Amino Acids (2008) 35: 129–137
DOI 10.1007/s00726-007-0612-2
Printed in The Netherlands

Amino Acids

Cloning and functional characterization of the HRASLS2 gene

R.-Y. Shyu[1], **Y.-C. Hsieh**[2,3], **F.-M. Tsai**[2], **C.-C. Wu**[4], and **S.-Y. Jiang**[2,3]

[1] Department of Internal Medicine, Buddhist Tzu Chi General Hospital, Taipei Branch, Sindian, Taipei, Taiwan, Republic of China
[2] Department of Research, Buddhist Tzu Chi General Hospital, Taipei Branch, Sindian, Taipei, Taiwan, Republic of China
[3] National Defense Medical Center, Department of Microbiology and Immunology, Taipei, Taiwan, Republic of China
[4] Department of Surgery, Tri-Service General Hospital, Taipei, Taiwan, Republic of China

Received July 10, 2007
Accepted September 6, 2007
Published online December 28, 2007;

Summary. The HRAS-like suppressor 2 (*HRASLS2*) gene belongs to the H-REV107 gene family involved in the regulation of cell growth and differentiation. *HRASLS2* is expressed at high levels in normal tissues of the small intestine, kidney, and trachea. We cloned *HRASLS2* cDNA from human SW480 colon cancer cells. Most wild-type, and some N- and C-terminal truncated HRASLS2 (HRASLS2ΔNΔC) were expressed as a granular pattern located at perinuclear region in HtTA cervical cancer cells, while truncation at the C-terminus only (HRASLS2ΔC) resulted in a diffuse pattern. Wild-type HRASLS2 significantly suppressed colony formation of HeLa and HCT116 cells. HRASLS2ΔNΔC significantly inhibited colony formation of HCT116 cells, but HRASLS2ΔC did not affect cell growth. HRASLS2 suppressed the RAS-GTP levels and total RAS protein by 44% and 25%, respectively in HtTA cells; however, the suppression was not observed in truncated HRASLS2 variants. In conclusion, the HRASLS2 protein suppressed growth and RAS activities of cancer cells, and the C-terminal hydrophobic domain appeared to be indispensable for both activities.

Keywords: HRASLS2 – Growth suppression – RAS – Tumor suppressor gene – Apoptosis – RARRES3 – HRASLS3

Abbreviations: β2-M, β2 microglobulin; EGFP, enhanced green fluorescent protein; FBS, fetal bovine serum; G-6PD, glucose 6-phosphate dehydrogenase; HRASLS2, HRAS-like suppressor 2; HRASLS3, HRAS-like suppressor 3; LDH, lactate dehydrogenase; LRAT, Lecithin:retinol acyltransferase; PBS, phosphate-buffered saline; RARRES3, retinoic acid receptor responder 3

Introduction

HRASLS2 (*HRAS-like suppressor 2*) is a member of the HREV107 type II tumor suppressor genes that belong to the Nlpc/p60 superfamily and LRAT (Lecithin:retinol acyltransferase)-like family (Anantharaman and Aravind, 2003). Proteins encoded by this gene family include *RARRES3/RIG1/TIG3* (DiSepio et al., 1998; Huang et al., 2000), *HRASLS3/HREV107* (Hajnal et al., 1994; Husmann et al., 1998), *HRASLS/A-C1* (Akiyama et al., 1999; Ito et al., 2001), *HRASLS2*, and *HRLP5*. The gene family is located at the region between chromosome 11q12.3 and 13.1 with the exception of *HRASLS*. With the exception of *HRLP5* that is predicted to encode a protein with 279 amino acids, cDNAs of the gene family encode proteins ranging from 162 to 168 amino acids with molecular weight of 18 kDa. The family proteins are evolutionarily conserved from virus to vertebrates (Hughes and Stanway, 2000; Anantharaman and Aravind, 2003), and contain an NC domain that is characterized by the presence of a well-conserved NCEHF motif (Marchler-Bauer et al., 2005). The exact role of this domain remains unclear, although a recent study demonstrated that a dodecapeptide containing the NCEHF motif of RARRES3 and HREV107 exhibited DNA binding and anti-proliferative properties (Simmons et al., 2006). In addition, the family proteins contain a hydrophobic membrane-anchoring domain at the C-terminus, which is involved in the endomembrane localization of the proteins (Sers et al., 1997; Akiyama et al., 1999; Tsai et al., 2007).

The HRASLS3, RARRES3, and HRASLS are involved in the regulation of cellular growth, apoptosis, and differentiation. The genes are expressed in normal tissues in a tissue-specific manner, and are downregulated in various cancer tissues (Duvic et al., 2000; Siegrist et al., 2001; Shyu et al., 2003; Lotz et al., 2005). The proteins exhibit growth suppressive activities when ectopically expressed in various types of cancer cells and RAS transformed fibroblasts (Hajnal et al., 1994; Sers et al., 1997; Akiyama et al., 1999; Huang et al., 2002; Higuchi et al.,

2003; Sturniolo et al., 2003; Lotz et al., 2005; Tsai et al., 2007). In addition, HRASLS3 and RARRES3 induce cellular apoptosis that is mediated through caspase-dependent and independent pathways (Sturniolo et al., 2003; Lotz et al., 2005; Tsai et al., 2007). *RARRES3* is expressed at high levels in differentiated tissues of normal and malignant tissues derived from skin (Duvic et al., 2000) and colorectum (Shyu et al., 2003). Terminal differentiation accompanied by the induction of cellular apoptosis and inhibition of cell growth has been observed in keratinocytes followed by induced *RARRES3* expression (Sturniolo et al., 2003). Therefore, RARRES3 appears to play an active role in the regulation of cellular differentiation in both normal and cancer cells.

The HREV107 family proteins regulate the activation of RAS and tissue transglutaminase. HRASLS3, RARRES3, and HRASLS exhibit activities to suppress RAS-mediated fibroblast transformation (Hajnal et al., 1994; Sers et al., 1997; Akiyama et al., 1999; Deucher et al., 2000). Subsequent analysis demonstrates that RARRES3 suppresses levels of total RAS and induces RAS proteins to localize to the Golgi apparatus in transfected cancer cells (Tsai et al., 2006, 2007). The suppression of RAS activation and induction of cellular apoptosis are both mediated by the Golgi-localized RARRES3. Besides exhibiting effects on RAS, RARRES3 activates tissue transglutaminase in human keratinocytes, and the activation is required for RARRES3-induced cellular apoptosis and differentiation (Sturniolo et al., 2003, 2005). Endomembrane localization of HREV107 family proteins is indispensable for their effects on RAS and tissue transglutaminase, since the truncation of C-terminal hydrophobic domain that leads to homogenous cytoplasmic localization of the family proteins results in complete devoid of the function (Sers et al., 1997; Akiyama et al., 1999; Deucher et al., 2000; Tsai et al., 2006, 2007). However, the C-terminal deleted murine H-rev107 and RARRES3 maintain the activities to suppress colony formation of RAS-transformed fibroblasts, albeit at weaker levels (Sers et al., 1997; Deucher et al., 2000). Therefore, the hydrophobic domain of HREV107 family proteins is involved in targeting the family proteins to distinct subcellular localization where they interact with effectors like RAS and transglutaminase. Whether the conserved NC domain is involved in the effects on RAS and transglutaminase remains unclear.

Although five members of the HREV107 families have been reported so far, the exact activities of HRASLS2 and HRLP5 remain to be investigated. Here, we investigated the expression profile of *HRASLS2* in normal tissues and cancer cell lines as well as the activities of HRASLS2 on cellular growth, apoptosis, and RAS activation in cancer cells. The results demonstrate that *HRASLS2* is expressed at high levels in small intestine, kidney, and trachea. The wild-type HRASLS2 exhibited growth suppressive and anti-RAS activities on HtTA colon cancer cells, and truncation at the C-terminal alone or N- and C-terminus resulted in the decrease or devoid of the activities.

Materials and methods

Cell culture

HeLa Tet-Off (HtTA) cervical cancer (obtained from Dr. T.-C. Chang, Department of Biochemistry, National Defense Medical Center, Taiwan), colon cancer (COLO205, CC-M1, CC-M2, LS174T, SW480, H3347 and HCT116), acute promyelocytic leukemia (HL-60), prostate cancer (Du-145) and gastric cancer (TSGH9201 and SC-M1) cells (obtained from Food Industry Research and Development Institute, Taiwan) were maintained in RPMI1640 medium supplemented with 25 mM HEPES, 26 mM $NaHCO_3$, 2 mM L-glutamine, penicillin (100 units/ml), streptomycin (100 μg/ml), and 10% fetal bovine serum (FBS) at 37 °C and in an atmosphere of 5% CO_2 in air. Human MCF-7 breast cancer cells (obtained from Food Industry Research and Development Institute, Taiwan) were maintained in DMEM medium supplemented with 2 mM L-glutamine, penicillin (100 units/ml), streptomycin (100 μg/ml) and 10% FBS at 37 °C and in an atmosphere of 5% CO_2 in air. Cells were routinely detected for free of mycoplasma by staining with the 4′,6-diamidino-2-phenylindole dye.

Transfection

Cells plated in culture dishes overnight were transfected with expression or control vectors using the method of liposome-mediated transfection. Briefly, plasmids and lipofectamine plus^{TM} (Life Technology, Germany) were diluted in Opti-MEM medium and then mixed at room temperature for 15 min. The DNA/lipofectamine plus complexes were then added into cells for 2.5 h at 37 °C. Cells were washed twice and cultured in medium containing 10% FBS.

Construction of expression vectors

The plasmid encoding the constitutively activated RAS^{G12V} was purchased from UMR cDNA Resource Center (Rolla, MO). The pRIG1-myc that encoded the RARRES3 fusion protein with myc and His epitopes has been described previously (Huang et al., 2002). The open reading frame of HRASLS2 cDNA, corresponding to nucleotides 57–545 of the sequence deposited in the NCBI database under the accession number NM_017878.1 was amplified from SW480 cells using 5′ (5′-AAGACATCATCTTGAA GGAAGG-3′) and 3′ (5′-TGGATTTATTGCCTTTCCCG-3′) primers. The cDNA fragment was first cloned into the TA cloning vector (Invitrogen, Carlsbad, CA), and then subcloned in-frame into *Eco*RI–*Hin*dIII sites of the pEGFP-C1 vector (Invitrogen) to produce pEGFP-HRSLS2. HRASLS2 cDNA amplified from pEGFP-HRASLS2 using 5′ (5′-GGG AATTCAGGATGGCTTTGGCCAG-3′) and 3′ (5′-CGAAGCTTTTGCC TTTCCCGCTTG-3′) primers, and then subcloned in-frame into *Eco*RI–*Hin*dIII sites of the pcDNA-myc-His vector (Invitrogen) to produce pHRASLS2-myc. To generate pHRASLS2ΔC-myc, the HRASLS2ΔC cDNA fragment, corresponding to amino acids 1–136, was amplified from pHRASLS2-myc using 5′ (5′-GGGAATTCAGGATGGCTTTG

GCCAG-3′) and 3′ (5′-CCCAAGCTTTGTCGTGACTGCACCAG-3′) primers and then subcloned in-frame into *Eco*RI–*Hin*dIII sites of the pcDNA–myc–His vector. To generate pEGFP-HRASLS2ΔC, the HRASLS2ΔC cDNA fragment was amplified using 5′ (5′-GGGAATT CTAGGATGGCTTTGGCCAGA-3′) and 3′ (5′-TCCCCCGGGTTATGT CGTGACTGCACC-3′) primers and then subcloned in-frame into *Eco*RI–*Sma*I sites of pEGFP-C1 vector. The HRASLS2ΔNΔC DNA fragment, corresponding to amino acids 20–136, was amplified using 5′ (5′-GGGAATTCAGGATGGCTATGCACACTGG-3′) and 3′ (5′-CCCAAG CTTTGTCGTGACTGCACCAG-3′) or 5′ (5′-CGGAATTCTAGGATGG GCTATGCACACTGG-3′) and 3′ (5′-TCCCCCGGGTTATGTCGTGA CTGCACC-3′) primer pairs and subcloned in-frame into *Eco*RI–*Hin*dIII sites of the pcDNA–myc–His vector or *Eco*RI–*Sma*I sites of pEGFP-C1 vector, respectively, to produce pHRSLS2ΔNΔC-myc or pEGFP-HRASLS2ΔNΔC. The cDNA sequences of fusion proteins were confirmed by DNA sequencing.

Western blotting

Cells were washed twice with phosphate-buffered saline (PBS) and total cellular lysates were prepared by lysing cells in lysis buffer (50 mM Tris–HCl pH 7.5, 150 mM NaCl, 1% Triton X-100, 1% Na deoxycholate, 0.1% SDS, 1 mM phenylmethylsulfonyl fluoride, 50 μg/ml aprotinin, 10 mg/ml leupectin, 10 mM β-glycerophosphate). Proteins were separated on 10 or 15% polyacryamide gels and transferred to polyvinylidene difluoride membranes. After blocking, blots were incubated first with anti-myc (Invitrogen), anti-EGFP (BD biosciences, Palo Alto, CA), or anti-actin (Sigma) monoclonal antibodies at 4 °C overnight and then incubated with horseradish peroxidase-conjugated goat anti-mouse antibodies at room temperature for 1 h. The ECL kit (Amersham, Bucks, U.K.) was used for substrate reaction.

Confocal and immunofluorescent analysis

Cells were plated on poly-L-lysine coated coverslips in 35-mm dishes at the density of 8×10^4 cells per dish in RPMI-1640 medium, containing 10% FBS overnight. Cells were then transfected with 1 μg of indicated EGFP-tagged HRASLS2 expression vector for 18 h. Cells were washed, fixed with 4% paraformaldehyde and analyzed with a laser scanning confocal microscope (Zeiss LSM510, Carl Zeiss Jena GmbH, Jena, Germany). The EGFP fusion proteins were detected at the excitation wavelength of 458 nm and the emission wavelength of 505–530 nm. Alternatively, expression of EGFP-tagged HRASLS2 fusion protein was observed using Olympus inverted microscope (Tokyo, Japan).

Analysis of cell death and apoptosis

Cells were plated in triplicate 6-well plates at the density of 6×10^4 cells per well in RPMI-1640 medium, containing 10% FBS overnight. Cells were transfected with 1 μg of various HRASLS2 expression or empty control vector and then refreshed with complete media immediate and 24 h after transfection. The Cytotoxicity Detection Kit (Roche Molecular Biochemicals, Germany) was used to detect the cell death by measuring the lactate dehydrogenase (LDH) activity in the culture medium. A 1000-μl sample of medium was removed well from each culture well every 24 h after transfection and then centrifuged at 1500 rpm at 4 °C for 10 min. One hundred microliter supernatant from each sample well was incubated with LDH reaction mixture at room temperature for 1 h. The absorbance of the sample at 492 and 650 nm was determined using a microplate reader. The percentage LDH release was defined as $[(A_{492}-A_{650})$ of HRASLS2 transfected cells$/(A_{492}-A_{650})$ of control transfected cells$] \times 100\%$. Chromatin condensation was used as a measure of cellular apoptosis. Briefly, cells in 6-cm dishes were transfected with 1.5 μg of EGFP or EGFP-HRASLS2 expression vector for 30 h and fixed with 4% paraformaldyhyde. After washing twice with cold PBS, cells were incubated at room temperature for 10 min with 0.2 μg/ml 4′,6-diamidino-2-phenylindole and 4% bovine serum albumin. The percentage of cells with chromatin condensation among 270 cells that showed green fluorescence was recorded using a fluorescence microscope (Olympus, Tokyo, Japan).

Colony formation assay

Cells were plated in triplicate in 60-mm dishes overnight and then transfected with HRASLS2 expression vectors or with control vector for one day. Cells were trypsinized and then plated triplicate into 6-well dishes at 1:20 to 1:30 dilutions for one day. Cells were then cultured in medium, containing 600 μg/ml G-418 for 10 to 14 days. Afterwards, cells were washed twice with PBS and incubated with solution containing 5% crystal violet, 1.9% formaldehyde, 50% ethanol, and 0.15 M NaCl at room temperature for 30 min. After washing and drying, colonies were recorded using the Kodak gel logic 100 image system and then calculated by the Kodak 1D Image Analysis software version 3.6.3 (Eastman Kodak, NY).

Semi-quantitative reverse transcription polymerase chain reaction (RT-PCR)

Human tissue total RNA was obtained from BD Biosciences. Total RNA from cancer cell lines was isolated using the TRIzol® reagent (Invitrogen), and cDNA was prepared using M-MLV reverse transcriptase (Invitrogen) and oligo $(dT)_{12-18}$. Semi-quantitative RT-PCR was used to analyze levels of HRASLS2 expression in normal tissues and cancer cells. The PCR reaction was conducted in a 25-μl reaction mixture containing 1 μl of cDNA, 10 mM Tris-HCl (pH 9.0), 50 mM KCl, 0.01% (w/v) gelatin, 0.1% Triton X-100, 2 mM $MgCl_2$, 20 μM dNTP, 100 nM primers for HRASLS2, glucose 6-phosphate dehydrogenase (G-6PD), or β2 microglobulin (β2-M) and 1 unit of Pro-Taq DNA polymerase (Protech Technology Enterprise Co, Taipei, Taiwan). The reaction mixtures were subjected to initial 95 °C incubation for 2 min and then amplified for 22 to 38 cycles (94 °C for 60 sec, 64 °C for 60 sec, and 72 °C for 60 sec) of amplification in a programmable thermal cycler (MJ Research Inc., Watertown, MA) following by initial 95 °C incubation for 2 min. Five microliter of the amplified product was analyzed by electrophoresis on a 2% agarose gel, and images were recorded using the Gel Logic 100 Imaging system (Kodak). The relative intensities of bands were quantified using the ImageGauge 4.0 software (Fujifilm, Tokyo, Japan). Primers used for amplification of HRASLS2 (5′-GGGAATTCAGGATGGCTTTGGCCAG-3′ and 5′-CGA AGCTTTTGCCTTTCCCGCTTG-3′), G-6PD (5′-GTGAAGCTCCCTG ACGCCTATGA-3′ and 5′-TGGTGCAGCAGTGGGGTGAAA-3′) and β_2-M (5′-CAGCAGAGAATGGAAATGCAA-3 and 5′-TGTTGATGTT GGATAAGAGAATT-3′) are indicated.

RAS activity assay

RAS activity was assessed with an RAS activation assay kit from Upstate Biotechnology (Lake Placid, NY) as described previously (Tsai et al., 2006). Briefly, cells in a 6-cm dish were washed with ice-cold PBS and then lysed in 200 μl of MLB buffer (25 mM HEPES [pH 7.5], 150 mM NaCl, 1% lgepal CA-630, 10 mM $MgCl_2$, 1 mM EDTA, 10% glycerol), containing protease inhibitors (20 μg/ml aprotinin, 20 μg/ml phenylmethylsulfonyl fluoride), and phosphatase inhibitors (2 mM NaF, 1 mM Na_3VO_4). After centrifugation at $14{,}000 \times g$ for 5 min, cell lysates containing 300 μg of protein were incubated at 4 °C for 45 min with 5 μl of agarose bound with glutathione S-transferase fusion protein corresponding to the human RAS-binding domain (RBD, residues 1–149) of RAF1. The samples were washed three times with MLB buffer containing protease and phosphatase inhibitors, the presence of activated RAS (RAS-GTP) was determined by Western blotting analysis with anti-RAS monoclonal antibody (Clone 10; Upstate Biotechnology).

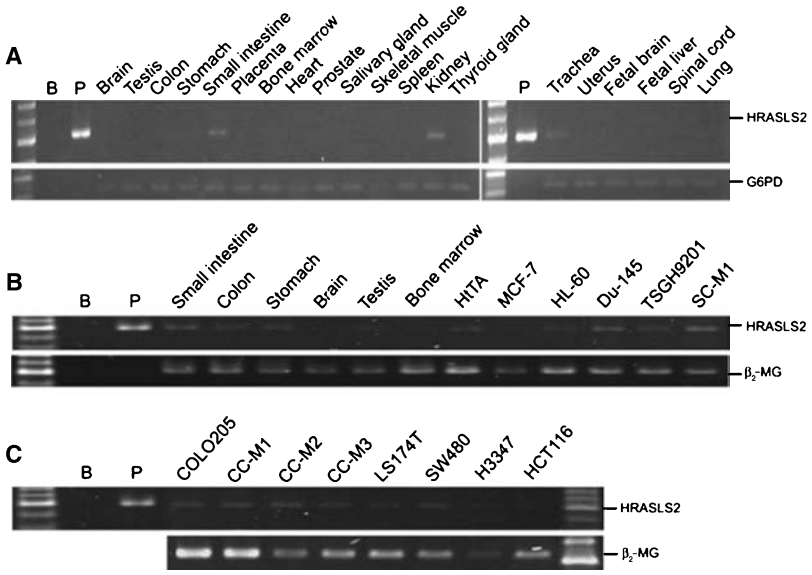

Fig. 1. Analysis of HRASLS2 expression in normal tissues and cancer cell lines. cDNA from total RNA of normal human tissues (**A**, **B**) or 20 cancer cell lines (**B**, **C**) was prepared. Expression of HRASLS2 was analyzed by semi-quantitative RT-PCR. Expression of G6PD (**A**) or β2-MG (**B**, **C**) analyzed by RT-PCR was served as the control. **B** without cDNA addition; P, addition of pHRASLS2-myc during PCR amplification

Results

HRASLS2 expression in normal tissues and cancer cell lines

The expression of *HRASLS2* was investigated in 20 normal tissues using semi-quantitative RT-PCR. High levels of *HRASLS2* cDNA with 504 bp in length was amplified from cDNA derived from normal tissues of small intestine, kidney, and trachea after 33 cycles of amplification (Fig. 1A, B). Low levels of *HRASLS2* expression were detected after 38 cycles of amplification in most tissues with the exception of testis, placenta, salivary gland, and fetal brain (data not shown). In addition, the expression of *HRASLS2* was detected in most cancer cell lines studied herein, including cancer cell lines derived from breast, colon, prostate, cervix, stomach, and leukemia (Fig. 1B, C). Among these, *HRASLS2* expression was the lowest in MCF-7 and HCT116 cells.

Cloning and expression of HRASLS2

The coding region of *HRASLS2* cDNA that encoded an 18 kDa HRASLS2 protein with 162 amino acids was amplified from SW480 colon cancer cells. HRASLS2 protein shares 59–65% amino acid sequence homology with other proteins (HRASLS3, RARRES3, HRASLS, and HRLP5) of the family (Fig. 2). The protein contains proline-rich sequences in the first 20 amino acids and a hydrophobic region located at the C-terminal 26 amino acids. Also, an NC domain located between amino acids 52 and 133 is shown.

```
          1                                                  50
HRASLS2
HRASLS3
RARRES3
 HRASLS       mafndc fslnyp
  HRLP5  mglspgaege yalrlpripp plpkpasrta gtgpkdqppa lrrsavphsg
Consensus

         51                                                  100
HRASLS2
HRASLS3
RARRES3
 HRASLS
  HRLP5  lnsiaplclc csvgfaalvq lpakqpppgt lcqgrsiqqg ckavvalctt
Consensus

        101                                                  150
HRASLS2                           --lar-r- rl------s- f--a------
HRASLS3                           -rapi-e- k--------- -f-r------
RARRES3                           --sphqe- k--------- l--e---l-i
 HRASLS                             gn- c------v-- ---q---l-l
  HRLP5  psqkadwssi pkpenegkli kqa-egk-r- r--------- i--e------
Consensus                          MA---P-P -PGDLIEIFR PGY-HWAIYV

        151                                                  200
HRASLS2 ---------- a--ia----- --l-a-t--- i--k---sv- a---n-----
HRASLS3 ---------- ---va----- --m-a-td-- i--k---y-- a-s-k-q---
RARRES3 -----i---- ---yp---ss --f-v---s- e--r-r-e-- v--cc-----
 HRASLS -----ini-- vdgip -sft -ak-vf-s-- l--mq--k-- v-n-t--i--
  HRLP5 e-dc------ ---efev-   -it-if--r- v--ysr-e-- lh-cswk---
Consensus GDGYVVHLAP PSE--GAGAA SV-S-LSNKA -VK-ELL-DV -GGD-YRVNN

        201                                                  250
HRASLS2 -h-dr-t--- sn--vk---- l----lp--- t-d------- h-----s--d
HRASLS3 -h-dk-s--- cs---q---- l-----l-k- t-e------- e-----a--d
RARRES3 sl-he-q-r- -ev--ss-k- m---kmk--i v-r------t q----ks-ck
 HRASLS -y-et-p--- -ee--k-s-f vi----a-n- lvn------t l----egv-e
  HRLP5 -l-gt-l--- -d---q-tkk m-nki-q--- ieg------- g-----p--q
Consensus K-D--Y-PLP V-KII-RAEE -VGQEV-YSL -S-NCEHFVN -LRYGV-RS-

        251                                  287
HRASLS2 --tg-vtt-g --agl-a-a- lv-il la-s -rerq
HRASLS3 --rdviiaas --gmg-a-m- li-vm fs-n -rqkq
RARRES3 --ek-kve-q --ta -qilv va-csfai-r yq-kata
 HRASLS -anr-ist-e fvtaavgvf- fl-lf pkgq ra-yy
  HRLP5 --eh-lmega k-ag   -vi sa   vvdsi -p-pita
Consensus OV--A---V- VA---L-A-S --G-----r- K-K----
```

Fig. 2. Multiple amino acid sequence alignment of the HRASLS2, RARRES3, HRASLS3, HRASLS, and HRLP5. Region of the NC domain is underlined. The similarities between five proteins were analyzed by T-COFEE program (http://us.expasy.org/cgi-bin/blasr.pl)

To analyze the expression pattern and function of the wild-type HRASLS2 and the protein with a N- and/or C-terminal truncation, expression vectors that synthesized full length, C-terminal truncated (HRASLS2ΔC), or N- and C-terminal truncated (HRASLS2ΔNΔC) HRASLS2 fusion protein containing myc or EGFP tag were constructed and transfected into HtTA cervical cancer cells. Myc-

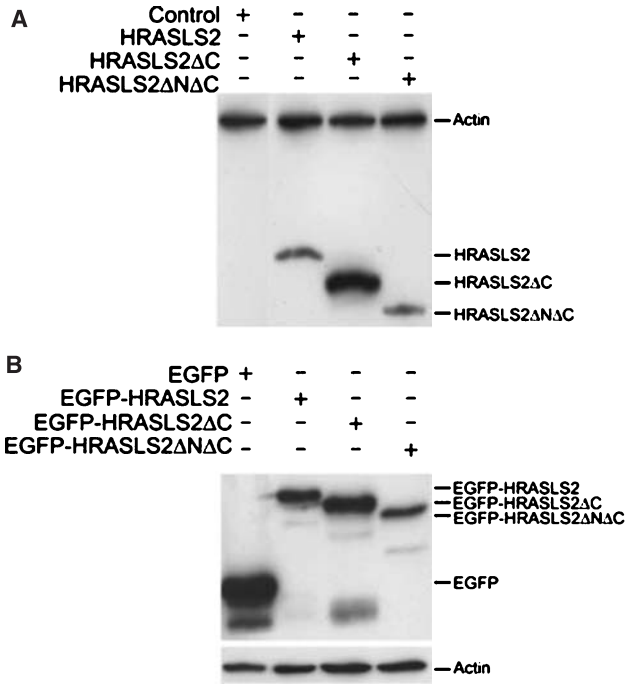

Fig. 3. Western blot analysis of HRASLS2 fusion proteins. HtTA cervical cancer cells were transfected with indicated myc- (**A**) or EGFP-tagged (**B**) HRASLS2 expression vectors or control vector for 24 h. Cytosols were prepared and proteins were separated on SDS-PAGE gels. Expression of HRASLS2 fusion proteins was analyzed by anti-myc (**A**) anti-EGFP (**B**) and anti-actin monoclonal antibodies

tagged HRASLS2, HRASLS2ΔC, or HRASLS2ΔNΔC fusion proteins with expected molecular weights of 20.3, 17.7, and 15.6 kDa, respectively, was detected in cytosol extracts prepared from HtTA cells followed by transfection for 24 h (Fig. 3A). Similarly, EGFP tagged HRASLS2, HRASLS2ΔC, or HRASLS2ΔNΔC fusion proteins with the expected molecular weight of 46.4, 43.1, or 41.0 kDa, respectively, were detected (Fig. 3B).

The distribution of wild-type and truncated HRASLS2 protein was analyzed in HtTA cells followed by transfection with various EGFP tagged HRALSLS2 expression vectors for 24 h. Images of fluorescent microscopy showed that the control EGFP and HRASLS2ΔC proteins appeared as diffused patterns and were distributed in both cytoplasm and nucleus with preferential nuclear localization (Fig. 4). In contrast, the wild-type HRASLS2 protein was present as a granular pattern in the cytoplasm with preferential perinuclear localization. HRASLS2ΔNΔC was primarily observed as a diffused pattern with equal distribution between cytoplasm and nucleus. However, less than 5% of HRASLS2ΔNΔC protein was present as granular pattern with a distribution pattern similar to the wild-type HRASLS2 protein.

HRASLS2 proteins suppress colony formation and induce cell death

Three members of the HREV107 protein family (i.e., RARRES3, HRASLS3, and HRASLS) exhibit growth suppressive activities (Hajnal et al., 1994; Sers et al., 1997; Akiyama et al., 1999; Huang et al., 2002; Higuchi et al., 2003; Sturniolo et al., 2003; Lotz et al., 2005; Tsai et al., 2007). Therefore, we investigated the involvement of myc-tagged wild-type and truncated HRASLS2 fusion proteins in the growth and death of cancer cells. The formation of G-418 resistant colonies was evaluated followed by transfection for 14–20 days. Expression of the myc-tagged wild-type HRASLS2 and RARRES3 resulted in significant ($P < 0.01$) inhibition of colony formation by 54 and 74%, respectively, on HeLa cells (Fig. 5A). Colony formation of the HCT116 colon cancer cells was also significantly inhibited by 46.4% followed by transfection with the wild-type HRASLS2 expression vector (Fig. 5B). The HRASLS2ΔNΔC exhibited a weaker activity as compared to the wild-type protein, and significantly suppressed the colony formation of HCT116 cells by 22.8%. In contrast, the C-terminal truncated HRASLS2ΔC increased colonly formation of HeLa cells or had no effect on the colony formation of HCT116 cells. The wild-type and truncated HRASLS2 had no significant effects on colony formation of MCF-7 cells (Fig. 5C).

Furthermore, MCF-7 cells were transfected with EGFP or EGFP-HRASLS2 expression vector. When resistant colonies, after maintaining in G-418 containing medium for 20 days, were evaluated for fluorescence-positive clones, fluorescence-positive clones were rarely observed in cells transfected with EGFP-HRASLS2 expression. However, less than 10 cells expressed the protein within the fluorescence-positive clone in EGFP-HRASLS2 transfected cells, which is in contrast to almost all fluorescence-positive cells detected within the positive clone derived from EGFP-transfected cells. Therefore, wild-type HRASLS2 suppressed colony formation of HeLa, HCT116, and MCF-7 cells. HtTA cells are G-418 resistant, and therefore were not analyzed for the effects of HRASLS2 on colony formation.

Effects of HRASLS2 fusion proteins on cellular death were analyzed by measuring the release of LDH from supernatants of transfected HtTA cells. Wild-type HRASLS2 significantly enhanced LDH released by 57–90% followed by transient transfection for 24–72 h with medium refreshed daily (Fig. 6A). The enhancement of LDH release was related to the increase in transfected HRASLS2 expression vector when LDH release was

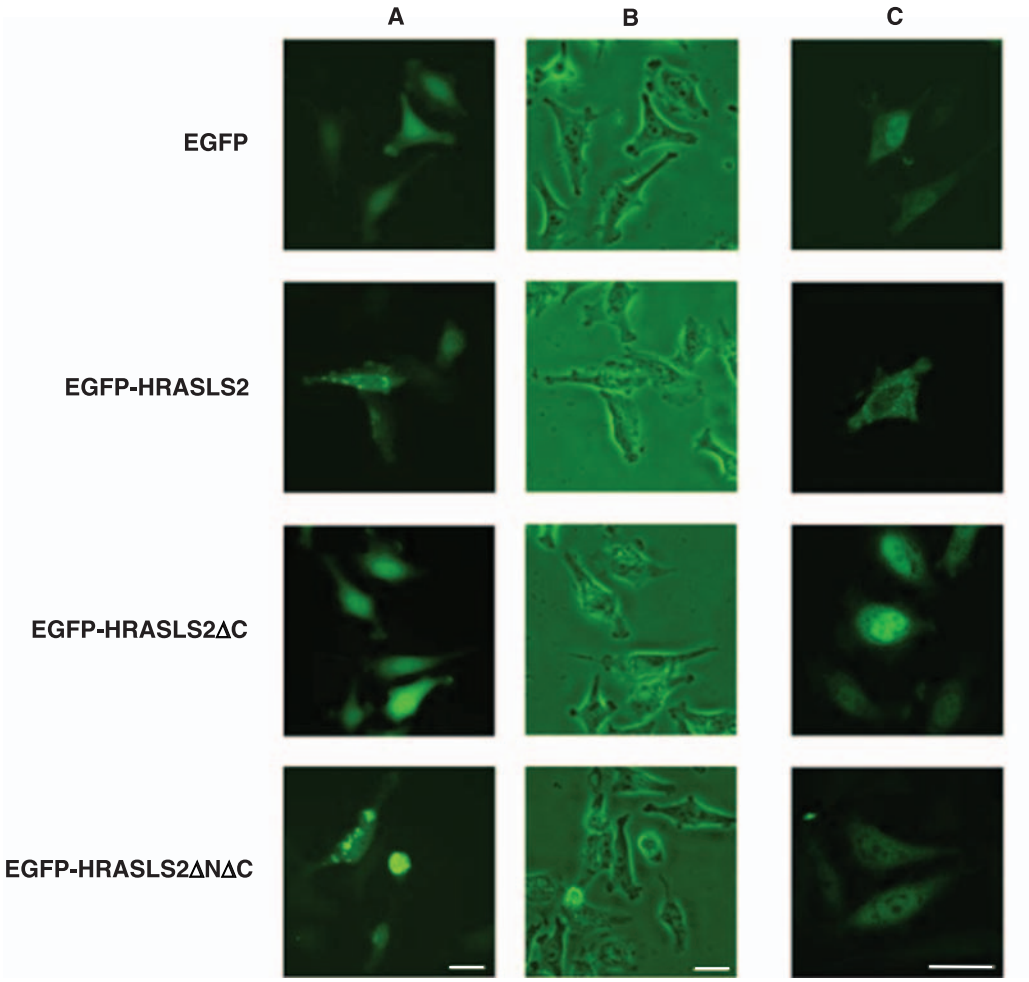

Fig. 4. Expression of HRASLS2 fusion proteins in HtTA cells. Cells were transiently transfected with EGFP or indicated EGFP-tagged HRASLS2 expression vectors for 24 h. Cells were fixed and analyzed for expression of HRASLS2 fusion proteins by fluorescent (**A** and **B**) or confocal (**C**) microscope. Scale bar: 30 μm (for an interpretation of the reference to colour in this figure, the reader is referred to the online version of this paper under www.springerlink.com)

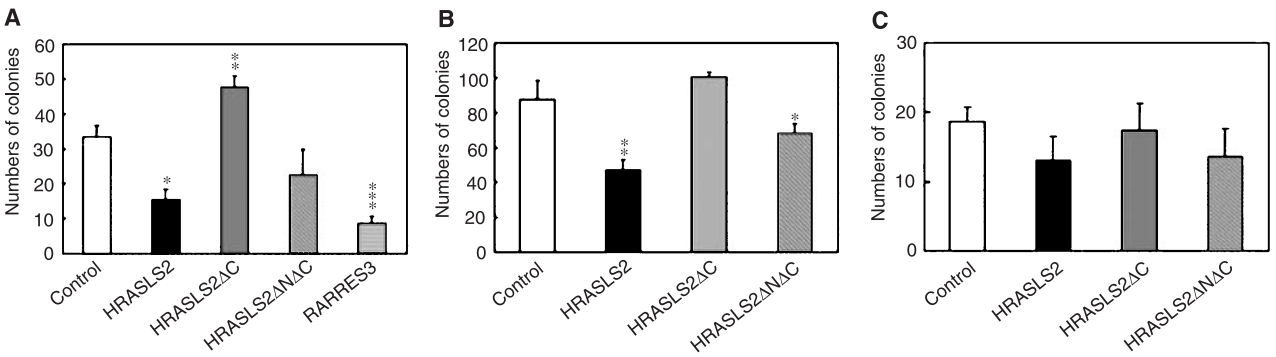

Fig. 5. HRASLS2 suppresses colony formation of cancer cells. HeLa (**A**), HCT116 (**B**), and MCF-7 (**C**) cells were plated in triplicate and then transfected with myc-tagged HRASLS2, HRASLS2ΔC, HRASLS2ΔNΔC, or RARRES3 expression vector or control vector overnight. Cells were cultured in medium containing G-418 for 14 (**A** and **B**) or 20 (**C**) days. Numbers of colonies with diameter larger than 1 mm were measured followed by crystal violet staining. Representative results of three independent experiments are shown and expressed as average and standard error of the means. Student *t*-test: $^{*}P < 0.05$; $^{**}P < 0.01$; $^{***}P < 0.001$

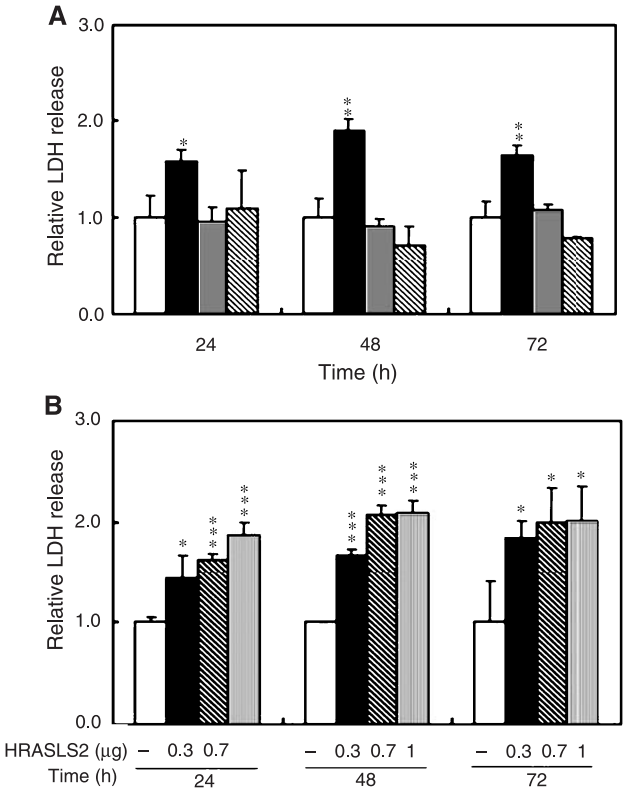

Fig. 6. HRASLS2 induces cell death of HtTA cells. Cells plated in triplicate in 6-well plates were transfected with 1 μg of myc-tagged HRASLS2 expression vectors (open bar: control; closed bar: HRASLS2; gray bar: HRASLS2ΔC; striped bar: HRASLS2ΔNΔC) (**A**) or 0–1 μg HRASLS2 expression vector (**B**). Culture supernatants were collected 24, 48, and 72 h after transfection, and cellular death was detected by measuring LDH release. Representative results of three independent experiments are shown and expressed as average and standard error of the means after normalization with the control group. Student *t*-test: $^{*}P<0.05$; $^{**}P<0.01$; $^{***}P<0.001$

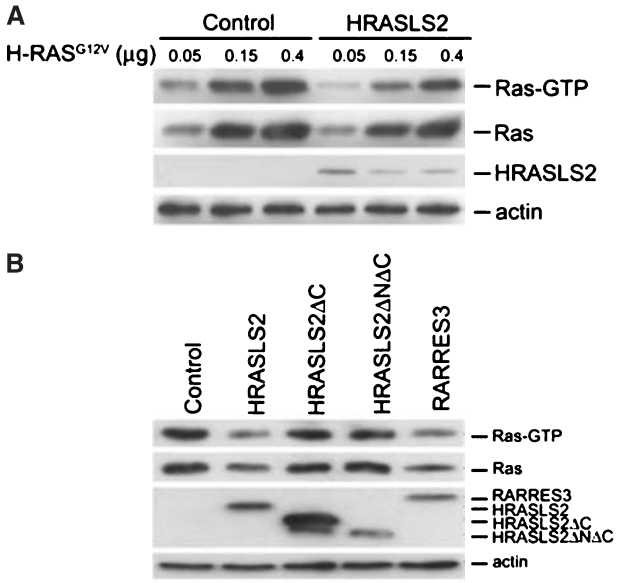

Fig. 7. HRASLS2 inhibited activation of the mutated H-RAS. (**A**) HtTA cells plated in 6-cm dishes were transfected with 1.5 μg of the HRASLS2 expression or control vector along with indicated amount of the activated H-RAS^{G12V} expression vector for 24 h. (**B**) Cells were transfected with 1.5 μg of the indicated wild-type or truncated HRASLS2 expression vectors along with 0.05 μg of the activated H-RAS^{G12V} expression vector for 24 h. Cellular lysates were prepared and the activated RAS (RAS-GTP) was pulled down by agarose-conjugated Raf-RBD, followed by Western blot analysis. The levels of total RAS, HRASLS2 and RARRES3 fusion proteins and actin were detected by Western blotting

analyzed 24 h after transfection (Fig. 6B). In contrast to the result of colony formation, HRASLS2ΔC and HRASLS2ΔNΔC did not increase the LDH release in HtTA cells (Fig. 6A). The wild-type HRASLS2 also exhibited proapoptotic activities on HtTA cells. Chromatin condensation was detected in 27.8% of HtTA cells expressed the EGFP-HRASLS2 followed by transfection for 30 h, as compared to 11.1% detected in cells expressed the EGFP protein (data not shown).

HRASLS2 suppresses the activation of mutated RAS

HRASLS3, HRASLS, and RARRES3 suppress colony formation of H-RAS-transformed fibroblasts. Previous studies from our laboratory showed that RARRES3, HRASLS3, and HRASLS2 inhibit EGF-stimulated endogenous RAS activation (Tsai et al., 2006). This study further analyzed effects of HRASLS2 on the activation of the constitutively activated H-RAS^{G12V} in the HtTA cervical cancer cells. Expression of HRASLS2 resulted in 52% suppression of RAS-GTP levels in HtTA cells transfected with 0.05 μg of H-RAS^{G12V} expression vector (Fig. 7A), and the suppression was declined with the increase in H-RAS^{G12V} expression. Similar to the results shown previously (Tsai et al., 2006), expression of RARRES3 protein resulted in 55 and 36% suppression of RAS-GTP levels and total RAS protein, respectively, and HRASLS2 suppressed RAS-GTP levels and total RAS protein by 44 and 25%, respectively (Fig. 7B). Both truncated HRASLS2 proteins (i.e., HRASLS2ΔC and HRASLS2ΔNΔC) had no effect on the level of RAS-GTP and total RAS protein.

Discussion

The *HRASLS2* gene belongs to the HREV107 gene family. This study shows that the *HRASLS2* is expressed at high levels in normal tissues of small intestine, kidney, and trachea. cDNA of the gene is cloned from SW480 colon cancer cells. Wild-type *HRASLS2* expresses as a

granular pattern at the perinulear region, suppresses colony formation and RAS activation, as well as induces cell death of cancer cells. Truncation of the C-terminal hydrophobic domain results in loss in the suppressive activities of HRASLS2.

Expression of the HREV107 family proteins in various normal tissues has been investigated. This study demonstrated that the HRASLS2 is expressed at the highest levels in normal tissues of small intestine, kidney, and trachea. The HRASLS3 is predominantly expressed in testis, ovary, skeletal muscle, and heart (Sers et al., 1997, 2002). The RARRES3 is primary expressed in skin, colon, lung, and liver tissues (DiSepio et al., 1998; Duvic et al., 2000; Huang et al., 2000; Shyu et al., 2003). The HRASLS is primarily expressed at skeletal muscle and testis (Ito et al., 2001). Thus, tissue-specific expression of HREV107 family genes supports the idea that each gene product is associated with a tissue-specific distinct role. Expression of HRASLS3 and RARRES3 is shown to decrease in various cancer cells or cancer tissues (Duvic et al., 2000; Siegrist et al., 2001; Kaneda et al., 2002; Sers et al., 2002; Shyu et al., 2003). This study did not observe consistent low levels of HRASLS2 in cancer cell lines as compared to the expression in normal tissues. However, HRASLS2 is shown to decrease expression in tissues of renal cell carcinoma (Lenburg et al., 2003). Future research is required to elucidate the exact role of HRASLS2 in various tumors.

Although HRASLS2 belongs to the HREV107 family proteins, activity of the protein has not been investigated. Among HREV107 family proteins, HRASLS3, RARRES3, and HRASLS are shown to exhibit activities to inhibit colony formation and induce cellular apoptosis in vitro or suppress tumor formation in vivo (Hajnal et al., 1994; Sers et al., 1997, 2002; Akiyama et al., 1999; Deucher et al., 2000). Also, RARRES3 induces terminal differentiation of human keratinocytes (Sturniolo et al., 2003). Similarly, results from this study show that the myc- or EGFP-tag wild-type HRASLS2 exhibits growth-suppressive and pro-apoptotic activities on several types of cancer cells, which is supported by the decrease in colony formation and the increase in LDH release and chromatin condensation followed by expression of recombinant HRASLS2. The wild-type HRASLS2 is expressed at the perinuclear region. The HRASLS2ΔC is expressed in diffused pattern and has the loss of growth suppressive activity. Therefore, the C-terminal hydrophobic domain that presumably targeted the protein to the endomembrane at the perinuclear region is indispensable for the growth-related suppressive function of HRASLS2 in cancer cells. However, this study observes that small fractions (less than 5%) of both N- and C-terminal truncated HRASLS2 (HRASLS2ΔNΔC) proteins are expressed at the perinuclear region in addition to a diffuse pattern of expression. This may explain the suppression of colony formation in HRASLS2ΔNΔC transfected HCT116 cells. The N-terminal 26 amino acid region of HRASLS2 contains proline-rich sequences and a conserved histidine residue (Hughes and Stanway, 2000). The difference in activities of colony formation between HRASLS2ΔC and HRASLS2ΔNΔC suggests a unique role of the N-terminal fragment region, which needs further investigation. Interestingly, a recent study shows that the N-terminal proline-rich region of HRASLS3 binds to the subunit of the protein phosphatase 2A (PR65α) and inhibits the enzyme activity (Nazarenko et al., 2007).

The HREV107 family proteins exhibit anti-RAS activities. This is supported by the inhibition of RAS-mediated transformation of fibroblasts followed by expression of HRASLS (Akiyama et al., 1999), HRASLS3 (Hajnal et al., 1994; Sers et al., 1997) and RARRES3 (DiSepio et al., 1998; Deucher et al., 2000). Recently, we have demonstrated that the suppression is mediated at the level of RAS. RARRES3 is co-precipitated with RAS and suppresses levels of total RAS (Tsai et al., 2006). In addition, RARRES3 alters the RAS subcellular distribution. The RAS protein is predominantly expressed in the Golgi apparatus where it co-localizes with RARRES3 (Tsai et al., 2007). Similar to RARRES3, this and our previous studies (Tsai et al., 2006) observed the inhibition of total and activated RAS^{G12V} followed by the expression of HRASLS2. The suppression is specific to the wild-type HRASLS2, and the anti-RAS activity is lost when the protein is truncated at the C-terminus or N- and C-terminus. We demonstrate a lack of direct correlation between anti-RAS and pro-apoptotic activities of RARRES3 (Tsai et al., 2007), which may explain our observation that the HRASLS2ΔNΔC protein does not exhibit anti-RAS activities, but exhibits weak activities in the suppression of colony formation of HCT116 cells. Therefore, the genuine role of the anti-RAS activities of the H-REV107 family proteins is worth investigating in future studies.

In conclusion, *HRASLS2* is highly expressed in normal tissues of the small intestine, kidney, and trachea. The cDNA of *HRASLS2* was cloned and expressed in cancer cells. HRASLS2 exhibited growth-suppressive and anti-RAS activities in human cancer cells. The C-terminal hydrophobic domain is essential for intracellular targeting and HRASLS2 activity. Remarkably, further deletion of the N-terminal domain resulted in a restoration of some of

the growth-suppressive activities of the HRASLS2ΔC protein. Future analysis of the structure-function relationship of HRASLS2 will be useful to pinpoint the role of the functional domain(s) of HRASLS2.

Acknowledgement

The authors thank the support from the Core Laboratory of the Buddhist Tzu Chi General Hospital, Taipei Branch. This study is supported in part by the National Science Council (NSC94-2314-B303-021, NSC94-2320-B303-006) and the Buddhist Tzu Chi General Hospital (TCRD-TPE-95-04, TCRD-TPE-95-07), Taipei, Taiwan, Republic of China.

References

Akiyama H, Hiraki Y, Noda M, Shigeno C, Ito H, Nakamura T (1999) Molecular cloning and biological activity of a novel Ha-Ras suppressor gene predominantly expressed in skeletal muscle, heart, brain, and bone marrow by differential display using clonal mouse EC cells, ATDC5. J Biol Chem 274: 32192–32197

Anantharaman V, Aravind L (2003) Evolutionary history, structural features and biochemical diversity of the NlpC/P60 superfamily of enzymes. Genome Biol 4: R11

Deucher A, Nagpal S, Chandraratna RA, Di Sepio D, Robinson NA, Dashti SR, Eckert RL (2000) The carboxy-terminal hydrophobic domain of TIG3, a class II tumor suppressor protein, is required for appropriate cellular localization and optimal biological activity. Int J Oncol 17: 1195–1203

DiSepio D, Ghosn C, Eckert RL, Deucher A, Robinson N, Duvic M, Chandraratna RA, Nagpal S (1998) Identification and characterization of a retinoid-induced class II tumor suppressor/growth regulatory gene. Proc Natl Acad Sci USA 95: 14811–14815

Duvic M, Helekar B, Schulz C, Cho M, Jackson B, Breuer-McHam J, Young J, Clayman G, Lippman SM, DiSepio D, Hager C, DiMao D, Hazarika P, Chandraratna RA, Robinson NA, Deucher A, Eckert RL, Nagpal S (2000) Expression of a retinoid-inducible tumor suppressor, Tazarotene-inducible gene-3, is decreased in psoriasis and skin cancer. Clin Cancer Res 6: 3249–3259

Hajnal A, Klemenz R, Schafer R (1994) Subtraction cloning of H-rev107, a gene specifically expressed in H-ras resistant fibroblasts. Oncogene 9: 479–490

Higuchi E, Chandraratna RA, Hong WK, Lotan R (2003) Induction of TIG3, a putative class II tumor suppressor gene, by retinoic acid in head and neck and lung carcinoma cells and its association with suppression of the transformed phenotype. Oncogene 22: 4627–4635

Huang SL, Shyu RY, Yeh MY, Jiang SY (2000) Cloning and characterization of a novel retinoid-inducible gene 1(RIG1) deriving from human gastric cancer cells. Mol Cell Endocrinol 159: 15–24

Huang SL, Shyu RY, Yeh MY, Jiang SY (2002) The retinoid-inducible gene I: effect on apoptosis and mitogen-activated kinase signal pathways. Anticancer Res 22: 799–804

Hughes PJ, Stanway G (2000) The 2A proteins of three diverse picornaviruses are related to each other and to the H-rev107 family of proteins involved in the control of cell proliferation. J Gen Virol 81: 201–207

Husmann K, Sers C, Fietze E, Mincheva A, Lichter P, Schafter R (1998) Transcriptional and translational downregulation of H-REV107, a class II tumour suppressor gene located on human chromosome 11q11-12. Oncogene 17: 1305–1312

Ito H, Akiyama H, Shigeno C, Nakamura T (2001) Isolation, characterization, and chromosome mapping of a human A-C1 Ha-Ras suppressor gene (HRASLS). Cytogenet Cell Genet 93: 36–39

Kaneda A, Kaminishi M, Yanagihara K, Sugimura T, Ushijima T (2002) Identification of silencing of nine genes in human gastric cancers. Cancer Res 62: 6645–6650

Lenburg ME, Liou LS, Gerry NP, Frampton GM, Cohen HT, Christman MF (2003) Previously unidentified changes in renal cell carcinoma gene expression identified by parametric analysis of microarray data BMC. Cancer 3: 31

Lotz K, Kellner T, Heitmann M, Nazarenko I, Noske A, Malek A, Gontarewicz A, Schafer R, Sers C (2005) Suppression of the TIG3 tumor suppressor gene in human ovarian carcinomas is mediated via mitogen-activated kinase-dependent and -independent mechanisms. Int J Cancer 116: 894–902

Marchler-Bauer A, Anderson JB, Cherukuri PF, DeWeese-Scott C, Geer LY, Gwadz M, He S, Hurwitz DI, Jackson JD, Ke Z, Lanczycki CJ, Liebert CA, Liu C, Lu F, Marchler GH, Mullokandov M, Shoemaker BA, Simonyan V, Song JS, Thiessen PA, Yamashita RA, Yin JJ, Zhang D, Bryant SH (2005) CDD: a conserved domain database for protein classification. Nucleic Acids Res 33: D192–D196

Nazarenko I, Schafer R, Sers C (2007) Mechanisms of the HRSL3 tumor suppressor function in ovarian carcinoma cells. J Cell Sci 120: 1393–1404

Sers C, Emmenegger U, Husmann K, Bucher K, Andres AC, Schafer R (1997) Growth-inhibitory activity and downregulation of the class II tumor-suppressor gene H-rev107 in tumor cell lines and experimental tumors. J Cell Biol 136: 935–944

Sers C, Husmann K, Nazarenko I, Reich S, Wiechen K, Zhumabayeva B, Adhikari P, Schroder K, Gontarewicz A, Schafer R (2002) The class II tumour suppressor gene H-REV107-1 is a target of interferon-regulatory factor-1 and is involved in IFNgamma-induced cell death in human ovarian carcinoma cells. Oncogene 21: 2829–2839

Shyu RY, Jiang SY, Chou JM, Shih YL, Lee MS, Yu JC, Chao PC, Hsu YJ, Jao SW (2003) RARRES3 expression positively correlated to tumour differentiation in tissues of colorectal adenocarcinoma. Br J Cancer 89: 146–151

Siegrist S, Feral C, Chami M, Solhonne B, Mattei MG, Rajpert-De Meyts E, Guellaen G, Bulle F (2001) hH-Rev107, a class II tumor suppressor gene, is expressed by post-meiotic testicular germ cells and CIS cells but not by human testicular germ cell tumors. Oncogene 20: 5155–5163

Simmons DP, Peach ML, Friedman JR, Green MM, Nicklaus MC, De Luca LM (2006) Evidence that sequence homologous region in LRAT-like proteins possesses anti-proliferative activity and DNA binding properties: translational implications and mechanism of action. Carcinogenesiss 27: 693–707

Sturniolo MT, Chandraratna RA, Eckert RL (2005) A novel transglutaminase activator forms a complex with type 1 transglutaminase. Oncogene 24: 2963–2972

Sturniolo MT, Dashti SR, Deucher A, Rorke EA, Broome AM, Chandraratna RA, Keepers T, Eckert RL (2003) A novel tumor suppressor protein promotes keratinocyte terminal differentiation via activation of type I transglutaminase. J Biol Chem 278: 48066–48073

Tsai FM, Shyu RY, Jiang SY (2006) RIG1 inhibits the Ras/mitogen-activated protein kinase pathway by suppressing the activation of Ras. Cell Signal 18: 349–358

Tsai FM, Shyu RY, Jiang SY (2007) RIG1 suppresses Ras activation and induces cellular apoptosis at the Golgi apparatus. Cell Signal 19: 989–999

Authors' address: Shun-Yuan Jiang, Department of Research, Buddhist Tzu Chi General Hospital, Taipei Branch, No 289 Chien-Kuo Rd, Sindian, Taipei 231, Taiwan, Republic of China,
Fax: +886-2-6628-9009, E-mail: syjiang@tzuchi.com.tw