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 cdf14 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., 1999; 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 IL1β 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/m², daily). Among of these patients, 4 were

| 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, Hemoglobin; S (m), Survival (months); n.a., Data is not available. **All patients have been treated with ATRA 45 mg/m², 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 specific products that melt at the different temperatures.
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

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

| Table 2. Primer sequences of selected genes. |
|---------------------------------------------|
| **rps9 housekeeping gene**                  | **CGTCTCGACCAAGAGCTGA** |
|                                           | **GTTGCTCTCATCAGAGCTGC** |
| **monocyte differentiation CD14 antigen precursor** | **GAGCCGCACAGGTTCCTG** |
|                                           | **GCTTGGGCAATGCTCAGTACC** |
| **il1β (Interleukin-1 beta precursor)**    | **GTGCTGAGTGACTCAATCC** |
|                                           | **TGACAGAGGGTTTTCTTGAAC** |
| **bcl2**                                   | **AGGAAGTGAACATTTCGGTGAC** |
|                                           | **GCTCAGTTCAGGACCAGGC** |
| **bax (BCL2-associated X protein)**        | **TGCTCAGGGTTTCTACCCAG** |
|                                           | **GGCGCAATATCTCCTG** |
| **mcl1 (Myeloid cell leukemia sequence 1)**| **GATGATCCATGTTTCCAGGC** |
|                                           | **CTCCACACACCTCAGCG** |
| **dapk1 (Death-associated protein kinase 1)** | **CAGTGGTGGTCCTAGGAAAG** |
|                                           | **GGGACTGCCACAAATGAGGC** |
| **Myc oncogene**                           | **GGCAAAAGGTCAGAGTTGG** |
|                                           | **GTGCATTTTCGGTTGTTGC** |
| **wt1 (Wilms tumour I)**                   | **CTGTCCCCACCTACAGATGCACAG** |
|                                           | **TCTTTTGAGCTGCTGTAACGA** |
| **bad (Bcl2 antagonist of cell death)**    | **GAGTGACGAGAGACTCCAGGC** |
|                                           | **TCCACAAATTCGTCATCAGCC** |
PCR amplifications and melting curve analysis were shown in Figure 1. Standard curves 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 (Brue et al., 1995; Hu et al., 1996; Ketley et al., 1997).

ATRA 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 bax 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 1)         | 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/gene 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 \textit{bcl2} to \textit{bax} may be more important for regulation of ATRA dependent apoptosis in this setting than the ratio of \textit{bcl2} to \textit{BAD}.

\textit{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, \textit{dapk1} expression were down regulated in patients 1, 2 and 3 but the ratios were very close to normal expression levels. On the other hand, \textit{wt1} gene levels were extremely down-regulated in patients 2, 3, 4.

\textit{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 \textit{bcl2} to \textit{bax} determines the \textit{ATRA} dependent apoptotic response. Either \textit{bcl2} family or other less known apoptosis related genes as \textit{wt1} will still be required to study in larger groups.

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\section*{References}

Adams J, Cory S. The Bcl-2 protein family: arbiters of cell survival. Science 1996;261:1322-6

Banker DE, Grouin 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 metod of RNA isolation by acide guanidium thiocyonate-phenol chloroform extraction. Anal Biochem 1994;216:223-6

Cole MD. The \textit{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 leukemia. Drugs 1995;50:897-923

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

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

Ketley NJ, Allen PD, Kelsey SM. Modulation of idarubicine-induced apoptosis in human acute myeloid leukemia blasts by all-trans retinoic acid, 1,25 (OH) \textit{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 EJBM, Wang K, Sedlak TW, Siöötes AW, de Witte T, Waksman G, Korsmeyer SJ. Hematopoietic malignancies demonstrate loss-of-function mutations of \textit{BAX}. Blood 1998;91:2991-7

Mitchell KO, Ricci MS, Miyashita T, Dicker DT, Jin Z, Reed JC, El-Deiry WS. \textit{Bax} is a transcriptional target and mediator of \textit{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 \textit{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 \textit{WT1} in leukaemia. Leukemia Lymphoma 1997;27:207-20

Savli H, Aalto Y, Nagy B, Knuttila S, Pakkala S. Gene expression analysis of 1,25 (OH) \textit{2}D3 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 \textit{Pseudomonas aeruginosa} by real-time quantitative RT-PCR. J Med Microbiol 2003;52:403-8

Schuhmacher M, Kohlhuber F, Holzel M, Kaiser C, Bartscher 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 Bioph Res 1999;259:523-6