Abnormal expression of apoptosis-related genes in haematological malignancies: overexpression of MYC is poor prognostic sign in mantle cell lymphoma

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Summary. The expression of apoptosis-related genes BCL2, BAX, BCL2L1, BCL2A1, MCL1, DAPK1 and MYC was studied by quantitative real-time polymerase chain reaction on total RNA samples from patients with acute lymphoblastic leukaemia (ALL, n = 16), acute myeloid leukaemia (AML, n = 27), chronic myeloid leukaemia (CML, n = 12), mantle cell lymphoma (MCL, n = 19) and chronic lymphoid leukaemia (CLL, n = 32). BCL2, BAX, BCL2A1, MCL1, DAPK1 and MYC were overexpressed in all patient groups. BCL2L1 was underexpressed in CLL and CML, but not in AML, ALL and MCL. MCL1 levels were significantly higher in CD13 and CD33-positive ALL, and in CD56-positive AML samples. BCL2, BCL2L1, BCL2A1 and MCL1 were overexpressed and DAPK1 was underexpressed in CLL samples with a 11q23 deletion. MYC overexpression was significantly associated with shorter overall survival in MCL (P < 0.01). AML patients with a normal karyotype showed a higher frequency of BCL2A1 overexpression (P < 0.001) than those with an abnormal karyotype.

Keywords: apoptotic genes, gene expression, haematological malignancies, quantitative real-time PCR, prognosis.

Apoptosis plays a critical role in the normal development of tissue homeostasis as well as in defence against infections. Deregulation of apoptosis has been shown to contribute to the development of cancer, autoimmune diseases and degenerative disorders (Reed, 1998, 1999, 2000). A number of genes are involved in the regulation of apoptosis, among them the family of BCL2 genes. They regulate cellular responsiveness to a wide variety of death-inducing stimuli, including growth factor deprivation, the presence of glucocorticoids, receptor antibodies, and radio- or chemo-therapeutical agents (Reed, 1998, 1999, 2000). Some of the BCL2 family members are antiapoptotic, such as BCL2, MCL1, BCL2A1 and BCL2L1, and others are pro-apoptotic such as BAX, BAK, BAD and BCLXS (Oltvai et al, 1993; Chao & Korsmeyer, 1998; Srivastava et al, 1999). The pro-and antiapoptotic proteins can form heterodimers and the ratio of these determines the execution of apoptosis (Oltvai et al, 1993; Adams & Cory, 1998; Schimmer et al, 2001). Another important regulator in apoptosis is the MYC oncogene. It has maintained a highly conserved sequence through evolution and encodes a transcription factor that plays an important role in a number of biological functions, including cell proliferation, differentiation and apoptosis (Cole, 1986).
Aberration of apoptosis has been observed in haematological neoplasms. Chromosomal translocations affecting apoptosis regulator genes, such as t(14;18) of the BCL2 gene, are well defined in haematological malignancies (Tsujimoto et al. 1985). The bcl2 protein inhibits apoptosis by forming dimers with bax and bak (Schimmer et al., 2001). It regulates cell death by controlling mitochondrial membrane permeability via inhibition of caspase activity and prevents the release of cytochrome c (Jacobson et al, 1993; Shimizu et al, 1999). BAX, which is a pro-apoptotic gene, induces the release of cytochrome c. Furthermore, the ratio of bcl2 to bax determines cell survival or death after an apoptotic stimulus (Oliva et al, 1993). BCL2L1 is a highly conserved gene encoding an alternative splicing of two isoforms with antagonistic functions, the shorter with a pro-apoptotic and the longer with an antiapoptotic function (Boise et al, 1993). Bcl2L1 forms dimers with bax and bak and has been shown to be an independent regulator of programmed cell death by preventing apoptosis (Boise et al, 1993; Korsmeyer, 1999). BCL2A1 prevents apoptosis induced by the p53 tumour suppressor protein (D’Sa-Eipper et al, 1996). MCL1 participates in myeloid cell differentiation and has sequence homology with BCL2 (Kozopas et al, 1993; Bae et al, 2000). Death-associated protein kinase (DAPK1) is a potential mediator of γ-interferon-induced cell death: when deregulated, it behaves as an oncogene or growth suppressor (Deiss et al, 1995). Overexpression of MYC has been found in numerous human tumours, including several haematological disorders with T- and B-cell lineage involvement (Marcu et al, 1992; Sakamuro & Prendergast, 1999).

Our recent studies of acute myeloid leukaemia (AML) and chronic lymphocytic leukaemia (CLL) using the microarray technology revealed altered expression of several apoptosis-related genes (Aalto et al, 2001; Larramendy et al, 2002).

In this study we determined the expression levels of seven genes (BCL2, BAX, BCL2L1, BCL2A1, MCL1, DAPK1, MYC) using quantitative real-time reverse transcription polymerase chain reaction (QRT-PCR). We set out to identify diagnostic and prognostic markers for acute lymphoid leukaemia (ALL), acute myeloid leukaemia (AML), chronic myeloid leukaemia (CML), mantle cell lymphoma (MCL) and chronic lymphocytic leukaemia (CLL).

PATIENTS AND METHODS

ALL, AML and CML samples
Bone marrow samples were obtained from 16 ALL patients, 27 AML patients and 12 CML patients at the Helsinki University Central Hospital, Helsinki, Finland. Table I shows the key data of these patients. Bone marrow aspirates were diluted 1:10 in RNA/DNA stabilization reagent for blood/bone marrow (Boehringer Mannheim GmbH, Mannheim, Germany) containing guanidinium thiocyanate and in Triton-X-100 (Boehringer) for simultaneous cell lysis and stabilization of nucleic acids according to the supplier’s instructions. Lysates were stored at −70°C until RNA extraction.

Mantle cell lymphoma (MCL)
Samples were obtained from 19 patients diagnosed at the Helsinki University Central Hospital (Table I). All patients were CD5+, CD19+, CD20+, CD23+ and cyclin D1+. The International Prognostic Index (IPI) was used for risk grouping of the patients (Blay et al, 1998).

CLL
Peripheral blood specimens were obtained from 32 patients referred to the CLL out-patient clinic at Tampere University Central Hospital (Tampere, Finland) (Table I). All had a blood lymphocyte count of 30 × 10^9/l or higher. These patients were diagnosed and staged according to standard clinical, morphological and immunophenotypic criteria and the Binet system. All patients had a CD19+/CD5+/CD23+ immunophenotype. The proportions of monocytes and polyclonal T and B lymphocytes were 1–13%, indicating that 87–99% of the isolated cells represented the leukaemic population.

Control samples
Bone marrows from four healthy individuals were used as controls for the ALL, AML and CML samples. References for MCL and CLL samples were CD19+ B cells, which were purified from the adenoid palatine tonsils of six healthy children and pooled. B cells were purified using microbeads conjugated to a monoclonal CD19 antibody (Miltenyi Biotec, Bergisch Gladbach, Germany). The proportion of T lymphocytes was less than 5%, indicating that 95% of the isolated cells represented B-lymphocyte population.

RNA isolation
AML, ALL, CML and control samples. Total nucleic acid was isolated from samples using the mRNA isolation kit for blood/bone marrow (Boehringer) based on magnetic glass particle technology following the manufacturer’s instructions.

CLL. Lymphocytes were isolated from patient blood using one-step density gradient centrifugation in Ficoll–Paque (Pharmacia Fine Chemicals, Uppsala, Sweden), and total RNA was extracted using the Trizol Reagent (Gibco BRL, Grand Island, NY, USA).

MCL. Total RNA was isolated from deep-frozen tissue specimens using an RNeasy Mini Kit (Qiagen, Valencia, CA, USA) according to the manufacturer’s instructions.

DNase I treatment
All RNA samples were treated with DNase I (Boehringer) to remove genomic DNA contamination from the preparations according to the manufacturer’s instructions. RNA quality and integrity were checked by electrophoresis using 1% agarose gel with ethidium bromide staining and UV transillumination. RNA concentrations were measured using a spectrophotometer at a wavelength of 260 nm.

QRT-PCR
QRT-PCR was performed on all samples to determine the expression of seven apoptosis-related genes (BCL2, BAX, BCL2L1, BCL2A1, MCL1, DAPK1, MYC) and one...
Table I. Patients divided according to clinicopathological features.

<table>
<thead>
<tr>
<th>Disease</th>
<th>Patients (n)</th>
<th>Mean age (years)</th>
<th>Karyotype/ altered gene expression</th>
<th>Risk group/FAB/ immunophenotype/ altered gene expression</th>
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<tr>
<td></td>
<td>M</td>
<td>F</td>
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<td>Mean age</td>
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<tr>
<td>Acute lymphoblastic leukaemia</td>
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<td>Acute myeloid leukaemia</td>
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<td>Chronic myeloid leukaemia</td>
<td>9</td>
<td>3</td>
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<tr>
<td>Mantle cell lymphoma</td>
<td>12</td>
<td>7</td>
<td>71</td>
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<tr>
<td>Chronic lymphoid leukaemia</td>
<td>23</td>
<td>9</td>
<td>64</td>
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HR, high risk; IR, intermediate risk; SR, standard risk; IPI, International Prognosis Index.

Gene expressions that showed statistically significant association with any features are marked for overexpression (↑) and underexpression (↓). Other clinicopathological parameters did not show statistically significant association with gene expression. Statistical tests were performed using the spss program package for Windows.
housekeeping gene for β-actin (ACTB) (Table II). Complementary DNA (cDNA) was synthesized using 0.5 μg of DNase-I-treated total RNA and a First Strand cDNA Synthesis kit for RT-PCR (Roche Diagnostics, Indianapolis, IN, USA). Gene-specific primers were designed and synthesized by TIB MOLBIOL (Berlin, Germany). PCR was performed in a LightCycler thermal cycler (Roche). Each PCR reaction consisted of 1 μl of DNA Master SYBR Green I mix (LightCycler-FastStart DNA Master SYBR Green I kit, Roche; containing Taq polymerase, dNTP, MgCl₂, and SYBR Green I dye). 1 μl of cDNA and 2.5–5 pmol of primers. The amplification programme included an initial denaturation at 95°C for 8 min, 45–55 cycles with denaturation at 95°C for 10 s, annealing at 58–62°C for 5 s and extension at 72°C for 20 s. Amplifications were followed by melting curve analysis using one cycle at 95°C for 0 s, 65°C for 10 s, and 95°C for 0 s at the acquisition step mode. A negative control without a cDNA template was run simultaneously with every assay. For each cDNA sample the PCR was run in duplicate. Standard curves were obtained using serial dilutions of the beta-globulin gene (DNA Control kit; Roche) according to the supplier’s instructions. The concentration of each gene product was determined on the basis of a kinetic approach using the LIGHTCYCLER software (Roche). The levels of ACTB were used for normalization of RNA quantity and quality differences in all samples.

Statistical analysis of data
Statistical analysis was performed using the Statistical Package for Social Sciences (SPSS) for Windows (Version 9.0), with the Student’s t-test and Levene’s test for the gene expression comparisons. The overall survival related to gene expression was analysed by the Kaplan–Meier method.

RESULTS
The expression levels of BCL2, BAX, BCL2L1, BCL2A1, MCL1, DAPK1 and MYC were determined by QRT-PCR (Table III). Figure 1A and B shows the ratios of expression levels in each sample. ALL, AML and CML samples were compared with RNA isolated from the bone marrow of healthy donors, whereas MCL and CLL samples were compared with RNA isolated from tonsillar B cells.

BCL2 and BAX were overexpressed in all patients (Table III). The BCL2/BAX ratio was highest in CLL (1.844), followed by ALL (0.895), MCL (0.44), AML (0.067) and CML (0.036). The BCL2L1 gene was underexpressed in CLL and CML, but the levels were higher than those of controls in AML, ALL and MCL. BCL2A1, MCL1, DAPK1 and MYC were overexpressed in all patient groups (Table III).

Risk groups and the gene expression
We observed statistically significant differences in the expression of MCL1 between high risk (HR) (53.35 ± 38.82 ng/μl, P < 0.05), intermediate risk (IR) (9.93 ± 4.32 ng/μl) and standard risk (SR) 10.43 ± 2.58 ng/μl groups of ALL. Gene expression levels in the MCL subgroups (IPI), and in the CLL subgroups (Binet) did not
Concentration of different gene products determined by quantitative real-time polymerase chain reaction (QRT-PCR) in groups of patients with haematological malignancy and healthy controls (ng/l)

<table>
<thead>
<tr>
<th>Gene</th>
<th>ALL (n = 16)</th>
<th>AML (n = 27)</th>
<th>CML (n = 12)</th>
<th>MCL (n = 19)</th>
</tr>
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<tbody>
<tr>
<td>BCL2</td>
<td>1.526 ± 0.8408</td>
<td>1.116 ± 2.16E+02</td>
<td>9.711 ± 0.6902</td>
<td>NS</td>
</tr>
<tr>
<td>BCL2L1</td>
<td>7.525 ± 0.8412</td>
<td>1.03 ± 2.30E+02</td>
<td>9.063 ± 0.3756</td>
<td>NS</td>
</tr>
<tr>
<td>BCL2L1</td>
<td>3.57 ± 0.4141</td>
<td>4.99 ± 1.3 ± 4.1E+02</td>
<td>2.87 ± 3.17</td>
<td>NS</td>
</tr>
<tr>
<td>MCL1</td>
<td>2.57 ± 0.6748</td>
<td>1.95 ± 0.6556</td>
<td>NS</td>
<td>4.83 ± 1.9513</td>
</tr>
<tr>
<td>MYC</td>
<td>7.53 ± 0.6902</td>
<td>1.15 ± 0.6902</td>
<td>NS</td>
<td>1.72 ± 0.6902</td>
</tr>
</tbody>
</table>

NS, not significant. The Student t-test was used to calculate significance levels.

In the present study, we analysed the expression of BCL2, BAX, BCL2L1, BCL2A1, MCL1, DAPK1 and MYC in different types of haematological malignancies by QRT-PCR using the SYBR Green I method for the quantification of PCR products. As controls, we used bone marrow samples from healthy donors for the ALL, AML and CML bone marrow samples. CD19 B cells were pooled from six normal adenoids and

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used as reference for the CLL and MCL samples. Neither control is a perfect means to verify whether or not gene expression differences in the cancer samples are attributable merely to the disease per se and not to the maturation stage or lineage involvement of haematopoietic cells. Nevertheless, although the stem cell from which the malignancy originates is unidentified, the selection of controls is a disputable issue. Therefore, the results must be interpreted with caution. For example, CD19+ B cells from children, which we used as controls for CLL and MCL, are likely to be active, and therefore less apoptotic, than CD19+ lymph nodes from adults or peripheral blood B cells. It should, however, be noted that the control we used was the same for all patients of each disease group. Thus the selection of controls did not have an effect on gene expression differences within the same disease, and any associations between gene expression and clinical parameters can be interpreted reliably.

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BCL2 was overexpressed in all patients. This gene is involved in both normal lymphoid development and lymphomas with the t(14;18) translocation (Kozopas et al., 1993). High levels of the bcl2 protein have been reported even in the absence of t(14;18), for example in CLL together with a high level of the mcl-1 protein (Gottardi et al., 1996) and in diffuse large B-cell lymphoma (Monni et al., 1999).

BAX showed the highest overexpression in MCL. Mutations in BAX have been documented in up to 20% of haematopoietic malignancies (Meijerink et al., 1998). The bcl2:bax ratio was found to be inversely related with drug-induced apoptosis in vitro and with clinical response to chemotherapy (Pepper et al., 1996). In our study, the ratio was highest in CLL, followed by ALL, MCL, AML and CML. The functions of BCL2L1 (underexpressed in CML and CLL) and DAPK1 (overexpressed in MCL) in haematological malignancies are still largely unknown, although BCL2L1 has been found to be overexpressed in about half of human cancers associated with resistance to radiotherapy and chemotherapy (Nicot et al., 2000).

Overexpression of MCL1 (in our CLL and CML patients) has been indicated to inhibit cell death in human cell lines less effectively than BCL2 (Krajewski et al., 1995) and to have an important role in the regulation of B cells (Lomo et al., 1997).

Our finding of overexpressed MYC and BCL2L1 in MCL is in concordance with two previous microarray analyses (Hofmann et al., 2001; Zhu et al., 2002). Furthermore, the present results and those by Zhu and colleagues give evidence that the overexpression of these genes is mainly associated with a blastoid morphology of MCL cells.

BCL2L1 and MCL1 were significantly overexpressed in CD13+CD33+ ALL patients. MCL1 levels in CD56+ AML patients were significantly higher than in CD56- patients. Recently, it was reported that high expression of MCL1 correlates with resistance to chemotherapeutical agents (Kaufmann et al., 1998). CD56 positivity is otherwise associated with a poor prognosis in AML (Di Bona et al., 2002). Whether a cause-and-effect relationship exists between these two phenomena is still open to question. An important role for MCL1 in the co-ordination of the BCL2 gene family in cell phenotype differentiation has recently been identified (Craig, 2002).

BCL2A1 levels in AML were significantly higher in patients with normal rather than abnormal karyotypes. It is generally acknowledged that approximately 40% of AML cases have a normal karyotype. To identify prognostic and follow-up markers for these patients, characterization of gene expression alterations is considered to be important. Our finding that overexpression of BCL2A1 is a characteristic phenomenon for a subtype of AML patients with a normal karyotype needs to be confirmed with a larger series of patients (Grimwade et al., 1998).

The antiapoptotic BCL2, BCL2L1, BCL2A1 and MCL1 genes were significantly overexpressed and the pro-apoptotic DAPK1 was underexpressed in CLL patients with a 11q23 deletion. The loss of 11q23 is one of the most common structural chromosomal changes in CLL and is associated with the progression of the disease (Zhu et al., 2000). The alterations in expression levels of anti- and pro-apoptotic genes we observed support the results of previous cDNA array studies by Aalto and colleagues who showed that patients with a 1q23 deletion form a subtype of CLL (Aalto et al., 2001).

The follow up for all patient groups other than MCL was too short to apply the Kaplan–Meier survival curve assessment. In this study, the most important novel clinical finding was the significant association of MYC overexpression with shorter overall survival in MCL. Many molecular and clinicopathological variables have been investigated in this lymphoma type but few prognostical markers have been found. Other than tumour-related factors, blastoid morphology has also been shown to be associated with poor prognosis (Bernard et al., 2001). In the present study six patients with blastoid morphology showed shorter overall survival and a higher frequency of MYC overexpression than patients with typical MCL morphology. However, these differences were not statistically significant. Recently, MYC overexpression has been observed in association with blastoid morphology (Bea et al., 1999; Hernandez et al., 1999; Zhu et al., 2002). However, survival had not been studied previously. Whether MYC overexpression is an independent prognostic marker in MCL needs to be studied in a larger patient group.

To conclude, the aberrant expression of the apoptotic genes was observed to be associated with immunophenotypic and cytogenetic findings and with overall survival in leukaemias and lymphomas.

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