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Research paper

Molecular cloning and mRNA expression analysis of antizyme inhibitor 1 in the ovarian follicles of the Sichuan white goose

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ABSTRACT

Antizyme inhibitor 1 (Azin1) plays critical roles in various cellular pathways, including ornithine decarboxylase regulation, polyamine anabolism and uptake and cell proliferation. However, the molecular characteristics of the *AZIN1* gene and its expression profile in goose tissues and ovarian follicles have not been reported. In this study, the *AZIN1* cDNA of the Sichuan white goose (*Anser cygnoides*) was cloned, and analyzed for its phylogenetic and physiochemical properties. The expression profile of *AZIN1* mRNA in geese tissues and ovarian follicles were examined using quantitative real-time PCR. The results showed that the open reading frame of the *AZIN1* cDNA is 1353 bp in length, encoding a 450 amino acid protein with a molecular weight of 50 kDa. Out of all tissues examined, *AZIN1* expression was highest in the adrenal gland and lowest in breast muscle. There was also a high expression of *AZIN1* in the cerebellum and isthmus of oviduct. With follicular development, *AZIN1* gene expression gradually increased, and its expression in F1 was significantly higher than in F5 ($P < 0.05$). *AZIN1* expression was also significantly higher in the POF1 than in the other follicles ($P < 0.05$), and there was a low mRNA expression of *AZIN1* in atretic follicles. The results of *AZIN1* expression profiling in ovarian follicles suggest that *AZIN1* may play an important role in the progression of follicular development, potentially through regulating polyamine levels.

1. Introduction

Polyamines, including putrescine, spermidine, and spermine, are involved in regulating fundamental cellular processes, including DNA replication, cell proliferation, apoptosis, reproduction, and ion channel gating (Lefevre et al., 2011; Pegg and Casero, 2011; Lopez et al., 2014). Elevated polyamine levels can cause cell growth and differentiation in eukaryotes, but abnormal polyamine concentrations are also associated with the development of cancer (He et al., 2014). Therefore, the regulation of homeostasis of intracellular polyamines is critical.

Cellular polyamines originate from exogenous and endogenous sources. In terms of endogenous biosynthetic mechanisms, the antizyme inhibitor (Azin) molecule binds to ornithine decarboxylase antizyme (Oaz), negating its effect on ornithine decarboxylase (Odc) and positively regulating cellular polyamines (Mangold, 2006; Kahana, 2009; Murakami et al., 2014). Azin also induces the uptake of extracellular polyamines by sequestering Oaz (Kanerva et al., 2010).

There are two Azin proteins known to date, Azin1 and Azin2. The *AZIN1* mRNA is distributed in most tissues of mouse and participates in polyamine metabolism and cell growth (Levillain et al., 2012). The *AZIN2* mRNA appears in brain and testes, with detectable levels also found in the murine adrenal gland, pancreas, lung, heart, and kidney (Lopez-Contreras et al., 2006; Ramos-Molina et al., 2012). Azin1 has been shown to act as a major regulator of Odc activity and polyamine levels during cell growth and transformation. However, studies have also shown that polyamines can inhibit Azin1 via a negative feedback loop (Ivanov et al., 2008; Murakami et al., 2009; Ivanov et al., 2010). A further study suggested that this inhibition mainly occurs at the translational level through a short upstream open reading frame (ORF) with a non-AUG start codon (Murakami et al., 2014).

Many studies have shown that Azin1 is closely related to tumorigenesis. A recent study has shown that Azin1 can increase polyamine synthesis through binding and suppressing Oaz, increasing cell transformation and tumorigenesis (Olsen and Zetter, 2011). Another study has demonstrated that *OAZ* overexpression suppresses estrogen receptor alpha expression, which should inhibit cell growth (Zhu et al., 2012). In our previous study, *OAZ1* mRNA expression was significantly greater in the ovaries of

Abbreviations: aa, amino acid(s); bp, base pair(s); cDNA, DNA complementary to RNA; OAZ, ornithine decarboxylase antizyme (s); OAZ1, ornithine decarboxylase antizyme 1; AZIN, antizyme inhibitor (s); AZIN1, antizyme inhibitor 1; AZIN2, antizyme inhibitor 2; ODC, ornithine decarboxylase; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; ORF, open reading frame; F1, largest follicle; F2, second largest follicle; F3, third largest follicle; F4, fourth largest follicle; F5, fifth largest follicle; SYF, small yellow follicle; SWF, small white follicle; POF, postovulatory follicle (POF1–POF4); AF, atretic follicle; LH, luteinizing hormone; DFMO, α-difluoromethylornithine.

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Table 1
Oligonucleotide primers used for studies of *AZIN1* gene cloning and expression.

Target gene	Primer sequence (5′–3′)		Amplicon size (bp)
AZIN1	Forward:	ATGAAAGGATTTCTCGAGGATGC	1353
	Reverse:	TTAAGCTGAAGTGGAAAAGCGG	
AZIN1-S	Forward:	GCTCTTACTGCACATTGCCACA	180
	Reverse:	TGAATGTACGTTTGCAGTTCCTTG	
GAPDH	Forward:	GTGGTGCAAGAGGCATTGCTGAC	86
	Reverse:	GCTGATGCTCCCATGTTCGTGAT	

laying geese than that of pre- laying geese, suggesting that *OAZ1* functions during ovarian development and function, potentially through the inhibition of polyamine biosynthesis (Kang et al., 2009). However, whether Azin1 regulates animal reproduction through regulating polyamine levels was not clear.

In this study, the *AZIN1* sequence of the Sichuan white goose was characterized. *AZIN1* expression profiling in different tissues and hierarchical follicles was also performed using quantitative real-time PCR (qRT-PCR). These data could improve our knowledge of *AZIN1* functions and molecular mechanisms during avian follicular development.

2. Material and methods

2.1. Experimental geese and tissue collection

Three healthy female Sichuan white geese were selected randomly from a local breeding farm and were handled in accordance with the guidelines used at the farm. All of the geese were fed under uniform standard management in conditions of nature light. The geese were killed by exsanguination. The heart, liver, spleen, lung, kidney, adrenal gland, thigh muscle, breast muscle, cerebrum, cerebellum, pineal gland, hypothalamus, pituitary gland, retina, spinal cord, infundibulum of oviduct, magnum of oviduct, isthmus of oviduct, shell gland of oviduct, ovary (not including the follicles) and follicles were collected. The follicles were separated from the ovary and weighed to identify the small white follicles (SWFs), small yellow follicles (SYFs), hierarchical follicles (from F5 to F1), postovulatory follicles (from POF1 to POF4). All of the follicles were cut open transversely along the stigma to completely eliminate the yolk material. The follicles were washed with the ice-cold sterile saline, paying attention to ensuring that there was no adherent yolk material.

2.2. Extraction of total RNA and cDNA synthesis

Total RNA was prepared from these samples by the Trizol reagent method (Takara Bio Inc., Dalian, China), and then stored at −80 °C until analysis. cDNA was synthesized using PrimeScript® RT reagent Kit (Takara Bio Inc., Dalian, China), according to the manufacturer's instructions. Briefly, the 10 μL reaction consisted of 2.0 μL of total RNA, 2.0 μL of 5× PrimeScript® Buffer, 0.5 μL of PrimeScript® RT Enzyme Mix, 0.5 μL of Random 6 mers, 0.5 μL of oligo dT Primer and 4.5 μL of RNase Free H_2O. Thermal cycling was performed for 15 min at 37 °C, and then 5 s at 85 °C.

2.3. Primer design and amplification of the AZIN1

According to the reported cDNA sequence of the *Anas platyrhynchos AZIN1* (GenBank accession number: NM_001291578.1), the primers of *AZIN1* were designed to amplify the full coding region of *AZIN1* using Primer Premier 5.0. The primers *AZIN1*-S and *GAPDH* were used for qRT-PCR. The primers are listed in Table 1. The 25 μL reaction consisted of 1 μL of cDNA, 1 μL of each primer (10 μmol/L), 10.5 μL sterile Milli-Q water and 12.5 μL prime STAR (Takara Bio Inc., Dalian, China). The PCR was performed by running the following program: 98 °C for 10 s, 58 °C for 15 s, 72 °C for 80 s, 35 cycles. The PCR products were gel-purified and ligated into the pGEM-T Easy Vector (Promega, USA), which was transformed into *E. coli* DH5α competent cells. Positive clones that contained the expected-size inserts were screened by colony PCR and then sequenced by Invitrogen.

2.4. Bioinformatic analysis

The *AZIN1* mRNA sequence was analyzed using the NCBI ORF Finder computational tool (http://www.ncbi.nlm.nih.gov/gorf/gorf.html). The analyses of the nucleotide and amino acid sequence homologies were performed using NCBI Blast (http://blast.ncbi.nlm.nih.gov/). A phylogenetic tree was constructed using the MEGA program based on the neighbor-joining method with 1000 bootstrap replicates. The ProtScale (http://web.expasy.org/protscale/) web-based programs were used to predict the physiochemical properties of the Azin1 protein. The PSORT II web-based program (http://psort.hgc.jp/form2.html) was used to predict the subcellular distribution of the Azin1 protein. The N-glycosylation sites, phosphorylation sites and the secondary structure of the Azin1 protein were predicted using NetNGlyc 1.0 Server, Netphos 2.0 Server and HNN (http://npsa-pbil.ibcp.fr), respectively.

2.5. Gene expression analysis

The expression profiling of *AZIN1* mRNA in different tissues and in various follicles were assessed using qRT-PCR. The qRT-PCR was performed in a 96-well iCycler CFX96 (Bio-Rad Laboratories, USA) using SYBR® Premix Ex Taq™ (Takara Bio Inc., Dalian, China). Briefly, the 50 μL reaction consisted of 1 μL of cDNA, 25 μL of SYBR® Premix Ex Taq™, 2 μL of 10 μmol/L of PCR forward primer and PCR reverse primer, and 22 μL of nuclease-free water. PCR was carried out on the Cycler system in 1 cycle of 95 °C for 10 s, followed by 40 cycles of 95 °C for 5 s, 58 °C for 30 s and 72 °C for 30 s, and then an 80 cycle melting curve was performed. Starting temperature was 62 °C and increasing by 0.5 °C every 10 s to determine the primer specificity. The relative quantitation of gene expression was performed in three replicates for each sample. Threshold and Ct (threshold cycle) values were determined automatically by the CFX-96 Real-time PCR Detection software, using default parameters. The relative levels of expression for *AZIN1* were calculated relative to *GAPDH* using the $2^{-\Delta\Delta Ct}$ method.

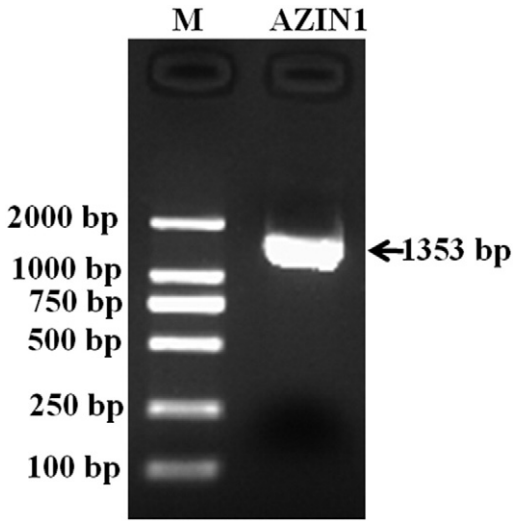

Fig. 1. Representative electrophoresis image of RT-PCR products for the *AZIN1* gene from the Sichuan white goose. The 1353 bp amplicon of *AZIN1* was separated on 1.0% agarose gels, stained with ethidium bromide, examined with ultraviolet light and visualized with a Gel-Pro Imager (Media Cybernetics, Silver Spring, MD, USA). A 2000 bp molecular weight marker (M) was used.