Expression of Apoptotic Inhibitors Survivin and Aven in Adult Acute Myeloid Leukemia

1EL-Bordiny MM, 2Hanafi SHM, 3Ghandour AH, 1Sorour A and 4Saad AA.

1Department of Clinical Pathology, Faculty of Medicine, Alexandria University, 2Genetic Engineering Institute, Menoufia University, 3Department of Internal Medicine, Faculty of Medicine, Alexandria University, 4Department of Biotechnology, Institute of Graduate Studies and Research, Alexandria University.

Survivin, and Aven are members of the inhibitor of apoptosis (IAP) proteins family. They are reported to be over-expressed in many cancers including leukemia. Previous studies suggested that Survivin is implicated in both control of apoptosis and regulation of cell division. The aim of this study was to examine the expression of the two apoptotic inhibitors - Survivin and Aven-at the messenger (m)RNA level in acute myeloid leukemias (AML) in relation to the clinical and hematological findings. Thirty adult patients with acute myeloid leukemia (AML) and 10 healthy subjects were studied. Patients were subjected to complete blood picture, bone marrow examination and immunophenotyping. Based on the French American British classification, they were subdivided to M1, M2, M3, M4 and M5. A reverse transcriptase-polymerase chain reaction (RT–PCR) was used to investigate the expression of Survivin, and Aven mRNA. The AML patients showed expression of Survivin (206 bp) but not Aven mRNA. In contrast, neither Survivin nor Aven were expressed in the control group. Out of the 30 patients, 23 patients (76.6 %) showed detectable levels of Survivin expression (p<0.001). Quantitative analysis revealed differences in expression levels of Survivin mRNA between different FAB subtypes. Three subtypes (M1, M4 and M5) showed a slightly higher expression level of Survivin mRNA (mean values of 0.56, 0.57 and 0.81 respectively) than the other two subgroups (M2 and M3) (mean range 0.04 and 0.1 respectively). A significant correlation was found between Survivin expression level and peripheral blood (PB) blast % in M5 subtype (p=0.02). It is concluded that, Survivin is an important antiapoptotic signal in acute myeloid leukemia. Up-regulation of Survivin expression in AML may be involved in its pathogenesis and may provide a useful tool for prognosis.

Apoptosis is a morphologically distinct genetic program of cellular suicide which provides a vital protective mechanism against the development of neoplasia by removing cells with DNA damage. It also plays a central role to the homeostasis of adult tissues by maintaining the balance between cell production and cell elimination. Inhibition of apoptosis thus confers a survival advantage on cells harboring genetic alterations and may promote acquisition of further mutations to cause neoplastic progression and also contribute to the development of resistance to chemotherapy (Kerr et al., 1994 and Meterissian., 1997).

A large number of evidences show that many molecules such as p53 and bcl-2 are involved in the regulation of apoptosis during tumorigenesis. The inhibitor of apoptosis proteins (IAPs), which are widely expressed in all kinds of malignancies, is encoded by the highly-conservative anti-apoptosis gene family and plays an important role in the regulation of apoptosis through caspase-dependent and caspase-independent mechanisms. (Miller 1999 and Altieri, 2001). In humans, several members of the IAP family have been described: HIAP1, HIAP2, XIAP, NIAP, Survivin and, more recently, Livin and Aven (Ambrosini et al., 1997, Lu et al., 1998 and Ashhab et al., 2001)

Survivin is a 16.5-kDa protein with a single baculovirus inhibitor of apoptosis (IAP) repeat (BIR) domain (Ambrosini et al., 1997). It has been mapped to chromosome 17q25 (Hou 2006). Survivin is expressed during embryonal development but is absent
Expression of Apoptotic Inhibitors in Acute Myeloid Leukemia

in most normal, terminally differentiated tissues. Survivin has also been detected widely in a variety of human tumors, including breast, colon, pancreas and prostate carcinoma, neuroblastoma, melanoma and non-Hodgkin’s lymphoma (Ambrosini et al., 1997). Studies performed by immunohistochemistry described presence of Survivin in a variable percentage of tumors, ranging from 30% of gastric cancers to 90% of melanomas (Lu et al., 1998 and Grossman et al., 1999). Most of these studies found a positive correlation between Survivin expression and prognosis of disease, which is more evident in neuroblastoma and in colorectal cancer, where a multivariate statistical analysis revealed that Survivin expression is an independent prognostic factor for disease progression (Kawasaki et al., 1998). Most data obtained from these studies suggest that survivin expression in cancer appears to be associated with unfavorable clinical and pathological parameters, such as poor prognosis with progressive diseases and shorter patient survival rates (Fengzhi 2003).

Survivin plays a role in the proliferation and survival of normal hematopoietic cells. Survivin expression is aberrantly enhanced in most cancers and hematopoietic malignancies (Xiu et al., 2004). Some hematological studies examined by immunocytochemistry the expression of survivin in bone marrow cells from acute myeloid leukemia (AML) cases and patients with myelodysplastic syndrome (MDS) and confirmed the high incidence of survivin expression in AML. They also suggested that survivin abnormal expression also in MDS may play a role in promoting aberrantly increased cell viability and contribute to the altered homeostatic balance between cell growth and cell death. (Invernizzi et al., 2004 and 2006). The expression of Survivin and its splicing variants was also examined by quantitative real-time RT-PCR method in patients with acute lymphoblastic leukemia (ALL) and chronic lymphocytic leukemia (CLL), and was found to be differentially regulated in ALL and CLL cells. (Nakagawa et al., 2004)

Aven is another anti-apoptotic 38.5 KDa protein, that suppresses apoptosis induced by Apaf-1 and caspase-9 (Chau et al., 2000). Aven gene is located on chromosome 15q1. Aven expression has recently been examined in children with acute lymphoblastic leukemia (ALL) for possible correlation with clinical features at diagnosis and treatment outcome and found to be higher in patients with unfavorable cytogenetic abnormalities, and in relapsed patients in the standard-risk group (Choi et al., 2006). Aven is less widely studied in cancer cells but its anti-apoptotic feature is known (Chau et al., 2000).

The present study aimed to examine the expression of both Survivin and Aven messenger RNA (mRNA) in cases of acute myeloid Leukemias with different FAB subtypes and to evaluate any possible association between their expression and relevant clinicopathological features.

Patients and Methods

Sample collection

This study included a total of 30 patients with newly diagnosed Acute Myeloid Leukemia admitted to the hematology unit in the Main Alexandria University hospital. The recruited patients were 14 men and 16 women; aged 24-58 years (mean (42±9) years). Ten healthy volunteers, with matched age and sex ratio, were included in the control group. Informed consents were obtained from all participants.

All patients were diagnosed using routine procedures, including morphology, cytochemistry. In addition to immunophenotyping, for CD34, CD13, CD33, CD14, CD19, CD7, cCD3 were performed for all patients.
Detection of Survivin and Aven mRNA using RT–PCR

- **Messenger (m)RNA extraction:**
The total RNA was extracted using TRIZOL reagent. Peripheral blood samples were obtained into vacutainer EDTA tubes. White blood cell (WBC) counting was performed in Sysmex KX-21 cell counter. Red cells were removed by centrifugation after lyses with red blood cell lysis buffer. The WBCs were suspended with lysis buffer and mRNA isolations were performed using Trizol® reagent (Invitrogen).

- **Reverse transcription and complementary (c)DNA synthesis:**
RNA (0.1-0.5 µg) was reverse transcribed using Revert Aid™ first strand cDNA synthesis Kit (Fermentas) according to the manufacturer’s guidelines. Briefly reverse transcription was carried out in a 20 µl reaction containing 1µl Revert Aid M-MuLV Reverse Transcriptase enzyme, 10 mM of each dNTP, 1µl of random hexamer primer, and 1µl RiboLock Ribonuclease inhibitor. The reaction mixture was incubated at 25°C for 10 min, then at 42°C for one hour followed by incubation at 95°C for 10 minutes to stop the reaction. cDNA samples were stored at –20°C until RT PCR was performed.

- **Primers:**
Sequences of the primers used are as follows (Gazzaniga et al., 2003 and Paydas et al., 2003):
- β-actin upstream, 5′-TTAGCTGTGCTCGCGCTACTCTC-3′;
- β-actin downstream, 5′-GTCGGATTGATGAAACCAGACA-3′;
- Survivin upstream, 5′-CAGATTTGAATCGCGGGACCC-3′;
- Survivin downstream, 5′-CCAAGTCTGGCTCGTTCTCAG-3′;
The survivin mRNA, GenBank accession number is NM 001168.
The aven mRNA GenBank accession number is AF283508.

- **Quantitative analysis of RT-PCR Products:**
RT-PCR products were electrophoresed through 3.0% agarose gel, stained with ethidium bromide, and visualized using UV transilluminator. We used the image analysis software program, Totallab, to accurately determine band intensity. For each sample we determined the ratios of surviving / β-Actin mRNA (Gazzaniga et al., 2003 and Paydas et al., 2003).

**Statistical analysis**
All the results were analyzed by SPSS software. (version 10). P value less than 0.05 was judged statistically significant. Hematological data of cases including WBCs, and Blast percentages are expressed as mean ± standard deviation (SD). Chi and Fisher exact test were performed for testing the significance of results. Student’s t test was used for comparison of independent variables. Comparison of mean values of studied variables among different subgroups was done using ANOVA test. Pearson’s correlation coefficient was used to quantify the relationship between the variables under study.

**Results**

**Patients’ characteristics and clinical data**
Our study group consisted of 30 patients with AML and 10 healthy subjects as a control group. Fourteen of our patients were males and sixteen were females; age range was
between 24 and 58 years (mean 42.87 ±9.8). According to French-American-British (FAB) classification, 8 cases (27%) were M1, while 5 cases were M2 (17%), 3 cases (10 %) were M3, 7 cases (23%) were M4, and 7 cases (23%) were M5. White blood cells count, Peripheral blood and bone marrow Blast percentages of AML samples are listed in Table 1.

**Table 1. Clinico-pathological characteristics of AML cases.**

<table>
<thead>
<tr>
<th>AML</th>
<th>WBCs (x10^9/L)</th>
<th>PB Blast (%)</th>
<th>BM Blast %</th>
</tr>
</thead>
<tbody>
<tr>
<td>Total (n=30)</td>
<td>51.45±27.52</td>
<td>49.03±27.93</td>
<td>60.50±26.83</td>
</tr>
<tr>
<td>M1 (n=8)</td>
<td>66.95±29.77</td>
<td>86.50±6.57</td>
<td>93.13±2.36</td>
</tr>
<tr>
<td>M2 (n=5)</td>
<td>42.90±26.59</td>
<td>43.60±±10.04</td>
<td>61.40±6.15</td>
</tr>
<tr>
<td>M3 (n=3)</td>
<td>44.80±27.36</td>
<td>30.67±1.15</td>
<td>39.00±1.15</td>
</tr>
<tr>
<td>M4 (n=7)</td>
<td>30.15±6.73</td>
<td>26.43±4.39</td>
<td>33.29±4.50</td>
</tr>
<tr>
<td>M5 (n=7)</td>
<td>64.00±27.30</td>
<td>40.57±29.80</td>
<td>56.86±29.95</td>
</tr>
</tbody>
</table>

AML: Acute Myeloid Leukemias
WBC: White Blood Cells
PB: Periferal Blood
BM: Bone Marrow
Data are presented as means ± standard deviation (SD)

---

**RT-PCR**

- Survivin mRNA expression in AML patients:

Expression of *Survivin* mRNA was detected by RT-PCR in AML patients. The gene products of β Actin and Survivin were 168bp and 206bp, respectively as presented in Figure 1. Out of 30 patients in subcategories M1 through M5 AML of FAB classification; only 23 patients (76.6 %) showed detectable levels of Survivin expression, while none of the control group showed Survivin expression. Survivin expression in AML cases compared to controls was significantly different \((P=0.000)\) as statistically calculated by Chi squared test using data presented in Table 2.

Differences in expression between patient’s subgroups were observed, Table 2. The expression of Survivin mRNA varied in correlation with the subgroup as indicated in Table 2. For M1 AML cases seven out of eight patients were positive for Survivin expression, similarly all M4 and M5 cases were positive while only one case of M2 and another of M3 showed bands of Survivin mRNA (Table 2). The differences in expression between subtypes were statistically significant \((P=0.001)\) as calculated by chi squared test.

---

![Figure 1: The expression of Survivin mRNA in AML cases. The gene product of Survivin was 206bp, and the gene product of β Actin was 168bp. M: ϕ x174/ HaeIII DNA marker, C: negative control, 1-10: different AML samples.](image-url)
The relative intensity of Survivin expression was analyzed quantitatively in AML samples showing expression bands. The expression level was calculated as a ratio between Survivin mRNA and β-Actin mRNA and the expression levels were plotted in Figure 2. Different expression levels of Survivin mRNA could be observed between different pathologic grades; Three of the 5 subgroups of AML patients (M1, M4, M5) had higher mean levels of Survivin mRNA expression of (mean range: 0.56-0.81) as compared to subgroups M2 and M3 (mean range:0.04 and 0.1 respectively) (Table 2).

Levels of Survivin expression varied even within the same diagnostic subcategory. The highest peak of Survivin expression among all AML subcategories was that of M5 as presented in Table 2. An independent Student t test was performed to compare the levels of Survivin mRNA in highly expressive subgroups (M1, M4 and M5) with that of the low expressive subgroups (M2, M3), the difference was statistically significant (t= 7.2, p=0.000). In addition one way ANOVA test between each individual AML subgroup showed high significant difference (p=0.000, Table 2).

Table 2. Levels of Survivin gene expression in AML cases.

<table>
<thead>
<tr>
<th>AML cases</th>
<th>Survivin Expression</th>
<th>Survivin level</th>
<th>ANOVA</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>-ve</td>
<td>+ve</td>
<td>Mean</td>
</tr>
<tr>
<td>Total (n=30)</td>
<td>7</td>
<td>23</td>
<td>0.49</td>
</tr>
<tr>
<td>M1 (n=8)</td>
<td>1</td>
<td>7</td>
<td>0.56</td>
</tr>
<tr>
<td>M2 (n=5)</td>
<td>4</td>
<td>1</td>
<td>0.04</td>
</tr>
<tr>
<td>M3 (n=3)</td>
<td>2</td>
<td>1</td>
<td>0.1</td>
</tr>
<tr>
<td>M4 (n=7)</td>
<td>0</td>
<td>7</td>
<td>0.57</td>
</tr>
<tr>
<td>M5 (n=7)</td>
<td>0</td>
<td>7</td>
<td>0.81</td>
</tr>
</tbody>
</table>

SD: standard deviation, SE: standard error
** Statistically highly significant P<0.001.

Figure 2: Survivin expression levels in different types of AML subgroups. N.B.: Samples of the same value are shown as one plot.

- Correlation between Survivin expression levels and hematological parameters:
  Correlation analysis between the hematological parameters and Survivin expression levels in different AML subgroups is displayed in Table 3.

A significant positive correlation was found between Survivin expression level and peripheral blood (PB) blast % in M5 subgroup only (r = 0.8, p=0.02). A positive correlation was also found between Survivin expression level and bone marrow (BM) blast % but it did not reach the level of significance ( r =0.69, p=0.08). On the other hand the Survivin expression level did not correlate with WBCs count in any subgroup of AML cases.
Table 3: Correlations between levels of Survivin expression and other hematological parameters in AML cases.

<table>
<thead>
<tr>
<th>AML cases</th>
<th>Survivin Expression and WBCs</th>
<th>Survivin Expression and PB blast %</th>
<th>Survivin Expression and BM blast %</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>r</td>
<td>p</td>
<td>r</td>
</tr>
<tr>
<td>Total</td>
<td>0.130</td>
<td>0.494</td>
<td>0.134</td>
</tr>
<tr>
<td>M1</td>
<td>0.455</td>
<td>0.258</td>
<td>0.357</td>
</tr>
<tr>
<td>M2</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>M3</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>M4</td>
<td>0.299</td>
<td>0.515</td>
<td>0.470</td>
</tr>
<tr>
<td>M5</td>
<td>0.228</td>
<td>0.622</td>
<td>0.826*</td>
</tr>
</tbody>
</table>

r: pearson’s correlation, p: Sig. (2-tailed)

* Correlation is significant at the 0.05 level (2-tailed).

Figure 3: The negative expression of Aven mRNA in AML cases. The expected gene product of Aven was 213bp, and the gene product of β Actin was 168bp. M: φ x174/ HaeIII DNA marker, C: negative control, 1-5: different AML samples.

- Aven mRNA expression in AML patients.

Expression of Aven mRNA was not detected by RT-PCR in AML patients as presented in figure 3. None of the examined samples showed diagnostic bands for Aven although all samples were positive for the internal control house keeping gene β actin.

Discussion

Recently, there has been great interest in apoptosis, or programmed cell death, the mechanism by which cells essentially suicide (Miller and Marx, 1998). Many inhibitors of apoptosis are known to contribute to tumorigenicity and increased spread of tumor cells (Sandler et al 2002). Survivin is described as a member of the inhibitor of apoptosis protein (IAP) family (Ambrosini et al 1997). This gene exists on chromosome 17q and inhibits apoptosis by blocking the effects of caspase-9, which is activated in extrinsic and intrinsic pathways (Ambrosini et al 1997, Reed, 2001 and Tajiri et al., 2001). Survivin is expressed in many malignant tumors, including breast, lung, stomach, colon and pancreatic cancers, bladder tumors, malignant lymphoma, and neuroblastoma (Nasu et al., 2002). It is not
usually present in normal tissues and is rarely found in mature tissues (Reed, 2001). Thus, Survivin expression is likely to be an important prognostic factor in tumor malignancy.

Aven has recently been identified as an anti-apoptotic protein. Higher levels of Aven mRNA are seen in childhood acute lymphoblastic leukemia than in control patients, suggesting that Aven expression can predict prognosis in childhood ALL (Choi, 2006).

The prognostic value of Survivin and Aven in hemopoietic neoplasias has not been as widely studied as in solid tumors. Although the data are limited, the prognostic value of Survivin has been studied in some hemopoietic malignancies such as high-grade lymphomas and leukemias (Adida et al., 2000a, b, Carter et al., 2001, Granziiero et al., 2001, Kamihira et al., 2001, and Mori et al., 2002). While Aven expression is less frequently studied in hemopoietic neoplasias.

We therefore used reverse transcription-polymerase chain reaction (RT-PCR) to investigate the expression of Survivin and Aven mRNA in a group of AML cases. The fidelity of mRNA extraction and RT was tested using oligonucleotide primers for β-actin a “housekeeping” gene. The β-actin primers - as described by Raff et al., (1997) - do not amplify genomic DNA and therefore provide absolute evidence that RT has been successful.

In the present study 30 patients of Acute myeloid leukemia -subdivided into M1-M5 FAB subtypes and 10 control subjects were examined by RT-PCR for Survivin and Aven expression. The expression of Survivin mRNA was detected in 23 patients out of 30 (76.6%), while none of the control group showed detectable expression. These results are in a good agreement with Adida et al. (2000b) who reported Survivin expression in 60% of a series of 125 cases of de novo AML.

These results are supported by the study of Carter et al. (2001) demonstrating expression of Survivin protein Western blot analysis in acute myeloid cell lines and in primary AML samples. As opposed to western blot analysis and immunohistochemistry, the PCR based approach used in our study is exquisitely sensitive and may detect gene transcripts even in a single cell or cell cluster with a comparable specificity (Shibata 1992).

Other studies proved that Survivin was expressed in all AML cell lines and most of the leukemia cells but not in normal peripheral blood mononuclear cells and/or bone marrow cells (Carter et al., 2001, Mori et al 2002, Kamihira et al., 2001, Morai et al., 2001 and Shinozawa et al., 2000). Similarly, Paydas et al (2003)) showed Survivin expression to be higher in study group of AML and ALL cases (in 35 patients, 54%) than in controls. In some studies Survivin-expressing leukemias have been found to have a worse prognosis compared with non-expressors (Adida et al., 2000a, Kamihira et al., 2001, Morai et al., 2001, and Mori et al. 2002).

In the current study, the expression of Survivin mRNA among AML subtypes showed two distinct groups; a highly expressing group (M1, M4, M5) as 7 patients out of 8 in M1 cases and all cases in M4 and M5 were positive for Survivin expression. The second group is a lower expressing one (M2 and M3) as 1 patient out of 5 and 1 patients out of 3, respectively, showed positive expression bands. There was a statistically significant difference between expressing cases in different FAB subtypes (p=0.001). This variation within the different subtypes of AML indicates that the
expression of Survivin mRNA might reflect the disease progression.

The Survivin expression was previously studied by immunohistochemistry in bone marrow cells from patients with chronic myelomonocytic leukemia (CMML) to evaluate possible abnormalities in comparison with other myelodysplastic (MDS) and myeloproliferative syndromes (Invernizzi et al. 2006). This study by Invernizzi et al (2006) found that in CMML there was no correlation between Survivin expression and blast cell percentage, FAB or WHO subgroup.

Additionally our present study analyzed quantitatively the Survivin mRNA and β-actin mRNA within AML samples subtypes. The highly expressing subgroups of AML patients (M1, M4, M5) had higher mean levels of Survivin mRNA expression of (mean value: 0.56 and 0.81 respectively) as compared to subgroups M2 and M3 (mean value: 0.04 and 0.1 respectively). Levels of Survivin expression was significantly different within the same diagnostic subcategories (p=0.000). In accordance with our study, Mori et al (2004) found that Survivin expression was lower in patients with M3 acute promyelocytic leukemia than in patients with other types of acute leukemia.

Our findings demonstrate that in M5 subgroup of patients a significant correlation exists between Survivin expression level and PB blast %, and also a non significant correlation exists with BM blast %. This may provide a useful information for the diagnosis and prognosis of AML subgroups. In another study, Survivin expression was found to correlate with lower WBCs count and favourable/intermediate cytogenetics in B-cell lymphomas (Adida et al, 2000a).

Our study revealed the absence of Aven mRNA in both AML cases and controls, this finding is contradictory to the study of Paydas et al (2003) who examined Aven expression by quantitative real-time PCR in AML and ALL patients and found a mean level of expression of 0.54 for Survivin and a mean level of 0.07 for Aven.

In conclusion, Survivin is an important anti-apoptotic signal in acute myeloid leukemia. Our findings suggest that up-regulation of Survivin expression in AML may be involved in its pathogenesis. Survivin may provide a useful tool for diagnosis and prognosis in acute myeloid leukemias. It is also concluded that Aven may not be a useful prognostic factor in acute myeloid leukemias.

References

8. Choi J; Hwang Y K; Sung KW; Kim D H; Yoo K H; Jung H L; Koo H H; Aven. Overexpression: Association With Poor Prognosis In Childhood Acute Lymphoblastic Leukemia. Leukemia Research, 30, 1019-1025, 2006


14. Invernizzi R ; Travaglino E ; Benatti Ch; Malcovati L ; Della Porta M; Cazzola M ; Ascali E. Survivin Expression, Apoptosis And Proliferation In Chronic Myelomonocytic Leukemia Eur. J. Haematol., 76, 494-501, 2006

15. Invernizzi R ; Travaglino E; Lunghi M; Klersy C ; Bernasconi P ; Cazzola M ; Ascali E. Survivin Expression in Acute Leukemias and Myelodysplastic Syndromes. Leukemia and Lymphoma, 45, 2229 – 223, 2004


