



The Porcine FoxO1, FoxO3a and FoxO4 Genes: Cloning, Mapping, Expression and Association Analysis with Meat Production Traits

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ABSTRACT : FoxO1, FoxO3a and FoxO4 belong to the FoxO gene family, which play important roles in the PI3K/PKB pathway. In this study, we cloned the porcine FoxO1, FoxO3a and FoxO4 sequences and assigned them to SSC11p11-15, SSC1p13 and SSC xq13 using somatic cell hybrid panel (SCHP) and radiation hybrid panel (IMpRH). RT-PCR results showed that these three genes are expressed in multiple tissues. Sequencing of PCR products from different breeds identified a synonymous T/C polymorphism in exon 2 of FoxO3a. This FoxO3a single nucleotide polymorphism (SNP) can be detected by *AvaII* restriction enzyme. The allele frequencies of this SNP were investigated in Dahuabai, Meishan, Tongcheng, Yushan, Large White, and Duroc pigs. Association of the genotypes with growth and carcass traits showed that different genotypes of FoxO3a were associated with carcass length and backfat thickness between 6th and 7th ribs (BTR) and drip loss ($p < 0.05$). (**Key Words :** Pig, FoxO1, FoxO3a, FoxO4, Mapping, Association Analysis)

INTRODUCTION

Forkhead-box O (FOXO) transcriptional factors are members of the large forkhead (fkh) family protein. Since the *Drosophila* fkh gene was cloned in 1984, many FOX family genes have been identified in various species (Kaestner et al., 2000). The name forkhead was firstly derived from the *Drosophila* fkh gene product (Paul and Boudewijn Burgering, 2004). Through phylogenetic analysis, forty-three human FOX family proteins are classified into 17 subfamilies (FOXA-FOXQ) (Masuko and Katoh, 2004). Among these families, the FOXO subgroup contains four members, FOXO1 (FKHR), FOXO3a (FKHRL1), FOXO4 (AFX) and FOXO6 (Tran et al., 2003; Masuko and Katoh, 2004). The FOXO6 gene is different from other members by lacking a highly conserved region containing multiple phosphorylation sites (Jacobs et al., 2003).

The FOXO protein is the direct substrate of the protein kinase AKT. It could mediate the IGF-1 effects and influence the effects of the PI3K/AKT pathway. Activation of AKT leads to the inactivation of the FOXO family of

transcription factors (Gustavo et al., 2005). These proteins can upregulate a series of target genes, thereby perform different biological functions (Eric and Anne, 2005). In cultured myotubes undergoing atrophy, the activation of PI3K/AKT pathway decreases, and leads to activation of FOXO and atrogen-1 induction. IGF-1 treatment or AKT overexpression inhibits FOXO and atrogen-1 expression (Sandri et al., 2004; Stitt et al., 2004). Both MuRF1 and MAFbx were shown to encode E3 ubiquitin ligases (Bodine et al., 2001; Gustavo, 2005). These ligases are the components which confer substrate specificity, modulating the ubiquitination of these different substrates with the activation of the ubiquitin-proteasome pathway (UPP), resulting in the degradation of muscle protein. The UPP is one of the most important protein catabolism pathways in mammals, through this pathway, it could mediate specific protein degradation (Jünger et al., 2003; Kramer et al., 2003). For example, FoxO1 transgenic mice have less skeletal muscle mass, down-regulated type I fiber genes and impaired glycemic control (Kamei et al., 2004).

Meat production is one of the most important economic traits in the pig. Characterization of genes related to protein deposition and degradation in the muscle or fat will help to understand the mechanism of muscle growth and find useful markers in porcine molecular breeding (Yang et al.,

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Table 1. Primer pairs designed for amplification and sequencing

Gene	GenBank	Primer sequences (5'-3')	Primer location	Temperature value (°C)	Size (bp)
FoxO1	DQ243692	F1-PL1-GGCGAAGACCGCTTTACAA	Exon 2	58	824
		F1-PR-CTCCGATTAGTTCCTCCTGTC	Exon 3		
		F1-PL2-CTGGTCACTTACTTGCCTTGT	Intron2		
FoxO3a	DQ243691	F3a-PL1-GACTTGGACCTGGACATGTTCAACGAG	3'UTR	62	1,090
		F3a-PL2-TCCATTATCCGTAGCGAACTA	3'UTR		
	DQ243691	F3a-PL3-TGTTTCATCCTCCTTCCCCTTGC	3'UTR	62	95
		F3a-PR3-ACAGGCGTGAACGCTTGAGA	3'UTR		
	DQ288236	F3a-PL4 - CAGACGACAGTCCCTCCCA	Exon 2	62	1,153
		F3a-PR4-1-GCCTGCTTAGCACCAAGTAA	Exon 2		
	DQ243691	F3a-PL4 - CAGACGACAGTCCCTCCCA	Exon 2	60	423
		F3a-PR4-2-CGAAGCCCTTGGTGGTGTGA	Exon 2		
	DQ383425	F3a-PL5-CTGCGTGCCCTACTTCAA	Exon 1	60	291
		F3a-PR5-TAGTGACTGTCGTCTGCGGA	Exon 2		
FoxO4	DQ243693	F4-PL1-TTAGGGTGGTAAAGTTAGGCA	3'UTR	58	108
		F4-PR1-GCACACGCTTCCACATAACCC	3'UTR		
	DQ383426	F4-PL2-GCTTTACGAGTGGATGGTC	Exon 1	60	192
F4-PR2-GATTTGCCGCTCTTGCT		Exon 2			

2005; Wang et al., 2006; Guan et al., 2006). In this study, we cloned three porcine FoxO genes, mapped them to chromosomes, find a SNP in FoxO3a and detected its association with some production traits.

MATERIALS AND METHODS

Isolation of the porcine FoxO1, FoxO3a and FoxO4 sequences

Human cDNA sequences of these three genes from NCBI (GenBank accession nos. NM_001455, NM_002015 and NM_005938) were used to do BLAST analysis (www.ncbi.nlm.nih.gov/blast/) in the EST (expressed sequence tag) non-human and non-mouse databases to obtain homologous porcine ESTs. The consensus sequences of porcine ESTs were used to design primers by Primer 5.0 for amplification of the porcine genes. The primer sequences, product sizes, and NCBI accession numbers were listed in Table 1. The PCR products were cloned and sequenced to verify their identities with the corresponding ESTs.

Chromosomal localization of the porcine FoxO1, FoxO3a and FoxO4 genes

The porcine somatic cell hybrid panel (Yerle et al., 1996) and INRA-University of Minnesota porcine radiation hybrid panel (IMpRH) consisting of 118 hybrid clones (Yerle et al., 1998) was employed for localization of FoxO1, FoxO3a, and FoxO4 to the pig chromosomes. For FoxO1, F1-PL1 and F1-PR were used to amplify an intron, this intron was sequenced and used to design F1-PL2. F1-PL2 and F1-PR primers (Table 1) were used to map this gene. For FoxO3a, 3' UTR sequence was obtained using the rapid

amplification of cDNA ends (RACE) method. RACE was performed according to the instructions of the SMARTTM RACE cDNA Amplification Kit (Clontech Inc, Palo Alto, CA, USA). The PCR products of RACE were purified with Wizard PCR Preps DNA Purification System (Promega, Madison, WI, USA) and cloned into the pGEM T-easy vector (Promega), then sequenced and used to design primer pair F3a-PL3 and F3a-PR3 to map this gene. For FoxO4, the primer pair F4-PL1 and F4-PR1 was designed from the EST contig. DNA from each hybrid panel was amplified in 10 µl reaction volume containing 25 ng of hybrid DNA, 1×PCR buffer, 3 pmol each primer, 75 µM each dNTP, 0.5 U Taq DNA polymerase. The PCR profiles are 94°C for 3 min; 35 cycles at 94°C for 30 s, 58 or 62°C for 30 s, and 72°C for 20 s; with a final extension for 5 min at 72°C. The PCR products were detected on a 3.0% agarose gel stained with 0.5 µg/ml ethidium bromide. The PCR typing was done twice for each gene. The results were analyzed by the tools provided at <http://www.toulouse.inra.fr/lgc/pig/hybrid.htm> for SCHP (Yerle et al., 1996) and <http://imprh.toulouse.inra.fr/> for RH mapping (Milan et al., 2000).

Expression pattern of the porcine FoxO1, FoxO3a and FoxO4 genes

Gene expression patterns were determined by RT-PCR. PCR primer pairs were F1-PL2 and F1-PR for FoxO1, F3a-PL5 and F3a-PR5 for FoxO3a, F4-PL2 and F4-PR2 for FoxO4 (Table 1). Total RNA was extracted from adult Landrace pig heart, liver, spleen, lung, kidney, skeletal muscle, fat, gut, small intestine, stomach, placenta, endometrium, and ovary. Reverse transcription was performed as described by Pan et al. (2003). PCR

Table 2. Chromosomal assignment results of the three porcine genes

Human		Porcine RH mapping results					Porcine SCHP assignment results (error risk <0.5%)			
Gene symbol	Acc. No.	Chromosome location	Retention (%)	Closest markers	Dist (cR)	LOD score	Chromosome position	Regional assignment	Probability	Correlation
FoxO1	NM_001455	13q14.1	36	SW1632	91	3.50	11p	11p11-15	1	0.9250
FoxO3a	NM_002015	6q21	29	SW301	26	14.72	1p13	1p13	0.8769	0.8528
			31	SW781	42	10.08				
FoxO4	NM_005938	xq13.1	33	SW1835	67	5.9	xq13	xq13	0.8169	1

Table 3. Genotypic and allele frequencies of FoxO3a in different breeds

Breeds	Number of animals	Genotype			Allele frequency	
		TT	TC	CC	T	C
Dahuabai	31	17	5	9	0.63	0.37
Meishan	22	12	6	4	0.68	0.32
Tongcheng	22	9	11	2	0.66	0.34
Yushan	28	6	16	6	0.50	0.50
Large White	21	8	11	2	0.64	0.36
Duroc	35	35	0	0	1	0

conditions were 4 min at 94°C followed by 28 cycles of 30 s at 94°C, 30 s at 60°C, 25 s at 72°C, and a final extension of 5 min at 72°C. Amplification of β-actin cDNA was performed as a positive control. Ten microlitre PCR products were used to detect the expression profile in agarose gel.

SNP identification

Pooled pig genomic DNA from Duroc, Erhualian and Tongcheng pigs was amplified and sequenced to identify SNPs. PCR-restriction fragment length polymorphism (PCR-RFLP) analysis and sequencing of PCR products were used to confirm the SNP. The PCR reactions for genotyping were performed in a volume of 25 µl of 1×PCR buffer, consisting of 30 ng of genomic DNA, 10 pmol of each primer, 100 µM of each dNTP, 1.5 mM MgCl₂ and 2.0 Units Taq DNA Polymerase (Promega, Madison, WI). The PCR profiles are 3 min at 94°C followed by 35 cycles of 15 s at 94°C, 15 s at 64°C, 15 s at 72°C, and a final extension of 5 min at 72°C.

Allele frequencies of the porcine FoxO3a gene in different breeds

The allele frequency analysis included 159 unrelated animals from six breeds (Table 3), including Dahuabai pigs from Guang Dong province (31), Small meishan pigs from Jiangsu province (22), Tongcheng pigs from Hubei province (22), Yushan pigs from Jiangxi province (28), Large White (21), and Duroc pigs (35).

Association analysis of the porcine FoxO3a gene

The animals used in the association analysis of FoxO3a included 218 pigs: Tongcheng (55), Denmark Landrace (28), Large White (27), and crossbreds of Large White (Landrace × Tongcheng, 50) and Landrace (Large White × Tongcheng,

58). Sixteen traits were recorded, including average daily gain (ADG) from birth to 90 kg (AB), dressing percentage (DP), percentage of leaf fat (PLF), percentage of leaf and caul fat (PLC), backfat thickness at the shoulder (BTS), last rib backfat thickness (LRB), average backfat thickness (ABT), backfat thickness between 6th and 7th ribs (BTR), longissimus dorsi area (LDA), percentage of ham in the carcass (PHC), proportion of lean + bone of the ham (PLBH), loin pH, loin marbling (LM), water loss, loin muscle drip loss (LDL), and intramuscular fat (IMF).

Considering the genotype frequencies are significantly different between each combination, two-step analysis method is utilized to evaluate the association between genotype and trait. In the first step, a preliminary generalized linear model without genotype item is used to eliminate systemic effects. The preliminary linear model is:

$$Y_{ijkl} = \mu + B_i + S_j + C_k + \epsilon_{ijkl}$$

where Y_{ijkl} is the $ijkl$ th observation, μ is the mean, B_i is the effect of i th batch, S_j is the effect of j th sex, C_k is the effect of k th population, and ϵ_{ijkl} is the residual corresponding to the observation with $\text{var}(\epsilon) = I\sigma_\epsilon^2$. The residual in the preliminary linear model is virtually inauthentic because it consists of the effect of genotypes. In the second step, a simpler linear model is used to separate the genotypic effects from the inauthentic residual:

$$Y_{ij} = \mu + G_i + e_{ij}$$

where Y_{ij} is the inauthentic residual of the ij th individual obtained from the preliminary model, μ is the mean, G_i is the effect of the i th genotype, and e_{ij} is the residual excluding the genotypic effects with $\text{var}(e) = I\sigma_e^2$. All analyses mentioned above are implemented using the

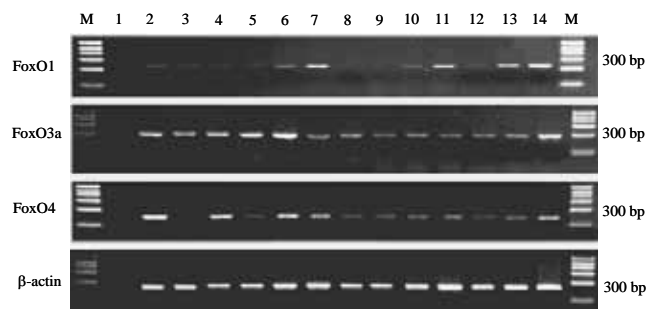


Figure 1. RT-PCR of FoxO1, FoxO3a and FoxO4 in heart, liver, spleen, lung, kidney, skeletal muscle, fat, gut, small intestine, stomach, placenta, endometrium, and ovary. Lane 1 is the negative control; Lanes 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, 13, and 14 represent 13 different tissues from heart, liver, spleen, lung, kidney, skeletal muscle, adipose, tongue, small intestine, stomach, placenta, ovary and endometrium respectively.

PROC GLM procedure in SAS software package (SAS Inst. Inc., Cary, NC).

RESULTS AND DISCUSSION

Chromosomal localization and expression of the porcine FoxO1, FoxO3a and FoxO4 genes

We assigned FoxO1, FoxO3a and FoxO4 to SSC11p11-15, SSC1p13 and SSC xq13 using SCHP. The two-point analysis from RH mapping revealed that the FoxO1 is closely linked to SW1632 (LOD = 3.50, 91cR) on SSC11. The FoxO3a gene was significantly linked to two markers, SW301 (LOD = 14.72, 26 cR) and SW781 (LOD = 10.08, 42 cR) on porcine SSC1. The FoxO4 gene was significantly linked to SW1835 (LOD = 5.9, 67 cR) on SSCx (Table 2). The homologous human genes were mapped to chromosomes 13, 6, and x. Our results are in agreement with previous comparative mapping results as human chromosomes 13, 6 and x share syntenic groups with porcine chromosomes 11, 1 and x respectively.

RT-PCR analysis of total RNA showed that the three genes were expressed in various tissues (Figure 1). All three genes were expressed in skeletal muscle, indicating the UPP pathway may be activated in muscle protein synthesis and degradation. However, the expression level of the FoxO3a and FoxO4 in muscle is relatively low. FoxO signaling

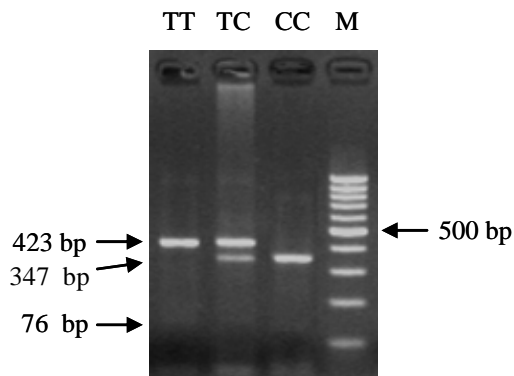


Figure 2. PCR-RFLP genotyping of FoxO3a.

regulates the expression of multiple atrophy-related genes, thus the low expression of these genes may indicate that the proteins in the muscle samples in this study are not under serious degradation. The expression in ovary, placenta and endometrium indicating FoxO transcriptional factors may also play important roles in reproduction.

Polymorphism detection and association analysis

One synonymous SNP (T to C) was detected in exon 2 of the FoxO3a gene. PCR products were digested with *AvaII* restriction enzyme to distinguish different alleles (423 bp for allele 1, 347 and 76 bp for allele 2 (Figure 2)). Allele frequencies for the *AvaII* polymorphism (Table 3) are significantly different in Tongcheng pigs compared with Yushan pigs ($p < 0.05$) and Dahuabai ($p < 0.01$), and between the Large White with Small Meishan ($p < 0.05$) and Duroc pigs ($p < 0.01$). The FoxO3a *AvaII* polymorphism showed significant association with carcass length, backfat thickness between 6th and 7th ribs (BTR) and drip loss. The carcass length with CC genotype was significantly shorter than those of pigs with the TT ($p = 0.014$) genotype and TC genotype ($p = 0.025$). The BTR with CC genotype was significantly higher than those of pigs with the TT ($p = 0.049$) genotype and TC genotype ($p = 0.012$). The PLBH with TT genotype was significantly higher than those of pigs with the TC genotype ($p = 0.002$) (Table 4).

Gene pathways determining whether muscle proteins are synthesized or degraded are crucial in muscle mass formation. It is generally agreed that a dynamic balance of

Table 4. Association analyses of FoxO3a *AvaII*-RFLP genotypes with carcass length, BTR and drip loss

Genotype	Number of animals	Carcass length	BTR	MS	Drip loss
TT	87	75.53±0.10	3.43±0.10	2.31±0.10	2.23±0.10
TC	105	75.47±0.09	3.30±0.09	2.64±0.09	1.81±0.09
CC	16	74.89±0.24	3.94±0.24	2.37±0.24	2.15±0.24
p-value					
TT-TC		0.63	0.34	0.02*	0.002**
TT-CC		0.01*	0.04*	0.83	0.74
TC-CC		0.03*	0.01*	0.29	0.18

* $p < 0.05$, ** $p < 0.01$.

anabolic and catabolic processes exists in muscle growth (Trevor et al., 2004). Studies showed that AKT1/FOXOs/Atrogin-1(MAFbx)/MuRF1 singling network plays an important role in the progression of skeletal muscle atrophy (Bodine et al., 2001; Gustavo et al., 2005). Transduction of FOXO3a activated the atrogin-1 promoter in both cultured myocytes and mouse heart, indicating FOXO3a activates atrophy-related genes that retards or prevents hypertrophy (Skurk et al., 2005). In high-fat diet-induced obesity, up-regulated FOXO3a transcription factor was also observed (Relling et al., 2006). Thus, FoxO3a may be important in both muscle and fat growth. In our study, we found a mutation in the exon 2 of FoxO3a gene. Association analysis showed that different genotypes of this mutation are related to carcass length and backfat thickness in the pig, indicating this gene may be a candidate gene for muscle growth in the pig. The SNP is a marker rather than a causative mutation for the meat production since it didn't cause amino acid change of the FOXO3a protein. However, our results need to be confirmed in the other populations as our population is small in sample size.

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