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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 *mcl 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 *il1β* 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.

Keywords: *APL*; apoptosis; ATRA; gene expression; real-time PCR; t(15;17)

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.,* 199l; 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 extremly 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*β 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^2, daily). Among of these patients, 4 were

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

Patient	Age/sex	BP	WBC (109/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, Hemoglobine; S (m), Survival (months); n.a., Data is not available. **All patients have been treated with ATRA 45 mg/m^2, daily.

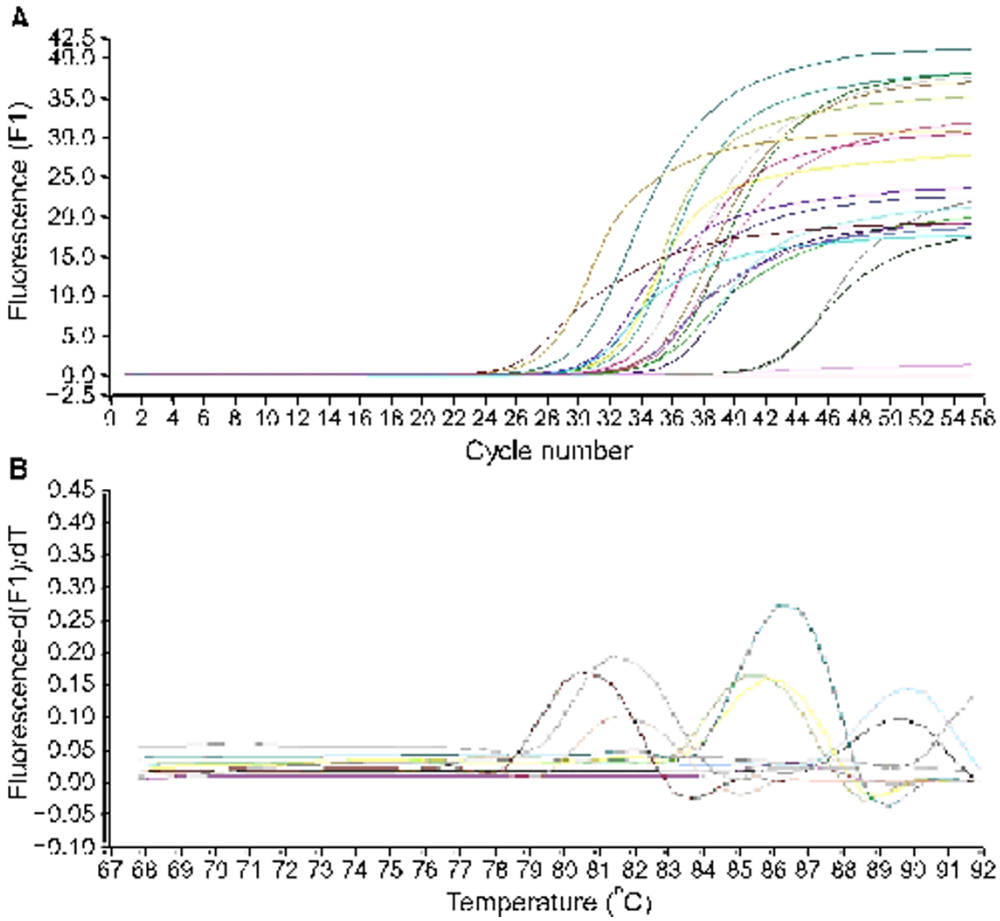

Figure 1. (A) LightCycler assisted standard analysis of the studied genes. On line fluorescence curves of PCR amplifications and exponential increases in fluorescence intensities are shown as a measurement for the quantity of amplified fragments. (B) Specific melting curve analysis of the apoptosis related genes in our study. The melting peaks at 87°C for RPS9, 90°C for CD14, 86°C for IL1β 1, and seven apoptosis related genes between 81°C to 92°C indicate the spesific products that melt at the different temperatures.

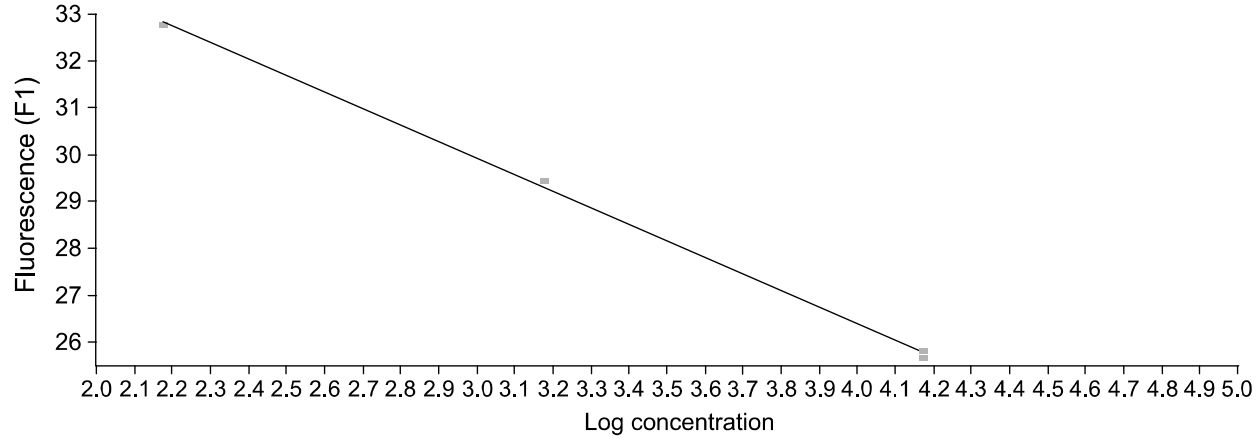

Figure 2. Standart curve analysis of the serial dilutions of the RPS9 housekeeping gene *(Slope: -2.701, Intercept:33.80, Error: 0.0349, R: -1.00).*

Table 2. Primer sequences of selected genes.

rps9 housekeeping gene	CGTCTCGACCAAGAGCTGA GGTCCTTCTCATCAAGCGTC
monocyte differentiation CD14 antigen precursor	GAGCCGCACAGGTTCCTG GCTTGGGCAATGCTCAGTACC
il1β (Interleukin-1 beta precursor)	GTGCTGAATGTGGACTCAATCC TGACAGAGGAGGGTTTCTTAGAAC
bcl2	AGGAAGTGAACATTTCGGTGAC GCTCAGTTCCAGGACCAGGc
bax (BCL2-associated X protein)	TGCTTCAGGGTTTCATCCAG GGCGGCAATCATCCTCTG
mcl1 (Myeloid cell leukemia sequence 1)	GATGATCCATGTTTTCAGCGAC CTCCACAAACCCATCCCAG
dapk1 (Death-associated protein kinase 1)	CAGTGTTGTTGCTCTAGGAAG GGGACTGCCACAAATGATGAGC
Myc oncogene	GGCAAAAGGTCAGAGTCTGG GTGCATTTTCGGTTGTTGC
wt1 (Wilms tumour I)	CTGTCCCACTTACAGATGCACAG TCTTTTGAGCTGGTCTGAACGA
bad (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 centrifugation. 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. Standart 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 *IL1β* 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
Myc oncogene	8q24	V00568	0.60	0.57	0.3	0.38	286
il1β (interleukin-1 beta precursor)	2q13-q21	K02770	5.7	4.9	34	0.98	30.4
monocyte differentiation CD14 antigen precursor	5q31.1	M86511	2.7	302	66	0.1	26.1
wt1 (Wilms tumour I)	11p13	NM 024426	0.65	0.0005	0.0	0.0008	1,341
mcl1 (Myeloid cell leukemia sequence 1)	1q21	L08246	0.0001	2.4	0.08	0.15	2.9
bcl2	18q21.3	M14745	0.65	0.16	0.03	0.16	9.3
dapk1 (Death-associated protein kinase 1)	9q34.1	P53355	0.69	0.78	0.02	0.98	42.5
bad (Bcl2 antagonist of cell death)	11q13.1	AK023420	0.21	3.4	0.002	0.0014	6.1
bax (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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