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A Semi-Quantitative RT-PCR to Assess Differential Expression Levels of TCF3 Gene in Two Chinese Indigenous Chicken Breeds

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Abstract: Semi-Quantitative Polymerase Chain Reaction (RT-PCR) was applied to quantify and compare variations in avian TCF3 expression level between two Chinese indigenous chicken breeds for different tissues. Six primer combinations in addition to β-actin as an internal control were used. The expression level of TCF3 was greater in Jinghai breed than Suqin breed for different tissues. The variability of gene expression between two breeds showed 18.55, 37.85 and 66.15% higher expression in Jinghai breed than Suqin breed for kidney, lung and spleen tissues, respectively. Differences were found to be significant ($p<0.05$) only for expression level in lung and spleen tissues. Significant effect of sex upon TCF3 expression was detected for both breeds.

Key words: TCF3, semi-quantitative RT-PCR, gene expression, chicken breeds, jinghai breed, suqin bread

INTRODUCTION

The Transcription Factor TCF3 (E2A; ITF1; immunoglobulin transcription factor 1) contributes to transcriptional regulation in many cell lineages. However, it is essential for the development of B lymphocytes (Bain *et al*., 1997; Zhuang *et al*., 1994) regulating somatic hypermutation (Meyer and Mufti, 2000) and stimulates Ig hypermutation (Schoetz *et al*., 2005). TCF3 is a good candidate since it binds to all Ig enhancer and play a critical role in other Ig modification events, such as Ig gene rearrangement and class switching (Conlon and Meyer, 2004). Furthermore, TCF3 is also play a role in peripheral B-lymphocytes initiation, differentiation and function, it is highly expressed in germinal centre B cell where Class Switching Region (CSR) and Somatic Hypermutation (SHM) are thought to occur (Quong *et al*., 1999). By alternative splicing the TCF3 gene codes for two proteins, E12 and E47, that are members of the basic Helix-Loop-Helix (bHLH) family of transcription factors. E12 and E47 are expressed in many cell types but form a unique homodimeric complex only in B lineage cells (Shen and Kadesch, 1995; Bain *et al*., 1993). These factors have been shown to function in a transcription hierarchy and also in a combinatorial manner, to establish the expression of the genes that comprise the B lineage differentiation program (Kee and Murre, 2001). Genetic and biochemical studies have demonstrated that the bHLH transcription factors encoded by TCF3 play an indispensible role in the initiation, differentiation and function of B-lymphocytes. However, how TCF3 controls B-cells specific gene expression and differentiation program are not fully understood (Zhuang *et al*., 1999).

Reverse Transcription-Polymerase Chain Reaction (RT-PCR) is highly sensitive and specific method useful for the detection of rare transcript or for analysis of samples available in limiting amount (Carding *et al*., 1992) and amplifies cellular RNA for use in analyzing gene expression (Raemaekers, 1996; Reidy *et al*., 1995).

In most cases, when RNA analysis is required, a qualitative study is not sufficient to deliver a satisfactory answer. A common question is the quantification of specific RNA transcripts and the detection of any variation in their expression levels under different experimental conditions (Marone *et al*., 2001). RT-PCR is most sensitive technique for mRNA detection and quantitation currently available compared with the two other commonly used techniques for quantifying mRNA levels, Northern blot analysis and RNase protection assay. It can be used to quantify mRNA levels from much smaller samples, regardless, RT-PCR can be highly variable and may not accurately reflect gene activity

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(Bustin, 2002). For semi-quantitative RT-PCR to be meaningful, the PCR products must be measured during the exponential phase of amplification and a suitable housekeeping gene (β-actin; GAPDH; 18s RNA; tRNA) as internal control is used to normalize sample -to-sample variation (Suzuki *et al*., 2000). Housekeeping genes play another role to monitor the efficiency of fewer amplification cycles resulting from qRT-PCR compared with basic PCR (Gaudette and Crain, 1991; Murphy *et al*., 1990). The objectives of the study aimed to explore semi-quantitative RT-PCR technique to detect TCF3 expression, quantitation and expression variations in different tissues of two Chinese indigenous chicken breeds.

MATERIALS AND METHODS

Experimental animal and sample collection: Two Chinese indigenous chicken breeds; Jinghai yellow chicken from Haimen Poultry Co. and Suqin yellow chicken from Yangzhou Poultry Institute were examined. Thirty sex-pooled individuals of each breed at 16 weeks of age were collected randomly and slaughtered. Immediately of slaughtering 2-5 g samples from kidney, lung and spleen were collected in 2 mL Eppendrof nuclease-free tube and directly stored in liquid nitrogen container to provide storage temperature-196°C. Samples were transferred to the central laboratory and stored in deepfreeze refrigerator (- 80°C) for further studying.

RNA extraction and purification: Total RNA was extracted from homogenized tissue (50-100 mg) using Trizol reagent (Sangon, Shanghai, China) according to single step method early reported (Chomczynski and Sacchi, 1987). Precipitated RNA was resuspended in 20 μL of 100% deionized formamide. RNA quality and quantity were assessed by agarose gel electrophoresis and UV spectrophotometer, respectively (Ausubel *et al*., 1989). Quantitation of RNA samples was determined spectrophotometrically by measuring the absorbance at 260_{nm} ($A_{260} = 1 \geq 40$ μg mL^{-1}) according to the following equation (www.qiagen.com):

Concentration of RNA sample (μg mL^{-1}) = 40X A_{260} X dilution factor

Table 1: Sequence of primers used in RT-PCR reactions

Primer name	Sequence, 5' to 3'
P1	TACCAGCTGCATTCAGGAGAG
P2	TCCTCCTCATCCTCATCTTG
P3	GATAAGGCCTCGTCTGTACTG
P4	TCAGGTTTGGATGAAAGACCGGGA
P5	AGCCCACCATTCAACTCTCCTGAA
P6 (β-actin)	TGCCAGGGTACATTGTGGTA
P7 (β-actin)	TGCGTGACATCAAGGAGAAG

RNA samples were equalized and adjusted to 1 μg uL^{-1}. DNase 1 treatment, followed by phenol extraction and ethanol precipitation was applied to remove traces of contaminating DNA (Sambrook *et al*., 1989) prior to cDNA synthesis.

Reverse Transcription (RT): Total RNA (2 μg) was reverse transcribed in a final reaction volume of 20 μL using ReverAid™ Moloney Murine Leukemia Virus (MMuLV) reverse transcriptase (Fermentas Life Science, Shanghai, China) and $Oligo(dT)_{18}$ primer. The first strand cDNA was synthesized at 42°C for 1 h and terminated by heating at 70°C for 10 min. cDNA samples stored in deepfreeze at -20°C.

Primer sequences: Primers sequences were determined using Primer Premier v5.0 software and Primer Quest™ (www.idtdna.com) (Table 1). For specificity, sequence alignment of all primers was examined with Genbank using Blast program available online (www.ncbi.nlm.nih.gov.). Primers were selected when sequences showed homology to the same gene of interest.

Semi-quantitative RT-PCR: Semiquantitative RT-PCR was performed for all samples (30 kidney samples, 30 lung samples and 30 spleen samples for each breed) to measure gene expression. 2 μL of respective 5X fold diluted cDNA was used in 50 μL total reaction mixtures containing 5 μL 10 X PCR plus Mg^{++} buffer (Shenergy Biocolor BioScience and Technology Co. Shanghai, China), 1 μL (4×2.5 mM each) dNTP mixture, 2 μL (10 pmol) of each gene specific primers and β-actin primers (Sangon, Shanghai, China) and 1 μL (5unit μL^{-1}) Taq DNA polymerase (Shenergy Biocolor BioScience and Technology Co. Shanghai, China). PCR reactions were performed on Thermocycler 9600 (Biometra). PCR cycling parameters were initially started a 94°C denaturation for 5 min, 30 sec at 59°C and 1 min at 72°C, followed by 30 sec at 94°C, 30 sec at 59°C and 1 min at 72°C for 30 cycles and elongation period for 10 min at 72°C. The PCR program for the expression of E12 and E47 factors in serial dilutions was 94°C denaturation for 10 min, followed by 30 sec at 94°C, 30 sec at 55°C and 3 min at 72°C for 30 cycles and 10 min at 72°C. The conditions were chosen so that no RNAs analyzed reached a plateau at the end of the amplification, they were in the exponential phase of amplification.

Gel electrophoresis: The PCR products (6 μL sample +2 μL loading dye) were loaded onto Ethidium Bromide-stained 1.7% Agarose (Biowest-Regular) in 1xTBE. The gel was run in 1xTBE buffer at a constant power of 100v until the xylene cyanole dye reached two-third of the gel.

1 kbp and 100bp DNA ladder molecular weight marker (Takara, Dalian,China) were run on every gel to confirm expected molecular weight of the amplification products for E12 and E47 expression and TCF3 expression level in different samples, respectively .

Gel images acquisition and quantitative analysis: Images of the RT-PCR ethidium bromide-stained agarose gels were acquired with Transilluminator UV/White 2020D camera (Kodak ds digital science, Japan). Quantification of the bands was performed by ImageQuant 1D sofware (Amersham Biosciences, USA). Bands intensity was expressed as relative absorbance units. Calibration (Vol. (μg)) was calculated between RNA samples to be determined and β-actin as a reference to normalize for initial variations in sample concentrations to compare the expression levels across all samples.

Statistical analysis: RT-PCR signals calibrated through ImageQuant software were subjected to SPSS v 14.0 software. The RT-PCR ratio values were analyzed using one-way ANOVA and correlation procedures to estimate significance variance within and between breeds for TCF3 expression level in different organs.

RESULTS AND DISCUSSION

Expression pattern of TCF3: Splice variant specific primers (P_1, P_2 and P_3) were used in semi-quantitative RT-PCR on serial diluted cDNA to examine the differential expression between E12 and E47. The result indicated that the relative amounts of E12 and E47 expressed are very similar (Fig. 1). Genetic, biochemical and functional studies have demonstrated that E12 and E47 differ only in their highly homologous DNA binding domain (Murre *et al*., 1989). The size of the cDNA full length message of the two factors has been reported to be similar and about 2.4 kb (Conlon and Meyer, 2004). Obviously, the relative expression amount of E12 or E47 could be and indicator of the overall expression pattern of the transcriptional factor TCF3. However, TCF3 contributing to gene expression in a variety of tissues but it have being of particular importance for lymphocytes (Quong *et al*., 1999; Meyer and Mufti, 2000).

Semi-quantitative RT-PCR could be a fairly reliable technique to detect the different expression of a candidate gene in different tissues. Amplification can vary depending on factors such as RNA integrity, Reverse Transcriptase (RT) efficiency, sample-to-sample variation, amplification efficiency and variation in cDNA sample loading. Using the same sample size, assessing RNA integrity and equalizing RNA concentration prior to RT

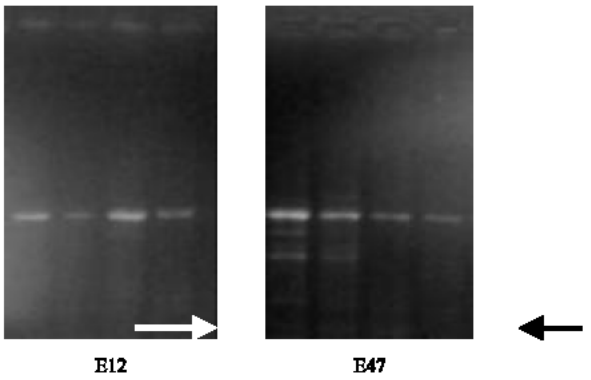

Fig. 1: Semi-quantitative reverse transcriptase polymerase chain reaction (RT-PCR) analysis of E12 and E47 splice variants expression on serial diluted cDNA (0.20, 0.25, 0.40, 0.50 and 1), ascending

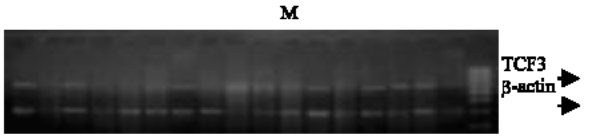

Fig. 2: Semi-quantitative reverse transcriptase polymerase chain reaction (RT-PCR) analysis confirming TCF3 target gene (701 bp) and ß-actin (233 bp) as internal control, lane M: 100 bp DNA ladder marker

are some of the basic consideration steps in semi-quantitative RT-PCR. However, normalization or calibration to an internal control (reference gene) is essential to balance sample to sample variation within the RT and PCR reactions.

Co-amplification of candidate gene TCF3 and β-actin with two primer sets (P_4, P_5, P_6 and P_7) in a duplex PCR reaction was performed (Fig. 2). Calibration values (T/C) measured as (Vol. (ug)) of the relative level of amplification product of TCF3 gene (T) over β-actin (C) showed that the relative expression level of TCF3 was greater in Jinghai yellow chicken than Suqin yellow chicken for different organs. The T/C calibrated values (ug) were 6.07±1.84, 5.39±3.27 and 7.51±6.06 for Jinghai breed and 5.12±3.57, 3.91±2.48 and 4.52±3.12 for Suqin breed for kidney, lung and spleen tissues, respectively. The increasing expression levels account 18.55, 37.85 and 66.15% for kidney, lung and spleen tissues, respectively. The degree of expression divergence in lung and spleen tissues between two breeds was found to be significant ($p<0.05$), however, expression level differences in lung tissues between two breeds were not significant (Fig. 3).

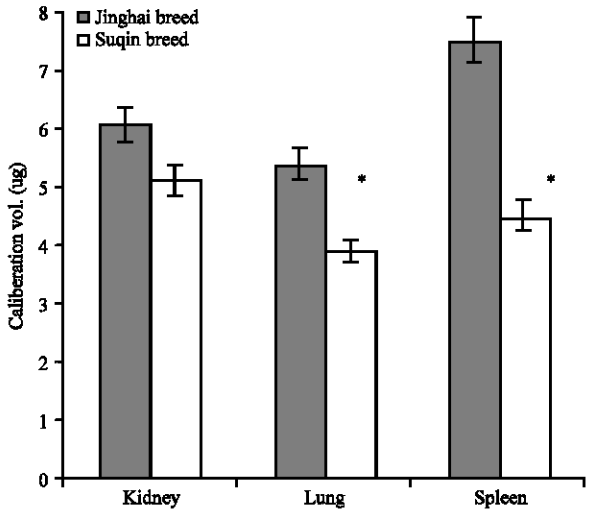

Fig. 3: Relative expression of transcriptional factor TCF3 in kidney, lung and spleen of the two chicken breeds, Jinghai yellow chicken and Suqin yellow chicken. The data shows the mean +/- SEM of all samples (19 kidney, 16 lung and 14 spleen samples, and 21 kidney, 18 lung and 18 spleen samples for Jinghai and Suqin, respectively). Values are expressed as Volume (ug) after calibration. *: Significant difference ($p<0.05$)

The results showed that sex affected TCF3 expression significantly ($p<0.05$) in both breeds, the expression level was substantially higher in males than females. Although the trend was occurred for the expression level in all tissues, differences were found to be significant only for kidney and lung tissues. Semi-quantitative RT-PCR results showed that differential expression of avian TCF3 can be detected in different tissues and the level of expression can be considered as a novel genetic marker for avian genetic further studies. Greatest expression level of TCF3 was reported for lung, spleen and ileum tissues of chicken (Conlon and Meyer, 2004) and in T cells, brain, muscles, kidney and spleen tissues of catfish (Hikima *et al*., 2005). Additionally, Mekki *et al*. (2006) reported significantly ($p<0.05$) higher expression level of TCF3 in kidney tissues than lung tissues of some Chinese indigenous duck breeds.

TCF3 expression level is detected in wide variety of normal primary tissues, however, in all of these, the level of expression varies considerably from cell to cell and high level expression is restricted to functionally relevant compartments or cell type (Rutherford and LeBrun, 1998) and the wide expression pattern may not reflect protein level since TCF3 is regulated post-transcriptionally factor (Quong *et al*., 1999; Meyer and Mufti, 2000).

Monitoring gene expression by measuring mRNA levels in different tissues and generate a measurable signal to quantify, semi-quantitative RT-PCR assay can be a sensitive method to detect subtle changes in gene expression (Bustin, 2002) and differences of calibrated values may reflect true changes in expression rather than RNA loading. However, semi-quantitative RT-PCR has been known to be 1.000-10.000 folds more sensitive than the more traditional RNA plotting techniques (Byrne *et al*., 1988; Morcharla *et al*., 1990) to detect difference in gene expression and may identify candidate genes involved, often a direct link cannot be made between a physiological response and genetic marker due to missing biochemical information (Bustin, 2002). It is possible that TCF3 functional divergence can be, in part attributed to expression divergence.

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