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Detecting Splicing Variants of *FOXP2* and its Protein Expression in Chicken Brain

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ABSTRACT

The whole cDNA sequence of chicken *FOXP2* gene was obtained by 3' and 5' RACE and the length was 3839 bp, getting four splicing variants for protein isoforms (707 AA, 706AA, 687AA, 684AA). The four splicing variants were common to Zang chicken, White Plymouth Rock, Beijing fat chicken, White leghorns and Rhode Island Red by the detection of a combination of PCR and sequencing and according to two main splicing variants, two antibodies were produced to be used to detect equivalent expression in chicken brain such as cortex, striatum, mesencephalon, thalamus and hypothalamus, in White leghorns (male and female) by immunohistochemistry. The results showed that different *FOXP2* mRNA and protein expression had no spatial difference.

Key words: Chicken, *FOXP2*, splicing variant, immunohistochemistry

INTRODUCTION

Forkhead proteins are important transcriptional regulators that are involved in pattern formation during vertebrate development as well as in tissue specific gene expression (Accili and Arden, 2004; Carlsson and Mahlapuu, 2002; Dirksen and Jamrich, 1992, 1995; El-Hodiri *et al.*, 2001; Erickson, 2001; Kaufmann and Knochel, 1996; Lai *et al.*, 2001; Lai, 1990; Lehmann *et al.*, 2003; Li and Vogt, 1993; Tseng *et al.*, 2004). FOXP2 (Forkhead box protein 2) is a transcription factor mainly consisting of three parts: a polyglutamine tract, a zinc-finger motif and a forkhead DNA-binding domain. *FOXP2* gene has several variants to code different protein. *FOXP2* is very conservative and its expression was most in brain. In human, mutations in this gene result in impaired linguistic and grammatical skills that together with diminished control of complex face and mouth movements, lead to disrupted speech (Hurst *et al.*, 1990; Lai *et al.*, 2001; Vargha-Khadem *et al.*, 1998).

Despite the importance of the *FOXP2*, there have been no comparative analyses of different FOXP2 protein expression from splicing variants. It is difficulty to find special different antibodies to make a distinction between the splice variants. To determine if the different protein from splicing variants was expressed in brain, we developed two special antibodies that specifically recognized the two kinds of proteins. We used them for immunocytochemical staining of chicken brain and combined PCR and sequencing to detect mRNA and protein expression.

MATERIAL AND METHODS

Source of animals and tissue: Experimental Chicken were all 180 days and collected from chicken farm of China Agriculture University, including Rhode Island Red (male), Zang chicken(male), White Plymouth Rock(male), Beijing Fat chicken(male), White leghorns(male) and White leghorns(female), procured brain tissue including striatum, thalamus, mesencephalon and cortex. Some were stored at -80°C until analysis and some were soaked in polymer formaldehyde solution.

Total RNA isolation and cDNA synthesis: Total RNA from chicken brain tissue was isolated using TRIzol reagent (invitrogen) and chloroform, according to manufacturer instructions. The RNA was run through a purification process, using an RNeasy mini kit (Qiagen). The quality and concentration of the RNA were determined by measuring absorbance at 260 and 280 nm and RNA integrity was confirmed by agarose gel electrophoresis. RNA was dissolved in diethylpyrocarbonate (DEPC) treated water and stored at -70°C until analysis. Approximately 1 μg total RNA was used to synthesize the first strand cDNA, using an MMLV-RT kit (Promega), according to the manufacturer protocol.

3' RACE (rapid amplification of cDNA Ends) and 5' RACE: According to the FOXP2 information of mouse (NM_212435.1), human (NG_007491.2) and bird (AY549149.1) from GenBank database, the primer 1a and primer 1b were designed, respectively for 3' RACE and 5' RACE by homology (Table 1). RNA PCR Kit ver 3.0 was used in reverse transcription reaction for 3' RACE according to the instruction of the kit (TaKaRa). The product of RACE was tested by 1.5% agarose gel electrophoresis. SMART RACE Kit was used in reverse transcription reaction for 5' RACE according to the instruction of the kit (Clontech). The product of 5' RACE PCR was tested by 1.5% agarose gel electrophoresis.

Designing of primer 2 and primer 3: Two pairs of primers were designed to detect 51 bp deletion in *FOXP2* gene sequence. Then a product length of 261 bp would be got if DNA template had no 51 deletion. Meanwhile, the template with 51 bp deletion would get a PCR product of 412 bp because the upstream of primer 3 just crossed the 51 bp deletion (Fig. 1).

Antibody production: Two polyclonal antibodies directed against distinct polypeptide regions within the C-terminus of FOXP2 were employed for our studies to check the protein expression of two main splicing variants (Mohkam *et al.*, 2011; Abdolalizadeh *et al.*, 2008). A commercially available primary antibody could not recognize FOXP2 from different splicing variants and was not suitable to use in chicken, so we developed own antibody in rabbit against FOXP2 according to the results of RACE (Spichkina and Petrov, 2009). One FOXP2 antibody (antibody 1) was made

Table 1: Primers in this study

Primer	Primer sequence (5'→3')	Annealing temperature (°C)	Product length (bp)
Primer 1a	CAGTTCTAAATGCAAGGCGAGAC		
Primer 1b	GCAGCAGCAAGCCGTTAT		
Primer 2	GCAAGAGCAGTTACATCTTC	54.0	261
	CTGTAAAAGCAGCTGTCTT		
Primer 3	CGTTATGTTGCAGCAGCTTTT	64.5	412
	TAGCCCTCCGTGTTTAATG		
Primer 4	GCAGCAGCAAGCCGTTAT	63.0	700
	CCAACGCATGTTCATTGTTAAGG		

GCAAGAGCAGTTACATCTTC [1]
... CGTTATGTTGCAGCAGCAACAACTACAAGAGTTTTACAAGAAACAGCAAGAGCAGTTACATCTTCAGCTTTT... [2]
CGTTATGTTGCAG CAGCTTTT [3]

Fig. 1: Location of the primer 2 and primer 3, Note: 1. upstream of primer 2; 2. part of *FOXP2* sequence; 3. upstream of primer 3; the underlined parts were location of 51 bp deletion (Table 1)

against the 12-aa sequence CLQEFYKKQQEQ in the C-terminal region of FOXP2 which was coded by the part of the 51 bp sequence so this antibody was to identify the protein isoforms from variant I (707AA) or variant II (706A) without 51 bp deletion. Another FOXP2 antibody (Antibody 2) was made against the 15-aa sequence SPELEDDREIEEEPC in the N-terminal region of FOXP2 and this antibody recognized all FOXP2 isoforms. The peptides were chosen on the basis of its antigenicity (DNASTAR) and dissimilarity between family members FOXP1 and FOXP4. Similarity of the amino acid sequence to other proteins was excluded by comparisons with family members FOXP1 and FOXP4, as well as by protein blast (blastp) analysis (BLAST). The peptide was conjugated to keyhole limpet hemocyanin and rabbits were immunized with the peptide and complete adjuvant (Sigma-Genosys). Antibody was purified on an affinity purification column with the peptide (Sigma-Genosys).

Immunostaining: For demonstrating the presence and location of FOXP2 in tissue sections, chicken brain tissues were fixed in 4% paraformaldehyde overnight at 4°C, embedded in paraffin and Paraffin-embedded tissues were sliced at 6 mm thickness using a microtome (Leica 2016, Germany). The slides were subjected to immunohistochemical analysis with immunostaining kit, Histostain-Plus Mouse Primary (Invitrogen, USA) according to the manufacturer's recommendations (El-Kott *et al*., 2006; Kabbinejadian *et al*., 2008).

After being washed in PBS, the sections were incubated with 10% goat non-immune serum (Invitrogen, USA) at room temperature for 20 min. The washed sections were then reacted orderly with Primary antibody incubation for 60 min, Antibody 1 or Antibody 2 for 10 min, Streptavidin-HRP solution for 10 min and all steps were in room temperature and Rinsed in wash buffer between steps, respectively. Followed by incubation with biotinylated second antibody (Invitrogen, USA) at 37°C for 15 min and after being washed in PBS, the sections were incubated with streptavidin-peroxidase (HRP) (Invitrogen, USA) at 37°C for 15 min. Finally, the slides were washed with PBS and stained with DAB kit (Invitrogen, USA). After being washed fully with water for 5 min, the slides were stained with hematoxylin and eosin and then examined under a confocal laser scanning microscope (Nikon i 50, Japan) (Iqbal *et al*., 2002).

RESULTS

RACE: The RACE method was usually used to complete the cloning of the 5' and 3' ends of the open reading frame. The cDNA length of FOXP2 is 3839 bp and ORF (Open Read Frame, ORF) coded 707AA (Fig. 2). Two deletion sequences of 51 bp (CAGCAACAACTACAAGAGTTTTA CAAGAAACAGCAAGAGCAGTTACATCTT) and 3 bp (CAG) were found, respectively and the two deletions brought out four kinds of alternative splicing which coded four different kinds of proteins: variant I (707AA),variant II (706A),variant III (686AA) and variant IV (685AA). The difference

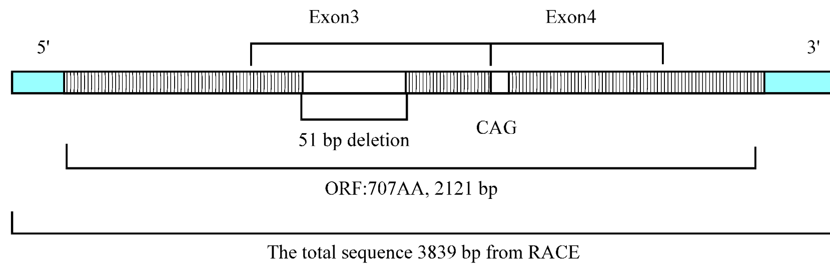

Fig. 2: The results of RACE

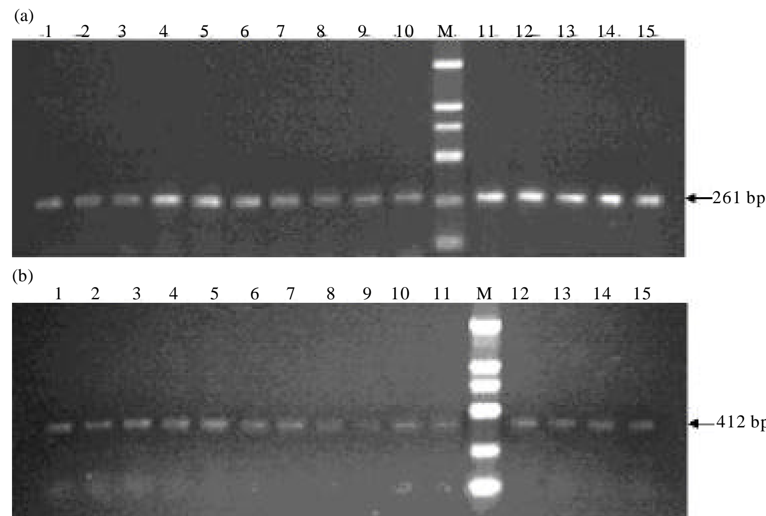

Fig. 3(a-b): (a) The 2% agarose gel electrophoretic tests of 51 bp deletion and (b) No 51 bp deletion and in various chicken breeds by PCR (reverse transcription-polymerase chain reaction). Note: M represents DL2000 Plus DNA Ladder marker (from up to down: 2000 bp, 1000 bp, 750 bp, 500 bp, 250 bp, 150 bp); 1-15 represent different samples (male): Rhode Island Red (1-3), Zang chicken (4-6), White Plymouth Rock (7-9), Beijing Fat chicken (10-12), White leghorns (13-15)

of variant I and variant II was a change from glutamine (Q) to arginine (R) and more one glutamine (Q) among poly (Q) caused by an insertion of CAG. The different of variant III (686AA) and variant IV (685AA) was the same as the one of variant I and variant II. Variant I had more an 51 bp insertion (17AA) than variant III and so did the variant II than variant IV.

In this Fig. 2, the first deletion of 51 bp existed among the exon3 and the CAG was in the beginning of the exon4.

Assaying four variants in some chicken breeds: Two pairs of primers (primer 2 and primer 3 in Table 1) were used to assay the 51 bp deletion. If Primer 2 was used to amply cDNA samples and the length of PCR product was 261 bp, it was the variant I or variant II (51 bp deletion). For the same reason, if the PCR product was 412 bp by Primer 3, it was the variant III or variant IV (existing 51 bp). Two percent agarose gel electrophoretic tests showed that 51 bp deletion (Fig. 3a) and no 51 bp deletion (Fig. 3b) coexisted in all the samples which were cDNA of corpus striatum.

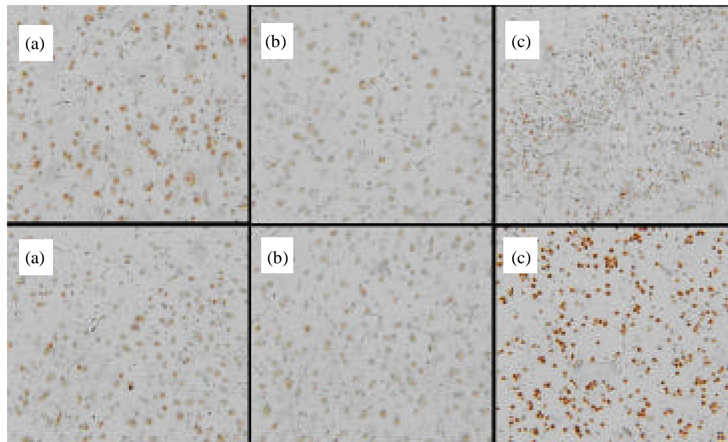

Fig. 4(a-f): Protein expression of FOXP2 from splicing variants without 51 bp deletion was detected at male chicken brain in White leghorns such as (a) Cortex, (b) Striatum, (c) Mesencephalon, (d) Thalamus, (e) Hypothalamus and (f) Female chicken striatum

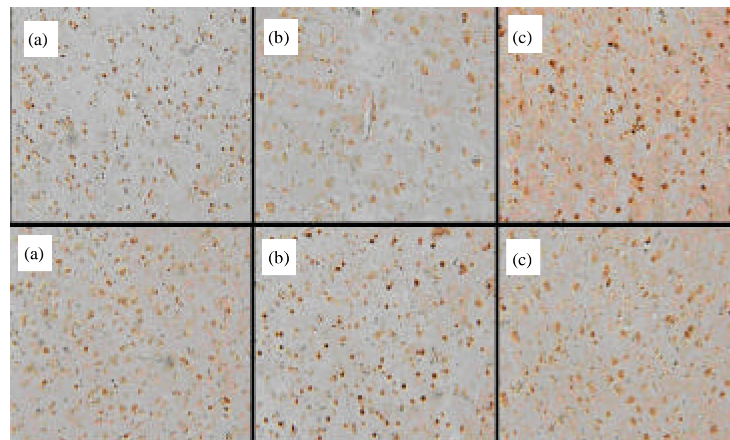

Fig. 5(a-f): Protein expression of FOXP2 from splicing variants with and without 51 bp deletion was detected at male chicken brain in White leghorns such as (a) Cortex, (b) Striatum, (c) Mesencephalon, (d) Thalamus, (e) Hypothalamus and (f) Female chicken striatum

A combination of PCR and sequencing detected ACG deletion in striatum of some chicken breeds including Rhode Island Red, Zang chicken, White Plymouth Rock, Beijing Fat chicken, White leghorns and a pair of primer 4 was used. The results showed that ACG deletion existed in all these breeds.

Checking FOXP2 by immunohistochemistry staining: Antibody 1 was used to check FOXP2 in the male and female chicken brain including cortex, striatum, mesencephalon, thalamus and hypothalamus (Fig. 4). So did antibody 2 (Fig. 5). The results showed that the FOXP2 with 51 bp deletion and without 51 bp deletion were both expressed in these sections of chicken brain.

DISCUSSION

FOXP2 gene is very conservative as a transcription factor. There are some variants because of the change number of Q of Poly Q (Polyglutamine) or the different amino acid in different species (Zhang *et al*., 2011). There were more 2 Qs and 6 Qs in each place of Ploy Q, respectively and more 25 AA in human than in chicken (Enard *et al*., 2002). Two amino acids, 274T/A and 346S/N were different between human and rat and human has more 2 Qs than rat. FOXP2 in chicken is more 4 Qs than in zebra finch and they have different five amino acids, 24G/S, 42T/S, 79T/S, 80D/E and 576R/G (Haesler *et al*., 2004). Mammals and birds are quite different species and they went in separate way more than 300 million years ago but only five amino acid of the FOXP2 protein are different between zebra finch and mice, eight between zebra finch and human; songbirds and human have more than 98% identity of the protein (Haesler *et al*., 2004). In the different amino acid, 576R/G is located in the forkhead DNA-binding domain which maybe implies the main reason that zebra finch is a kind of songbird but chicken is not.

Zebrafish and Mouse *FOXP2* is expressed in the diencephalon, thalamus, cerebellum, hindbrain and spinal cord (Ferland *et al*., 2003; Lai *et al*., 2003; Teramitsu *et al*., 2004). This expression pattern is not obviously similar to that of other known genes (Goyal *et al*., 2010; Raj *et al*., 2009). Zebrafish *FOXP2* is also expressed in the heart but we have not detected embryonic expression in the spleen, kidney or gut, unlike its mouse and songbird orthologs (Haesler *et al*., 2004; Shu *et al*., 2001). In humans, *FOXP2* expresses in subcortical regions (Lai *et al*., 2003; Teramitsu *et al*., 2004) which in the KE family show abnormalities when imaged by use of functional MRI (Liegeois *et al*., 2003; Watkins *et al*., 2002). Nevertheless, the previous studies were not related to the mRNA expression of each splicing variants of *FOXP2*.

In this study, two antibodies were developing according to two FOXP2 proteins, Immunostaining indicated that protein expression of *FOXP2* from splicing variants with or without 51 bp was at the cortex, striatum, mesencephalon, thalamus and hypothalamus of chicken brain. This study showed that the two FOXP2 proteins had no spatial expression specificity and that different splicing variants was translated into different protein isoforms which span similar ranges of tissue expression, though they might have different quantity and different function.

The way of using different antibodies to detect differential splicing was not so much in reports but Prescott and Chamberlain (2011) had used this way to study SNAP25a and SNAP25b (Differential splicing of the SNAP25 gene) which expressed differently in their development and region in human and rat brain. These differences might show that alternatively spliced isoforms had distinct functions in Complex neural pathways in the brain (Prescott and Chamberlain, 2011). However in this study, we did not find any different expression and function between different splicing of *FOXP2*, so more research was needed to show their each function in neural system of brain.

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