New Tumor Metastasis Suppressor Gene from Korean Tiger Shark (*Scyliorhinus torazame*)

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New tumor suppressor gene, *snm23*, homologous to human *nm23*/NDP kinase (human nucleoside diphosphate kinase) gene whose product has a tumor metastasis inhibitory activity, was first cloned from Korean tiger shark (*Scyliorhinus torazame*) skin cDNA library constructed by using a λ ZAP-II cDNA synthesis kit. About 1×10^5 plaques were screened and several positive plaques were isolated and confirmed by second screening. The phagemid containing a positive clone from the Uni-Zap XR vector was excised *in vivo* and the gene containing the tumor metastasis suppressor protein was named as *snm23*. Cloned gene, *snm23*, was sequenced with ABI-PRISM 310 Genetic Analyzer. The nucleotide and deduced amino acid sequences of *snm23* have shown an open reading frame consisting of 450 base pairs that correspond to a protein of 150 amino acid residues, with a calculated molecular mass of 16.8 kDa. Sequence comparison of *snm23* with human *nm23* /NDP kinase was performed by using Blast protein data base of National Center for Biotechnology Information. In order to determine tissue specificity, reverse transcription-polymerase chain reaction (RT-PCR) was used. Good expression level of *snm23*/NDP kinase was detected at the tissues from skin, cartilage, and liver of Korean tiger shark.

Key words: tumor metastasis suppressor, nm23/NDP Kinase, *Scyliorhinus torazame*, molecular cloning

Introduction

Molecular biology and genetic manipulation techniques have the potential to play a major role in improving the efficiency of fish production and availability. Recombinant DNA technology allows the isolation of genes encoding proteins with biological functions useful to human health, particularly in the areas of disease therapeutics such as cancers (Neel and Kumar, 1993).

A cascade of cellular, biochemical, and genetic events is known to occur in the development and progression of tumors leading to malignancy and ultimately to metastasis. Tumor metastasis, the movement and colonization of cells from a tumor to other organs remain a leading cause of death for cancer patients. A noble gene, nm23, a candidate angiogenesis suppressor gene or metastasis suppressor gene, encodes a human 17 kDa nuclear and cytoplasmic protein containing 152 amino acids (Leone *et al.*, 1991; Marshall, 1991; Liotta *et al.*, 1991). *Nm23* genes have been implicated in the control of tumor metastasis (Steeg *et al.*, 1988; Chen *et al.*, 1994; De la Rossa *et al.*, 1995; Jong and Ma, 1991). *Nm23* genes encode nucleoside diphosphate (NDP) kinases, enzymes that synthesize nucleoside triphosphates by transferring a phosphate from ATP to the corresponding nucleoside diphosphates (Marx, 1990; Biggs *et al.*, 1990; Sommer and Song, 1994; Ouatas *et al.*, 1997).

For many types of tumors, there is an inverse relationship between the level of *nm23* expression and metastatic potential (De la Rossa *et al.*, 1995; Wang *et al.*, 1993). Overexpression of nm23 in highly metastatic tumor cells reduces their metastatic potentials, indicating that nm23 genes can suppress tumor metastasis (Marx *et al.*, 1993; Leone *et al.*, 1991; Stahl *et al.*, 1991; Florenes *et al.*, 1992; Kantor *et al.*, 1993; Hähnel *et al.*, 1994). Although it was originally proposed that NDP kinase participates in the maintenance of physiological levels of nucleoside triphosphates other than ATP, recent evidence suggests that it may also participate in the regulation of growth, development, and signal transduction processes. It has also been proposed that NDP kinase may interact with signal transducing GTP-binding proteins (Randazzo *et al.*, 1991).

In the current study, we report the complete nucleo-

tide and deduced amino acid sequence and the characterization of new tumor metastasis suppressor gene, *snm23*, homologous to human *nm23*/NDP kinase gene product as determined by molecular cloning from Korean tiger shark (*Scyliorhinus torazame*).

Materials and Methods

Materials

Alive Korean tiger shark (*Scyliorhinus torazame*) was obtained from Chagalchi fish market (Pusan, Korea). Total RNA isolation kit was purchased from Ultraspec™, BIOTECX. Restriction enzymes were purchased from New England Biolabs Inc., Promega, and United States Biochemicals Corp. λ ZAP-II cDNA synthesis kit was purchased from Stratagene. T4 DNA ligase and *Taq* DNA polymerase were purchased from United States Biochemicals Corp.

Total RNA isolation from shark skin tissue

Total RNA from shark skin was isolated using Total RNA isolation kit (Ultraspec™, BIOTECX). 100 mg of fresh shark skin tissue was homogenized in 1 mℓ of denaturation reagent using power driven homogenizer (Polytron™) for 30 sec at 25,000 rpm. Homogenized sample was kept on ice for 5 min and 200μℓ of chloroform was added, shaken vigorously for 15 sec, and kept on ice 5 min. Then, sample was centrifuged at 13,000 rpm for 15 min and aqueous phase was carefully transferred to new tube. Half volume of isopropanol was added and mixed with inverting. 0.05 volume of RNA Tack™ resin was added and vortexed for 30 sec. Sample was centrifuged for 1min and the pellet was washed with 75% ethanol and dried and resuspended in DEPC treated water.

Shark genomic DNA isolation

Approximately, 50~100 mg each (wet weight) of shark skin tissues were cut and 5 mℓ of lysis buffer (100 mM Tris-HCl, pH 8.5, 0.2% SDS, 5 mM EDTA, and 200 mM NaCl) was added and homogenated with Polytron homogenator at 25,000 rpm for 30 sec. Proteinase K was added to 100 μg/mℓ concentration and incubated at 55℃ for 6 hours. 800 μℓ of phenol (pH8.0) was added, vigorously mixed, and centrifuge at 13,000 rpm for 10 min. The upper phase was carefully transferred to a new microcentrifuge tube and extracted one more time with phenol. 800 μℓ of isoprophanol was added to the solution, mixed carefully, and centrifuged 5 min at 5,000 rpm. The pellet was washed with 800 μℓ of 70% ethanol twice and resuspended in 100μℓ TE buffer [0.1 M Tris-HCl (pH 8.0), 1mM EDTA (pH 8.0)] containing 1 M NaCl.

Primer synthesis and DIG-labeled probe preparation

Conserved sequence of *nm23* of human and several mammals was determined using NCBI (National Center for Biotechnology Information) nucleotide and protein sequence database and oligonucleotide [5'-GA (C/T) TC (C/T) AAGCCTGG (G/T/C) ACCAT (C/A) CG (T/G/C) G-3'] for screening probe was synthesized from Bioneer Inc. Probe was labeled with DIG (digoxigenin) oligonucleotide 3'-end labeling kit (Boehringer Mannheim). Total volume (20 μℓ) was mixed with 10 X labeling buffer, 6.25 mM $CoCl_2$ solution, 100 pmol oligonucleotide, 50 μM of DIG-ddUTP solution, and 50 units terminal transferase, incubated at 37℃ for 15 min, and then transferred to ice. Reaction mixture was mixed with 1 μℓ glycogen and 200 μℓ of 0.2 mM EDTA solution (pH 8.0). The labeled oligonucleotide was precipitated with ethanol and dissolved in 50 μℓ water.

Screening of nm23/NDP kinase from shark cDNA library

Titer of cDNA library was determined as described in Stratagenes cDNA construction protocol (Kim *et al.*, 1997). 100 μℓ of XL-1 Blue stock cell was inoculated to 2 mℓ of fresh LB/tetracyclin medium and incubated to 0.5 at OD_{600}. 1 μℓ of 4×10^6 pfu/mℓ phage stock was added and incubated another 15 min with gentle agitation. Culture was mixed with 5 mℓ of top agar (preheated to 50℃) and poured to LB plate. The plate was incubated at 42℃ for 3 hours, transferred at 37℃, incubated until the plaques reached a diameter not exceeding 1.5 mm. Then, the plate was placed at 4℃ for 1 hr. Using sterile forceps, a nitrocellulose filter was placed onto the LB soft top agar for 2 min. Then, filter was carefully peeled off, floated on top of DNA denaturing solution for 30 sec, and immersed for 5 min. The filter was removed and immersed in neutralizing solution for 5 min. The filter was briefly rinsed in 2×SSC, and placed on a Whatman 3 MM paper to dry. UV crosslinker was used to fix DNA to the filter.

The filter was incubated with hybridization buffer (5× SSC, 0.1% N-laurolsarcosine, 0.02% SDS, 1% blocking reagent) for 30 min and the denatured DIG-labeled probe was added and incubated another 16 hours at 55℃ with gentle agitation. The hybridization solution was transferred to new tube and the filter washed with 2× SSC, 0.1% SDS for 5 min at room temperature twice. The filter was washed twice in 0.1×SSC, 0.1% SDS for 15 min at 68℃ under agitation. The membrane was briefly rinsed in maleic acid buffer and incubated in 1× blocking solution for 30 min. Then, the membrane was incubated for 30 min in diluted anti-DIG antibody conjugate to 75 mU/mℓ and washed twice for 15 min in maleic acid buffer. The membrane was equilibrated with detection buffer for 2 min and 45 μℓ of NBT and 35 μℓ of BCIP were added to 10 mℓ of detection buffer and incubated for color development.

Reverse Transcription-Polymerase Chain Reaction (RT-PCR)

For DNA amplification, polymerase chain reaction (PCR) was performed in 25 μℓ volumes containing 2.5 μℓ 5 x *Taq* DNA polymerase buffer (USB), 100 μM each of dATP, dGTP, dCTP, dTTP, 5 pmol primer, 200 ng of template DNA and 2.5 mM $MgCl_2$ and 1.0 unit Tag DNA polymerase as described in (Innis and Gelfand, 1990). Amplification was performed in a Ericom DNA Delta Cycler I programmed for 45 cycles of 1 min denaturation at 95℃, 1 min annealing at 48℃ and 2 min extension at 72℃ (Kim and Richardson, 1993; 1994; Chun *et al.*, 1997).

In order to perform reverse transcription polymerase chain reaction, total RNA was isolated from skin, cartilage, and liver from Korean shark. Titan™ one tube RT-PCR system (Boehringer Mannheim) was used. Master mix 1 contained 0.2 mM dNTPs, 5 mM DTT, 100 pmol upstream (snm23-1; 5'-GACCTCAAGGACAAGC-3') and downstream (snm23-2; 5'-TCCATCCCTCACTCG-3') primers, template RNA, and 5 u of RNase inhibitor. Master mix 2 consisted of 5×RT-PCR buffer and enzyme mix. Mix 1 and mix 2 were added to a 0.2 mℓ thin-walled PCR tube on ice. Then, the sample was placed in a thermocycler (GeneAmp PCR system 2400, Perkin Elmer) and incubated for 1 hr at 50℃ for reverse transcription following with thermocycling. A temperature profile was one prereaction at 94℃ for 2 minute and 40 cycling reaction at 94℃ for 30 seconds, 50℃ for 1 min, and 72℃ for 2 min. Twenty microliters of the reaction products were separated by electrophoresis through 1.5% agarose gels and stained with Etidium bromide.

In vivo excision of positive clone

The positive plaque from the agar plate was cored and transferred to a microcentrifuge tube containing 500 μℓ of SM buffer and 20 μℓ of chloroform. The tube vortexed to release the phage particles into the SM buffer. XL1-Blue cell was grown overnight culture in LB broth, supplemented with 0.2% maltose and 10 mM $MgSO_4$ at 37℃. 20 μℓ of XL1-Blue cells at an OD_{600} of 1.0 were combined with 250 μℓ of phage stock (containing $>1\times10^5$ phage particles) and 1 μℓ of the K408 helper phage ($>1\times10^6$ pfu/μℓ). The tube was incubated at 37℃ for 15 min and 3 mℓ of LB broth was added and incubated for 2.5~3 hours at 37℃ with shaking. The tube was heated at 65℃ for 20 min and then spun at 1000×g for 15 min. The supernatant was decanted into a new tube and mixed with 200 μℓ of freshly grown XL1-Blue cells (OD_{600}=1.0) and 100 μℓ of the phage supernatant. The tube was incubated at 37℃ for 15 min. 200 μℓ of the mixture was plated to LB-ampicillin agar plate and incubated overnight at 37℃.

DNA Sequencing

Sequencing reaction was performed using ABI PRISM™ DNA sequencing kit (Perkin Elmer) and sequence was analyzed using ABI 310 Genetic Analyzer. Referring to the ABI PRISM™ DNA sequencing kit and user's manual (Perkin Elmer) for detailed procedures.

Results and Discussion

This is the first report on the identification and molecular cloning of the tumor metastasis suppressor protein gene from fish, tiger shark, as a homologue of human *nm23*/NDP kinase gene product. *Nm23* genes are implicated in tumor metastasis control through as yet unknown mechanisms (Marx, 1990; Marshall, 1991; Chang *et al.*, 1994). Nm23 genes are also involved in the cell proliferation (Hsu *et al.*, 1995; Hildebrandt *et al.*,

1995), differentiation (Okabe-Kado *et al.*, 1995; 1997), motility (Kantor *et al.*, 1993; Wagner *et al.*, 1997), and development (Caligo *et al.*, 1995; Leung and Hightower, 1997), as indicated by high degree of homology between *nm23* genes. Proposed cellular roles of such enzymes include involvement in control of microtubule polymerization and modulation of G-protein activity (Randazzo *et al.*, 1991; Wagner *et al.*, 1997).

In order to identify the tumor metastasis suppressor protein genes from Korean tiger shark, degenerate oligonucleotide primers encoding the part of the conserved domains were prepared and used as probe in the screening for a Korean tiger shark skin cDNA library constructed by using a λ ZAP-II cDNA synthesis kit as described under "Materials and Methods." Conserved sequence of *nm23* of human and several mammals was determined using NCBI (National Center for Biotechnology Information) nucleotide and protein sequence database. To this end, degenerate oligonucleotide probe was labeled with digoxigenin (DIG) and used as screening probe. Approximately, 1×10^5 plaques were screened as described under Materials and Methods section. Several positive phaques were isolated and confirmed by the second screening. The phagemid containing a positive clone was excised *in vivo* from the Uni-Zap XR phage vector, and the gene containing the tumor metastasis suppressor protein was named as *snm23*.

The nucleotide sequence of the complete cDNA encoding the shark tumor metastasis suppressor protein gene, *snm23*, is shown in Fig. 1. The sequence contains a 44-bp 5'-untranslated region followed by a coding region of 450-bp that corresponds to a protein of 150 amino acid residues and a 3'-untranslated region of 132-bp. Cloned cDNA encodes a protein of 150 amino acids that contains single polypeptide. The predicted molecular mass of the cloned shark *nm23*/NDP kinase of Korean tiger shark was 16.8 kDa that is very similar to the 17 kDa of human *nm23*/NDP kinase. As shown in Fig. 1, the shark cDNA clone contains an in-frame termination codon (TGA) at 424-426. A polyadenylation signal, AATAAA, can be found 16-bp upstream from the polyadenylation.

An alignment of the amino acid sequences of the shark and human *nm23*/NDP kinase is shown in Fig. 2. Sequence comparison of *snm23* with human *nm23*/NDP kinase was conducted by using Blast protein data base of National Center for Biotechnology Information (NCBI). By this analysis shark *nm23*/NDP kinase shows 66% sequence identity and 85% sequence similarity with human *nm23*/NDP kinase. The *nm23*/NDP kinase genes possess several of the major functional domains associated within NDP kinase activity. In general, the shark *nm23*/NDP kinase gene might be divided into three regions according to the sequence comparison on the functional domains; the amino-terminal, central, and carboxyl-terminal regions. The N-terminal regions are conserved among the *nm23*/NDP kinases. Especially, the putative ATP binding site is located at the N-terminal region. The proposed consensus sequence of ATP-binding sites; Gly-X-Gly-X-X-Gly (Gilles *et al.*,1991; Hanks *et al.*, 1988; Harris *et al.*,1994), is presented as Gly^{12}-Val-Gln-Arg- Gly-Leu-Ile-Gly^{19}. The conserved motif of the catalytic site to protein kinases (Ala-X-Lys) in which the lysine residue is essential for catalytic activity (Buechler and Taylor, 1988) is also conserved as Ala^{123}-Lys-Lys^{125} at the carboxy-terminal region.

The analysis of hydropathicity of the amino acid sequence calculated by the method of Kyte and Doolittle (1982) showed that shark *snm23*/NDP kinase is, on the average, hydrophilic but possesses a few hydrophobic regions, each of which is composed of 5-10 amino acid residues as shown in Fig. 3D. Predictions of secondary structure by the method of Chou and Fasman (1978) indicated that several of α-helices were spanned but single β-sheet potential was calculated (Fig. 3A and 3B). As shown in Fig. 3C, the shark *snm23*/NDP kinase consists of the acidic amino acid residues in the C-terminal region, indicating that the C-terminal region of the enzyme may be important for protein-protein interaction in the cellular regulation.

In order to determine the expression specificity in the shark tissues, reverse transcription-polymerase chain reaction (RT-PCR) was performed as described under "Materials and Methods" using the isolated total RNA from shark tissues as a template. As shown in Fig. 4, the DNA banding patterns resulted from RT-PCR provided evidence for the good expression level of *snm23*/NDP

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                                               1/1
AATTCGGCACGAGGGGCAGCCGAGGCCACACAACAAACAGCAAGATG GAA CGC ACG CTC ATT GCC GTC AAG
                                              M   E   R   T   L   I   A   V   K

 33/11                                         63/21
AGC GAT GGA GTT CAG AGA GGT CTT ATC GGA GAG ATC ATC AAG CGA TTT GAA CAT CGA GGT
 S   D   G   V   Q   R   G   L   I   G   E   I   I   K   R   F   E   H   R   G

 93/31                                         123/41
TTC AAA CTC ATT GGG TTG AAG ATG GTG AAG CCT ACC GAA GAT TTA GCC AAA CAT CAT TAT
 F   K   L   I   G   L   K   M   V   K   P   T   E   D   L   A   K   H   H   Y

 153/51                                        183/61
ATC GAC CTC AAG GAC AAG CCC TTT TAC GCT GGA CTT TGT AAA TTC ACC TCT GCT GGT CCC
 I   D   L   K   D   K   P   F   Y   A   G   L   C   K   F   T   S   A   G   P

 213/71                                        243/81
TTC GTA GCG ATG TGC TGG GAA GGT CAG AAT ATT GTG AAG ATG GGC AGA GAC ATG ATG GGT
 F   V   A   M   C   W   E   G   Q   N   I   V   K   M   G   R   D   M   M   G

 273/91                                        303/101
GAG ACA AAC CCA GCA GAT TCC AAG CCA GGA ACC ATA CGA GGA GAC CTT TGT GTT CAA GTA
 E   T   N   P   A   D   S   K   P   G   T   I   R   G   D   L   C   V   Q   V

 333/111                                       363/121
GGA AGG AAT ATT ATC CAT GGC AGT GAC TCG CTG GAA ACA GCG AAG AAG GAG GTC GCC CTG
 G   R   N   I   I   H   G   S   D   S   L   E   T   A   K   K   E   V   A   L

 393/131                                       423/141
TGG TTC AAG CCT GAG GAA CTG ATT GAG TGG AAG TCC TGT GCG GAG GTT TAT ACC TAC GAG
 W   F   K   P   E   E   L   I   E   W   K   S   C   A   E   V   Y   T   Y   E

TGAGGGATGGATGTGTGCCCAGGTGCCTGCGAGCGATAGGGTCTGTGTGGGCTTCGGAACCCCCTTTTTCTGTGTACA

AACCAGAATAAAACCTGCTGGAAATATCAAAAAAAAAAAAAAAAAAAAAAAAA
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Fig. 1. The nucleotide sequence of the *nm 23*/NDP kinase cDNA (*upper*) and deduced amino acid sequences (*lower*) of Korean tiger shark.
The nucleotide sequence is numbered on the left above the sequence and the deduced amino acid sequence on the right. Asterisk represents the stop codon. Solid underlined nucleotides in the 3′-untranslated region represent the possible polyadenylation signal. *Dotted underlined* nucleotides indicate that the primer sites of the probe preparation for reverse transcription-polymerase chain reaction (RT-PCR).

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Shark  nm23       1 MERTLIAVKSDGVQRGLIGEIIKRFEHRGFKLIGLKMVKPTEDLAKHHYIDLKDKPFYAG 60
human nm23-H2     4 LERTFIAIKPDGVQRGLVGEIIKRFEQKGFRLVAMKFLRASEEHLKQHYIDLKDRPFFPG 63
                     *** ** * ******* ********  ** *   *     *   * ******* **  *

Shark  nm23      61 LCKFTSAGPFVAMCWEGQNIVKMGRDMMGETNPADSKPGTIRGDLCVQVGRNIIHGSDSL 120
human nm23-H2    64 LVKYMNSGPVVAMVWEGLNVVKTGRVMLGETNPADSKPGTIRGDFCIQVGRNIIHGSDSV 121
                     * *     ** *** *** * ** ** * **************** * ************

Shark  nm23     121 ETAKKEVALWFKPEELIEWKSCAEVYTYE 149
human nm23-H2   122 KSAEKEISLWFKPEELVDYKSCAHDWVYE 152
                      * **  *********   ****    **
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Fig. 2. Alignment of predicted amino acid sequence comparison of the shark and human *nm23*/NDP kinases.
Identical amino acid residues between the nm23/NDP kinases are marked as *Asterisk*. By this analysis shark nm23 shows 66 % sequence identity and 85 % sequence similarity.

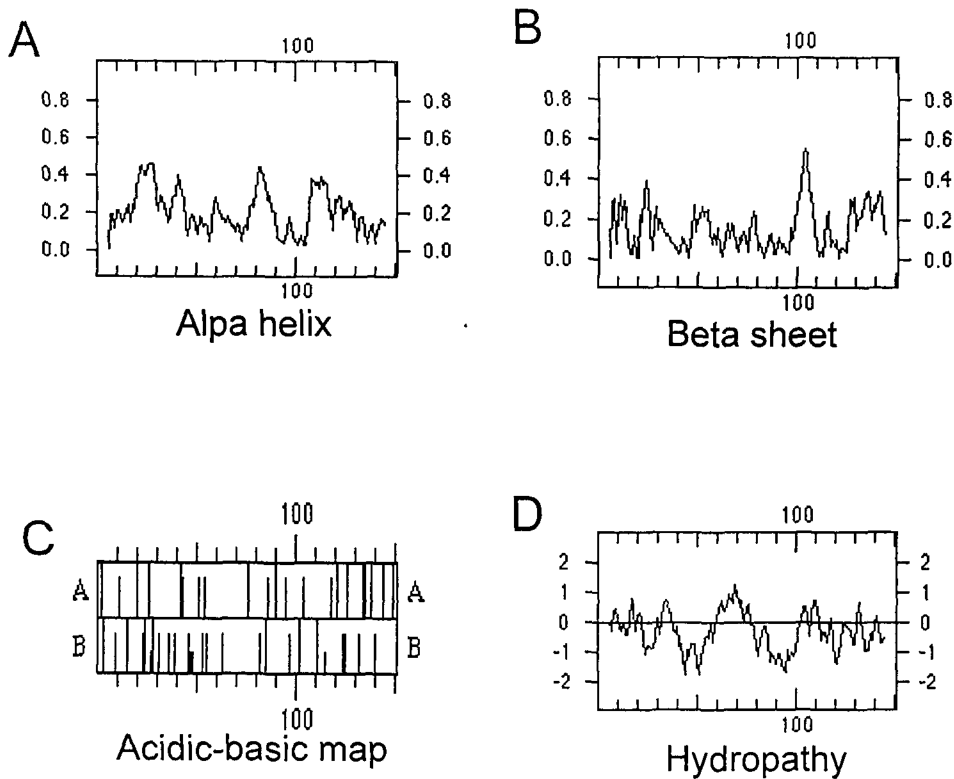

Fig. 3. Hydropathicity profile and prediction of secondary structure of the deduced amino acid sequence of shark *nm23*/NDP kinase.
Hydropathicity values were calculated by the method of Kyte and Doolittle (1982). The α-helical and β-sheet potentials were calculated by the method of Chou and Fasman (1978).

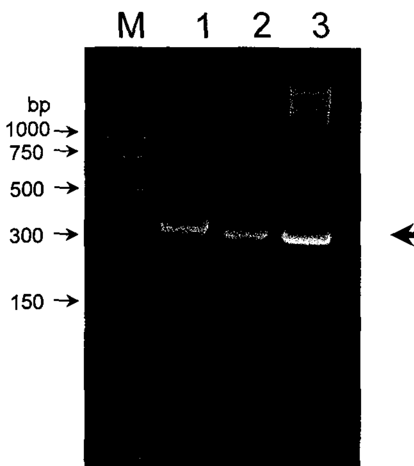

Fig. 4. Patterns of mRNA expression of shark tissues by reverse transcription polymerase chain reaction.
Lane M indicates a molecular weight marker. The number above the lanes corresponds to total RNA template for RT-PCR isolated from the shark skin (1), cartilage (2), and liver (3).

kinase at the investigated tissues from skin, cartilage, and liver of Korean tiger shark.

The present study suggests that it may also be useful in investigating the noble shark *nm23*/NDP kinase gene functioning to prevent neovascularization and tumorigenicity. The isolation of the shark *nm23*/NDP kinase and the identification of its biological activity have provided new insights into the molecular biology of tumor metastasis.

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