Identification of Differentially Expressed Genes in the Longissimus Dorsi Muscle Tissue between Duroc and Erhualian Pigs by mRNA Differential Display*

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ABSTRACT : In order to identify differentially expressed mRNAs (which represent possible candidates for significant phenotypic variances of muscle growth, meat quality between introduced European and Chinese indigenous pigs) in the longissimus dorsi muscle tissue between adult Duroc and Erhualian pigs, mRNA differential display was performed. Five 3′ anchor primers in combination with 20 different 5′ arbitrary primers (100 primer sets) were used and nearly 5,000 cDNA bands were examined, among which 10 differential display cDNAs were obtained, cloned and sequenced. Six of the 10 cDNAs showed similarity to identified genes from GenBank and the other 4 had no matches in GenBank. Differential expression was tested by Northern blot hybridization and could be confirmed for 2 cDNAs. The method used in this study provides a useful molecular tool to investigate genetic variation that occurs at the transcriptional level between different breeds. *(Asian-Aust. J. Anim. Sci. 2003. Vol 16, No. 7 : 1066-1070)*

Key Words : Pig, Longissimus Dorsi Muscle, mRNA Differential Display

INTRODUCTION

The mRNA differential display first described by Liang and Pardee is a fast and efficient method for isolating and characterizing altered gene expression in different cell types (Liang et al., 1992; Liang et al., 1994; Bauer et al., 1993). Theoretically, it can detect all expressed genes in a cell, compare multiple cell types simultaneously, display all differences, and detect over- and under-expression of genes. Therefore, the mRNA differential display technique has become a widely used methodology for identifying differentially expressed genes due to those advantages comparing to other conventional techniques.

There are significant phenotypic variances between Chinese indigenous pig breeds and introduced European pig breeds. Introduced European pigs have higher growth rate, lean meat percentage and food conversion efficiency, whereas Chinese indigenous pigs have high prolificacy and superior meat quality (Johns, 1998). Phenotypic differences are mainly arisen from genetic divergence. To better understand the molecular mechanism of those differences, a preliminary study was conducted using the mRNA differential display technique to isolate and identify differentially expressed genes in the longissiumus dorsi muscle between Duroc and Erhuanlian pigs. Duroc and Erhualian pigs were chosen in our study because they are extremely representatives for muscle growth and other phenotypic differences.

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MATERIALS AND METHODS

Animals

Duroc pigs were from the animal husbandry and veterinary medicine research institute of Hubei Province and Yamei farm of Henan Province. Erhualian pigs were from the Changshu city state-operated animal breeding farm of Jiangsu Province.

Longissmus muscle collection and RNA isolation

Longissimus dorsi muscles were collected from adult pigs at slaughter and stored in liquid nitrogen. Total RNA was isolated using TRIzol Reagent total RNA extraction kit (GIBCO, USA). After extraction, total RNAs were treated with RNase-free DNase I (Promega, USA) and precipitated with ethanol.

Differential display

Reverse transcription was performed using four pools of total RNA (2 from 6 Erhualian pigs and 2 from 6 Duroc pigs, 3 individuals/pool). 2 μg of total RNA were used for reverse transcription. Reaction buffer includes 1 μM anchor primers (all primers were kindly provided by the USDA supported U.S. Pig Genome Coordination Project, http://www.genime.iastate.edu), 300 U M-MLV reverse transcriptase (Promega, USA) and 500 μM dNTP mix

(Sangon, China). Total RNA and primers were added in a 0.5ml RNase-free tube. The tube was heated to 70°C for 5 minutes to melt secondary structure within the template and cooled immediately on ice to prevent secondary structure from reforming. Then the tube was spin briefly to collect the solution at the bottom. The other components were added in order, mixed gently and incubated at 37°C for 1 hour. Then incubated at 95°C for 5 minute in order to inactive reverse transcriptase. The PCR amplification was performed following Doss (1996) with some modifications. In a total 25 μl reaction volume include 1 μM of each primer (anchor and arbitrary), 2 μl of first strand products, 2.5 mM $MgCl_2$, 200 μM dNTP and 2 U Taq DNA polymerase. The parameter of PCR was 94°C for 30s, 40°C for 2min, 72°C for 30 s, 40 cycles followed by a final extension step at 72°C for 5 min. PCR products were electrophoresis at once or stored at -20°C.

PCR products were separated on 6% nondenaturing polyacrylamide gel. The plates were pretreated with binding solution and SigmaCote (Sigma, USA). After the gel was prepared, 0.5×TBE was poured and electrophoresis was carried out at 30 W of constant power for 3 h.

After bands were visualized by silver staining followed Neilan et al. (1994), the gel was dried at room temperature.

The same procedure was repeated to confirm the results to those primer sets that gave differential display bands in the first time test.

Recovery, reamplification, cloning and sequence analysis of DD-bands

The interest bands were excised from the dried gel using a sharp razor blade, placed in 50 μl ddH_2O and boiled for 10 minutes. Then 2 μl of the upper liquid was used for reamplification. The conditions of reamplification were same as the first PCR except the final volume was 100 μl.

The reamplified cDNA fragments were visualized on 2% agarose gel containing 0.5 μg/ml ethiduim bromide and recovered with Wizard prep PCR DNA purification system (Promega, USA). Recovered products were subcloned using TA-cloning kit (Promega, USA). Two different clones of each differential display bands were subjected to sequence by Bioasia company (Shanghai, China). The obtained sequences were compared with those available in GenBank using BLAST for homologies.

Northern blot analysis

Differential expression of the cDNAs was confirmed using Northern blot. 20 μg total RNA from 7 individuals of these two pig breeds was separated on a 1.2% agarose-formaldehyde gel and transferred onto nylon membrane (Sambrook et al., 1989). Probes were labelled with [α-^{32}P]dCTP (Yahui company, Beijing, China) using the purified PCR product of plasmids as the template by the Prime-a-Gene labelling system (Promega, USA). The nylon membrane was prehybridized and hybridized following the standard procedure. The membrane was washed once for 15 min with 2×SSC, 0.1% SDS at room temperature, then washed for 15 min with 0.2×SSC, 0.1%SDS at 42°C and 55°C respectively. An X-ray film was exposed with intensifying screen at -80°C. The same membrane was stripped and used for the control.

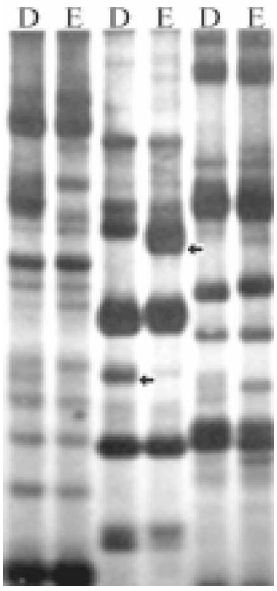

Figure 1. Part of the results of differential display. D represents Duroc pigs and E represents Erhualian pigs. Arrows indicate the differential display cDNAs.

RESULTS

Differential display

In this study, a total of 100 combinations derived from 20 arbitrary primers and 5 anchor primers ($T_{11}AG$, $T_{11}GT$, $T_{11}AC$, $T_{11}CG$, $T_{11}CA$) were used to display total RNA from the longissimus dorsi muscle between the Duroc and Erhualian adult pigs. Out of nearly 5,000 bands examined, 10 differential display cDNAs (ESThp1, ESThp2...ESThp10) were reproducibly found and excised from the gels and reamplified by PCR. Figure 1 shows a part of display result.

Sequence analysis

The length of these 10 differential display cDNA fragments ranged from 158 to 502 base pairs (bp; average length=272 bp). Two independent clones from each ESTs were sequenced. In all cases except one, sequences of 2 different clones of each EST were identical. For ESThp9, the result of sequencing revealed two different clones corresponded to different cDNA inserts with similar length

Table 1. Information of differential display ESTs

EST	Size (bp)	DD direction	Matching sequences	E-value	Identity	bp overlap/total bp
ESThp1	238	Duroc>Erhualian	No matches			
ESTh2	244	Duroc>Erhualian	Porcine CoII	e-103	100%	192/192
ESThp3	158	Duroc>Erhualian	No matches			
ESThp4	343	Duroc>Erhualian	Porcine ATPase 6	e-160	97%	309/316
ESThp5	341	Duroc>Erhualian	Porcine 12s rRNA	e-161	98%	301/305
ESThp6	304	Duroc>Erhualian	Porcine NADH2	e-137	98%	258/261
ESThp7	178	Duroc>Erhualian	No matches			
ESThp8	502	Duroc>Erhualian	Porcine MyHC	0.0	99%	442/444
ESThp9-1	195	Duroc>Erhualian	No matches			
ESThp9-2	196	Duroc>Erhualian	Porcine NADH4	e-96	99%	156/174
ESThp10	291	Duroc>Erhualian	Porcine NADH4	e-130	99%	240/241

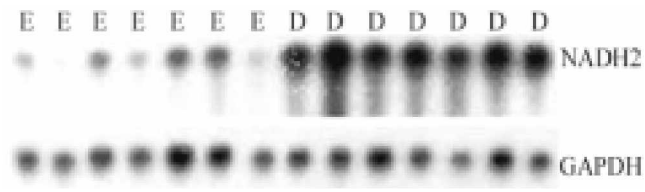

Figure 2. The Northern blot result of ESThp6 (NADH2). D represents Duroc pigs and E represents Erhualian pigs. GAPDH was used as the control for quantitative analysis.

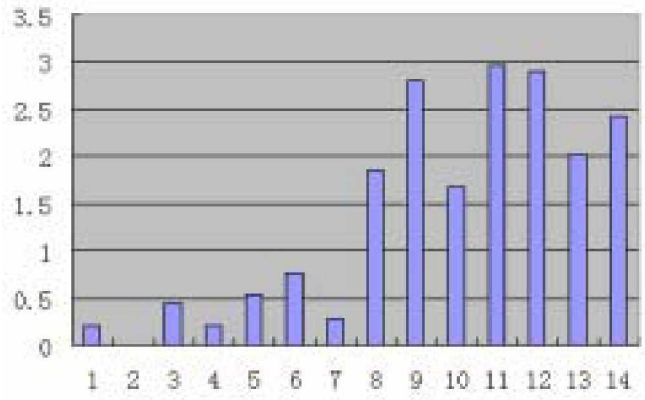

Figure 3. The bar graph of the quantitative values of NADH2/GAPDH. 1 to 7 represent Erhualian pigs, 8 to 14 represent Duroc pigs.

(195 bp for ESThp9-1 and 196 bp for ESThp9-2). Analysis of the sequence data revealed that each clone of these cDNAs had the anchor primer on one extremity and the arbitrary primer at the other. These sequences were compared with those available in GenBank to search for homologies. The criteria for scoring a sequence as having a significant match were similarity≥70% or E-value≤$1e^{-10}$ in an overlapping region of at least 70 bp (Davoli et al., 1999). This search allowed identification of ESThp2 as porcine cytochrome c oxidase subunit II (CoII), ESThp4 as porcine ATPase 6, ESThp5 as porcine 12 s rRNA, ESThp6 as porcine NADH dehydrogenase subunit 2 (NADH2), ESThp8 as porcine mRNA for myosin heavy chain (MyHC), ESThp9-2 and ESThp10 as porcine NADH dehydrogenase subunit 4 (NADH4). ESThp1, ESThp3, ESThp7 and ESThp9-1 had no match in the EST database and GenBank. These four sequences have been submitted to GenBank and the accession numbers are BI596262-BI596265. The detailed information about the 10 differential display ESTs was indicated in Table 1.

Confirmation by Northern blot analysis

Differential expression of the differential display ESTs was tested by Northern blot analysis. The first EST, ESThp1, was found to be differentially expressed between the breeds (Figure 2 and 3). ESThp6 (porcine NADH dehydrogenase subunit 2) also showed substantially higher expression in Duroc pigs (Figure 4 and 5). For the other ESTs, ESThp2, ESThp3, ESThp4, ESThp5, ESThp7, ESThp8, ESThp9-1, ESThp9-2 and ESThp10, there was no differential expression between the breeds.

DISSCUSION

Before 1992, the conventional techniques for comparing study of different cell types were subtractive or differential hybridization (Lauren et al., 1997). Although potentially powerful, these techniques had some drawbacks. Compare to these techniques, the advantages of differential display are obvious: only a small amount of mRNA is needed, the results can be obtained easily and confirmed quickly, multiple samples can be displayed simultaneously and it has high sensitivity. Consequently, differential display has become widely used in many research fields after its development.

In many studies, after differential display was established, isotopes were used to detect the differential display bands and made the process very complex. To circumvent this problem, many researchers have tried other non-radioactive methods and made some achievements (Roger et al., 1997; Ponsuksili et al., 2000). In our study, the silver staining was used to visualize differential

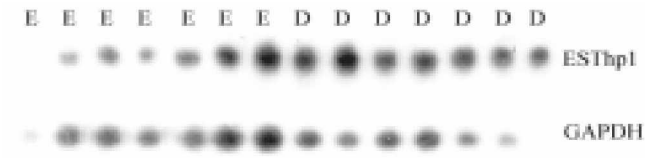

Figure 4. The Northern blot result of ESThp1. D represents Duroc pigs and E represents Erhualian pigs. GAPDH was used as control for quantitative analysis.

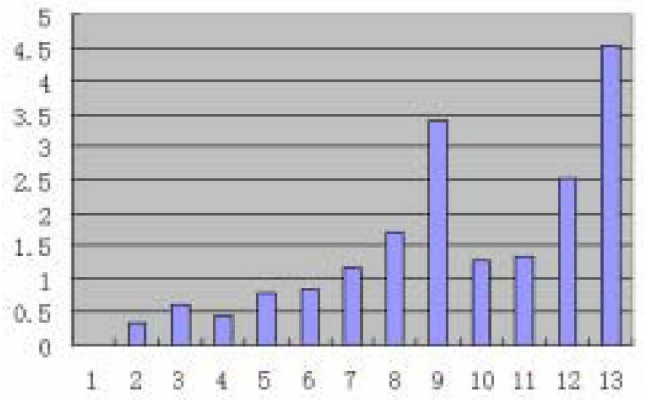

Figure 5. The bar graph of the quantitative values of ESThp1/GAPDH. 1 to 7 represent Erhualian pigs, 8 to 13 represent Duroc pigs (number 14 which represents the last Duroc pig in Fig. 4 is not included because there was no hybridization signal for GAPDH and so the value is infinite).

expressed bands. It is very convenient and could produce reproducible results.

The major pitfall of differential display is the very high frequency of false positive (Li et al., 1996). In our experiments, to reduce the effect of individual sequence polymorphism, RNAs were prepared from pools from several animals. Even though the false positive rate still reached 80% in the present study.

Porcine skeletal muscle genes play a major role in determining muscle growth and meat quality (Davoli et al., 1999). To identify possible candidate genes that represent for these traits, differential display was used in a previous study between F_2 animals of a resource population and German Landrace pigs before (Ponsuksili et al., 2000). Janzen et al. (2000) also used this technique to identify genes in longissmus dorsi muscle that were differentially expressed between a line of pigs selected for increased 200 d weight and a random selected control line. But no researchers have investigated differential gene expression in skeletal muscle tissue that may result in the obvious phenotypic differences between different pig breeds in the above traits. We selected two extremely representative pig breeds (Duroc and Erhualian) in our study. The results indicated the differential display method is a feasible model for identifying differential display ESTs in longissimus dorsi muscles between different pig breeds.

In the present study, most of the ESTs were homologous to porcine mitochondrial gene. It is in agreement with the results of Davoli et al. (1999), who reported that the most frequent cDNAs expressed in porcine skeletal muscle were the mitochondrial genes.

Northern blots were performed in order to confirm the differential display bands. Differential expression of EST hp 1 was confirmed and it indicated that this cDNAs might play an important role in influencing the differences of muscle growth and meat quality between Duroc and Erhualian pigs. Porcine NADH2 gene also showed substantially higher expression in Duroc pigs. The levels of some mitochondrial mRNAs are regulated in different tissues by hormone factors, such as thyroid hormone, androgen, estrogen, glucocorticoids and corticotropin (Raikhinstein et al., 1993). NADH2 gene has a higher level of expression in the longissimus dorsi muscle of Duroc than in Erhualian indicated that there might be some hormone in different concentration level in this tissue between these two pig breeds. There were no differential expression for other ESTs, this scenario might because one allele have bound the arbitrary primer used in a given experiment, but mRNA levels are the same.

The method used in this study provides a useful molecular tool to investigate genetic variation that occurs at the transcriptional level between different breeds. However, further investigation involving obtaining full-length cDNA of ESThp1 and predicting amino acids sequences, identifying which hormone and how it was contributing to this difference of NADH2 expression will be conducted.

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