A single nucleotide polymorphism of porcine *MX2* gene provides antiviral activity against vesicular stomatitis virus

Keisuke Sasaki[a], Pullop Tungtrakoolsub[a], Takeya Morozumi[b], Hirohide Uenishi[c,d], Manabu Kawahara[a,*], and Tomomasa Watanabe[a]

[a]Laboratory of Animal Breeding of Reproduction, Graduate School of Agriculture, Hokkaido University, Sapporo 060-8589, Japan.

[b]Animal Research Division, Institute of Japan Association for Techno-innovation in Agriculture, Forestry and Fisheries, 446-1 Ippaizuka, Kamiyokoba, Tsukuba, Ibaraki 305-0854, Japan

[c]Animal Genome Research Unit, Agrogenomics Research Center, National Institute of Agrobiological Sciences, 2 Ikenodai, Tsukuba, Ibaraki 305-8602, Japan

[d]Animal Immune and Cell Biology Research Unit, Division of Animal Sciences, National Institute of Agrobiological Sciences, 2 Ikenodai, Tsukuba, Ibaraki, 305-8602, Japan

Corresponding author:

Dr. Manabu Kawahara, PhD

Laboratory of Animal Breeding and Reproduction, Graduate School of Agriculture, Hokkaido University, Sapporo 060-8589, Japan

Tel. & Fax: +81 11 706 2541

E-mail: k-hara@anim.agr.hokudai.ac.jp

Abstract

The objective was to determine if single nucleotide polymorphisms (SNPs) in porcine *MX2* gene affect its antiviral potential. MX proteins are known to suppress the multiplication of several viruses, including influenza virus and vesicular stomatitis virus (VSV). In domestic animals possessing highly polymorphic genome, our previous research indicated that a specific SNP in chicken *Mx* gene was responsible for its antiviral function. However, there still has been no information about SNPs in porcine *MX2* gene. In this study, we first conducted polymorphism analysis in 17 pigs of *MX2* gene derived from 7 breeds. Consequently, a total of 30 SNPs, of which 11 were deduced to cause amino acid variations, were detected, suggesting that the porcine *MX2* is very polymorphic. Next, we classified *MX2* into 8 alleles (A1–8), and subsequently, carried out infectious experiments with recombinant VSVΔG*-G to each allele. In A1–5 and A8, position 514 amino acid (514 aa) of MX2 was glycine (Gly), which did not inhibit VSV multiplication. Whereas, in A6 and A7, 514 aa was arginine (Arg), which exhibited the antiviral ability against VSV. These results demonstrate that a SNP at 514 aa (Gly-Arg) of porcine MX2 plays a pivotal role in the antiviral activity as well as that at 631 aa of chicken Mx.

Keywords

MX2, Pig, SNP, Vesicular stomatitis virus, Antiviral activity, Mismatched PCR-RFLP

Introduction

MX gene is one of the interferon-stimulated genes, and can be induced by antiviral interferon alpha/beta (Haller and Kochs 2002; Horisberger et al. 1983). Some MX proteins are known to inhibit the multiplication of several viruses, including influenza virus and vesicular stomatitis virus (VSV) (Frese et al. 1996; Jin et al. 1999; Kanerva et al. 1996; Landis et al. 1998; Schneider-Schaulies et al. 1994; Zhao et al. 1996). Unlike birds, mammals typically have 2 isoforms of *MX* genes, *MX1* and *MX2*. In human, these 2 *MX* genes are identified as *MxA* and *MxB* (Aebi et al. 1989; Lindenmann 1964; Staeheli and Sutcliffe 1988).

The MX protein contains 4 conserved domains: G domain (GD) (Pitossi et al. 1993), self-assembly sequences (SAS) (Nakayama et al. 1993), central interactive domain (CID) (Flohr et al. 1999), and GTPase effector domain (GED) (Schwemmle et al. 1995). In particular, both CID and GED play key roles in viral inhibition (Flohr et al. 1999; Zurcher et al. 1992). In human MxA and mouse MX1, binding of CID to GED during viral recognition leads homo-oligomerization and subsequent conformational changes in these proteins (Gao et al. 2010; Haller and Kochs 2002; Schumacher and Staeheli 1998). Thus, the interaction between CID and GED appears to be essential for the antiviral ability of MX proteins.

In chicken, we had previously found that the antiviral ability of chicken Mx protein against influenza viruses and VSV was the results of a single amino acid polymorphism at position 631, which was located in a conserved domain of GED (Ewald et al. 2011; Ko et al. 2002; Sasaki et al. 2013; Sironi et al. 2008). In swine, *MX1* showed 2 natural variants, and the antiviral activity of one of these variants was lost because of a frameshift in the GED (Asano et al. 2002; Nakajima et al. 2007). In Landrace pig, the MX2 protein could inhibit the multiplication of influenza virus (Morozumi et al. 2009). However, unlike for chicken *Mx*, little is known the single nucleotide polymorphisms (SNPs) in the porcine *MX2*.

Here, we attempted to identify SNPs of porcine *MX2* gene in 7 breeds, and to further investigate the antiviral ability against VSV on the basis of polymorphisms detected in this study. We determined a SNP responsible for the antiviral ability of porcine MX2 protein. Furthermore, for feasible identification of genotypes harboring a SNP that could confer virus resistance, we developed a PCR-Restriction Fragment Length Polymorphism (RFLP) analysis using mismatched primers.

Nowadays, a number of infectious diseases involving influenza, foot-and-mouth disease (FMD), and porcine reproductive and respiratory syndrome (PRRS), threaten swine industry. These viruses are members of RNA viruses involving VSV, suggesting that pig breeding strategy focused on a single nucleotide polymorphism of porcine MX2 might be effective for the production of virus-resistant pigs.

Material and methods

Preparation of peripheral blood from 7 breeds of pigs

Seven porcine breeds were used in this study: Duroc (DR), Jinhua (JH), Landrace (LR), Large White (LW), Meishan (ME), Mexican Hairless (MH), and Middle Yorkshire (MY). Peripheral bloods from 17 pigs were provided from the National Livestock Breeding Center (Fukushima and Ibaraki Prefectures, Shizuoka Prefectural Livestock Institute, and Gifu Prefectural Livestock Institute). The number of individuals belonging to the different breeds was as follows: 3 Duroc (DR1–3), 2 Jinhua (JH1, and 2), 2 Landrace (LR1, and 2), 5 Large White (LW1–5), 3 Meishan (ME1–3), 1 Mexican Hairless (MH), and 1 Middle Yorkshire (MY).

In vitro culture of porcine mononuclear cells and mouse established cell line

In this study, we prepared pig peripheral blood mononuclear cells and mouse BALB/c 3T3 cultured cells (RCB0163). The procedures of cell cultures were performed as previously described (Jin et al. 1999; Jin et al. 1998; Sasaki et al. 2013). In brief, pig peripheral blood cells were isolated from whole blood using HISTOPAQUE-1077 (Sigma-Aldrich, St. Louis, MO) and cultured for 42 h. Thereafter, to induce

MX2 mRNA expression, we added 1000 U/mL of human recombinant interferon-alpha (2b), INTRON-A (Schering-Plough, Kenilworth, NJ) to the medium and the cells were further cultured for 6 h.

RT-PCR of complete MX2 cDNA

Extraction of total RNAs, and reverse transcription (RT)-PCR to total cDNAs of porcine *MX2* cDNA were performed as previously described (Sasaki et al. 2013). We used the primer sets pMX2 160F (5'-GATTGAAGCCACCAGCTCAC-3') and pMX2 2349R (5'-ACTGGGGTTCTTTGGGGAGT-3') for PCR. The cycling profile comprised an initial denaturing step of 5 min at 95°C, followed by 35 cycles at 95°C for 0.5 min, 60°C for 1 min, 72°C for 2.5 min, and a final extension at 72°C for 5 min.

Molecular cloning and sequencing of MX2 cDNA

The complete *MX2* cDNAs amplified by RT-PCR were cloned into pGEM-T Easy Vector (Promega) with T4 Ligase (Promega). We cloned porcine *MX2* cDNA constructs from 17 individuals of each pig stain (DR, JH, LR, LW, ME, MH and MY). For the purpose of avoiding any possible artifacts, we prepared 8 identical cDNA constructs for 6 individuals (LR1, LW1, ME1, ME2, MH, and MY), and 10 identical those for 11 individuals (DR1, DR2, DR3, JH1, JH2, LR2, LW2, LW3, LW4, LW5, and ME3).

We next confirmed the sequences of each porcine *MX2* cDNA insert within the plasmid using an ABI PRISM 310 Genetic Analyzer (PerkinElmer Inc., Waltham, MA). The determined nucleotide sequences were compared to that found in the database (GenBank ID: AB259856) corresponding to the porcine *MX2* genomic DNA sequence of a Landrace breed (Morozumi et al. 2009).

Construction of MX2 mRNA expressing cells

According to the determined nucleotide sequences as shown in the result section (Table 3), we classified porcine *MX2* gene into 8 alleles type (A1–8). For evaluating the antiviral activity, the *MX2* cDNA constructs derived from each allele (i.e., A1 [allele 1. 1 from LW1], A2 [allele 2 from LR2], A3 [allele 3 from LW5], A4 [allele 4 from DR2], A5 [allele 5 from DR3], A6 [allele 6 from JH1], A7 [allele 7 from ME1], and A8 [allele 8 from ME2]) were subcloned into the *Not*1 site of the pCI-neo vector (pCI-neo Mammalian Expression vector; Promega). We transfected these *MX2* constructs within the pCI-neo vector into 3T3 cells and generated stable cell lines expressing porcine *MX2* mRNA. Before the transfection of *MX2* cDNA constructs, we confirmed the absence of any artifacts caused in the previous cloning process by nucleotide sequencing. The complete experimental procedures were described in our previous reports (Ko et al. 2002; Sasaki et al. 2013). Next, we confirmed the mRNA expression of

porcine *MX2* and mouse *Gapdh* in the cloned cells by RT-PCR. RT-PCR for mouse *Gapdh* was conducted using the primer pairs of Gapdh 327F (5'-GTCGTGGAGTCTACTGGTGTC-3') and Gapdh 566R (5'-GAGCCCTTCCACAATGCCAAA-3').

Infection with recombinant VSV

Recombinant VSV (VSVΔG*-G) carrying the green fluorescent protein (GFP) gene instead of the G protein gene was used as previously described (Ko et al. 2002; Sasaki et al. 2013). Using stable 3T3 cell lines, we analyzed the antiviral activity of porcine MX2 protein against VSVΔG*-G infection. The relative infectivity of VSVΔG*-G in 3T3 cell clones was determined by counting the number of GFP-expressing cells in 10–20 microscopic fields (Ko et al. 2002; Sasaki et al. 2013; Takada et al. 1997). As negative controls, the relative infectivity of nontransfected 3T3 (3T3) and empty vector-transfected (pCI-neo) cells were examined.

3-dimentional structure prediction

We predicted 3-dimentional (3D) structure for all 8 MX2 alleles using SWISS-MODEL (http://swissmodel.expasy.org/). The predicted models in the range of 90–706 amino acid residues were

constructed by reference to the 3D structure of human MxA (PDB IB: 3szrA, Sequence identity: 53%). Experimental procedures were followed to previous studies (Arnold et al. 2006; Guex and Peitsch 1997; Schwede et al. 2003).

Mismatched Restriction Fragment Length Polymorphism (RFLP) analysis

To identify the amino acid polymorphism at 514 aa, we conducted mismatched PCR-RFLP analysis. Porcine genomic DNA was first prepared from the nuclei of peripheral blood mononuclear cells. The mononuclear cells were dissolved in the lysis buffer containing 50 μg/mL of proteinase K, from which the genomic DNAs were extracted using the phenol/chloroform method and used for experiment. Next, we amplified intron 10 and exon 11 of the *MX2* gene obtained from the genomic DNAs. The primer sets used for PCR were as follows: intron 10 F primer (5'-AAAGGCCTGTGACATGGTTC-3') and exon 11 R mismatched primer (5'-ACCAACGACCGTCTGCAGCATCT-3'). Since the amplified region contains the SNP corresponding to 514 aa, digestion with the restriction enzyme *Xho*1 (TOYOBO, Japan) would enable discrimination between Gly and Arg at 514 aa of the porcine MX2 protein. The cycling profile comprised an initial denaturing step of 5 min at 94°C, followed by 35 cycles at 94°C for 1 min, 58°C for 1 min, and 72°C for 1 min, and a final extension at 72°C for 5 min. After amplification, a 214-bp

amplicon was digested with *Xho*1 at 37°C for 3 h. The digested DNA fragments were electrophoresed through 3% agarose gel and visualized with ethidium bromide.

Statistical analysis

Statistical analysis of all data for comparison was carried out using the Fisher protected least significant difference (PLSD) test based on Statview (Abacus Concepts, Inc., Berkeley, CA). Experimental data are presented as means ± standard errors. *P* values of <0.01 were considered statistically significant.

Results

Sequencing and typing of porcine MX2

We first analyzed and determined the nucleotide sequences of open reading frame (ORF) of porcine *MX2* cDNA (n = 17) derived from 7 breeds (i.e., DR, JH, LR, LW, ME, MH, and MY). According to the nucleotide sequences in the database (Genbank ID: AB259856), we designated the first nucleotide in exon 1 of porcine *MX2* gene as position 1 nucleotide. In addition, we found the start codon at position 194–196 nucleotide (22962–22964 in the database), and the stop codon at position 2327–2329 (46985–

46987 in the database). From these evaluations, the complete ORF of porcine *MX2* consisted of 2136-bp length, and porcine MX2 protein encoded 711 amino acids, consistent with the previous study (Morozumi et al. 2009). Subsequently, we found SNPs at 30 nucleotide positions by polymorphism analysis, among which 25 were heterogenic (Table 1). Moreover, we compared the predicted amino acid sequences of MX2 on the basis of the obtained nucleotide sequences with that of the reference breed. Next, we numbered all 13 alleles found in the nucleotide sequencing (i.e., allele 1.1–3, 2.1, 3.1, 4.1–4, 5.1, 6.1, 7.1, and 8.1). The obtained nucleotide sequences of each allele were deposited to public database, DDBJ (URL: http://www.ddbj.nig.ac.jp/), and their accession numbers were described in Table 1. In terms of amino acid sequences, 19 of the 30 SNPs were silent, but the other 11 SNPs were deduced to cause amino acid substitutions, suggesting that porcine *MX2* gene is highly polymorphic. These amino acid substitutions were located at position 68, 131, 218, 254, 376, 377, 386, 514, 518, 543, and 706 of MX2 (Supplemental Table S1). Eight out of 11 amino acid substitutions caused by the SNPs were found in the conserved domains of porcine MX2, that is in the SAS (at amino acid position 131), CID (at amino acid position 376, 377, 386, 514, 518, and 543), and GED (at amino acid position 706) (Fig. 1, Supplemental Table S1). However, the substitutions at amino acid position 68, 218, and 254 were not located within any conserved domains. According to these amino acid substitutions, we classified porcine MX2 protein into

8 alleles type (A1–A8) (Table 2). A1–A5 were found in DR, LR, LW, MH, and MY, while A4 and A6–A8 were identified in the breeds of JH and ME (Table 2, Supplemental Table S1).

Antiviral activity of porcine MX2 protein

In order to evaluate the antiviral activity of MX2 derived from A1–A8, we prepared mouse 3T3 cells transfected with the *MX2* cDNA construct of each allele and confirmed the *MX2* expression (Fig. 2a). According to the number of infected cells, we carried out the infectivity relative to nontransfected 3T3. As shown in Fig. 2b, the stable cell lines expressing *MX2* mRNA of A1–A5 and A8 showed 95.1–107.4% relative infectivity, similar to those of the negative controls 3T3 and pCI-neo. However, the cell lines transfected with the cDNA of A6 and A7 showed significantly lower relative infectivity (31.2% and 30.5%, respectively) than that of 3T3 and pCI-neo (100.0% and 100.9%, respectively). Subsequently, uninfected cells in all lines showed 0% of relative infectivity (Data not shown). Taken together, the relationship between the antiviral ability against VSV and the newly detected amino acid substitutions at 11 positions were represented by the negative (A1–A5 and A8) and positive (A6 and A7) groups. Thus, these results demonstrated that a specific amino acid substitution at 514 aa (Gly in A1–A5 and A8, and Arg in A6 and A7) causes a difference in the antiviral ability against VSV.

3D structure prediction for porcine MX2 alleles

We investigated the effects of the polymorphisms on 3D structures of porcine MX2 proteins in all 8 alleles (A1–A8) by using a protein structure homology-modeling server, SWISS-MODEL (Supplemental Figs. S1–2). The results revealed the characteristic structures in A7 and A8, in which the 2 beta-sheets were distinctly represented by SWISS-MODEL. Meanwhile, the corresponding regions in the other alleles (A1–A6) did not construct sheet structures (Supplemental Fig. S1a, b). We next focused on changes of amino acid residues caused by polymorphisms (Supplemental Fig. S2). The polymorphisms at 218, 254, 518, and 706 aa did not cause any changes in side chains (data not shown), whereas those at 376, 377, and 386 aa in CID appeared to cause alterations of side chains (Supplemental Fig. S2b, c, d). The side chains in A6 and A7 showed distinctive patterns comparing those at 514 aa in the other alleles (A1–A5 and A8). The side chain of hydrophobic Gly at 514 aa in A1–A5 and A8 folded into protein structure, whereas those of hydrophilic Arg in A6 and A7 projected out of protein (Supplemental Fig. S2a). These results suggested that the structural change caused by only the SNP at 514 aa affected antiviral activity against VSV.

Identification of the amino acid variation at position 514 specific to positive antiviral MX2 by mismatched PCR-RFLP analysis

We attempted to develop a method to identify the SNP corresponding to 514 aa of porcine MX2 by mismatched PCR-RFLP analysis. The 214-bp PCR products amplified with mismatched primers was digested by the restriction enzyme of *Xho*1 at 37°C for 3 h (Supplemental Fig. S3a). When *MX2* gene encodes Gly (GGG) at 514 aa, the amplicons can be digested by *Xho*1, and therefore, the digested DNA fragments would be 191 bp in length. On the contrary, when 514 aa is Arg (AGG), the amplicons is non-digestible by *Xho*1, resulting in 214 bp. As expected, the products of DR3, LR1, and LW3 were 191 bp in length (Supplemental Fig. S3b), implying that DR3, LR1, and LW3 *MX2* did not have antiviral activity against VSV. By contrast, 214-bp products from ME1 and ME2 were expected to be positive for antiviral activity. Thus, a mismatched PCR-RFLP analysis would feasibly enable us to identify the amino acid mutation of Arg at position 514 specific for the antiviral *MX2* gene.

Discussion

MX protein inhibits the growth of a variety of RNA viruses including influenza virus and VSV (Jin et al. 1999; Zhao et al. 1996). In swine, Landrace MX2 at the nuclear membrane also shows antiviral

ability against influenza virus (Morozumi et al. 2009). Additionally, this study demonstrated the positive antiviral ability of porcine MX2 against VSV. Yet, there is no evidence of a connection between SNPs and the antiviral ability of porcine *MX2* gene. On the other hand, in chicken *Mx* gene, a specific coding SNP (cSNP) corresponding to 631 aa was significantly responsible for its antiviral activity against influenza virus and VSV (Ko et al. 2002). Hence, we hypothesized that cSNPs in the porcine *MX2* gene would also affect its antiviral function. In this study, we first investigated SNPs in porcine *MX2* gene derived from 7 pig breeds (i.e., DR, JH, LR, LW, ME, MH, and MY) and identified 8 independent alleles (A1–A8). Furthermore, we analyzed the differences in the antiviral ability depending on each cSNP in porcine *MX2* by the infectious experiment with recombinant VSV. Strikingly, we found that MX2 from A6 and A7 possessing Arg at 514 aa exhibited positive antiviral responses to VSV. This result demonstrates that a cSNP at 514 aa (Gly-Arg) plays a pivotal role in the antiviral activity of porcine MX2, as well as that at 631 aa of chicken MX (Fig. 2, Table 2). This is strongly supported by the finding that (i) the MX2 in A6 exhibits antiviral activity against VSV but not in A4, and (ii) the amino acid substitution between A4 and A6 only presents at 514 aa (Fig. 2, Table 2).

We confirmed that the cSNP at 514aa of porcine MX2 was located in CID (Fig. 1, Table 2). In humans, the monoclonal antibody 2C12 against CID of human MxA impaired the interaction between

MxA and viral nucleocapsids, resulting in the lack of antiviral activity against Thogoto virus (Flohr et al. 1999). This observation indicates that amino acid substitutions in CID prevent the interaction between MX proteins and viral components, which is consistent with our observations. Therefore, the Gly-to-Arg substitution at 514 aa of porcine MX2 appears to contribute to antiviral activity against VSV through the interaction between MX2 proteins and viral components.

Except for the cSNP at 514 aa, we detected 7 cSNPs in the conserved domains of SAS, CID, and GED in porcine *MX2* genes, although these were not associated with antiviral activity against VSV. We also had similar observations that cSNPs in the conserved domains did not affect antiviral ability against VSV in chickens (Sasaki et al. 2013). In chicken Mx, we found two cSNPs at 185 aa in SAS and at 632 aa in GED, which were not associated with antiviral ability at all (Sasaki et al. 2013). Alternatively, a single amino acid polymorphism at 631 aa altered the antiviral ability against VSV. This result indicated that single amino acid substitution in the conserved domains was sufficient for causing MX proteins to acquire antiviral ability against the particular strain of virus. However, our data could not eliminate the possibility that the cSNPs other than that at 514 aa might not be associated with the antiviral ability against other viruses.

We also constructed 3D structure models for all 8 alleles (A1–A8) of MX2 proteins by reference to

human MxA. The results revealed that the MX2 3D models in A7 and A8 constructed 2 beta-sheets in GED (574–624 residues), whereas those in other alleles (A1–A6) were not represented by SWISS-MODEL (Supplemental Fig. S1b). These structural changes in A7 and A8 might be caused by the polymorphisms at both or either 376 aa in A7 and 131 aa in A8 (Table 2). However, since the MX2 in A8 did not show any antiviral activity against VSV (Fig. 2), we concluded that these beta-sheet structures were not associated with antiviral activity of porcine MX2 against VSV. We further focused on the patterns of the side chains caused by the polymorphisms. When the side chain patterns caused by the polymorphisms were compared among A1–A8, outcome could be classified into 4 types (Supplemental Fig. S2). The side chains at 514 aa in A6 and A7 obviously showed projecting pattern comparing those in the other alleles (Supplemental Fig. S2a). Likewise, the polymorphisms at 376, 377, 386 aa in CID caused the projecting patterns (Supplemental Fig. S2b, c, d), which might be involved in antiviral activity of MX2 against viruses except VSV. Taken together with the result of the infection examination (Fig. 2), our results raised a possibility that the SNP at 514 aa was involved in binding of CID to GED for a conformational change in porcine MX2 as well as in human MxA (Haller and Kochs 2002; Flohr et al. 1999; Schumacher and Staeheli 1998).

In early studies, a number of *MX2* sequences have been reported (GenBank ID: CU467746,

DQ444963, AB258432, AK345001, AY897395, and M65088), including reference sequence in this study (GenBank ID: AB259856). By comparing these sequencing data, we detected 6 SNPs as shown in Supplemental Table S2. Although 4 of them were synonymous, the other 2 SNPs were predicted to accompany amino acid substitutions. These 2 cSNPs could cause substitutions from alanine to serine at 38 aa (AK345001), and from phenylalanine to serine at 345 aa (AY897345), respectively (Supplemental Table S2). However, since cSNPs at both 38 and 345 aa are not located in any conserved domains, these 6 SNPs are unlikely to affect the antiviral ability of MX2.

Furthermore, through mismatched PCR-RFLP analysis, we could determine whether the *MX2* gene encodes Gly or Arg in the site corresponding to 514 aa (Supplemental Fig. S3). The result showed the distribution of the genetic variation in the examined breeds. We also found that only the Chinese breeds JH and ME, but not European breeds, possessed the VSV-resistant *MX2* gene (Table 2, Supplemental Table S1). These results suggested the possibility of breeding anti-VSV pigs by crossing Chinese breeds. Virus infection causes serious illness or death in pigs, which are also infectious to humans. Therefore, selective breeding of virus-resistant pigs would be beneficial for the livestock industry.

Conclusion

Our research classified the coding region of porcine MX2 into 13 alleles. Moreover, according to

amino acid substitutions, we typed them into 8 allele (A1–A8). Finally, we revealed that the specific cSNP corresponding to 514 aa was crucial for the antiviral activity of porcine MX2 protein against VSV.

Acknowledgement

We thank Dr. Ayato Takada for kindly providing VSVΔG*-G. We are also grateful to the National Livestock Breeding Center (Fukushima and Ibaraki Prefectures, Shizuoka Prefectural Livestock Institute, and Gifu Prefectural Livestock Institute) for offering pig peripheral bloods.

Conflicts of interest

We proclaim that there is no conflict of interest.

References

Aebi M, Fah J, Hurt N, Samuel CE, Thomis D, Bazzigher L, Pavlovic J, Haller O, Staeheli P (1989) cDNA structures and regulation of two interferon-induced human Mx proteins. Mol Cell Biol 9:5062–72

Arnold K, Bordoli L, Kopp J, Schwede T (2006) The SWISS-MODEL workspace: a web-based

environment for protein structure homology modeling. Bioinformatics 2:195–201

Asano A, Ko JH, Morozumi T, Hamashima N, Watanabe T (2002) Polymorphisms and the antiviral property of porcine Mx1 protein. J Vet Med Sci 64:1085–9

Ewald SJ, Kapczynski DR, Livant EJ, Suarez DL, Ralph J, McLeod S, Miller C (2011) Association of Mx1 Asn631 variant alleles with reductions in morbidity, early mortality, viral shedding, and cytokine responses in chickens infected with a highly pathogenic avian influenza virus. Immunogenetics 63:363–75

Flohr F, Schneider-Schaulies S, Haller O, Kochs G (1999) The central interactive region of human MxA GTPase is involved in GTPase activation and interaction with viral target structures. FEBS Lett 463:24–8

Frese M, Kochs G, Feldmann H, Hertkorn C, Haller O (1996) Inhibition of bunyaviruses, phleboviruses, and hantaviruses by human MxA protein. J Virol 70:915–23

Gao S, von der Malsburg A, Paeschke S, Behlke J, Haller O, Kochs G, Daumke O (2010) Structural basis of oligomerization in the stalk region of dynamin-like MxA. Nature 465:502–6

Guex N, Peitsch MC (1997) SWISS-MODEL and the Swiss-PdbViewer: an environment for comparative protein modeling. Electrophoresis 15:2714–23

Haller O, Kochs G (2002) Interferon-induced mx proteins: dynamin-like GTPases with antiviral activity. Traffic 3:710–7

Horisberger MA, Staeheli P, Haller O (1983) Interferon induces a unique protein in mouse cells bearing a gene for resistance to influenza virus. Proc Natl Acad Sci U S A 80:1910–4

Jin HK, Takada A, Kon Y, Haller O, Watanabe T (1999) Identification of the murine Mx2 gene: interferon-induced expression of the Mx2 protein from the feral mouse gene confers resistance to vesicular stomatitis virus. J Virol 73:4925–30

Jin HK, Yamashita T, Ochiai K, Haller O, Watanabe T (1998) Characterization and expression of the Mx1 gene in wild mouse species. Biochem Genet 36:311–22

Kanerva M, Melen K, Vaheri A, Julkunen I (1996) Inhibition of puumala and tula hantaviruses in Vero cells by MxA protein. Virology 224:55–62

Ko JH, Jin HK, Asano A, Takada A, Ninomiya A, Kida H, Hokiyama H, Ohara M, Tsuzuki M, Nishibori M, Mizutani M, Watanabe T (2002) Polymorphisms and the differential antiviral activity of the chicken Mx gene. Genome Res 12:595–601

Landis H, Simon-Jodicke A, Kloti A, Di Paolo C, Schnorr JJ, Schneider-Schaulies S, Hefti HP, Pavlovic J (1998) Human MxA protein confers resistance to Semliki Forest virus and inhibits the amplification

of a Semliki Forest virus-based replicon in the absence of viral structural proteins. J Virol 72:1516–22

Lindenmann J (1964) Inheritance of Resistance to Influenza Virus in Mice. Proc Soc Exp Biol Med 116:506–9

Morozumi T, Naito T, Lan PD, Nakajima E, Mitsuhashi T, Mikawa S, Hayashi T, Awata T, Uenishi H, Nagata K, Watanabe T, Hamasima N (2009) Molecular cloning and characterization of porcine Mx2 gene. Mol Immunol 46:858–65

Muller M, Winnacker EL, Brem G (1992) Molecular cloning of porcine Mx cDNAs: new members of a family of interferon-inducible proteins with homology to GTP-binding protiens. J Interferon Res 2:119–29

Nakajima E, Morozumi T, Tsukamoto K, Watanabe T, Plastow G, Mitsuhashi T (2007) A naturally occurring variant of porcine Mx1 associated with increased susceptibility to influenza virus in vitro. Biochem Genet 45:11–24

Nakayama M, Yazaki K, Kusano A, Nagata K, Hanai N, Ishihama A (1993) Structure of mouse Mx1 protein. Molecular assembly and GTP-dependent conformational change. J Biol Chem 268:15033–8

Pitossi F, Blank A, Schroder A, Schwarz A, Hussi P, Schwemmle M, Pavlovic J, Staeheli P (1993) A

functional GTP-binding motif is necessary for antiviral activity of Mx proteins. J Virol 67:6726–32

Sasaki K, Yoneda A, Ninomiya A, Kawahara M, Watanabe T (2013) Both antiviral activity and intracellular localization of chicken Mx protein depend on a polymorphism at amino acid position 631. Biochem Biophys Res Commun 430:161–6

Schneider-Schaulies S, Schneider-Schaulies J, Schuster A, Bayer M, Pavlovic J, ter Meulen V (1994) Cell type-specific MxA-mediated inhibition of measles virus transcription in human brain cells. J Virol 68:6910–7

Schumacher B, Staeheli P (1998) Domains mediating intramolecular folding and oligomerization of MxA GTPase. J Biol Chem 273:28365–70

Schwede T, Kopp J, Guex N, Peitsch MC (2003) SWISS-MODEL: An automated protein homology-modeling server. Nucleic Acids Res 13:3381–5

Schwemmle M, Richter MF, Herrmann C, Nassar N, Staeheli P (1995) Unexpected structural requirements for GTPase activity of the interferon-induced MxA protein. J Biol Chem 270:13518–23

Sironi L, Williams JL, Moreno-Martin AM, Ramelli P, Stella A, Jianlin H, Weigend S, Lombardi G, Cordioli P, Mariani P (2008) Susceptibility of different chicken lines to H7N1 highly pathogenic

avian influenza virus and the role of Mx gene polymorphism coding amino acid position 631. Virology 380:152–6

Staeheli P, Sutcliffe JG (1988) Identification of a second interferon-regulated murine Mx gene. Mol Cell Biol 8:4524–8

Takada A, Robison C, Goto H, Sanchez A, Murti KG, Whitt MA, Kawaoka Y (1997) A system for functional analysis of Ebola virus glycoprotein. Proc Natl Acad Sci U S A 94:14764–9

Uenishi H, Eguchi T, Suzuki K, Sawazaki T, Toki D, Shinkai H, Okumura N, Hamasima N, Awata T (2004) PEDE (Pig EST Data Explorer): construction of a database for ESTs derived from porcine full-length cDNA libraries. Nucleic Acids Res 32(Database issue):D484–8

Zhao H, De BP, Das T, Banerjee AK (1996) Inhibition of human parainfluenza virus-3 replication by interferon and human MxA. Virology 220:330–8

Zurcher T, Pavlovic J, Staeheli P (1992) Mechanism of human MxA protein action: variants with changed antiviral properties. EMBO J 11:1657–61

Figure legends

Fig. 1 Schematic representation of the amino acid substitutions detected in porcine MX2 protein

Arrows indicate the substituted amino acid position located in the conserved domains. N and C mean *N*-terminus and *C*-terminus, respectively. aa, amino acid; GD, G domain; SAS, self-assembly sequences; CID, central interactive domain; GED, GTPase effector domain; LZ2, leucine zipper motif 2.

Fig. 2 Antiviral activity toward all the alleles of porcine MX2 protein against VSV

a RT-PCR analysis of each porcine *MX2* cDNA construct within pCI-neo vector-transfected into 3T3 cells. *Gapdh* expression was used as the positive control. **b** The infectivity of nontransfected 3T3 cells (3T3) is expressed as 100.0%. The relative infectivity of each cell line (A1–A8) was determined by dividing by that of 3T3. pCI-neo represents 3T3 cells transfected empty pCI-neo vector. The values are shown as mean ± standard error of the mean. Significance levels at $P < 0.01$ (*) compared with 3T3 are indicated.

Table 1. Nucleotide substitutions of porcine *MX2* cDNA

Breeds	Nucleotide position																													Allele number	Accession number
	396	403	556	584	625	652	676	829	845	910	953	1084	1165	1168	1279	1319	1323	1349	1443	1525	1630	1733	1745	1788	1821	1888	1987	1996	2011	2309		
Database	A	A	C	G	C	C	C	G	A	G	A	G	T	C	A	G	A	T	T	C	C	G	A	A	A	A	A	G	G	G	1. 1	AB259856
LW1, LR1, MH, MY	A	A	C	G	C	C	C	G	A	G	A	G	T	C	A	G	A	T	T	C	C	G	A	A	A	A	A	G	G	G	1. 1	AB854066
LW2, LW3, LW4	A	A	C	G	C	C	C	G	A	G	A	G	T	C	A	G	A	T	T	C	C	G	A	A	A	A	A	G	G	G	1. 1	AB854066
	A	A	C	G	C	C	C	T	A	G	A	A	C	C	A	G	A	T	T	C	T	G	A	A	A	A	A	G	A	G	1. 2	AB854067
DR1	A	A	C	G	C	C	C	T	A	G	A	A	C	C	A	G	A	T	T	C	C	G	A	A	A	A	A	G	G	G	1. 1	AB854066
	A	A	C	G	C	C	C	T	A	T	A	A	C	C	A	G	A	T	T	C	C	G	A	A	A	A	A	G	G	G	1. 3	AB854068
LR2	A	A	C	G	C	C	C	G	A	G	A	G	T	C	A	G	A	T	T	C	C	G	A	A	A	A	A	G	G	G	1. 1	AB854066
	A	A	C	G	C	C	C	G	A	G	A	G	T	C	A	G	A	T	T	C	C	G	A	C	A	A	A	A	A	T	2. 1	AB854069
LW5	A	A	C	G	C	C	C	G	A	G	A	G	T	C	A	G	A	T	T	C	C	G	A	A	A	A	A	G	G	G	1. 1	AB854066
	A	A	C	G	C	C	C	G	A	G	G	G	T	C	A	G	G	T	T	C	C	G	G	A	A	A	A	G	G	G	3. 1	AB854070
DR2	A	A	C	G	C	C	C	G	A	G	A	G	T	C	A	G	A	T	T	C	C	G	A	A	A	A	A	G	G	G	1. 1	AB854066
	A	C	T	G	C	A	C	G	A	T	A	G	T	C	A	G	A	A	T	C	C	G	A	A	A	A	A	G	A	G	4. 1	AB854071
DR3	A	A	C	G	C	C	C	G	A	G	A	G	T	C	A	G	A	A	T	C	C	G	A	A	A	A	A	G	G	G	4. 2	AB854072
	A	C	T	G	C	A	C	G	G	G	A	G	T	C	A	G	A	A	C	C	C	G	A	A	A	G	G	G	G	G	5. 1	AB854075
JH1, JH2	A	C	T	G	C	A	C	G	A	G	A	G	T	C	A	G	A	A	T	C	C	G	A	A	A	A	G	G	G	G	4. 3	AB854073
	A	C	T	G	C	A	C	G	A	G	A	G	T	C	A	G	A	A	T	T	C	A	A	A	A	A	G	G	G	G	6. 1	AB854076
ME1, ME2	A	C	C	G	T	A	T	G	A	G	A	G	C	A	G	T	A	A	T	C	C	A	A	A	A	A	G	G	G	G	7. 1	AB854077
ME3	A	C	C	G	T	A	C	G	A	G	A	G	T	C	A	G	A	A	T	C	C	G	A	A	A	A	G	G	G	G	4. 4	AB854074
	G	C	C	A	T	A	C	G	A	G	A	G	T	C	A	G	A	A	T	C	C	G	A	A	G	A	G	G	G	G	8. 1	AB854078

Database represents the nucleotide sequences of a Landrace breed's *MX2* gene (GenBank ID: AB259856). The number of pig means the individual number in each breed. *MX2* cDNA from DR1–3, JH1 and 2, LR2, LW2–5, and ME3 encoded heterozygous alleles, shown as double lines.
DR, Duroc; JH, Jinhua; LR, Landrace; LW, Large White; ME, Meishan; MH, Mexican Hairless; MY, Middle Yorkshire.

Table 2. Alleles identified in porcine MX2 protein

Allele	Amino acid position (Nucleotide position)											Antiviral activity against VSV
	68 (396)	131 (584)	218 (845)	254 (953)	376 (1319)	377 (1323)	386 (1349)	514 (1733)	518 (1745)	543 (1821)	706 (2309)	
1	Gln	Val	Thr	Thr	Gly	His	Phe	Gly	Thr	Gln	Ala	Negative
2	Gln	Val	Thr	Thr	Gly	His	Phe	Gly	Thr	Gln	Ser	Negative
3	Gln	Val	Thr	Ala	Gly	Arg	Phe	Gly	Ala	Gln	Ala	Negative
4	Gln	Val	Thr	Thr	Gly	His	Ile	Gly	Thr	Gln	Ala	Negative
5	Gln	Val	Ala	Thr	Gly	His	Ile	Gly	Thr	Gln	Ala	Negative
6	Gln	Val	Thr	Thr	Gly	His	Ile	Arg	Thr	Gln	Ala	Positive
7	Gln	Val	Thr	Thr	Trp	His	Ile	Arg	Thr	Gln	Ala	Positive
8	Arg	Met	Thr	Thr	Gly	His	Ile	Gly	Thr	Arg	Ala	Negative

Ala, alanine; Arg, arginine; Gln, glutamine; Gly, glycine; His, histidine; Ile, isoleucine; Met, methyonine; Phe, phenylalanine; Ser, serine; Thr, threonine; Trp, tryptophan; Val, valine; VSV, vesicular stomatitis virus.

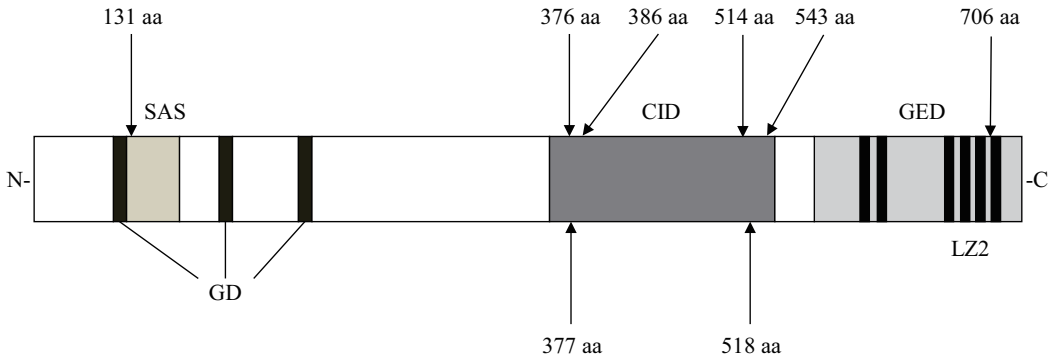

131 aa
376 aa
386 aa
514 aa
543 aa
706 aa
SAS
CID
GED
N-
-C
LZ2
GD
377 aa
518 aa

Figure 1.

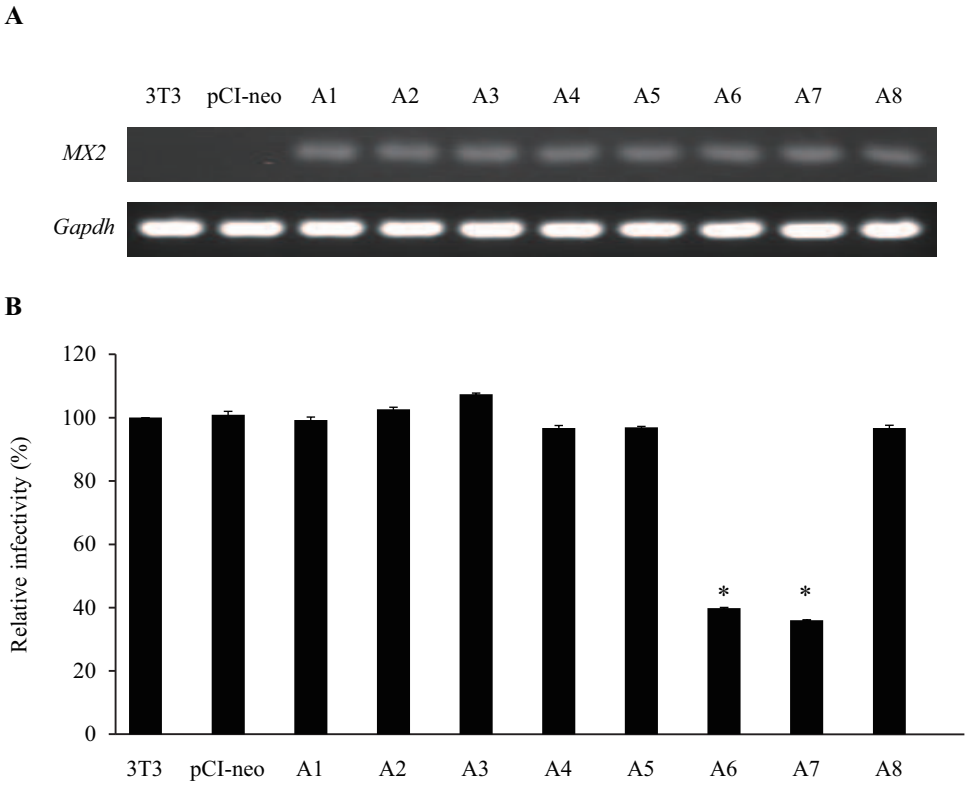

A
3T3
pCI-neo
A1
A2
A3
A4
A5
A6
A7
A8
MX2
Gapdh
B
Relative infectivity (%)
120
100
80
60
40
20
0
*
*
3T3
pCI-neo
A1
A2
A3
A4
A5
A6
A7
A8

Figure 2.

SUPPLEMENTAL INFORMATION

Supplemental Figures S1–3
&
Supplemental Tables S1–2

Article Title: A single nucleotide polymorphism of porcine *MX2* gene provides antiviral activity against vesicular stomatitis virus

Journal Name: Immunogenetics

Authors Names: Keisuke Sasaki[a], Pullop Tungtrakoolsub[a], Takeya Morozumi[b], Hirohide Uenishi[c,d], Manabu Kawahara[a,*], and Tomomasa Watanabe[a]

Corresponding Author: Dr. Manabu Kawahara, PhD

Affiliations: [a]Laboratory of Animal Breeding of Reproduction, Graduate School of Agriculture, Hokkaido University, Sapporo 060-8589, Japan.

[b]Animal Research Division, Institute of Japan Association for Techno-innovation in Agriculture, Forestry and Fisheries, 446-1 Ippaizuka, Kamiyokoba, Tsukuba, Ibaraki 305-0854, Japan

[c]Animal Genome Research Unit, Agrogenomics Research Center, National Institute of Agrobiological Sciences, 2 Ikenodai, Tsukuba, Ibaraki 305-8602, Japan

[d]Animal Immune and Cell Biology Research Unit, Division of Animal Sciences, National Institute

of Agrobiological Sciences, 2 Ikenodai, Tsukuba, Ibaraki, 305-8602, Japan

E-mail address of the Corresponding Author: k-hara@anim.agr.hokudai.ac.jp

Supplemental Figure Legends

Supplemental Fig. S1 3D structure prediction for porcine MX2 allele

a 3D models for all 8 MX2 alleles (A1–A8) were constructed using SWISS-MODEL, based on the template of human MxA (PDB ID: 3szrA).Note that 2 beta-sheets (574–624 amino acid residues) in both A7 and A8 were distinctly represented, but not in the other alleles (A1–A6). **b** Magnified images focusing on the range of 574–624 amino acid residues in A6–A8.

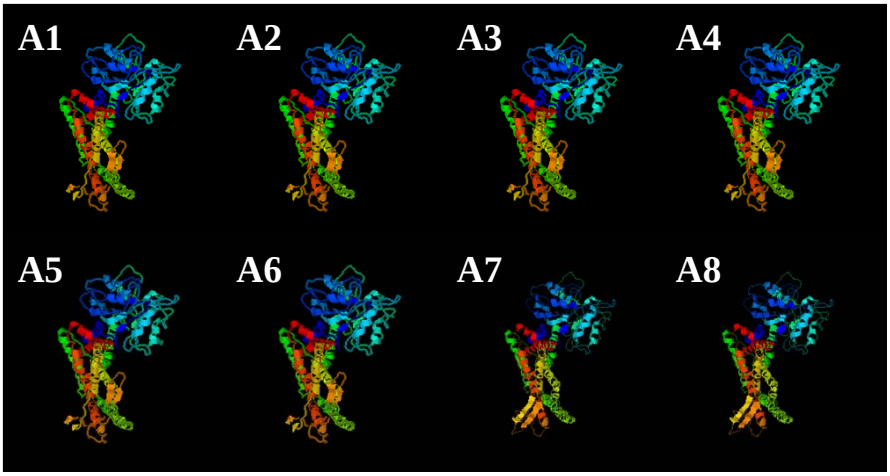
Supplemental Fig. S2 Variation of residue side chains caused by polymorphisms in CID.

3D structures images focusing on amino acid residues that were caused by polymorphisms in CID. Each characteristic amino acid residue was represented; the polymorphism at 514 aa for A4 and A6 (**a**), at 376 aa for A6 and A7 (**b**), at 377 aa for A3 and A4 (**c**), and at 386 aa for A1 and A4 (**d**). Arg, arginine; Gly, glycine; His, histidine; I, isoleucine; Phe, phenylalanine; Trp, tryptophan.

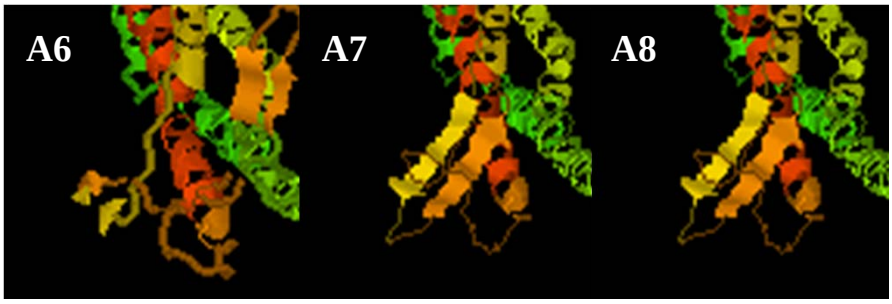
Supplemental Fig. S3 Mismatched PCR-RFLP analysis of porcine *MX2* gene targeting on the 514 aa polymorphism

The method and results of mismatched PCR-RFLP analysis are described in the respective sections in the text. **a** Nucleotide sequences of porcine *MX2* gene in the genomic DNA. Underlined characters show both the intron 10 F and exon 11 R primers used for mismatched PCR-RFLP analysis. Normal characters indicate introns, bold indicate exon 11. The region folded by square represents the digestion site of the restriction enzyme *Xho*1. When the nucleotide at position 1733 codes guanine, PCR amplicons are digested by *Xho*1 into fragments of 191 bp in length (GGG). In the case of adenine, the amplicons were not digested and appear as fragments of 214 bp in length (AGG). **b** Mismatched PCR-RFLP analysis of porcine *MX2* gene targeting on the 514 aa polymorphism. In the lanes LW3, LR1, and DR3, the PCR amplicons were digested and appeared as fragment of 191 bp in length. In lanes ME1 and ME2, the amplicons were not digested and appeared as fragments of 214 bp. The lanes of LW3+ME1 and LR1+ME2 shows simulated heterozygous patterns, resulting in 2 bands at 191 and 214 bp in length. M indicates DNA marker.

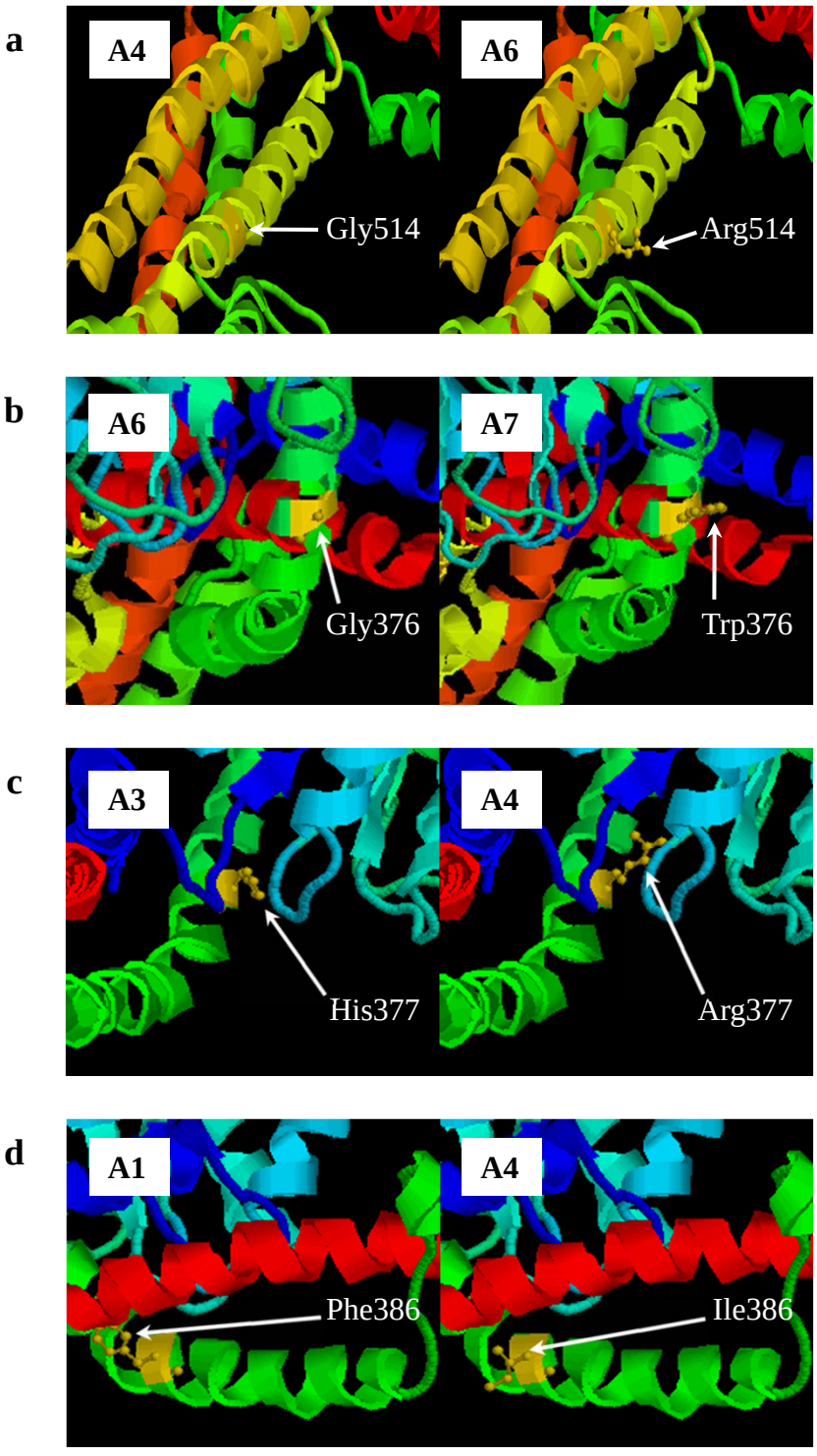
a



b

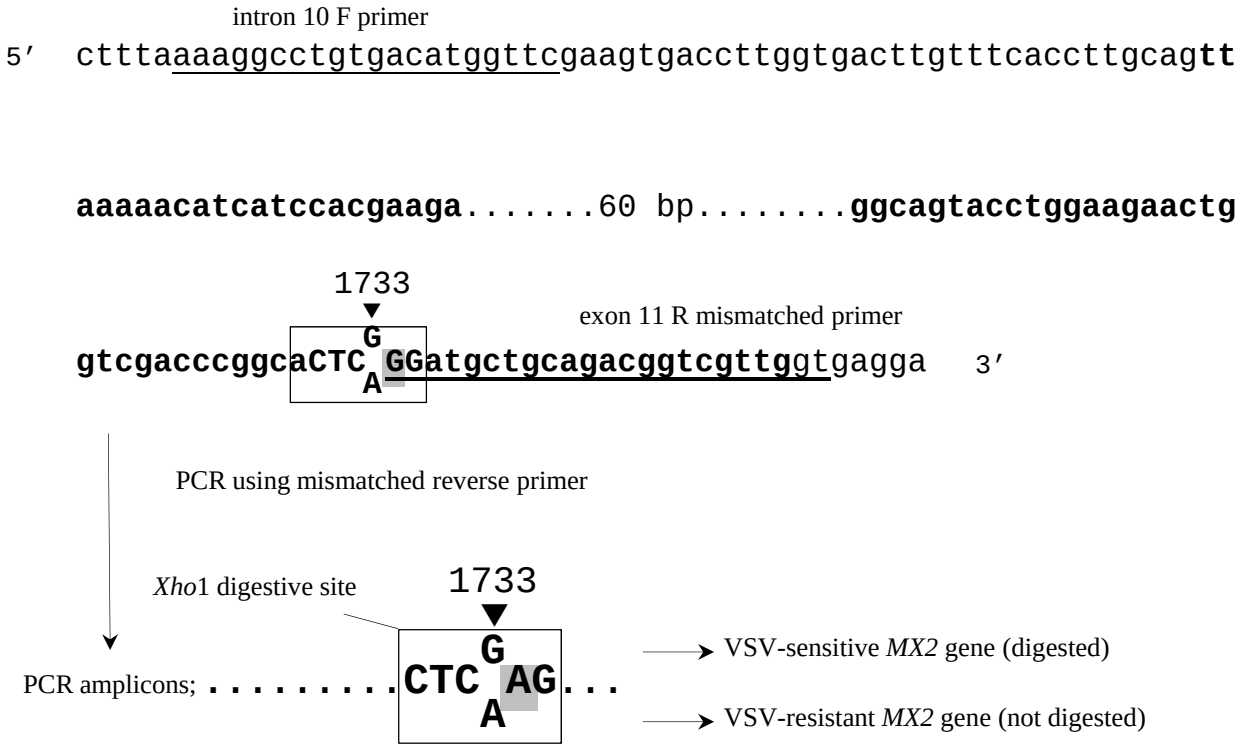


Supplemental Fig. S1

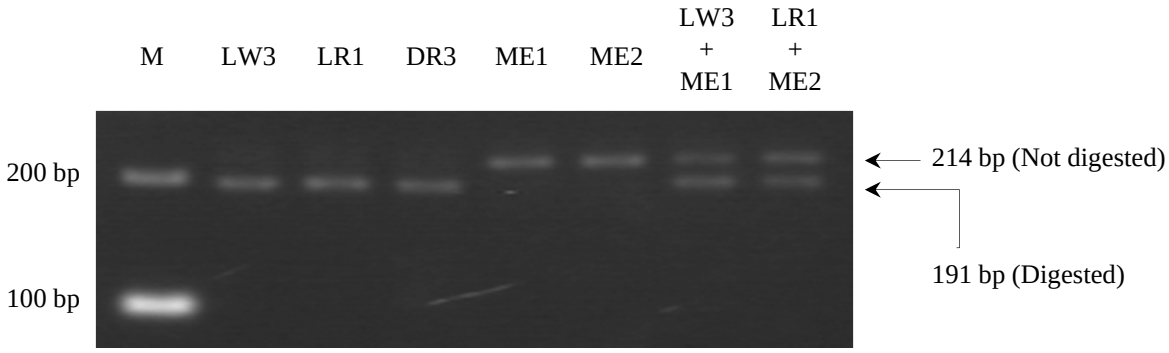


Supplemental Fig. S2

a



b



Supplemental Fig. S3

Supplemental Table S1. Amino acid substitutions of porcine MX2 protein

Breed	Amino acid position (nucleotide position)											Allele
	68 (396)	131 (584)	218 (845)	254 (953)	376 (1319)	377 (1323)	386 (1349)	514 (1733)	518 (1745)	543 (1821)	706 (2309)	
Database	Gln	Val	Thr	Thr	Gly	His	Phe	Gly	Thr	Gln	Ala	
LW1, LW2, LW3, LW4, LR1, DR1, MH, MY	Gln	Val	Thr	Thr	Gly	His	Phe	Gly	Thr	Gln	Ala	1/1
LR2	Gln	Val	Thr	Thr	Gly	His	Phe	Gly	Thr	Gln	Ala/Ser	1/2
LR5	Gln	Val	Thr	Thr/Ala	Gly	His/Arg	Phe	Gly	Thr/Ala	Gln	Ala	1/3
DR2	Gln	Val	Thr	Thr	Gly	His	Phe/Ile	Gly	Thr	Gln	Ala	1/4
DR3	Gln	Val	Thr/Ala	Thr	Gly	His	Phe	Gly	Thr	Gln	Ala	4/5
JH1, JH2	Gln	Val	Thr	Thr	Gly	His	Phe	Gly/Arg	Thr	Gln	Ala	4/6
ME1, ME2	Gln	Val	Thr	Thr	Gly	His	Phe	Gly	Thr	Gln	Ala	7/7
ME3	Gln/Arg	Val/Met	Thr	Thr	Gly	His	Phe	Gly	Thr	Gln/Arg	Ala	4/8

Database represents the predicted amino acid sequences of Landrace *MX2* gene (GenBank ID: AB259856).
Diagonals represent that the position is heterozygous alleles. The number of pig means the individual number in each breed.
DR, Duroc; JH, Jinhua; LR, Landrace; LW, Large White; ME, Meishan; MH, Mexican Hairless; MY, Middle Yorkshire.
Ala, alanine; Arg, arginine; Gln, glutamine; Gly, glycine; His, histidine; Ile, isoleucine; Met, methionine; Phe, phenylalanine; Ser, serine; Thr, threonine; Trp, tryptophan; Val, valine.

Supplemental Table S2. Allele typing of previously reported porcine *MX2*

GenBank ID	Nucleotide position (Amino acid position)						Protein allele type	References
	305	403	676	1084	1165	**1227**		
	(38)	(70)	(161)	(297)	(324)	**(345)**		
AB259856	**G**	A	C	G	T	**T**	A1	Morozumi et al. 20209
	(Ala)	(Pro)	(Cys)	(Thr)	(Arg)	**(Phe)**		
CU467746	**G**	A	C	G	T	**T**	A1	Submitted (16-MAY-22008)
	(Ala)	(Pro)	(Cys)	(Thr)	(Arg)	**(Phe)**		
DQ444963	**G**	A	C	G	T	**T**	A1	Unpublished
	(Ala)	(Pro)	(Cys)	(Thr)	(Arg)	**(Phe)**		
AB258432	**G**	A	C	G	T	**T**	A1	Morozumi et al. 2009
	(Ala)	(Pro)	(Cys)	(Thr)	(Arg)	**(Phe)**		
AK345001	**G**	A	C	G	T	**C**	Not identified in this study	Uenishi et al. 2004
	(Ala)	(Pro)	(Cys)	(Thr)	(Arg)	**(Ser)**		
AY897395	**T**	C	T	A	C	**T**	Not identified in this study	Unpublished
	(Ser)	(Pro)	(Cys)	(Thr)	(Arg)	**(Phe)**		
M65088	**G**	A	T	A	C	**T**	A1	Muller et al. 1992
	(Ala)	(Pro)	(Cys)	(Thr)	(Arg)	**(Phe)**		

Normal characters indicate SNPs found in this study. Bold characters indicate SNPs found in previously reports.
Ala, alanine; Arg, arginine; Cys, cysteine; Phe, phenylalanine; Pro, proline; Ser, serine; Thr, threonine.