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Screening Genes Related to Breast Blister (Keel Cyst) in Chicken by Delta Differential Display

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ABSTRACT

In this study, screening and identification differentially displayed genes related to breast blisters (keel cysts) in the "Gushi chickens×Ankao" F_2 resource population. Delta Different Display Reverse Transcription-Polymerase Chain Reaction (DDRT-PCR) in combination with Single Strand Conformation Polymorphism (SSCP) is a very sensitive tool for analyzing gene expression of isolated liver tissue from birds. The special primer pair was designed to identify the differential displayed band and a semi-quantitative Reverse Transcription-Polymerase Chain Reaction (RT-PCR) was performed simultaneously. We now are able to directly characterize genetic differences in breast blisters and the healthy birds. The new ESTs was submitted to GenBank (Accession number: GW317166, GW317167, GW317168, GW317169, GW317170, GW317171, GW836603). Several of the differentially expressed fragments were messenger RNA. Six fragments showed interesting homologies to known sequences from the databases. One fragment did not show any homologies to known sequences. This lays a foundation for further study on the mechanism of differential gene expression in breast blisters and the healthy chickens. It is the first time to demonstrate in this study that Tumor Susceptibility Gene 101 (TSG101) is related to breast blisters (keel cysts) of poultry. Furthermore, GW317167 is suggested to contribute to the development of breast blisters (keel cysts), thus may be candidates of new targets of tumor susceptibility genes.

Key words: Breast blister (keel cyst), DDRT-PCR, F_2 resource population, SqRT-PCR, SSCP, ESTs

INTRODUCTION

Breast blisters (keel cysts) are an important factor affecting the carcass grade of broilers. In broiler chickens suffering from breast blisters, a clear, sticky fluid appears between the keel bone and the skin of the breast, swelling over the keel bone with bruising and discolouration. When this bursa becomes inflamed by trauma or infection, fluid or exudate accumulates and appears as a fluid-filled blister 1-3 cm in diameter (Zhao *et al*., 2009; Lewis and Weaver Jr., 1974; Oikawa *et al*., 1993; Castaneda *et al*., 2005; Kahn and Line, 2010; Alsobayel and Al-Miman, 2010). Inflammation of the sternal bursa along the keel bone which may, in chronic cases, give way to scar tissue (May and Cox, 1970). The incidence of breast blisters is affected by many factors, such as

age, body weight, sex, size of rearing site, breed and its distance from the processing plant and various weather conditions (Maye, 1980; Islam *et al.*, 2004).

Genetic factors, pressure, irritation, breast feather-covering or friction to the keel contribute to the development of breast blisters (Huang *et al.*, 2006; Dobson, 1966; Miner and Smart, 1975; Reed *et al.*, 1966). A breast cyst in an 8-week-old broiler, fluid cysts make up less than 5% of trimming. Breast cysts in birds may also be associated with infectious synovitis or chronic arthritis. In both cases, birds lay with their breast on the floor (Herenda and Franco, 1996). Broiler carcasses are often trimmed during evisceration to remove damaged areas of the carcass. A survey was conducted into down-grading due to breast blisters at a large processing plant. The condition is not fatal but morbidity may reach more than 50% (McMullin, 2004).

The research on breast blisters (keel cysts) was not at the molecular level to this day. In this study, screening genes related to breast blisters (keel cysts) in resource population of genetic background clearly by differential display for the first time (Liang and Pardee, 1992; Wu *et al.*, 2005). This study was aimed to explain the pathogenesis of breast blisters (keel cysts) from the perspective of molecular biology.

MATERIALS AND METHODS

Chickens: The F_2 resource population from the Henan Innovative Engineering Research Center of Poultry Germplasm Resources were used in this study. The complete resource population included 38 grandparents, 66 F_1 parents and 864 F_2 chickens. The F_2 resource population was generated from 23 Gushi chicken (2 cocks, 21 hens) representing a slow-growing Chinese native chicken and 15 Ankao broilers (4 cocks, 11 hens) representing a fast-growing broiler. The F_2 resource population consist of 4 cross families (Ankao broilers were male parent) and 3 reciprocal cross families (Gushi chicken were male parent), a total of 864 birds. All Chickens were managed in cage according to standard practices.

In this study, there is a contraposition between the birds suffering from breast blisters and the healthy birds of full-sib. The birds suffering from breast blisters and the healthy birds are one-to one. Each family selected 2 pairs, a total of 28 chickens. One pair of the experimental observations belong to the same family, their shapes are basically similar and the sex is the same. At the age of 84 days, 864 F_2 chickens were dissected for liver. Liver was collected from the tested chickens and immediately immersed in liquid nitrogen and then stored at -80°C before use.

Total RNA extraction and cDNA synthesis: The total RNA samples was extracted from chicken liver for the DDRT-PCR prescreening using the Trizol reagent (TAKARA BIO.CO.) according to the manufacturer's protocol. The RNA quality and yield were assessed spectrophotometrically using ratios of A_{260}/A_{280} and by agarose gel electrophoresis. Prior to the reverse transcription reaction, RNA were treated with 1 μL of DNase (10 unit μL^{-1}) for 30 min at 37°C, phenol extracted, ethanol precipitated and resuspended in nuclease-free water (RNase free DNase ,Beijing SBS Genetech Co.) at a concentration of 2 μg μL^{-1} and stored at -80°C until use. Reverse-transcribed for the generation of cDNA using a first-strand cDNA synthesis kit with oligo (dT) primer (Fermentas life Sciences) following the manufacturer's instructions.

Differential display procedure: Following this first strand cDNA synthesis, a PCR reaction was performed in a total volume of 50 μL containing 6 μL of the reverse transcription product, 2 μL of

Table 1: Primers used in this study

Primers	Primer sequences (5'-3')	Primers	Primer sequences (5'-3')
T1	CATTATGCTGAGTGATATCTTTTTTTTTAA	P1	ATTAACCCTCACTAAATGCTGGGGA
T2	CATTATGCTGAGTGATATCTTTTTTTTTAC	P2	ATTAACCCTCACTAAATCGGTCATAG
T3	CATTATGCTGAGTGATATCTTTTTTTTTAG	P3	ATTAACCCTCACTAAATGCTGGTGG
T4	CATTATGCTGAGTGATATCTTTTTTTTTCA	P4	ATTAACCCTCACTAAATGCTGGTAG
T5	CATTATGCTGAGTGATATCTTTTTTTTTCC	P5	ATTAACCCTCACTAAAGATCTGACTG
T6	CATTATGCTGAGTGATATCTTTTTTTTTCG	P6	ATTAACCCTCACTAAATGCTGGGTG
T7	CATTATGCTGAGTGATATCTTTTTTTTTGA	P7	ATTAACCCTCACTAAATGCTGGTGG
T8	CATTATGCTGAGTGATATCTTTTTTTTTGC	P8	ATTAACCCTCACTAAATGGAGCTGG
T9	CATTATGCTGAGTGATATCTTTTTTTTTGG	P9	ATTAACCCTCACTAAATGTGGCAGG

P primer and 2 μL T primer (Wu *et al*., 2005) (Table 1), 30 μL of Taq DNA polymerase (promage). Forward primers and reverse primers were the combination of P_xT_y. The condition for the PCR amplification reactions were: 94°C for 6 min, 40°C for 5 min, 68°C for 5 min, 94°C for 2 min, 40°C for 5 min, 68°C for 5 min (3 cycles); 94°C for 1 min, 60°C for 1 min, 68°C for 2 min (28 cycles); a final extension at 68°C for 7 min. Subsequently, 10 μL of the PCR products were mixed for with 2 μL of loading dye, denatured for 10 min at 100°C and loaded onto a 12% non-denaturing polyacrylamide sequencing gel. The gel, run at constant power (120 W) for 5 h and then the banding patterns of the DDRT-PCR products were revealed with sliver-stained method (Bassam *et al*., 1991; Gao *et al*., 2009).

Bands corresponding to differentially expressed band were excised from the PAGE gel and placed in Eppendorf tubes containing 100 μL H_2O for 10 min. Gel slices were then boiled for 10 min, spun down at 10,000 rpm for 2 min and then extract supernatant. The supernatant were used for re-amplification in a PCR reaction with the corresponding primers as previously indicated. To avoid normalization due to the "Cot effect" (Mathieu-Daude *et al*.,1996a), we reduced the number of PCR to 25 cycles.

PCR products were purified from agarose gel using a Gel Extraction Kit (Tiangen Bio co.) and the purification were identified by Single Stranded Conformation Polymorphism (SSCP). Each fragment subcloned into the pUCM-T vector (Shanghai Sangon Bio Co.). The PCR-positive colonies were selected for sequencing (TAKARA BIO.CO). Subsequently the sequences were analyzed using the BLAST search software at the National Center for Biotechnology Information (NCBI).

Gene expression by semi-quantitative RT-PCR analysis: Total RNA from liver tissues of 8 breast blisters and 8 healthy chickens was quantitated by spectrophotometry and their integrity was evaluated on a 1% agarose gels. Reverse transcription and PCR followed the instructions of the manufacturer (RevertidTM First Strand cDNA Synthesis Kit, Fementas, MBI, USA). First strand cDNA was synthesized from 3 μg total RNA from each individual animal oligo $(dT)_{18}$ primer and the M-MuLV reverse transcriptase. The mRNA expression levels for the selected genes were analysed by semi-quantitative reverse transcription PCR analysis with specific primer. The specific primer (Table 2), a total of 7, were designed in order to analysis gene expression between breast blisters and healthy chickens by the sequences of differential bands. PCR reactions were initiated at 95°C for 5 min, followed by 40 cycles of 95°C for 30 sec, 52°C for 30 sec, 72°C for 50 sec and terminated at 72°C for 10 min. The products of PCR were electrophoresed in a 3.0% agarose gel electrophoresis.

Table 2: RT-PCR primers information

Sequences	Forward primers (5'-3')	Reverse primers (5'-3')	Fragment size (bp)
GW317166	TTTTCGTTTTCGTTTGTGG	TAACCCTCACTAAATCGGTC	147
GW317167	TAGAGGAAGAAATATGCAAC	AACCCTCACTAAATGCTG	201
GW317168	TGCTGGGGAGAGAGGGATTC	ATGCTGGGGAGGAGGGTG	141
GW317169	AATGCTGCGGAGAGAGGGAT	TGCTGGGGAGGAGGGTGAG	93
GW317170	TTTCATTTCGTTTGTGG	TACCTCACTAAATCGGTC	157
GW317171	TAGAGAAGACCATATGCAAC	AACCTCACTAAATGCTG	158
GW836603	ATGCTGCGGAGAGAGGGTAT	TCTGGGGAGGAGGGTGAAG	157

RESULTS

Incidence rate of the "Gushi chickens×Ankao" F_2 resource population: In this study, the incidence of breast blisters reach more than 10.4% in 864 chickens of "Gushi chickens×Ankao" F_2 resource population. In the seven families, the highest incidence rate reach 20.9% and the lowest incidence rate was only 2.27%. In the same feeding conditions, the incidence rate was 5 times between families.

Isolations of differentially expressed cDNA in liver: In order to run the assay efficiently, 81 pairs of primers that were able to amplify more bands were chosen to amplify cDNA from paired tissues. As shown in Fig. 1, the comparison showed that the certain DNA bands between 100 and 200 bp were displayed. Eventually, many differentially expressed bands were targeted. The arrows indicate differentially expressed fragments used for further analysis (Fig. 1).

Re-amplification and SSCP analysis: Using the differential display approach, ESTs of approximately 100-200 bp were obtained from livers and a number of differentially expressed fragments were identified. The differentially expressed bands resamplified using the appropriate combination of primers and analyzed with Single Strand Conformation Polymorphism (SSCP). The migration of delta DDRT-PCR products by the SSCP gel relies on both the conformation of the single stranded DNA and the molecular weight, products of the same size but different sequence can be separated (Fig. 2).

Semi-quantitative RT-PCR analysis of ESTs: The results of differential display and identification of ESTs as differentially expressed gene in the liver between breast blisters and healthy chickens by semi-quantitative reverse transcription polymerase chain reaction (RT-PCR). The expressions of ESTs between breast blisters and healthy chickens are showed in Fig. 3.

DNA sequencing and homological analysis: The characterized delta DDRT-PCR sequences were submitted to the NCBI databank with the accession numbers presented in Table 3. Among these seven sequences, six sequences fragments were revealed highly homologous to known sequences from the databases. In contrast, one fragment was identified as unknown sequences, it showed no significant hit in GenBank having a potential to be unique genes. These fragments will be further analyzed. The putative functions of the genes were predicted by sequence homologies of genes which are available in the GenBank database.

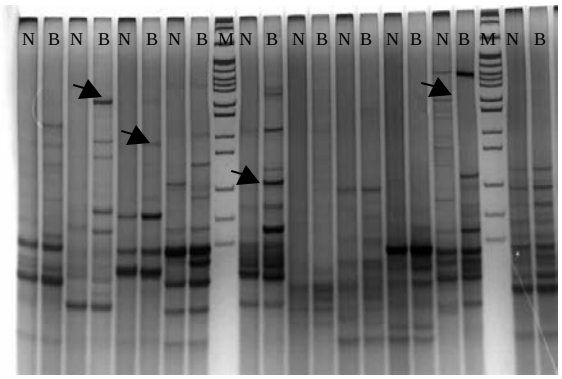

Fig. 1: Differential analysis of expressed bands between normal chickens and breast blisters of chickens. Parts of the differentially expressed bands were showed by arrows. N: Normal chickens; B: Breast blisters and M: Marker 100 bp

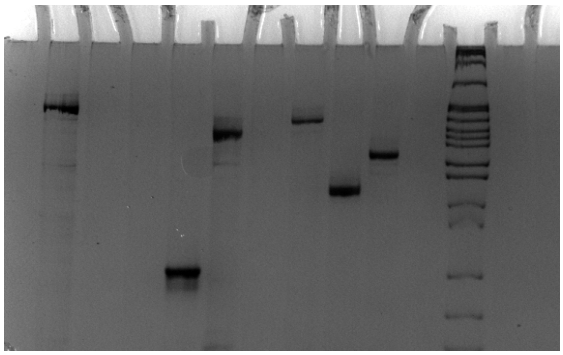

Fig. 2: Confirmation of delta DDRT-PCR results by SSCP. The products of delta DDRT-PCR were electrophoresed in a 12% polyacrylamide gel electrophoresis. It shows that the bands in the DDRT-PCR are truly differentially expressed.The band was excised and cloned into the pUCM-T Vector System (Shanghai Sangon Bio Co.)

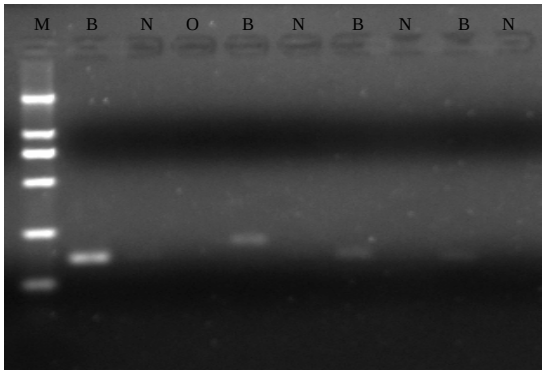

Fig. 3: Agarose gel electrophoresis (3.0%) showing the products of semi-quantitative RT-PCR.N and B represent healthy chickens and breast blisters chickens, respectively. M: Molecular weight standards, Marker DL2000 DNA Ladder (2,000, 1,000, 750, 500, 250 and 100 bp) (TaKaRa). O: The negative control amplified with H_2O as template

Table 3: Homology results of 7 bands according to the GenBank database

Serial No.	Accession No.	Definition (just highest homology)	Identities (%)
GW317166	AC171006.3	Gallus gallus BAC clone CH261-172K16 from chromosome ul, complete	97
GW317169	XM_003206262.1	Meleagris gallopavo tumor susceptibility gene 101 (TSG101), mRNA	98
GW317171	NM_204542.2	Gallus gallus nuclear receptor subfamily 1, group H, member 3 (NR1H3), mRNA>emb|AJ851708.1| Gallus gallus mRNA for hypothetical protein, clone 19n22	97
GW317168	XM_001233574.1	Gallus gallus similar to mitochondrial complex I subunit NDUFS5 (LOC771510), mRNA	100
GW317170	GU261719.1	Gallus gallus isolate YP20092 breed Chigulu mitochondrion, complete genome	100
GW317167		No significant homology	
GW836603	XM_003206414.1	Meleagris gallopavo oxysterols receptor LXR-alpha-like (LOC100548441), mRNA	92

DISCUSSION

DDRT-PCR is a powerful methodology to compare gene expression in cells or tissues under different physiological conditions (Liang and Pardee, 1992; Fawzi, 2007). This technique, originally described by Liang and Pardee (1992) has since been used extensively to identify gene expression, diagnose genetic diseases, analyze differences in gene expression and so on. The rationale for DDRT-PCR is based on the amplification, by the polymerase chain reaction, of the 3' terminal portion of mRNAs using different combinations of arbitrary primers followed by visualization of the fragments by gel electrophoresis. Radioactive profiles of mRNAs expressed under a normal and an experimental condition are generated and compared side by side using polyacrylamide gel electrophoresis. DNA sequencing further identifies transcripts represented by bands that are differentially expressed (Bratanich and Blanchetot, 2006). Delta DDRT-PCR method is an improved mRNA differential display (Malabadi and Nataraja, 2007; Matar *et al*., 2009).

Delta differential display PCR is a novel technique that was conceived to allow the identification and molecular cloning of differentially expressed genes. Using this method, a large number of genes can be analyzed with only a small quantity of mRNA (Pathak and Kanungo, 2007).

This method of DDRT-PCR can be highly repetitive in detecting differential expression of RNA. The results can be repeated more than 95% and more than 85% of the differentially expressed bands can be confirmed by SSCP and specific PCR. Herein, the application of the DDRT-PCR methodology was used to identify breast blisters related genes in this study. The method of Single-Strand Conformation Polymorphism (SSCP) analysis is a very suitable for verifying results in a system where only small amounts of RNA are available (Schneider and Zelck, 2001; Qiong *et al*., 2011; Halabian *et al*., 2008; Onsori *et al*., 2007).

Based on conformational intrastrand differences in DNAs sequences, SSCP also facilitates separation of co-migrating complex DDRT-PCR products of the same size (Mathieu-Daude *et al*., 1996b; Aboul-Ata *et al*., 2010).

We performed SSCP to confirm the differential expression of selected fragments. After reamplification, false-positive fragments could be easily distinguished from true differentially expressed signals. DDRT-PCR in combination with SSCP is a very sensitive tool for analyzing gene expression of isolated liver tissue from birds. This proved to be a effective and rapid method to obtain differentially expressed cDNA fragments. We now are able to directly characterize genetic differences in breast blisters and the healthy birds.

This study aimed to provide information on the breast blister of candidate genes associated with economic traits in birds.

We selected and identified the differentially expressed cDNA fragments (GW317169) which was homologous to the Meleagris gallopavo tumor susceptibility gene (TSG101) for future studies according to the important function of this gene and isolated chicken TSG101 gene fragments with corresponding knowledge of the known Meleagris gallopavo TSG101 gene by bioinformatics methods. It is the first time to demonstrate in this study that tumor susceptibility gene 101 (TSG101) is related to breast blisters (keel cysts) of poultry.

Three the differentially expressed fragments (GW317171, GW317168 and GW836603) were messenger RNA. The sequence (GW317168) was analyzed using BLAST software in the NCBI GenBank databases, similar to Gallus gallus mitochondrial complex I subunit NDUFS5 (LOC771510). Significant matches were returned for the differentially expressed genes (100%).

Since, GW317167 has no homology with any known genes, they are considered as new genes in breast blisters. Furthermore, GW317167 is suggested to contribute to the development of breast blisters (keel cysts), thus may be candidates of new targets of tumor susceptibility genes. GW317167 is only expressed in pathological changes but not in normal chickens. Consequently, the gene may be regarded as important indicator in breast blisters, but its exact functions haven't been elucidated. This study provides evidence that seven genes expressed differentially between breast blisters and the healthy birds.

This study lays a foundation for further study on the mechanism of differential gene expression in breast blisters and the healthy chickens. Future work is to investigate the functional importance of the breast blister related gene by analyzing its spatial expression profiles and sequence characteristics. These data should help us to find key genes for the treatment and prevention of breast blisters.

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