

## Real-time PCR analysis of the apoptosis related genes in ATRA treated APL t(15;17) patients

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Abbreviations: APL, acute promyelocytic leukemia; ATRA, all-trans retinoic acid; FAB, French-American-British; PCR, polymerase chain reaction; REST, relative expression software tool

### Abstract

All-trans retinoic acid (ATRA) treatment of the acute promyelocytic leukemia (APL) have subsequently resulted in cell apoptosis, but the molecular mechanism of this effect remains elusive. In order to understand a possible involvement of genes regulating apoptotic signal pathways, expression levels of *bcl2*, *bax*, *dapk1*, *myc*, *bad*, *wt1*, and *mcl1* genes were analyzed during ATRA treatment in five APL patients with t(15;17) using Real-time PCR (LightCycler). Two samples from each patient were compared to each other: primary diagnostic sample and a sample taken at remission. Effect of the ATRA treatment was demonstrated by the concomitant induction of *cd14* and *il1 $\beta$*  genes in four patients. Also other apoptosis related genes were found down-regulated in general but especially the down regulated levels of *wt1* and *bax* attract attention. Result suggested that ATRA dependent apoptosis of APL was under the control of both internal and external pathways without relationships to the amount of the blast populations. Ratio of *bcl2* to *bax* may be more important for this regulation than the ratio of *bcl2* to *bad*. Either *bcl2* family or less known apoptosis related genes as *wt1* will still be required to further studies in this setting.

### Introduction

All-trans retinoic acid (ATRA) dependent differentiation induction is used in the treatment of the acute promyelocytic leukemia (APL) successfully. It has been clearly demonstrated in pre-clinical studies that cells induced to differentiate subsequently die via apoptosis (Martin *et al.*, 1991; Gillis *et al.*, 1995; Lo Coco *et al.*, 1998).

Apoptosis might be induced by external signals at the surface of the cell. Fas and the TNF receptors transmit a signal to the cytoplasm that leads to activation of caspase 8. *Myc* expression can activate this signalling pathway prematurely (Hoffman *et al.*, 2002). In the second mechanism, a group of *bcl2* family proteins protect or initiate apoptosis. The pro- and anti-apoptotic *bcl2* proteins can make heterodimers and the ratio of these determines the execution of cell death. Among these members *bcl2* is anti-apoptotic while *bax* and *bad* are pro-apoptotic (Oltvai *et al.*, 1993; Adams and Cory, 1998; Srivastava *et al.*, 1999; Mitchell *et al.*, 2000; White *et al.*, 2001).

*Mcl1* is also described as a differentiation-related mitochondrial anti-apoptotic factor related to erk signal transduction pathway. There may be other genes in different pathways leading to apoptosis which are not yet fully characterized. Among these genes, *dapk1* is a potential mediator cell death, and over expression of *wt1* may lead to apoptosis (Deiss *et al.*, 1995; Townsend *et al.*, 1999; Mitchell *et al.*, 2000).

Apoptosis has been traditionally studied by characteristic cleavage of DNA into nucleosomal fragments. Unfortunately, this method is unable to help in studying different genes simultaneously and not very sensitive.

In our study we analysed the expression of the apoptosis related genes during the ATRA treatment in five APL patients with t(15;17) using an extremely sensitive technique known as quantitative Real-Time PCR (LightCycler, Roche Diagnostics GmbH, Germany). We have used SYBR Green I dye binding method and determined the expression levels of seven apoptosis related genes (*bcl2*, *bax*, *mcl1*, *dapk1*, *myc*, *bad* and *wt1*). Differentiation effect of the ATRA

treatment was aimed to demonstrate by *cd14* and *IL1 $\beta$*  genes. Two samples from each patient were compared to each other: primary diagnostic sample in diagnosis and a sample taken at complete remission.

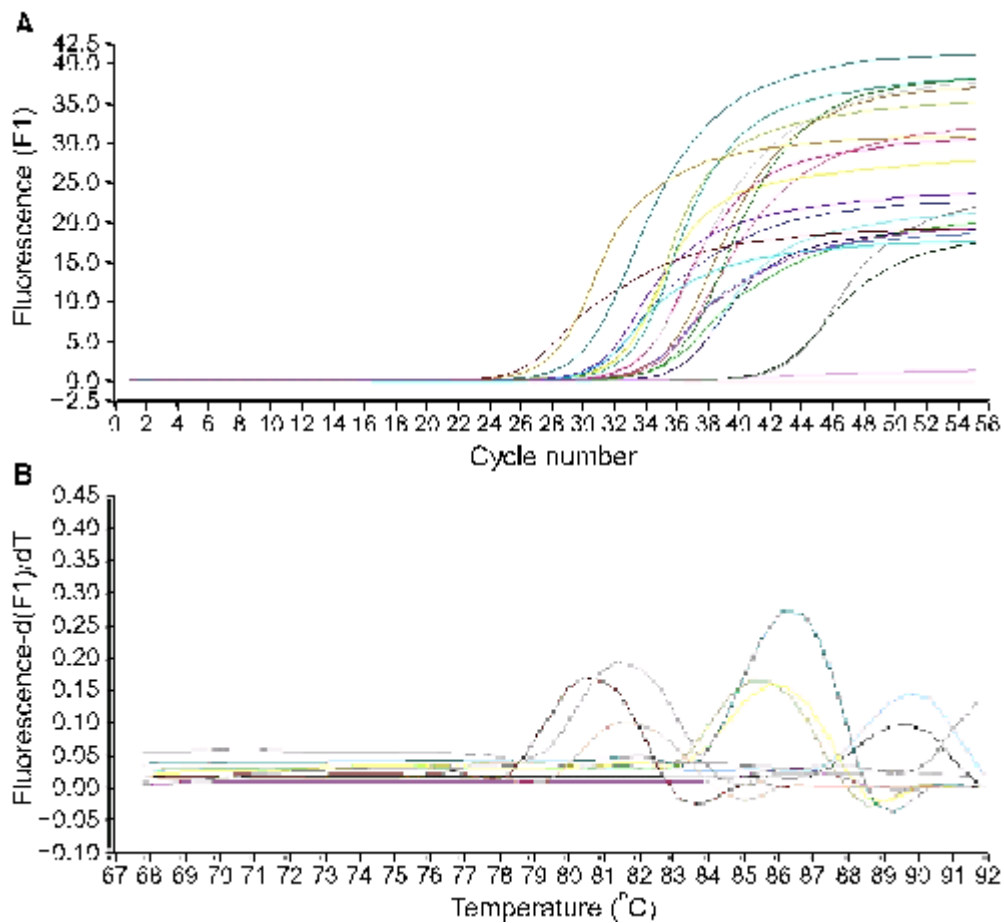
## Materials and Methods

Our study was based on bone marrow aspirates from 5 APL patients having t(15;17) and treated with ATRA (45 mg/m<sup>2</sup>, daily). Among of these patients, 4 were

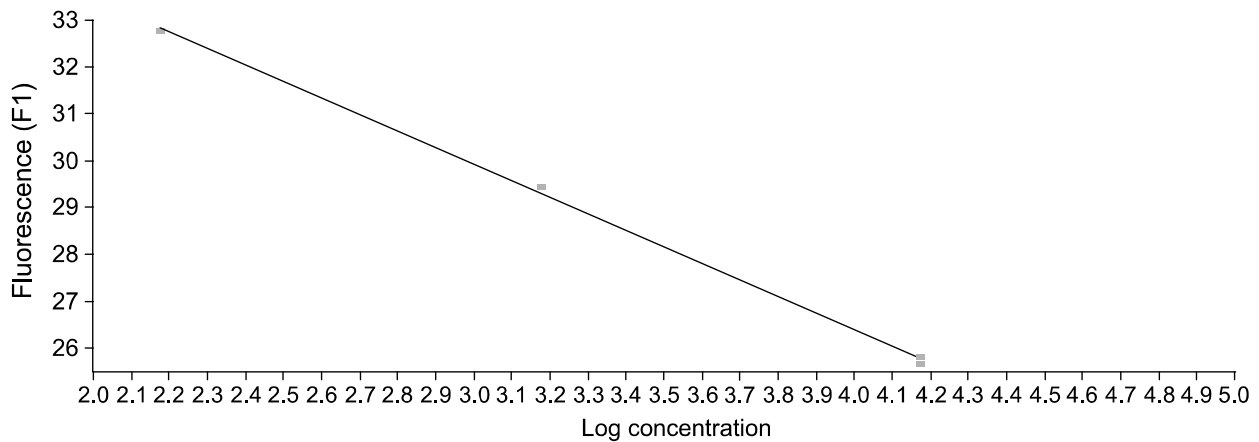
**Table 1.** \*\*Data of 5 acute promyelocytic leukemia t(15;17) patients.

Patient	Age/sex	BP	WBC (10 <sup>9</sup> /L)	Hb (g/d)	Blast count at diagnosis	S (m)
1	19 (F)	bcr1/2	7,000	10	18%	14+
2	69 (F)	bcr1/2	2,600	10	50%	16+
3	44 (M)	bcr1/2	2,500	5.1	95%	47+
4	29 (M)	bcr3	39,500	6.7	80%	12+
5	2 (M)	bcr1/2	10,000	8	20%	n.a.

Abbreviations: BP, Breakpoint; WBC, White Blood Cells; Hb, Hemoglobin; S (m), Survival (months); n.a., Data is not available. \*\*All patients have been treated with ATRA 45 mg/m<sup>2</sup>, daily.



**Figure 1.** (A) LightCycler assisted standard analysis of the studied genes. On line fluorescence curves of PCR amplifications and exponential increases in fluorescence intensities are shown as a measurement for the quantity of amplified fragments. (B) Specific melting curve analysis of the apoptosis related genes in our study. The melting peaks at 87°C for RPS9, 90°C for CD14, 86°C for IL1 $\beta$  1, and seven apoptosis related genes between 81°C to 92°C indicate the specific products that melt at the different temperatures.



**Figure 2.** Standart curve analysis of the serial dilutions of the RPS9 housekeeping gene (Slope: -2.701, Intercept:33.80, Error: 0.0349, R: -1.00).

**Table 2.** Primer sequences of selected genes.

<i>rps9</i> housekeeping gene	CGTCTCGACCAAGAGCTGA GGTCCTTCTCATCAAGCGTC
monocyte differentiation CD14 antigen precursor	GAGCCGCACAGGTTCCCTG GCTTGGGCAATGCTCAGTACC
<i>il1β</i> (Interleukin-1 beta precursor)	GTGCTGAATGTGGACTCAATCC TGACAGAGGAGGGTTTCTTAGAAC
<i>bcl2</i>	AGGAAGTGAACATTTCCGGTGAC GCTCAGTTCCAGGACCAGGc
<i>bax</i> (BCL2-associated X protein)	TGCTTCAGGGTTTCATCCAG GGCGGCAATCATCCTCTG
<i>mcl1</i> (Myeloid cell leukemia sequence 1)	GATGATCCATGTTTTTCAGCGAC CTCCACAAACCCATCCCAG
<i>dapk1</i> (Death-associated protein kinase 1)	CAGTGTTGTTGCTCTAGGAAG GGGACTGCCACAAATGATGAGC
<i>Myc</i> oncogene	GGCAAAAGGTCAGAGTCTGG GTGCATTTTCGGTTGTTGC
<i>wt1</i> (Wilms tumour 1)	CTGTCCCACCTTACAGATGCACAG TCTTTTGAGCTGGTCTGAACGA
<i>bad</i> (Bcl2 antagonist of cell death)	GAGTGAGCAGGAAGACTCCAGC TCCACAAACTCGTCACTCATCC

in adult (average age: 27) and 1 was in childhood age (2). Diagnosis of APL is based on FAB criteria's. All of the patients were positive for PML-RAR alpha fusion transcripts detected by RT-PCR (Table 1) The samples were obtained from the University of Istanbul and all the treatments have been performed between 1998-2001. Mononuclear cells from bone marrow aspirates were isolated by ficoll-hypaque centrifuga-

tion. Total RNA was extracted by using guanidium thiocyanate-phenol-chloroform extraction method as previously described (Chomznsky *et al.*, 1987). RNA samples were treated with DNase I and quantitative Real-time PCR was performed as we described previously (Savli *et al.*, 2002; Savli *et al.*, 2003). Primer sequences of the selected genes have been shown in Table 2. On line fluorescence curves of

PCR amplifications and melting curve analysis were shown in Figure 1. Standard curve analysis of the serial dilutions of the RPS9 housekeeping gene was shown in Figure 2.

Obtained gene expression values were normalized using a housekeeping gene in both patient and healthy control groups. For this aim *rps9* was used which belongs to a gene family accepted more reliable than either of the classical housekeeping genes, in human and mouse malignant cell lines (Bhatia *et al.*, 1994; Zhong *et al.*, 1999). A new software tool was used, named REST (relative expression software tool), and compared treated and non-treated samples of each patient. The mathematical model used is based on the PCR efficiencies and the crossing point deviation between the samples (Pfaffl *et al.*, 2002).

## Results and Discussion

Specific results were indicated that induction of the apoptosis related genes takes place in parallel with the induction of differentiation (Table 3). This is demonstrated by the concomitant induction of *cd14* and *IL1 $\beta$*  genes in four of the five patients while *myc* was down-regulated. *Myc* is a well known oncogene who has many functions in the cell cycle and these findings were suggested that external pathway of the

apoptosis is involved in the regulation of ATRA dependent affect on APL (Cole *et al.*, 1986; Schumacher *et al.*, 2001).

It has been informed that ATRA can induce differentiation and reduce intracellular *bcl2* levels without altering the susceptibility to drug-induced apoptosis and ATRA seems to increase chemo sensitivity by down regulation of *bcl-2* in cell line (Bruel *et al.*, 1995; Hu *et al.*, 1996; Ketley *et al.*, 1997).

ATRA can also down regulate *bcl2* expression in native AML blasts for a subset of patients independent of their FAB classification (Pisani *et al.*, 1997). Here we demonstrated that *bcl2* gene levels were down-regulated in four of the five patients and these findings were in concordance with the previous literature. Pro and anti-apoptotic *bcl2* proteins can make heterodimers where the ratio determines the sensitivity of leukemic cells to apoptosis. In some reports the *bcl2* to *bax* ratio has been inversely related with drug induced apoptosis *in vitro* and clinical response to chemotherapy. (Martin *et al.*, 1991; Banker *et al.*, 1997; Decaudin *et al.*, 1997; Pepper *et al.*, 1997; Adams and Cory, 1998; Meijerink *et al.*, 1998; Hoffman *et al.*, 2001).

We found that *bcl2* to *bad* ratios were 3.09, 0.04, 15, 114, and 1.5 while *bcl2* to *bax* ratios were 16, 160, 17.6, 1.6, and 23.2 after the ATRA treatment, for patients 1 to 5 respectively. This suggests that the

**Table 3.** Validation of relative gene expression by quantitative fluorescent PCR.

GENES	Chromos. location	Accession number (GenBank)	*Ratio patient 1	*Ratio patient 2	*Ratio patient 3	*Ratio patient 4	*Ratio patient 5
<i>Myc oncogene</i>	8q24	V00568	0.60	0.57	0.3	0.38	286
<i>il1<math>\beta</math></i> (interleukin-1 beta precursor)	2q13-q21	K02770	5.7	4.9	34	0.98	30.4
<i>monocyte differentiation</i> <i>CD14 antigen precursor</i>	5q31.1	M86511	2.7	302	66	0.1	26.1
<i>wt1 (Wilms tumour I)</i>	11p13	NM 024426	0.65	0.0005	0.0	0.0008	1,341
<i>mcl1</i> (Myeloid cell leukemia sequence 1)	1q21	L08246	0.0001	2.4	0.08	0.15	2.9
<i>bcl2</i>	18q21.3	M14745	0.65	0.16	0.03	0.16	9.3
<i>dapk1</i> (Death-associated protein kinase 1)	9q34.1	P53355	0.69	0.78	0.02	0.98	42.5
<i>bad</i> (Bcl2 antagonist of cell death)	11q13.1	AK023420	0.21	3.4	0.002	0.0014	6.1
<i>bax</i> (BCL2-associated X protein)	19q13.3-q13.4	L22474	0.04	0.001	0.0017	0.1	0.4

\*Ratio: Observed expression of the genes in ATRA treated patient samples/genes in non treated patient samples (The levels of housekeeping gene *rps9* were used as internal controls for normalization of RNA quantity and quality differences in all samples)

ratio of *bcl2* to *bax* may be more important for regulation of ATRA dependent apoptosis in this setting than the ratio of *bcl2* to *BAD*.

*Mcl1* gene ratios were found under-expressed in three patients (1, 3, and 4) while only slightly up-regulated in patients 2 and 5. Also, *dapk1* expressions were down regulated in patients 1, 2 and 3 but the ratios were very close to normal expression levels. On the other hand, *wt1* gene levels were extremely down-regulated in patients 2, 3, 4.

ATRA dependent apoptosis of APL cells were observed under the control of the both internal and external pathways without relationship to the amount of the blast populations. Our data was confirming the hypothesis that the ratio of *bcl2* to *bax* determines the ATRA dependent apoptotic response. Either *bcl2* family or other less known apoptosis related genes as *wt1* will still be required to study in larger groups.

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### References

Adams J, Cory S. The Bcl-2 protein family: arbiters of cell survival. *Science* 1998;281:1322-6

Banker DE, Groudine M, Norwood T, Appelbaum FR. Measurement of spontaneous and therapeutic agent-induced apoptosis with Bcl-2 protein expression in acute myeloid leukemia. *Blood* 1997;89:243-55

Bhatia P, Taylor WR, Greenberg AH, Wright, JA. Comparison of glyceraldehyde-3-phosphate dehydrogenase and 28S-ribosomal RNA gene expression as RNA loading controls for northern blot analysis of cell lines of varying malignant potential. *Anal Biochem* 1994;216:223-6

Bruel A, Benoit G, De Nay D. Distinct apoptotic responses in maturation sensitive and resistant t(15;17) acute promyelocytic leukemia NB4 cells. 9-cis retinoic acid induces apoptosis independent of maturation and Bcl-2 expression. *Leukemia* 1995;9:1173-84

Chomczynsky P, Sacchi N. Single step method of RNA isolation by acide guanidium thiocyanate-phenol chloroform extraction. *Anal Biochem* 1987;152:66

Cole MD. The *myc* oncogene: its role in transformation and differentiation. *Annual Rev Genet* 1986;20:361-84

Decaudin D, Geley S, Hirsch T, et al. Bcl-2 and Bcl-XL antagonize the mitochondrial dysfunction preceding nuclear apoptosis induced by chemotherapeutic agents. *Cancer Res* 1997;57:62-7

Deiss LP, Feinstein E, Berissi H, Cohen O, Kimchi A. Identification of a novel serine/threonine kinase and a novel 15-kD protein as potential mediators of the gamma interferon-induced cell death. *Gene Dev* 1995;9:15-30

Gillis JC, Goa KL. Tretinoin. A review of its pharmacodynamic and pharmacokinetic properties and use in the management of acute promyelocytic leukaemia. *Drugs* 1995;50:897-923

Hoffman B, Amanullah A, Shafarenko M, Liebermann DA. The proto-oncogene *MYC* in hematopoietic development and leukemogenesis. *Oncogene* 2002;21:3414-21

Hu ZB, Minden MD, McCulloch EA. Post-transcriptional regulation of bcl-2 in acute myeloblastic leukemia: significance for response to chemotherapy. *Leukemia* 1996;10:410-6

Ketley NJ, Allen PD, Kelsey SM. Modulation of idarubicin-induced apoptosis in human acute myeloid leukemia blasts by all-trans retinoic acid, 1,25 (OH) 2 vitamin D3, and granulocyte-macrophage colony-stimulating factor. *Blood* 1997;90:4578-87

Lo Coco F, Nervi C, Avvisati G, Mandelli F. Acute promyelocytic leukemia: a curable disease. *Leukemia* 1998;12:1866-80

Martin SJ, Bradley JG, Cotter TG. HL-60 cells induced to differentiate towards neutrophils subsequently die via apoptosis. *Clin Exp Immunol* 1990;79:448-553

Meijerink JPP, Mensink EJB, Wang K, Sedlak TW, Sløetjes AW, de Witte T, Waksman G, Korsmeyer SJ. Hematopoietic malignancies demonstrate loss-of-function mutations of *BAX*. *Blood* 1998;91:2991-7

Mitchell KO, Ricci MS, Miyashita T, Dicker DT, Jin Z, Reed JC, El-Deiry WS. *Bax* is a transcriptional target and mediator of *MYC*-induced apoptosis. *Cancer Res* 2000;60:6318-25

Oltvai ZN, Milliman CL, Korsmeyer SJ. Bcl-2 heterodimerizes *in vivo* with a conserved homolog, *Bax*, that accelerates programmed cell death. *Cell* 1993;74:609-19

Pepper C, Hoy T, Bentley DP. Bcl-2/*Bax* ratios in chronic lymphocytic leukaemia and their correlation with *in vitro* apoptosis and clinical resistance. *Brit J Cancer* 1997;76:935-8

Pfaffl MW, Horgan GW, Dempfle L. Relative expression software tool (REST) for group-wise comparison and statistical analysis of relative expression results in real-time PCR. *Nucleic Acids Res* 2002;30:e36

Pisani F, Del Poeta G, Aronica G. *In vitro* down-regulation of bcl-2 expression by all-trans retinoic acid in AML blasts. *Ann Hematol* 1997;75:145-7

Pritchard-Jones K, King-Underwood L. The Wilms tumour gene *WT1* in leukaemia. *Leukemia Lymphoma* 1997;27:207-20

Savli H, Aalto Y, Nagy B, Knuutila S, Pakkala S. Gene expression analysis of 1,25 (OH) 2D3 dependent differentiation of HL-60 cells: a cDNA array study. *Brit J Haematol* 2002;118:1065-70

Savli H, Karadenizli A, Kolayli F, Gundes S, Ozbek U, Vahaboglu H. Expression stability of six housekeeping genes: a proposal for resistance gene quantification studies of *Pseudomonas aeruginosa* by real-time quantitative RT-PCR. *J Med Microbiol* 2003;52:403-8

Schuhmacher M, Kohlhuber F, Holzel M, Kaiser C, Burtscher H, Jarsch M, Bornkamm GW, Laux G, Polack A, Weidle UH, Eick D. The transcriptional program of a human B cell line

in response to *Myc*. *Nucleic Acids Res* 2001;29:397-406

Srivastava RK, Sasaki CY, Hardwick JM, Longo DL. Bcl-2-mediated drug resistance: inhibition of apoptosis by blocking nuclear factor of activated T lymphocytes (NFAT)-induced Fas ligand transcription. *J Exp Med* 1999;190:253-65

Townsend KJ, Zhou P, Qian L, Bieszczad CK, Lowrey CH, Yen A, Craig RW. Regulation of *MCL1* through a serum response factor/Elk-1-mediated mechanism links expression of a viability-promoting member of the *BCL2* family to the induction of hematopoietic cell differentiation. *The J Biol Chem* 1999;274:1801-13

White MK, McCubrey JA. Suppression of apoptosis: role in cell growth and neoplasia. *Leukemia* 2001;15:1011-21

Zhong H, Simons JW. Direct comparison of GAPDH, beta-actin, cyclophilin, and 28S rRNA as internal standards for quantifying RNA levels under hypoxia. *Biochem Biophys Res* 1999;259:523-6