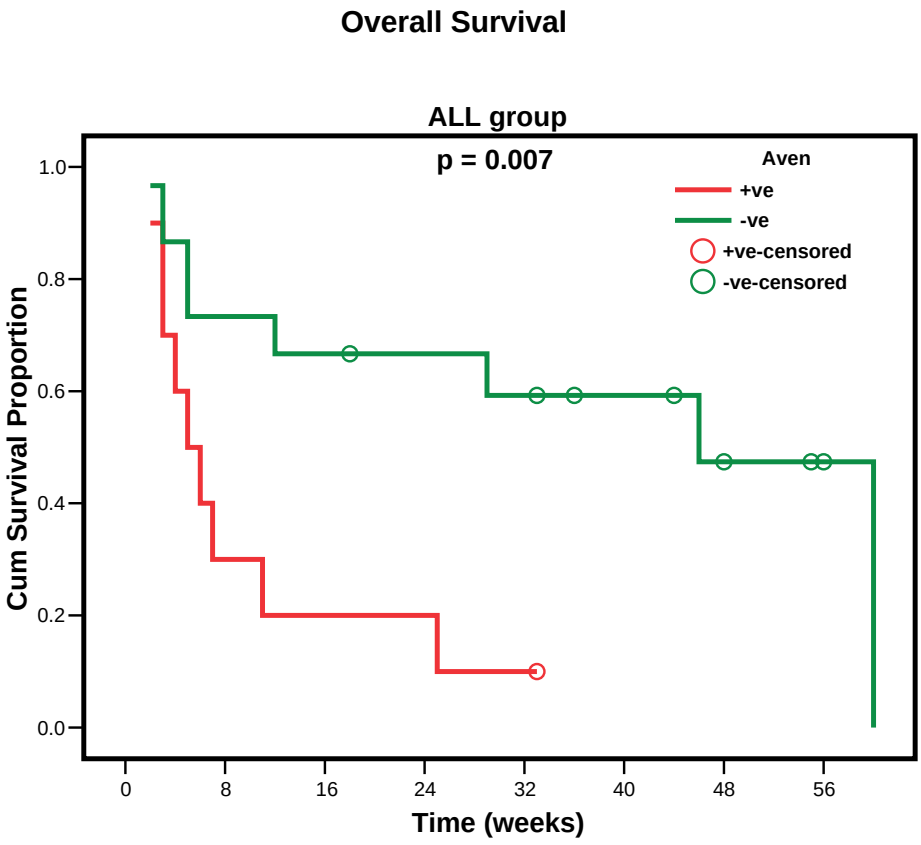


Figure (7): overall survival with both Aven and Survivin positive in acute leukemia.

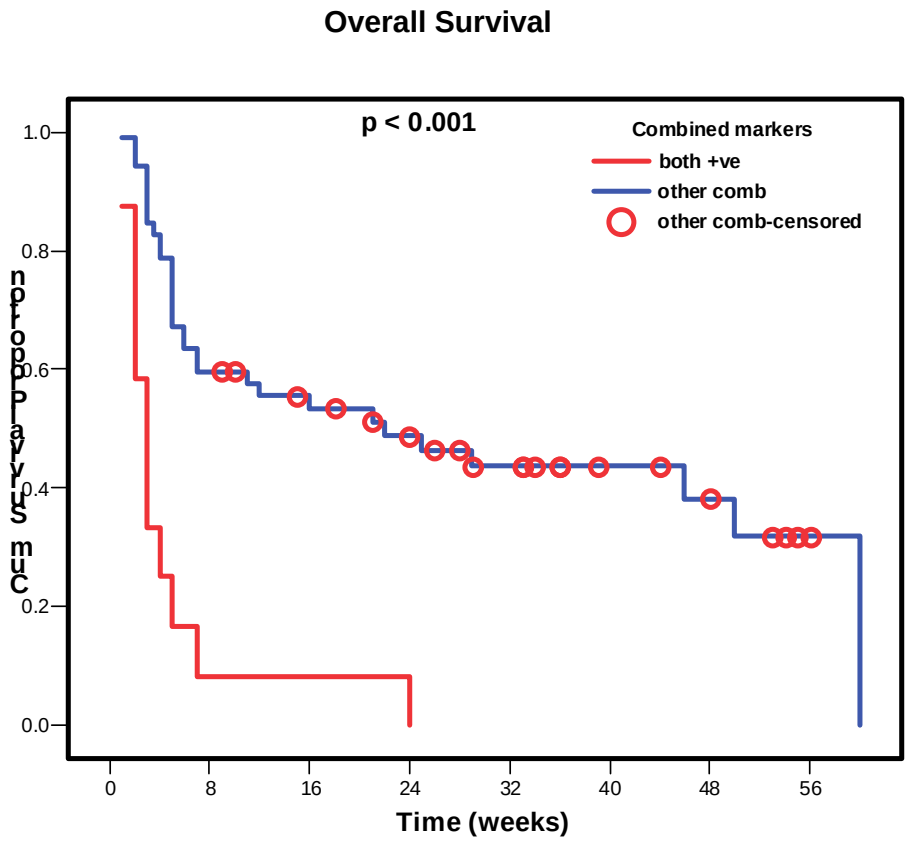


Figure (8): overall survival with both Aven and Survivin positive in AML.

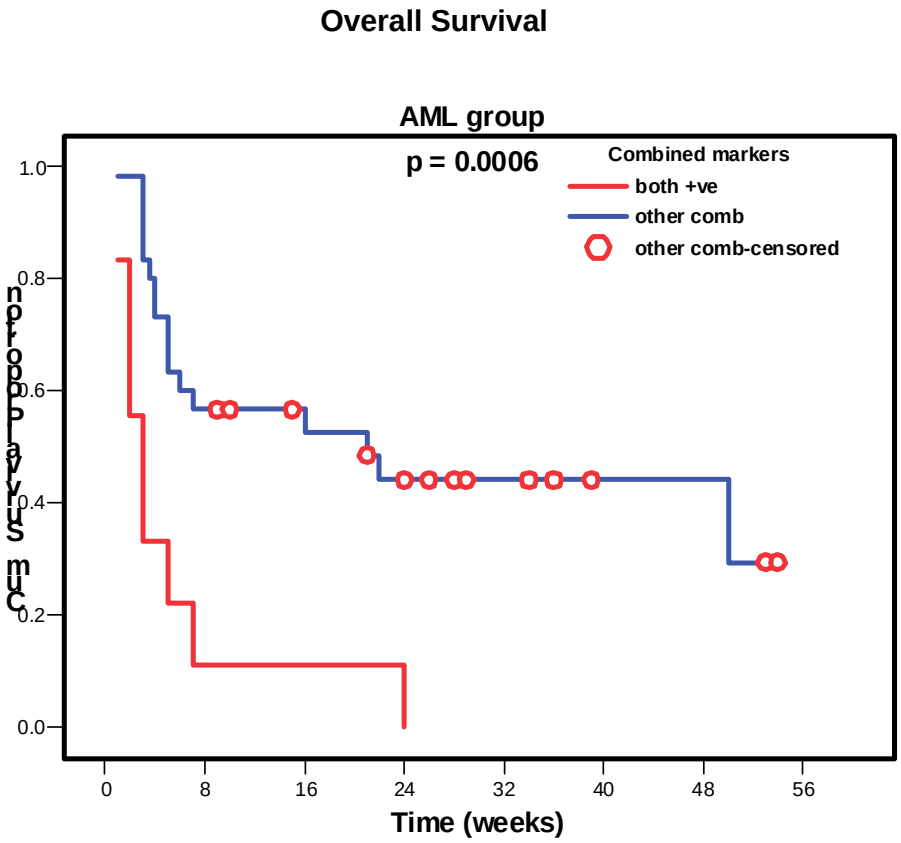
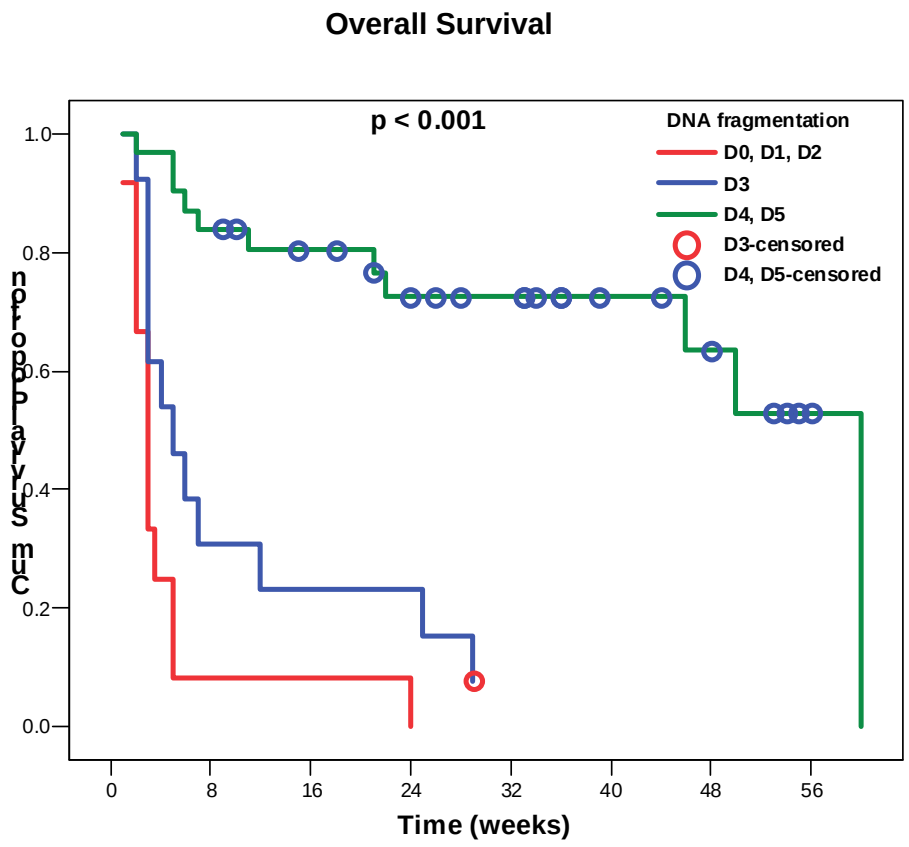


Figure (9): overall survival with DNA fragmentation in acute leukemias:



D0, D1, D2 (group I)
D3 (groupII)
D4, D5 (groupIII)