

Mutation in porcine Zip4-like zinc transporter is associated with pancreatic zinc concentration and apparent zinc absorption

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Abstract

The aim of the present study was to analyse the sequence variability of the porcine *Zip4-like* Zn transporter gene and the association of identified sequence variants with average daily gain, apparent Zn absorption, plasma Zn concentration and Zn concentration in the liver and pancreas. For the purpose of the study, two different sample sets were used. Set one, which was used for sequencing and association analysis, included mRNA from intestinal tissue from thirty-five piglets of a feeding trial. Sample set two consisted of forty-six samples of genomic DNA from sperm or tissue of wild boars and several pig breeds and was used to genotype animals of different breeds. The sequence analysis of porcine *Zip4-like* complementary DNA in sample set one revealed the presence of seven nucleotide substitutions. Of these, six were synonymous, whereas a substitution of A with C in exon IX (XM_001925360 c.1430A > C) causes an amino acid exchange from glutamic acid to alanine (p.Glu477Ala). The association analysis revealed no influence of the six synonymous substitutions on Zn values, but the non-synonymous nucleotide exchange significantly increased Zn concentration in the pancreas and apparent Zn absorption of the piglets in week 2 of the feeding trial. The parentage of the piglets and the genotyping results in sample set two suggest a breed-specific presence of the A allele in Piétrain for this amino acid substitution. These results indicate that genotype influences the Zn absorption abilities of individual animals, which should be taken into consideration in animal breeding as well as for the selection of experimental animals.

Key words: Zip4: SNP: Zinc absorption: Pigs

Zn is an essential trace element which is involved in several metabolic pathways. About 2800 proteins of the human proteome contain Zn and up to 10% of the human coding DNA encodes for Zn-containing proteins⁽¹⁾. The functional information available until now suggests that a major part of these proteins is involved in the regulation of gene expression⁽¹⁾. Furthermore, Zn plays an important role as a catalytic centre of several metalloenzymes and as a structural part in protein formations such as the Zn finger⁽²⁾.

Plants, especially cereals, often contain significant amounts of phytic acid, a Zn-complexing agent. Therefore, Zn from plant feedstuff is often poorly available to monogastric animals and humans. Due to this, diets for livestock animals normally need to be supplemented for Zn⁽³⁾.

On the other hand, Zn can cause environmental problems if its emission via manure is too high⁽⁴⁾. Therefore, the European Commission has limited the Zn concentration in livestock diets to 150 mg Zn/kg diet (Regulation (EC) No. 1334/2003). Among

livestock animals, growing piglets have with 100 mg Zn/kg the highest requirement for Zn if it is calculated per kg feed intake^(3,5,6). This high requirement does not leave much room for safety margins in view of the European Union regulation. Accordingly, there is a general agreement that these animals need a highly available source of Zn. Much research has been done to investigate the bioavailability of several organic bound Zn sources for piglets^(7–14), but these studies do not deliver consistent indications that organic binding can significantly improve Zn absorption.

To the best of our knowledge, no research has investigated the influence of genes and their variants on Zn absorption in livestock animals. Moreover, it might also be worthwhile investigating animal-related factors of Zn absorption, such as the genetic variability of the genes encoding for Zn transporter proteins.

There are two different families of Zn transporter proteins: the ZnT and the Zip family. Transporters of the ZnT family

Abbreviations: cDNA, complementary DNA; DE, Deutsches Edelschwein; DL, Deutsche Landrasse; PHD, Profile network from HeiDelberg; Pi, Piétrain.

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facilitate Zn transport from the cytoplasm into cell organelles or the extra-cellular space, whereas transporters of the Zip family transport Zn from the extra-cellular space or cell organelles into the cytoplasm⁽¹⁵⁾. The gene of one transporter of the Zip family, the Zn transporter Zip4 gene (*Zip4*), also known as *SLC39A4*, was discovered in the context of acrodermatitis enteropathica, a severe, autosomal recessive inherited Zn deficiency in humans^(16,17). Therefore, Zip4 is known to play a major role in intestinal Zn absorption. It is expressed in tissues related to Zn absorption and re-absorption such as the gastrointestinal tract^(16,17), kidneys⁽¹⁶⁾ and the visceral yolk sac⁽¹⁸⁾.

In humans, several mutations of *SLC39A4* are known that lead to severe Zn deficiency syndromes, which might be lethal if untreated⁽¹⁹⁾. Among livestock animals, there are similar inherited Zn deficiencies known for cattle⁽²⁰⁾ and goats⁽²¹⁾. The inherited Zn deficiency in cattle was also shown to be related to mutations in *SLC39A4*⁽²⁰⁾. Besides these described forms of severe inherited Zn deficiency, no further information on the genetic factors influencing the efficiency of Zn absorption exists, nor have there been any sequence variants described in porcine Zn transporter genes until now.

The porcine gene coding for the Zip4 Zn transporter (*Zip4-like*) is located on chromosome 4, from position 490 789 to 495 010 (GenBank accession no. NC_010446). Like the human *Zip4*⁽¹⁷⁾, the porcine *Zip4-like* consists of twelve exons and eleven introns. The sequence of the porcine mRNA transcript is also available (GenBank accession no. XM_001925360), whereas no genetic variation is recorded in the dbSNP database (<http://www.ncbi.nlm.nih.gov/projects/SNP>, last accessed on 11 August 2011), nor has such a variation been published elsewhere for this pig gene.

Therefore, the aim of the present study was (i) to analyse the sequence variability of the porcine *Zip4-like* gene and (ii) to test for association of identified sequence variants with average daily gain, apparent Zn absorption, plasma Zn concentration and Zn concentration in the liver and pancreas measured in a previous feeding trial.

Materials and methods

Animals and samples

This study used two different sample sets. Sample set one included mRNA extracted from intestinal tissue originating from thirty-five piglets (barrows) of a previous feeding trial (F Siebert and J Pallauf, unpublished results), which primarily investigated the bioavailability of different Zn sources. This sample set was used to screen the *Zip4-like* mRNA for sequence variants and to test their possible influence on Zn absorption.

Sample set two was used to check one of the identified polymorphisms in the Zip4-like coding region for its presence in different pig breeds and wild boars. For this purpose, the second sample set consisted of forty-six samples of genomic DNA from sperm or tissue (muscle or testicular tissue) from the domestic pig breeds Piétrain (Pi) (*n* 9, including five

boars siring the piglets of the feeding trial), Deutsche Landrasse (DL) (*n* 9), and Deutsches Edelschwein (DE) (*n* 5); the commercial crossings Pi × (DE × DL) (*n* 8); Pi × Duroc (*n* 5); Pi × (Duroc × DE) (*n* 1), and from wild pigs (*Sus scrofa scrofa*, *n* 9). With the exception of three wild sows, all animals were boars. All animals of sample set two, except the wild boars and the boars siring the piglets of the feeding trial, were kept on the research and teaching unit 'Oberer Hardthof' of the Justus-Liebig University, Giessen.

Phenotyping

During the previous feeding trial, thirty-five Pi × DAN-Breed hybrid weaned, castrated male piglets with an average age of 27 d and 6.46 (SEM 0.56) kg mean live weight were used. The animals were acquired from a commercial farm and were the offspring of five different boars. Animals were allocated to one of four experimental groups and received a diet based on maize and soyabean meal (20.2 mg native Zn/kg diet) supplemented with 50 mg Zn from one of four different Zn sources (glycine Zn chelate hydrate, Zn methionine hydroxyl analogue chelate, Zn acetate dehydrate and ZnSO₄·7H₂O) for 28 d. Animals were kept in stainless steel metabolism cages to facilitate total quantitative collection of urine and faeces during two metabolism periods, one in week 2 and one in week 4 of the trial. During the collection periods, faeces and urine were collected twice daily, weighed, and the aliquots were stored at -20°C. Feed intake was recorded daily and the animals were weighed weekly. Blood samples were taken on day 0, day 14 and day 28. At the end of the trial, the animals were killed by exsanguination after stunning and samples of the liver and pancreas for Zn analysis and samples of the small intestine for DNA and mRNA extraction were taken. All samples were cooled on dry ice immediately after sampling and were stored at -70°C before analyses. The animal trial was reported to the veterinary office of the Regional Council Giessen (V54-19 c 20/15 c GI19/3 Nr. A 18/2009).

Diets were dried at 103°C overnight and ashed at 550°C in a muffle furnace. Faecal samples were freeze-dried and ground before ashing at 550°C. Ash was solved in 3 M-HNO₃, filtered and diluted by a factor of 10. Organ samples were wet-ashed by incubation with concentrated HNO₃. After incubation, the solution was filtered and diluted with *aqua bidest.*, to an acid concentration of 2.8 M. Directly before measurement, 2.5 ml of each urine sample were mixed with 1.8 ml 69% HNO₃ and 5.7 ml *aqua bidest.*, and centrifuged to remove suspended particles.

Zn in faeces, organs and diet was measured by inductively coupled plasma-atomic emission spectrometry (Unicam 701 Emission Spectrometer; Unicam Limited). Zn in urine and bone was measured by flame atomic absorption spectrophotometry (AAS; Thermo S2 AA System, Thermo Electron Corporation). To ensure the quality of measurement, reference probes were measured after every ten samples. Apparent Zn absorption was calculated as Zn intake during sampling periods minus excretion via faeces, expressed as percentage of intake.

Extraction of mRNA, complementary DNA synthesis and extraction of DNA

Extraction of total mRNA from the intestinal mucosal tissue was done with a modified protocol of the acid guanidinium thiocyanate phenol chloroform extraction⁽²²⁾. The volume of the reagents was downscaled by 40% compared to the original protocol (for economic reasons) and 100 mg of each tissue were used. Concentration and purity of the mRNA were determined photometrically and RNA integrity was checked by a denaturing agarose gel electrophoresis⁽²²⁾. After this, the RNA samples were treated with DNase and copied to complementary DNA (cDNA) with the Revert Aid kit (MBI Fermentas), according to the manufacturer's protocol.

DNA was extracted from the liver and testicles with the Invisorb DNA Tissue HTS96 Kit (Invitex GmbH) following the kit protocol.

PCR amplification and sequencing of Zip4-like complementary DNA and genomic DNA fragments

For PCR amplification of the complete coding region of the porcine *Zip4-like* cDNA covered with four fragments, and for amplification of a part of the genomic sequence from intron eight to intron nine, reaction mixes with final concentrations of 1 × GoTaq FlexiBuffer, 0.2 mM-dNTP, 2 mM-MgCl₂, 0.67 mM-forward primer, 0.67 mM-reverse primer and 1 U GoTaq polymerase (Promega) were used. After denaturation at 94°C for 1.5 min, thirty-five amplification cycles were performed including denaturation at 94°C for 15 s, annealing at a fragment-specific temperature for 20 s, and extension at 72°C for 1 min, followed by a final 5 min extension at 72°C. Fragment-specific PCR details are given in Table 1.

Amplified PCR products were purified with the MSB[®] Spin PCRapace system (Invitex GmbH) and sequenced using the Big Dye Terminator version 1.1 cycle sequencing kit and the forward PCR primer. Sequencing reactions were run on a 3130 Genetic Analyzer, following the manufacturer's recommendations for sequencing kit and instrument (Applied Biosystems). If the results were unclear, sequencing was repeated with the reverse PCR primer. Sequences were analysed by visual evaluation of resulting chromatograms supported by ChromasPro version 1.33 (Technelysium Private Limited).

Sequence analysis

For identification of sequence variants, multiple alignments of the *Zip4-like* cDNA sequences obtained from the thirty-five animals of sample set one and the GenBank sequence XM_001925360 (*S. scrofa* Zn transporter *Zip4-like* (LOC100156896) mRNA) as a reference were generated using the ChromasPro software version 1.33 (Technelysium Private Limited).

The same software was used to genotype one of the identified polymorphisms in the *Zip4-like* coding region in sequences from sample set two by comparing these with the GenBank sequence NC_010446 (*S. scrofa* breed mixed chromosome 4, Sscrofa9.2).

For alignments of *Zip4-like* cDNA and translated protein sequences with sequences from other species (GenBank accession numbers: *Bos taurus*: NM_001046067; *Homo sapiens*: NM_017767.2; *Pongo abelii*: XM_002819543; *Rattus norvegicus*: NM_001101021; *Mus musculus*: NM_028064.2; *Canis familiaris*: XM_539217.2), the 'ClustalW' function of Chromas Pro was used.

SIFT Blink analysis (http://sift.jcvi.org/www/SIFT_Blink_submit.html) was used to assess whether an identified amino acid exchange might be deleterious for protein function⁽²³⁾. Additionally, the BLOSUM62 matrix was consulted to determine whether this amino acid substitution is to be regarded as conservative or non-conservative⁽²⁴⁾. Finally, the possible effect of an identified amino acid exchange on the predicted protein secondary structure was tested with the Profile network from HeiDelberg (PHD) method (http://npsa-pbil.ibcp.fr/cgi-bin/npsa_automat.pl?page=/NPSA/npsa_phd.html)⁽²⁵⁾.

Statistical analysis

All nucleotide substitutions identified in sample set one were analysed for potential influence on the piglet production data, Zn plasma concentration as well as apparent Zn digestibility by the GLM procedure of the SPSS 19 statistical software (IBM Corporation). The statistical model included genotype and diet fed as fixed effects. Where it improved the model, the interaction of genotype and diet was included in the model. We used the model

$$y_{ijk} = \mu + \text{diet}_i + \text{genotype}_j + \text{diet}_i \times \text{genotype}_j + e_{ijk},$$

Table 1. Details of PCR amplification and sequencing of *Zip4-like* gene fragments

Amplified region*	Type of template	Forward and reverse primer (5'–3')	Fragment size (bp)	Annealing temperature (°C)	GenBank accession no. of sequence used for primer design
I†–II–III†	cDNA	gct gac ctt gct gtc ctc a ctc ccg gaa cac aaa gtc c	556	61	XM_001925360 (mRNA)
III†–IV–V–VI–VII–VIII†	cDNA	gtg tgg acc tgc ctc agc cgc tag ttg cat gga cac g	899	65	XM_001925360 (mRNA)
VII†–VIII–IX–X–XI–XII†	cDNA	gga ggc ctc tat gcc ttc tt acc ttc ctg ggt ggg aga c	896	65	XM_001925360 (mRNA)
8†–IX–9†	Genomic DNA	cac tcc cag ctc cca ctc ta ctg cac tgc gca cca aac	229	63	NC_010446 (DNA)

cDNA, complementary DNA.

* Roman numerals: exons; arabic numerals: introns.

† Partially amplified.

Table 2. Identified nucleotide substitutions in the porcine *Zip4-like* coding region and frequencies in sample set one

Region	Nucleotide substitution*	Amino acid change	GenBank no.	Frequency in sample set 1
Exon IV	c.711C > T	–	JF346411	0.111
Exon V	c.918C > A	–	JF346412	0.083
Exon V	c.960C > T	–	JF346413	0.153
Exon V	c.963C > T	–	JF346414	0.028
Exon VIII	c.1323C > T	–	JF346415	0.014
Exon IX	c.1430A > C	p.Glu477Ala	JF346416	0.743

* Nucleotide substitutions and their numbering referring to GenBank sequence XM_001925360. Position +1 corresponds to the first nucleotide of the ATG translation initiation codon according to the *Zip4* protein sequence (XP_001925395.2).

where y_{ijk} = the dependent variable (e.g. average daily gain, Zn concentration in plasma, liver and pancreas, apparent Zn absorption), $diet_i$ = the diet fed in the feeding trial, $genotype_j$ = the genotype of each tested SNP (e.g. SNP 1430: cc and ac) and e_{ijk} = the error term. All variables were normally distributed.

Results

The sequence analysis of the complete porcine *Zip4-like* cDNA in the thirty-five animals of sample set one, derived from the feeding trial, revealed the presence of seven nucleotide substitutions compared to the reference sequence (Table 2). The length of the sequenced region was 2038 bp in total.

The nucleotide substitutions are spread quite evenly from exon IV to IX and were not identified in a homozygous form in the samples. The frequencies of the substituted alleles compared to the reference sequence varied from 0.01 to 0.74 (Table 2). In this study, six of the nucleotide substitutions were synonymous, whereas a substitution of A with C in exon IX (XM_001925360 c.1430A > C) causes an amino acid exchange from glutamic acid to alanine (p.Glu477Ala). Remarkably, C is the major allele and had by far the highest frequency (0.74) compared to the other identified nucleotide substitutions in our samples, while A was the allele of the used GenBank reference sequence (XM_001925360).

The association analysis revealed a significant influence of the identified non-synonymous nucleotide substitution XM_001925360 c.1430A > C on two of the investigated traits

(Table 3). The least square means of Zn concentration in the pancreas and of apparent Zn absorption (week 2) were significantly higher for animals with the AC genotype compared to the CC genotype. R^2 for apparent Zn absorption is 0.48 and for Zn concentration in the pancreas 0.36, respectively, which indicates that a considerable part of the variation is explained by the different genotypes.

The other nucleotide substitutions did not have a significant effect on the analysed traits (Table 3). The results from the bioinformatical analysis with the SIFT Blink procedure for the porcine amino acid sequence XP_001925395.2 implied that the amino acid exchange p.Glu477Ala would not be deleterious for protein function. Nevertheless, the BLOSUM62 matrix revealed a negative score (–1) and therefore indicated this amino acid exchange as rather deleterious. Furthermore, the PHD method predicted an alpha helix structure for the amino acid residues at positions 473–475 when glutamic acid is present at position 477, instead of a random coil structure when alanine is present. However, this change is predicted for only three positions within a random coil stretch over approximately sixty positions, and the prediction accuracy is lower than 83% for positions 473 and 474.

Sequencing exon IX in sample set two showed that three of the five Pi sires of the piglets in sample set one had the heterozygous AC genotype and the other two sires the CC genotype of variant XM_001925360 c.1430A > C. Additionally, we identified two other Pi pigs that were heterozygous AC. All other pigs of sample set two, including the nine wild boars, were homozygous CC. Furthermore, the alignment with exon IX of other species revealed that nucleotide C is found in most

Table 3. Influence different genotypes of the *Zip4-like* nucleotide substitution (XM_001925360, c.1430A > C) on average daily gain (ADG), zinc concentration in plasma, liver and pancreas and apparent zinc absorption in piglets (Least squares mean (LSM) with their standard errors)

Genotype...	AC (n 18)		CC (n 17)		P^*			
	LSM	SE	LSM	SE	Genotype	Diet	Interaction	
ADG (g/d)	0.263	0.014	0.265	0.014	0.906	0.091	0.665	
Plasma Zn (mg/l)	Day 0	0.592	0.020	0.643	0.021	0.095	0.856	0.787
	Day 14	0.557	0.018	0.514	0.019	0.119	0.093	0.839
	Day 28	0.649	0.031	0.692	0.032	0.345	0.033	0.546
Liver Zn (mg/g DM)	Day 28	151.5	7.33	139.4	7.15	0.248	0.932	0.305
Pancreas Zn (mg/g DM)	Day 28	128.8	4.65	107.2	4.90	0.004	0.525	0.384
Apparent Zn absorption (% of intake)	Week 2	30.97	1.72	23.86	1.79	0.008	0.139	0.914
	Week 4	34.30	1.71	32.70	1.79	0.523	0.042	0.077

* P values for significance of genotype, Zn source and their interaction.

<i>Sus scrofa</i> 1	GTG <u>GCA</u> GAA GAG AGC CCG GAG CTG CTG AAC CCG GAG CCC CGG AGA CTG CGC CCG
	V <u>A</u> E E S P E L L N P E P R R L R P
<i>Sus scrofa</i> 2	GTG GAA <u>GAA</u> GAG AGC CCG GAG CTG CTG AAC CCG GAG CCC CGG AGA CTG CGC CCG
	V E E E S P E L L N P E P R R L R P
<i>Bos taurus</i>	GTG GCG GAG GAG AGC CCC GAG CTG CTG AGC CCG GAG CCC CGG AGA CGG AGC CCA
	V <u>A</u> E E S P E L L S P E P R R R S P
<i>Homo sapiens</i>	GTG GCG GAG GAG AGC CCG GAG CTG CTG AAC CCG GAG CCC AGG AGA CTG AGC CCA
	V <u>A</u> E E S P E L L N P E P R R L S P
<i>Pongo abelii</i>	GTG GCA GAG GAG AGA CCG GAG CTG CTG AAC CCT GAG CCC AGG AGA CTG AGC CCA
	V <u>A</u> E E R P E L L N P E P R R L S P
<i>Rattus norw.</i>	GTG ACA GAG GAG ACC CCG GAA CTA CTG AAC CCA GAC ACC CGG CGA CTG AGA GCA
	V T E E T P E L L N P E T R R L R A
<i>Mus musculus</i>	GTG GCA GAG GAG ACC CCG GAA CTA CTG AAC CCA GAG ACC CGG CGA CTG AGA GCA
	V <u>A</u> E E T P E L L N P E T R R L R A
<i>Canis fam.</i>	GTG GCG GAG GAG AGC CCG GAG CTG CTG AGC GCG GAG CCC CGG AGA CTG AGC CCA
	V <u>A</u> E E S P E L L S A E P R R L S A

Fig. 1. Alignment of the mRNA and protein sequences for exon IX of the Zip4 transporter in different species (GenBank accession numbers: *Sus scrofa* 2: XM_001925360; *Bos taurus*: NM_001046067; *Homo sapiens*: NM_017767.2; *Pongo abelii*: XM_002819543; *Rattus norvegicus* (*Rattus norw.*): NM_001101021; *Mus musculus*: NM_028064.2; *Canis familiaris* (*Canis fam.*): XM_539217.2). The shaded areas indicate homologue regions. *Sus scrofa* 1: sequence obtained in this study. The underlined letter indicates the nucleotide substitution in this sequence compared to the reference sequence *Sus scrofa* 2: XM_001925360. (A colour version of this figure can be found online at <http://www.journals.cambridge.org/bjn>)

species which therefore have the same amino acid (alanine) in this position of the Zip4 peptide chain (Fig. 1). Only the rat showed threonine in this position (GenBank accession no. NM_001101021).

Discussion

In the present study, seven nucleotide substitutions were identified in the porcine *Zip4-like* coding region compared to the porcine GenBank sequence XM_001925360. One of these nucleotide substitutions (c.1430A > C) was non-synonymous and the association analysis revealed a significant influence on apparent Zn absorption in the first metabolism period and on pancreatic Zn concentration. The absence of significant associations of the other identified sequence variants with the analysed traits was not unexpected as an influence of synonymous nucleotide substitutions in coding regions on the gene function is less likely, although not impossible, e. g. when located in binding sites for splicing enzymes.

The database of genetic variation of the National Center of Biotechnology (<http://www.ncbi.nlm.nih.gov/snp>, last accessed on 11 August 2011) did not contain any SNP for the porcine *Zip4-like* gene. To our knowledge, there has not been any other study investigating the genetic variation in the porcine Zip4 transporter.

The fact that there is a significant difference between the genotypes CC and AC in apparent Zn absorption in the first metabolism period (second week of the trial) but not in the second period (fourth week of the trial) might be caused by the different Zn requirement of the piglets. Even though the Zn concentration in the diet was constantly at 70 mg/kg during the whole feeding trial, the Zn requirements of the piglets decreased from 100 mg/kg during the first metabolism period to 80 mg/kg during the second period^(5,6). Therefore, the relative Zn supply increased from the first to the second metabolism period. From other species it is known that Zip4 expression is up-regulated during Zn deficiency⁽²⁶⁾. Therefore, the transporter-facilitated Zn uptake plays a predominant role in situations of suboptimal Zn supply.

However, when Zn supply is adequate, other uptake mechanisms such as intracellular uptake can influence the Zn absorption⁽²⁷⁾. Furthermore, in a situation of excess Zn supply, significant amounts of Zn are excreted via the enterocytes and pancreatic secretions into the intestine that will influence the results of apparent Zn absorption⁽²⁸⁾.

Zn has important functions in the synthesis, storage and secretion of insulin and influences its conformational integrity⁽²⁹⁾. Therefore, the maintenance of an appropriate pancreatic Zn concentration in the pancreas is crucial for the organism. Additionally, the pancreas plays an important role in Zn metabolism⁽³⁰⁾. Significant amounts of Zn are excreted via the secretion of pancreatic juices into the gut and the concentration of Zn in the pancreatic juices is Zn dependent (summarised by Rofe *et al.*⁽³¹⁾). This might explain why there is a genotype-dependent difference in Zn concentration in the pancreas but not in the liver or in the plasma Zn.

The nucleotide substitution XM_001925360 c.1430A > C, which leads to an amino acid substitution (p.Glu477Ala), showed a high frequency in the present sample. In none of the sample sets was there any animal with the homozygous genotype AA of c.1430A > C. In sample set one, eighteen of thirty-five animals had the heterozygote AC genotype. All piglets of this sample set were Pi × DAN-Breed hybrids. In sample set two, only five of forty-six animals were heterozygous for the SNP c.1430A > C. All five animals were purebred Pi pigs, and three of these were sires of the feeding trial piglets. Additionally, all nine genotyped wild boars had the CC genotype. All this led to the assumption that C might be the wild-type allele. Furthermore, the alignment of the porcine exon IX with other species in Fig. 1 shows that alanine is the predominant amino acid in this position across the analysed species. However, other amino acids such as threonine in the rat can also be found at that position.

Even though our sample sets are too small and do not include enough purebred and unrelated animals of each breed for definite conclusions, these results indicate that the A allele of the c.1430A > C substitution is notably more prevalent in Pi than in the other investigated breeds and in wild

boars. Animals with the AC genotype showed a significantly better apparent Zn absorption in the first metabolism period compared with the animals of the CC genotype. From human studies it is known that, in situations with suboptimal Zn supply, the amount of Zn supply is closely related to growth rate in children⁽³²⁾. As pigs of the Pi breed have been selected intensively for a high growth rate, they might have been selected indirectly for improved Zn absorption. In several studies on quantitative trait loci for average daily growth rates and body weight at slaughter in modern European and Asian pig breeds as well as in wild-type pigs^(33–35), quantitative trait loci have been found in regions on the porcine chromosome 4 where the *Zip4-like* gene is located.

As the exact mechanism of Zn transport by the Zip4-transporter proteins is not known, it is not yet possible to determine whether the amino acid exchange (p.Glu477Ala)

directly influences the biological function of the transporter. Alanine and glutamic acid are both strong alpha-helix creators⁽³⁶⁾, so their exchange may not have a strong effect on the secondary structure of the protein. Additionally, the results of the bioinformatical analysis with the SIFT Blink procedure, the BLOSUM62 matrix and the PHD method did not indicate a strong effect of the amino acid substitution p.Glu477Ala on protein structure and function. However, alanine has a neutral hydrophobic side chain while the side chain of glutamic acid is negatively charged, which might make a difference in the transport abilities of Zip4.

In humans, there are more than thirty different variants known to cause acrodermatitis enteropathica. They are spread evenly over the whole gene and include any type of mutation such as nonsense and missense mutations, deletions of different sizes, insertions with frameshifts and splice-site changes (reviewed by Schmitt *et al.*⁽¹⁹⁾). They often appear

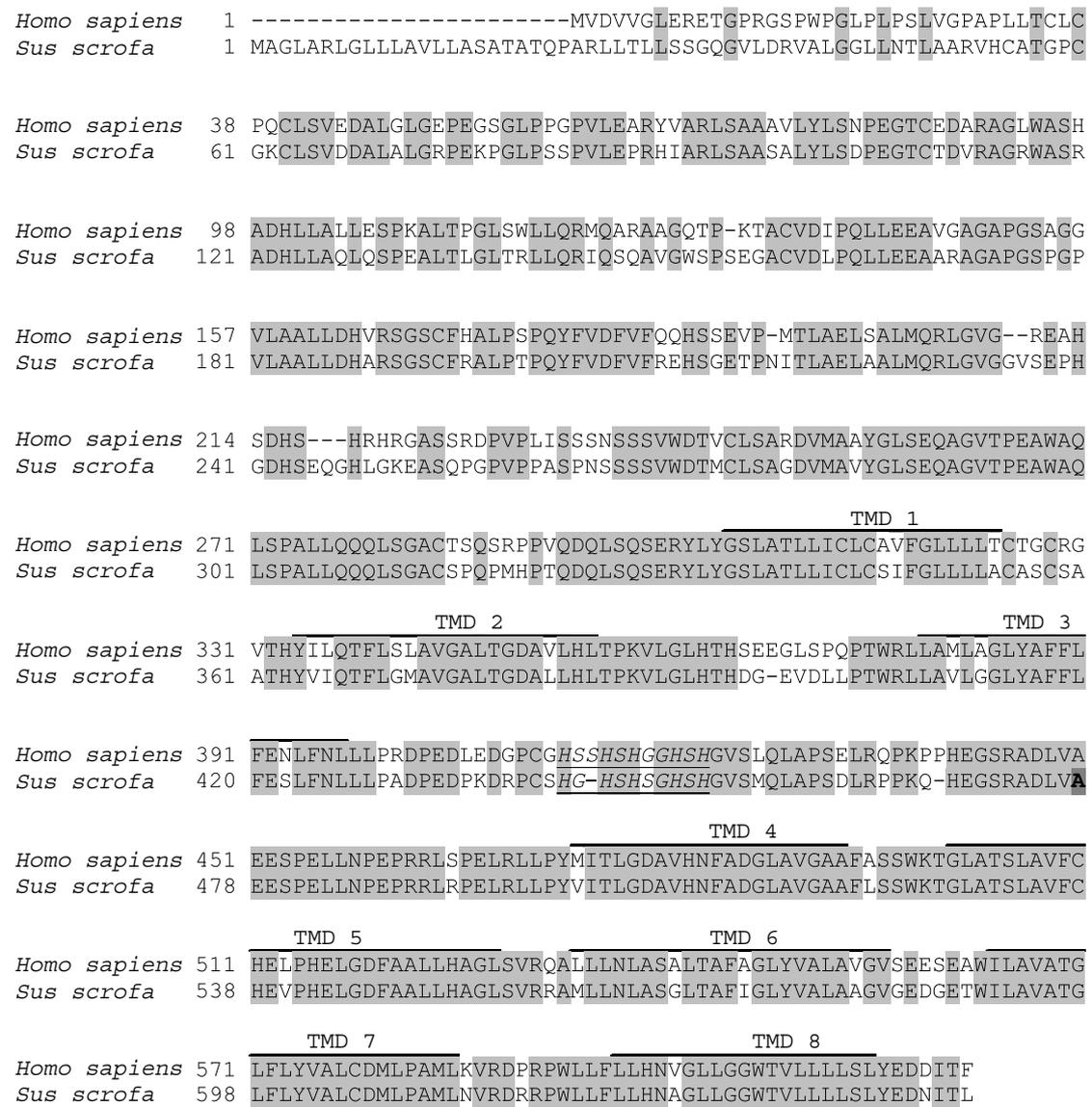


Fig. 2. Sequence alignment of the complete porcine and human Zip4 proteins (reference sequences: *Sus scrofa*: XP_001925395.2; *Homo sapiens*: NP_060237). The shaded amino acids are conserved between human and pig Zip4. The eight transmembrane domains (TMD) are indicated by a solid line above the sequences⁽¹⁸⁾. The histidine-rich region is indicated by italics, and the position of the nucleotide substitution p.Glu477Ala with a bold letter.

in highly conserved regions of the gene, such as the transmembrane domains. However, these mutations lead to severely reduced Zn absorption, which causes clinical Zn deficiency symptoms that may be lethal if untreated. The p.Glu477Ala amino acid substitution lies in an area between the histidine-rich region and the fourth transmembrane domain (Fig. 2). This region might be less crucial for protein function than the transmembrane domains.

Even though it is not clear whether the amino acid substitution itself is responsible for the difference in apparent Zn absorption and pancreatic Zn concentration or if it is linked to another causal sequence variant, the polymorphism could be used as a marker to genotype experimental animals with regard to their Zn absorption abilities. This can be a powerful tool to reduce the inter-individual variance in experiments dealing with Zn absorption.

Furthermore, the approach of investigating the genetic aspect of Zn absorption efficiency offers interesting new possibilities in the field of animal breeding and nutrition. So far, research on Zn bioavailability has only investigated different dietary Zn sources. Our results show that in the conflict between providing an adequate Zn supply to the animal on the one hand and environmental issues on the other hand, it might be worthwhile considering the genetic compounds that influence Zn absorption. The estimated frequency of 0.26 for the A-allele in the crossbred animals of sample set one would be high enough to be considered in breeding programmes after associations with economic parameters are evaluated.

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References

- Andreini C, Banci L, Bertini I, *et al.* (2006) Counting the zinc-proteins encoded in the human genome. *J Proteome Res* **5**, 196–201.
- Cousins RJ (1996) Zinc. In *Present Knowledge in Nutrition*, 7th ed., pp. 293–306 [EE Ziegler and LJ Filer, editors]. Washington, DC: ILSI Press International Life Sciences Institute.
- McDowell LR (2003) *Minerals in Animal and Human Nutrition*, 2nd ed. Amsterdam: Elsevier.
- Jondreville C, Revy PS & Dourmad JY (2003) Dietary means to better control the environmental impact of copper and zinc by pigs from weaning to slaughter. *Livest Prod Sci* **84**, 147–156.
- National Research Council (1998) *Nutrient Requirements of Swine*, 10th ed. Washington, DC: National Academy Press.
- Gesellschaft für Ernährungsphysiologie (2008) *Recommendations for the Supply of Energy and Nutrients to Pigs*. Frankfurt/Main: DLG-Verlag.
- Hill DA, Peo ERJ & Lewis AJ (1987) Influence of picolinic acid on the uptake of 65 zinc-amino acid complexes by the everted rat gut. *J Anim Sci* **65**, 173–178.
- Wedekind KJ, Lewis AJ, Giesemann MA, *et al.* (1994) Bioavailability of zinc from inorganic and organic sources for pigs fed corn-soybean meal diets. *J Anim Sci* **72**, 2681–2689.
- Schell TC & Kornegay ET (1996) Zinc concentration in tissues and performance of weanling pigs fed pharmacological levels of zinc from ZnO, Zn-methionine, Zn-lysine, or ZnSO₄. *J Anim Sci* **74**, 1584–1593.
- Case CL & Carlson MS (2002) Effect of feeding organic and inorganic sources of additional zinc on growth performance and zinc balance in nursery pigs. *J Anim Sci* **80**, 1917–1924.
- Revy PS, Jondreville C, Dourmad JY, *et al.* (2002) Bioavailability of two sources of zinc in weanling pigs. *Anim Res* **51**, 315–326.
- Carlson MS, Boren CA, Wu C, *et al.* (2004) Evaluation of various inclusion rates of organic zinc either as polysaccharide or proteinate complex on the growth performance, plasma, and excretion of nursery pigs. *J Anim Sci* **82**, 1359–1366.
- Buff CE, Bollinger DW, Ellersieck MR, *et al.* (2005) Comparison of growth performance and zinc absorption, retention, and excretion in weanling pigs fed diets supplemented with zinc-polysaccharide or zinc oxide. *J Anim Sci* **83**, 2380–2386.
- Hollis GR, Carter SD, Cline TR, *et al.* (2005) Effects of replacing pharmacological levels of dietary zinc oxide with lower dietary levels of various organic zinc sources for weanling pigs. *J Anim Sci* **83**, 2123–2129.
- Lichten LA & Cousins RJ (2009) Mammalian zinc transporters: nutritional and physiologic regulation. *Annu Rev Nutr* **29**, 153–176.
- Kury S, Dreno B, Bezieau S, *et al.* (2002) Identification of *SLC39A4*, a gene involved in acrodermatitis enteropathica. *Nat Genet* **31**, 239–240.
- Wang K, Zhou B, Kuo YM, *et al.* (2002) A novel member of a zinc transporter family is defective in acrodermatitis enteropathica. *Am J Hum Genet* **71**, 66–73.
- Dufner-Beattie J, Wang FD, Kuo YM, *et al.* (2003) The acrodermatitis enteropathica gene *ZIP4* encodes a tissue-specific, zinc-regulated zinc transporter in mice. *Biol Chem* **278**, 33474–33481.
- Schmitt S, Kury S, Giraud M, *et al.* (2009) An update on mutations of the *SLC39A4* gene in acrodermatitis enteropathica. *Human Mutat* **30**, 926–933.
- Yuzbasiyan-Gurkan V & Bartlett E (2006) Identification of a unique splice site variant in *SLC39A4* in bovine hereditary zinc deficiency, lethal trait A46: an animal model of acrodermatitis enteropathica. *Genomics* **88**, 521–526.
- Krametter-Froetscher R, Hauser S & Baumgartner W (2005) Zinc-responsive dermatosis in goats suggestive of hereditary malabsorption: two field cases. *Vet Dermatol* **16**, 269–275.
- Chomczynski P & Sacchi N (1987) Single-step method of RNA isolation by acid guanidinium thiocyanate phenol chloroform extraction. *Anal Biochem* **162**, 156–159.
- Kumar P, Henikoff S & Ng PC (2009) Predicting the effects of coding non-synonymous variants on protein function using the SIFT algorithm. *Nat Protoc* **4**, 1073–1082.
- Henikoff S & Henikoff JG (1992) Amino-acid substitution matrices from protein blocks. *Proc Natl Acad Sci U S A* **89**, 10915–10919.

25. Rost B & Sander C (1993) Prediction of protein secondary structure at better than 70% accuracy. *J Mol Biol* **232**, 584–599.
26. Weaver BP, Beattie JD, Kambe T, *et al.* (2007) Novel zinc-responsive post-transcriptional mechanisms reciprocally regulate expression of the mouse Slc39a4 and Slc39a5 zinc transporters (Zip4 and Zp5). *Biol Chem* **388**, 1301–1312.
27. Dibley MJ (2001) Zinc. In *Present Knowledge in Nutrition*, 8th ed., pp. 329–343 [BA Bowman and RM Russell, editors]. Washington, DC: ILSI Press International Life Sciences Institute.
28. Pekas JC (1966) Zinc 65 metabolism – gastrointestinal secretion by pig. *Am J Physiol* **211**, 407–413.
29. Chausmer AB (1998) Zinc, insulin and diabetes. *J Am Coll Nutr* **17**, 109–115.
30. McClain CJ (1990) The pancreas and zinc homeostasis. *J Lab Clin Med* **116**, 275–276.
31. Rofe AM, Winters N, Hinskens B, *et al.* (1999) The role of the pancreas in intestinal zinc secretion in metallothionein-null mice. *Pancreas* **19**, 69–75.
32. Gibson RS, Hess SY, Hotz C, *et al.* (2008) Indicators of zinc status at the population level: a review of the evidence. *Br J Nutr* **99**, S14–S23.
33. Nagamine Y, Haley CS, Sewalem A, *et al.* (2003) Quantitative trait loci variation for growth and obesity between and within lines of pigs (*Sus scrofa*). *Genetics* **164**, 629–635.
34. Liu G, Kim J, Jonas E, *et al.* (2008) Combined line-cross and half-sib QTL analysis in Duroc–Pietrain population. *Mamm Genome* **19**, 429–438.
35. Edwards DB, Ernst CW, Raney NE, *et al.* (2008) Quantitative trait locus mapping in an F2 Duroc × Pietrain resource population: II. Carcass and meat quality traits. *J Anim Sci* **86**, 254–266.
36. Christen P & Jaussi R (2005) *Biochemie*. Berlin: Springer.