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Avian E2A Expression Level Variation Associated with Phenotypic Variation of Two Chinese Indigenous Duck Breeds, (*Anas platyrhynchos*)

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Abstract: Semi-quantitative polymerase chain reaction (RT-PCR) was applied to quantify and compare variations in avian E2A expression level between two Chinese indigenous duck breeds for different tissues. Six primer combinations in addition to β-actin as an internal control were used. The expression level of E2A was significantly ($p<0.05$) higher in kidney tissues than lung tissues for both Gao you and Kun shan indigenous duck breeds. The variability of gene expression between two breeds showed 34.35% and 60.70% higher expression in Gao you breed than Kun shan breed for kidney and lung tissues, respectively. Differences were found to be significant ($p<0.05$) only for expression level in lung tissues. No significant effect of sex upon E2A expression was detected. The association between E2A relative expression level and body weight was found to be positive and moderate for the genetically lean breed (Gao you), while it was negative and has low values for genetically heavy breed (Kun shan).

Key words: Avian E2A, duck breeds, gene expression, semi-quantitative RT-PCR

Introduction

The transcription factor E2A contributes to transcriptional regulation in many cell lineages. However, it is essential for the development of B lymphocytes (Zhuang *et al.*, 1994; Bain *et al.*, 1997), regulating somatic hyper mutation (Meyer and Mufti, 2000) and stimulates Ig hyper mutation (Schoetz *et al.*, 2005). E2A 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, E2A 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 hyper mutation (SHM) are thought to occur (Quong *et al.*, 1999). By alternative splicing the E2A 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 (Bain *et al.*, 1993; Shen and Kadesch, 1995). 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 E2A play an indispensable role in the initiation, differentiation, and function of B-lymphocytes. However, how E2A 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 (Reidy *et al.*, 1995; Raemaekers, 1996).

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 (Bustin, 2002). For semi-quantitative RT-PCR to be meaningful, the PCR products must be measured during the exponential phase of amplification (Dukas *et al.*, 2000) and a suitable housekeeping gene (β-actin, GAPDH, 18s RNA and 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 (Murphy *et al.*, 1990; Gaudette and Crain, 1991). The objectives of the study aimed to explore semi-quantitative RT-PCR technique to detect E2A expression, quantitation and

Table 1: Phenotypic and carcass characteristics of Gao you and Kun shan indigenous duck breeds

Breed	Weight (mean ± std) g					
	Live body	Liver	Gizzard	Proventriculus	Breast muscles	Leg muscles
Gao you	1737.91±164.37 (30)	33.16±4.49 (30)	76.22±10.43 (30)	5.79±1.30 (30)	74.24±9.63 (30)	76.80±1087 (30)
Kun shan	2147.91±228.99 (30)	44.00±6.36 (30)	73.48±9.44 (30)	6.88±1.43 (30)	91.65±13.23 (30)	77.35±11.31 (30)

Numbers between parentheses indicated the numbers of sex-pooled individuals.

expression variations in different tissues of different breeds associated with phenotypic variations.

Materials and Methods

Experimental animal and sample collection: Two Chinese indigenous duck breeds (*Anas platyrhynchos*); named Gao You and Kun Shan maintained in Taizhou Poultry Company as closed flock were examined. Management conditions were typically same to all of them before the commencement of the experiment. At 16 weeks of age thirty mixed-sex individuals were randomly selected and slaughtered. 2-5 gram samples from kidney and lung 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 deep freeze refrigerator (-80°C) for further studying.

Phenotypic measurement: Phenotypic and carcass characteristics comparison between two breeds was based on the measurement of live body weight and different organs weight (heart, liver, gizzard, proventriculus, breast muscles and leg muscles) (Table 1).

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 20µl of 100% deionized form amide. 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) according to the following equation (www.qiagen.com):

Concentration of RNA sample (µg/ml) = 40 X A_{260} X dilution factor

RNA samples were equalized and adjusted to 1 µg/ul. 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.

Table 2: Sequence of primers used in RT-PCR reactions

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

Reverse transcription (RT): Total RNA (2 µg) was reverse transcribed in a final reaction volume of 20 µl using Moloney Murine Leukemia Virus (MMLV) reverse transcriptase (Bio Basic Inc., Ontario, Canada) and Oligo $(dT)_{18}$ primer. The mixture of total RNA and Oligo $(dT)_{18}$ primer (0.5 µg/µl) was incubated at 70°C for 5 min. 5X reaction buffer, RNaese inhibitor (20 u/µl) and dNTP mix (10mmol/L) were added on ice and the mixture incubated for another 5 min at 37°C. 1 µl of MMLV reverse transcriptase (20 u/µl) was added. The mixture was incubated at 37°C for 60 min and the reaction was terminated by heating at 70°C for 10 min. cDNA samples stored in deep freeze at -20°C.

Primer sequences: Primers sequences were determined using Primer premier v5.0 software and Primer Quest™ (www.idtdna.com) (Table 2). 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 and 30 lung samples for each breed) to measure gene expression. 2µl of respective 5 fold diluted cDNA was used in 50 µl total reaction mixtures containing 5 µl 10 X PCR plus Mg^{++} buffer (Shenergy Biocolor BioScience & Technology Co. Shanghai, China), 1 µl (10 mM each) dNTP mixture, 2 µl (10 pmol) of each gene specific primers and β-actin primers (Sangon, Shanghai, China) and 1µl (5unit/µl) Taq DNA polymerase (Shenergy Biocolor BioScience & Technology Co. Shanghai, China). PCR reactions were performed on Thermocycler 9600 (Biometra). PCR cycling parameters were initially started a 94°C denaturation for 5 minutes, 30 seconds at 52°C and 1 minute at 72°C, followed by 30 seconds at 94°C, 30

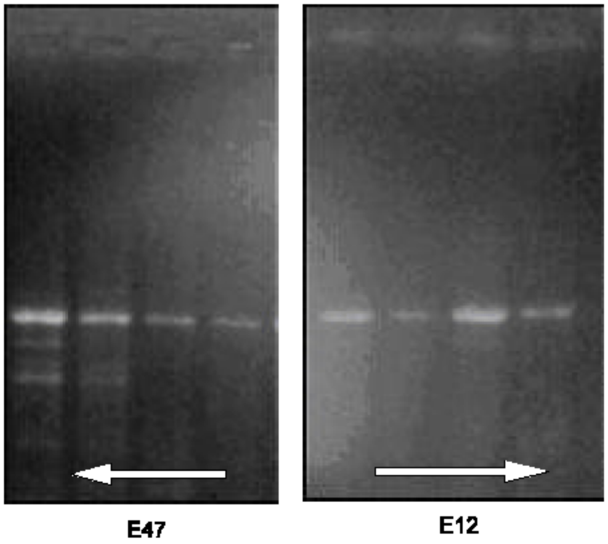


Fig. 1: Semi-quantitative reverse transcriptase polymerase chain reaction (RT-PCR) analysis of E12 and E47 splice variants expression on serial diluted cDNA (0.20, 0.25, 0.5 and 1), M: 1kbp marker (5 µ*l*). ⟹ = ascending.

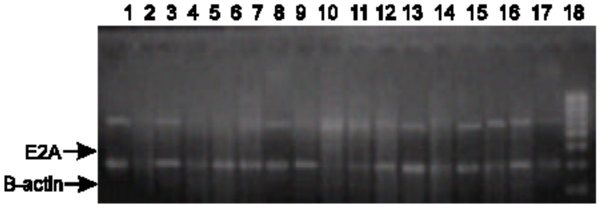


Fig. 2: Semi-quantitative reverse transcriptase polymerase chain reaction (RT-PCR) analysis confirming E2A target gene (701 bp) and β-actin (233 bp) as internal control, lane 18: 100 bp DNA ladder marker.

seconds at 52°C and 1 minute at 72°C for 30 cycles and elongation period for 10 minutes at 72°C. The PCR programme for the expression of E12 and E47 factors in serial dilutions was 94°C denaturation for 10 minutes, followed by 30 seconds at 94°C, 30 seconds at 55°C and 3 minutes at 72°C for 30 cycles and 10 minutes 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 E2A expression 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 E2A expression in different organs and association between phenotype and gene expression levels.

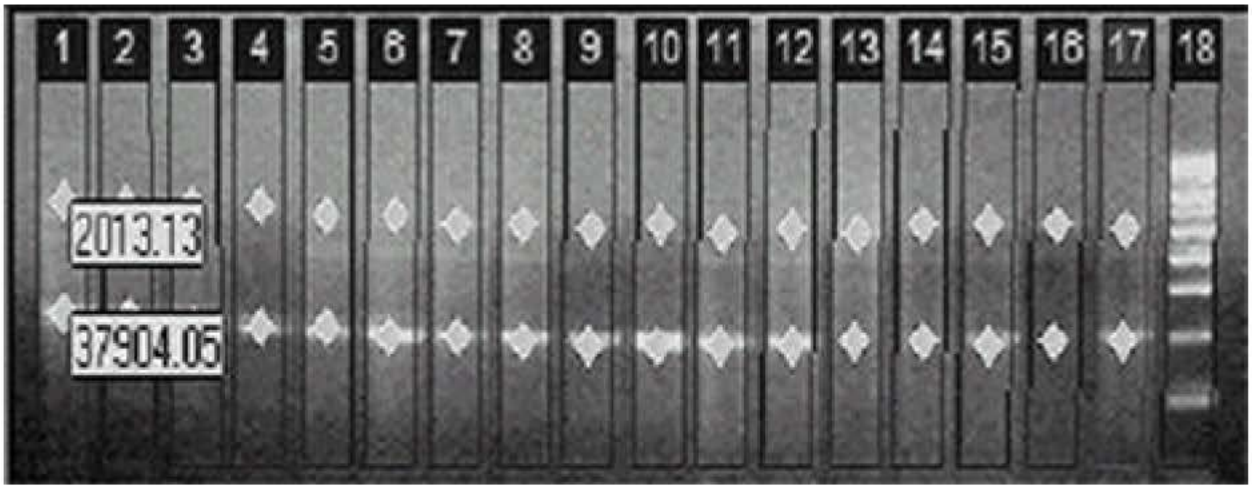
Results

Serial diluted cDNA, 0.25, 0.05 and 1 were used in a semi-quantitative RT-PCR with specific splice variant primers P_1, P_2 and P_1, P_2 to detect the differential expression level between E12 and E47, respectively. The two splice variant gave similar expression message (Fig. 1).

Assessment of E2A expression variation within breeds (between organs) and between breeds was applied with two pair of specific primer sets and β-actin as internal control in duplex PCR reaction (Fig. 2). Images were subjected to ImageQuant software for calibration (Fig. 3) and the intensities of bands were measured as volume (µg). The relative level of amplification of E2A product (T) over β-actin (c) was determined.

Statistical analysis showed significantly ($p<0.05$) higher expression of E2A in kidney tissues than lung tissues for both two breeds (Fig. 4), however, significant differences were obtained only for expression level in lung tissues between two breeds.

Phenotypically, Kun shan live body weight (2147.91±228.94) g was significantly ($p<0.05$) greater than Gao you live body weight (1737.91±164.37) g (Fig. 5). Correlation between E2A relative expression level and body weight for Gao you breed was 0.41318 and 0.27523 for kidney and lung tissues, respectively, while for Kun shan breed the association was negative and account (-0.07844) and (-0.15705) for kidney and lung tissues, respectively.



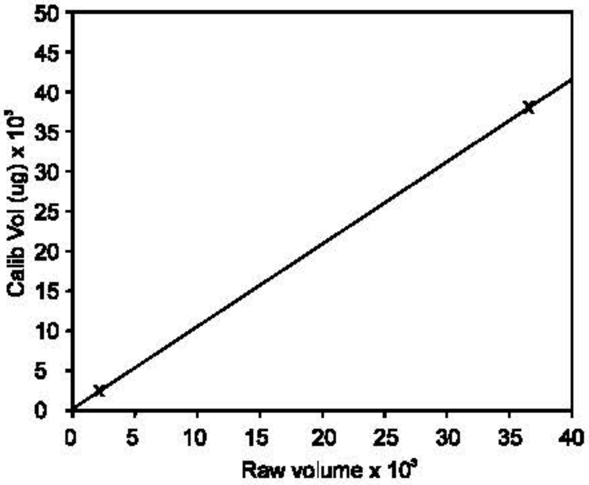


Fig. 3: Calibration (Vol. (µg)) of E2A target gene (T) and β-actin (c) as an internal control (lane 1 to lane 17) to normalize sample-to-sample variation. M: 100 pb DNA ladder marker.

Discussion

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. 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 E2A. However, E2A contributing to gene expression in a variety of tissues but it has 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 in amplification efficiency and variation in cDNA sample loading. Using the same sample size, assessing RNA integrity and equalizing RNA concentration prior to RT 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 E2A 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 E2A gene (T) over β-actin (c) showed that the relative expression level of E2A was greater in kidney tissues than in lung tissues for both breeds. The increasing expression level account 32.98% and 59.06% for Gao you and Kun shan indigenous duck breeds, respectively. The degree of expression divergence between tissues within breeds was found to be significant ($p<0.05$). However, the expression level of E2A was 34.35% and 60.70% higher in Gao you breed than Kun shan breed for kidney and lung tissues, respectively, significant differences ($p<0.05$) were obtained only for the expression level in lung tissues. The results showed that sex affected E2A expression insignificantly. Semi-quantitative RT-PCR results showed that differential expression of avian E2A can be detected in different tissues and the level of expression can be considered as a novel genetic marker for avian genetic further studies.

E2A 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

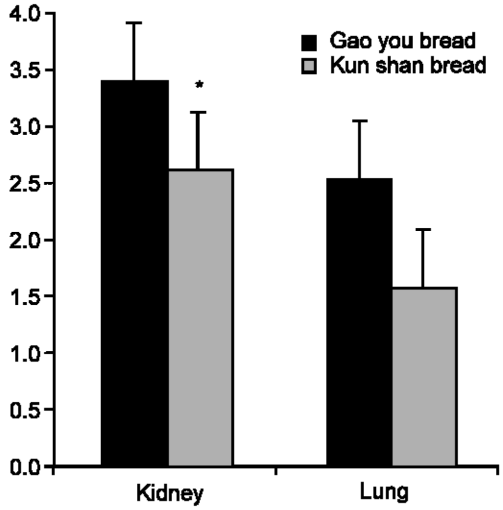


Fig. 4: Relative expression of transcriptional factor E2A in kidney and lung of the two duck breeds, Kun shan breed and Gao you breed. The data shows the mean ± SEM of all samples (20 kidney and 23 lung samples, and 22 kidney and 26 lung samples for Kun shan and Gao you, respectively). Values are expressed as Volume (ug) after calibration. *: Significant difference ($p \leq 0.05$).

Fig. 5: Live body weight (g) of sex-polled Gao you and Kun shan Chinese indigenous duck breeds represented as mean ± SEM (n= 30, for each breed). *: significant differences ($p<0.05$).

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 E2A 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 E2A functional divergence can be, in part attributed to expression divergence.

The live body weight of Kun shan breed was significantly ($p<0.05$) greater than Gao you breed. The association between E2A expression levels and body weight in Gao you breed were positively moderate, 0.413 and 0.275 for kidney and lung tissues, respectively. Whereas in Kun shan breed were -0.078 and -0.157 for kidney and lung tissues, respectively. It is clear that E2A expression level in different organs in genetic lean indigenous duck breed was higher and positively correlated with body weight, in contemporary to its expression in the genetically heavy breed. Genome expression studies have shown numerous differences in transcript abundance both within and between closely related species (Enard *et al.*, 2002; Whitney *et al.*, 2003). In some instance, genetic variation in gene expression has been associated with phenotype variation (Oleksiak *et al.*, 2002; Wayne and McIntyre, 2002; Schadt *et al.*, 2003). However, gene expression differences correlated with a phenotype may or may not contribute to the phenotype (Fay *et al.*, 2004).

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