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Molecular Cloning and Expression Analysis of *Vps26A* Gene from Deer Antler Tip of Different Growth Stages

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Authors' Contribution
YX and DL designed the experiment. HL collected the tissue samples. YL performed the experiment. JZ and BL analyzed the data. YX wrote the article.

Key words
Antler, Vps26A gene, Cloning, Real-time quantitative RT-PCR.

ABSTRACT

Retromer complex plays a crucial role in retrograde transport of the recycle proteins from the endosome to Golgi, and Vps26A protein is an important component of the retromer complex. In the present study cDNA sequence of the full coding region of the *Vps26A* gene was successfully cloned from antler tip of the Sika deer (*Cervus nippon hortulorum*). The Vps26A cDNA contains an open reading frame of 984bp encoding a polypeptide with 327 amino acids. The deduced molecular mass and isoelectric point of Vps26A protein were 38.2 kDa and 6.13, respectively. Glutamic acid had the largest proportion (10.4%) in the primary structure. Homologous sequence alignment and phylogenetic tree analysis indicated that theVps26A protein of sika deer was highly similar to that of *Bos taurus*. Expression analysis by real-time quantitative RT-PCR revealed that *Vps26A* gene had a higher expression level at day 90 than those obtained at day 60 and 30.

INTRODUCTION

Antlers are bony appendages of the deer head which are the only organ of regeneration in Mammals. Antlers are male secondary sexual characteristic which regularly cast and re-grow each year. The developing antlers are covered with velvet-like skin which grow at the fastest rate among all the mammalian organs/tissues. Antler growth process is divided into growth period and ossification period. In the growing period the maximal elongation rate can be up to 1-2 cm per day, which is more rapid than the growth rate of any mammal bone tissue. The growth rate of antler tip cells is 30 times faster than that of a tumor cell, however displays no sign of canceration. Thus, it has attracted a great deal of attention by many scientists (Brockes and Kumar, 2005; Odelberg, 2005; Price and Allen, 2004). It is an ideal tissue/sample for studying the proliferation, growth and differentiation of mammalian cells. The development of antlers is co-regulated by some genes. Thus, defining the expression properties of the genes would lead to a deeper understanding of deer antler growth and development mechanisms. Vps26A protein is a component of the retromer complex which is a peripheral membrane protein complex. The mammalian retromer complex consists of SNX1/2, SNX5/6, Vps26, Vps29 and Vps35 (Brockes and Kumar, 2005). The retromer complex is essential for normal cell function, and it is involved in recycling of the proteins from the endosomes to trans-Golgi network or plasma membrane. Functionally, the retromer has been linked to prominent neurodegenerative diseases such as Alzheimer's and Parkinson's (Trousdale and Kim, 2015; Wang and Bellen, 2015; Small and Petsko, 2015). Some studies also show that the retromer complex is involved in some specific developmental processes (Wang and Bellen, 2015).

In the present study, we have successfully cloned the sequence of full coding region of *Vps26A* gene in the reserve mesenchyme of antler tip samples, and studied the expression levels in different growth periods. The results provide a basis for further study of biological function of the *Vps26A* gene and provides a theoretical foundation for subsequent investigations perhaps in the field of cancer therapy for mammals.

MATERIALS AND METHODS

Collection of tissue samples

The tips of growing antler (about 30, 60 and 90 days)

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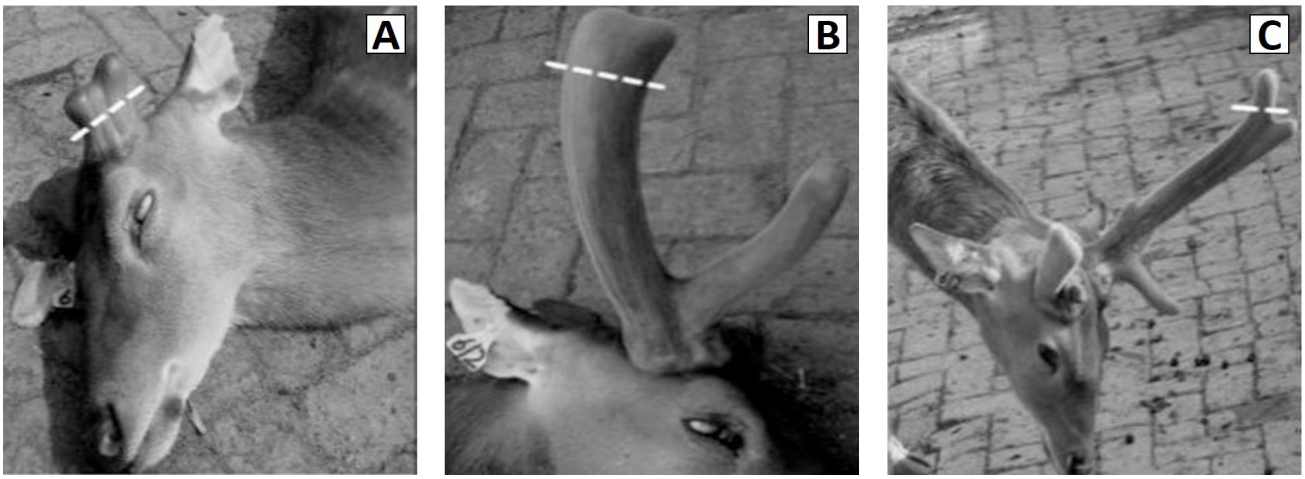

Fig. 1. Deer antlers from different growth periods. A, deer antler of 30 days; B, deer antler of 60 days; C, deer antler of 90 days.

were collected from an adult sika deer. 30 days sample was the left antler tip, 60 days sample was the right antler tip and when the left antler tip continued to grow to three branched velvet antler was used as 90 days sample (Fig. 1). The distal 5 cm of the tips were removed, and reserve mesenchyme was collected (Li *et al*., 2002). Samples were immediately preserved in liquid nitrogen.

Total RNA isolation

Total RNA was extracted from samples obtained in different growing periods by using TRIZOL reagent (Invitrogen, America) as instructed in its manual. DNA content of the extract was removed by incubating the total RNA with RNase-free DNase I (Promega, America) at 37°C for 30min. The total RNAs were electrophoresed in 1.0% agarose gel for 20 min. First-strand cDNA synthesis was performed using M-MLV reverse transcriptase (Rever Tra Ace -α- TM, Japan) with oligod (T) 20.

Amplification of Vps26A cDNA

Briefly, based on analysis of the conservative amino acid sequences, the primers pair (F- primer: 5'-AATGAGTTTTCTTGGAGGA-3' and R-primer: 5'-CAAACCTAAATCTCAACGG-3') was designed to amplify cDNA of *Vps26A* gene according to *Vps26A* gene sequences in homologous species. The RT-PCR was performed using the primers and cDNA sample produced from the tissue samples in rapid growing period. Positive colonies of *Vps26A* gene were selected to carry out PCR identification, bacterial amplification and sequencing.

Bioinformatics analysis

Homology of the cloned sequence was analysed by using the NCBI BLAST facility. The*Vps26A* gene sequence was analyzed and compared using the BLAST and ORF search programs in the GenBank database. The multiple sequences alignment of Vps26A protein set was performed with DNAStar7.10. The signal peptide site was predicted by Signal P3.0, and the Vps26A protein MW and PI were computed by ProtParam tool. A phylogenetic tree based on evolutionary distances was constructed from amino acid sequences with MEGA 6.0 using the neighbor-joining method.

Expression analysis of Vps26A gene

The expression pattern of *Vps26A* gene in different developmental periods was examined by quantitative real-time polymerase chain reaction. QRT-PCR analysis was performed by using a SYBR Premix EX TaqTM II (TaKaRa, Japan) on a Chromo 4 Real-Time PCR Detector (BIO-RAD, USA). In the real-time RT-PCR study, specific primers (Vps26A-F: 5'-TTTTTGGTCCCATTTGTGAGA-3' and Vps26A-R: 5'-GCCTCTTTCCAGGTTGCTT-3') were used to amplify a 168bp fragment with the cDNA from reserve mesenchyme of different growth periods. β-Actin was used as a the reference (β-Actin-F: 5'-GCGTGACATCAAGGAGAAGC-3' and β-Actin-R: 5'-GGAAGGACGGCTGGAAGA-3',173bp). PCR reaction was performed in a total volume of 25 μL containing 12.5 μL 2 × SYBR Green Master Mix (TARAKA), 1.5 μL (each) Vps26A-F and Vps26A-R primers (10 mM), 2 μL template, and 7.5 μL DEPC-water. The thermal profile for SYBR Green real-time PCR was 95 °C for 30s, followed by 40 cycles of 95 °C for 30 s, and 58 °C for 30 s, and 72 °C for 1 min. Each sample was performed in three technical replicates. DEPC-water for the replacement of template was used as negative control. The relative expression was calculated according to $2^{-\Delta\Delta Ct}$ method (Livak and Schmittgen, 2001; Zhai, 2017).

Fig. 2. Nucleotide and deduced amino acid sequences of Vps26A cDNA from sika deer (ATG is the initiation codon; * is the stop codon).

RESULTS AND DISCUSSION

Sequencing and bioinformatics analysis of Vps26A gene

The *Vps26A* gene encodes a polypeptide of 327 amino acids (Fig. 2). The calculated molecular mass of the mature protein is 38.2 kDa and isoelectric point is 6.13. Vps26A protein belongs to non-secretory protein without signal peptide. Sequence analysis revealed that the putative amino acid sequence of Vps26A is very similar to the other Vps26A proteins from other species (Fig. 3). Based on the amino acid sequences of Vps26A proteins, a phylogenetic tree was constructed using the neighbor-joining method (Fig. 4), which showed that Vps26A of sika deer was mostly related to *Bos taurus,* whereas it was most distant to *Gallus gallus and Alligator sinensis*. The analysis was consistent with the order of those species in the traditional taxonomy. Homology analysis showed that Vps26A proteins are highly conserved among different species. It implies that *Vps26A* gene would be an important stabilizing agent for various cell function.

Expression analysis of Vps26A gene

Differences in the expression level of Vps26A mRNA was detected in different periods (about 30, 60, 90 days). Day 60 is equivalent to the rapid growth period and day 90 is equivalent to the ossification period. Result showed that the gene has the lowest expression level in day 30. In the other periods, 60 days1.32, 90 days 2.00 (Fig. 5), indicating that the gene may play a regulatory role in cartilage formation.

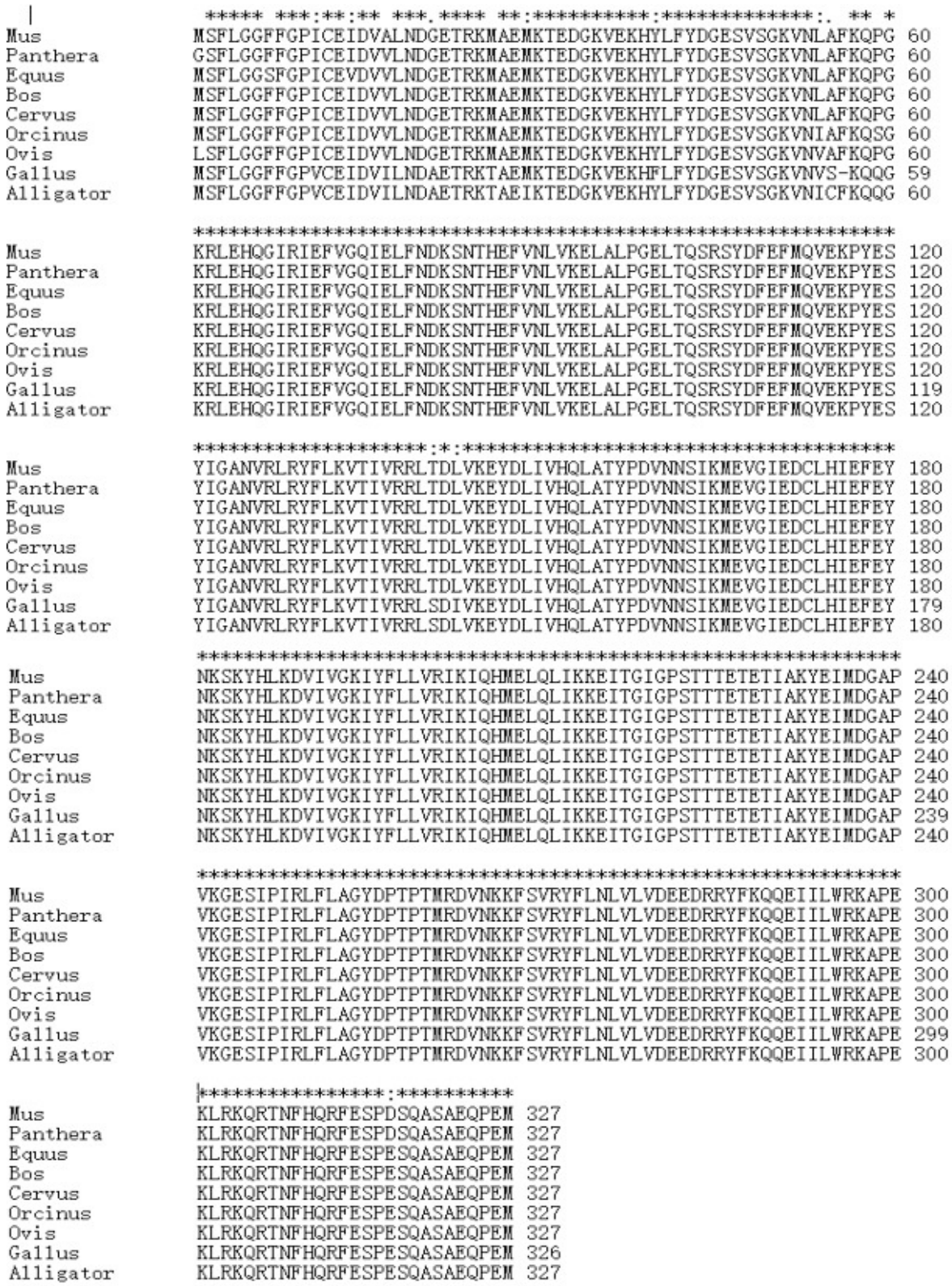

Fig. 3. Multiple alignment of *Vps26A* protein sequences from different species.

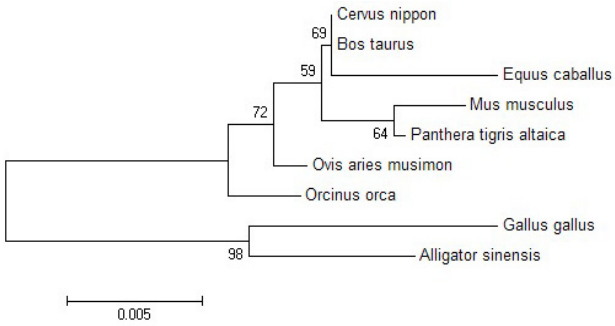

Fig. 4. The phylogenetic tree of Vps26A proteins from different species. Phylogenetic analysis based on the Vps26A amino acid sequences from various animals. The phylogenic tree for the Vps26A proteins was constructed using the neighbor-joining method. The origins and accession numbers of the Vps26A sequences were: *Bos taurus* (NP_001068923.1); *Equus caballus* (XP_005613841.1); *Mus musculus* (NP_598433.1); *Panthera tigris altaica* (XP_015399148); *Ovis aries musimon* (XP_011980964.1); *Orcinus orca* (XP_004280845.1); *Alligator sinensis* (XP_006023201.1); *Gallus gallus* (XP_421577.2).

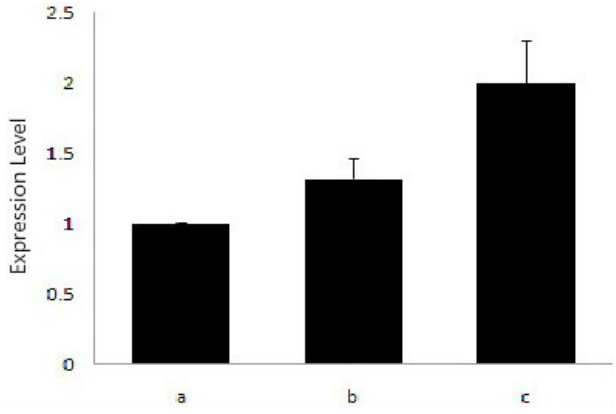

Fig. 5. Expression of *Vps26A* gene in different growth periods from sika deer antler. a, 30 days; b, 60 days; c, 90 days.

Retromer, consisting of two biochemically distinct sub-complexes, regulates the retrieval of cargo from endocytic system to TGN (Seaman *et al*., 1998; Harbour *et al*., 2010). Vps26A protein is a component of the retromer complex. Retromer complex is highly conserved among various eukaryotes including mammals and plants composed of similar subunits (Oliviusson *et al*., 2006; Shimada *et al*., 2006). The core retromer complex, consisting of trimer of Vps26, Vps29 and Vps35 recognizes the membrane bound receptor whereas the SNX sub-complex helps it to associate with the membrane (Arighi *et al*., 2004; Bonifacino and Hurley, 2008). Vps26 of mammalian cells contain two paralogues of the protein, Vps26A and Vps26B, and they bind to different retromer cargo molecules (Bugarcic *et al*., 2011; Kerr *et al*., 2005). But Vps26A is more important than Vps26B since it has greater range of binding partners and the greater severity of defects seen in cells without Vps26A (Trousdale and Kim, 2015).

The Retromer complex is involved in the Wnt signaling pathway by Wntless which is a kind of WNT signaling receptor. Recently, some studies have identified the Wntless and retromer complex as important components of the WNT signaling pathway. In the absence of retromer, Wntless is degraded in lysosomes and Wnt secretion is impaired (Eaton, 2008). Previous researches have already showed that Wnt signaling plays an important role in the early development of animal embryos, organ formation, tissue regeneration and other physiological processes. Wnt signaling pathway can significantly promote the proliferation of rat mesenchymal stem cells *in vitro* (Olivares-Navarrete *et al*., 2011), and the Wnt signaling pathway plays an important role in the osteogenic differentiation of mesenchymal stem cells (Yang *et al*., 2003). In addition, the retromer complex is essential for removal of the apoptotic cells. Retromer plays a role in the regulation of CED-1Which is mediated by retromer from phagocytosis to the surface of the phagocytic cells membrane. Without retromer complex, CED-1 will be transported to the lysosomal and degradated during the apoptotic process of the cell (Collins, 2008).

The results of the present study indicated that the expression level of *Vps26A* gene in reserve mesenchyme varies significantly during the development. The expression level of *Vps26A* gene was up-regulatedin in the process of growth and development of deer antler.*Vps26A* gene has a higher expression level at 90 days than 30 days and 60 days. It may be proposed that the gene plays an important role in cartilage development process. As a component of the retromer complex, the Vps26A may also be related to the clearance of apoptotic cells. However, specific mechanisms associated with these functions require further research.

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Statement of conflict of interest

Authors have declared no conflict of interest.

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