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Abnormal Intracellular Localization of Bax with a Normal Membrane Anchor Domain in Human Lung Cancer Cell Lines

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Proapoptotic Bax is a member of the Bcl-2 family proteins, which have a key role in regulating programmed cell death. The intracellular localization and redistribution of Bax are important in promoting apoptosis. Bax contains a BH3 domain heterodimerizing with Bcl-2 and a hydrophobic transmembrane segment to be inserted in specified organelle membranes. In this study, Bcl-2 showed cytoplasmic localization in all of ten human lung cancer cell lines tested. Interestingly, Bax was localized in the nucleus in 7 cell lines, although Bax lacks nuclear import signals. This may allow cancer cells to escape from apoptosis. Why Bax is able to exist in the nucleus is still unclear. We hypothesized that mutation in the BH3 domain and/or transmembrane segment of Bax possibly causes intracellular Bax distribution. We analyzed the sequence of the *bax* gene in these cell lines and found only a silent point mutation at codon 184 (TCG→TCA) in the transmembrane segment in all cell lines. This finding indicates that changes in cellular localization of Bax in lung cancer cell lines do not depend on *bax* mutation and that Bax is possibly translocated into the nucleus without any mutation. This is the first report showing that Bax with the normal amino acid sequence can be localized in the nucleus in established lung cancer cell lines without any treatment of the cells.

Key words: Bax localization — BH3 domain — Transmembrane segment

Of the Bcl-2 family proteins, some members including Bcl-2, Bcl-XL, Bcl-W, Mcl-1 and A1 promote cell survival, while other members including Bax, Bad, Bid, Bak, Bcl-XS, Bik, Bim and HRK potentiate apoptosis.[1] This family is specifically defined by four regions that share amino acid sequence homology, designated BH1, BH2, BH3, and BH4.[2,3] Mutational analysis has indicated the importance of BH1 and BH2 for the antiapoptotic function of Bcl-2 and Bcl-XL, as well as the binding to Bax,[4] while the BH4 domain is vital for the death-repressor function. The BH3 domains of Bax and Bak are critical for promoting cell death and dimerization with Bcl-2 and Bcl-XL.[5,6] Most Bcl-2 family proteins contain a single predicted transmembrane segment at their extreme carboxylic terminal region, which is presumed to function in anchoring these proteins to organelle membranes.[7]

Bax has been suggested to target organelle membranes,[8] particularly mitochondria,[3] using the C-terminal hydrophobic region. By means of an immuno-histochemical technique[9] and subcellular fractionation[10] Bax has been detected in the cytosol. A recent study showed that Bax requires Bcl-2 to associate with organelle membranes[11] and the BH3 domain of Bax is critical for heterodimerization with Bcl-2.[4,5] Alteration in the subcellular distribution of Bax is important, as it suggests that Bax redistribution may have a role in apoptosis.[12–15] Most of the previous studies have indicated that Bax translocates to the mitochondria and becomes a membrane-bound form following the induction of apoptosis.[10,12,16,17] On the other hand, the nuclear localization of Bax during induction of apoptosis has been reported in many kinds of cells, such as immortalized lung cancer cells during hyperthermia treatment,[18] fibroblasts after both γ-irradiation and topotecan treatment,[19] some dying neurons in hippocampus and entorhinal cortex after kainic acid administration in the rat,[20] and human colorectal carcinoma cells after growth factor deprivation.[13] We also found that Bax was localized in the nucleus in seven of ten cell lines without any apoptotic stimulus, although it lacks the nuclear localizing sequence (NLS) in the primary structure.[21] Ions and other small molecules passively diffuse through ~10 nm aqueous channels in the nuclear pore complexes. Most macromolecules are too large to pass through these diffusional channels and are transported through a central gated channel in an ATP- and temperature-dependent manner.[10] If 21-kDa Bax is distributed through the aqueous channels of 10 nm or less in the nuclear pore complexes, like ions and other small molecules, Bax should exist equivalently in both the cytoplasm and the nucleus, as was observed in one cell line. However, as we found that Bax was localized only in the nucleus in three cell lines, we considered that Bax should be translocated into the nucleus by an active transport mechanism after translation from mRNA in the cytoplasm. The question then arose, does Bax newly acquire NLS due to Bax mutation?

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The *bax* gene encodes six exons and shows a complex pattern of alternative mRNA splicing. Six different Bax variants have thus far been characterized: 21-kDa Baxα[21] and 15.8-kDa Baxδ[22] that have a putative transmembrane domain, and 24-kDa Baxβ, 4.5-kDa Baxγ,[21] 28-kDa Baxω[23] and 18-kDa Baxε[24] that lack the hydrophobic transmembrane segment. *In vivo* studies suggest that a single protein product predominates for each gene, and Baxα is the predominant transcript in the human lung.[18, 21, 23] Moreover, we detect Bax as a 21-kDa single band by western blotting. Therefore, Bax that we detected in this study should be Baxα.

In this study, we focused on the association of Bax localization with Bax mutation in human lung cancer cell lines. Therefore, we analyzed the sequence of the *bax* gene in these cell lines to test if the nuclear localization of Bax is a result of *bax* mutation.

MATERIALS AND METHODS

Cell lines Ten human lung cancer cell lines used were squamous cell carcinomas (NPC-2, NPC-5, PC-10, and QG-56), adenocarcinomas (PC-3, NPC-4, and NPC-8), and small cell carcinomas (NPC-1, PC-6, and QG-90).[25] The cells were cultured in RPMI 1640 medium (Gibco, Grand Island, NY) containing 10% fetal bovine serum (Gibco) (complete medium) in a humidified atmosphere of 5% CO_2 at 37°C. The medium was replaced with fresh complete medium every three days.

Antibodies The anti-human Bcl-2 mouse monoclonal antibody (Bcl-2 mAb; Oncogene Science, Cambridge, MA) and anti-human Bax rabbit polyclonal antibody (Bax pAb; PharMingen, San Diego, CA) were developed using synthetic peptides corresponding to the amino acid residues 41–54 of human Bcl-2 and 43–61 of human Bax as immunogens, respectively.

Protein extraction Cells were harvested by scraping, washed twice with ice-cold phosphate-buffered saline (PBS), and lysed with a lysis buffer [10 m*M* Tris (pH 7.4), 150 m*M* NaCl, 1% Triton X-100, 1% Na-deoxycholate (Kanto Chemical, Tokyo), 0.1% sodium dodecyl sulfate (SDS) (Kanto Chemical), 5 m*M* ethylenediaminetetraacetic acid (EDTA), 1 m*M* phenylmethylsulfonyl fluoride (Sigma, St. Louis, MO), 0.28 U/ml aprotinin (TaKaRa Shuzo, Otsu), 10 μM leupeptin (Sigma), 1 m*M* benzamidine (Sigma), and 7 μg/ml pepstatin A (Sigma)] on ice for 30 min then centrifuged at 15 000 rpm for 30 min.

Western blotting Western blotting was performed by the method of Krajewski *et al.*,[9] with minor modification. Briefly, samples containing 100 μg protein were subjected to SDS-polyacrylamide gel electrophoresis (SDS-PAGE, 12% gels). The proteins were transferred to Hybond-P (polyvinylidene difluoride; PVDF membrane, Amersham, Buckinghamshire, UK) membranes, which were blocked overnight at 4°C with 5% non-fat milk in PBS containing 0.1% Tween 20 (TPBS). The membranes were then incubated for 1 h at room temperature with anti-human Bcl-2 mouse mAb (1:200 dilution) or anti-human Bax rabbit pAb (1:1000 dilution). They were washed twice with TPBS, then incubated for 1 h at room temperature with horseradish peroxidase (HRP)-linked anti-mouse immunoglobulin (Ig) (1:300 dilution; Amersham) for Bcl-2 or HRP-linked anti-rabbit Ig (1:300 dilution; Amersham) for Bax. The immunoblots were developed using ECL western blotting reagents (Amersham) and the chemiluminescence was visualized and quantified using a Lumino image analyzer (LAS1000, Fuji Film, Tokyo).

Immunofluorescence Cells (1×10^4/chamber) were preincubated in the complete medium on an 8-chamber slide (Nunc, Naperville, IL) for 48 h to allow them to grow exponentially and were fixed with 3% paraformaldehyde for 15 min at room temperature and with 70% methanol for 5 min at −20°C. The fixed cells were washed with PBS containing 0.1% bovine serum albumin and 0.05% Tween 20 (PBT) and were incubated with 1% rabbit serum or 1% swine serum for 30 min at room temperature to block the non-specific binding of antibodies. Then, the cells were exposed to anti-human Bcl-2 mAb (1:40 dilution) or anti-human Bax pAb (1:1000 dilution) at 4°C overnight. They were washed with PBT, then exposed further to fluorescein isothiocyanate (FITC)-conjugated rabbit anti-mouse Ig for Bcl-2 (1:30 dilution; Dako, Glostrup, Denmark) or FITC-conjugated swine anti-rabbit Ig for Bax (1:30 dilution; Dako) for 30 min at room temperature in the dark. Control cells were stained only with FITC-labeled second Ab. Finally, double-stranded DNA was stained with 100 ng/ml propidium iodide for 20 min at room temperature in the dark. The samples were observed using a confocal laser-scanning microscope (MRC-1024, Bio-Rad Microscience Division, Watford, UK) equipped with a computer (NEC, Tokyo).

DNA extraction After removal of the medium, the cells were washed once with ice-cold PBS. DNA was extracted from the cells (4×10^6 cells/tube) using a Puregene DNA isolation kit (Gentra System, Minneapolis, MN), according to the manufacturer's instructions.

***Bax* gene amplification by PCR (polymerase chain reaction)** Six exons of the *bax* gene were amplified using a PCR reagent system kit (GIBCO BRL, Life Technologies, Tokyo) and appropriate nucleotide primers under the reaction conditions in Table I, as described by Chou *et al.*,[26] with a minor modification. Namely, PCR was performed in 100 μl mixtures containing PCR buffer [200 m*M* Tris-HCl (pH 8.4), 500 m*M* KCl, and 15 m*M* $MgCl_2$], 0.2 m*M* of each dNTP, 0.5 μM of each primer, 200 ng of DNA, and 2.5 U of *Taq* DNA polymerase. Thirty-five cycles of denaturation at 94°C for 1 min, annealing for 2 min either at 54°C for exon 1, at 58°C for exons 2–3 and

Table I. Sequences of Oligonucleotide Primers and Conditions Used in PCR Amplification of *Bax* Gene

Primers		Sequence	T_m (°C)[b]	bp[c]
bax exon 1[a]	Sense	5′-CGTTCAGCGGGGCTCTCA-3′	54	207
	Antisense	5′-CAGGCCGGTAGGAAGGAT-3′		
bax exon 2, 3[a]	Sense	5′-CCCCTAGAACCCAAGAGTC-3′	58	400
	Antisense	5′-GGCTGAGAGTCCTGTGTCC-3′		
bax exon 4	Sense	5′-TCCCCAGGTCCTCACAGAT-3′	58	209
	Antisense	5′-TCTCCTGCAGGATGATTGC-3′		
bax exon 5	Sense	5′-CAGGCAGTGGGGACAAGGTT-3′	62.5	192
	Antisense	5′-GCGGTGGTGGGGGTGAGGAG-3′		
bax exon 6	Sense	5′-CCCCTGGCCGAGTCACTGAA-3′	60	237
	Antisense	5′-AATGCCCATGTCCCCCAATC-3′		

a) 5% dimethyl sulfoxide was added.
b) Optimum annealing temperature.
c) Amplicon size expected, in base pairs (bp).

exon 4, at 62.5°C for exon 5, or at 60°C for exon 6, and elongation at 72°C for 3 min were performed using a programmable GeneAmp PCR system 2400 (Perkin-Elmer, Applied Biosystems, Foster City, CA). The first denaturation step and the last elongation step were extended to 4 and 12 min, respectively. The PCR products were separated by electrophoresis on agarose (Type II, Medium EEO, Sigma) gels [3% gel in Tris acetate/EDTA buffer (TAE)] for 45 min and visualized by ethidium bromide staining to detect the amplicon size. The molecular weight standard used was 100 bp marker (Bio-Rad Laboratories, Hercules, CA).

Sequencing analysis For sequencing, the PCR products were separated by electrophoresis on low-melting-temperature agarose (NuSieve GTG agarose, FMC BioProducts, Vallensbaek Strand, Denmark) gels (1% gel in TAE buffer containing 0.1 m*M* EDTA) at 4°C for 45 min and were visualized by staining with ethidium bromide. Bands of interest were excised from the gel and the DNA was recovered according to the standard protocol of phenol/chloroform extraction and ethanol precipitation.[27] The purified PCR products were sequenced directly using an "ABI PRISM BigDye" terminator cycle sequencing kit (Perkin-Elmer, Applied Biosystems, Chiba). The extension products were purified using "Centri-Sep" spin columns (Princeton Separations, Inc., Adelphia, NJ) and then were electrophoresed using an ABI PRISM 310 Genetic Analyzer (Perkin-Elmer, Applied Biosystems).

RESULTS

Expression levels of Bcl-2 and Bax protein in lung cancer cell lines We measured the expression levels of anti-apoptotic Bcl-2 protein and proapoptotic Bax protein because the Bax/Bcl-2 ratio is a very important factor to determine if the cell will survive or die.[4, 21] Levels of Bcl-2 varied relatively widely (0.2 to 1.9 arbitrary unit) among cell lines as compared with Bax levels (0.1 to 1.4 arbitrary unit) (Fig. 1). Peripheral lymphocytes expressed a little Bax and abundant Bcl-2, while immortalized lung cancer cells expressed Bax at five- to thirteen-fold higher levels than normal lymphocytes. NPC-1 and NPC-5 cells expressed large amounts of both Bcl-2 and Bax. The PC-3 cells, however, expressed only a little Bcl-2, in spite of a relatively high expression of Bax, which resulted in a high Bax/Bcl-2 ratio (3.9–7.9 times) compared with the ratios of other cell lines.

Intracellular Bcl-2 and Bax localizations Intracellular localizations of Bcl-2 and Bax were examined in lung cancer cells using a confocal laser-scanning microscope (Table II). Bcl-2 was localized in the cytoplasm in all cell lines (Fig. 2, a, b, c, d). However, Bax was localized in the nucleus and/or the cytoplasm (Fig. 2, e, f, g, h): (i) localized only in the cytoplasm in PC-10 and QG-56 squamous cell carcinomas and NPC-8 adenocarcinoma; (ii) localized only in the nucleus in PC-3 adenocarcinoma and NPC-1 and QG-90 small cell carcinomas; (iii) localized either in the nucleus or the cytoplasm in NPC-2 squamous cell carcinoma, NPC-4 adenocarcinoma and PC-6 small cell carcinoma; and (iv) localized in both the nucleus and the cytoplasm in NPC-5 squamous cell carcinoma. Thus, the cytoplasmic and/or nuclear localization of Bax was unrelated to histologic cell type.

***Bax* gene amplification** Bax consists of 192 amino acid residues and has BH1, BH2, and BH3 domains and a transmembrane segment at the C-terminus (Fig. 3a). Genomic DNAs of ten lung cancer cell lines were screened for mutation in the *bax* gene by PCR and a direct sequencing strategy. The amplicon sizes of the PCR products of exons 1, 2–3, 4, 5, and 6 were 207, 400, 209, 192, and 237 bp, respectively (Table I, Fig. 3b).

BH3 domain sequencing The third exon of *bax* con-

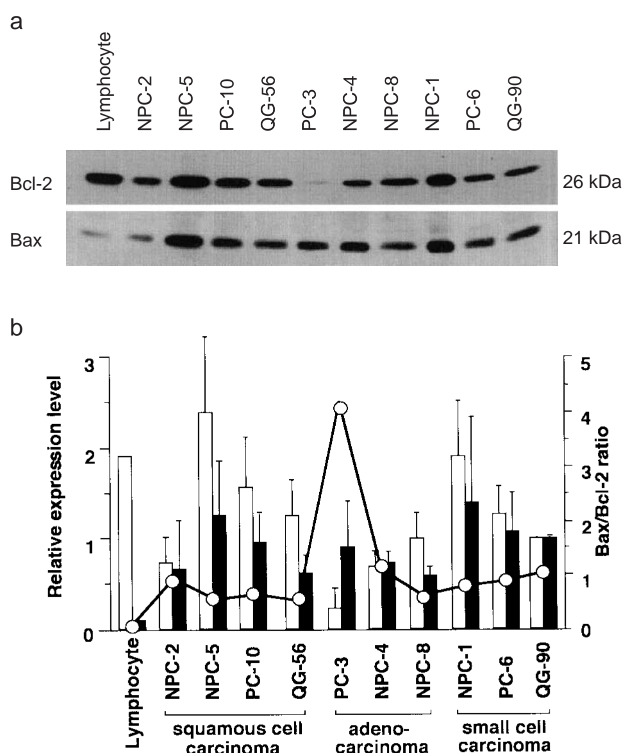

Fig. 1. Bcl-2 and Bax expression levels in human lung cancer cell lines. a) Cell lysates containing 100 μg/ml total protein were subjected to SDS-PAGE followed by western blotting using anti-human Bcl-2 mAb and anti-human Bax pAb. b) The levels of Bcl-2 (□) and Bax (■) in western blot bands were measured using a densitometer and the Bax/Bcl-2 ratio (○) was calculated. Data are mean values±SD of three independent experiments.

Table II. Distribution of Bcl-2 and Bax Proteins in Human Lung Cancer Cell Lines

Cell line	Bcl-2 localization[a]	Bax localization[a]	Histologic cell type[b]
NPC-8	C	C	Ad
PC-10	C	C	Sq
QG-56	C	C	Sq
NPC-1	C	N	SCC
PC-3	C	N	Ad
QG-90	C	N	SCC
NPC-2	C	N or C	Sq
NPC-4	C	N or C	Ad
PC-6	C	N or C	SCC
NPC-5	C	N and C	Sq

a) C, cytoplasm; N, nucleus.
b) Ad, adenocarcinoma; Sq, squamous cell carcinoma; SCC, small cell carcinoma.

tained the BH3 domain spanning the codons 59–73, which is necessary for homodimerization, heterodimerization and proapoptotic activity.[4] Also, exon 3 contains a stretch of eight consecutive deoxyguanosine residues, the poly (G)8 tract ATG GGG GGG GAG at codons 38–41 relative to the ATG start codon, which was proposed as a potential site for heterozygous frameshift mutation involving a single nucleotide deletion or insertion (Table III).[28–35] Sequencing analysis of exon 3 after PCR amplification with the sense and antisense primers showed no mutation in the BH3 domain or the poly (G)8 tract (data not shown).

Transmembrane domain sequencing We focused on the C-terminal coding region of the *bax* gene because Bax

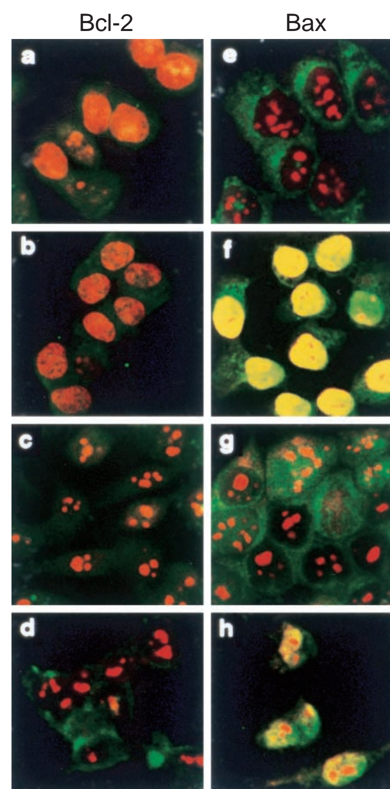

Fig. 2. Bcl-2 and Bax intracellular localizations. Cells were fixed and Bcl-2 (left) and Bax (right) were stained as described in "Materials and Methods." Images represent Bcl-2 cytoplasmic localization in a) PC-10, b) PC-3, c) NPC-4, and d) NPC-5 cells. Green fluorescence and red fluorescence are specific for Bcl-2 and DNA, respectively. Bax localizations are different among the cell lines; Bax is localized in e) the cytoplasm of PC-10; f) the nucleus of PC-3; g) the nucleus or the cytoplasm of NPC-4; and h) both the nucleus and the cytoplasm of NPC-5. The green fluorescence is Bax and red fluorescence is DNA, while the overlap of green and red fluorescence gives the yellow color.

protein, at its extreme C-terminus, contains a single transmembrane segment that functions in anchoring Bax into organelle membranes.[7] We initially considered that exon 6

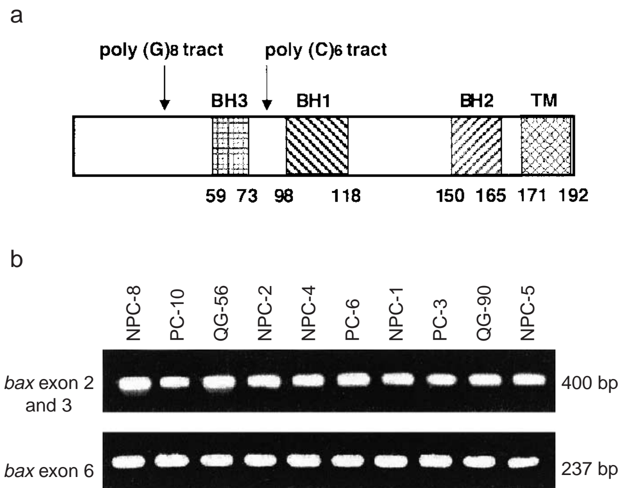

Fig. 3. a) Schematic representation of the different Bcl-2 homology (BH) domains and transmembrane (TM) segment of Bax. The arrows point to the positions of the eight consecutive deoxyguanosine stretch, the poly (G)8 tract, (spanning nucleotides 114–121 of exon 3) and the six cytosine stretch, the poly (C)6 tract (at nucleotides 260–265). b) Genomic DNAs prepared from lung cancer cells were amplified using *bax* exons 2,3 and *bax* exon 6 primers. The PCR products were separated by electrophoresis on 3% agarose gel and were visualized by ethidium bromide staining. The numbers on the right side are the amplicon sizes.

Table III. Summary of *Bax* Frameshift Mutations (%) at the (G)8 Tract in Tumors

Tumors[a]	Mutations (%)	Reference[b]
MMP$^+$colorectal	51	28)
MI$^+$ gastric	33	29)
MI$^+$ endometrial	12	29)
MI$^+$ colorectal	41	29)
MMP$^+$ gastric	64	30)
MI$^+$ gastric carcinomas	67	31)
HNPCC adenocarcinomas	54.5	32)
HNPCC adenomas	15.4	32)
MMP$^+$ HNPCC	52	33)
Hematopoietic malignancy	21	34)
DG75 and Jurkat cells of human hematopoietic malignancies	recorded in both	35)

a) MMP$^+$, tumor with microsatellite mutator phenotype; MI$^+$, tumor with microsatellite instability; HNPCC, hereditary nonpolyposis colorectal cancer.
b) Reference number (see reference list).

of *bax* containing the transmembrane domain at codon 172–192 might be frequently mutated in lung cancer cell lines. Therefore, the PCR products of exon 6 were sequenced using the sense primer of exon 6. A silent point mutation in codon 184 (TCG→TCA) was found (Fig. 4a) in all cell lines tested. The two codons code for the same amino acid, serine. To confirm this silent point mutation, the PCR products were sequenced using the antisense primer of exon 6, and we found that the nucleotide sequence at codon 184 was changed from AGC to AGT (Fig. 4b).

Mutation analysis of the entire *bax* sequence Bax lacks the NLS in the primary structure,[21] and we confirmed that Bax had no NLS using PSORT II software (Institute of Molecular Biology, Osaka University). While the NLS had not been identified within the primary structure of Bax, we found that Bax was localized in the nucleus in 7 of 10 cell lines. To determine if *bax* newly expresses an

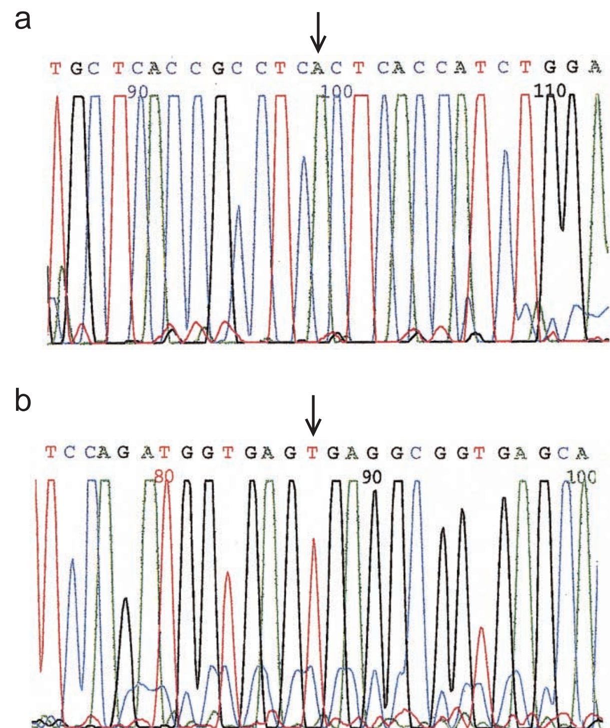

Fig. 4. Nucleotide sequence of the transmembrane domain of *bax* surrounding codon 184 from PC-3 cells. The PCR band of exon 6 was eluted from the low-melting-temperature agarose gel and sequenced using an ABI PRISM 310 Genetic Analyzer. a) The sense strand was sequenced using a sense primer of *bax* exon 6. b) The antisense strand was sequenced using the antisense primer of *bax* exon 6. The arrows point to the nucleotide change at codon 184 of the sense strand (TCG→TCA) and antisense strand (AGC→AGT).

NLS resulting from several mutations in the gene encoding Bax, we examined whole *bax* sequence in 10 cell lines, but we found neither mutation in these sequences nor expression of a new NLS.

DISCUSSION

Apoptosis-inducing proteins are normally coexpressed with antiapoptotic proteins, and the relative ratios between antiapoptotic (e.g. Bcl-2) and proapoptotic proteins (e.g. Bax) determine whether the cell will survive or die.[4, 21] Previous studies indicated that, in many cancers, expression levels of Bcl-2 increase, while Bax protein levels decrease.[36–40] Our data show that immortalized lung cancer cells express Bax at five- to thirteen-fold higher levels than normal lymphocytes. As the Bax expression itself, at least at physiological levels, is not lethal to cells,[12] our results support recent observations that a high expression of Bax without any apoptotic stimulus does not induce apoptosis.[21, 41] For example, sympathetic neurons expressing high levels of Bax mRNA will not undergo apoptosis unless deprived of growth factors.[41] Even cells stably over-expressing Bax proliferate normally, and Bax only accelerates cell death after receiving an external signal, such as interleukin-3 withdrawal.[21] Therefore, the lung cancer cell lines may proliferate normally and survive even if they contain Bax at a high Bax/Bcl-2 ratio compared with normal lymphocytes.

In this study we found a normal localization of Bcl-2 in lung cancer cell lines, which is consistent with the cytosolic localization in other studies.[9, 42] Conversely, Bax had abnormal nuclear localization in seven cell lines: NPC-1, NPC-2, PC-3, NPC-4, NPC-5, PC-6, and QG-90. The nuclear localization of Bax may endow cancer cells with resistance to apoptosis and afford a growth advantage to these cell lines. Our question was, might the nuclear localization of Bax be due to a mutation in the BH-3 domain? Mutant Bax missing its BH3 domain fails to dimerize with Bcl-XL or Bax.[43, 44] Other *bax* mutations, such as single amino acid substitutions within the BH3 domain, result in a decrease in the ability of Bax to dimerize.[34] To test the possibility that lung cancer cells have a mutation in the BH3 domain of Bax resulting in intracellular Bax localization by disrupting the association between Bax and Bcl-2 in lung cancer cell lines, we analyzed the nucleotide sequence of the BH3 domain; however, we found no mutation. Frequent frameshift mutations of *bax* in simple repeated sequences within the coding sequence exist in some cell lines and gastrointestinal cancer, as shown in Table III.[28–35] Therefore, we examined *bax* mutations at the repetitive sequences within its coding region. Two regions of the gene, poly (G)8 and poly (C)6 tracts, were analyzed. No mutation was observed in either tract in any of the cell lines. Frameshift mutation should result in the production of a truncated Bax protein.[29] The absence of Bax expression in some cell lines of human hemopoietic malignancies[35] and colorectal cancers[28, 32] is due to insertion or deletion of a single residue in the (G)8 tract within the *bax* coding sequence. This alters the reading frame and results in premature termination of Bax translation. In human lung cancer cell lines, we easily detect Bax as a 21-kDa single band in all cell lines by western blotting using the antibody that recognizes the epitope from amino acid 43 to amino acid 61, just after the (G)8 tract, which also proved that this area of *bax* had no frameshift mutation.

Table IV. Summary of Somatic *Bax* Mutation in Tumors

Tumors[a]	Exon	Domain	Nucleotide	Codon	bp alteration	Amino acid alteration
Gastrointestinal[33]	6		506	169	ACG to ATG	Thr to Met
Gastric[30]	2		84–86	29	GGG to GGGG	frameshift
Colorectal[30]	3	BH3	174	58	AAG to AAA	Lys to Asn
Colorectal[30]	3	BH3	203	68	GAC to GTC	Asp to Val
Colorectal[30]	4		266–267	89	CGA to CGTA	frameshift
Colorectal[30]	4		276	92	TTT to TTA	Phe to Leu
Gastric[30]	5	BH2	453	151	TGG to TGA	Trp to stop codon
Gastric[30]	6	BH2	495	165	TTT to TTA	Phe to Leu
Gastric[30]	6		505	169	ACG to GCG	Thr to Ala
Colorectal[30]	6		505	169	ACG to GCG	Thr to Ala
Colorectal[30]	6		506	169	ACG to ATG	Thr to Met
Colon[30]	6		506	169	ACG to ATG	Thr to Met
Hematopoietic[49]	3		199	67	GGG to AGG	Gly to Arg
Hematopoietic[49]	4	BH1	323	108	GGC to GTC	Gly to Val
Hematopoietic[49]	intron 5		508	in Baxβ	CGT to TGT	Arg to Cys

a) Reference number (see reference list).

Another essential region of Bax is the C-terminal hydrophobic segment spanning the last 21 amino acids of exon 6. The C-terminal sequence consists of a hydrophobic α-helix to be inserted into host membranes.[15] Deletion of the C-terminal eliminates the ability of Bax to associate with organelles and inhibits the Bax redistribution during apoptosis.[12] The amino acid serine at codon 184 (Ser184) is most important in regulating Bax subcellular localization, and mutation of Ser184 causes Bax to remain in either of two subcellular sites, the cytosol or the mitochondria, depending on the amino acid substitution.[14] Therefore, we speculated that a mutation in the transmembrane segment might disturb the intracellular Bax localization in lung cancer cell lines. We found a silent point mutation in codon 184 in all cell lines, but this mutation has no effect on the amino acid sequence because the two codons are both translated into serine. As Bax had a normal transmembrane segment in all cell lines, but showed various intracellular localizations, we concluded that the C-terminal domain might not determine Bax nuclear localization.

The selectivity of nuclear transport resides in nuclear import signals, which only nuclear proteins contain.[45] A typical signal peptide sequence for the nuclear import signal is -Pro-Pro-Lys^+-$\underline{Lys}^+$-Lys^+-Arg^+-Lys^+-Val-.[46] This sequence consists of four to eight amino acid residues that are rich in positively charged amino acids, such as lysine and arginine and usually contains proline. A mutation in a single amino acid ($\underline{Lys}^+$ to Thr) prevents nuclear transport and causes the mutant protein to remain in the cytoplasm.[46] Theoretically, the active nuclear transport of protein is thought to be mediated by the NLS and cognate transport factors.[47] Therefore, binding of Bax protein to nuclear pore complexes may be necessary for Bax to translocate into the nucleus. But Bax lacks the NLS in the primary structure.[21] So, we examined whether Bax may newly express the NLS resulting from accumulation of several mutations in the gene encoding Bax, but found no mutation creating a new NLS in the whole *bax* sequence. However, because projects to discover new monopartite or bipartite NLS are in progress, [47] the possibility that Bax may have an undiscovered NLS can not be ruled out. Phosphorylation influences both localization and the activity of the apoptosis promoter Bad,[6] and phosphorylation of Bcl-2 may regulate its function.[48] Although Ser184 appears important in the control of Bax localization and toxicity, no phosphorylation of Bax has been found before or after cell death.[14] Because we detected only a single band of Bax protein at 21 kDa, we considered that Bax was neither phosphorylated nor dephosphorylated, and that the nuclear localization of Bax did not depend on the phosphorylation pathway.

The mutations of the *bax* gene, as listed in Tables III and IV, may have an important role in the multistep pathogenesis of hematological malignancies,[34, 35, 49] the genesis of endometrial, gastric, and colorectal cancers with microsatellite instability,[29] human adenocarcinomas with microsatellite mutator phenotype,[28] and adenoma-carcinoma transition in hereditary non-polyposis colorectal cancer (HNPCC) tumorigenesis,[32, 33] because mutant Bax lacks its function as a promoter of apoptosis in these cancers. The above studies showed that the disruption of the *bax* gene may be an important cause of carcinogenesis in a variety of cancers. However, Japanese HNPCC patients having microsatellite instability,[50] and pancreatic cancer patients with a high incidence of microsatellite instability[29] showed no mutation in the *bax* gene. Our results are consistent with these findings. Possible explanations for these findings are that the *bax* mutation may not have an important role in the development of lung cancer and that a transporter of Bax to the nucleus, which has stronger affinity for Bax than Bcl-2 and mitochondrial membrane, may exist.

We found that all the cell lines examined expressed high levels of wild-type Bax protein and that the transmembrane domain of Bax had no role in determining the nuclear Bax localization in established lung cancer cell lines. Therefore, we conclude that the nuclear localization of Bax may be induced by a mechanism other than *bax* mutation, and further study is needed to clarify the native conformation of Bax in lung cancer cell lines and the possible post-translation modification of nucleus-associated Bax.

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