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Bax mutations in cell lines derived from hematological malignancies

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Many genes are involved in cell cycle control, DNA repair and induction of cell death. Alterations in these genes have been responsible for the development of cancer as well as for resistance to cancer therapy. Recently, an emerging family of *bcl2*-like genes has been identified that plays a role in the regulation of cell death. Its members are highly conserved in several domains which have been shown to be important for homodimerization or heterodimerization. The ratio between BAX/BCL2 heterodimers and BAX/BAX homodimers appears to be pivotal in deciding the life or death of a cell. We recently detected mutations in evolutionary highly conserved domains of the *bax* gene in cell lines derived from hematologic malignancies. Similar artificially generated mutations in other *bcl2*-like family members *bcl2, bclxl*, or *ced9* have been shown to alter their function. This suggests a role for *bax* mutations in the multistep pathogenesis of hematological malignancies.
Keywords: bax; p53; mutation; apoptosis; resistance

Introduction

Cell proliferation and cell death are two important processes involved in embryonal development, tissue homeostasis and regeneration. One of the genes involved in the regulation of cell death is the human *bcl2* gene, which can protect against cell death induced by a variety of death signals.[1,2] Recently, an emerging family of related proteins has been identified.[3] The protein products of *bcl2*,[1] *bclxl*,[4] *mcl1*[5] can protect against apoptosis whereas the protein products *bax*,[6] *bclxs*,[4] *bad*[7] *and bak*[8–10] make cells more sensitive for apoptosis induction. The regulation of the cell death pathway by the *bcl2*-like family members is very conserved throughout evolution. The human *bcl2* gene can partially substitute for *ced9* in nematode *Caenorhabditis elegans* in preventing developmental cell death in *ced9* loss-of-function mutants.[11]

The different members of the *bcl2*-like family can form homodimers or heterodimers with other members of the same family, possibly in a head-to-tail configuration.[12] Three conserved domains, box 1 to 3 as defined by Hengartner and Horvitz,[11] seem to be important for dimerization. Mutation analysis of the two C-terminal homology domains, called *bcl2* homology domain 1 (BH1, which equals box 2) and BH2 (which equals box 3), demonstrates that several amino acid residues are crucial for dimerization.[13] For *bcl2* and *bax* it has been demonstrated that their protein ratio determines the cells susceptibility for cell death following a death signal. BAX/BAX homodimers renders a cell more vulnerable for apoptosis and BCL2/BAX heterodimers provides protection.[6,13] The model is more complex: first, the *bax* gene encodes for several protein products of which BAXα and BAXβ seem to be the most important ones. BAXα is a membrane-bound protein whereas BAXβ is a cytoplasmic protein.[6] Second, other family members are also involved and complex interactions among these proteins may dictate complex set points unique to each cell.[14] Third, most family members are also expressed in a tissue-specific manner, like *bclx* which seems to be predominantly expressed in neurons and thymocytes.[4] Fourth, expression patterns of specific family members can be modulated by external signals,[15] and therefore affect the specific set point within target cells.

Upon genotoxic stress, an intracellular defense program is activated to facilitate repair prior to distribution of mutations over daughter cells. This damage response pathway is regulated by the *p53*-gene.[16,17] Upon sustaining damage by chemotherapeutic agents or γ-radiation, the *p53* expression is upregulated by a post-transcriptional mechanism and immediately blocks the cell cycle at the G1 to S phase transition[16] and facilitates repair. For this, the *p53* gene upregulates the expression of the *waf1/cip1* gene[18] and the *gadd45* gene.[19] If DNA damage is too severe, P53 can induce cell death.[20]

Induction of apoptosis in a *p53*-dependent pathway is regulated by members of the *bcl2*-like family. P53-induced apoptosis is preceded by a decrease of *bcl2* gene expression and an increase of *bax* gene expression.[21,22] The P53 protein can directly modulate *bcl2* and *bax* expression by a *cis*-acting *p53* negative response element located in the 5′ untranslated region of the *bcl2* gene[23] and a *p53* binding-site located in the promotor region of the *bax* gene.[24]

Inactivation of the *p53*-dependent cell death pathway can contribute to resistance to therapy as well as to oncogenesis itself. In nearly all human neoplasms, mutations have been found in the *p53* gene with concomitant loss of the normal allele.[25,26] Germ-line muations in the *p53* tumor suppressor gene as found in families with Li–Fraumeni syndrome have been associated with increased risk for cancer development.[27] Mutations in the *p53* gene result in loss of the tumor suppressor activity and enhancement of oncogenic activity by dimerization of mutant P53 with wild-type P53 to form an inactive dimer or tetramer.[28] This lowers the DNA binding capacity of the P53 complex to target genes and subsequent transcriptional activation.[29] *In vivo* experiments as well as correlative studies have shown that mutations in the *p53* gene also contribute to resistance to treatment.[17,30,31]

Deregulation of downstream genes in the *p53*-dependent cell death pathway can also contribute to oncogenesis and increased resistance to treatment. In many cases of human follicular lymphoma, the *bcl2* gene is overexpressed due to a translocational event. As an effect of *bcl2* overexpression, cells become less sensitive for induction of apoptosis and therefore have a longer lifespan with a simultaneous increased risk for the accumulation of secondary mutations.[1] In mice, overexpression of *bcl2* results in the expansion of a polyclonal B cell compartment, which occasionally dedifferentiates into a high-grade monoclonal disease in which the *c-myc* gene is frequently rearranged.[32] Overexpression of *bcl2* has also been correlated with poor prognosis to therapy in patients with non-Hodgkin's lymphoma, acute myeloid leukemia and prostate cancer.[2] Since BCL2 and BAX have opposite effects on induction of apoptosis following a death signal, it seems reasonable that mutations which disturb BAX function exert a similar effect on cells as BCL2 overexpression. This way, aberrations in the *bax* gene could be involved in the genesis of cancer. It

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also seems likely that these mutations contribute to enhanced resistance to chemotherapy.

In this report we describe the presence of *in vivo bax* mutations in cell lines derived from hematological malignancies. The frequency of the mutations is relatively high in the small number of cell lines tested. The potential role of these mutations in the pathogenesis of malignancies is discussed.

Materials and methods

Cell lines

Cell lines of different hematopoietic background were used in the present study and include three human T-ALL cell lines: CEM, Jurkat and HPB-ALL, a human Burkitt lymphoma cell line Daudi, and two human B cell lines JM and KM3.

RNA-isolation

RNA was isolated according to a method developed by Chirgwin *et al.*[33]

Oligonucleotides

Sense oligonucleotides used were bax-exon 3: 5′ GTCCACCAAGAAGCTGAGCG 3′, bax-exon 4: 5′ GCCCTTTTCTACTTTGCCAGC 3′; Antisense oligonucleotides used were: bax-exon 5: 5′ TCCAGCCCAACAGCCGCTC 3′; bax-intron 5.2: 5′ GACACGTAAGGAAAACGCATTAT 3′; bax-exon 6: 5′ GCACTCCCGCCACAAAGATG 3; bax-exon 6.2: 5′ TCAGCCCATCTTCTTCCAGAT 3′. The C-terminal coding region of *baxα* or *baxβ* cDNA was amplified using bax-exon 3 primer in combination with bax-exon 6.2 primer or bax-intron 5.2 primer, respectively.

RT-PCR amplification

One microgram of total RNA was reverse transcripted in the presence of 40 mM Tris-HCl (pH 8.3), 75 mM KCl, 3 mM $MgCl_2$, 10 mM DTT, 625 μM of dATP, dTTP, dCTP and dGTP, 5 μg/ml oligo dT, 1000 U/ml RNase inhibitor RNasin (Promega, Madison, WI, USA), 10 000 U/ml Moloney murine leukemia virus reverse transcriptase (MMLV-RT) (Gibco BRL, Gaithersburg, MD, USA) in a total volume of 20 μl. Reactions were overlaid with 60 μl mineral oil (Sigma, St Louis, MO, USA), and were subsequently incubated at 20° C for 10 min, at 42° C for 45 min and at 95° C for 10 min. Samples were amplified by polymerase chain reaction (PCR) in a DNA thermal cycler (Perkin Elmer Cetus, Norwalk, CT, USA), in the presence of 0.001% gelatine, 65 mM KCl, 28 mM Tris-HCl (pH 8.3), 2.6 mM $MgCl_2$, 500 μM of dATP, dTTP, dCTP and dGTP, 300 pmol/ml of oligonucleotides, and 25 U/ml *Taq* DNA polymerase (Gibco BRL) in a total volume of 100 μl. Amplification started with an initial denaturation step at 94° C for 5 min, followed by 30 cycles at 94° C for 1.5 min, 59° C for 2 min and 72° C for 2 min. After the last cycle, extension phase was prolonged for 10 min at 72° C, and the reactions were cooled to 40° C.

Sequencing

PCR products were sequenced according to the method developed by Innis *et al*,[34] with minor modifications. *Baxα* PCR product was sequenced using oligonucleotides bax-exon 4, bax-exon 5 or bax-exon 6. *Baxβ* PCR product was sequenced using oligonucleotides bax-exon 4, bax-exon 5 or bax-exon 6. *Baxβ* PCR product was sequenced using oligonucleotides bax-exon 4, bax-exon 5 bax-intron 5.

Antibodies

Bcl2 antibody 124 (Dako, Glostrup, Denmark) was a mouse IgGl, kappa. Bax antiserum P-19 (Santa Cruz Biotechnology, Santa Cruz, CA, USA) is a polyclonal rabbit antiserum raised to a peptide corresponding to amino acids 43–61 of the mouse bax gene, but also reacts with human bax. Horseradish peroxidase conjugated goat anti-mouse IgGl (Southern Biotechnology Association, Birmingham, AL, USA) and peroxidase-conjugated goat anti-rabbit (Jackson Immuno Research, West Grove, MI, USA) were used as secondary antibodies.

SDS PAGE and immunoblotting procedure

Cells were washed once with phosphate-buffered saline (PBS) and resuspended at a concentration of 2×10^7 cells/ml in PBS. Cells were boiled after addition of 2 × SDS sample buffer. One microliter of these extracts was applied to a 12.5% polyacrylamide gel using the fast system (Pharmacia, Uppsala, Sweden). After electrophoresis the samples were blotted by diffusion for 1 h at 60° C onto a 0.45 μm nitro-cellulose membrane (Schleicher and Schuell, Dassel, Germany) in blotting buffer (25 mM Tris, 192 mM glycine (pH 8.3), 20% methanol). Blocking buffer for immunological detection was prepared by heating 0.5% NIP 552 blocking reagent (Amersham, Bucks, UK) for 1 h at 65° C. Blocking buffer was cleared by centrifugation and Tween-20 was added to a final concentration of 0.05%. The membrane was blocked for 1 h at room temperature (RT) in blocking buffer. The first antiserum was added to a final concentration of 2 μg/ml. The membrane was washed three times in PBS containing 0.05% Tween-20 and incubated for 1 h at RT in 3000-fold diluted secondary antibody in blocking buffer. After five washes with PBS containing 0.05% Tween-20, blots were developed using the ECL detection system (Amersham) and used for exposure on an X-ray film.

Results and discussion

mRNA of six hematopoietic cell lines were screened for mutations in the *bax* gene by an RT-PCR strategy. We focussed on the C-terminal coding region of the *bax* gene in which the BH1 and BH2 domains are located, important for heterodimerization of BAX with BCL2 or BCLXL.[7,13] The region containing the N-terminal homology domain Box 1[11] was not screened, although this region may also be important for dimerization of *bcl2*-like family members.[12] We found three mutations in two out of six cell lines (33%). Since we did not check the N-terminal coding region of the bax gene, the frequency may be underestimated.

Cell line HPB-ALL, which was derived from a patient with T cell acute lymphoblastic leukemia (T-ALL), expressed *bax* mRNA in which a G to A mutation (not shown) was present at position 199 (relative to the ATG start codon) as well as the normal allele. This mutation leads to the substitution of a glycine residue by an arginine residue (G67R). Alignment of this region to other *bcl2*-like family members (Figure 1a) demonstrates that this glycine residue is very conserved throughout evolution. This domain is located between the conserved Box 1 and Box 2 regions,[11] and the functional significance of this region has not been determined. A second C to T mutation was present in intron 5 at position 508 (not shown) in the same cell line. The unmutated allele was also expressed on mRNA level. Since this intron is alternatively spliced, this mutation only affects the BAXβ variant.[6] By this mutation an arginine residue is sustituted by a cysteine residue. It is not known if expression of BAXβ like BAXα renders a cell more vulnerable for induction of cell death, and therefore it is difficult to draw any conclusion regarding this mutation. It is not known if both mutations occur in the same allele.

The Burkitt lymphoma cell line Daudi, expressed *bax* mRNA containing a G to T mutation in BH1 on position 323, but also expressed the normal allele (Figure 2). By this mutation, a glycine residue is substituted by a valine residue. This BAX G108V mutation is located in BH1[13] (also defined as Box 2[11]) at a position which is completely conserved in all known *bcl2*-like family members (Figure 1b). Mutation analysis for the *bcl2* gene[13] and the *bclxl* gene[7] clearly demonstrates that substitution of this particular glycine residue by glutamate or alanine, respectively, results in complete loss of heterodimerization capacity with BAX and concomitant loss of cell death repressor function. In another mutation analysis, the nematode *C. elegans* paradoxically demonstrates a gain-of-function of CED9 upon substitution of the same glycine residue by glutamate. Although BCL2 normally can substitute for CED9 in *C. elegans*,[11] this mutation renders BCL2 completely ineffective for rescue of CED9 loss-of-function mutations. Interestingly, the *bcl2* gene isolated from a chicken B cell lymphoma cDNA bank coded for a valine instead of a glycine residue at the same position,[35] and it was speculated that this mutation contributed to lymphoma genesis.[36] Mutating this glycine residue has not only different effects in different proteins of the *bcl2*-like family members, but its effect is also dependent on the nature of the amino acid substitution.

What are the consequences of mutations within the *bax* gene? We hypothesize that the mutation in the *bax* gene as found in the cell line Daudi is accompanied with a loss-of-function of BAX, since gain-of-function would lead to a dramatic increase of cell death. The protein data also argue in favor of this suggestion (Figure 3). Normal bone marrow cells as well as the cell lines CEM, Jurkat, JM, HPB-ALL and KM3 all express BAX as well as BCL2, although there are differences in the relative BAX/BCL2 ratio as measured by our assay. The situation is different in cell line Daudi, since we did not detect any BCL2 expression whereas BAX expression seems to be abundant. Therefore, BAX homodimers probably will be abundant as well, and this cell line would be extremely sensitive for apoptosis induction by normal BAX function which is not the case. It would be thrilling to know in what way these mutations affect BAX function. Do they make BAX oncogenic by more effective heterodimerization with BCL2 or BCLXL or by less effective homodomerization? Do they act in a dominant negative way on the normal allele by $BAX_{(mutant)}$/$BAX_{(wildtype)}$ homodimerization and concomitant loss-of-func-

a

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CED9       116 MRVMGTIFEKKHAENFE 132   (C. elegans)
CED9       116 MRSLGTIFEKRHAEMFE 132   (C. briggsae)
BCL2        91 LRQAGDEFSRRYQRDFA 107   (Chicken)
BCL2       .94 LRRAGDDFSRRYRRDFA 110   (Mouse)
BCL2        97 LRQAGDDFSRRYRGDFA 113   (Human)
BAX         63 LKRIGDELDSN--MELQ  77   (Human)
BCLX1/s     90 LREAGDEFELRYRRAFS 106   (Human)
BCLX        86 LRDAGDEFELRYRRAFS 102   (Chicken)
MCL1       213 LRRVGDGVQRNHETVFQ 229   (Human)
BAK         78 LAIIGDDINRRYDSEFQ  94   (Human)
                   Δ
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b

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LMW5-HL   76 ELFKDLI-NWGRICGFIV--FSA  95 (African Swine Virus)
BHRF1     88 EIFHRGDPSLGRALAWMA--WCM 108 (Epstein Barr Virus)
CED9     159 AQTDQCPMSYGRLIGLIS--FGG 179 (C. elegans)
CED9     159 SSNTPCPMSYGRLIGLIS--FGG 179 (C. briggsae)
BCL2     130 ELFRDGV-NWGRIVAFFE--FGG 149 (Chicken)
BCL2     130 ELFRDGV-NWVRIVAFFE--FGG 149 (Chicken*)
BCL2     133 ELFRDGV-NWGRIVAFFE--FGG 152 (Mouse)
BCL2     136 ELFRDGV-NWGRIVAFFE--FGG 155 (Human)
BAX       98 DMFSDGNFNWGRVVALFY--FAS 118 (Human)
BCLX1    130 ELFRDGV-NWGRIVAFFS--FGG 149 (Human)
BCLX     125 ELFHDGV-NWGRIVAFFS--FGG 144 (Chicken)
MCL1     233 HVFSDGVTNWGRIVTLIS--FGA 253 (Human)
BAK      117 SLFESGI-NWGRVVALLG--FGY 136 (Human)
BAD      138 PPNLWAAQRYGRELRRMSDEFEG 160 (Mouse)
A1        77 KEFEDGIINWGRIVTIFA--FGG  97 (Mouse)
                        Δ
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Figure 1 Alignment of *bax* gene domains which were mutated in cell lines HPB-ALL and Daudi with other *bcl2*-like family members. (a) alignment of *C. elegans* or *C. briggsae* CED9,[11] chicken BCL2,[39] mouse BCL2,[40] human BCL2,[41] human BAX,[6] human BCLXs and BCLX1,[4] chicken BCLX,[4] MCL1[42] and BAK.[9] The BAX G67R mutation as found in cell line HPB-ALL is indicated by a triangle. The most conserved amino acid residues among the genes are indicated in bold (b) Alignment of the BH1 domain for *bcl2*-like genes as used in (a) supplemented with chicken BCL2*,[35] mouse BAD,[7] mouse A1,[43] Epstein–Barr virus BHRF1 and African swine virus LMW5-HL.[3] The BAX G108V mutation as found in cell line Daudi is indicated by a triangle. The most conserved amino acids are indicated in bold

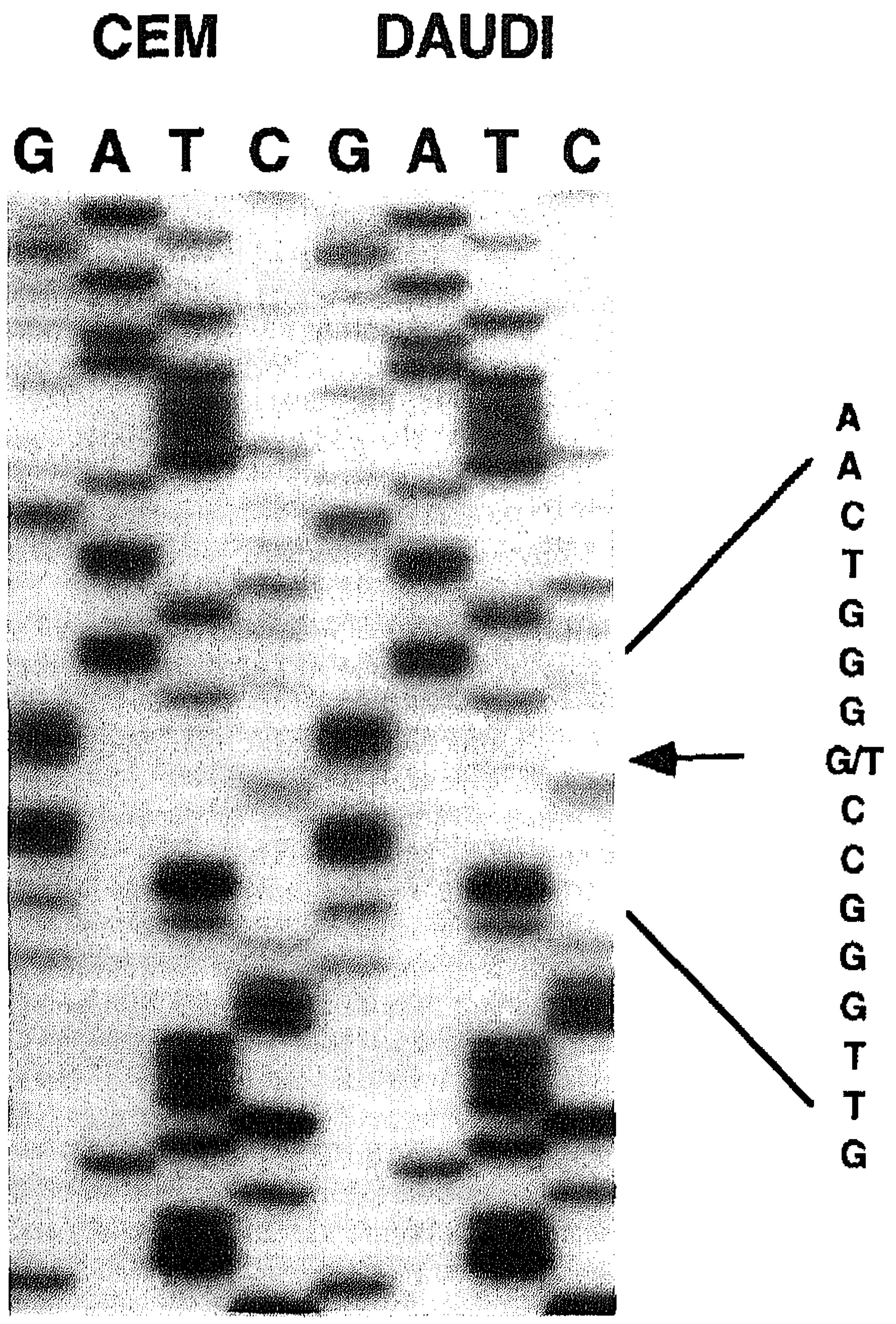

Figure 2 Sequence of the BH1 region of the *bax* gene from cell lines CEM and Daudi. The G to T mutation present in one of the *bax* alleles in the Daudi cell line results in the G108V mutation

tion? Functional studies will be necessary to provide an answer.

The Daudi cell line is derived from a Burkitt lymphoma with a rearranged and overexpressed *c-myc* gene. Overexpression of *c-myc* induces apoptosis in fibroblasts under conditions which normally promote G1 arrest, and it was hypothesized that secondary defects in tumorigenesis affect the cell death pathway.[37] It has been demonstrated that ectopic expression of *bcl-2* inhibits apoptosis induced by deregulated *c-myc* expression.[38] Since BCL2 and BAX have opposite effects on the cells susceptibility to undergo cell death, it is clear that mutations which disrupt BAX function could contribute to Burkitt lymphoma genesis in an identical manner. This may have been the case in the patient from which the Daudi cell line is derived.

Overexpression of BCL2 as a first genetic defect seems to be sufficiently tumorigenic in mice model.[32] Most human follicular lymphoma cases carry the t(14;18) in which the *bcl2* gene is deregulated. Therefore, it is important to investigate whether mutations in the *bcl2*-like family member *bax* also contribute to cancer development. Mutations in the *p53* gene and defects in genes which are part of the *p53*-dependent apoptotic machinery like *bcl2* provide increased resistance to chemotherapeutic agents as well as γ-radiation.[2,17,26,30,31] It therefore seems reasonable that mutations negatively affecting BAX function also provide increased resistance to treatment.

In conclusion, we found mutations in the *bax* gene in about 30% of the human hematological cell lines tested. Mutations in the conserved regions of the *bax* gene are probably associated with a loss-of-function of BAX, and may contribute as an oncogenic event to the pathogenesis of cancer as well as to increased resistance to treatment; so far these mutations have been found in hematological cell lines. In order to demonstrate the frequency and importance of these mutations, it is necessary to screen a large panel of tumor samples derived from hematological malignancies as well as other types of cancer.

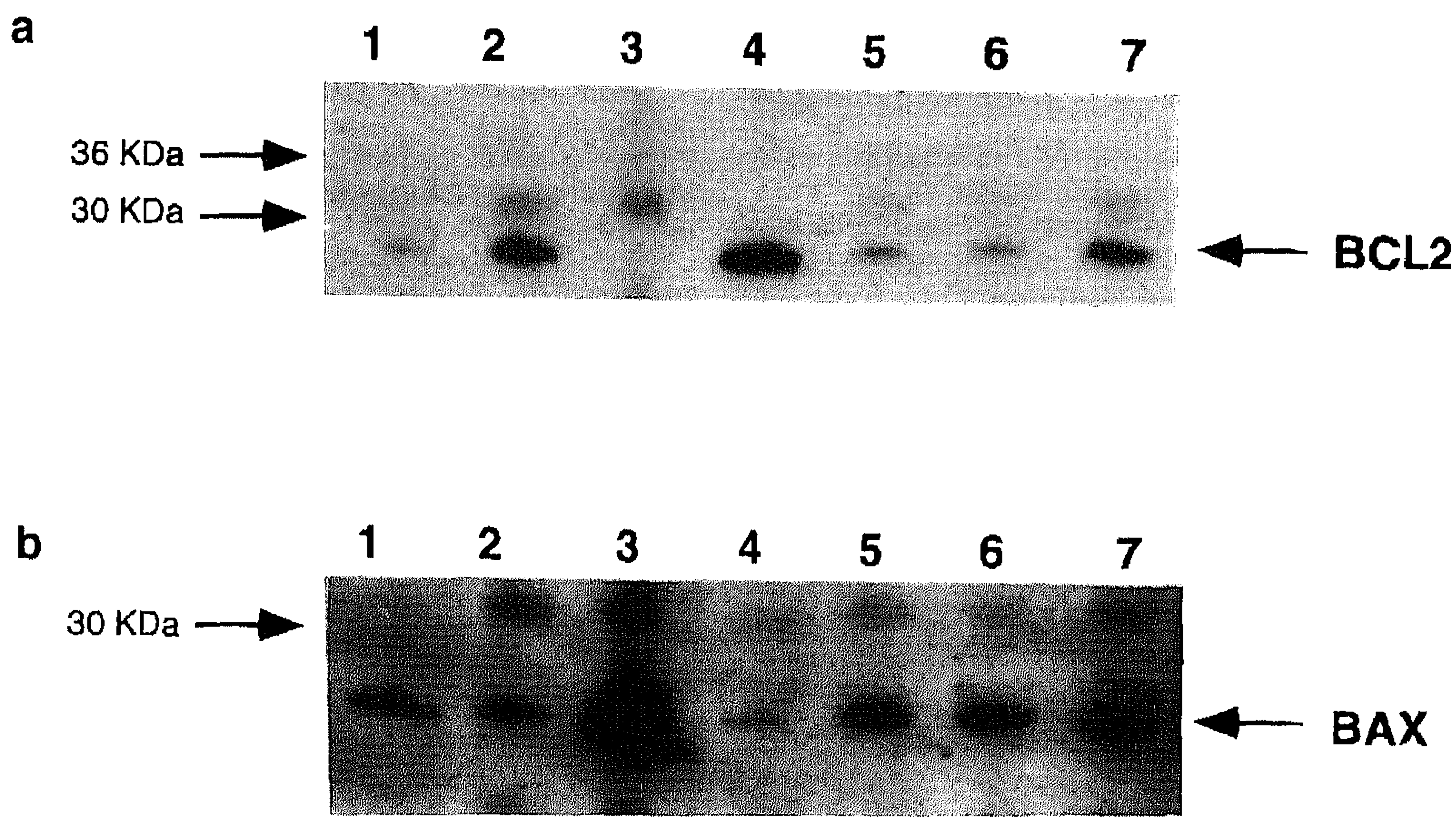

Figure 3 BCL2 and BAX detection by an immunoblot of cell lysates from 1: normal bone marrow cells; 2: HPB-ALL; 3: Daudi; 4: KM3; 5:CEM; 6: Jurkat; 7: JM. The immunoblot was consecutively screened for (a) BCL2 with moAb 124 (Dako) or (b) BAX with polyclonal P-19 antiserum (Santa Cruz Biotechnology)

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