Age-dependent Changes of Differential Gene Expression Profile in Backfat Tissue between Hybrids and Parents in Pigs

ZH. Q. Ren, Y. ZH. Xiong*, CH. Y. Deng, B. Zuo, Y. G. Liu and M. G. Lei

Agricultural Ministry Key Laboratory of Swine Breeding and Genetics, Huazhong Agricultural University
Wuhan 430070, P. R. China

ABSTRACT : Large White, an introduced European pig breed, and Meishan, a Chinese indigenous pig breed, were hybridized directly and reciprocally and a total of 260 pigs, including purebreds, Large White and Meishan, and their hybrids, White×Meishan (LM) and Meishan×Large White (ML) pigs, were bred in our laboratory. The mRNA differential display PCR (DD-PCR) was used to detect the age-dependent changes of differential gene expression in backfat tissue between hybrids and parents. Some measures were taken to reduce the false positives in our experiment. Among the total of 2,686 bands obtained, 1,952 bands (about 72.67%) were reproducible and eight patterns (fifteen kinds) of gene expression were observed. The percentage of differentially expressed genes between hybrids and parents is 56.86% at the age of four months and 57.71% at the age of six months. This indicated that the differences of gene expression between hybrids and their parents were very obvious. U-test was used to compare the patterns of gene expression between the age of four and six months, and results showed that bands occurring in only one hybrid and bands displayed in one hybrid and one parent were significantly different at $p<0.05$, and bands visualized in only two hybrids were significantly different at $p<0.01$. These indicated that differential gene expression between hybrids and parents changed at different ages. ***(Asian-Aust. J. Anim. Sci. 2005. Vol 18, No. 5 : 682-685)***

Key Words : Differential Gene Expression, Age-dependent Changes, Pig, DD-PCR, Backfat Tissue

INTRODUCTION

Fat tissue was not only the main location saving up fat, which could store energy, maintain animal heat, protect vital organisms, lighten physical pressure but also secreted many cytokines and cytokine-likes such as leptin (Luheshi et al., 1999), TNF-α (tumor necrosis factor alpha) (Kern et al., 1995), IL-6 (interleukin-6) (Yumoto et al., 1999), adiponectin (Yamauchi et al., 2001) and so on. Although the increase of cell number was found at forepart before and after birth in adipose tissue, cell volume increased basically. The increase of lipocyte volume was very quick at the age of four months but became slow after the age of six months in pigs. So it would be very meaningful that pigs at the age of four and six months were selected to detect the age-dependant changes of differential gene expression.

There are significant phenotypic variance between Chinese indigenous pigs and introduced pigs. Chinese pigs have higher prolificacy and superior meat quality, but lower growth rate and higher fat ratio compared to the introduced pigs (Mo et al., 2003). Many pig breeders have crossed indigenous pig breeds with European pig breeds to develop new pig breeds that have superior performance compared to their parents. However, the exogenous genes are not integrated to the hybrid's genome derived from parents, so the hybrids don't create new genes. This implies that gene expression varies probably under new genetic background (Romagnoli et al., 1990; Taffairs et al., 1995; Xiong et al., 1998). The mRNA differential display technique first described by Liang and Pardee (1992, 1993) is a fast, efficient method for detecting the difference of gene expression in different cell types or at different growth age. This technique possesses the advantages over other similar techniques as follows: more than two samples can be compared simultaneously, and only a small amount of total RNA is needed. In the present experiment, the aim is to detected the difference of gene expression of backfat tissue at the age of four and six months between purebreds, Large White and Meishan, and their hybrids, White×Meishan (LM) and Meishan×Large White (ML) pigs.

* Corresponding Author: Yuanzhu Xiong. College of Animal Science and Technology, Huazhong Agricultural University, Wuhan 430070, P. R. China. Tel: +86-27-87394184, Fax: +86-27-87287390, E-mail: xiongyzh@public.wh.hb.cn
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MATERIALS AND METHEDS

Animals

Six pigs for each type which included Large White, Meishan, LM and ML pigs at the age of four months and six months were randomly sampled from a total of 260 pigs bred in our laboratory for the construction of RNA pools, so 48 pigs in total were slaughtered.

Samples collection and total RNA extraction

Samples of backfat were collected from pigs at slaughter and stored in liquid nitrogen. Total RNA was

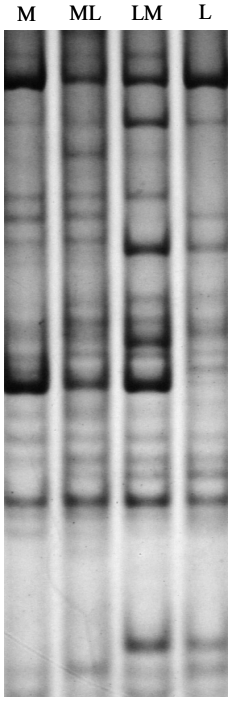

Figure 1. The result of sliver strain of differential display. M represents Meishan pig, ML represents the direct hybrid Meishan× Large White pig, LM represents the reciprocal hybrid Large White ×Meishan pig and L represents Large White.

isolated using TRIzol Reagent (Invotrigen, USA). After extraction, total RNA was treated with RNase-free DNase I (Promega, USA) to destroy potential contaminating genomic DNA and precipitated with ethanol.

Reverse transcription to synthesize cDNA

The reverse transcription reaction system required only 2 μg of total RNA pool derived from 6 pigs. In a sterile RNase-free microcentrifuge tube, 0.5 μg of the oligo $(dT)_{15}$ primer (Promega, USA) per mg of the mRNA sample was added to a total volume of 15 μl with nuclease-free ddH_2O. The tube was heated to 70°C for 5 min to denature within the template, and then cooled immediately on ice. After that, the tube was spun briefly to collect the solution at the bottom. The following components were added to the annealed primer/template: 5 μl of M-MLV 5×Reaction Buffer, 1.25 μl of 10 mM dNTPs, 25 units of RNasin® Ribonuclease Inhibitor (Promega, USA), 200 units of M-MLV RT (Promega, USA), nuclease-free ddH_2O to a final volume of 25 μl and mixed gently by flicking the tube. The tube was incubated for 60 min at 42°C, and terminated by heating 5 min with 75°C and then the efficiency of reverse transcription was checked on 1% agrose/EtBr gel.

Differential display PCR

Duplicate PCR of each cDNA was carried out simultaneously with ten arbitrary primers in combination with ten anchor primers. Thus 100 sets of primers were used. The reaction of 25 μl in total was: 1.0 μl cDNA, 0.5μl 10 mM mixed dNTPs, 2.5 μl 10×Taq DNA polymerase buffer, 1.5 μl 25 mM $MgCl_2$, 2.0 μl 10 μM anchor primer, 2.0 μl 10 μM arbitrary primer, 2.0 units of Taq DNA polymerase (1 U/1 μl), and 13.5 μl sterile water. The PCR was done as follows: 94°C for 5 min, 40°C for 5 min, 72°C for 5 min, 3 cycles of 94°C for 2 min, 40°C for 2 min, 72°C for 5 min, followed by 30 cycles of 94°C for 1 min, 60°C for 1 min, 72°C for 2 min, and then 72°C extension for 10 min.

Non-denaturing polyacrylamide gel electrophoresis and silver stain

The PCR amplified product was separated on 8% non-denaturing polyacrylamide gel (39:1). After that, silver stain was done as follows: the gel was fixed with 10% ethanol for 5 min, washed with 1% HNO_3 for 3 min, stained for 15 min using 0.1% $AgNO_3$ solution, rinsed in distilled water three times, developed in 3% Na_2CO_3 (with 1.5% formaldehyde), and terminated reaction with 3% acetic acid, then took photography and dried the gel.

Analysis of differentially expressed gene

The fragment no more than 1,000 bp was recorded in our experiment. The bands founded in gel were marked with "1"and nothing marked with "0". Only bands that can be repeated in duplicate PCR were used for analysis. Data was analyzed with U-test (Rowe, 1995).

RESULTS

The reaction conditions of DD-PCR and the efficiency of reproducible PCR

The uses of lower annealing temperature and arbitrary primers were key reasons to result in false positive, which was avoidless in DD-PCR amplification. In order to reduce false positive as little as possible, following measures were taken: (1) total RNA was treated with RNase-free DNase I to destroy potential contaminating genomic DNA; (2) the use of long anchor and arbitrary primers (31 and 26 bp); (3) the integration of high and low annealing temperature and (4) two duplicate PCRs for each combination of anchor and arbitrary primers. The bands that couldn't be repeated in duplicate PCR were discarded. 1,952 bands were amplified reproducibly in total of 2,686 ones. So the percentage of bands reproducible was 72.67%. The percentages of eight patterns were calculated according to the bands reproducible. The result of sliver strain was indicated in Figure 1.

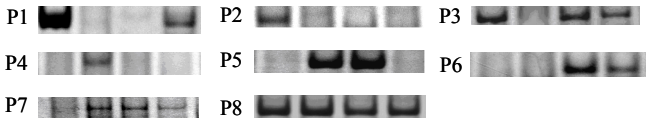

Figure 2. The patterns of gene expression between hybrids and their parents. P1: bands occurring in only parents; P2: bands occurring in only one parent; P3: bands observed in parents and one hybrid; P4: bands occurring in only one hybrid; P5: bands visualized in only hybrids; P6: bands occurring in one hybrid and one parent; P7: bands occurring in hybrids and one parent; P8: bands detected in both hybrids and parents. For each pattern, the first lane represents Meishan pigs, the second lane represents ML pigs, the third lane represents LM pigs and the last lane represents Large White pigs.

Analysis of patterns of gene expression between hybrids and parents

Eight patterns (fifteen kinds) of gene expression were observed at both four and six months old (Figure 2), which included: (1) bands occurring in only parents (1001) (pattern P1); (2) bands occurring in only one parent (0001 and 1000) (pattern P2); (3) bands observed in parents and one hybrid (1101 and 1011) (pattern P3); (4) bands occurring in only one hybrid (0100 and 0010) (pattern P4); (5) bands visualized in only hybrids (0110) (pattern P5); (6) bands occurring in one hybrid and one parent (1010, 0101, 1100 and 0011) (pattern P6); (7) bands occurring in hybrids and one parent (1110 and 0111) (pattern P7) and (8) bands detected in both hybrids and parents (1111) (pattern P8).

Analysis of differential gene expression between the age of four and six months

The percentage of the bands detected in both hybrids and parents was 43.14% at four months old and 42.29% at six months old. So the percentage of differentially expressed genes was 56.86% at four months old and 57.71% at six months old. The percentage of the bands occurring in only parents and of the bands visualized in only two hybrids were lower than others at the age of both four and six months. The percentage of each pattern was different between the age of four and six months, respectively, and U-test of the patterns of gene expression between the age of four and six months showed that bands occurring in only one hybrid and bands occurring in one hybrid and one parent were significantly different at $p<0.05$, and bands visualized in only two hybrids was significantly different at $p<0.01$ (Table 1).

DISCUSSION

The key problems of this technique were the reproducibility of display PCR and the high percentage of false positive. The display PCR conditions were optimized to minimally reduce false positive (Liang et al., 1994; Doss et al., 1996; Smith et al., 1997; Liu et al., 2004). In this experiment, the measures were taken to reduce false positive and the result showed that 72.67% bands could be reproducible. The percentage of bands reproducible increased about 4% more than that reported by Xie (2003) and about 6% reported by Wang (2004). But the use of long differential display primers and the integration of high and low annealing temperature must lose some information of differentially expressed mRNA, resulting in statistically inaccuracy. Ten 5' arbitrary primers in combination with ten 3'anchor primers (100 sets) were used in this experiment. All the patterns of gene differential expression between hybrids and parents were examined and the information of differentially expressed mRNA was farthest embodied.

In our experiment, the percentage of bands occurring in only parents and ones visualized in only two hybrids are lower than others at both the age of four and six months, in accordance with the report by Xie (2003). The possible reason that low percentage of these two extreme patterns was due to very close pedigree of parents analyzed by Xie (2003) couldn't explain the result in this experiment, because of distant pedigree between Large White and Meishan pigs. So the lower percentage of these two patterns was not probably related to the pedigree of parents. The percentage of differentially expressed genes between hybrids and parents was 56.86% at the age of four months and 57.71% at the age of six months. This indicated that the differences of gene expression between hybrids and their parents were very obvious. The hybrid couldn't create new genes compared to its parents, so it confirmed that the gene expression changed under new genetic background. U-test

Table 1. Patterns and percentage of gene expression profile at four and six months old hybrids LM, ML versus parents Large White, Meishan pigs

	P1[1]	P2	P3	P4	P5	P6	P7	P8
Four months old	1.15[2]	10.16	7.12	8.06	0.94	17.07	12.36	43.14
Six months old	1.79	8.12	5.02	11.11	5.38	13.50	12.78	42.29
u value	-1.13[3]	1.48	1.85	-2.20*	-5.48**	2.09*	-0.27	0.36

[1] The patterns of gene expression in this line, P1: bands occurring in only parents; P2: bands occurring in only one parent; P3: bands observed in parents and one hybrid; P4: bands occurring in only one hybrid; P5: bands visualized in only hybrids; P6: bands occurring in one hybrid and one parent; P7: bands occurring in hybrids and one parent; P8: bands detected in both hybrids and parents.

[2] The percentage of patterns of gene expression (unit: %) in this and next line.

[3] U-test value of patterns of gene expression between the age of four and six months.

* Value in this line is significantly different at $p<0.05$, ** Significantly different at $p<0.01$.

of the patterns of gene expression between the age of four and six months showed some patterns were significantly different. This implied that differential gene expression between hybrids and parents varied in the process of pig growth. This age-dependent changes may be provide some useful information for pig breeding. Among these patterns, bands visualized in only two hybrids was lower at four months than at six months old and was significantly different at $p<0.01$. This indicated that some genes in hybrids, LM and ML, suppressed at four months old were expressed at six months old or silent genes were activated.

All the eight patterns (15 kinds) of gene expression between direct and reciprocal hybrids and their parents were observed in this experiment. This implied gene expression and regulation of the hybrid were complicated. These patterns might have a different influence on the performance of trait at different age. However, this work was preliminary, and more research should be performed to isolate, functionally analyze the differentially expressed genes and make clear the reasons leading to gene differential expression, consequently, go deep into understanding age-dependant changes of differential gene expression between hybrids and parents.

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