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Apoptosis related protein 3 is a lysosomal membrane protein

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ABSTRACT

Apoptosis Related Protein 3 (APR3) is an important protein which is involved in retinoic acid-induced apoptosis, osteoblast differentiation and cervical squamous cell carcinoma progression. Although it was predicted to be a trans-membrane protein, its cellular localization is not clear. In this study, we analyzed APR3 with bioinformatic tools and found that APR3 contains a potential signal peptide, a transmembrane region and 3 N-glycosylation sites, all of which are characteristics of lysosomal proteins. Western blot with isolated lysosomes demonstrated that APR3 was mainly present in lysosomes, specially in the lysosomal membrane fraction, but not in endoplasmic reticulum. Concomitantly, double immunofluorescence confirmed that APR3 co-localized with lysosomal membrane protein, LAMP1, as well as lysosomal specific marker, Lyso-Tracker Red. Moreover, we showed that APR3 was highly expressed in the lung, liver, spleen, kidney and adipose tissue, but expressed at the low level in the heart, pancreas, stomach and intestine. Interestingly, APR3 expression was elevated in multiple hepatocellular carcinoma cell lines comparing to normal liver cells. Collectively, our results proved that APR3 is a novel lysosomal membrane protein and shed light on its possible functions.

1. Introduction

Apoptosis Related Protein 3 (APR3) was first identified to be deferentially expressed when human promyelocytic leukemia cells were induced for differentiation and apoptosis with retinoic acid (RA) [1]. Later, it was proved that RA induced APR3 expression not only in promyelocytic leukemia cells, but also in many other cell lines [2]. Thus, it was also termed as all-trans retinoic acid-induced differentiation factor (ATRAID). There were only limited reports about the cellular functions of APR3. It was shown APR3 inhibited Cyclin D1 expression and consequently caused cell G1/S arrest [2]. Another study demonstrated that APR3 interacted with NEL-like 1 (NELL-1) in sarcoma cells and cooperatively functioned with NELL-1 to repress Cyclin D1 expression and osteoblast differentiation [3]. Beside that, evidences also implied that APR3 might be involved in cancer progression. APR3 was overexpressed in cervical squamous cell carcinoma and its high expression was associated with lymph node infiltration [4].

APR3 was predicted to be a trans-membrane protein based on sequence similarity [2]. It contains a transmembrane domain in the middle, as well as a signal sequence and EGF-like domain at the N-terminus. Overexpression of GFP-tagged APR3 in MCF-7 cells drove the protein to the cell membrane [2]. However, when ARP3-specific antibody was used to map the protein in the immunofluorescence experiment, it was labeled perinuclearly in multiple cell lines [3]. Although it is accepted that APR3 is a transmembrane protein, which exact membrane system APR3 is localized at still needs to be addressed.

Lysosomes are a vesicular organelle encircled by a single lipid bilayer. Inside the lysosomal lumen are more than 60 different acidic hydrolases, such as cathepsins [5,6]. The major function of lysosomes is to degrade biological macromolecules, such as proteins, polysaccharides, lipids and nucleic acids delivered by endocytosis or autophagy. The lysosomal compartment is acidic, with a pH of 4.5–5.0 [7]. The acidic environment maintained by proton pumps on lysosomal membrane is pivotal for its functions. Most lysosomal hydrolases are pH sensitive and have optimal enzymatic activity at low pH.

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Defects of lysosomal functions have been observed in multiple diseases, including neurodegenerative disorders, cancer, and cardiovascular diseases [8]. One of the causes of lysosome-related diseases can be attributed to the accumulation of macromolecules in lysosomes. The other causes may lie behind the role of lysosomes in programmed cell death [7,8]. Lysosomes are a mediator of apoptosis, upstream of the mitochondrial signaling. Various stimuli can induce the leakage of lysosomal membrane, a phenomenon termed as lysosomal membrane permeabilization (LMP) [9]. LMP results in the release of cathepsins which will cleave precursors of apoptosis signaling molecules to their active form. A well-studied substrate of cathepsin is Bid. Cleavage of Bid lead to mitochondria outer membrane permeablization (MPTP), subsequently promote cytochrome C release to cytoplasm, activate Apaf-1 and caspase-9, initiate the cascade reaction of apoptosis-related factors, at last result in apoptosis. A better understanding of lysosomal components will help us learn more about lysosome function and related diseases. We have isolated lysosomal components and identified 2 peptides from APR3 by mass spectrometry in a previous study. Here we further provided evidences that apoptosis related protein-3 (APR3) is a lysosomal membrane protein.

2. Method and materials

2.1. Bioinformatic analysis

The signal peptide, transmembrane region, and N-glycosylation sites of APR3 was analyzed with softwares of SignalP, TMHMM, and NetNGlyc on Denmark Technology University (DTU) sever, respectively [10,11] (http://www.cbs.dtu.dk/services/SignalP/, http://www.cbs.dtu.dk/services/TMHMM/, http://www.cbs.dtu.dk/services/NetNGlyc/). Hydrophobility of APR3 was analyzed with ExPASy sever [12] (http://web.expasy.org/cgi-bin/protscale/protscale.pl?1).

2.2. Isolation of lysosomes

Four to six livers from 8 week old male B129 mice were washed with cold PBS for 3 times and carefully minced with scissors. They were then homogenized on ice in 4–5 X volume of cold 0.25 M sucrose complemented with proteinase inhibitors (PIs, containing 5 μg/ml Leupeptin, 5 μg/ml Pepstatin A, 0.1 mM PMSF), and centrifuged for 10 min at 1,000 g at 4 °C. The homogenization and centrifugation were repeated once, then the two supernatants were combined and adjusted concentration of $CaCl_2$ to 1 mM with 100 mM $CaCl_2$. After incubating for 5 min at 37 °C, the solution were centrifuged for 15 min at 15,000 g at 4 °C to pellet the crude organelles, which were resuspended with 6 ml 0.25 M cold sucrose and homogenized one more time. The homogenate was loaded on the top of 40% Percoll and centrifuged for 45 min at 60,000 g at 4 °C. Fractions of 0.5 ml were collected from the top and centrifuged for 1 h at 300,000 g at 4 °C with 0.5 ml 50% sucrose on the top to remove Percoll. Lysosomes at the interface were collected and resuspended with 10 ml 0.25 M sucrose. After centrifuging for 20 min at 25,000 g at 4 °C, the lysosome pellets were store at −70 °C for future uses.

2.3. Preparation of proteins from lysosomes

Isolated lysosomes were resuspended in lysis buffer (50 mM Tris–HCl, pH 8.0, 0.2 M NaCl, 1 mM EDTA) complemented with PIs. The solution was repeated to thaw at 37 °C and frozen in liquid nitrogen for 10 times to break down lysosomes. It was then centrifuged for 30 min at 355,000 g at 4 °C. The soluble lysosomal proteins are in the supernatant while the lysosomal membrane proteins are in the pellet. The pellet was then dissolved in cold 0.1 M Na_2CO_3 (pH = 11.0), votexed for 5 s and incubated on ice for 30 min. After centrifuging for 30 min at 355,000 g at 4 °C, the supernatant which contained lysosome associated proteins was collected, and the pellet which contained lysosome integral proteins was subject to further dissolving in membrane protein buffer (6 M urea, 1% octyl-beta-glucopyranoside, 50 mM Tris–HCl, pH 8.5, 0.1% SDS, complemented with PIs). All the protein samples were stored at −20 °C for further uses.

2.4. Preparation of protein samples from cultured cells and tissues

Cultured cells were trypsinzed, washed with cold PBS and lysed with RIPA buffer. The lysis was centrifuged for 30 min at 15,000 g at 4 °C and the supernatant was collected for further analysis. When mouse tissues were used, about 100 mg of samples were minced and added to 1 ml RIPA buffer with 1 mM PMSF. The solution was sonicated for 8 s for 3 times to break down cells, and centrifuged for 5 min at 15,000 g. The supernatant was saved for further Western blot analysis.

2.5. Western blot

Protein samples were quantified according to the instruction of BCA kit (Thermo Scientific BCA 23227, USA). They were then separated with SDS-PAGE and transferred onto PVDF membrane. The membrane was blocked with 5% milk and incubated with the primary and secondary antibodies subsequently. The primary antibodies used are as follows: anti-APR3 (1:250, Abcam, UK), anti-Calnexin (1:1000, Abcam, UK), anti-LAMP1 (1:1000, clone H4A3, DSHB, USA), Anti-GAPDH (1:5000, Kangcheng, Shanghai, China). After incubation with the secondary antibodies, the membrane was developed with ECL and imaged with Bio-rad LAS-4000.

2.6. Total RNA purification, reverse transcription and real-time PCR

One hundred milligrams of tissues from 8 week old B129 male mice were used to purify total RNAs with TRIzol (Invitrogen, USA) as described in the manufacture's instruction. Reverse transcription was performed with Reverse Transcription Kit (Takara, Japan). The synthesized cDNAs were used for real-time PCR with PrimeScript™ RT reagent Kit and SYBR® Premix Ex Taq™ (Perfect Real Time, from TaKaRa, Japan). Primers used for real-time PCR are as follows: APR3 S (ATTTGTCCAGAGTGGCTGCT)/APR3 R (GAGTAAACCCACGGAAGGTG); GAPDH S (GGCACAGTCAAGGCTGAGAATG)/GAPDH R (ATGGTGGTGAAGACGCCAGTA).

2.7. Immunofluroesence

Mouse fibroblasts were isolated, cultured and stored as described (Experimental Instructions of Animal Cell Culture). They were inoculated on coverslips in 24-well plates when they reached logarithmic phase and cultured until reached 40–50% confluence. After fixed with 4% paraformaldehyde, the cells were permealized with 0.1% Saponin, incubated with anti-LAMP1(1:200) and anti-APR3 (1:100) antibodies, respectively. After incubation with corresponding secondary antibodies, the coverslips were counterstained with DAPI and observed under confocal microscopy. When Lyso-Tracker Red was used to label lysosomes, the cultured cells were washed with DMEM first and incubated with Lyso-Tracker Red (1:5000 diluted in DMEM) at 37 °C for 2 h. The cells were then fixed and stained with anti-APR3 antibodies as described above.

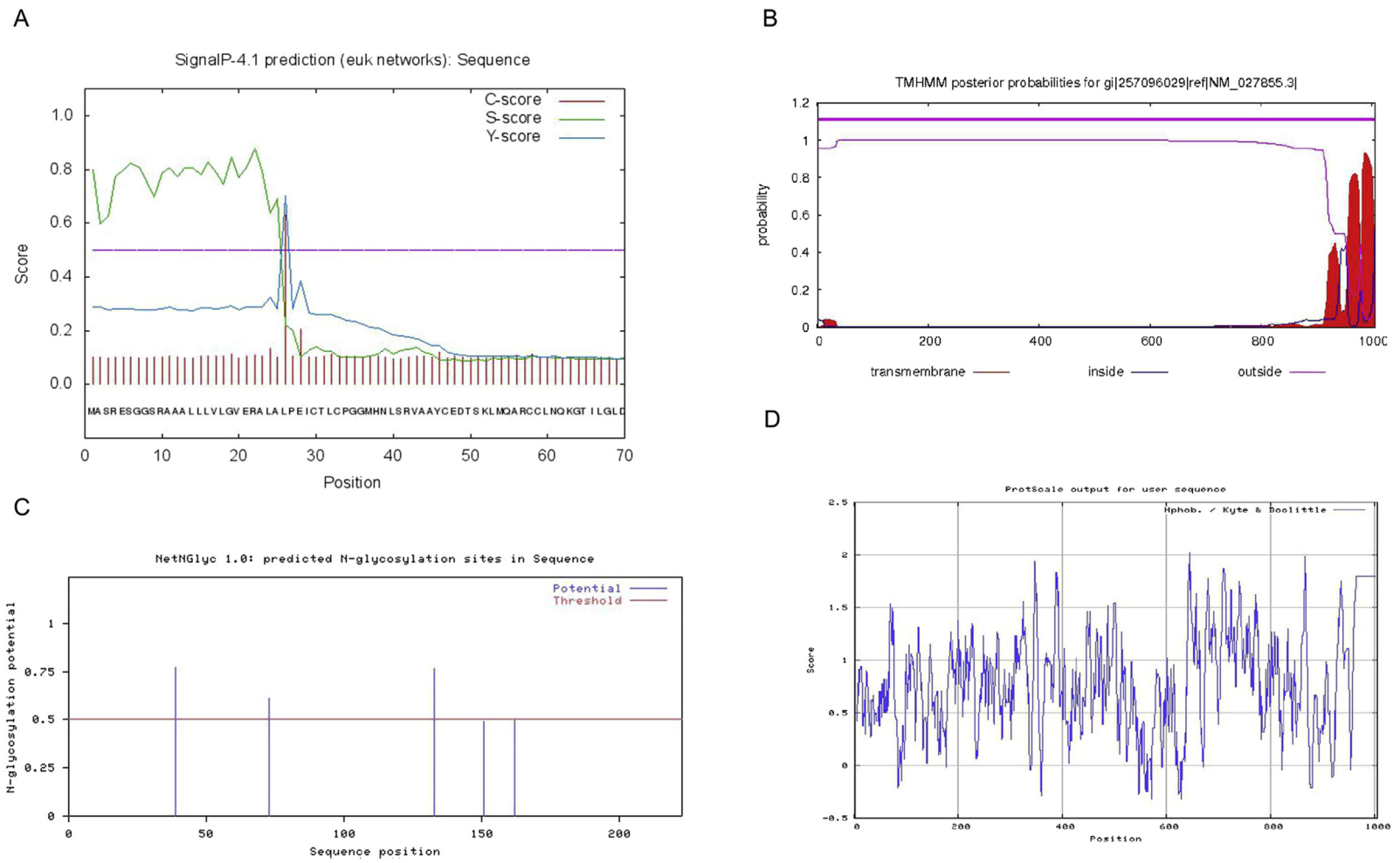

Fig. 1. Bioinformatics analysis of APR3 disclosed a hydrophobic protein with a signal peptide, transmembrane region, and 3 N-glycosylation sites. A. The potential signal peptide of the first 25 residues of APR3. B. The possible transmembrane region in APR3 located inside the residues 192–214. C. Three possible N-glycosylation sites at positions 39, 73, 133. D. The hydrophobicity of APR3 reveal by ExPASy.

2.8. Statistical analysis

Quantitative data were presented as mean ± standard deviation. Data with Gaussian distribution were compared with student *t* test or one way ANOVA. It is considered to be statistical different when $p < 0.05$, and significantly statistical different when $p < 0.001$. All the data were analyzed with SPSS19.0.

3. Results

3.1. Bioinformatic analysis

Lysosomal membrane proteins are usually hydrophobic, and contain transmembrane regions to anchor themselves, a signal peptide for its transportation to lysosomes after synthesis, and N-glycosylation sites to protect them from degradation by the hydrolases inside the lumen [13,14]. Therefore, we used bioinformatic tools to reveal if APR3 shared these characteristics. SignalP assay disclosed the presence of a potential signal peptide in the first 25 residues of APR3 (MASRESGGSRAAALLLVLGVERALA), with the cleavage site at 25–26 position (Fig. 1A). TMHMM software revealed that there are two possible transmembrane regions in APR3 located inside the residues 192–214 "FSLLMFFGILGSTTLAISILLWG" (Fig. 1B), and NetNGlyc predicted 3 possible N-glycosylation sites at 39, 73, 133 position (Fig. 1C). At last, APR3 is highly hydrophobic, lipophilic protein, as ExPASy has revealed (Fig. 1D).

3.2. Lysosome isolation

After differential centrifugation and Percoll density gradient centrifugation, different organelles distributed in their own density layers (Fig. 2A). To determine the distribution of lysosomes, we collected 0.5 ml fractions successively from the top of the tube after 40% Percoll density gradient ultracentrifugation (Fig. 2B). We obtained 23 fractions totally. After Percoll removal step, we obtained

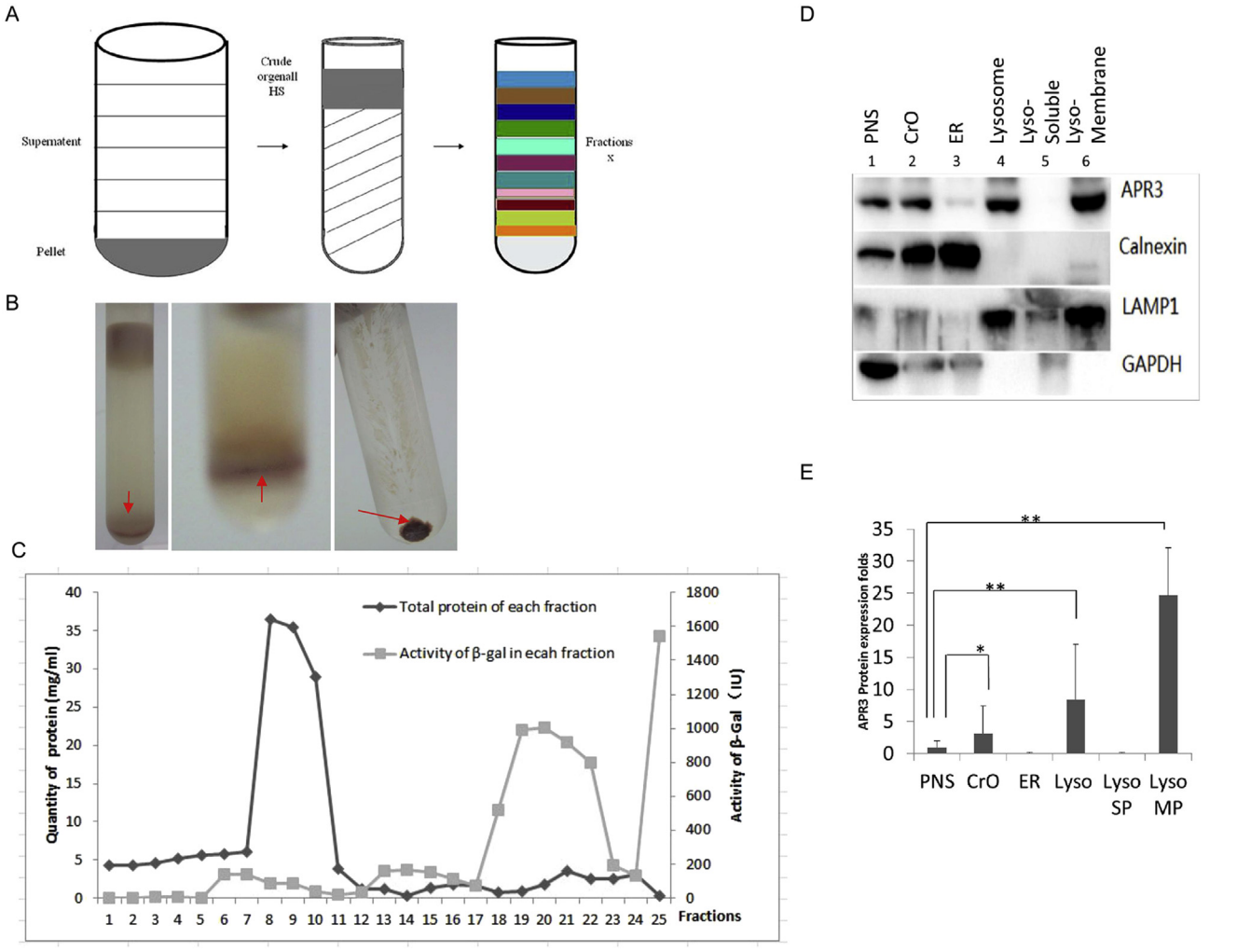

Fig. 2. Western blot with isolated organelles demonstrates APR3 is a lysosomal membrane protein. A. A schematic diagram showing the steps of differential centrifugation and Percoll density gradient centrifugation. After differential centrifugation at 15,000 g (the left), the pellet contains crude organelles. Crude organelles were loaded on 40% Percoll solution (the middle) and centrifuged. Different organelles will distribute in their own density layers (the right) after 60,000 g for 45 min. B. Representative pictures of Percoll denstiy gradient centrifugation. The left picture shows lysosome (red arrow) layer after Percoll density gradient centrifugation. The upper layer in the picture consist of big organelles, including ER, Golgi apparatus and mitochodria. The middle picture shows lysosomes (red arrow) at the interface of sucrose and Percoll phases. The right picture shows purified lysosomes (red arrow). C. β-gal activity analysis demonstrated the lysosomal fractions after Percoll density gradient centrifugation. The pale gray line represents β-gal enzymatic activity of each fraction. The dark gray line represents total proteins of each fraction. The protein amount peaks at 8–10 fractions while the β-gal activity peaks at 19–22 fractions. D. Western blot of isolated organelles. Lane 1: PNS, post nuclear supernatant; 2: CrO, crude organelle (the pellet after centrifugation at 15000 g); 3: ER (pooled fractions 8–10); 4: lysosomes, (pooled lysosomal fractions 19–22); 5: Lysosomal soluble proteins as described in the methods. 6: Lysosomal membrane proteins as described in the method. E. The quantitative results of figure D. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

lysosomal soluble proteins and lysosomal membrane proteins. The lysosomal membrane protein was further divided into lysosomal associated membrane proteins and lysosomal integral membrane protein, and designated as fractions 24 and 25. To determine which fractions are enriched of lysosomes the most, we measured the activity of β-galactosidase (β-gal) in every fractions, an enzyme that is abundantly present in lysosomes [13,14]. Our results showed that the peak of β-gal enzymatic activity appeared in fractions 19–22 as well as fraction 25 (Fig. 2C). This results indicate that lysosomes are mainly in fractions 19–22 which is corresponding to the brown pellets in Fig. 2B.

3.3. APR3 is a lysosomal membrane protein

After purification of lysosomes and other organelles, we examined APR3's subcellular distribution with Western blot. As expected, GADPH is present mainly in the post nuclear supernatant (PNS), while the endoplasmic reticulum (ER) protein Calnexin is present in PNS, crude organells (CrO), and ER fractions, but not in lysosomal fractions (Fig. 2D and E). On the other side, LAMP1, a known lysosomal membrane protein [13,14], is mainly present in lysosome, specifically in lysosomal membrane protein fraction (Fig. 2D and E). These results demonstrated that the isolated organelles are pure and representative. Further, we showed that APR3 was present in PNS, CrO, and lysosomes (Fig. 2D and E). There were only very weak signals of APR3 in the ER fraction (Fig. 2D and E). In lysosomes, APR3 is absent from the soluble protein fractions, but appears high in the fraction of lysosomal membrane proteins. These results suggested that APR3 is a lysosomal membrane protein.

3.4. APR3 co-localized with LAMP1 and lysosomal markers in cells

To further confirm the subcellular localization of ARP3, we stained APR3 with a APR3 specific antibody and observed its location under confocal microscopy. Immunofluorescence results show that, APR3 signals (green) is absent from nucleus and plasma membrane (Fig. 3A,C,E,G). In cytoplasm, APR3 signals appear to be punctate, mainly around the perinuclear region (Fig. 3A and E). When co-stained with LAMP1 (red, Fig. 3B and F), the known lysosomal membrane protein, we observed that APR3 signals overlapped with LAMP1 signals (orange, Fig. 3D and H). Next, we labeled lysosomes with Lyso-Tracker Red first and stained with anti-APR3 antibody. Lyso-tracker Red is a red fluorescent probe with weak alkalinity [13,14]. It can permeate cell membrane freely and be held up selectively in acidic organelles, thus is widely used to label lysosomes. As shown in Fig. 4, after labeled with Lyso-Tracker Red (Fig. 3J) and stained APR3 with green fluorescence (Fig. 3I), we observed similar results as described above and APR3 co-localized with Lyso-Tracker Red (Fig. 3I–K,M). These results suggested that APR3 is indeed a lysosomal protein and associated with lysosome membrane.

3.5. APR3 expression patterns among organs and cell lines

Until now, there is no information available concerning the organ distribution of APR3. This hinders its functional analysis. We used real-time PCR and Western blot to examine its distribution among important organs. As shown in Fig. 4A and B, the real-time PCR results matched well with our Western bolt overall. APR3 is

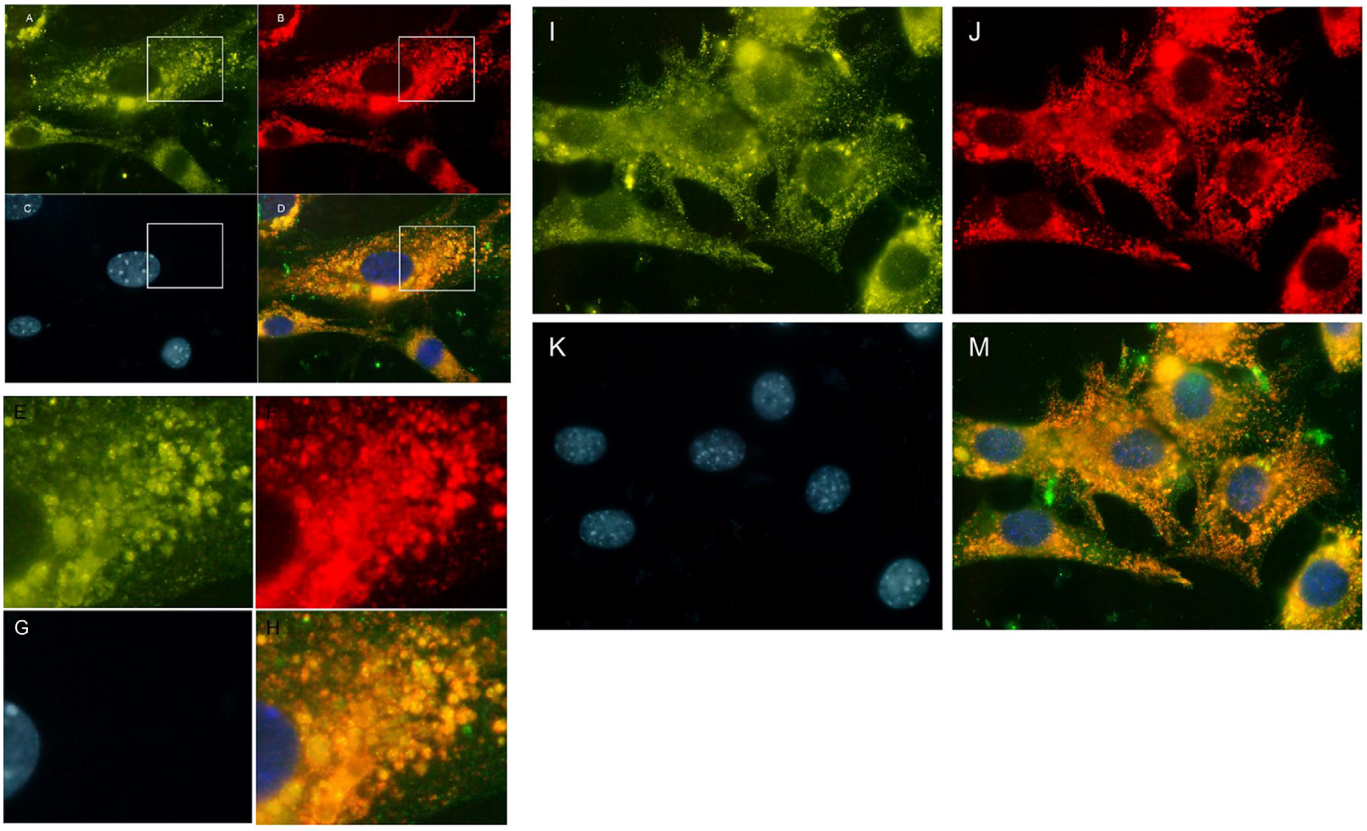

Fig. 3. Immunofluorescence confirm that APR3 is a lysosomal protein in NIH 3T3 fibroblasts. Anti-APR3 specific antibody (green, figure A) was used to visualize the location of APR3. LAMP1 (red, figure B) and DAPI (blue, figure C) was used to label lysosome and nuclei, respectively. APR3 and LAMP1 co-localizes together after the 2 signals merged (orange, figure D). Figures E, F, G, H are the pictures at a higher magnification showing the boxed region of figures A, B, C, and D. Lyso-Tracker Red (red, figure J) was also used to mark lysosomes and its signals overlapped with APR3 (green, figure I) after merged (orange, figure M). DAPI was used to stain nuclei (blue, figure K). (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

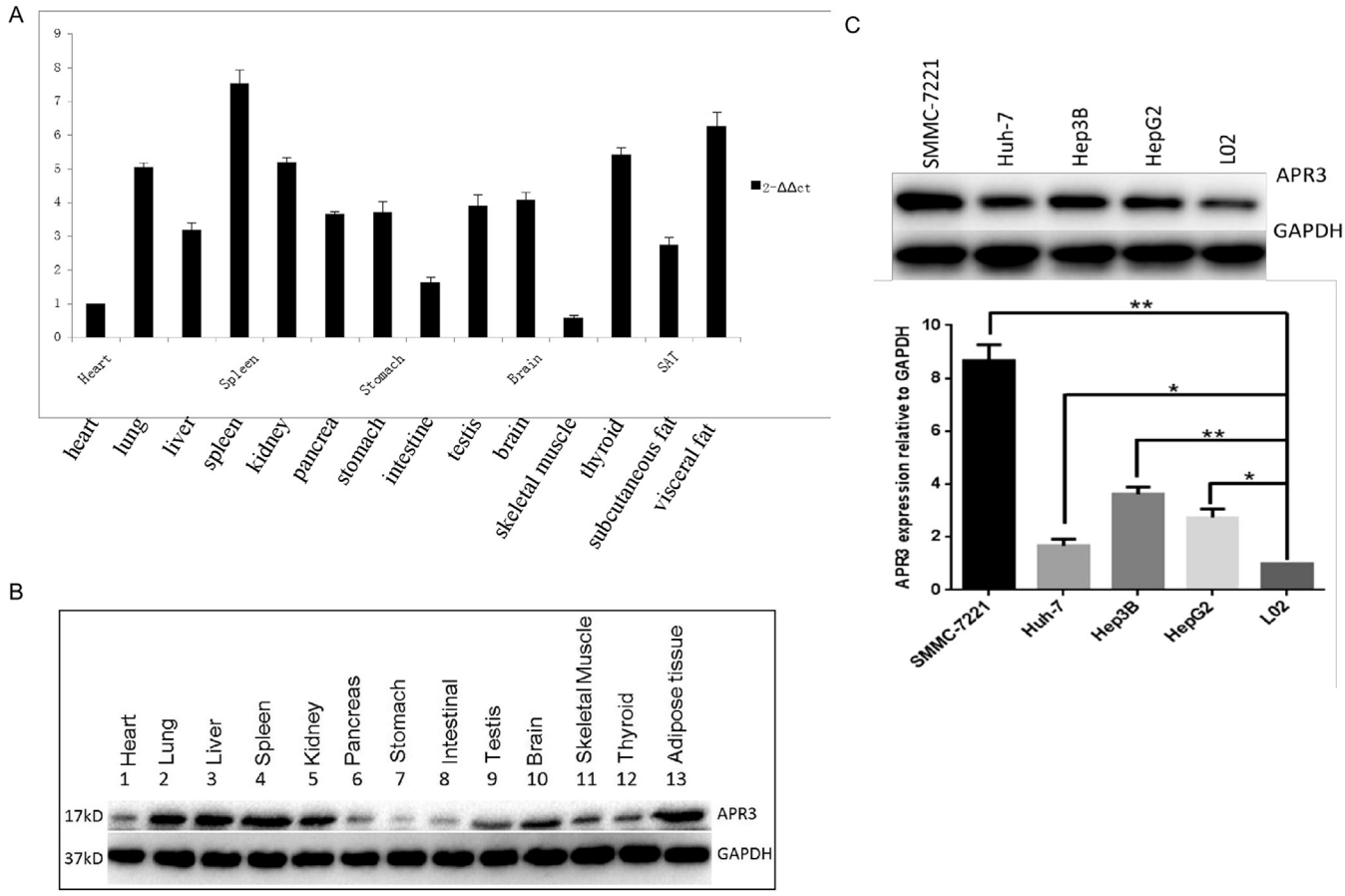

Fig. 4. The expression patter of APR3 in rat organs and human liver cell lines. A. Real-time PCR of APR3 mRNAs in various rat organs. The expression of APR3 was normalized with GAPDH mRNA. The results are from three independent experiments. SAT: subcutaneous adipose tissue, VAT: visceral adipose tissue. Data are shown as mean ± SD. B. Western blot of APR3 in various rat organs. C. Western blot of APR3 in different human liver cell lines. Huh-7, HepG2, Hep3B and SMMC-7221 are hepatocellular carcinoma cells, L02 are normal liver cells. *:$P < 0.05$,**:$P < 0.01$.

highly expressed in the lung, liver, spleen, kidney and adipose tissue, but has very little expression in the heart, pancreas, stomach and intestine (Fig. 4A and B). We then examined APR3 expression in various cell lines, including SMMC-7221, Huh-7, HepG2, Hep3B and L02 cells. L02 cells, a immortal hepatic cell line, were used as normal cell control, whereas SMMC-7221, Huh-7, HepG2 and Hep3B cells were all epithelial-like hepatocellular carcinoma cells (HCCs). Except HepG2, all other 3 cell lines are tumorigenic in immunosuppressed mice. To compare the relative APR3 expression in these cells, We used Thermo Scientific Lysosome Enrichment Kit, a kit for crude preparation of lysosomes from cultured cells, to isolated lysosomes from cultured cells and probed APR3 with Western blot. Thermo Scientific Lysosome Enrichment Kit was used because it could enrich lysosomes proteins and keep GAPDH in the samples at the same time. Therefore, APR3 expression could be normalized with GAPDH in Western blot. As shown in Fig. 4C, the expression of APR3 is low in normal liver L02 cells. However, its expression is significantly elevated in Huh-7, HepG2, Hep3B and SMMC-7221 cells. This result indicated that APR3 possibly play a role in HCC malignancy.

4. Discussion

Until now, about 60 lysosomal membrane proteins have been disclosed. There are 86 more proteins that are suggested to be enriched in lysosomal membrane components, which could be candidates of lysosomal membrane proteins [15]. At least 21 proteins are functionally unknown and enriched in lysosomal membrane component when using proteomic techniques to analyze lysosomal membrane component [16]. Characterization of these proteins will largely expand our knowledge on lysosome functions and its related diseases. In this study, we demonstrated that APR3 is a novel lysosomal membrane protein, and predicted its molecular characteristics and potential functions.

Bioinformatics analysis showed APR3 shares common characteristics with most lysosomal membrane proteins (Fig. 1A–D). It is hydrophobic, and contains multiple glycosylation sites, at least one transmembrane region, as well as a signal peptide that can guide their transportation to lysosomes after synthesis. Its transportation from Golgi apparatus to lysosome may rely on it tyrosine-based motif (YXXØ), similar as NPLK, YTAI, and YKCM [17,18].

Human APR3 gene is located in chromosome 2p23.3. Its mouse ortholog is located in chromosome 5 and shares 84% homology with its human counterpart. To determine the APR3 subcellular localization, we employed Percoll and sucrose density gradient centrifugation to purify lysosomes from mouse livers (Fig. 2). We compared APR3 distribution in various organelles by Western blot and showed that APR3 has the same subcellular distribution as LAMP1, a lysosomal membrane protein (Fig. 2D and E). Moreover, APR3 is only present in the membrane associated fraction, but not in the soluble fraction (mainly non-membrane protein inside the lysosomal lumen). This indicates that APR3 is a lysosomal membrane component.

We further confirmed this conclusion with double immunofluorescence staining (Fig. 3). Our results showed that APR3 signals co-localized with the LAMP1 signals as well as lysosomal tracers, Lyso-Tracker Red. This conclusion is also supported by previous proteomics experiment in which lysosomes isolated from mouse and rat livers were subject to mass spectrometry analyis and APR3 was identified [15,19]. Despite the overexpressed, GFP-tagged APR3 is located on MCF-7 cell plasm membrane in a previous study [2], the excessive exogenous proteins might not reflect its true cellular location, and caused false results. Zou et al. [3] used confocal microscopy to map APR3's location subcellularly and found that APR3 was located at the perinuclear region in human osteosarcoma Saos2, U2OS cells, or non-tumor Cos-7 and HEK-293T cells. This result does not conflict with ours as lysosomes are mainly distributed around the perinuclear area [20,21].

At present, the role of APR3 is still not very clear. So far there is no APR3 homologs identified, which makes it difficult to predict its biological functions. As a lysosomal membrane protein, APR3 shows some similarities with LAMP-1/2. Their C-termini are inside the lysosome lumen whereas the N-termini are facing to the cytoplasm; their N-glycosylation sites are all close to the N-termini; they all have one transmembrane helices. As the most abundant proteins in the lysosomal membrane, LAMP-1/2 account for about half amount of lysosomal membrane proteins [22,23]. LAMP-1/2 amount increased in the surface of highly metastatic cancer cells and they could sever as ligands of Selectin to mediate cell adhesion and recognition when present on the cell surface. The presence of LAMP-1 on cell surfaces may be also related to the differentiation status of the cells [24]. LAMP-2 plays a vital role in autophagy. LAMP-2 deficient mice accumulated extensive autophagic vacuoles in various tissues [25], and the degradation of long half-life proteins in the liver was severely impaired. The mice showed abnormal cardiocyte ultrastructure and significantly-reduced myocardial contractility, exhibiting syndromes of Danon's disease.

Based on the similarities between APR3 and LAMP-1/2, we speculate APR3 may be involved in the following functions: firstly, it may help maintain the integrity of the lysosomal membranes by forming the lysosomal membrane structure to protect the lysosomal membrane from degradation; secondly, it may mediate endocytosis or autophagy; thirdly, it may play a role in transportation of substances between lysosomes and cytoplasm; fourthly, it may participate in regulation of apoptosis or autophagy [22,23].

To further elucidate the biological functions of APR3, we examined the organ distribution of APR3. We found that APR3 are highly expressed in the lung, liver, spleen, kidney and adipose tissue, but expressed at low level in the heart, pancreas, stomach and intestine. We also showed that APR3 expression is elevated in hepatocellular carcinoma cells comparing to normal liver cells. These results suggest that APR3 may play a role in HCC progression. Evidences have shown that APR3 expression was regulated by NF-κB [26], whose function in HCC is still controversial [27]. Whether APR3 plays a role in HCC and what is the signaling pathway involved need further investigation. More experiments are undergoing to address the biological functions of APR3.

To summarize, we used multiple approaches to demonstrate that APR3 is a novel lysosomal membrane protein. We also showed the expression pattern of APR3 in various organs and cell lines. These results increased our understanding of APR3 as well as lysosomal functions and its related diseases.

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Conflict of interests

The authors declare that there is no conflict of interest.

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None.

Transparency document

Transparency document related to this article can be found online at http://dx.doi.org/10.1016/j.bbrc.2015.03.127.

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