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TRAIL in the mandarin fish *Siniperca chuatsi*: Gene and its apoptotic effect in HeLa cells

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HeLa cells

Abstract Tumor necrosis factor (TNF)-related apoptosis-inducing ligand (TRAIL) is one of the TNF superfamily members, participating in many biological processes including cell proliferation and apoptotic death. In this study, a TRAIL gene was cloned from a perciform fish, the mandarin fish *Siniperca chuatsi*, a major cultured fish in China's aquaculture, and is named as SCTRAIL for *S. chuatsi* TRAIL. The full-length cDNA of SCTRAIL is 1359 bp, encoding a 283-amino-acid protein. This deduced protein contains the Cys_{231}, a 23-mer fragment of transmembrane region, a glycosylation site and a TNF family signature, all of which are conserved among TRAIL members. SCTRAIL gene consists of six exons, with five intervening introns, spaced over approximately 9 kb of genomic sequence. Southern blotting demonstrated that the SCTRAIL gene is present as a single copy in mandarin fish genome. A 620 bp promoter region obtained by genome walking contains a number of putative transcription factor binding sites, such as Oct-1, Sp-1, NF-1, RAP-1, C/EBPalp, NF-κB and AP-1. The SCTRAIL is constitutively expressed in all the analyzed tissues, as revealed by RT-PCR, which is confirmed by Western blotting analysis using polyclonal antibody against bacteria-derived recombinant SCTRAIL protein. As an apoptosis-inducing ligand, the overexpression of SCTRAIL but not the mutant SCTRAIL-C203S in HeLa cells induced changes characteristic of apoptosis, including chromatin condensation, nucleus fragmentation, DNA ladder, and increase of sub-G0/G1 cells in FACS analysis.

Introduction

The tumor necrosis factor (TNF) ligand superfamily is an important group of cytokines regulating the pathway of inflammation, apoptosis, and cell proliferation [1]. Over 19

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members in this family have been identified so far in human [2]. In fish, several members in this family have been reported; for example, TNFα was reported in Japanese flounder (*Paralichthys olivaceus*) [3], rainbow trout (*Oncorhynchus mykiss*) [4], brook trout (*Salvelinus fontinalis*) [5], carp (*Cyprinus carpio*) [6] and sea bream (*Dicentrarchus labrax*) [7], and a FasL-like protein, Fas ligand in channel catfish (*Ictalurus punctata*) [8].

TNF-related apoptosis-inducing ligand (TRAIL) is a member in the TNF superfamily, which was first identified on the basis of its sequence homology to other members of the TNF superfamily in 1995 [9]. Human TRAIL, also known as Apo-2L [10], is a typical type II transmembrane protein and shows the highest homology to RANK ligand. In comparison to other members of TNF family, TRAIL has two unique characteristics: one is that TRAIL can selectively induce apoptosis in tumor or transformed cells, but not in normal cells, highlighting its potential therapeutic application in cancer treatment [11], and the second is that TRAIL mRNA is expressed constitutively in a wide range of tissues, whilst the expression of other members is tightly regulated and often transiently expressed in activated cells [1]. In relation to fish TRAIL, there have been only two reports: they are related to TRAIL-like gene in zebrafish (*Danio rerio*) and brook trout [12], and TRAIL gene in grass carp (*Ctenopharyngodon idella*) [13]. Despite the understanding of the gene and its expression, the special apoptotic function of piscine TRAIL has not been verified.

In China, the mandarin fish or the so-called Chinese perch, *Siniperca chuatsi* (Basilewsky) (Perciformes) has a relatively high market value, and is widely cultured throughout the country [14]. Continuous effort has recently been made to understand its immune system [15—17]. In this study, the cloning and expression analysis of the TRAIL gene from the mandarin fish were conducted, and its apoptotic function after overexpression was revealed in cancer cells.

Materials and methods

Fish and cell line

A total of 20 mandarin fish, weighing about 200 g each, obtained from Niushan Lake in Wuhan, Hubei Province of China, were maintained in aquarium with aerated water for at least 2 weeks before the start of any experiment.

The cervical carcinoma cell line, HeLa, was maintained in RPMI 1640 medium supplemented with 10% heat-inactivated foetal calf serum (Invitrogen) containing penicillin (100 units ml^{-1}) and streptomycin (100 $\mu g\,ml^{-1}$). For

Table 1 Primers for PCR amplification and analyses

Name	Sequence (5′—3′)	Note
UPM-long	CTAATACGACTCACTATAGGGCAAGCAGTGGTATCAACGCAGAGT	RACE-PCR and expression analysis
UPM-short	CTAATACGACTCACTATAGGGC	
F1	AGACCCCTGCTGGCAGGTCA	
R1	AGGCACCACCAGCTCTCCATC	
F2	CAGAGGCAGATTTCATCGGCAG	
F3	CCCAAAGTCGCAGCCCATGTCA	
R2	GCCCCTCCCACCACGAAATCTT	
R3	AAGCCCTGTCTGCCATCACGAG	
AP-1	GTAATACGACTCACTATAGGGC3	Genomic sequence and promoter region amplification
AP-2	ACTATAGGGCACGCGTGGT	
R4	GGTGAAGTGCAGCACGGTGATGGT	
F5	CTGCTGCAGACTGTGGTCGTCA	
R5	ACGCTGCTCCTGGCAAACG	
F6	GAGACGTTTGCCAGGAGC	
R6	AAGCCCTGTCTGCCATCA	
F7	CGTGATGGCAGACAGGGCTT	
R7	GCGTAGACGTAGTAGAGGCCG	
F8	CGGCCTCTACTACGTCTACGC	
R8	CTGATGAGAAACGCCCCAAAGA	
F9	GTGAGCTCCTACCCGGTTCC	Probe synthesis for Southern blotting
R9	CTGATGAGAAACGCCCCAAA	
F10	TCAGCGTTACCAGAGGCAGAT	RT-PCR
R10	CCCTCCCACCACGAAATCTT	
F11	GACATCAAGGAGAAGCTGTGCT	
R11	ATGCTGTTGTAGGTGGTCTCGT	
F12	CCTGTGGATCCTACCAGAGGCAGATTTCATCG	Expression vector construction
R12	TCGACAAGCTTTCCTGAGTCCTGCGTCTGAT	
F13	CGATAGCTAGCGTGATGGCAGACAGGGCTTC	
R13	CGCCTGGTACCCGAGCTGATGAGAAACGC	

transfection, cells were seeded into a six-well plate at 2×10^5 cells per well, and 2 μg of DNA was transfected with Lipofectamine™ reagent (Invitrogen) according to the manufacturer's manual.

Cloning of SCTRAIL full-length cDNA by RACE-PCR

Total RNA was extracted from the head kidney of two fish with TRIzol Reagent (Invitrogen). The SMART cDNAs were synthesized and amplified using a SMART PCR cDNA Synthesis Kit (Clontech) by following the protocol. According to the zebrafish and trout EST sequences (GenBank database accession no. DR728061 and BX299019, respectively) obtained by searching the zebrafish and trout database with chicken TRAIL cDNA sequence, primers (F1/R1; Table 1) were designed to amplify a segment of SCTRAIL cDNA sequence from the SMART cDNAs. The PCR cycling conditions were one cycle of 94 °C for 5 min, 35 cycles of 94 °C for 30 s, 60 °C for 30 s, and 72 °C for 50 s, and then a final elongation step at 72 °C for 7 min.

RACE-PCR was performed to clone the full-length cDNA of SCTRAIL [18]. Briefly, gene specific primers (F2, F3, R2, R3; Table 1) were designed according to the obtained segment with the method described above. Nest-PCR was performed with the combination of adaptor primers (UPM) and R2/R3 for 5′-RACE-PCR, the combination of adaptor primers (UPM) and F2/F3 for 3′-RACE-PCR. The generated PCR

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                GACGGGGAGGGTAGAAGTCAAGGAGGCAGCTCTCTGCCATTGTTCTTGTTCT  52
GGAAGTCTGGGAACGGGACGGGACGACGCTGGTCGGGTACCGGACGGCGGGTCGGTCGGT 112
ATGACGGGCTCCGGTCCGAAGCTCGGGGTGCTGCTGCTGCTGGCGGTTCTGCTGCAGACT 172
  M  T  G  S  G  P  K  L  G  V  L  L  L  L  A  V  L  L  Q  T   20
GTGGTCGTCACCATCACCGTGCTGCACTTCACCACGGCTCTCAATTCGATGAAGGAGACG 232
  V  V  V  T  I  T  V  L  H  F  T  T  A  L  N  S  M  K  E  T   40
TTTGCCAGGAGCAGCGTTTCCTGCCTGACGGGCGCTGACCTGCAGAGCATCACGGCTGTA 292
  F  A  R  S  S  V  S  C  L  T  G  A  D  L  Q  S  I  T  A  V   60
CGGGGGGATCCATGCTGGCAGGTCACTCAGCAGCTTCACCTGCTCATTGAGAAGTCTCTG 352
  R  G  D  P  C  W  Q  V  T  Q  Q  L  H  L  L  I  E  K  S  L   80
TCTCAGCGTTACCAGAGGCAGATTTCATCGGCAGTCAGAGATGAAGTGTCTCGGGTTCTG 412
  S  Q  R  Y  Q  R  Q  I  S  S  A  V  R  D  E  V  S  R  V  L  100
CCCTCACTCGTGATGGCAGACAGGGCTTCACCTCGACCCAAAGTCGCAGCCCATGTCACT 472
  P  S  L  V  M  A  D  R  A  S  P  R  P  K  V  A  A  H  V  T  120
GGCAGCTTTGTGCCCAAACTGGAGCGAGAGGGAGGAGCTCCAGTCTCTGCTGGCCGTCGA 532
  G  S  F  V  P  K  L  E  R  E  G  G  A  P  V  S  A  G  R  R  140
GTTCAGGGCCAGAAGATTTCGTGGTGGGAGGGGCAGAAGGGGCTGGCTTTCCTCCAGGAC 592
  V  Q  G  Q  K  I  S  W  W  E  G  Q  K  G  L  A  F  L  Q  D  160
GTCCAGCTGGTGGACGGGGAGCTGGTGGTGCCGCAGCCCGGCCTCTACTACGTCTACGCC 652
  V  Q  L  V  D  G  E  L  V  V  P  Q  P  G  L  Y  Y  V  Y  A  180
CAGACCTACTTCAGACACACACACTCCCTGGAGGACGAGGGCGGGGACAGCGAGGAGGCG 712
  Q  T  Y  F  R  H  T  H  S  L  E  D  E  G  G  D  S  E  E  A  200
GAGGACAGAGGGAGACCCCTGTTGCAGTACGTCTATAAAAAGGTGAGCTCCTACCCGGTT 772
  E  D  R  G  R  P  L  L  Q  Y  V  Y  K  K  V  S  S  Y  P  V  220
CCCATCCTGCTGATGAAGACGAGCCGAACCTCCTGCTGGTCCCGGGGCTCCCAGTTTTCT 832
  P  I  L  L  M  K  T  S  R  T  S  C  W  S  R  G  S  Q  F  S  240
CTGCACTCCGCCCACCAGGGGGGGCTGTTCCCTCTCAGCAGCGGCGACCGCCTGTTTGTC 892
  L  H  S  A  H  Q  G  G  L  F  P  L  S  S  G  D  R  L  F  V  260
ACAGTGACCAACGCCTCTGCTGTGGACATGGACGAAAAAAGCAGCTTCTTTGGGGCGTTT 952
  T  V  T  N  A  S  A  V  D  M  D  E  K  S  S  F  F  G  A  F  280
CTCATCAGCTAGACGATCAGACGCAGGACTCAGGATCGCAGGTCGATCTGGGGAAATCAA 1012
  L  I  S  *                                                    283
CCAGATCTACAGACGCCATCATGTTTTTAATGTTTGTGTTGAACGCATCTGTCTGGTAAA 1072
ACACCAAATTATTGCAGCCTCTAGACTTCTCCTATTAATGAGCCCAGAAAATGAAAAGCT 1132
GCAACATAGCGACTCGCGGGGAAGCCTGTCTTTCTTTTTTCAAGATCACAAACCGAAAAC 1192
ATTTGACAACACTGGTTTCGTGGGCGGAGAAGAGACCGATATGGATTTGTGGCTGTGTGT 1252
ATGTGCGAGAGCTAATCAAACTCAACAAATATTTGTACTTGAGTGTATTAAATTTGGTGT 1312
GGCTTGGACTCGAAAAAGAAAAAAAAAAAAAAAAAAAAAAAAAAAAA              1359
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Figure 1 Nucleotide sequence and deduced amino acid sequence of mandarin fish *Siniperca chuatsi* TRAIL (SCTRAIL) with the GenBank accession no. being AY839276 and AAX77404, respectively. The nucleotides (upper row) and deduced amino acids (lower row) are numbered at the right side of sequences. The predicted transmembrane region is underlined. The start codon (ATG) is boxed and the stop codon (TGA) indicated by an asterisk. The polyadenylation signal is shown in bold near the 3′ end of the sequence.

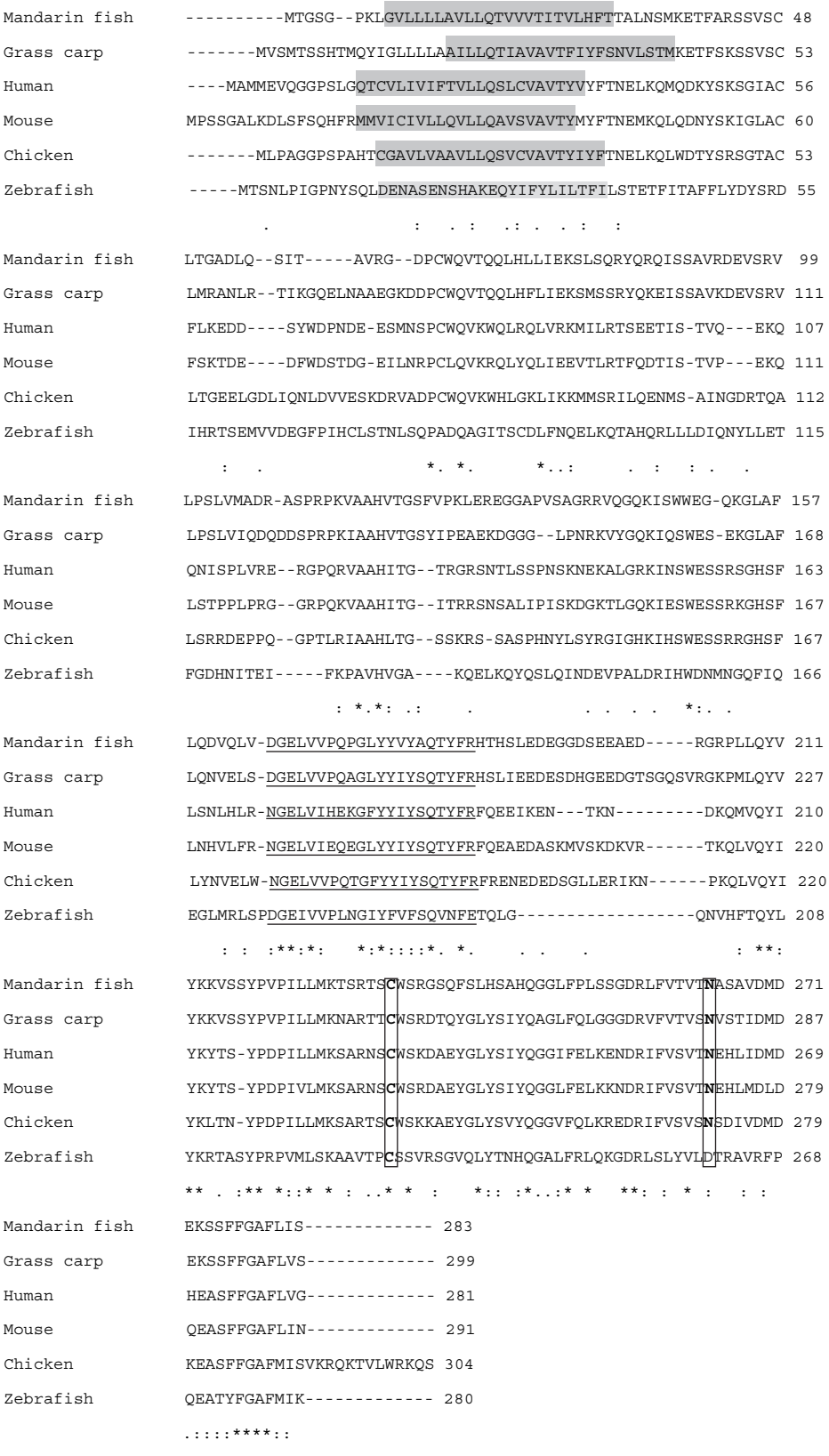

Figure 2 Multiple alignment of the mandarin fish *Siniperca chuatsi* TRAIL (SCTRAIL) deduced amino acid sequence (AAX77404) with those of grass carp (AY697730), human (NP_003801), mouse (AAC52345), and chicken (NP_989710), zebrafish (XP_689994). Identical (*) and similar (: and .) residues identified by the CLUSTALW program are indicated. The shadow marks the transmembrane region. The underlined nucleotides show the TNF superfamily signature [LV]-x-[LIVM]-x3-G-[LIVMF]-Y-[LIVMFY]2-x2-[QEKHL]. The conserved cysteine and putative glycosylation site are boxed.

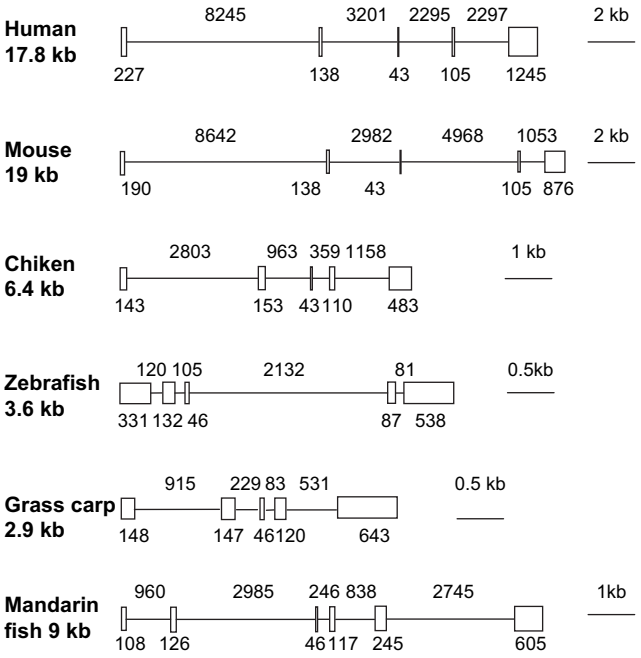

Figure 3 Comparison of mandarin fish *Siniperca chuatsi* TRAIL (SCTRAIL) genome structure with previously reported TRAIL genes. Exons are represented by boxes and introns are shown by lines. Numbers indicate the length of exons and introns in base pairs.

products were sequenced and assembled to get the full-length cDNA of SCTRAIL. Nest-PCR cycling conditions were described as following: When using SMART cDNA as template, the first run was one cycle of 94 °C for 5 min, seven cycles of 94 °C for 30 s, 66 °C for 30 s, and 72 °C for 90 s, 32 cycles of 94 °C for 30 s, 62 °C for 30 s, and 72 °C for 90 s, followed by one cycle of 72 °C for 7 min. The second run used the first run's product as template with the cycling conditions similar to the first. All the primers used for PCR were designed with the software Primer premier 5.0.

The PCR products were isolated using the Omega agarose purification kit, and were cloned into pMD-18-T Vector (Takara) by following the protocol. The selected clones were sequenced using the dideoxy chain-termination method on an automatic DNA sequencer (ABI Applied Biosystems Model 377).

Cloning of SCTRAIL genomic sequence and the promoter region

The genomic DNA was purified from the fin of two healthy mandarin fish using Wizard® Genomic DNA Purification Kit (Promega). Based on the cloned SCTRAIL cDNA sequence, primers (R4, R5, R6, R7, R8 and F5, F6, F7, F8; Table 1) were designed to obtain the full-length SCTRAIL gene sequence. The 5′-flanking region was obtained using a genome walking approach by constructing genomic library with a Universal Genome Walker™ kit (Clontech). The adaptor primers for genome walking were AP-1 and AP-2 (Table 1). The genomic sequence was obtained by PCR and the 5′-flanking region by Nest-PCR with primers described in Table 1.

Southern blotting

Genomic DNA was digested at 37 °C overnight with 2 units *Xba*I, *Sac*I, *Eco*RI, *Xho*I (Takara) per microgram DNA. Restricted DNA (10 μg per lane) was separated in a 0.7% agarose gel, and transferred onto Hybond-N^+ nylon membrane using upward capillary transfer. Transferred DNA was hybridized with a 206 bp probe generated using the PCR DIG Probe Synthesis Kit (Roche) with primers F9 and R9 (Table 1). Following an overnight hybridization, blots were washed twice for 5 min each with 2×SSC containing 0.1% SDS at room temperature and twice for 15 min each with 0.1×SSC containing 0.1% SDS at 60 °C. After Anti-Digoxigenin-AP (Roche) incubation and CSPD (Roche) chemiluminescent reaction, the hybridized bands were visualized by exposing to the X-ray film.

Sequence analysis

The BLAST programs from National Center for Biotechnology Information were used to search for homologous sequences. The putative signal peptides and transmembrane regions were predicted using the SOSUI (http://expasy.pku.edu.cn). Multiple alignments were generated with the CLUSTAL 1.8 program. Phylogenetic tree was generated for the data set based on the principle of parsimony by the PROPARS program in PHYLIP [19]. The SCTRAIL intron/exon organization structure was determined by alignment of the full-length cDNA with the genomic DNA. The sequence of the 5′-flanking region was analyzed by TRANSFAC software for potential transcriptional factor binding sites [20] and the transcriptional start

Table 2 Exon—intron boundaries of TRAIL gene in the mandarin fish *Siniperca chuatsi*

Exon		5′-splicing donor		3′-splicing acceptor		Intron	
No.	Size (bp)					No	Size (bp)
1	108	AATTCG	**gt**gagg	gtgc**ag**	ATGAAG	1	960
2	126	GAGAAG	**gt**gtgg	ctgc**ag**	TCTCTG	2	2985
3	46	TCAGAG	**gt**gaga	tgtt**ag**	ATGAAG	3	246
4	117	GAGGAG	**gt**gagt	ccac**ag**	CTCCAG	4	838
5	245	AAAAAG	**gt**gaga	cgcc**ag**	GTGAGC	5	2745
6	605						

Sequences in exons are shown in uppercase letters and in introns in lowercase letters. The consensus gt/ag splice sequences are shown in bold.

ACGACGGCCAGTGCCAAGCTTGCATGCCTGCA -701
Oct-1
GGTCGACGATTACTATAGGGCACGCGTGGTCGACGGCCCGGGCTGGTACTGGGAACAGCAAAAATACTGC -631
Sp-1
GCAGAACCCTCAAGCTCCCAGGCCTCTGGTAGAAGACCCGAGCTCGAAGGACGAGACCACCCGCGGAGGG -561
Sp-1 Sp-1
TGAAGGACAAAGTTTTTTTTTATATATATATGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTAAGCAA -491

GTATATGAATTTATCTAATAGGGATTTAATATAAGTGATTAAAACTTTAGTTTATGTGAGTTTAAACTCT -421
NF-kappaB AP-1 C/EBPalp
ATAACATCTTAATAAAAGTCATGTACGTGTATGTGCCAAAAATCAGCTGCTGTGGCACTGCTGTGCATGT -351
NF-1
TTAAAAACATTATATCTGATCATATTCTGAGGTAAAATGTGTTTTTCTCACAGTAAAGCTCAGTACAGAG -281
RAP1
ATCTACAGCAAAGATATGAGCGTTCACAATAAATAAGAAGGATATCTGTGTTTTTCCAGCGCAGAAATGA -211
C/EBPalp
AACAATAATCTCTCCAGGAGAAATTCATAGAGGGAAAAAGCTCTTGTTCTCCAGGCCGGCCCCGCCTCCT -141
Sp-1
CCTCCTCCTCCTCCTCCTGCTTGCAGGGGACGGGGAGGGTAGAAGTCAAGGAGGCAGCTCTCTGCCATTG -71
Sp-1
TTCTTGTTCTGGAAGTCTGGGAACGGGACGGGACGACGCTGGTCGGGTACCGGACGGCGGGTCGGTCGGT -1

ATGACGGGCTCCGGTCCGAAGCTCGGGGTGCTGCTGCTGCTGGCGGTTCTGCTGCAGACTGTGGTCGTCA 70

Figure 4 Nucleotide sequence of the 5′-upstream region of mandarin fish *Siniperca chuatsi* TRAIL (SCTRAIL) gene. Putative transcription factor core and matrix sequences are shadowed. Predicted transcriptional start region is underlined and determined using the Neural Network Promoter Prediction Program (http://www.fruitfly.org/seq_tools/promoter.html).

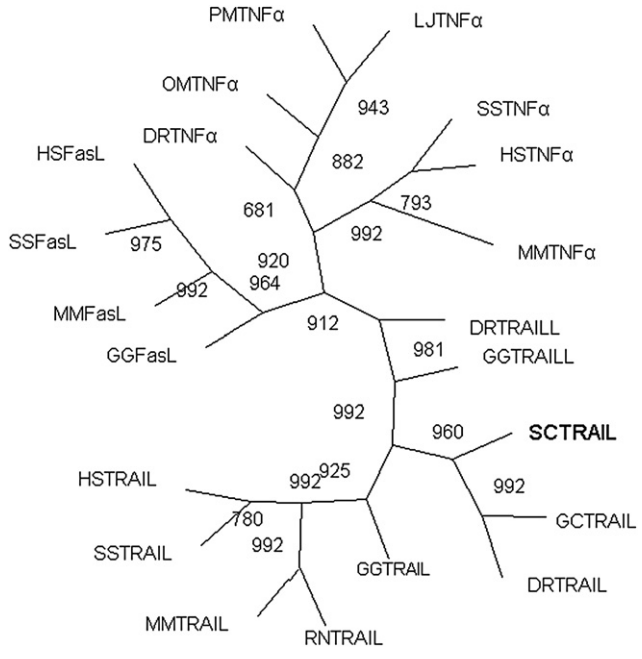

Figure 5 Phylogenetic relationship of members in the TNF superfamily. Mandarin fish *Siniperca chuatsi* TRAIL (SCTRAIL) is indicated in bold. The tree is constructed with PHYLIP. The default option, in which all characters are equally weighted, was in effect. The bootstrap confidence values shown at the nodes of the tree are based on 1000 bootstrap replications. All the GenBank Accession numbers of amino acid sequences and their brief names are listed in Table 3.

region was predicted using the Neural Network Promoter Prediction Program (http://www.fruitfly.org/seq_tools/promoter.html).

Tissue distribution of SCTRAIL mRNA by RT-PCR

Total RNAs were extracted from different tissues of three healthy mandarin fish using TRIzol Reagent (Invitrogen) and then digested by DNase I (RNase-free, Takara) to avoid genomic DNA contamination. RNA (5 μg) from different tissues was reverse-transcribed with PowerScript™ Reverse Transcriptase (Clontech). Semi-quantitative RT-PCR was performed with the primers for SCTRAIL (F10/R10; Table 1) and the housekeeping gene β-actin (F11/R11; Table 1). The reaction was completed in a thermocycler with the following thermoprofiles: 94 °C for 3 min, then 30 cycles with 94 °C for 30 s, 60 °C for 30 s, 72 °C for 30 s. Upon the completion of PCR, the reaction was incubated at 72 °C for an additional 7 min. The RT-PCR product was analyzed by 2% agarose gel electrophoresis and documented with a Gel Documentation System.

Expression vector construction

For the construction of pET-32a-SCTRAIL, the cDNA sequence encoding amino acid 84—283 of SCTRAIL, which is similar to the functional segment of human TRAIL [9], was amplified by PCR with a pair of primers containing BamHI and HindIII digestion sites (F12/R12; Table 1) and cloned into the multiple clone sites of pET-32-a(+) vector (Merk) previously digested with *Bam*HI and *Hind*III.

For the construction of pEGFP-N3-SCTRAIL, the cDNA encoding 114—281 aa of SCTRAIL, which encodes the

Table 3 TNF superfamily members used for phylogenetic tree construction and multiple sequence alignments

Species	Protein	Brief name	Accession no.
Homo sapiens	TNFα	HSTNFα	NP_000585
	Fas ligand	HSFasL	NP_000630
	TRAIL	HSTRAIL	NP_003801
Mus musculus	TNFα	MMTNFα	NP_038721
	Fas ligand	MMFasL	NP_034307
	TRAIL	MMTRAIL	AAC52345
Rattus norvegicus	TRAIL	RNTRAIL	AAM49797
Sus scrofa	TNFα	SSTNFα	CAB63852
	TRAIL	SSTRAIL	NP_001019867
	Fas ligand	SSFasL	NP_998971
Gallus gallus	TRAIL-like	GGTRAILL	NP_989922
	Fas ligand	GGFasL	CAI64582
	TRAIL	GGTRAIL	NP_989710
Danio rerio	TNFα	DRTNFα	NP_998024
	TRAIL	DRTRAIL	XP_689994
	TRAIL-like	DRTRAILL	AAG47640
Oncorhynchus mykiss	TNFα	OMTNFα	CAB92316
Lateolabrax japonicus	TNFα	LJTNFα	AAR02413
Pagrus major	TNFα	PMTNFα	AAP76392
Ctenopharyngodon idella	TRAIL	GCTRAIL	AY697730
Siniperca chuatsi	TRAIL	SCTRAIL	AAX77404

apoptogenic receptor-binding moiety when compared with human TRAIL, was amplified by PCR reaction using appropriate primers (F13/R13; Table 1) to introduce *Nhe*I and *Kpn*I restriction enzyme sites, the amplified fragment was digested by the corresponding restriction enzyme, and cloned into the pEGFP-N3 vector, the mutant SCTRAIL-C230S was constructed by KOD plus DNA polymerase according to the user manual (TOYOBO). The recombinant plasmids were then transformed into competent *Escherichia coli* and amplified in the hosts.

Expression and purification of the fusion protein Trx—SCTRAIL in *E. coli*

E. coli BL21 (Rosetta-gami DE3, Merk) transformed with the recombinant expression vector was grown at 25 °C in LB medium containing four antibiotics (34 μg ml^{-1} chloramphenicol, 12.5 μg ml^{-1} tetracycline, 15 μg ml^{-1} kanamycin, 100 μg ml^{-1} ampicillin) until the optical density (OD_{600}) reached 0.6. Expression was induced by the addition of IPTG to a final concentration of 0.5 mM and incubation for another 8 h at 25 °C. The bacteria were then harvested by centrifugation for 5 min at 10,000g and then re-suspending in PBS. After ultrasonic treatment and centrifugation for 5 min at 10,000g, the soluble form of Trx—SCTRAIL in the supernatant was purified according to the manufacturer's introduction with metal-affinity chromatography on columns with His—Bind Resin (Novagen) under native conditions. The purified protein was checked on 12% SDS-PAGE.

Preparation of polyclonal antibody against recombinant SCTRAIL and Western blotting

Polyclonal antibody against the purified protein was raised in mouse. Nine mice were injected subcutaneously with purified protein (20—50 mg each) emulsified in complete Freund's adjuvant (Sigma). After the first injection, three times of booster injections were done with the same antigen amount but using incomplete Freund's adjuvant (Sigma) with intervals of 1 week. The mice were bled 2 weeks after the last booster and serum samples were stored at −80 °C.

For Western blotting analysis, samples prepared from different organs were ran on 12% SDS-PAGE gel, and were electrophoretically blotted to a nitrocellulose membrane. The membrane was blocked with 2.5% fat-free dry milk in TS buffer (100 mM NaCl, 100 mM Tris-base pH 7.5), followed by incubation with antiserum against the rSCTRAIL at a dilution of 1:200 in TS buffer containing 1.0% fat-free milk and 0.05% Triton X-100 at 4 °C for 1 h. The membrane was washed three times for 15 min each in TS buffer and then incubated with 1:200 diluted alkaline phosphatase-conjugated goat anti-mouse IgG (Sino-American). After washing it three times for 10 min each in TS buffer, detection was performed using BCIP/NBT.

Apoptosis assay

Cell cycle analysis

Nuclei of fixed cells were stained with propidium iodide and treated by RNase A. Cells with sub-G1 DNA content were detected by fluorescence-activated cell sorter (FACS) after staining with propidium iodide. Cells in sub-G1 phase were regarded as the percentage of apoptotic cells.

DNA fragmentation

Cells were harvested and washed with phosphate-buffered saline (PBS), resuspended in 50 μl lysis buffer (0.25% Nonidet P-40, 1 mM EDTA, 5 mM Tris—HCl, pH 8.0). After treatment with RNase A (DNase-free, 400 mg ml^{-1}, 37 °C for 1 h), samples were treated with Proteinase K (400 mg ml^{-1}, 37 °C for 1 h) and DNA fragments were separated in agarose gel

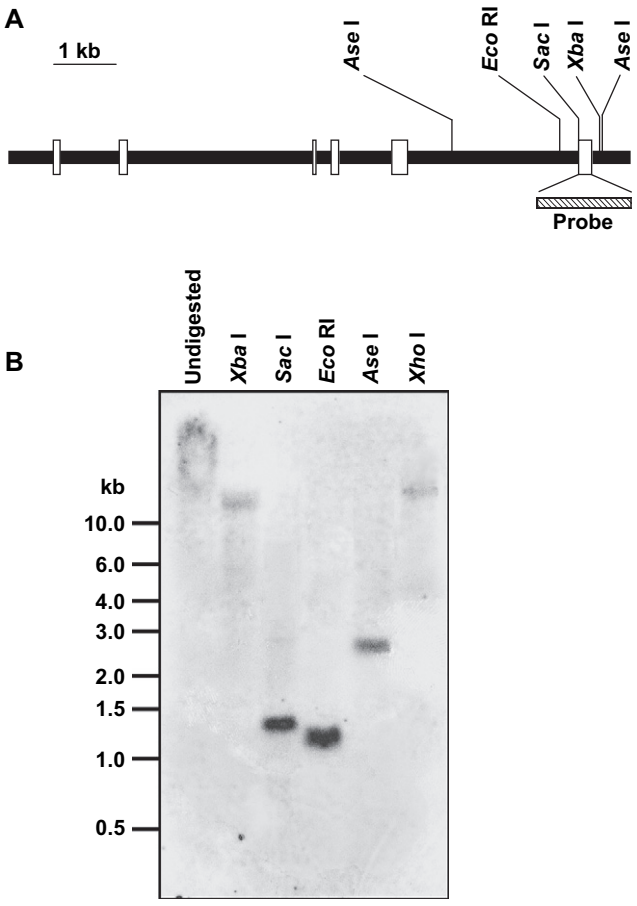

Figure 6 Southern blotting analysis. (A) Restriction map of mandarin fish *Siniperca chuatsi* TRAIL (SCTRAIL) genomic sequence. The thick black bars and empty boxes represent the introns and exons, respectively. (B) Genomic Southern blotting analysis of SCTRAIL DNA fragment. Approximately 10 μg of genomic DNA from mandarin fish was digested with enzymes and separated by 0.7% agarose electrophoresis, followed by transferring onto Hybond-N^+ nylon membrane. Southern hybridization was performed with a 208 bp PCR product as probe, which was labelled with the PCR DIG Probe Synthesis Kit.

(1.5% agarose, Tris—Acetate EDTA (TAE) buffer), as previously described by Du et al.[21].

Morphological change

Morphological changes of apoptotic cells were visualized and photographed under fluorescence microscope after cells were stained with DAPI (Sigma).

Results

Analysis of the full-length cDNA and protein sequences of SCTRAIL

Full-length cDNA of SCTRAIL was obtained by RACE-PCR (Fig. 1). It has 1359 base pairs (bp) in length, with an open reading frame (ORF) of 852 bp encoding a protein of 283 amino acids (aa), a 5′-untranslated region (UTR) of 112 bp, and a 3′-UTR of 395 bp containing a polyadenylation signal (ATTAAA) and a poly(A) tail.

The deduced SCTRAIL protein shares similarities to that of human, mouse, chicken, grass carp and a putative zebrafish TRAIL with the identity of 36%, 38%, 40%, 62%, and 60%, respectively. Amino acid sequence alignment revealed a strong cross-species conservation among TRAIL proteins (Fig. 2). Typical conserved clusters of amino acid residues were found in the SCTRAIL protein, e.g. the Cys_{231} which is similar to residue 230 in human TRAIL [22], the conserved transmembrane region and an N_{264} for a glycosylated site [23], and the existence of a conserved TNF family signature [LV]-x-[LIVM]-x3-G-[LIVMF]-Y-[LIVMFY]2-x2-[QEKHL] [1].

Genomic organization and the 5′-upstream region of SCTRAIL gene

A 9753 bp genomic DNA sequence covering the entire coding region of SCTRAIL was amplified using the specific primer (Table 1) and subsequently compared with SCTRAIL cDNA sequence to clarify the exon—intron organization. As shown in Fig. 3, SCTRAIL gene is composed of six exons and five introns. Table 2 indicates the nucleotide sequences surrounding the exon—intron boundaries. All exon—intron junctions follow the consensus rule of the splice acceptor-AG/GT-splice donor for splicing [24]. According to the gene organization (Fig. 3), SCTRAIL is in accordance with those of human, mouse, chicken and zebrafish, and each species has the similar size for exons but very different size for introns. Interestingly, an additional intron was found in SCTRAIL, which separates the last exon in other species into two exons.

The 620 bp SCTRAIL promoter region upstream of the translation initiation codon was obtained by genome walking. The sequence analysis by TRANSFAC software reveals that typical ''TATA'' or ''CAAT'' boxes are absent from this putative promoter. However, consensus sequences for several transcription regulatory factor binding motifs were identified in this promoter region, including binding sites for Oct-1, Sp-1, NF-1, RAP-1, C/EBPalp, NF-κB and AP-1 (Fig. 4).

Phylogenetic analysis

A phylogenetic tree was constructed with the sequences of TRAIL proteins from mammals, birds, and fish (Fig. 5, Table 3). The results showed that SCTRAIL is clustered with the putative zebrafish TRAIL and grass carp TRAIL.

Southern blotting analysis

Southern blotting was performed to determine the number of SCTRAIL genes in the genome. As shown in Fig. 6, when digested with different restriction enzymes, only one band was observed in each lane (Fig. 6B), suggesting that the SCTRAIL gene is present as a single copy in mandarin fish genome.

Distribution of SCTRAIL mRNA in different organs

RT-PCR analysis demonstrated a constitutive expression of the SCTRAIL in all studied tissues of the mandarin fish, with predominant expression in head kidney, spleen, kidney, and intestine, but with a relatively low level of expression in gill and muscle (Fig. 7A).

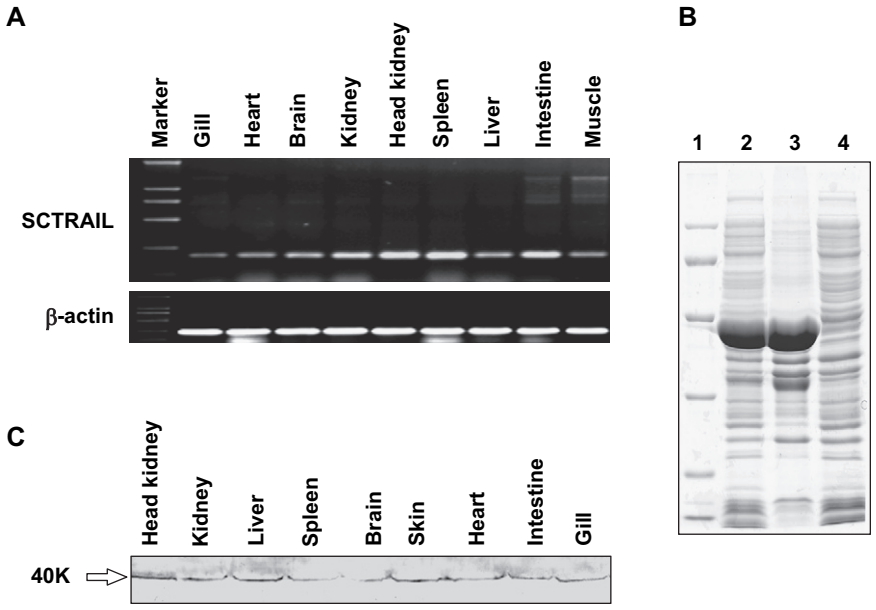

Figure 7 Distribution of mandarin fish *Siniperca chuatsi* TRAIL (SCTRAIL) in different organs of the fish. Single strand cDNAs and total proteins were prepared from gill, heart, brain, kidney, head kidney, spleen, liver, intestine, and muscle, from head kidney, kidney, liver, spleen, brain, skin, heart, intestine, and gill of healthy mandarin fish, respectively. (A) Distribution of SCTRAIL mRNA as revealed by semi-quantitative RT-PCR analysis; β-actin was used as a positive control to normalize the sample; the marker is DL2000 (Takara). (B) SDS-PAGE analysis of recombinant SCTRAIL obtained by prokaryotic expression in *E. coli* DE3 which was transformed with pET-32a-SCTRAIL carrying mandarin fish TRAIL, and the positive clones containing the recombinant plasmid were cultured until the OD_{600} reached 0.6—1; part of the samples were taken out as control (Lane 4), and the remains were induced with IPTG for additional 8 h at 25 °C. Following the harvest, ultrasonic treatment, and centrifugation, the pellet (Lane 3) and supernatant (Lane 2) were analyzed by SDS-PAGE. Lane 1 shows the protein molecular maker with the molecular weight of 97.4, 66.2, 43, 31, 20.1, and 14.4 kDa, respectively. (C) Distribution of SCTRAIL protein analyzed by Western blotting. The equal amount of total proteins from different organs were loaded separately onto gel as antigen to perform Western blotting with the polyclonal antibody to SCTRAIL core protein as primary antibody, and the goat anti-mouse IgG-conjugated with alkaline phosphatase as the secondary antibody. The membrane was washed and visualized by NBT/BCIP.

Recombinant expression of SCTRAIL in *E. coli*

The cDNA sequence encoding amino acid 84—283 of SCTRAIL was cloned into expression vector pET-32a, fused downstream of Trx—Tag keeping the correct reading frame and orientation. The fusion protein was expressed under IPTG inducement. Compared with the uninduced sample, there is a predominant extra protein band with the molecular weight of 42 kDa in the IPTG induced samples when analyzed by SDS-PAGE (Fig. 7B). The molecular weight of Trx—Tag is 20 kDa, and the weight of the SCTRAIL truncation (84—283 aa) is 22 kDa, so the theoretical molecular weight of the fusion protein is 42 kDa, and the result shown in Fig. 7B is in accordance with this prediction.

Distribution of SCTRAIL protein in different organs

The expression of SCTRAIL protein in different organs including head kidney, kidney, liver, spleen, brain, skin, heart, intestine and gill from healthy mandarin fish were detected by Western blotting. As indicated in Fig. 7C, a broad distribution of SCTRAIL protein was detected in all organs, with predominant expression in organs such as in head kidney, kidney and gonad, but a very low level of expression in brain. There existed one main form in all examined organs with the molecular weight of about 40 kDa, similar to that in human [31].

Overexpression of SCTRAIL induced apoptosis characteristics in HeLa cells

Apoptosis-inducing feature of SCTRAIL was assessed by cell cycle analysis and 17.8% of the SCTRAIL transfected HeLa underwent apoptosis, while in the GFP or SCTRAIL-C230S transfected controls, only 3.6% and 3.9% cells lies in the sub-diploid phase (Fig. 8A).

DNA gel electrophoresis showed that in the non-transfected and the GFP transfected HeLa cells, the DNA appeared as sharp band, indicating the cell DNA kept intact; while in the SCTRAIL transfected HeLa cells, the DNA was fragmented and a DNA ladder observed (Fig. 8B).

The GFP and SCTRAIL were expressed as a fusion protein, so the existence of green fluorescence in transfected cells represented the successful recombinant expression of SCTRAIL. Morphological changes of apoptosis could be obviously investigated under fluorescence microscopy after DAPI staining. When observed at high magnification, extreme chromatin condensation and nucleus fragmentation were observed from part of the transfected cells, while in

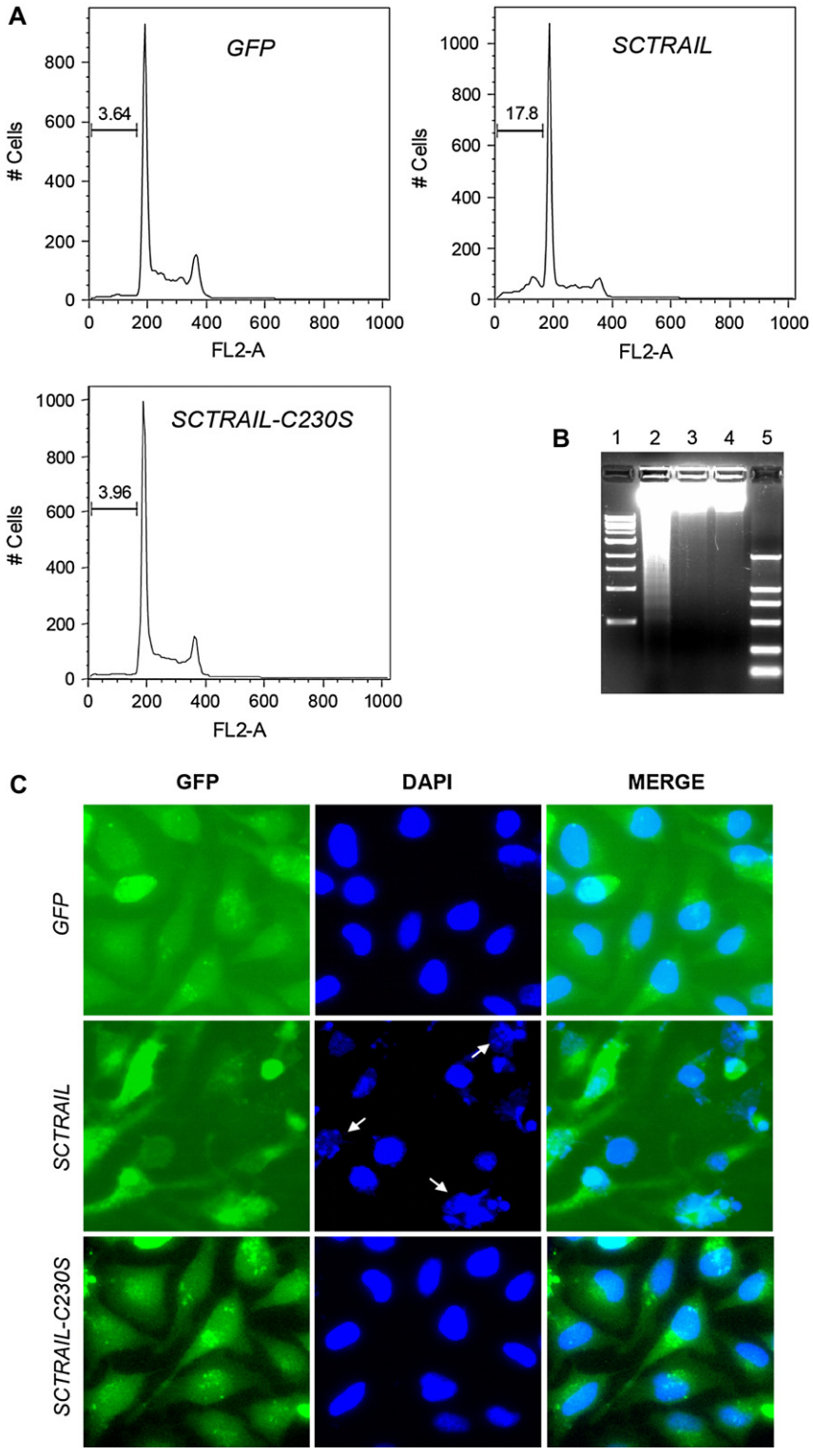

Figure 8 Overexpression of mandarin fish *Siniperca chuatsi* (TRAIL) SCTRAIL induced apoptosis in the cultured HeLa cells. HeLa cells were transfected with the expression vector (pEGFP-N3), the recombinant vector (pEGFP-N3-SCTRAIL) or the mutant SCTRAIL-C230S, and cell apoptosis was assessed by FACS analysis, DNA gel electrophoresis and fluorescence microscopy. (A) FACS analysis of the transfected cells. DNA content of apoptotic cell is below diploid (G0/G1), about 17.8% of the cells undergo apoptosis in the SCTRAIL transfected HeLa cells. (B) DNA ladder indicated by the DNA gel electrophoresis. Lanes 1 and 5 represent the DNA molecular marker NEB 1 kb DNA ladder and Takara DL2000, respectively; Lanes 2—4 show the DNA extracted from GFP-STRAIL transfected, GFP transfected and control HeLa cells; note the DNA ladder in Lane 2. (C) Fluorescence microscopic observation of the transfected HeLa cells. The successful expression and transfection effect were indicated by green fluorescence, and morphological changes of the cell nucleus were shown by the DAPI staining with the arrow indicating the fragmented nuclei of the apoptotic cells.

the N3-GFP or SCTRAIL-C230S transfected cells, the nucleus was intact (Fig. 8C).

Discussion

Since the finding of TRAIL gene in human [9], several studies have been carried out to understand its function [11] and in other vertebrates [23]. Although the TRAIL gene has been reported in zebrafish and grass carp [12,13], the present study is the first to illustrate that the TRAIL gene in fish has a function similar to its mammalian counterparts in inducing apoptosis in cancer cells.

Sequence characteristics and phylogenetic analysis showed that the cloned SCTRAIL is really the homologue of human TRAIL. Like other TRAIL proteins, SCTRAIL has the typical characters of TRAIL proteins. Firstly, SCTRAIL is a type II membrane protein in that it has an internal transmembrane domain without leader sequence. Secondly, SCTRAIL has an N-terminal domain, which is not conserved across other species, while the C-terminal domains show significant conservation among different species. Thirdly, in human TRAIL protein, Cys_{230} is not only required for TRAIL function, but also for the modulation of TRAIL apoptotic activity by forming an intermolecular disulfide bridge [22], and the cloned SCTRAIL also has this important conserved amino acid site (Fig. 2). Furthermore, the construction of a phylogenetic tree of other TNF members with the introduction of the putative protein of SCTRAIL using PHYLIP showed that SCTRAIL protein and other TRAIL members from different species clustered together and had the closest relationship with grass carp TRAIL (Fig. 5).

The genomic structure of SCTRAIL comprised six exons and five introns, which is similar to those reported in other species. The difference is that there exists a fifth intron, which separates the last exon exited in other species into two parts in the mandarin fish. The genomic size of human TRAIL gene spans approximately 18 kb, while SCTRAIL is only 9 kb. The difference in genomic sequence size is mainly due to the variable sizes of their introns.

Like the promoter region of human TRAIL, SCTRAIL 5′-upstream region lacks a recognizable TATA box [26,27], but it has a number of putative transcription factor binding sites such as AP-1 and SP-1. In human TRAIL gene promoter, AP-1 supplies the binding site for interferon to regulate TRAIL surface expression [28—30]. Sp-1 plays a role in both basal and inducible TRAIL expression [26]. The promoter region of SCTRAIL also has an NF-κB binding site, which has an essential role for NF-κB in the regulation of TRAIL in human primary T lymphocytes [31]. All these important regulation sites existing in the SCTRAIL promoter region may imply that SCTRAIL has similar expression regulation mechanisms.

The mRNA and protein of SCTRAIL gene are ubiquitously distributed in all examined tissues, which is similar to the pattern observed in the expression of TRAIL in mammals [9,10,25,32]. Although the expression of SCTRAIL at the level of mRNA and protein as revealed by RT-PCR and Western blotting cannot be considered as quantification, the strong bands observed in head kidney, spleen, kidney, and intestine may to some extent imply that SCTRAIL may have some role in fish immune system, but this speculation needs to be tested.

To examine the apoptotic function of SCTRAIL, the *in vitro* cultured HeLa cell and Jurkat cell were treated with the soluble SCTRAIL obtained from prokaryotic expression in *E. coli*, but no apoptosis was detected in the treated cells (data not shown); this may be explained in two ways: one is that the prokaryotic expression system cannot supply well post-translational modification for eukaryotic gene and cannot produce an intact active SCTRAIL and another reason is that SCTRAIL may not have the apoptosis-inducing ability at all, unlike its mammalian counterpart [33]. The *in vitro* overexpression of SCTRAIL in eukaryotic cell, according to the method adopted by Long et al. [34] when investigating the function of zebrafish haematopoietic death receptor, induced typical apoptosis in transfected HeLa cells, however, the mutant plasmid SCTRAIL-C230S cannot induce the same phenomena, which indicated that apoptosis is specific and not a fusion protein artifact. All these demonstrated that the cloned SCTRAIL is the TRAIL-like gene with similar functions as its mammalian counterpart.

In conclusion, a TRAIL-like gene has been cloned and identified in the mandarin fish *S. chuatsi*. The *S. chuatsi* TRAIL (SCTRAIL) is similar to other TRAILs reported in other vertebrates in gene sequence, primary structure and constitutive expression. It is further demonstrated, for the first time, that fish TRAIL gene can induce cell apoptosis when overexpressed in cancer cells.

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