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An association between type A porcine endogenous retrovirus copy number and hematological parameters and gender in miniature pigs

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Pig is the most promising species for transplantation of organs and cells into humans, although implementation of xenotransplantation in clinical practice has been hindered by the risk of infecting the recipient with zoonotic infectious diseases. Porcine endogenous retroviruses (PERV) are capable of incorporating copies of DNA into the genome of a host cell. Based on the nucleotide sequence of the envelope gene (*env*), three main types of pig retrovirus, PERV-A, PERV-B and PERV-C, have been recognized, with PERV-A and PERV-B having the capability of infecting human cell lines *in vitro*. Selection for animals with low copy number of retroviruses in the genome using simple phenotypic indications is required for the widespread implementation of xenotransplantation. The objective of this study was to evaluate the correlation between PERV-A *env* gene copy number and hematological parameters, gender and coat color in miniature pigs of the Institute of Cytology and Genetics (ICG) SB RAS. Reference values for eighteen blood parameters of miniature pigs were determined, including white blood cell count (WBC), red blood cell count (RBC), platelet count (PLT), absolute (LYM#) and relative (LYM%) lymphocyte counts, absolute (MID#) and relative (MID%) monocyte, basophil and eosinophil counts, absolute (GRA#) and relative (GRA%) granulocyte counts, hematocrit (HCT) and thrombocrit (PCT), mean cell volume (MCV) and mean platelet volume (MPV). Males had significantly higher reference values for WBC, MID#, GRA# and red cell distribution width (RDW-CV) as compared to females. The mean corpuscular hemoglobin concentration (MCHC) and platelet distribution width (PDW-CV) were significantly higher in female animals. No correlation between PERV-A *env* gene copy number and the coat color of animals was detected, suggesting that retroviral insertion sites and genes that determine the coat color of miniature pigs, namely *KIT* (chromosome 8) and *MC1R* (chromosome 6), are either located far apart on same chromosome or on different chromosomes. The copy number of PERV-A *env* gene in males was lower than in females. Presence of multiple copies of PERV-A on the X-chromosome is the most probable cause of such gender-related differences in miniature pigs. Thus, male miniature pigs of ICG SB RAS should be the source of material for xenotransplantation.

Key words: xenotransplantation; miniature pigs of ICG SB RAS; porcine endogenous retrovirus; PERV; *envA* gene; gender; coat color; blood test.

Ассоциация числа копий эндогенных ретровирусов типа А с гематологическими показателями и полом у мини-свиней

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Свинья является наиболее перспективным видом для ксенотрансплантации органов и клеток человеку. Внедрение ксенотрансплантации в клиническую практику сдерживается возможным риском передачи реципиенту зоонозных инфекционных заболеваний. Эндогенные ретровирусы свиней (PERV) способны встраиваться в геном клетки в виде ДНК-копий. Три типа ретровирусов PERV – A, B и C – различаются по нуклеотидной последовательности гена *env*. PERV типа A и B могут инфицировать некоторые линии клеток человека *in vitro*. Для широкого внедрения ксенотрансплантации необходим поиск простых фенотипических признаков, по которым можно отбирать животных с наименьшим числом ретровирусов в геноме. Целью работы было выявление корреляции числа копий гена *envA* PERV с гематологическими показателями, полом и окраской у мини-свиней Института цитологии и генетики (ИЦиГ) СО РАН. Были определены референсные значения восемнадцати параметров крови для мини-свиней, включая абсолютное содержание лейкоцитов (WBC), эритроцитов (RBC) и тромбоцитов (PLT), абсолютное (LYM#) и относительное (LYM%) содержание лимфоцитов, абсолютное (MID#) и относительное (MID%) содержание моноцитов, базофилов и эозинофилов, абсолютное (GRA#) и относительное (GRA%) содержание гранулоцитов, гематокрит (HCT) и тромбокрит (PCT), средний объем эритроцита (MCV) и тромбоцита (MPV). Показатели WBC, MID# и GRA#, а также относительная ширина распределения эритроцитов по объему (RDW-CV) у самцов были достоверно выше, чем у самок. Самки превосходили самцов по средней концентрации гемоглобина в эритроцитарной массе (MCHC) и относительной ширине распределения тромбоцитов по объему (PDW-CV). Корреляционный анализ показал отсутствие связи между числом копий гена *envA* PERV на клетку и окраской животных. По-видимому, сайты инсерции ретровирусов у мини-свиней либо нахо-

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дятся на одной и той же хромосоме, но на значительном генетическом расстоянии от генов *KIT* (хромосома 8) и *MC1R* (хромосома 6), контролирующих окраску, либо локализованы с ними на разных хромосомах. Число копий гена *envA* PERV у самцов было ниже, чем у самок. Наиболее вероятной причиной гендерных различий является локализация нескольких копий PERV-A на X хромосоме мини-свиней. Таким образом, материал для ксенотрансплантации целесообразно брать у самцов миниатюрных свиней ИЦиГ СО РАН.

Ключевые слова: ксенотрансплантация; мини-свиньи ИЦиГ СО РАН; свиной эндогенный ретровирус; PERV; ген *envA*; пол; окраска; анализ крови.

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Today, in all countries of the world, the number of people with end-stage organ failure far exceeds the number of donor organs/cells available for transplantation. Xenotransplantation of organs/cells from pig to human offers a potential solution to this problem. Pig is the most promising species for xenotransplantation due to its anatomical and physiological similarity to humans, as well as for ethical and economic reasons (Ekser et al., 2015). However, the widespread implementation of xenotransplantation in clinical practice has been hampered by the risk of infecting the recipient with zoonotic infectious diseases.

Porcine endogenous retroviruses (PERV) are RNA-containing viruses that are capable of incorporating copies of DNA into the host cell's genome. Therefore, PERV cannot be eliminated by breeding pigs under specific pathogen-free conditions (Yudin et al., 2011). Since immunosuppressive therapy is mandatory after organ transplantation, the recipient has a high risk of developing PERV infection (Denner, 2016).

Based on the significant differences in the amino acid sequence of the receptor-binding domain of the *env* gene, which encodes a viral envelope protein, three main types of pig retrovirus, PERV-A, PERV-B and PERV-C, have been recognized. PERV-A and PERV-B infects not only porcine cell lines, but also several human cell lines *in vitro*, while PERV-C replicates only in porcine cells (Kimsa et al., 2014). Observations of patients treated with living porcine tissues or organs have not yet revealed the development of PERV infection in humans *in vivo* (Godehardt et al., 2015). It is not clear whether these data are due to real lack of virus production by pig cells or the result of effective inactivation of released virus particles by the immune system.

According to preliminary estimates, the pig genome contains 6 to 10 copies of replicable provirus, 30 to 50 full-length PERV copies and 100 to 200 loci containing partial PERV sequence (Niebert, Tonjes, 2005). The number of retroviral integration sites, generally, positively correlates with the level of viral mRNA expression (Ka et al., 2009). The existing methods for determining the copy number of PERV in the genome based on real-time PCR require special equipment and reagents, as well as highly skilled personnel. Identification of simple phenotypic indications allowing to select for animals with low copy number of retroviruses in the genome is required for the widespread implementation of xenotransplantation.

A unique breed of Siberian miniature pig was developed at the Institute of Cytology and Genetics (ICG) SB RAS for biomedical applications by crossing pigs of Vietnamese Potbellied breed, Large White and Landrace breeds, and wild boars of Central Asian subspecies (Tikhonov, 2010). Using material from miniature pigs, studies are carried out to test and produce bioprosthetic heart valves, blood vessels and pericardial flap for intracardiac surgery and angioplasty. A technique for obtaining chondrotransplants from newborn miniature pigs to treat cartilage tissue dystrophic and traumatic changes in idiopathic scoliosis has been developed. Yet, PERV retroviruses of all three types are present in the genomes of most miniature pigs (Aitnazarov et al., 2014).

The objective of this study was to evaluate the correlation between PERV-A *env* gene copy number and hematological parameters, gender and coat color in miniature pigs of ICG SB RAS.

Materials and methods

1-month-old miniature pigs were obtained from the Common Use Center for "Gene pools of fur and farm animals" of ICG SB RAS. Gender and coat color were visually determined. Venous blood was collected in tubes containing anticoagulant (EDTA K-2). Automatic blood analyzer Hemascreen 18P (Hospitex Diagnostics, Italy) was used to determine 18 hematological parameters. The study was conducted in strict compliance with the Helsinki Declaration on Humane Treatment of Animals.

Whole blood DNA isolation was performed with protease treatment and phenol extraction (Sambrook, Russel, 2006). The copy number of PERV-A *env* gene was determined by real-time PCR using standard samples prepared by the limiting dilution method (Aitnazarov et al., 2016) using Rotor-Gene Q (Qiagen, Netherlands). Amplification was carried out using a set of real-time PCR reagents with the SYBR Green I dye (Sintol, Russia) according to the manufacturer's protocol. For each DNA sample, PCR was performed at least three times. Data was processed with Rotor-Gene 6000 Series software, version 1.8.17.5. In the calculations, the genomic DNA amount was assumed equal in pig cells and human cells (6 pg DNA per cell). Since all investigated parameters, according to the Kolmogorov–Smirnov test, were normally distributed, a one-way analysis of variance and a correlation coefficient were used to assess the impact of factors. The arithmetic mean and standard error were calculated for each experimental group. The results were processed using STATISTICA 8 software.

Results and discussion

Data on PERV-A *env* gene copy number in blood cells from different groups of miniature pigs of ICG SB RAS are given in Table 1. The mean copy number for all studied animals (26.1, $n = 40$) significantly exceeds the value, which we previously obtained for pigs of the same breed (4.5, $n = 10$) (Aitnazarov et al., 2016). Influence of such factors as "boar genotype" and "sow genotype" on the PERV-A *env* gene copy number was insignificant ($F_{6.33} = 0.65$, $p = 0.69$ and $F_{14.25} = 1.08$, $p = 0.42$, respectively). The minimum/maximum values were 12.3/38.1 in the present study and 0.6/58.9 in the previous study, which confirms data from other authors stating significant differences between animals in this parameter (Liu et al., 2011). The observed differences might be related to a relatively small number of animals in our first study.

Table 1. PERV-A *env* gene copy number per cell in different groups of miniature pigs

Group	Number of animals	PERV-A *env* gene copy number per cell
Males	20	23.7 ± 1.5
Females	20	28.3 ± 1.3*
White	21	26.8 ± 1.5
Black	14	25.3 ± 1.8
Black-pied	5	24.3 ± 1.5

* $p < 0.02$ as compared to males.

Determination of retroviral genome copy number *in vivo* or *in silico* remains a difficult task. The alignment of primers used in our study to the Duroc pig genome, version Sscrofa10.2/susScr3 in the UCSC genomic browser, using Blat procedure allowed us to detect a total of seven copies: on chromosome 1 (two copies) and chromosomes 7, 8, 12, 13 and X (one copy each). However, this result may change when using another procedure and/or parameters for alignment. In addition to the individual differences mentioned above, it has been shown that the copy number of PERV retrovirus per cell may depend on the breed (Yu et al., 2007; Ma et al., 2010; Lee et al., 2011) and the examined organ (Zhang et al., 2010; Mazurek et al., 2013).

Earlier, based on the study of the prevalence of retrovirus in animals of different gender, we suggested that a copy(ies) of PERV-A is localized on the X-chromosome in pigs of the Large White breed of the Achinsk type (Aitnazarov et al., 2006). In our study, the copy number of PERV-A *env* gene in males was 16 % lower than in females ($p < 0.02$), which is about 4 copies per cell. The most likely cause of the observed differences is the localization of several PERV-A copies on the X-chromosome of miniature pigs. According to cytogenetic studies, PERV-A is localized on the X-chromosome in Australian Westran pigs, but is absent on the same chromosome in Large White and native Korean pig breeds (Jung et al., 2010).

A similar gender effect has been observed in other species of mammals. The copy number of *env* gene of endogenous human retrovirus, which has been associated with the development of multiple sclerosis, in peripheral blood mononuclear

Table 2. Values for hematological parameters and their correlation with PERV-A *env* gene copy number per cell in miniature pigs

Parameter	Males ($n = 20$)	Correlation coefficient, r	Females ($n = 20$)	Correlation coefficient, r
White blood cell count (WBC), $\times 10^9$ cells/L	14.97 ± 0.87	–0.09	12.15 ± 0.51##	0.23
Lymphocyte cell count (LYM#), $\times 10^9$ cells/L	9.16 ± 0.63	–0.06	8.26 ± 0.47	0.29
Monocyte, basophil and eosinophil count (MID#), $\times 10^9$ cells/L	3.63 ± 0.26	–0.13	2.58 ± 0.21##	0.04
Granulocyte count (GRA#), $\times 10^9$ cells/L	2.17 ± 0.29	–0.04	1.31 ± 0.15##	–0.17
Lymphocyte percentage (LYM%)	61.38 ± 2.53	–0.02	67.99 ± 2.35	0.17
Monocyte, basophil and eosinophil percentage (MID%)	24.03 ± 0.75	–0.09	21.18 ± 1.32	–0.08
Granulocyte percentage (GRA%)	14.60 ± 2.04	0.05	10.84 ± 1.19	–0.24
Red blood cell count (RBC), $\times 10^{12}$ cells/L	7.46 ± 0.20	–0.15	7.18 ± 0.19	–0.15
Hematocrit (HCT), %	66.27 ± 1.52	0.06	65.60 ± 0.19	–0.01
Mean cell volume (MCV), fL	89.30 ± 1.86	0.27	91.95 ± 0.19	0.31
Red blood cell distribution width (RDW-CV), %	24.76 ± 1.66	–0.19	20.29 ± 0.76##	–0.19
Mean cell hemoglobin (MCH), pg/cell	16.33 ± 0.40	0.19	17.12 ± 0.20	0.09
Mean corpuscular hemoglobin concentration (MCHC), g/L	182.60 ± 1.34	–0.11	186.40 ± 0.98##	–0.55*
Hemoglobin (HGB), g/L	121.20 ± 3.33	0.00	122.35 ± 2.37	–0.17
Platelet count (PLT), $\times 10^9$ cells/L	316.40 ± 24.65	–0.27	309.65 ± 16.37	–0.14
Mean platelet volume (MPV), fL	11.17 ± 0.35	0.23	11.85 ± 0.21	0.22
Thrombocytocrit (PCT), %	36.16 ± 3.45	–0.16	36.88 ± 0.22	–0.05
Platelet distribution width (PDW-CV), %	43.62 ± 1.89	0.04	48.76 ± 1.34##	–0.18

* $p < 0.01$; ## $p < 0.01$ as compared to males.

cells is significantly higher in women as compared to men (Garcia-Montojo et al., 2013). According to the authors, this may be due to localization of at least two copies of retrovirus on the human X-chromosome. In cats, on the contrary, the copy number of *env* gene of endogenous retrovirus is higher in males than in females (Tandon et al., 2007). This is probably due to the localization of 3–5 provirus copies on the cat Y-chromosome (Roca et al., 2005).

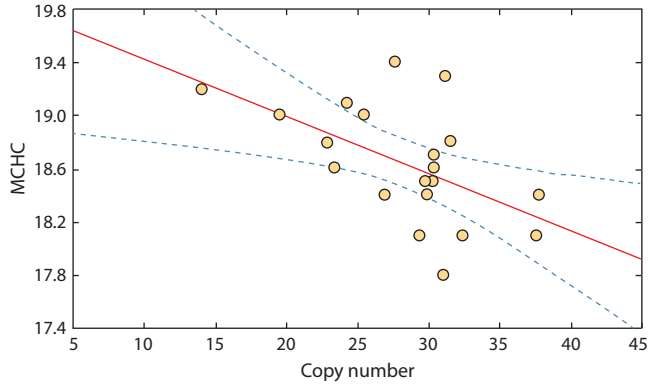
We did not identify reliable correlation between the PERV-A copy number per cell and the coat color of animals. In miniature pigs of ICG SB RAS and in pigs of other breeds, the white coat color is controlled by the *KIT* gene (genotypes I/I and I/i) located on chromosome 8. *MC1R* gene located on chromosome 6 controls black-pied (genotype E^p/E^p) and black (genotypes E^d/E^d, E^d/E and E^d/E^p) coat colors (Nikitin et al., 2016). It seems that retroviral insertion sites and genes that determine the coat color of miniature pigs are either located far apart on same chromosome or on different chromosomes.

A dilute viral (*dv*) coat-color mutation is known in inbred mice, caused by the insertion of murine leukemia virus Emv-3 into the intron of the *Myo5a* gene (Seperack et al., 1995). Insertion of retrovirus leads to alternative splicing and formation of a shorter form of myosin heavy-chain protein, which is encoded by *Myo5a* gene. It is believed that this protein is necessary for maintaining normal structure of dendrites and organelle transport in melanocytes. Feline leukemia virus is more common in animals with a solid, rather than spotted fur color (McMichael et al., 1986). Insertion of retrovirus into the 5′ region of the aromatase gene in the Sebright chicken breed leads to the development of plumage color of roosters similar to the plumage color of hens (McPhaul et al., 1991).

Reference values for 18 haematological parameters of miniature pigs of ICG SB RAS have been determined (Table 2). All blood parameters were in agreement with the accepted physiological norms (Kondrakhin, 2004) and data obtained from other breeds of pigs (Rispat et al., 1993; Ekser et al., 2012; Kawaguchi et al., 2012). The white blood cell count (WBC), monocyte, basophil and eosinophil count (MID#) and granulocyte count (GRA#), as well as the red blood cell distribution width (RDW-CV) were significantly higher in males than in females. Females had higher mean corpuscular hemoglobin concentration (MCHC) and platelet distribution width (PDW-CV) as compared to males.

A study of 23 blood parameters in Japanese micro mini pigs has shown that basophil and lymphocyte counts were higher in females, but neutrophil count was higher in male animals (Kawaguchi et al., 2012). In Yucatan miniature pigs, the only detected difference between males and females was the higher platelet count in males (Rispat et al., 1993). The observed gender differences in hematological parameters between pigs of different breeds might be due to the influence of both genetic and environmental factors (feed, external conditions, etc.), as well as due to the different ages of studied animals.

No correlation between hematological parameters and the copy number of PERV-A *env* gene in blood cells of male miniature pigs has been detected. In females, a significant negative correlation was found between the copy number and the mean corpuscular hemoglobin concentration (MCHC) (Figure). Possibly, a copy(ies) of an active form of PERV-A



Correlation between PERV-A *env* gene copy number in blood cells and the mean corpuscular hemoglobin concentration (MCHC, g/L) in female miniature pigs.

retrovirus causes disruption of genes that regulate hemoglobin level in females to a greater extent than in males.

It is known that hemoglobin level is on average 12 % lower in women than in men and this ratio is also observed in many species of mammals, birds and reptiles (Murphy, 2014). Since both genders have similar erythropoietin levels, it was hypothesised that oestrogens dilate and androgens constrict the renal microvasculature. Dilatation and vasoconstriction respectively increase and decrease the hematocrit in blood, providing a mechanisms for varying the red cell mass without changes in erythropoiesis.

Thus, material for xenotransplantation should be taken from male miniature pigs of ICG SB RAS, since they have a much lower copy number of PERV-A *env* gene as compared to females. It seems also promising to use some hematological parameters for this purpose, but this issue requires further investigation.

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Conflict of interest

The authors declare no conflict of interest.

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