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Cloning of *BNIP3h*, a member of proapoptotic *BNIP3* family genes

Mohammad Farooq[1], Younghee Kim[1], Sanguk Im[1], Eunjung Chung[1], Sunyoung Hwang[1], Miyoung Sohn[1], Moonkyu Kim[1] and Jungchul Kim[1,2]

[1] Department of Immunology, Kyungpook National University School of Medicine, Teagu 700-422, Korea
[2]Corresponding author: Tel, +82-53-420-6977; Fax, +82-53-423-4628; E-mail, moonkim@knu.ac.kr

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Abbreviation: *BNIP-3*, Bcl-2/19kda interacting protein-3; *BNIP3h*, *BNIP3* homolog; BH-3, Bcl-2 homology domain-3; TM, transmembrane domain

Abstract

Apoptosis is regulated by interaction of antiapoptotic Bcl-2 family proteins with various proapoptotic proteins, several of which are also members of the Bcl-2 family. *BNIP3* (formerly NIP3) is a proapoptotic mitochondrial protein classified in the Bcl-2 family based on limited sequence homology-3 (BH3) domain and COOH-terminal transmembrane domain. Sequence comparison of *BNIP3* has indicated that there are several *BNIP3* human homologs of this protein, like *BNIP3L*, *Nix* and *BNIP3*. We have cloned a new member of BNIP3 family from the cDNA library prepared from human dermal papilla cells and designated as *BNIP3h*. *BNIP3h* shows substantial homology with other BNIP3 family proteins. BNIP3h induced apoptosis from 24 h after transfection in MCF7 cell lines and its apoptosis inducing activity is extended until 72 h after transfection.

Keywords: Apoptosis, BNIP3 family proteins, BNIP3h

Introduction

Apoptosis is an essential physiological process of selective elimination of cells in multicellular organism. This process is invoked during normal organ development and tissue homeostasis and also during certain pathological conditions that result in degenerative diseases. Several regulatory components of the apoptotic pathway have been identified in various living organism including man.

The process of apoptosis is also initiated as defensive mechanism in cells infected by pathogenic agents, such as viruses. Several cellular and viral proteins related to Bcl-2 proto-oncoproteins are efficient inhibitors of apoptosis. The antiapoptotic Bcl-2 family proteins have been shown to complex with a number of cellular proteins (Reed *et al.,* 1997). Some of these proteins themselves are also members of the Bcl-2 family. These Bcl-2 family proteins generally promote apoptosis when ectopically overexpressed. The proapoptotic Bcl-2 family proteins share one or more conserved domains with Bcl-2 and related antiapoptosis proteins. All of the proapoptotic proteins share a common death effector domain designated BH-3. The BH-3 domain of proapoptotic Bcl-2 family proteins is indispensible for the execution of cell death and for heterodimerization with antiapoptosis proteins.

Yeast two hybrid screen of proteins that interact with E1B 19K identified several unique cDNAs named NIP1, NIP2 and NIP3 (Boyd *et al.,* 1994). All three proteins interact with discrete domains of E1B 19K protein and Bcl-2 that are involved in suppression of cell death. BNIP3 (Yasuda *et al.,* 1998a) is a mitochondrial protein that induces apoptosis, when transiently expressed. Several homologs of the BNIP3 protein have also been reported; BNIP3L (Matsushima *et al*., 1998), BNIP3α (Yasuda *et al.,* 1999), Nix (Chen *et al.,* 1999) and a BNIP3 homolog in *C. elegans* (Yasuda *et al.,* 1998b). All of these proteins retain the same intrinsic proapoptotic activity like BNIP3.

It has been shown that BNIP3 also contains a BH-3 domain (Yasuda *et al.,* 1998a). Although most BH-3 containing proapoptosis proteins induce rapid cell death when overexpressed, BNIP3 exhibited delayed level of proapoptotic activity (Yasuda *et al.,* 1999).

We have cloned a new member of BNIP3 from the cDNA library prepared from human dermal papilla cells and designated as BNIP3h that shows substantial homology and similar characteristics with other BNIP3 family proteins.

Material and Methods

cDNA library construction and BNIP3h cloning

Human dermal Papilla (DP) cells were cultured in Dulbecco's Eagle medium (DMEM) (Gibco BRL, Gaithersburg, MD, USA.) supplemented with penicillin (100 U/ml), streptomycin (100 μg/ml) and 10% fetal bovine

serum (Hyclone Laboratories Inc, UT, USA). cDNA library was constructed by using Zap cDNA synthesis kit (Stratagene, La Jolla, CA, USA) with 4 μg of poly A^+ RNA obtained from primary cultured human dermal papilla cells. The phage library was converted into a pBluescript phagemid cDNA library by *in vivo* excision by the ExAssist/SOLR system (Stratagene). In order to make a hair specific EST database, random clones were selected and 5' single-path sequencing reactions were performed with Sequenase™ kit (USB corporation, Cleveland, OH, USA) according to the manufacturer's protocol. The GenBank database was searched for homologous sequence using BLAST (Altschul *et al*., 1990). One of the clone (clone B764) has homology with BNIP3 family protein and we designated it BNIP3h.

Northern blot analysis with multiple human tissues

Human multiple tissue and human cancer cell line poly A^+ RNA blots were purchased from Clontech (East medow circle, CA, USA). To differentiate with other BNIP3 family genes, 3' untranslated region of BNIP3h gene probe was made by digesting the clone B764 with *Hind*-III and *Xho* I. And it was used for hybridization in human multiple tissue northern blot analysis. Human multiple tissue and cancer cell line blots were prehybrized at 68°C for 30 min in prehybridization solution (Express hybridization solution, Clontech). After one hour of hybridization with radiolabelled BNIP3h probe, membranes were washed twice with 2XSSC/0.05% SDS for 10 min each at room temperature and followed by two washes of 0.1XSSC/0.1% SDS at 50°C for 20 min each, and then membranes were subjected to exposure to film at −70°C for overnight.

Construction of expression plasmids

Plasmids designed to express BNIP3h were constructed by cloning the coding region into pcDNA3.1/*myc*-His expression vector (Invitrogen). In order to make cloning between the *Eco*R I and *Xho* I of the expression vector, an *Eco*R I site at 5'-prime and an *Xho* I site at 3'-prime was introduced by PCR, The orientation and sequence was confirmed by sequencing the entire insert.

Transient cell death assay

Apoptosis assay was carried out in MCF7 cell line. (ATCC, Maryland, USA). MCF7 cells were cultured in Dulbecco's Eagle medium (DMEM) (Gibco BRL) supplemented with penicillin (100 U/ml), streptomycin (100 μg/ml) and 10% fetal bovine serum. MCF7 cells were transiently transfected using Lipofectamine Plus reagent (Gibco BRL) with pcDNA3.1/*myc* His- BNIP3h expression plasmid. β-Galactosidase expressing plasmids was cotransfected to check the transfection efficiency. Twenty four and forty eight hours after transfection, the cells were fixed, stained with X-Gal and microscopically examined. 100 to 200 blue color cells were microscopically scored as live (flat) and apoptotic (round) and were photographed (Yasuda *et al*., 1998a)

Results

Isolation of *BNIP3h* in human dermal papilla cells

The B764 clone that contains *BNIP3h* sequence is composed of 2,144 nucleotides with an open reading frame of 660 bp (220 amino acids) extending from an ATG codon at position 75 to a TGA stop codon at position 735. It includes 75 nucleotides in the 5'-untranslated region, and 1411 nucleotides in 3'-untranslated region (Figure 1). The sequence surrounding the potential start codon is in agreement with Kozak's rule (Kozak, 1991). Amino acid sequence from residues 134 to 145 showed a motif similar to the BH-3 domain of *BNIP3*

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GGC ACG AGA GAG AGA GAG AGA GAG AGA GAG AGA GAG AGA GAG AGA GAG      48
AGC CGC AGT CCT GCC AGC TGT CCG ACA ATG TCG TCC CAC CTA GTC GAG      96
                                M   S   S   H   L   V   E           7
CCG CCG CCG CCC CTG CAC AAC AAC AAC AAC AAC TGC GAG GAA AAT GAG     144
 P   P   P   P   L   H   N   N   N   N   N   C   E   E   N   E      23
CAG TCT CTG CCC CCG CCG GCC GGC CTC AAC AGT TCC TGG GTG GAG CTA     192
 Q   S   L   P   P   P   A   G   L   N   S   S   W   V   E   L      39
CCC ATG AAC AGC AGC AAT GGC AAT GAT AAT GGC AAT GGG AAA AAT GGG     240
 P   M   N   S   S   N   G   N   D   N   G   N   G   K   N   G      55
GGG CTG GAA CAC GTA CCA TCC TCA TCC TCC ATC CAC AAT GGA GAC ATG     288
 G   L   E   H   V   P   S   S   S   S   I   H   N   G   D   M      71
GAG AAG ATT CTT TTG GAT GCA CAA CAT GAA TCA GGA CAG AGT AGT TCC     336
 E   K   I   L   L   D   A   Q   H   E   S   G   Q   S   S   S      87
AGA GGC AGT TCT CAC TGT GAC AGC CCT TCG CCA CAA GAA GAT GGG CAG     384
 R   G   S   S   H   C   D   S   P   S   P   Q   E   D   G   Q     103
ATC ATG TTT GAT GTG GAA ATG CAC ACC AGC AGG GAC CAT AGC TCT CAG     432
 I   M   F   D   V   E   M   H   T   S   R   D   H   S   S   Q     119
TCA GAA GAA GAA GTT GTA GAA GGA GAG AAG GAA GTC GAG GCT TTG AAG     480
 S   E   E   E   V   V   E   G   E   K   E   V   E   A   L   K     135
AAA AGT GCG GAC TGG GTA TCA GAC TGG TCC AGT AGA CCC GAA AAC ATT     528
 K   S   A   D   W   V   S   D   W   S   S   R   P   E   N   I     151
 CCA CCC AAG GAG TTC CAC TTC AGA CAC CCT AAA CGT TCT GTG TCT TTA     576
 P   P   K   E   F   H   F   R   H   P   K   R   S   V   S   L     167
AGC ATG AGG AAA AGT GGA GCC ATG AAG AAA GGG GGT ATT TTC TCC GCA     624
 S   M   R   K   S   G   A   M   K   K   G   G   I   F   S   A     183
GAA TTT CTG AAG GTG TTC ATT CCA TCT CTC TTC CTT TCT CAT GTT TTG     672
 E   F   L   K   V   F   I   P   S   L   F   L   S   H   V   L     199
GCT TTG GGG CTA GGC ATC TAT ATT GGA AAG CGA CTG AGC ACA CCC TCT     720
 A   L   G   L   G   I   Y   I   G   K   R   L   S   T   P   S     215
GCC AGC ACC TAC TGA GGG AAA GGA AAA GCC CCT GGA AAT GCG TGT GAC     768
 A   S   T   Y   *                                               220
CTG TGA AGT GGT GTA TTG TCA CAG TAG CTT ATT TGA ACT TGA GAC CAT     816
TGT AAG CAT GAC CCA ACC TAC CAC CCT GTT TTT ACA TAT CCA ATT CCA     864
GTA ACT CTC AAA TTC AAT ATT TTA TTC AAA CTC TGT TGA GGC ATT TTA     912
CTA ACC TTA TAC CCT TTT TGG CCT GAA GAC ATT TTA GAA TTT CCT AAC     960
AGA GTT TAC TGT TGT TTA GAA TTT GCA AGG GCT TCT TTT CCG CAA ATG    1008
CCA CCA GCA GAT TAT AAT TTT GTC AGC AAT GCT ATT ATC TCT TAA TTA    1056
GTG CCA CCA GAC TAG ACC TGT ATC ATT CAT GGT ATA AAT TTT ACT CTT    1104
GCA ACA TAA CTA CCA TCT CTC TCT TAA AAC GAG ATC AGG TTA GCA AAT    1152
GAT GTA AAA GAA GCT TTA TTG TCT AGT TGT TTT TTT TCC CCC AAG ACA    1200
AAA GGC AAG TTT CCC TAA GTT GAG TTT GAT AGT TTT TTA TTA AAA AAG    1248
AAA ACA AAA AAC AAA AAA AAA AGC CAA GGC ACA AAA AAA AAT ATC CTG    1296
GGC AAT AAA AAA AAA TAT TTT AAA CCT ACG TTT TCG ACG CAT TTT GTC    1344
TAG CTC TAT AGG CTT TTA ATT TTA AGA GCA CGT TAT AAA AGT ACT AGG    1392
CTA GTC AAA ATA AGA ATA AAG AAA GTA AAA TAA CAA TCA GCA GAT TTC    1440
ATA CTA GTA TGT TGT AAT GCT GTC TTT TCT AGG GTG TAG AAA TCT TTC    1488
TTT CTG ATA AGG AAC GTC TCA GGC TTG ACG AAA TAT ATG GAA ATT GCT    1536
TTT TTT GAG ATT TTT GCG TGT GTG TTT GAT ATT TTT TAA CCG AAT AAT    1584
TAG CTG CAT GTG AAT TTT TCA TGA CCT TCT TTT ACA TTT TTT ATT TTT    1632
TAT TTT TCT TTT ATT TTT TTT TTT TTT TTT TCT CTA AGA AAA CTT TGG    1680
AAT TAG GTT TCC AAT TTG TGA TGG GAA TTA CAG GCT TCT TGT TTT TAG    1728
GGG AAG CCA TCA CCT ATA ACT CTG AAA GCC TTT AAA ACT CTG AAG AGA    1776
ATT GTT TCA GAA AGT TAC CAA GCA CCT TGT GCA ACT TGG AAA AAC CAG    1824
AAC TTG GGT TGT GGG AAC AGT TGA ACA GCG GTT CTG AAA AGA AAT GCC    1872
AAT TTG TTT CCC TTC TGG ATC TCT CAA CTG AAT TAA TGT TTA CTG GTA    1920
ACA GTC TTC CCA AGG TGA TTC TGC AAC TGC CCA GGC ACT TTG GTC AAT    1968
TTT CTC AST GGT AGC CCT GTC TTT TCA GTT AAT GGG TAA AAC TCT TAA    2016
AGG TTC AAA GAA CAC CTC CAA CAA GAA TTC CTT CAG TGA AAT AAT ACT    2064
TGT TCG TTC AAT TTC TAA AAT GTG AAG GCT TTA AGG ACC AAA TTG TTA    2112
GAA AGC ATC AAG GSA TGG ACG AGT TAT CTC GA                        2144
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Figure 1. The nucleotide and deduced amino acids sequence of BNIP3h. Amino acids are numbered from the initiating methionine. Stop codon is indicated by an asterisk. The possible BH-3 domain is bold-underlined and transmembrane domains are underlined.

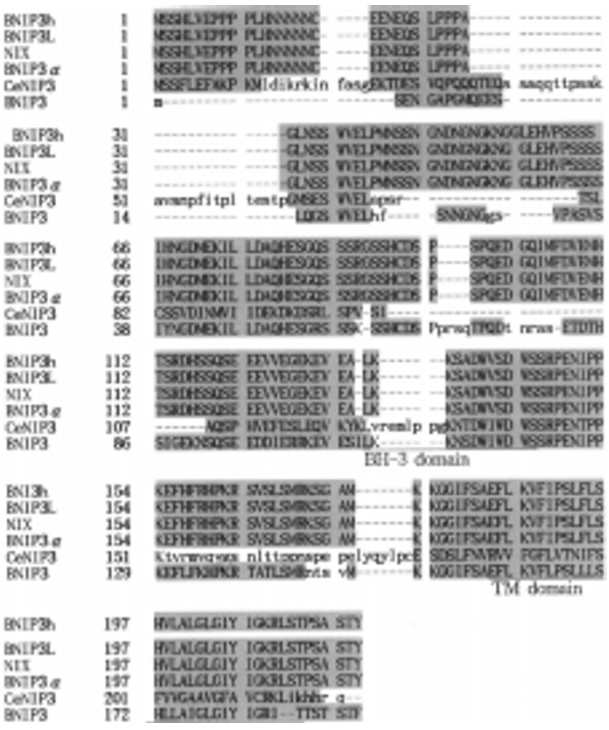

Figure 2. Identification of BNIP3h and homology to BNIP3 family proteins. Upper case letters show the identical and related amino acids between five proteins and lowercase letters show unidentical amino acids. The similar amino acids are shaded. The BH-3 and TM domains are underlined. The amino acids were aligned using the DIALIGN2.1 Program (B. Morgenstern, *et al.* 1996).

family proteins (Figure 2). It also possesses a potent carboxy terminal transmembrane domain (from residues 184 to 214) characteristic of many Bcl-2 and BNIP3 family proteins.

Interestingly, although the DNA sequence of 5'- and 3'-untranslated regions of *BNIP3h* were different from other BNIP3 families, the comparison of amino acid sequence showed that BNIP3h has the same amino acid sequence with *BNIP3L*, *NIX*, and *BNIP3* (Figure 2).

Tissue distribution of BNIP3h

The tissue distribution of BNIP3h mRNA in different human tissues was determined by Northern blot analysis. Two transcripts of 1.6 and 3.9 kb bands were detected when hybridized to multiple tissue human blot with 2.1 kb B764 clone as probe. These two transcripts were ubiquitously expressed in all tissues examined. BNIP3h mRNA was highly expressed in brain, heart, thymus and most abundantly in testis. A lower level of expression was detected in the liver, skeletal muscle and pancreas (Figure 3).

The expression pattern of BNIP3h was also determined in different human cancer cell lines. Human cancer cell line poly A^+ RNA blots (Clontech) was hybridized with a P^{32} labelled BNIP3h probe. As shown in Figure 3C, BNIP3h expressed in same two transcripts of 1.6 and 3.9 kb in all cancer cell lines examined but a lower level of expression in promyelocytic leukemia HL60, and Hela cell S3. However, the expression level was higher in chronic myelogenous leukemia K-562 and lympho-

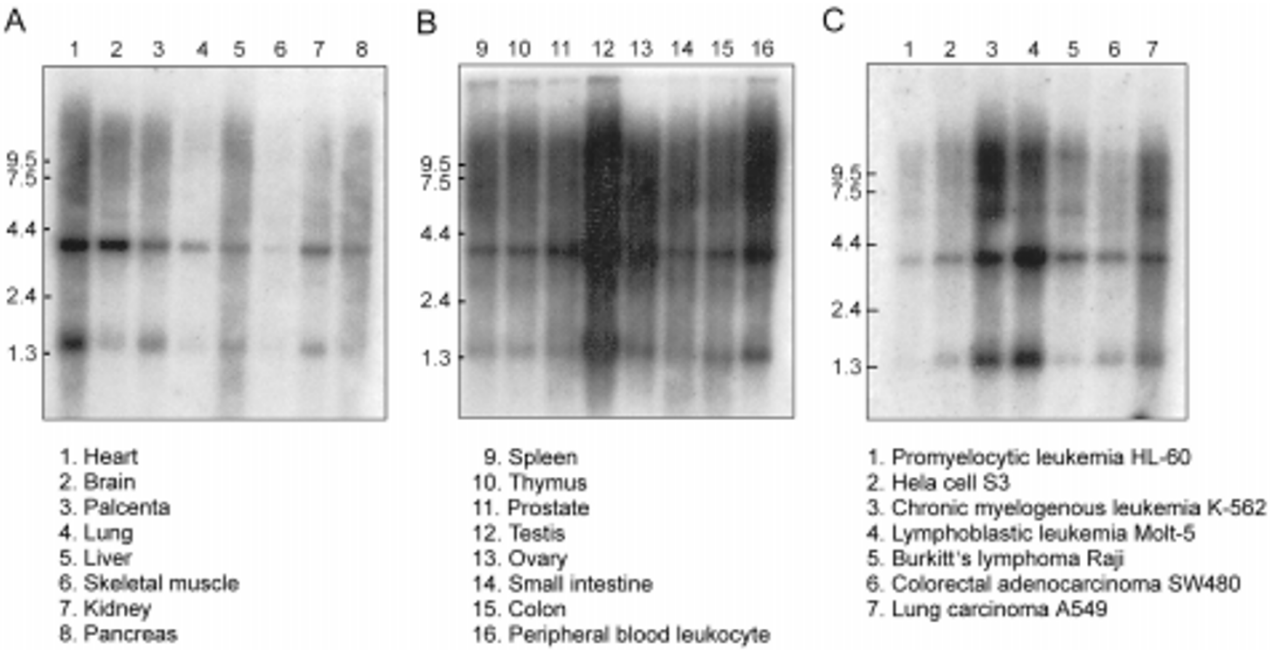

Figure 3. Expression of BNIP3h in human tissues and cancer cell lines. Multiple tissue northern blots (Clontech) with 2 μg of mRNA from several selected adult human tissues (A and B) and cancer cell lines (C) were hybridized with radiolabelled BNIP3h cDNA. The position of size markers is indicated in kilobases.

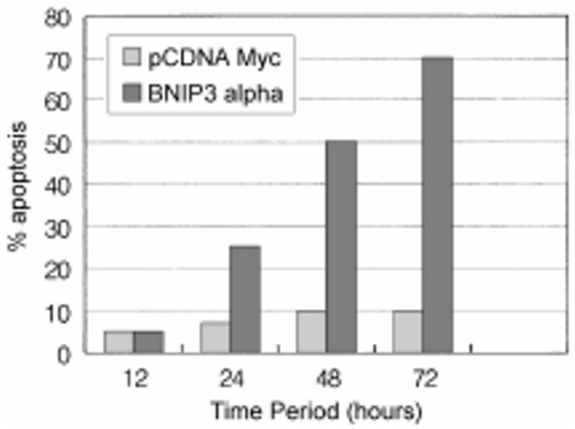

Figure 4. Proapoptotic activity of BNIP3h. MCF7 cells were transiently transfected either with pcDNA/*myc*-His (empty), or pcDNA/*myc*-His BNIP3h. The cells were fixed after 24 h, 48 h or 72 h and stained with β-Gal, and live and apoptotic cells were counted as described in Materials and Methods.

blastic leukemia MOLT-4. The identification of two transcripts suggests that the BNIP3h mRNA may be expressed as two alternatively spliced form or hybridized to other BNIP3 family genes.

Pro-apoptotic activity

About 20 to 25% of BNIP3h transfected MCF7 cells were observed as apoptotic after 24 h. And the percentage of apoptotic cell is increased to 70% after 72 h (Figure 4).

Discussion

BNIP3h is a member of proapoptotic Bcl-2 family protein having homology with other BNIP3 family proteins. BNIP3h contains a BH-3 domain which is more closely related to BNIP3, BNIP3, BNIP3L and Nix proteins than other BH-3 containing Bcl-2 family proteins such as Bik (Boyd *et al.,* 1995), BID (Wang *et al.,* 1996), Hrk (Inhora *et al.,* 1997) and BAD (Yang *et al.,* 1995). Most BH-3 containing proapoptotic proteins induce rapid cell death when overexpressed, however, as shown by Yasuda *et al*. (1998a) and Figure 4 in this study, BNIP3 and BNIP3h exhibited delayed proapoptotic activity. The substantial level of apoptosis was observed 48 h after transfection in MFC7 cells (Figure 4). It seems that these proteins do not possess a potent proapoptotic activity like other BH-3 containing proapoptotic proteins.

The sequence comparison of *BNIP3* and *BNIP3h* indicates that there are several homologs of these genes, like *BNIP3L* (Matsumhima M. *et al.,* 1998), *BNIP3* (Yasuda M. *et al.,* 1999), and *Nix* (Chen G. *et al.,* 1999). The proteins encoded by the *BNIP3L*, *Nix* and *BNIP3* genes had same amino acid sequences with *BNIP3h* (Figure 2). In a recent report (Chen G. *et al.,* 1999) *Nix* and *BNIP3L* cDNAs encode the same amino acids sequences but they have different chromosomal locations. *BNIP3L* have been mapped to chromosome 8p21 and *Nix* to chromosome 14 (14q11.2-q12). The conceptual amino acids sequence encoded by the *BNIP3h* cDNA is also identical to these two proteins but it has different nucleotides sequences at both 5'- and 3'-untranslated region. This supports the idea to have a unique name for the cDNA, which we have identified from human dermal papilla cells, as it is not the long form of any of BNIP3 family proteins. The chromosomal mapping of *BNIP3h* needs to be investigated.

Amino acids sequence analysis of *BNIP3h* has shown that residues from 184 to 213 (Figure 2) at carboxyl terminal are similar to the transmembrane domain of *BNIP3*. Previous indirect immunofluorescence analysis revealed that *BNIP3* and its homologs were primarily localized in mitochondria (Yasuda *et al.,* 1999, Matsushima M. *et al.,* 1998, Chen G. *et al.,* 1997) and carboxyl terminal transmembrane domain was thought to be responsible for the subcellular localization of these proteins. As *BNIP3h* possesses a transmembrane domain similar to other *BNIP3* family proteins, so it may also be localized to mitochondria. It was also shown (Imazu. *et al.,* 1999) that *BNIP3L* directly targets the mitochondria to induce apoptosis-associated mitochondrial changes including membrane potential loss and cytochrome C release.

Another attractive mechanism to regulate dimerization of *Bcl-2* family members is phosphorylation (Gajewski and Thompson, 1996). For example, Bad, a proapoptotic member of the Bcl-2 family, is phosphorylated by a putative kinase that can be activated by growth factor engagement (Zha *et al.,* 1996). The phosphorylated Bad loses the ability to bind Bcl-xL. Instead, it binds to 14-3-3, a protein that can interact with several signaling enzymes. The Bcl-xL dissociated from Bad, now can execute its antiapoptotic function. The possible mechanism for the induction of apoptosis by BNIP3h may be the phosphorylation by a putative kinase. As BNIP3h is rich in Ser/Thr residues, raising the possibility that activities of these proteins may be regulated by phosphorylation in response to apoptotic signals.

BNIP3h expressed ubiquitously in all normal and cancerous human tissues examined as two transcripts of 1.6 and 3.9 kb (Figure 3). These results suggest that in certain human tissues *BNIP3h* is differentially expressed, and thus may contribute to apoptosis with some degree of specificity.

In this study, *BNIP3h*, a homolog of BNIP3 family protein, has been identified and functionally characterized. *BNIP3h* encodes a proapoptotic protein and induces apoptosis in transfected cells. A possible direct binding activity of BNIP3h with E1B-19k and Bcl-2 and suppression of their antiapoptotic activity needs to be investigated. Among the various proapoptotic genes that have

been identified so far, the *BNIP3*, *BNIP3h*, *BNIP3, BNIP3L,* and *Nix* genes seem to be the first examples of human proapoptotic proteins that are homologous, underscoring the fact that these proteins may play a concerted role in human apoptosis pathway.

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