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Original Article

Analysis of *env* Subtypes of Porcine Endogenous Retrovirus in SNU Miniature Pigs

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All xenografts from pigs impose infection risk by porcine endogenous retrovirus (PERV). The purpose of this study was to investigate the *env* constructs with the comparison of the ratio of the competent form to the defective one of *env* in subtypes, PERV-A, PERV-B and PERV-C in different pig breeds. The results of PCR amplification of *env* represented that all env subtypes had more than two defective forms which cannot bind to host cells due to the absence of binding regions of *env* in miniature pigs, SNU and PWG, and farm pig breeds, Duroc, Yorkshire and Landrace. In addition, comparing the full sequences with the defective ones in three subtypes demonstrated that the present percentages of *env* sequences in defective PERV-A, PERV-B and PERV-C were approximately 50%, 38~45% and 4~11%, respectively, in SNU and PWG pigs whereas PERV-A and PERV-B occupied around 40 to 60% but PERV-C was not detected in farm pigs. Quantitative real-time PCR assays with primers and probes targeted to proline-rich region (PRR) of each *env* subtype were done to measure the copy numbers of each *env* subtype. When the reference was set with copy number of PERV-A, the ratio of those of PERV-B and PERV-C to the reference were 1.5 to 6.0 folds high in SNU and PWG pigs while 1.0 or less in farm pigs. These contradictory results of PERV-C constructs and copy numbers in SNU pigs suggests that many truncated or short defective sequences of PERV-C might be present in them.

Key Words: Xenotransplantation, Porcine endogenous retrovirus, Subtype, Proviral load, Genome construct

INTRODUCTION

The integrated virus has been regarded as a potential risk of infection in the field of xenotransplantation, since the existence of porcine endogenous retrovirus (PERV) was reported (1). It is still controversial whether PERV could impact on human recipient after transplantation (2, 3). PERV had the transmission ability to human cell line such as human embryonic kidney (HEK) 293 and HeLa and primary human cells *in vitro* (4~6). However, co-culture of human cell line and cells from SNU miniature pigs showed that the virus was non-replicable and non-productive in human cells (7). Also, PERV has not been reported the infection to human and non-human primates *in vivo* (6, 8, 9). Nevertheless, PERV could integrate into human genome (10) and no one expect the effect of virus after adaptation in human genome.

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PERV can be classed into subtypes, PERV-A, PERV-B and PERV-C by their *env* sequences which determines human-tropism (11). Although PERV-C is not human tropic and not detected in PK15 cell line (12), the exclusion of PERV-C is recommended in xenotransplantation because the recombination between PERV-A and PERV-C can result in the modification to human tropism (13, 9). The ENV glycoprotein is composed of surface (SU) and trans-membrane (TM) subunit (14). The specificity among PERV types are determined by receptor binding domain (RBD) which includes variable region A (VRA), variable region B (VRB) and proline-rich region (PRR) (15, 16). This domain is important not only in binding to host cells but also in determining the characteristics of the types.

The purpose of this study was to identify the various *env* constructs of PERV-A, PERV-B and PERV-C and compare their characteristics in different pig breeds.

MATERIALS AND METHODS

Animal samples

Whole blood samples were obtained from 18 SNU miniature pigs bred in Centers for Animal Resource Development (CARD) in Seoul National University College of Medicine and 11 PWG miniature pigs supplied by PWG Genetics Pte Ltd. Semen samples from 3 pigs of each farm pig breed, Duroc, Yorkshire and Landrace, were taken from the Green Cross Corp. in Korea. All animal experiments were performed after receiving approval of the Animal Care and Use Committee (IACUC: 12-0374-C2A2 (0)) of Clinical Research Institute in Seoul National University Hospital AAALAC accredited facility and according to the National Institutes of Health guidelines.

Isolation of genomic DNA

Pig peripheral blood mononuclear cells (PBMCs) were isolated by Ficoll-Paque (GE healthcare, Little Chalfont, United Kingdom), density-gradient centrifugation of buffy coat preparation and stored at -80℃ prior to use. Genomic DNA (gDNA) was extracted from PBMCs of SNU and PWG pigs and semen of farm pigs using DNA extraction kit (Qiagen, Hilden, Germany) according to the manufacturer's instructions. The final concentration of all gDNA was from 1 to 50 ng/μl for both PCR and quantitative real-time PCR (qPCR) amplification.

Table 1. PERV *env* specific primers and probes used in this study.

Primer/probe	Sequence	Amplicon size (bp)	Reference (GenBank Acc.. No.)
ENV ABC F	5'-ATGCATCCCACGTTAAGCCG-3'		
ENV A R	5'-TTAGCTTGGAAGGCCTTGGTA-3'	1,965	HQ540592
ENV B R	5'-CTAGAGGTCGATTTCTCCTTGGCT-3'	1,974	AJ293657
ENV C R	5'-CTAGCGGCCAGCTTCCCTGC-3'	1,917	AF417227
ENV A real-time F	5'-GATGGAACCTCCGGTTGCT-3'		
ENV A real-time R	5'-GTTCTTGGATTGGAGGTCCTTG-3'	68	HQ540592
ENV A real-time probe	JOE-5'-TAGGACCAAATAAGGGTTTG-3'-TAMRA		
ENV B real-time F	5'-TGCCGGTGCCCCAAT-3'		
ENV B real-time R	5'-TGGTAGGAATCAATCCAGTGGTAC-3'	81	AJ293657
ENV B real-time probe	JOE-5'-ACCTCGCTGCGGCC-3'-TAMRA		
ENV C real-time F	5'-ACCAGGCTCCATTCTAACTATTCG-3'		
ENV C real-time R	5'-CGTATTTGGTCCTATAGCCATTGG-3'	73	AF417227
ENV C real-time probe	JOE-5'-CTCAAAATAAACCAGCTGGAG-3'-TAMRA		

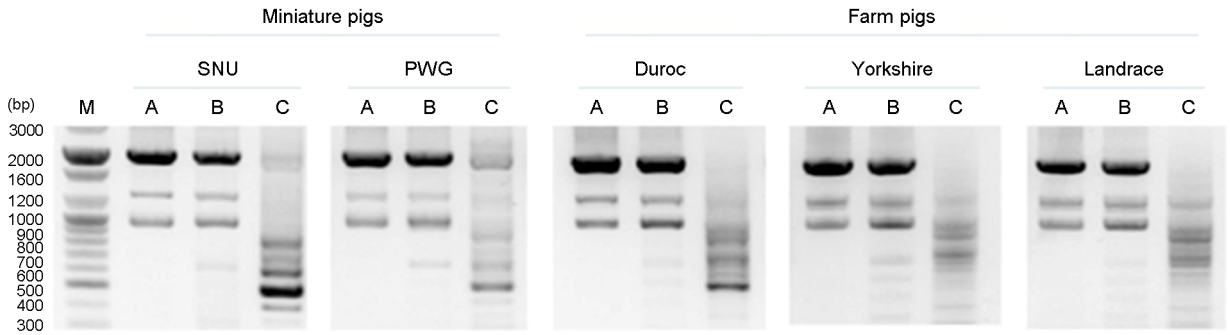

Figure 1. Amplified profile of *env* subtypes in miniature pigs (SNU and PWG) and farm pig breeds (Duroc, Yorkshire and Landrace). Gene amplification was done by PCR with the primers described in Table 1. All the experiments revealed the similar amplification patterns and the representative pictures were shown. (M) denotes molecular marker; (A), (B) and (C) denote PERV subtypes A, B and C, respectively.

Sequencing of *env* subtypes

Primers for the detection of the whole sequences of PERV *env* were selected in both ends of the gene. The full length of *env* was amplified each type of primers with using AccuPower PCR premix (Bioneer, Seoul, Korea). The conditions of PCR were 95℃ for 3 min for pre-denaturation and then repeated 30 cycles of 95℃ for 30s, annealing at 55℃ for 30s, and 72℃ for 2 min. The final elongation step was done at 72℃ for 5 min. All bands on agarose gel were purified using gel extraction kit (Qiagen, Hilden, Germany) and then directly sequenced with the respective primers in commercially available company). They were aligned by Blast and phylogenetically grouped by ProtDist on NCBI and NCBS, respectively.

Primers and labeled probes

Primer Express 3.0.1 (Applied Biosystems, Foster City, CA, USA) for real-time PCR was used to design primer and probe set within the consensus sequence of *env* gene from our previous data (GenBank access Nos. HM131061 - HM131078) and other PERV sequences in Genbank database (Table 1). The probe carried a 5' reporter dye, 4-5-Dichloro carboxyfluorescein (JOE), and a 3' quencher dye, 6-carboxytetramethyl-rhodamine (TAMRA). Primers and probes were designed not to amplify other subtype sequences among the subtypes for the prevention of cross amplification. The run method of real-time was 2 min at 50℃ and 10 min at 95℃ for holding stages, followed by 40 cycles of 15 sec at 95℃ and 1 min at 60℃.

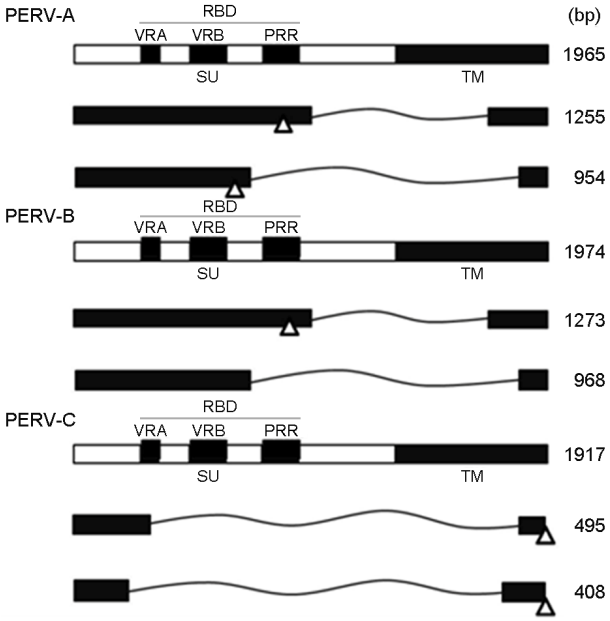

Figure 2. Schematic diagrams of constructs of PERV A, B and C *env* gene derived from SNU miniature pig. SU, surface; TM, transmembrane; RBD, receptor binding domain; VRA, variable region a; VRB, variable region b; PRR, proline-rich region; open triangle: stop codon.

DNA standards

PCR products of approximately 2 kb of PERV-A and PERV-B and 1.1 kb of PERV-C were cloned into pCR4-

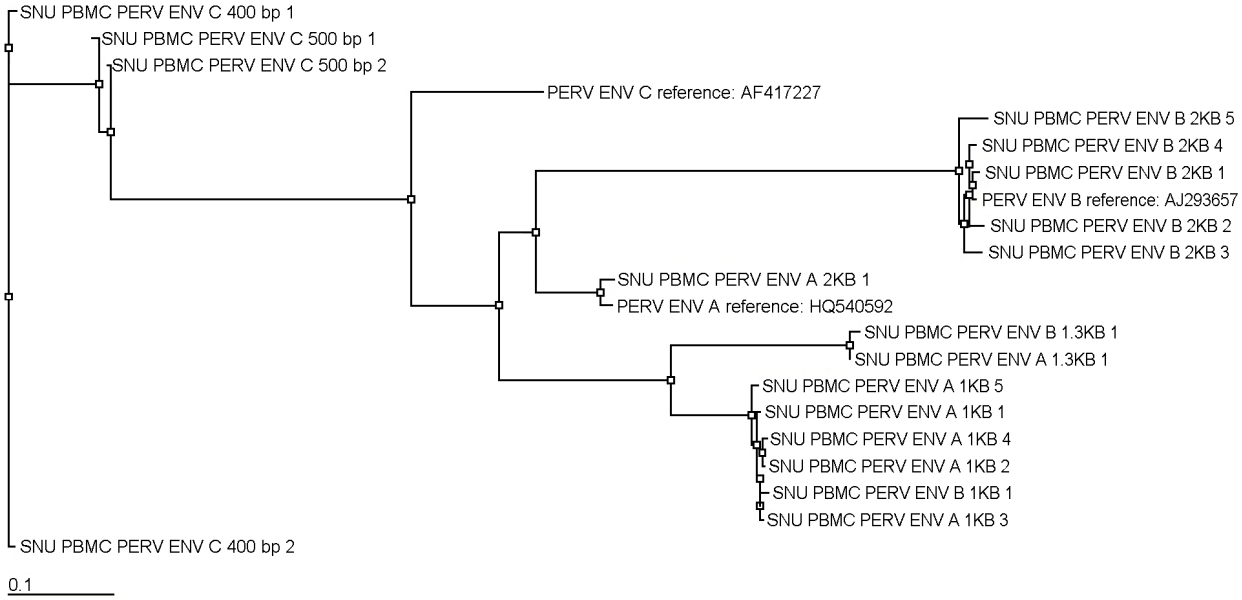

Figure 3. Pylogenetic relationship tree using neighbor-joining methods on the nucleotide sequences of complete and defective PERV *env* derived from SNU pigs.

TOPO vector using a TOPO TA cloning kit (Invitrogen, Calsbad, CA, USA) for positive target plasmid. To evaluate the positive plasmid, qPCR was performed on each positive clone using 3 types of primer and probe sets. To determine the linear range of amplification, a 10 fold serial dilution of env target plasmid ranging from 10 to 1 $\times$ 10^9 copies per reaction was examined with the respective primers and probe using the TaqMan universal PCR master mix (Applied Biosystems, Foster City, CA, USA).

Quantitation of *env* subtypes

To check the integrated PERV *env* gene, the quantitation of target DNA was performed by densitometry for the PCR amplicons and by real-time PCR for the absolute copy numbers. The density of PCR product of *env* full-length and defective forms on the agarose gel was calculated by Image Lap 3.0 (Bio-Rad, Hercules, CA, USA). Absolute copy number was calculated from the standard curve using primer and probe sets targeted specific region for each type.

RESULTS

Detection of PERV *env* subtypes in various pig breeds

PCR assay was performed to verify the presence of subtype-specific sequences in gDNA isolated from PBMC of miniature pigs, SNU and PWG, and farm pigs, Duroc, Yorkshire and Landrace (Fig. 1). The results of amplification showed all pigs have more than two defective forms in each type. PERV-A and PERVB-B have similar amplified patterns in all pig breeds whereas PERV-C has diverse smaller sizes in individual pigs.

Analysis of *env* constructs

To confirm whether the smaller amplicons had genuine PERV sequences, nucleotide sequences were determined in each size of amplified products. The blast analyses revealed that they were defective forms of PERV *env*. The structure of construct contained stop codons in the middle of the open reading frame (ORF) and most of defective forms did not have some parts of RBD region (Fig. 2). Phylogenetic tree showed that PERV-A sequences were well grouped

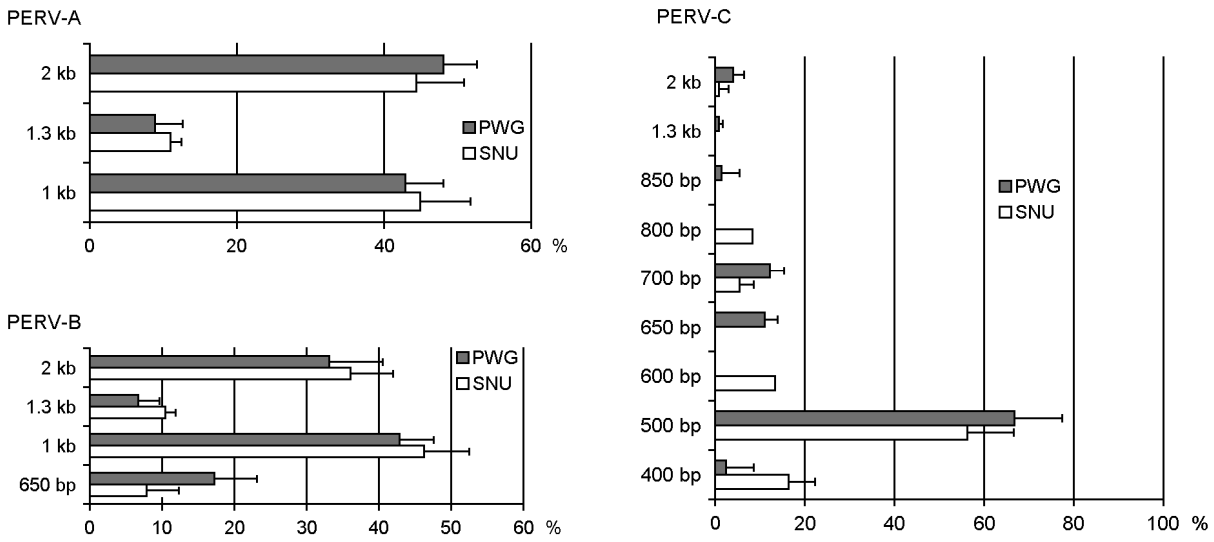

Figure 4. The proportion of the full length sequences and defective ones of *env* in 18 SNU and 11 PWG miniature pigs. The relative amount of each construct was measured by the densitometry of amplified PCR products on agarose gel. Results were shown with mean + standard deviation.

irrespective of their sizes, but PERV-B and PERV-C sequences were not well demarcated. Smaller sizes of PERV-B, 1.3 and 1.0 kb, were grouped into PERV-A (Fig. 3). Because their sequences were truncated where the place to distinguish the subtypes as shown in Fig. 2, grouping of sequences was different from expected subtypes.

Quantitation of the complete and defective forms in PERV *env*

Sizes of PERV-A constructs were 2.0, 1.3 and 1.0 kb, and PERV-B constructs had an additional size of 650 bp, while PERV-C constructs consisted of 9 sizes mainly under 1 kb in SNU and PWG pigs (Fig. 4). The defective forms of *env* in PERV-A and PERV-B constituted over 50% compared to whole sequences in SNU and PWG pigs. PERV-C also represented almost all the defective forms and the full sequences were difficult to detect. PWG pig had less than 5% of PERV-C full sequence but less than 1% in SNU pigs. Among the defective forms of PERV-C, construct with 500 bp occupied majority in SNU and PWG pigs.

Linear range of amplification of real-time PCR for PERV *env*

The linear range of real-time PCR assays was determined with absolute copy number of the serially diluted amount of the standard target *env* plasmid (Fig. 5). The standard curve of the PCR assay was derived from the logarithmic input template quantity (copies/μl) of each *env* subtype clones. The detection limits of PERV-A, PERV-B and PERV-C in the PRR domain by real-time PCR were 100, 10 and 100 copies/μl, respectively.

Quantitation of PERV *env* subtype

The comparison of the amplification ratio of the full length sequences in three subtypes demonstrated that the proportion of PERV-A, PERV-B and PERV-C were approximately 50%, 38~45% and 4~11%, respectively, in SNU and PWG pigs whereas PERV-A and PERV-B occupied over 99% and PERV-C was detected below 1% in farm pigs (Fig. 6A). On the other hand, the ratio of the copy numbers of PERV-B and PERV-C to that of PERV-A was 1.5~1.6 and 5.0~6.0, respectively, in SNU and PWG pigs while there was little amount of PERV-C in farm pigs (Fig. 6B).

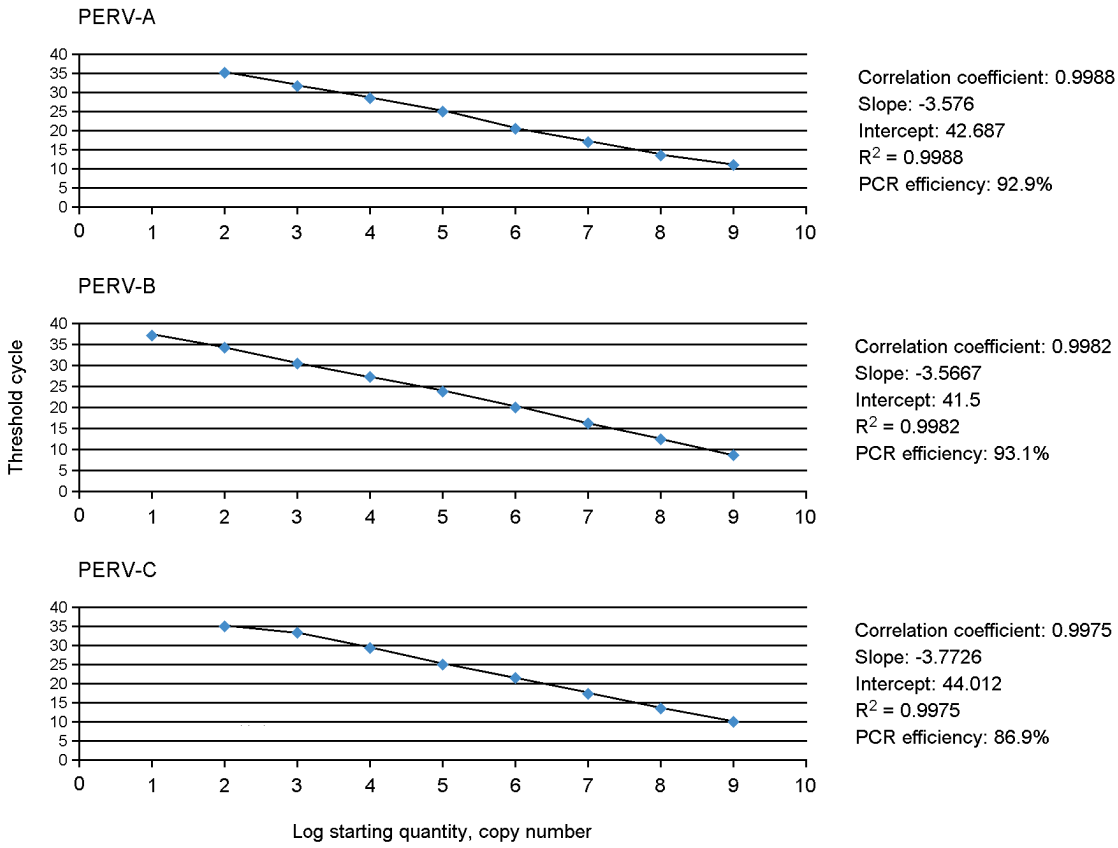

Figure 5. Linearity of standard curve of the real-time PCR assays for each *env* subtype.

DISCUSSIONS

These studies demonstrate the analysis of PERV-A, PERV-B and PERV-C in miniature and farm pigs. Occupations of complete sequences compared to defective forms were less than 50% and the defective constructs were deleted in the region of binding sites. Even though some defective forms have PRR, the coding transcript was ended in the binding domain due to many mutations. Previous studies of *env* RBD in C-terminus of SU subunit represent the importance of recognition and influence of infectivity to human (15) and of critical residues for human cell infection showed that the location was within PRR (16). These results mean that defective proviruses might have lower infectivity to host and not express functional proteins properly.

Analysis of PCR products with densitometry show that there was little amount of PERV-C but ratios of individual subtypes based on copy numbers of the target genes indicate the conflictive outcomes to PCR assay. The ratios of PERV-C to all subtypes by PCR assay in this study were similar to the reported results of PERV quantitation by qPCR in DNA and RNA from transgenic Polish Landrace pigs (17). One of some possibilities of contradictory results is the inaccuracy of primer sequences used in this study. But we selected the conserved sequences in PERV *env* collected from our previous deposited sequence data of SNU miniature pigs and recent GenBank data and the construct results would be correct. The quantity of PERV-C full sequence in farm breeds was not found as the report that some pigs such as Landrace were free of PERV-C (18). Moreover, the supporting idea for the results was carried out that inbreeding compared to wild boar increased the

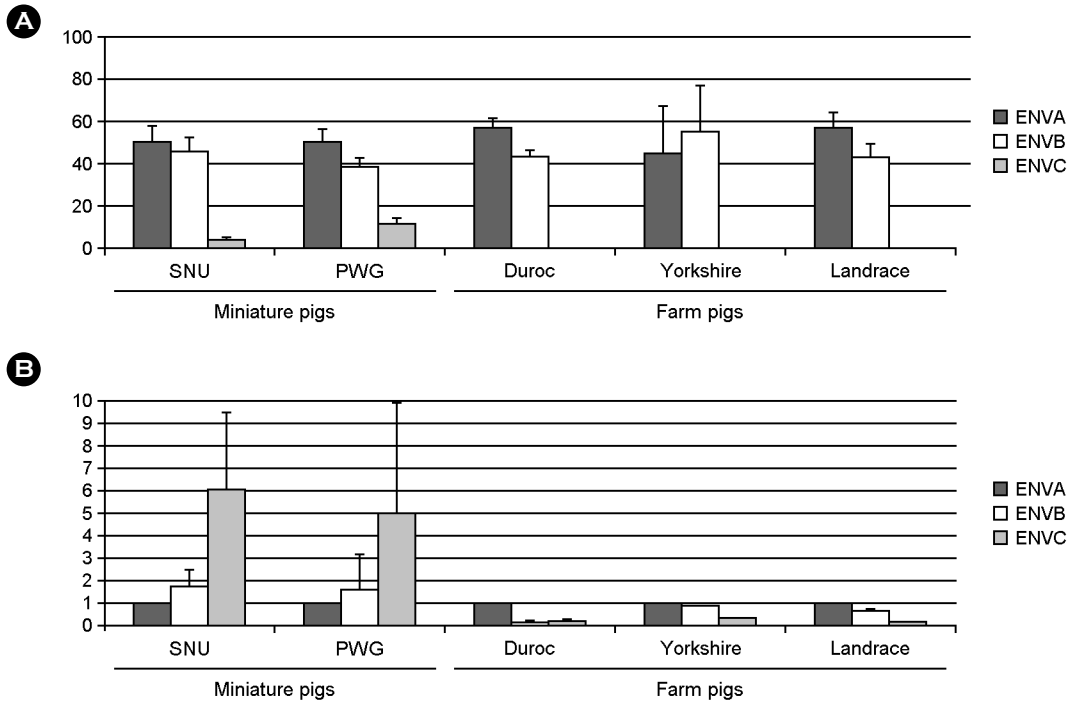

Figure 6. The proportion of the *env* A, B and C subtypes in various pig breeds. (A) the percentages of each env subtype by densitometry of PCR product on the agarose gel. (B) the ratio of copy numbers of PERV-B and PERV-C to that of PERV-A. Results were shown in mean + standard deviation in 18 SNU, 11 PWG, 3 Duroc, 3 Yorkshire, and 3 Landrace pigs.

copy number of PERV integration (19). However, another data suggests copy numbers were not related to pig inbreeding (20) and according to consensus statement (21), inbreeding in SPF strains is indispensable to prevent other infectious risks. When the results of genome structure above (Fig. 2) were applied to the results of real-time PCR assays targeted to *env* PRR, the defective forms might have not been amplified or other defective forms which do not have at the end part of *env* gene could have been amplified in the assays. Therefore, we assume that the presence of various forms of defective PERV *env* result in these consequences because of the truncated chromosomal mutations in the host. To confirm this assumption the further sequence information such as the whole genome sequencing should be done in the future.

Nevertheless, defective transcripts need to be examined if they have ability to cause oncogenesis especially in melanoma and leukemia. Human endogenous retrovirus (HERV) is similar to PERV in the terms of its structure and property that integrated long times ago to its own host. Rec and Np9 of the viral proteins which are derived from the deleted sequences in the middle of *env* gene of HERV type K were expressed in host cells and have hazardous potential like tumor (22~25). Therefore, the short transcripts of PERV *env* should be examined to exclude a significant risk factor as xenotransplantation donor in the future.

In conclusion, the contradictory results of PERV-C constructs and copy numbers in SNU pigs suggests that many truncated or short defective sequences of PERV-C might be present in them. Further characterization of PERV subtypes would give insight in the selection of suitable breeds as pig donors for xenotransplantation.

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