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Low expression of *APAF-1XL* in acute myeloid leukemia may be associated with the failure of remission induction therapy

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Apoptotic protease activating factor 1 (APAF-1) has a critical role in the regulation of apoptosis. In the present study, the mRNA expression analysis of different *APAF-1* transcripts (*APAF-1S, APAF-1LC, APAF-1LN*, and *APAF-1XL*) was analyzed in bone marrow samples from 37 patients with acute myeloid leukemia (newly diagnosed, with no previous treatment). *APAF-1XL* and *APAF-1LN* transcripts (with and without an extra WD-40 repeat region, respectively) were detected in all samples, although the major form expressed was *APAF-1XL* in 65% of the samples (group 1), while 35% of the samples expressed primarily *APAF-1LN* (group 2). Only 46% of the patients presented complete remission in response to remission induction therapy (represented by less than 5% marrow blasts and hematological recovery), all but 2 cases being from group 1, 21.6% did not attain complete remission (only 1 case from group 1), and 32.4% of the patients died early. Lower expression of *APAF-1XL* (*APAF-1XL/APAF-1LN* ratio <1.2) was associated with a poor response to therapy (P = 0.0005, Fisher exact test). Both groups showed similar characteristics regarding white blood cell counts, cytogenetic data or presence of gene rearrangements associated with good prognosis as *AML1-ETO*, *CBFB-MYH11* and *PML/RARA*. Since it has been shown that only the isoforms with the extra WD-40 repeat region activate procaspase-9, we suggest that low procaspase-9 activation may also be involved in the deregulation of apoptosis and chemotherapy resistance in acute myeloid leukemia.

Key words: *APAF-1*; Acute myeloid leukemia; Apoptosis; Chemotherapy resistance

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Introduction

Disturbance of the apoptotic mechanism is implicated in many human diseases, especially cancer (1), and failure to activate the apoptotic machinery can result in resistance to the cytotoxic effects of multiple chemotherapeutic agents (2,3). For the majority of anticancer drugs, apoptosis appears to be initiated by the cytochrome c/apoptotic protease activating factor 1 (APAF-1)/caspase-9 pathway. Cytochrome c can be released from mitochondria into the cytosol after the induction of apoptosis by many different stimuli. Upon its release and in the presence of dATP or ATP, APAF-1 forms a complex with procaspase-9. In turn, procaspase is cleaved into its active form, leading to the activation of caspase-3, the executioner of apoptosis (4-6).

A critical role for APAF-1 in the regulation of apoptosis was confirmed by the analysis of APAF-1-deficient mice in which abnormalities were observed in several tissues, particularly the brain, and characterized by the lack of developmental cell death (7,8). The original APAF-1 described by Xiadong Wang's group (9) was identified as a mammalian homologue of *C. elegans* CED-4. This 130-kDa protein comprises three functional domains, the N-terminal caspase recruitment domain (CARD) followed by a putative ATPase domain and a C-terminal domain with a 12 WD-40 repeat region (WDR).

Several investigators have described multiple *APAF-1* splice variants. Alternative splicing can create an NH_2-terminal 11-amino acid insert between the CARD and ATPase domain or an additional COOH-terminal WDR between the fifth and sixth WDRs. The APAF-1XL and APAF-1LN isoforms have the insertion between the CARD and ATPase domains, and APAF-1XL also presents the additional WDR (10,11). *In vitro* assays have shown that only the forms containing the extra WDR were able to activate procaspase-9 in a cytochrome c- and dATP-dependent fashion, while the NH_2-terminal insert was not required (12).

Recent findings have provided insight into the regulation of the balance between apoptosis and cell proliferation signals, and have suggested that some genes and pathways may have an important role in the pathogenesis of acute myeloid leukemia (AML). AML is a lethal malignant disease characterized by the abnormal proliferation and differentiation of myeloid progenitor cells. The transformation to leukemogenesis requires multiple steps, including defects in the growth and differentiation of hematopoietic cells (13).

Analysis of apoptotic gene expression in patients with AML has revealed that tumor suppressor genes are down-regulated and protooncogenes are up-regulated (13) and that several cellular signaling pathways acting either on cell cycle regulation or on apoptosis are altered. Deregulation was found in pathways that contribute to genomic stability and regulate cell cycle progression, and transcriptional alterations were also identified. Abnormalities were observed in the regulation of caspases by up-regulation of CASP8 and by the altered expression of the BCL2-related pathway (14). Svingen et al. (15) demonstrated that the levels of APAF-1 protein vary between acute lymphoblastic leukemia and AML patient samples and did not find a correlation with prognostic factors or response to induction therapy (15). However, Jia et al. (16) demonstrated that APAF-1 deficiency induces resistance to apoptosis in human leukemic cells. Moreover, Furukawa et al. (17) described the methylation silencing of *APAF-1* in AML cell lines and in a few AML patient samples but no studies have reported its relationship with prognosis (17). Thus, the role of APAF-1 in AML remains poorly characterized.

The aim of the present study was to characterize the expression of different *APAF-1* transcripts in untreated AML bone marrow samples and to correlate the expression of these mRNAs with the clinical outcome of these patients. The relative expression of these transcripts was determined by RT-PCR band densitometry, a technique that allows a reproducible comparison between *APAF-1LN* and *APAF-1XL* transcripts. A strong association between low *APAF-1XL* mRNA expression and poor response to chemotherapy was detected.

Subjects and Methods

Subjects

Thirty-seven patients with AML (1 biphenotypic, 1 M0, 10 M1, 5 M2, 6 M3, 12 M4, 1 M5B, and 1 M6) (18) and 10 normal bone marrow donors participated in this study. The diagnosis of AML was based on clinical and laboratory data. Informed written consent was obtained from all individuals, and the study was approved by the Ethics Committee of the General Hospital of the State University of Campinas. Median age was 49 years (range: 19–86 years). The male:female ratio was 2.3:1. The median rate of leukemic blasts in patient bone marrow samples was 85% (range: 34 to 97%). All patients were untreated and were enrolled at the time of initial therapy. Thirty-four patients presented primary AML and 3 patients secondary AML after myelodysplasia. Remission induction therapy included anthracyclines, cytarabine and 6-thioguanine for patients under 60 years and etoposide, mitoxantrone and 6-thioguanine for patients over 60 years. For patients with the M3 subtype, the induction treatment included all transretinoic acid and mitoxantrone.

Complete remission was determined when bone marrow became normocellular, containing less than 5% blasts. Relapse was diagnosed when bone marrow contained >5% leukemic blasts or when leukemic infiltration occurred at any other site.

RNA extraction, cDNA transcription and RT-PCR assays

Total RNA from bone marrow samples was prepared using TRIzol® Reagent (Invitrogen, USA) and cDNA was synthesized with the SuperScript II™ reverse transcriptase and oligo (dT) primer. Reverse-transcribed RNA was submitted to PCR in a 50-µL reaction mixture using high fidelity Taq Platinum® (Invitrogen). The relative amounts of *APAF-1* cDNAs with or without the NH_2-terminal 11-amino acid insert were determined using the specific primers N1, 5'-GTAAGAAATGAGCCCACTC-3' and N2, 5'-TACTCCA CCTTCACACAG-3', and the following PCR conditions: 33 denaturation cycles of 30 s at 94°C, 40 s annealing at 52°C, and a 30-s extension at 72°C, followed by a 5-min extension at 72°C. The relative amounts of APAF-1 cDNAs with or without the additional COOH-terminal WDR were determined using the specific primers C1, 5'-CAGCTGAT GGAACCTTAAAGC-3' and C2, 5'-GTCTGGTCATCA GAAGATGTC-3' (12), and the following PCR conditions: 33 denaturation cycles of 30 s at 94°C, 50-s annealing at 58°C, and 50-s extension at 72°C, followed by a 5-min

extension at 72°C. In order to ensure that the RT-PCR signal was linear with respect to RNA input, we performed kinetic analysis by varying the number of amplification cycles (25, 30, 33, 35, 38) and the amount of cDNA input (0.25, 0.5, 0.75, 1.0, 1.25 µg), considering that the same amount of RNA was reverse-transcribed for each sample (Figure 1). On the basis of this analysis, we used 33 cycles for both pairs of primers and 1 µg cDNA. PCR amplification was carried out in duplicate and water was used as negative control. PCR products were run on 1.5% agarose gels and analyzed by staining with ethidium bromide. Band quantification was performed using the Kodak Digital Image 1D software, version 3.0.2. For most samples, experiments were repeated twice.

RT-PCR was also performed for the detection of other recognized prognostic markers in AML as *PML-RARA, AML1-ETO, CBFB-MYH11* rearrangements using the following primers: 1) Inv16C1: 5'CAGGCAAGGTATATTTGAAGG3', Inv16M1: 5'CTCTTCTCCTCATTCTGCTC', and Inv16nest: 5'GAAATGGAGGTCCATGAGCT3'; 2) PML-RAR: 5'-ACCGATGGCTTCGACGAGTTC-3', 5'-AGCCCTTGCAGCCCTCACAG-3', 5'-AGCGCGACTACGAGGAGATG-3', and 5'-CCATAGTGGTAGCCTGAGGACT-3'; 3) AML1 gene: 5'-AGCTTCACTCTGACCATCAC-3', ETO gene: 5'TGAACTGGTTCTTGGAGCTCCT-3', and 8,21 probe ETO gene: 5'-TTCACAAACCCACCGGAAGTA-3'.

Cell cultures, induction of apoptosis and Western blot

In order to determine if the expression of APAF-1 transcripts is related to apoptosis, we used KG-1 and HL-60 cells as a model since *APAF-1XL* is expressed differently in these cell lines. The induction of apoptosis was performed by incubation of KG-1 and HL-60 cell lines with 100 mM ethanol with or without serum (5%, v/v, heat-inactivated horse serum and 10%, v/v, fetal calf serum) for 24 h (19). Total protein was then extracted and submitted to SDS-PAGE, on a 15% polyacrylamide Laemmli system (20). Western blot was performed using a caspase-9 antibody (Santa Cruz Biotechnology, USA). Inactive caspase-9 is 47 kDa and after hydrolysis, a 35-kDa band should be detected.

Statistical analysis

Correlation between mRNA expression and clinical response to remission induction therapy was assessed using the Fisher exact test. The association of *APAF-1* isoforms with survival was determined by comparing Kaplan-Meier survival curves constructed for the two different groups by the log-rank test. Overall survival was measured from diagnosis to death or date of the last follow-up visit. Patient follow-up continued up to January 31, 2007.

Results

Relative expression of APAF-1 transcripts in AML blasts and normal bone marrow

PCR analysis using N1 and N2 primers revealed only the *APAF-1* transcript with the 11-amino acid NH_2-terminal insertion in all samples. The same was observed in normal controls. PCR analysis using primers C1 and C2 showed two products in all samples, although their relative amounts varied among samples (Figure 2). Only two *APAF-1* transcripts, *APAF-1XL* and *APAF-1LN*, were detected in the 37 AML patient samples examined.

The results of analysis by real-time PCR were not reproducible (data not shown). However, taking advantage of the fact that both transcripts can be efficiently and simultaneously amplified in the same tube, the *APAF-1XL/APAF-1LN* mRNA ratio was calculated based on the quantification of the 430- and 301-bp band, respectively. Duplicate analysis showed a coefficient of variation lower than 20% (median = 6.4%; range: 0 to 19%) for quantitation of the bands indicating the reliability of the method. This method has the advantage of permitting the comparison of the two transcripts in a same sample.

In 24 samples (65%), the predominantly expressed

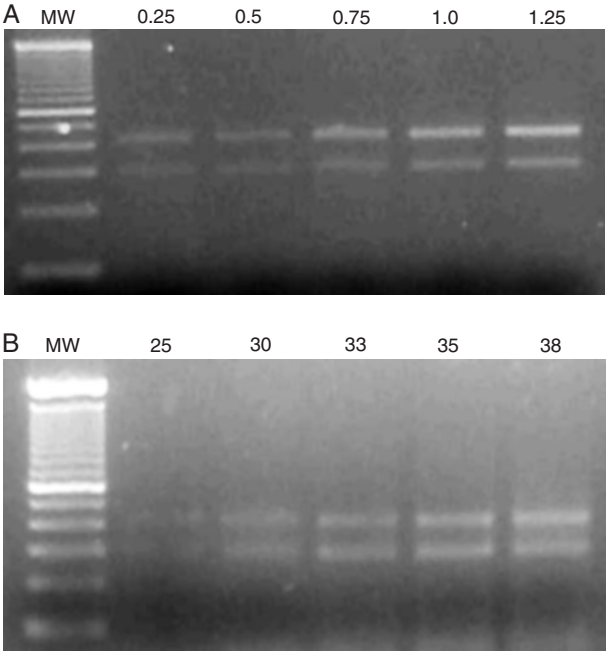

Figure 1. Standardization of RT-PCR conditions by kinetic analysis of APAF-1 transcript amplification. *A*, Increasing amounts of cDNA were submitted to 33 cycles of amplification (from 0.25 to 1.25 µg). *B*, cDNA (1 µg) was submitted to different numbers of cycles of amplification (from 25 to 38). MW = molecular weight (100-bp ladder).

PCR product contained the extra WDR (*APAF-1XL/APAF-1LN* ratio ≥1.2), and in 13 samples (35%), the expression of both products was similar or with a predominance of the transcript lacking the extra WDR (*APAF-1XL/APAF-1LN* ratio ≤1.15; Figure 2, Table 1). The analysis of 10 normal bone marrow samples showed a median *APAF-1XL/APAF-1LN* ratio = 1.07 (range = 0.92-1.38). Approximately equal amounts of *APAF-1XL* and *APAF-1LN* (containing only the NH_2-terminal insertion) were detected in normal bone marrow, colon and spleen tissues, while tissues such as brain, kidney, stomach, and skeletal muscle expressed more *APAF-1XL* (12).

Low expression of *APAF-1XL* is associated with failure of remission induction therapy

Patients were divided into two groups according to

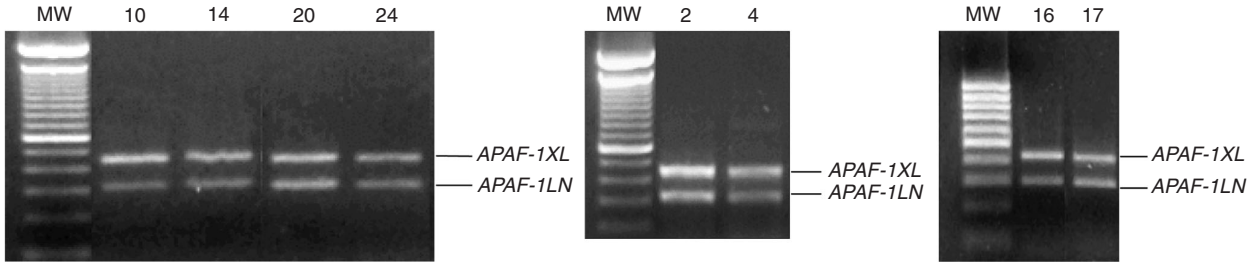

Figure 2. RT-PCR of *APAF-1* transcripts from patients with acute myeloid leukemia numbered as shown in Tables 1 and 2. MW = molecular weight (100-bp ladder).

Table 1. Relationship of *APAF-1XL/APAF-1LN* ratio with the response to the first or second cycle of remission induction therapy.

Patient number	Age (years)	AML (FAB)	Leukocytes (N/mm^3)	Cytogenetics	*AML1-ETO*	*PML-RARA*	*CBFB-MYH11*	*APAF-1XL/APAF-1LN* ratio	Remission
1	60	M1	na	nd	nd	nd	nd	1.27	Yes
2	54	M4	26760	46,XY	negative	negative	negative	1.97	Yes
3	51	M4	7260	46,XY	negative	negative	negative	1.36	Yes
4	51	M4	123000	46,XX	negative	negative	negative	1.49	Yes
5	76	M4	11820	46,XY	negative	negative	positive	1.29	Yes
6	47	M1*	6750	45,XY,8q-,-21	nd	nd	nd	1.34	Yes
7	20	M1	27370	46,XX	negative	negative	negative	1.88	Yes
8	24	M3	24800	46,XY	negative	positive	negative	3.16	Yes
9	41	M1	2800	46,XX	negative	negative	negative	1.58	Yes
10	18	M0	16800	nd	negative	negative	negative	1.65	Yes
11	86	M1	8430	nd	negative	negative	negative	1.24	Yes
12	44	M6	na	nd	nd	nd	nd	1.34	Yes
13	44	biphenotypic	na	nd	nd	nd	nd	1.33	Yes
14	58	M4	12200	nd	nd	nd	nd	1.31	Yes
15	43	M4	6830	nd	nd	nd	nd	1.34	Yes
16	83	M4	26450	46,XX	negative	negative	positive	1.76	No
17	34	M1	12890	nd	nd	nd	nd	0.89	Yes
18	31	M3	28630	nd	negative	positive	negative	1.05	Yes
19	58	M4	11690	nd	nd	nd	nd	0.78	No
20	22	M2	2100	46,XY	negative	negative	negative	1.05	No
21	31	M1	8630	nd	nd	nd	nd	1.08	No
22	70	M2	112000	47,XY,+11	negative	negative	negative	1.07	No
23	30	M5B	6810	nd	negative	negative	negative	1.08	No
24	36	M2*	2310	45,XY,-5,17p+[6]/46,XY[19]	negative	negative	negative	1.15	No
25	52	M1	na	nd	negative	negative	negative	1.08	No

Data are reported for the 25 patients who survived. Patients marked with an asterisk had a secondary acute myeloid leukemia (AML) after myelodysplasia. *APAF-1XL/APAF-1LN* ratio result is represented as mean of at least two experiments. FAB = French American British classification; *AML1-ETO, PML-RARA, CBFB-MYH11* = fusion transcripts due to gene rearrangements. na = not available; nd = not done.

APAF-1XL/APAF-1LN ratio, with group 1 expressing mostly *APAF-1XL*, ranging from 1.20 to 3.16 and group 2 expressing *APAF-1XL/APAF-1LN,* ranging from 0.78 to 1.15. Comparison of the two AML groups showed no difference regarding age, AML subtypes, white blood cell counts, cytogenetic data, presence of *PML-RARA, AML1-ETO, CBFB-MYH11* gene rearrangements, expression of CD34, HLA-DR, lymphoid markers. However, in response to the first or second cycle of remission induction therapy, 17 (46%) patients obtained complete remission (less than 5% marrow blasts and hematological recovery), all but 2 of these cases were in group 1, while 8 (21.6%) did not attain complete remission (only 1 case in 8 were from group 1) and 12 (32.4%) patients died early (equally distributed in both groups). To our knowledge, the data reported herein show for the first time that levels of *APAF-1XL* lower than *APAF-1LN* are associated with a poor response to remission induction therapy (Fisher test: P = 0.0005, 95%CI: 3.09-2458.81, odds ratio = 39.59; Table 2).

Lack of correlation between APAF-1 transcript expression and overall survival

To investigate a possible correlation between *APAF-1* mRNA expression and overall survival we divided the patients into two groups with an *APAF-1XL/APAF-1LN* ratio = 1.15 as the cut-off. Mean survival was 12.8 months for the group with a ratio >1.15 and 11.5 months for the group with a ratio ≤1.15; however, the difference was not statistically significant (P = 0.54; see Figure 3). Thus, although APAF-1 transcript expression appears to be an important marker of the response to chemotherapy, it is not associated with patient survival.

Correlation between APAF-1 transcript expression and caspase-9 activation

To determine if lower amounts of APAF-1XL are associated with lower caspase-9 activation, we induced apoptosis in HL-60 and KG-1 cells, and determined caspase-9 activation and the *APAF-1XL/APAF-1LN* expression ratio. Apoptosis was induced by incubating the cell lines with 100 mM ethanol with or without FBS for 24 h (19), and Western blot was performed using caspase-9 antibody, as described in Subjects and Methods.

The KG-1 cell line had a high expression of *APAF-1LN* and a very low expression of *APAF-1XL*, with the opposite occurring in HL-60 cells, which contain high amounts of APAF-1XL (Figure 4A). Densitometric analysis of the bands showed, as expected, higher amounts of cleaved caspase-9 in the HL-60 cell line, and a predominance of the inactive form in the KG-1 cell line (Figure 4B,C). These data indicate a direct relationship between higher *APAF-1XL* mRNA levels and the activation of caspase-9 at the protein level.

Discussion

Different transcripts of the APAF-1 gene are produced by alternative splicing, which is a widespread process used by higher eukaryotes to regulate gene expression and permit protein diversification. A reliable method for measuring the expression levels of splice variants is important for the determination of the significance of each variant. Several methods are commonly used to quantify alternative transcripts (21) and all present limitations: Northern blot requires large amounts of RNA, a moderate to high abundance of the transcript and is time consuming. RNAse protection is also time consuming and unable to detect low abundance transcripts. Semi-quantitative PCR requires quantification in the exponential phase, which is different for every sample because it depends on the initial target

Table 2. Correlation between the relative expression of APAF-1 transcripts and response to remission induction therapy.

Group	Remission	
	Yes	No
1 (ratio >1.15)	15	1
2 (ratio ≤1.15)	2	7

Group 1: predominantly expressing *APAF-1XL* (*APAF-1XL/APAF-1LN* ratio >1.15) and group 2: predominantly expressing *APAF-1LN* or both isoforms equally (*APAF-1XL/APAF-1LN* ratio ≤1.15). P = 0.0005 (Fisher test).

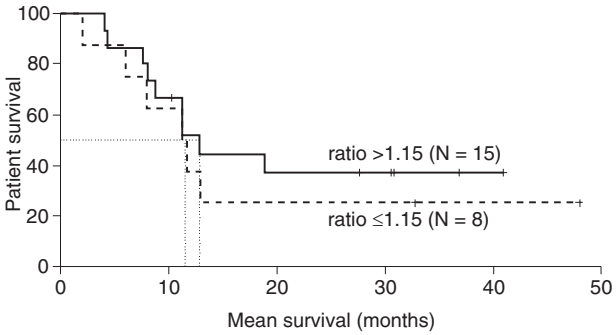

Figure 3. Kaplan-Meier plot of overall survival according to the ratio *APAF-1XL/APAF-1LN*. Overall survival of patients presenting an *APAF-1XL/APAF-1LN* ratio >1.15 (solid line, N = 15) versus patients presenting an *APAF-1XL/APAF-1LN* ratio ≤1.15 (dashed line, N = 8). There was no significant difference between groups (log-rank test).

concentration. Competitive PCR is a labor-intensive method limited to a variable target-to-competitor ratio. Lately, real-time PCR has been used with reliable results (22); however, a well-validated boundary-spanning primer is necessary. Moreover, the method requires multiple internal control genes for normalization of the reaction.

In the present study, the relative expression of *APAF-1* transcripts was measured by semi-quantitative PCR, which allowed us to efficiently amplify both transcripts in the same reaction tube. Other investigators have used this same approach (12,22). Furthermore, our analysis intended to demonstrate the relationship between the two transcripts in the same sample, rather than compare each transcript in several samples; thus, this method allowed us to approximately determine the *APAF-1XL/APAF-1LN* ratio. Our results showed that two *APAF-1* transcripts were present in all AML patients, although in varying amounts. All samples examined exclusively showed the transcript corresponding to the insertion of the 11 amino acids between the CARD and ATPase domains. Regarding the COOH-terminal, the presence of both transcripts, with and without the extra WDR, was observed. Thus, the mRNA transcripts found in patient cells were both *APAF-1XL* and *APAF-1LN*. Indeed, the results clearly showed the association of the *APAF-1* transcript mRNA expression with the response to induction treatment. All but two patients, presenting predominantly the *APAF-1XL* transcript, responded to the first or second cycle of remission induction therapy. Patients with the highest expression of *APAF-1XL* had a tendency to higher survival, which, however, was not statistically significant.

When we induced apoptosis in cell cultures of the HL-60 line, which presents the highest amounts of *APAF-1XL*, efficient caspase-9 activation was observed but not in the KG-1 line which has relatively less *APAF-1XL* and more *APAF-1LN*. This association between the levels of a specific transcript of the APAF-1 gene and caspase-9 activation suggests how the result of alternative splicing could modulate an important biological process such as apoptosis.

Quantitation of the various molecules involved in the apoptotic pathways has been studied to predict the response of leukemia patients to chemotherapy. High AF1q expression was associated with poor survival in pediatric AML (23). FAK expression correlates with enhanced migratory properties, drug resistance, high leukocytosis, and reduced survival (24), and low or absent expression of the FADD protein in leukemic cells at diagnosis is a poor independent prognostic factor (25). In addition to their prognostic significance, a better understanding of the biologic mechanism of these molecular changes may help identify new targets for cancer therapy.

In AML, the expression level of molecules involved in the mitochondria-mediated pathway of apoptosis also provides important prognostic information. Although the prognostic value of the expression of BCL-2 antiapoptotic and

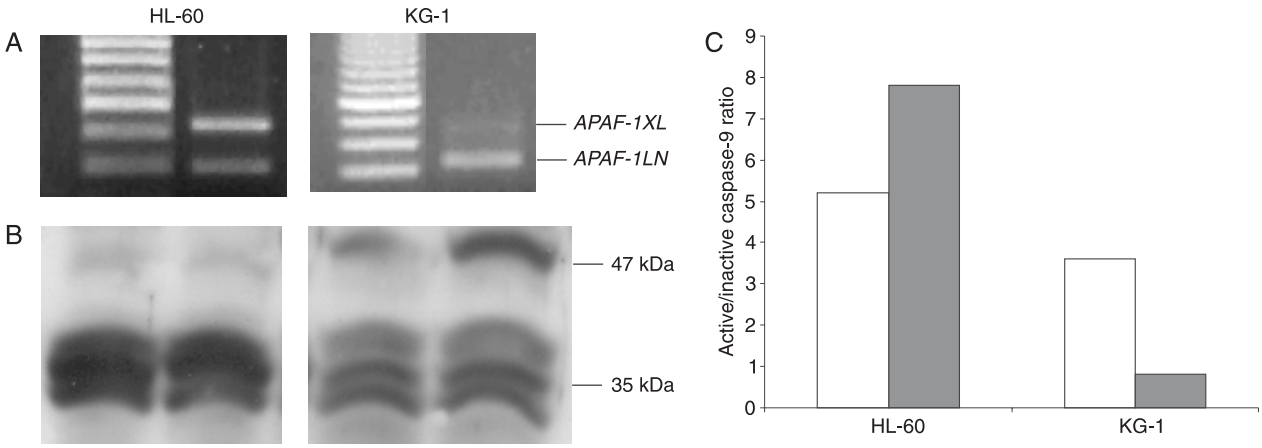

Figure 4. *A*, Expression of APAF-1 transcripts in HL-60 and KG-1 cell lines and its relation to caspase-9 activation. *B*, The molecular weight of inactive caspase-9 is 47 kDa, and the molecular weight of the active cleaved form is 35 kDa. Apoptosis was induced by incubating the KG-1 and HL-60 cell lines with 100 mM ethanol with or without serum (5%, v/v, heat-inactivated horse serum and 10%, v/v, fetal calf serum) for 24 h. Total protein was extracted and submitted to SDS-PAGE on a 15% polyacrylamide Laemmli system. Western blot was performed using a caspase-9 antibody (Santa Cruz Biotechnology, Inc.). *C*, Active/inactive caspase-9 ratio obtained by densitometric scanning.

BAX proapoptotic molecules is controversial (26-29), the BAX to BCL-2 ratio is of prognostic value in AML patients (30).

Benedict et al. (12) have shown that only the APAF-1 isoforms with the extra WDR activate procaspase-9. Thus, we suggest that in AML a low expression of *APAF-1XL* may possibly be correlated with a lower procaspase-9 activation, corroborating the deregulation of apoptosis and chemotherapy resistance.

Several studies have demonstrated a strong relationship between APAF-1 expression and response to chemotherapy and progression or prognosis of many neoplastic diseases such as melanoma, Burkitt lymphoma, chronic lymphoid leukemia, colorectal cancer, and cervical cancer (31-35). Moreover, hypermethylation of the APAF-1 gene may be an independent prognostic factor in acute lymphoblastic leukemia (36). In addition to these findings, the results herein presented showed, for the first time, that APAF-1 expression is also associated with response to chemotherapy in acute myeloid leukemia.

Despite the small number of patients studied, the present results strongly suggest that APAF-1 plays a role in their response to chemotherapy and may have an impact on the survival of AML patients. Thus, multicentric studies with a larger number of patients should be encouraged in order to test the impact of *APAF-1XL* as a prognostic marker in AML.

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