Molecular characterization of human reticulon 3 as a potential marker during the differentiation of human neuroblastoma SH-SY5Y cells*

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Abstract Neuroendocrine-specific protein (NSP)-reticulons are endoplasmic reticulum-associated protein complexes, which are localized in the endoplasmic reticulum (ER) and identified as markers for neuroendocrine differentiation. In the present study, human reticulon 3 gene (*hRTN3*) was cloned and its expression pattern in a variety of tissues was investigated. Truncated hRTN3s corresponding to the C-terminal (hRTN3-C) domain were expressed and purified. *hRTN3* mRNA was down-regulated during the differentiation of human neuroblastoma cell line SH-SY5Y induced by all-*trans*-retinoic acid (RA), which suggests that, like other members of the reticulon family, *hRTN3* is a potential marker for neuroendocrine differentiation.

Keywords: human reticulon 3, expression, differentiation.

In the last decade, a novel gene family encoding the membrane reticulon (RTN) proteins of endoplasmic reticulum (ER) has been identified. They might play a role in vesicular formation, packaging of secretary products[1] or regulation of intracellular Ca^{2+} levels[2]. These neuroendocrine-specific proteins (NSPs) or reticulons are clinically used as independent markers to distinguish small cell lung cancer (SCLC) from non-SCLC[3,4]. NSPs are present in the SCLC tumors while non-SCLC tumors are generally negative[4]. The NSPs can also be used to distinguish non-SCLC tumors from non-SCLC with neuroendocrine differentiation[4]. NSP, the first member of the neuroendocrine-specific protein family, was cloned by Roeroek et al. using monoclonal antibodies to proteins expressed specifically in small cell lung carcinomas and neuroendocrine tissues[5]. Three transcripts of 3.4, 2.3 and 1.8 kb were identified and found to be alternatively spliced mRNAs originating from one gene[6]. A 66-residue lumenal/extracellular domain of Nogo-A/RTN4-A was found to inhibit axonal extension and collapse dorsal root ganglion growth cones, thus shed some light on the failure of axonal regeneration in the adult central nervous system[7,8]. Recently, RTN-xs/RTN4-B was demonstrated to interact with both Bcl-$_{XL}$ and Bcl-2 on endoplasmic reticulum and thus reduce their anti-apoptotic activity[9].

hRTN3 was firstly isolated during a subtraction cloning between macula and peripheral retina[10]. The mRNA for this NSP/RTN-like gene was found to be approximately threefold more abundant in macula than in peripheral retina. Later, *hRTN3* was found to be underexpressed in chemotherapy-resistant human ovarian cancer cell lines by cDNA microarray[11]. Recently, mouse ortholog (*mRTN 3*) of *hRTN3* was cloned and characterized[12]. The highest expression of *mRTN3* was observed in brain, especially in neurons. However, like most of the other members of *RTN* family, the physiological role of mammalian reticulon 3 is still not fully understood.

In the present paper, we report the cloning and expression pattern of *hRTN3*, prokaryotic expression of the truncated protein, and down-regulation of hRTN3 during the differentiation of human neuroblastoma cell line SH-SY5Y induced by all-*trans*-retinoic acid (RA).

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1 Materials and methods

1.1 Isolation of *hRTN3* cDNA

A total of 895 clones randomly selected from fetal brain λZAP express cDNA library were sequenced on ABI 377 autosequencer. Then 11 clones containing complete 5′ terminal region of the open reading frame (ORF) were identified and inserted into pBK-CMV plasmid. The full-length cDNA sequences of the 11 clones were constructed by primer walking. *hRTN3* cDNA is one of them, its nucleic acid sequence and deduced peptide sequence were searched against the sequences in GenBank using Gapped BLAST program.

1.2 Northern blot analysis

The multiple tissue Northern (MTN™) blot membrane of mRNA from 16 different tissues was purchased from Clontech Ltd. An amplified 436 bp of *hRTN3* cDNA was used as the probe. Hybridization was performed according to the manufacturer's instructions.

1.3 Expression and purification of recombinant hRTN3 C-terminal

PCR with a set of primers (Forward: 5′-GAAGATCTCTCATTTTCAGTCRCCCGATTGTC-3′ Reverse: 5′-GCGTCGACTGGGGCAGGAAGATAGGATGAG-3′) produced a fragment corresponding to part of the 3′-end of *hRTN3* cDNA that encodes the last 46 amino acids. The fragment was sequenced and inserted into pGEX4T-1 vector (Amersham Pharmacia Biotech). *E. coli* BL21 harboring the recombinated plasmid was grown in 1 L LB medium with 50μg/mL kanamycin and 1 mmol/L isopropyl-β-D-thiogalactopyranoside at 37 ℃ for 4 hours. GST fusion proteins were bound to glutathione-sepharose beads (Amersham Pharmacia Biotech) and purified according to the manufacturer's guide. The concentration of the protein solution was determined with BCA™ kit (PIERCE).

1.4 Cell culture

Human neuroblastoma cell line SH-SY5Y was cultured at 37 ℃ in humified atmosphere with 5% CO_2 in RPMI 1640 supplemented with 10% fetal calf serum.

SH-SY5Y cells were induced to differentiate with 20 mmol/L all-*trans* RA. Morphological changes of SH-SY5Y were detected microscopically. Cells were harvested on the 0th, 1st, 2nd, 3rd, 4th, 5th, 6th, 7th and 8th days of induction respectively. Total poly (A)$^+$ RNAs were extracted by Trizol reagent (Invitrogen) and transferred to Hybond-N™ nylon membrane (Amersham, Buckinghamshire, UK) according to the manufacturer's guide.

2 Results and discussion

2.1 Identification of *hRTN3*

The full-length cDNA of *hRTN3* was 2527 bp in length (GenBank accession number: AF119297), containing an open reading frame of 136~847 nt. Its deduced amino acids were shown in Fig. 1. There were two predicted hydrophobic regions at the C-terminal. An endoplasmic reticulum retrieval motif also exists.

Multiple alignments showed that the amino acids at the C-terminal of the protein shared high homology with other members of *RTN* family (Fig. 1). Whereas the first 42 amino acids at the N-terminal of the protein were not homologous to the known members of *RTN* family.

2.2 Tissue expression pattern

Northern hybridization under high stringency conditions revealed that *hRTN3* has three different transcripts, with the length of 2.3 kb, 2.6 kb and 4 kb respectively (Fig. 2). The expression level of *hRTN3* was significantly high in brain compared to other tissues. As shown in Fig. 2, the band of 2.3 kb was more dominant in non-brain tissues, but bands of 2.6 kb and 4 kb showed a high selectivity in brain tissue.

2.3 Expression and purification of recombinant C-terminal of hRTN3 protein

The hRTN3C-GST fused protein was effectively expressed after induction with 1 mmol/L IPTG. The expressed protein was purified by glutathione-sepharose column chromatography. The analysis of the proteins by SDS-PAGE is shown in Fig. 3.

2.4 Down-regulation of *hRTN3* mRNA during differentiation of human neuroblastoma cell line SH-SY5Y induced by all-*trans* RA

Human neuroblastoma cell line SH-SY5Y was induced to differentiation by all-*trans* RA as reported with an apparently elongated process (Fig. 4). During this process, the expression level of *hRTN3* mRNAs decreased, which was especially remarkable from the fourth day of induction (Fig. 5).

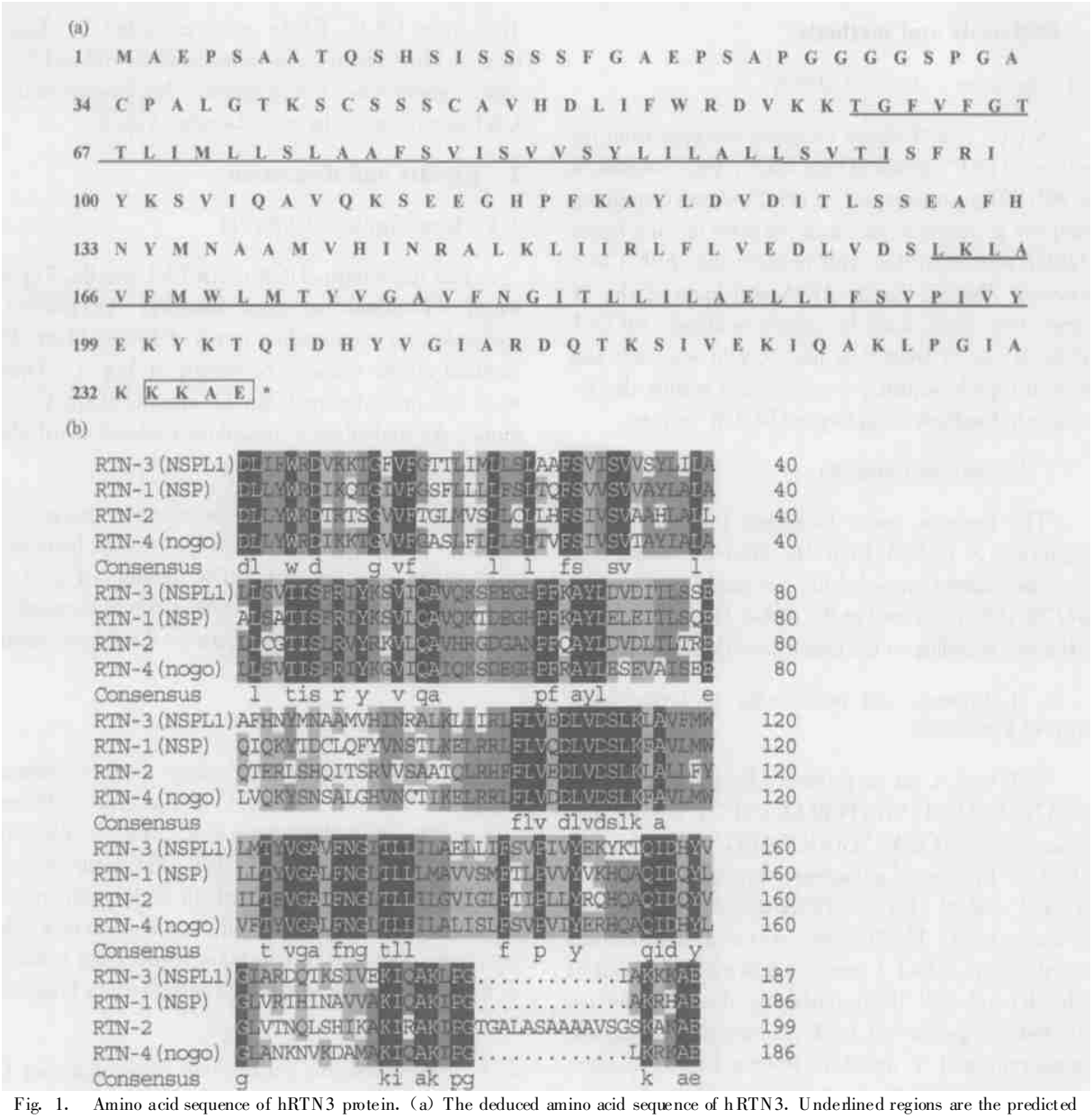

Fig. 1. Amino acid sequence of hRTN3 protein. (a) The deduced amino acid sequence of hRTN3. Underlined regions are the predicted transmembrane region; * is a stop codon; boxed is the retrieval motif of ER. (b) Multiple alignment of the amino acid sequences of C-terminal between hRTN3 and some other members in RTN family. Four shading colors are assigned to the three levels of homology: black, 100% homology; dark grey, between 75% and 100%; light grey, between 50% and 75%; white, less than 50%.

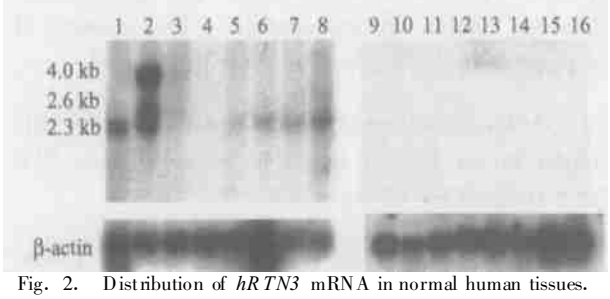

Fig. 2. Distribution of *hRTN3* mRNA in normal human tissues. 1, heart; 2, brain; 3, placenta; 4, lung; 5, liver; 6, skeletal muscle; 7, kidney; 8, pancreas; 9, spleen; 10, thymus; 11, prostate gland; 12, testis; 13, ovary; 14, small intestine; 15, colon; 16, periphery blood cells. Blots were reprobed with the cDNA of β-actin for the loading control.

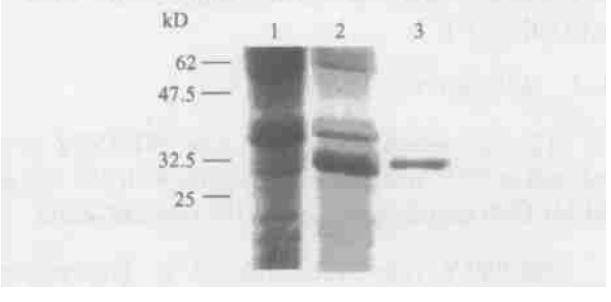

Fig. 3. Purification of the recombinant C-terminal of hRTN3 protein. 1, pGEX 4T-1-hRTN3C plasmid uninduced; 2, pGEX 4T-1-hRTN3 plasmid induced; 3, the purified protein hRTN3C-GST.

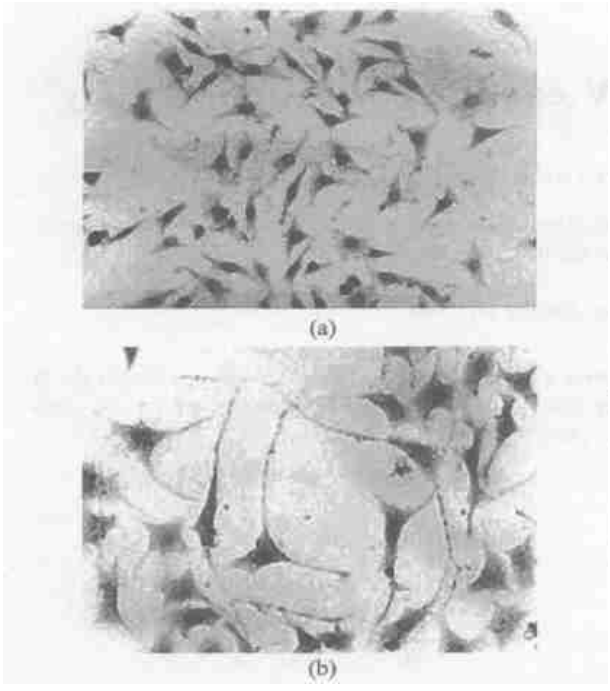

Fig. 4. Morphological characterization of neuroblastoma cell line SH-SY5Y during the differentiation induced by all-*trans* RA. (a) Before the induced differentiation. (b) During the induced differentiation.

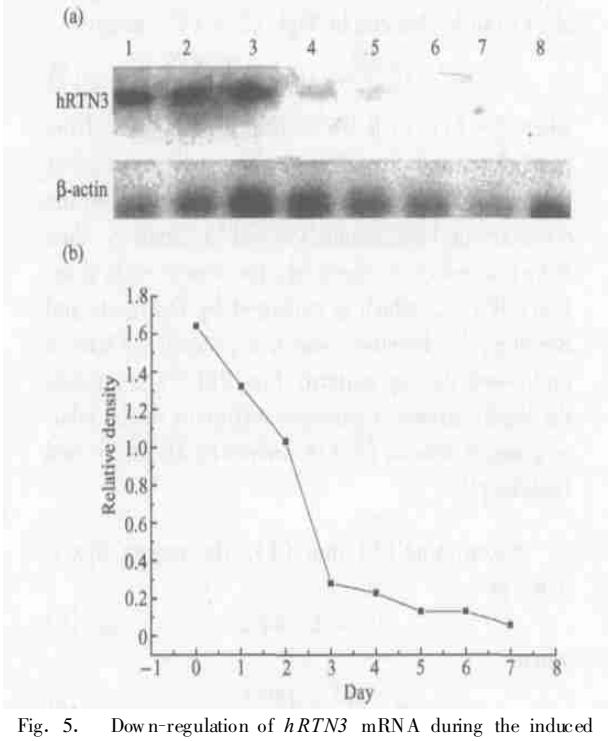

Fig. 5. Down-regulation of *hRTN3* mRNA during the induced differentiation of SH-SY5Y. (a) Northern blots containing poly $(A)^+$ RNA from SH-SY5Y cells. (b) The variation of *hRTN3* mRNA during the induced differentiation. 1 to 8 lanes in (a) represent the total RNA of SH-SY5Y on 0~7th days of induction.

All-*trans*-RA plays a crucial role in normal cellular differentiation and programmed cell death. RA regulates neuroblastoma growth and differentiation *in vitro*, and has shown activity against human neuroblastoma *in vivo*[13, 14]. It has been demonstrated that incubation with RA causes arrest of proliferation and neurite extension in SH-SY5Y cells[15, 16]. Our results indicate that human reticulon 3 is down-regulated during the differentiation of human neuroblastoma cell line SH-SY5Y induced by all-*trans*-RA, which suggests that, like other members of the reticulon family, *hRTN3* is a potential marker for neuroendocrine differentiation.

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