Mol Biol Rep (2009) 36:751–756
DOI 10.1007/s11033-008-9239-y

Full-length coding sequences, polymorphism and chromosomal localizations of the porcine *EDG4* and *EDG7* genes

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Received: 23 October 2007 / Accepted: 26 March 2008 / Published online: 1 April 2008

Abstract *EDG4* and *EDG7* are identified as cellular receptors for lysophosphatidic acid (LPA), belonging to the endothelial cell differentiation gene (EDG) family of G protein-coupled receptors (GPCR) which play an important role in the function of LPA. In this study, we presented the complete coding sequences of porcine *EDG4* and *EDG7* genes. The nucleotide sequences and the predicted protein sequences share high sequence identity with other mammals. Spatial expression analysis by reverse transcriptase-polymerase chain reaction (RT-PCR) revealed that porcine *EDG4* and *EDG7* genes are mainly expressed in brain, liver, spleen, lung, kidney, large intestine, small intestine, but absent in muscle tissues. Radiation hybrid mapping data indicated that *EDG4* and *EDG7* map to q2.1 of pig chromosome 2 (SSC2) and q2.6–3.2 of pig chromosome6 (SSC6), respectively. A T/C single nucleotide polymorphism (SNP) in the coding sequence of porcine *EDG4* was identified. A PCR-restriction fragment length polymorphism (PCR-RFLP) method was employed to genotype this locus among Guizhou Miniature, Guangxi Miniature, Laiwu, Wuzhishan, Tongcheng, Landrace and Yorkshire pigs. The association analysis suggested that the EDG4 genotype was associated with carcass length ($P < 0.05$) and drip loss percentage ($P < 0.05$) in the experimental population consisting of Tongcheng, Landrace, Yorkshire and two crossbred porcine populations (Wang et al. Biochem Genet (1–2):51–62, 2007).

Keywords Swine · Physical mapping · Polymorphism · Association analysis · *EDG4* · *EDG7*

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Introduction

Lysophosphatidic acid (LPA) is a simple bioactive phospholipid with activities for many cell types, stimulates cell proliferation, migration, tumor-cell invasion, actin stress fiber formation and survival [1]. In addition, LPA induces cellular shape changes, increases endothelial permeability and inhibits gap-junctional communication between adjacent cells, and acts through specific G protein-coupled [2].

EDG4 and *EDG7* were identified as cellular receptors for lysophosphatidic acid (LPA), which belong to the endothelial cell differentiation gene (EDG) family of G protein-coupled receptors (GPCR) and play an important role in the effect of LPA. *EDG4* couples with three types of G proteins, $G_{i/o}$, G_q, and $G_{12/13}$, to mediate LPA-induced cellular signaling and plays a role in LPA stimulation of ovarian tumor growth [3–5]. *EDG7* can mediate pleiotropic LPA-induced signaling that includes phospholipase C (PLC) activation, Ca^{2+} mobilization, and mitogen-activated protein kinase (MAPK) activation [3]. In neuroblastoma cells, overexpression of *EDG7* leads to neurite elongation [2].

Pigs and humans share similar anatomical, physiological, and pathological characteristics; the minipig is considered as an important experimental animal model of human disease and even a xenotransplantation donor [6, 7]. The knowledge of pig lysophosphatidic acid receptors,

therefore, will contribute to the understanding and development of porcine models to study the biology of LPA in vivo. Here, we describe the full-length coding sequences of porcine *EDG4* and *EDG7*, their expression patterns, chromosome assignments and polymorphisms.

Materials and methods

Sample collection

Fifteen tissues, including brain, liver, spleen, lung, kidney, large intestine, small intestine, stomach, ovary, uterus, testicle, thymus, lymph, dorsal muscles and muscle of leg, were obtained from mature Wuzhishan minipigs to do the spatial expression analysis. The genetic variability analysis within the porcine *EDG4* and *EDG7* sequence employed genomic DNAs that represented five indigenous Chinese breeds (Wuzhishan Miniature, Guangxi Miniature, Guizhou Miniature, Laiwu, and Tongcheng pigs) and two introduced commercial breeds (Yorkshire and Landrace).

cDNA clone and sequence analysis

Human mRNA sequence of *EDG4* and *EDG7* (GenBank accession numbers NM_004720.4 and NM_012152.1) were compared to all sequences available for pig in the expression sequence tags (EST) databases using the BLAST algorithm (http://www.ncbi.nlm.nih.gov/BLAST). We selected the porcine ESTs that shared more than 80% sequence identity to the corresponding human cDNA in order to assemble the potential porcine gene using the DNAStar program (Madison, WI, USA). To verify and clone the cDNA sequence of porcine *EDG4* and *EDG7*, RT-PCR was performed using Taq polymerase (Fermentas, Vilnius, Lithuania), cDNA pool derived from different porcine tissues as the template, and specific primers (Table 1). The predominant PCR product was purified and subsequently cloned into the

Table 1 Primers employed in these experiments

Gene name	Primer name	Primer and sequences (5′–3′)	Binding region	Size (bp)	Annealing temperature (°C)
EDG4	SNP PL[a]	GCCAGTGCTACTACAATGAG	Exon 2	729	60
	SNP PR	CCCAGAATGATGACAACAG	Exon2		
	Genomic 1 PL[b]	GCCAGTGCTACTACAATGAG	Exon 2	729	60
	Genomic 1 PR	CCCAGAATGATGACAACAG	Exon2		
	Genomic 2 PL[b]	CCTGTTGGATACAAGCCTGACG	Exon2	1,535	61
	Genomic 2 PR	TCAGCATCACGGCATGAGTA	Exon3		
	Genomic 3 PL[b]	ATCATTCTGGGAGCATTCGT	Exon3	537	61
	Genomic 3 PR	CAACTCTTTCCTGGGTGCTTA	Exon3		
	Genomic 4 PL[b]	CACCCAGGAAAGAGTTGAAT	Exon3	366	62
	Genomic 4 PR	TACTCCCTTCATAGACCTCC	3′UTR		
	Expression PL[c]	CACCCAGGAAAGAGTTGAAT	Exon3	366	62
	Expression PR	TACTCCCTTCATAGACCTCC	3′UTR		
EDG7	Genomic 1PL[b]	GTGAGCGGATGTTCAGTTCT	Exon1	756	59
	Genomic 1PR	GCCATCACCGTCTTCATTAG	Exon1		
	Genomic 2PL[b]	TGGTTCTGCTACTGGACGGC	Exon2	1,005	61
	Genomic 2PR	ACACCGAGCTACTAGTCATG	3′UTR		
	Expression PL[c]	TGGTTCTGCTACTGGACGGC	Exon2	311	62
	Expression PR	AGGGGCACCCATAGCTCAGG	3′UTR		
	CDS PL[d]	GTGAGCGGATGTTCAGTTCT	Exon1	1,801	61
	CDS PL	ACACCGAGCTACTAGTCATG	3′UTR		
	Beta-actin[e]	GCCGTGATCTCCTTCTGCA	Exon2	248	62
	Beta-actin	CTTCCAGCCCTCCTTCCTGG	Exon4		

[a] Primers for polymorphism genotyping

[b] Primers for amplifying genomic DNA to search polymorphisms

[c] Primers for various tissue expressions

[d] Primers for amplifying coding region of the *EDG7*

[e] Primers for endogenous control in spatial expression analysis

pEGM-T-Easy vector (Promega, Madison, WI) prior to sequencing.

Chromosome mapping by IMpRH

Radiation hybrid mapping was performed using the INRA-University of Minnesota 7,000 rads radiation hybrid panel (IMpRH), consisting of 118 hamster-porcine hybrid cell lines [7]. The PCR for RH mapping was performed in a total volume of 10 μl 1× PCR buffer (Fermentas), containing 25 ng panel DNA, 0.3 μM each primer (Table 1), 75 μM each dNTP, 1.5 mM MgCl2, and 1.0 U Taq DNA polymerase (Fermentas). The PCR profile consisted of 5 min at 95°C, 30 cycles of 30 s at 94°C, 30 s at 62°C, and 30 s at 72°C, and a final 5-min extension at 72°C. Data analysis was performed on the IMpRH web site (http://www.toulouse.inra.fr/lgc/pig/RH/IMpRH.htm) [8].

Spatial expression analysis

Gene expression patterns were determined by RT-PCR. Total RNA was extracted from each sample using the Trizol reagent (Invitrogen, San Diego, CA) and treated with RNase-free DNase (Fermentas), according to the manufacturer's instructions. RNA concentration was estimated, and the same amount of RNA from each targeted tissue sample of different individuals was mixed to form the RNA pool, then reverse-transcribed into cDNA using M-MLV reverse transcriptase (Promega) and oligo (dT) primers, followed by PCR using gene-specific primers (Table 1). PCR conditions were: 5 min at 95°C followed by 28 cycles of 30 s at 94°C, 30 s at 62°C, 30 s at 72°C, and a final extension of 5 min at 72°C. The housekeeping gene, beta-actin, was used as an endogenous control in all the PCR amplifications. The resulting PCR products were analyzed by agarose gel electrophoresis to determine the expression profile.

Polymorphism detection and statistical analysis

After alignment of the pig *EDG4* and *EDG7* mRNA sequence with the human mRNA sequence (GenBank accession no. NM_004720.4 and NM_012152.1), respectively, primer pairs for amplification of exon, intron and untranslated region UTR regions were designed based on the mRNA sequence employing the genomic DNA as templates, which were used to facilitate the identification of SNPs. Genomic DNA fragments were amplified by PCR in a volume of 20 μl 1× PCR buffer (Fermentas) containing 50 ng porcine genomic DNA, 0.3 μM each primer, 75 μM each dNTP, 1.5 mM MgCl2, and 2.0 U Taq DNA polymerase (Fermentas). The PCR parameters were 5 min at 95°C followed by 30 s at 94°C, 30 s at 60°C, and 30 s at 72°C for 32 cycles, followed by a final extension of 5 min at 72°C. After sequencing 12 unrelated individuals from six porcine breeds (Wuzhishan, Guangxi, Laiwu, Tongcheng, Yorkshire and Landrace), we confirmed the PCR products. Then 12 comparative sequencings revealed genetic variation which was detected by PCR-RFLP using the *Mbo*I restriction enzyme.

The animals used for association analysis were 189 pigs from three pure-bled experimental populations, including Tongcheng (T, $n = 63$), Landrace (L, $n = 21$), Yorkshire (Y, $n = 26$), and two crossbred populations, including L (♂) × YT (♀) (LYT, $n = 37$) and Y (♂) × LT (♀) (YLT, $n = 42$). Tongcheng pigs were slaughtered at 75 kg body weight; the other pigs were slaughtered at 90 kg body weight. Phenotypic data were collected in a 1-year serial slaughter experiment at the Animal Husbandry Bureau of Tongcheng County (Hubei Province, China). Production traits, including carcass traits (intramuscular fat, backfat, carcass length, and eye area), percentage of backfat thickness (carcass), and meat quality traits (meat color score, marbling score, tenderness, drip loss, and fat) were measured according to the standard Chinese meat industry carcass measurements. According to the population, the general linear model (GLM) procedure of SAS (SAS Institute, Cary, NC) was employed to evaluate the association between the genotypes and traits recorded [9]: $Y_{ijkl} = \mu + C_i + G_j + B_k + S_l + (CB)_{ik} + (BS)_{kl} + e_{ijkl}$, where μ is the population mean; Y_{ijkl} is the phenotypic value of the target trait on $ijkl$th animal; C_i is the effect of jth combination ($i = 1, 2, 3, 4, 5$); G_j is the effects of ith genotype ($j = 1, 2, 3$); B_k is the effect of kth batch ($k = 1, 2$); S_l is the effect of lth sex ($l = 1, 2$); $(CB)_{ik}$ is the effects of interaction ith combination × kth batch; $(BS)_{kl}$ is the effects of interaction kth batch × lth sex; e_{ijkl} is the error term.

Results and discussion

Molecular characterization of porcine *EDG4* and *EDG7* genes

We cloned and sequenced the partial cDNAs and did sequence alignments with the human genomic DNA sequences which came from GenBank (accession nos. NC_000019.8 and NC_000001.9). We found that the porcine *EDG4* and *EDG7* genes all contain three exons and two introns. Analysis of the partial cDNA sequences of the porcine *EDG4* and *EDG7* revealed the following: (1) the partial cDNA of porcine *EDG4* consists of 1,621 bp that contains an open reading frame (ORF) of 1,056 bp encoding a protein of 351 residues with a calculated molecular mass of 39.3 kDa and an isoelectric point (pI) of 9.78, and contains a 3′-untranslated region (3′-UTR) of

563 bp. (2) The partial cDNA of porcine *EDG7* consists of 1,801 bp; computer analysis revealed a 1,065 bp ORF flanked by a 708 bp 3′-UTR. The porcine *EDG7* gene is predicted to encode a polypeptide of 354 amino acids with a molecular mass of 40.4 kDa and a pI of 9.36. The sequences of porcine *EDG4* and *EDG7* were deposited in GenBank (accession nos. EF546799 and EF546800).

Human-porcine sequence similarity between the coding sequences of *EDG4* and *EDG7* is 88% for both genes. The corresponding proteins reveal 95% and 85% similarity of amino acid sequences between human and pig suggesting that *EDG4* and *EDG7* are evolutionarily conserved in large mammals.

Spatial expression analysis of porcine *EDG4* and *EDG7* genes

RT-PCR was performed to determine the mRNA expression of *EDG4* and *EDG7* in various pig tissues. The housekeeping gene, beta-actin was used for endogenous control. As shown in Fig. 1, Porcine *EDG4* mRNA was expressed in all the tissues except spleen and muscle. The highest expression was detected in large intestine. Varied amounts of *EDG4* transcript was detected in brain, liver, small intestine and lymph. The expression of porcine *EDG7* was most abundant in spleen and lymph, with less expression in lung, ovary, thymus and liver. We hardly detected any expression of *EDG7* in brain, kidney, stomach, large intestine and muscle. To our surprise, the expression of porcine *EDG4* was at the highest level in the large intestine; however, the expression of porcine *EDG7* was undetected in this tissue. Also in small intestine, *EDG4* expression is much higher than *EDG7*. In mouse, the *EDG4* exhibited highest expression in testis, brain and kidney while *EDG7* was abundant in testis, kidney, heart and lung [10, 11]. The large intestine was not included in the mouse study. In addition, the expression of the two genes in the porcine testis is not as abundant as their mouse counterparts. Therefore, the spatial mRNA expression differences between porcine *EDG4* and *EDG7* genes, and even among species, deserve further study.

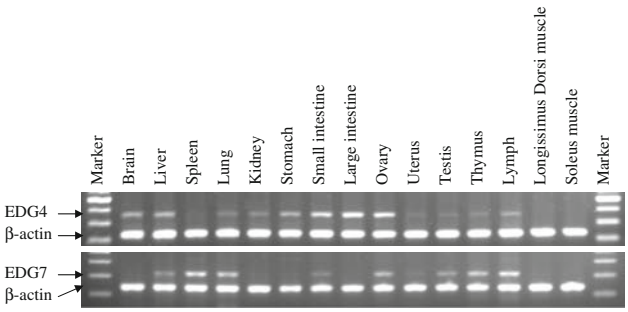

Fig. 1 mRNA expression analysis of porcine *EDG4* and *EDG7* in brain, liver, spleen, lung, kidney, large intestine, small intestine, stomach, ovary, uterus, testicle, thymus, lymph, longissimus dorsi muscle and soleus muscle

Chromosomal localization of the porcine *EDG4* and *EDG7* genes

Chromosomal localizations of porcine *EDG4 and EDG7* genes were determined by PCR screening of INRA-University of Minnesota 7,000 rads radiation hybrid panel (IMpRH) with the gene specific primers (Table 2). We mapped *EDG4* on porcine chromosome 2 (SSC2q2.1) closest to marker SW834 (LOD score 9.32) and *EDG7* to porcine chromosome 6 (SSC6q2.6–3.2) closest to marker UOX (LOD score 16.09) (Table 2). In the human *EDG4* and *EDG7* genes have been mapped to HSA chromosome 19 and 1 (HAS19 and SHA1), respectively [10, 11]. Our mapping of porcine *EDG4* and *EDG7* improves the human–pig comparative map for HSA19 with SSC2 and HSA 1 with SSC6, respectively [12].

Polymorphism and association analysis

The whole regions of porcine *EDG4* and *EDG7* genes were screened for polymorphisms except for 5′UTR and the first

Table 2 Chromosomal assignments of porcine *EDG4* and *EDG7* genes

Gene symbol	GenBank accession no. (pig)	Primer sequences (5′–3′)	Size (bp)	Human cytogenetic position[a]	Porcine IMpRH mapping results				
					Retention (%)	Closest marke	Dist (cR)	LOD score	Porcine cytogenetic position[b]
DG4	EF546799	CACCCAGGAAAGAGTTGAAT TACTCCCTTCATAGACCTCC	366	19p12	21	Sw834	0.53	9.32	2q2.1
EDG7	EF546800	AAGATAACGGCAACCGAGGC AGGGTCACAGGACATCAAGC	511	1p22.3-p31.1	25	UOX	0.19	16.09	6q2.6–3.2

[a] The cytogenetic location in human come from the website (http://www.ncbi.nlm.nih.gov/locuslink/)

[b] The position deduced from the closest marker available on the cytogenetic map [8, 13]

intron of both genes by comparative sequencing using primers provided in Table 1. Comparative sequence analysis of 12 unrelated individuals from six porcine breeds (Wuzhishan, Guangxi, Laiwu, Tongcheng, Yorkshire, Landrace) revealed one conservative SNP in the coding region at nucleotide position 236 (c. 236C > T) of the porcine *EDG4* mRNA sequence (GenBank accession no. EF546799), which does not alter the amino acid sequence of the protein. Another SNP was found at nucleotide position 1194 (c.1194C > T) in the 3′UTR of porcine *EDG7* mRNA sequence (GenBank accession no. EF546800).

For further analyses, we genotyped the SNP (c. 236C > T) of *EDG4* by PCR-RFLP in 259 unrelated pigs, representing seven indigenous and introduced commercial breeds. PCR amplification using primers *SNP* PL and *SNP* PR (Table 1) yielded a fragment containing the SNP (c. 236C > T) which can be discriminated by the *Mbo*I restriction enzyme (Fig. 2). Among our sample of 259 unrelated pigs, genotyping results showed great variation in allele frequency between Chinese indigenous and introduced commercial breeds, and allele T was dominant in the Chinese indigenous Guizhou breed and Guangxi breed (Table 3). We performed a preliminary association

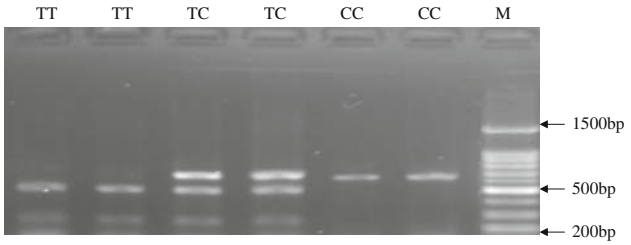

Fig. 2 PCR-RFLP analysis of 236C > T SNP in porcine *EDG4* gene locus. The SNP in the 729bp PCR fragment could be recognized by the *MboI* enzyme (CC 729bp, TT 509/220bp, CT729/509/220bp). Marker: 100–1500bp

Table 3 Allele frequencies of different pig breeds at the polymorphism in the EDG4 locus at nucleotide position 236 (c. 236C > T) of the porcine EDG4 mRNA sequence (GenBank accession No. EF546799)

Breeds	No.	Genotype			Allele frequency	
		CC	TT	TC	T	C
Laiwu	37	30	1	6	0.11	0.89
Tongcheng pigs	63	20	11	32	0.43	0.57
Guizhou miniature	41	0	33	8	0.90	0.10
Guangxi miniature	43	1	31	11	0.85	0.15
Wuzhishan miniature	30	11	4	15	0.38	0.62
Landrace	20	10	4	6	0.35	0.65
Yorkshire	25	14	8	3	0.38	0.62
Total	259					

Table 4 Association analyses of the *EDG4* SNP (c. 236C > T) with carcass length and drip loss in pig[a]

Genotype	Number of animals	Carcass length (cm)	Drip loss (%)
CC	106	75.5 [b] ± 0.27	1.85 [b] ± 0.13
TC	60	74.2 [c] ± 0.39	2.28 [c] ± 01.6
TT	23	75.4 [b] ± 0.64	1.82[b] ± 0.22

[a] Three plural breed population Tongcheng pigs, Landrace, Yorkshire, and two crossbred populations: L (♂) × YT (♀) (LYT) and Y (♂) × LT (♀) (YLT)

[b, c] Values (mean ± SE) with the same lowercase letter show no difference among genotypes ($P > 0.05$); values with different lowercase letters are significantly different among genotypes ($P < 0.05$)

study to determine whether this polymorphism was related to some carcass traits and meat quality in pig. As a result, the carcass length (minimum) of pigs with TC genotype was significantly shorter then that of pigs with CC ($P = 0.025$) and TT ($P = 0.047$) genotypes. The drip loss percentage of pigs with CC and TT genotypes differed significantly from that of pigs with TC ($P = 0.023$) and ($P = 0.021$) genotypes respectively (Table 4). Though the SNP does not cause any amino acid alteration, it may be linked to other loci controlling the traits of interest. Further association study is needed to investigate whether the *EDG4* genotypes do really affect the traits.

In conclusion, we cloned the full-length coding sequences of porcine *EDG4* and *EDG7* genes, assigned them to porcine chromosomes firstly. Two SNPs were identified in the genes and a SNP (c. 236C > T) located in coding sequence of *EDG4* gene was associated with the carcass length (minimum) and drop loss percentage in our experimental populations. The results provide a base for further research of porcine *EDG4* and *EDG7*.

Acknowledgements The authors wish to express their thanks to Dr Martine Yerle of INRA for providing the RH panel and H. Wang for his helps. This research was supported by the State Platform of Technology Infrastructure (2005DKA21101) National High Science and Technology Foundation of China (20060110Z1039), National Natural Science Foundation of China (30500300), Key Project of National Basic Research and Developmental Plan of China (G2006CB102105), The Key Project of National Natural Science of China (30330440) and Key Project of Scientific Research Foundation of Ministry of Human Resources of China for Returned Chinese Scholars.

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