Evaluation of two promising genes from the target region of SSC13 with susceptibility towards the ETEC F4ac adhesion in pigs

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ABSTRACT

Enterotoxigenic Escherichia coli (ETEC) expressing F4 fimbria is the major pathogenic bacteria causing diarrhoea in neonatal and post-weaned piglets. Based on our previous GWAS results from 301 pigs in a two generation family-based population, two promising candidate genes (HEG1 and ITGB5) from a 0.65 Mb region on pig chromosome 13 for susceptibility to ETEC F4ac infection were investigated for the presence of possible causative mutations. A total of 23 polymorphisms in their coding regions were identified, and two previous uppermost GWAS significant SNPs (ALGA0072075; GenBank NC_010455.4:g.145009857 A > G) were also chosen for genotyping in the three pig breeds. The genotyping data and association analysis results showed that a C to T polymorphism in exon7, and a G to T polymorphism in exon14 of the HEG1 gene significantly increased the effects on the ETEC F4ac adhesion traits ($p = 2.853E^-11$; $p = 1.410E^-09$). Although the identified polymorphisms were not causal mutations, these results indicate that the HEG1 gene is an important adhesion molecule, and it could serve as a genetic marker for selecting ETEC F4ac-resistant pigs in breeding programmes.

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Introduction

Susceptibility to enterotoxigenic Escherichia coli (ETEC) with F4 fimbriae is dominantly inherited in pigs, potentially leading to diarrhoea and death of neonatal and recently-weaned piglets. Some piglets are resistant to ETEC F4 infection and their resistance are determined by the existence of a specific receptor for ETEC F4ab/F4ac (Jacobsen et al. 2011). The ability to rapidly identify pigs carrying the receptor variants that increase F4ab/F4ac susceptibility, and to select pigs that do not have the variant, is a feasible way to control piglet diarrhoea and to improve the health of the pig population (Niu et al. 2011). In the past few years, the potential candidate genes of the ETEC F4ac receptor (F4acR) have been mapped on SSC13 (Rampoldi et al. 2011). Several interesting candidate genes that included MUC4, MUC13, MUC20, TFRC and ACK1 in the target region have been investigated, and genetic markers significantly associating with F4ab/F4ac adhesion phenotypes in some pig populations have been reported ( Jacobsen et al. 2011; Ren et al. 2012). However, the responsible gene and the causal variant of F4ab receptor are unknown.

Based on our previous GWAS results of the loci controlling F4ab/F4ac susceptibility in pigs, we identified a target region that approximately corresponds to a 0.65 Mb from position 101,305,796 to 101,955,862 in the published pig genomic map in Ensembl (Sscrofa10.2) (Fu et al. 2012). To further clarify genetic basis of ETEC F4ac susceptibility and to obtain additional confirmatory evidence of F4ac receptor, two reported functional genes (HEG1 and ITGB5) from the target region were chosen as promising positional candidate genes underlying F4ac susceptibility in pigs.

Materials and methods

Animal populations and determination of phenotypes

The animals used for this study were from a two generation family-based population, consisting of 301 purebred piglets of three breeds, among which 67
were Landrace offspring of 4 boars and 13 sows, 161 were Yorkshire offspring of 7 boars and 29 sows, and 73 were Songliao Black (a Chinese native breed) offspring of 3 boars and 13 sows (Table 1). All 301 piglets were used in association analysis and phenotyped for F4ac susceptibility using an in vitro adhesion test. These procedures have been described in detail in our previous report (Li et al. 2007). Briefly, the collected of the brush border cells and bacterial suspension were mixed, combined with 0.4 mg/mL mannose, and incubated for 30 min at room temperature. A drop of the mixed suspension was examined by phase contrast microscopy to determine the extent of bacterial adhesion. The presence of more than five bacterial cells adhering to the brush border membrane was scored as negative for adhesion, and the presence of less than five bacterial cells was scored as positive for adhesion.

**Design of primers and identification of polymorphisms**

Based on genomic sequences in the Ensemble database (Sscrofa 10.2), the sequences of HEG1 [Ensembl Gene ID: ENSSSCG00000002002] and ITGB5 [Ensembl Gene ID: ENSSSCG00000002002] were obtained. Primers were designed to span intron sequences flanking the exons using Primer3 software (http://frodo.wi.mit.edu) (see Supplementary material Addition file 1). Thirty phenotyped-samples with an equal DNA concentration (50 ng/μl) from 15 resistant large white piglets and 15 susceptible large white piglets were selected for the preparation of a DNA pool to investigate the genetic variations. After performing polymerase chain reaction (PCR), the PCR products were sequenced directly, and SNPs were identified using Chromas 2.3.1 (Technelysium, South Brisbane, Australia) and DNAMAN 6.0 software (Lynnnon, San Ramon, CA).

**Genotyping and association analysis**

SNPs were genotyped by matrix-assisted laser desorption/ionisation time-of-flight mass spectrometry (MALDI-TOF MS) (Sequenom MassARRAY, Biao Miao Biological Technology Co., Ltd., China). The correlations between the different genotypes of individual pigs and the different ETEC F4 adhesion phenotypes were evaluated using a Chi-square test and Chi-square goodness of fit was adapted. The analysis was executed using the proc freq procedure of SAS 9.2 software (SAS Institute 2009), and the significance level was set at .05. A standardised linkage disequilibrium (LD) value ($r^2$) was performed using Haploview 4.2 software (Gabriel et al. 2002).

**Results and discussion**

After sequencing assays, 9 and 14 mutations were found in the coding regions of the ITGB5 and HEG1 genes, respectively (Table 2). Combined with two previous uppermost GWAS significant SNPs (ALGA0072075; GenBank NC_010455.4:g.145009857 A > G), a total of 25 SNPs were genotyped for each animal in three pig breeds. The results of the associated analysis of the SNPs with the ETEC F4ac adhesion phenotype are presented in Table 3. Fifteen of the SNPs significantly associated with the ETEC F4ac adhesion phenotype ($p < .05$), and a C to T polymorphism in exon7 (HEG1-SNP13) and a G to T polymorphism in exon14 (HEG1-SNP14) of the HEG1 gene were the effects that associated most with the ETEC F4ac adhesion traits ($p = 2.853E − 11$; $p = 1.410E − 09$).

Under the assumption that the causal mutations are in a strong linkage disequilibrium (LD) with the significant SNPs, potential functional genes within the LD region for F4ab and F4ac susceptibility.

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**Table 1. Family structure in three pig populations.**

| Breed           | No. piglet | No. boars | No. sows |
|-----------------|------------|-----------|----------|
| Landrace        | 67         | 4         | 13       |
| Yorkshire       | 161        | 7         | 29       |
| Songliao Black  | 73         | 3         | 13       |

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**Table 2. Position of the 25 SNPs from target region for F4ab and F4ac susceptibility.**

| SNPs         | Mutation | Position, bp |
|--------------|----------|--------------|
| ITGB5-SNP1   | C > T    | 13: 145109683|
| ITGB5-SNP2   | G > A    | 13: 145109692|
| ITGB5-SNP3   | A > G    | 13: 145109696|
| ITGB5-SNP4   | G > A    | 13: 145109711|
| ITGB5-SNP5   | C > T    | 13: 145112133|
| ITGB5-SNP6   | A > C    | 13: 145121375|
| ITGB5-SNP7   | T > C    | 13: 145121386|
| ITGB5-SNP8   | A > G    | 13: 145125901|
| ITGB5-SNP9   | G > A    | 13: 145128051|
| HEG1-SNP1    | C > T    | 13: 144800485|
| HEG1-SNP2    | A > G    | 13: 144800488|
| HEG1-SNP3    | A > G    | 13: 144800521|
| HEG1-SNP4    | G > A    | 13: 144798590|
| HEG1-SNP5    | G > A    | 13: 144798837|
| HEG1-SNP6    | A > G    | 13: 144798933|
| HEG1-SNP7    | T > C    | 13: 144799008|
| HEG1-SNP8    | C > T    | 13: 144799015|
| HEG1-SNP9    | C > T    | 13: 144788953|
| HEG1-SNP10   | C > T    | 13: 144788775|
| HEG1-SNP11   | A > G    | 13: 144783582|
| HEG1-SNP12   | A > G    | 13: 144783594|
| HEG1-SNP13   | C > T    | 13: 144779944|
| HEG1-SNP14   | G > T    | 13: 144737152|
| ALGA0072075  | A > G    | 13: 144832256|
| MUC13-SNP1   | A > G    | 13: 145009857|
regions covered should be identified. The LD patterns of the 25 SNPs for ETEC F4ac adhesion traits showed that almost all of them had a high LD ($r^2$) level. Three LD blocks were identified according to the criteria of Gabriel et al., and 14 SNPs of the HEG1 gene were in a block (Figure 1). Outside of the three blocks, there was merely one SNP (ITGB5-SNP9, GenBank NC_010455.4:g.145128051 G $>$ A), which could be a long-distance LD marker, i.e. it had a strong LD but a long physical distance from the causative mutation of ETEC F4ac susceptibility.

Infection by enterotoxigenic Escherichia coli (ETEC) F4ac is a major cause of death in piglets. Identification of the causative gene will be greatly benefit breeding programmes. In a previous report, we found that a novel 12 bp deletion in the ITGBS gene strongly associated with Escherichia coli F4ac adhesion and increased the susceptibility of pigs to infection (Liu et al. 2015). Although the deletion of the ITGBS gene may be an important factor in determining the susceptibility of young pigs to infection with ETEC F4, it is possible that variants of other genes on the surface of gut epithelial cells also play a role. For instance, we characterised the HEG1 gene for exonic mutations and found that the two SNPs of the HEG1 gene had a higher significant effect on the ETEC F4ac adhesion.

Heart of Glass (HEG1), a transmembrane receptor, is one of the genes essential for cardiovascular development in humans. In vascular endothelial cells, cell-cell contacts are formed by adherens and tight junctions

| Table 3. Associations of the 25 SNPs with ETEC F4ac adhesion phenotypes in three pig breeds. |
|-----------------------------------------------|
| SNPs                        | Chi-square value | p Value |
| ITGBS-SNP1                  | 9.330            | .0094** |
| ITGBS-SNP2                  | 1.276            | .5280   |
| ITGBS-SNP3                  | 6.265            | .0436   |
| ITGBS-SNP4                  | 6.332            | .0422** |
| ITGBS-SNP5                  | 5.380            | .0679   |
| ITGBS-SNP6                  | 1.002            | .6060   |
| ITGBS-SNP7                  | 1.785            | .4100   |
| ITGBS-SNP8                  | 5.781            | .0555   |
| ITGBS-SNP9                  | 2.320            | .3130   |
| HEG1-SNP1                   | 0.230            | .8910   |
| HEG1-SNP2                   | 3.310            | .1910   |
| HEG1-SNP3                   | 6.800            | .0330*  |
| HEG1-SNP4                   | 3.947            | .1390   |
| HEG1-SNP5                   | 20.230           | 4.047E−05** |
| HEG1-SNP6                   | 3.946            | .1390   |
| HEG1-SNP7                   | 13.390           | .0012** |
| HEG1-SNP8                   | 16.000           | .0003** |
| HEG1-SNP9                   | 20.950           | 2.823E−05** |
| HEG1-SNP10                  | 20.950           | 2.823E−05** |
| HEG1-SNP11                  | 22.270           | 1.459E−05** |
| HEG1-SNP12                  | 22.250           | 1.474E−05** |
| HEG1-SNP13                  | 48.560           | 2.853E−11** |
| HEG1-SNP14                  | 40.760           | 1.410E−09** |
| ALGAA072075                 | 24.506           | 4.771E−06** |
| MUC13-SNP1                  | 20.175           | 4.160E−05** |

*p $< .05$;

**p $< .01$.  

Figure 1. Linkage disequilibrium (LD) pattern for 25 SNPs of target region on SSC13. Values in boxes are LD ($r^2$) between SNP pairs and the colour reflects the degree of LD.
HEG1 has been reported to anchor to endothelial-specific Rap1-binding protein (Rasip1) at endothelial cell-cell junctions to support vascular integrity (de Kreuk et al. 2016). Rap1 is a small GTPase that regulates epithelial and EC adhesion (Pannekoek et al. 2014). HEG1 plays a profound role in cell-cell adhesion in humans, and thus may also impact the adhesion of ETEC F4ac to porcine intestinal epithelial cells. In spite of there not being any obvious causal mutations for ETEC F4ac susceptibility in the HEG1 gene so far, our results indicate that follow-up functional validations should be performed by focussing on the identified significant mutations of the HEG1 gene.

Conclusions

23 polymorphisms in the coding regions of two promising candidate genes (HEG1 and ITGB5) were identified and genotyped in three pig populations. The further association analysis results showed that a C to T polymorphism in exon7, and a G to T polymorphism in exon14 of the HEG1 gene significant increased the effects on the ETEC F4ac adhesion traits ($p = 2.853E^{-11}$; $p = 1.410E^{-09}$). These results indicate that the HEG1 gene is an important adhesion molecule, and it could serve as a genetic marker for selecting ETEC F4ac-resistant pigs in breeding programs.

Disclosure statement

The authors report no conflicts of interest. The authors alone are responsible for the content and writing of this article.

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