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ORIGINAL ARTICLE

Characterization of Clones of Human Cell Line Infected with Porcine Endogenous Retrovirus (PERV) from Porcine Cell Line, PK-15

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돼지 세포주 PK-15에서 유래한 돼지내재레트로바이러스에 감염된 사람세포주 클론의 특성 분석

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Background : Porcine endogenous retroviruses (PERVs) form part of the chromosomes of all pigs. Since they can be produced as infectious virion and infect human cells, safety issues on PERVs infection to human are still controversial and is one of main hurdles of xenotransplantation using pig cells or organs. It has been reported that the established porcine cell line, PK-15, produces PERVs and can infect the human cell lines. Therefore, clonal analysis on human cell line infected with PERV is a prerequisite to characterize the infectivity to human cells and to investigate the harmfulness of PERVs to human.

Materials and Methods : For the characterization of PERV that originates from porcine cell line, PK-15, full length PERV cloning from genomic DNA of PK-15 was performed and partial sequences of both ends were achieved. Cell clones from human cell line, 293, persistently infected with PERVs from PK-15 were established by the method of limiting dilution. Nested PCR and direct sequencing of PCR products in each clone were carried out so as to confirm the PERV genomes in each clone. The growth rate of each clone was checked using cell counting and 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay, the infectivity by reverse transcriptase (RT) assay, and genetic analysis by karyotyping.

Results : A total of 12 genomic PERV clones could be retrieved; 1 with full length, 4 with defective forms, and others with irrelevant sequences. Intact PERV was thought to be able to infect 293 and the PERV-infected cell clones were selected by limiting dilution. PCR results confirmed that nine cell clones were infected with PERV, and sequence alignment data on PCR products of pol region from PK-15 and human cell clones with PERV showed very similar results. Cell counting and MTT assay for growth kinetics of each clone indicated that two clones showed reduced growth rate. However, it was difficult to verify the effect of PERV infection on the cell growth because of the presence of many genetic alterations in 293 parental cells. No RT activities were detected in the culture supernatant from PERV-infected 293 cell clones.

Conclusion : The sequences of PERVs were detected in human cell clones after PERV infection, but PERV virions could not be detected from the culture supernatant by RT assay.

Key Words : Porcine endogenous retrovirus, PK-15, Xenotransplantation, Xell clone, Genetic analysis

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Introduction

Xenotransplantation using pig cells or organs is one of the solutions to overcome the shortage of donor organs for allotransplantation (1), but the potential risk of diseases by zoonosis has not been clearly solved (2). Porcine endogenous retroviruses (PERVs) are one of the zoonotic microorganisms. PERVs exist in the genomic DNA of all pigs, and three replication-competent subtypes, A, B, and C, have been reported (3-6). Some of them are capable of infecting human cells and their genomes can be integrated into human chromosomes, transcribed, translated, and replicated in a variety of human cells. Therefore, infectivity of PERV to human cells is one of main hurdles of xenotransplantation using pig cells or organs (7) and thus, safety issues on PERV infection to human still remain controversial. It has been revealed that PERVs are spontaneously produced from established porcine kidney cell line, PK-15 (8), and infect human cell lines *in vitro* (9). There have been no reports on the effect of PERV infection on functional alteration at genetic or cellular level after the integration of PERV genome to human cells. Therefore, clonal analysis of human cell line infected with PERV is a prerequisite to characterize the infectivity to human cells and to investigate the harmfulness of PERVs to human.

Methods and Materials

1. Cell lines

Porcine cell line, PK-15 (ATCC CCL-33), and human embryonic kidney cell line, HEK293 (ATCC CRL-1573), were cultured with Dulbecco's Modified Eagle's Medium (DMEM) supplemented with 10% fetal bovine serum and penicillin/streptomycin at 37℃ in 5% CO_2 incubator.

2. Cloning of full length genome

Polymerase chain reaction (PCR) with primer pairs, 5-ACGTGCTAGGAGGATCACAGGCTGC-3 and 5-GTTGTCTAAGTACCATGATCTGGACTGCAC-3 (10) (Cosmo Genetech, Seoul, Korea), was performed to generate a 7.2-kb product in genomic DNA from PK-15. Amplification was carried out in 50 uL of Takara LA Taq polymerase (Takara, Japan) with Gene cycler (Bio-rad, USA). PCR products were gel-purified with QIA quick gel extraction kit (Qiagen, Hilden, Germany), ligated into the Topo XL cloning vector, and introduced into Top10 electrocompetent cells (Invitrogen, USA) following manufacturers manuals. Colonies with vector containing the insert were selected and plasmid DNA was prepared with the Qiagen mini prep kit (Qiagen, Hilden, Germany).

3. Cell cloning

293 cells were infected with the low-speed centrifuged culture supernatant from PK-15 for one day, and detached from culture dish and re-seeded in 96-well culture plates with the amount of 0.5 cells/well. After 10-14 days culture, wells containing only one cell cluster were selected under the inverted microscope observation.

4. Nested PCR

PCR with outer primer pairs, 5-GCATTCAGTACTACTACAAC-3 and 5-ATTGGACAGGAACTAGGATG-3(9) (Cosmo Genetech, Seoul, Korea), was performed to generate a 536-kb product in genomic DNA from PERV infected HEK 293 clones. For nested PCR, 1 μL of outer PCR mixture were used as the template for PCR with inner primer pairs, 5′-GCTACAACCATTAGGAAAACTAAAAG-3′ and 5′-AACCAGGACTGTATATCTTGATCAG-3′(9) (Cosmo Genetech, Seoul, Korea). PCR was carried out using Gene cycler (Bio-rad, USA). Nested PCR was performed as follow; one cycle at 95℃ for 10 minutes, 30 cycles at 95℃ for 30 seconds, 55℃ for 30 seconds and 72℃ for 45 seconds, and one cycle at 72℃ for 5 minutes.

5. Sequencing

The sequences of the cloned full-length PERV DNA from pre-selected colonies were obtained by using M13 forward and reverse sequencing primer with commercial DNA sequencing services (Cosmo Genetech, Seoul, Korea).

6. Karyotyping

Each cloned cells were cultured with DMEM supplemented with 10% fetal bovine serum, treated with 75 mM KCl for 30 minutes at 37℃, spread onto clean slide glass,

and dried in the dry oven for 30 minutes at 90℃. The slides were stained with Giemsa and observed under light microscope. The cytogenetic properties were analysed according to guidelines of Hamchoon Institute of Fertility & Genetics.

7. MTT assay

Same numbers of each cloned cells were seeded and cultured with DMEM supplemented with 10% fetal bovine serum and penicillin/streptomycin in 96well plates. They were treated with thiazolyl blue tetrazolium bromide (Sigma, USA) at day 0, 1, 2, 3, 4, 5, and 6. After incubation at 37℃ for 4 hours, clarification was done by centrifugation and dimethyl sulfoxide (DMSO) was added to dissolve insoluble purple formazan product. Color change was measured at 540 nm by VICTOR3 (PerkinElmer, USA) and the viability of each clone was calculated as compared with the control cells.

8. Reverse transcriptase (RT) assay with real time PCR

The culture supernatants from each cloned cells were harvested and cleared by centrifugation at 10,000×g for 5 minutes and filtered through 0.45 mm filters to remove cells and debris. 1% Triton ×100 was added to each supernatant and freezing-thawing was repeated twice. MS2 phage RNA, reverse primer, RNA inhibitor, and reaction buffers (Roche, Germany) were added to each supernatant or MMLV RT (Invitrogen, USA) for positive control. Mixtures were incubated for 30 minutes at 37℃ for cDNA synthesis. Realtime PCR with primer pairs and probe, 5′-CTCGGGTTTCCGTCTTGCT-3, 5′-TTTCACCTCCAG TATGGAACCA-3′ and 5′FAM-AACGCAAGTTCTTC AGCGAAAAGCACG-3′TAMRA (Bionics, Seoul, Korea), was performed to measure of reverse transcriptase activity by ABI Prism 7900HT (AppliedBiosystems, USA). The initial denaturation step was 2 minutes at 95℃, followed by 40 cycles of 10 s at 95℃, 10 s at 55℃, and 10 s at 72℃.

Results

1. Molecular cloning of full length genomic DNA of PERV in PK-15

Both end sequencing of cloned full-length PERV DNA from pre-selected twelve clones was performed by using M13 forward and reverse sequencing primer. Considering the sequences and the size of inserted PERV genome on agarose gel, one clone was presumed to contain the full-length PERV genome, 4 clones with defective genomes, and others with irrelevant sequences. In clones with defective genomes, the deletion patterns were categorized into 2 groups according to the deleted site in genome (Fig. 1). One group had defective genes in *pol* and *env* region in clone 5 and 7, and the other in *gag* and pol region in clone 4 and 9.

2. Cell cloning of 293 cells infected with PERV from PK-15

After infecting with PERV, 293 cells were cloned by limiting dilution. Cell clones containing PERV-specific genes were screened with *pol* amplification by PCR. Nine cell clones were confirmed to be infected with PERV by nested PCR (Fig. 2A). The sequences of *pol* region in PCR products were acquired in 7 human cell clones containing PERV. Alignment data showed that sequences in JK3, JK4, JK5, and JK7 were the same with the reference se-

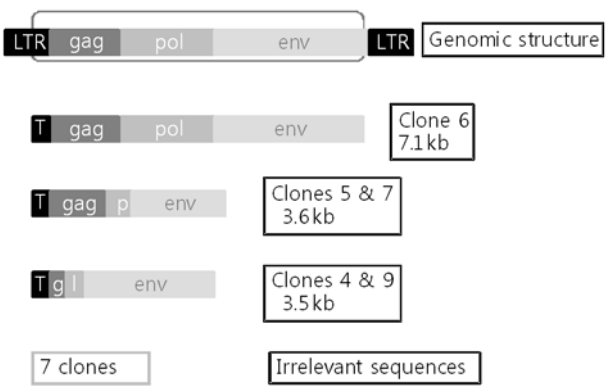

Figure 1. Schematic construct of full length PERV genomic clones from PK-15 determined by partial sequencing of both ends. The sequencing of PERV DNA in Topo XL vector was performed by using M13 forward and reverse sequencing primer. Characters such as g, p, and l stand for the partial sequences of *gag, pol* and *pol*, respectively. LTR: long terminal repeat.

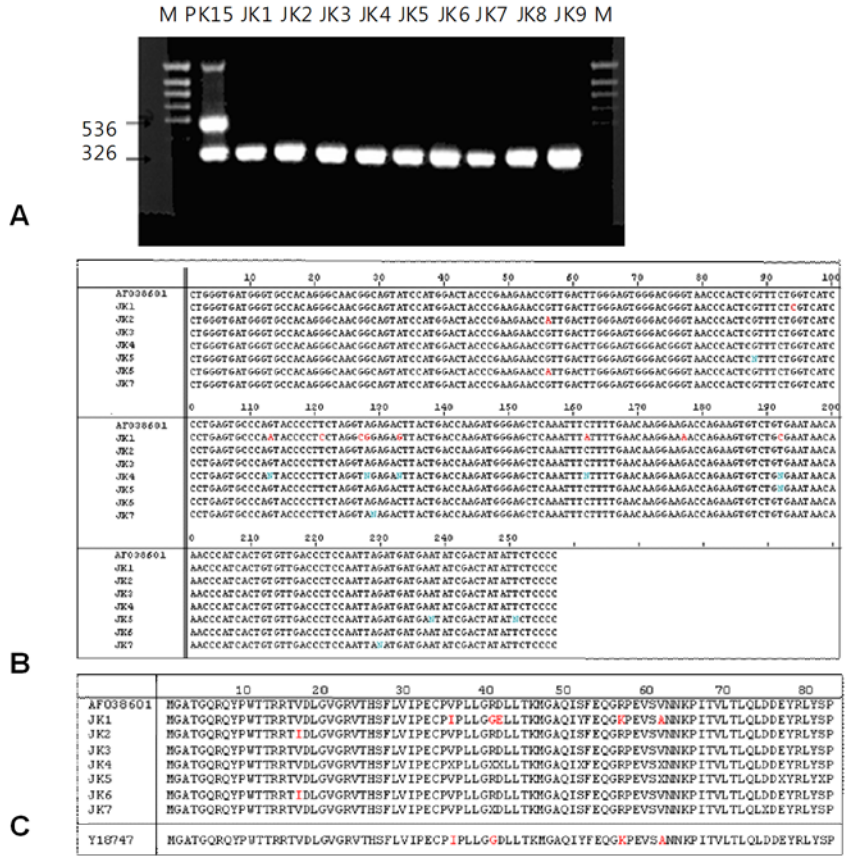

Figure 2. Identification of PERV *pol* in PERV PK-15-infected 293 clones. (A) Nested PCR results of *pol* region on agarose gel. (B) Alignment of nucleotide sequences by direct sequencing of PCR products. (C) Alignment of amino acids translated from nucleotide sequences of (B).

quence of PK-15 (GenBank access No. AF038601) among 257 nucleotides. Only 1 nucleotide in JK2 and JK6, and 9 nucleotides in JK1 were different from the reference sequence (Fig. 2B). Translated amino acid sequences of JK1 were different in 5 positions from the reference sequence while those of JK2 and JK6 were different only in one position (Fig. 2C).

3. Karyotyping and genetic analysis

Karyotypes for 293 cell and PERV-infected clones revealed 3n with three X chromosomes and were denoted 69, XXX (Fig. 3A and 3B). The number of chromosomes varied from 64 to 69. However, their structural abnormality was similar (Table 1). Genetic alteration was different from clone to clone.

4. Growth kinetics of PERV-infected 293 clones

Cell counting and MTT assay of each clone indicated that the growth rate of JK2 and JK3 was different from that of 293 parental cells ($P<0.05$) while that of the others showed no difference (Table 2).

5. Measurement of virion in culture supernatant by RT assay

RT activities were not detected in the culture supernatants from PERV-infected human cell clones by ultrasensitive RT assay, which demonstrated the absence of PERV virions.

Discussion

Nine 293 cell clones containing PERV genome were established by PERV infection and limiting dilution, and

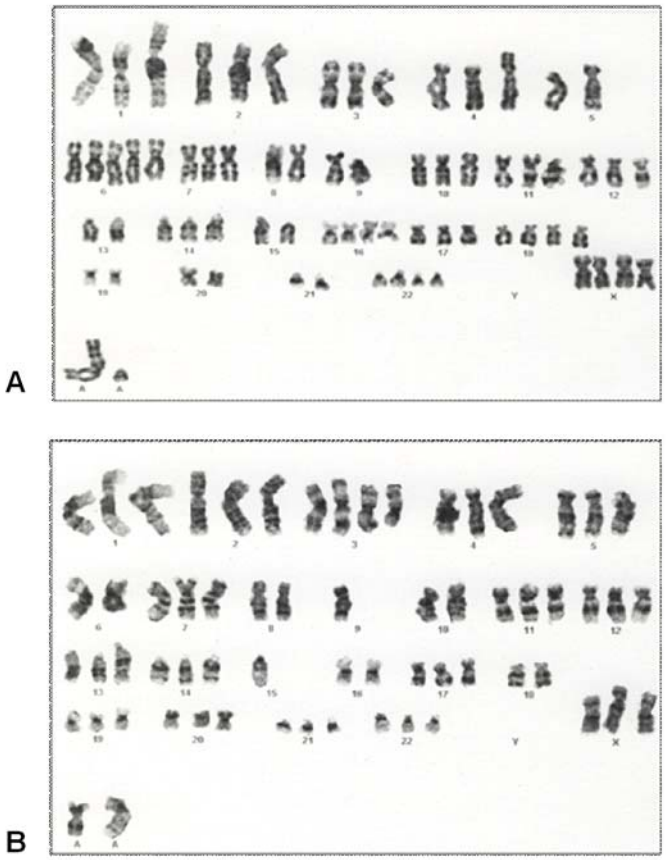

Figure 3. Karyotypes for 293 and PERV PK-15-infected 293 clone. Samples were stained with Giemsa after treated with trypsin and observed under light microscope and analysed in the cytogenetic properties. (A) karyotype for 293, (B) karyotype for representative PERV PK-15-infected 293 clone among nine clones. Data showed that karyotype for clone contained 3n with three X chromosomes.

the characteristics of clones were analysed at cellular and molecular level. Analysis of cloned full length PERV genomes in PK-15 revealed that four among five clones consisted of defective genes in *gag* or *pol* (Fig. 1). These findings explain that copy numbers of *gag* and *pol* are not 1:1 in PK-15 cells and DNA from pig cells (11). However, the proportion of non-defective PERV genome present in pig cells has not been reported yet. The mechanism of obtaining defective genes in pig cells would be recombination at genomic level or RNA splicing at RNA level, but the exact explanation should be elucidated in the future experiments.

Direct sequencing of PCR products from 7 clones showed that 0 to 9 nucleotides among 257 nucleotides were different from reference PK-15 PERV sequences (GenBank accession No. AF038601), which ruled out the possibility of cross-contamination of PCR products during experimental procedure and confirmed the presence of PERV genome in each cell clone. When the nucleotide sequences were translated into amino acids, one amino acid was different from PK-15 reference (valine(V) to isoleucine(I)) in JK2 and JK6, while five in JK1. However, JK1 had the same amino acid sequences except in one position as compared with other reference sequence (GenBank accession No. Y18747) (Fig. 2C). It is not a surprising result because different PERV subtypes or sequences are commonly present even in a single pig cell.

Presence of complete virions in the culture supernatant from the cell clones containing PERV genome was tested with ultra-sensitive RT assay. This assay can detect 10^{-2} RT unit in the standard control sample, the sensitivity of which is higher than the conventional RT assays. RT

Table 1. Genetic Analysis of PERV PK-15-infected 293 Cell Clones

Clone	Genetic analysis
293	69, XXXX, der(1)t(1;?)(1q?;?),?i(1),der(4)t(4;?)(p?;?),−5,+6,−8,−9,−13, −15,+16,+18+−19,−20,−21,−22,+2mar
Clone JK1	69, X, add(X)(q?),der(1)t(1;?)(1q?;?),der(1)t(1;?)(1q?;?),?i(1)(1p?), −2,der(2)t(2;?)(q?;?),+3,+3,+3,der(3)t(3;?),der(4)t(4;?)(p?;?),+6,−7,−8,−9,−10, −13,der(13)t(13;?)(q?;?),add(13)(?p),−15,−18,+4mar
Clone JK2	66, X, add(X)(q?),der(1)t(1;?)(1q?;?),−2,del(3)(q?),der(4)(4;?)(p?;?),−6,−8,−10,−14, −16,−18,t(20;?13)(p?;q?),+5mar
Clone JK3	63, XX, add(X)(q?),der(1)t(1;?)(1q?;?),der(1)t(1;?)(1q?;?),+del(3)(q?), der(4)t(4;?)(p?;?),−6,−8,−9,−9,−10,der(13)t(13;?)(q?;?),−15,−15,−16,−18,+2mar
Clone JK4	66, XXX, add(X)(q?),der(1)t(1;?)(1q?;?),der(1)t(1;?)(1q?;?), −2,del(3)(q?),der(4)t(4;?)(p?;?),−6,der(8)t(8;?)(p?;?),−10,+der(11)t(11;?)(?p;?), −13,der(13)t(13;?)(q?;?),−15,−18,−19,+t(20;?13)(p?;q?),−21,+2mar 66, XX, del(1)(q?),der(1)t(1;?)(1q?;?),−2,+der(3)(q?),+der(3)t(3;?)(p?;?), −5,del(7)(q?),der(8)t(8;?)(p?;?),−9,−11,−13,der(13)t(13;?)(q?;?), +14,der(16)(16;?15)(q;p?),−18,+t(20;?13)(p?;q?),+2mar
Clone JK5	NA*
Clone JK6	NA
Clone JK7	68, XXX, der(X)t(X;?)(p?;?),der(1)t(1;?)(1q?;?),der(1)t(1;?)(p?;?), +3,der(4)t(4;?)(p?;?),−5,−8,+9?,−10,−13,der(13)t(13;?)(q?;?),−15,+16,+16,−18, −19,−19,+3mar
Clone JK8	67, XXX, der(1)t(1;?)(1q?;?),der(1)t(1;?)(p?;?),der(1)t(1;?)(1q?;?),+1,+3,der(4)t(4;?)(p?;?), der(7)t(7;?)(q?;?),der(8)t(8;?)(p?;?),−10,−13,der(13)t(13;?)(q?;?), −15,der(15)t(15;?)(?p;?),−18,−20,der(22)t(22;?)(?p;?),+2mar
Clone JK9	65, XXX, der(1)t(1;?)(1q?;?),der(1)t(1;?)(p?;?),der(1)t(1;?)(1q?;?),+1,del(3)(q?), der(4)t(4;?)(p?;?),der(8)t(8;?)(p?;?),−9,−10,−13,der(13)t(13;?)(q?;?),−15, −18,t(20;?13)(p?;q?),−21,+mar

*NA : not analysed

Table 2. Growth Kinetics of 293 and PERV PK-15-infected 293 Clones*

		Time of culture (days)						*P* value
		1	2	3	4	5	6	
	HEK293	1.00	5.31±4.68	8.02±4.98	16.91±8.89	28.43±0.73	34.05±0.42	
	1	1.00	3.65±2.34	5.92±4.06	14.94±12.01	21.23±20.24	19.24±17.53	0.10
	2	1.00	2.30±0.37	4.52±0.13	12.24±3.09	22.75±2.28	21.63±0.40	<0.05
	3	1.00	2.56±1.48	5.49±0.42	10.78±5.20	24.17±20.87	28.48±23.39	<0.05
PERV	4	1.00	3.07±2.11	6.44±0.83	17.90±12.81	27.54±17.39	33.40±14.64	0.18
PK-15-infected	5	1.00	4.48±1.23	6.45±1.04	16.38±1.76	20.97±2.16	21.81±5.39	0.12
293 clone	6	1.00	3.91±3.56	8.66±0.80	18.13±6.55	34.17±23.23	50.62±16.79	0.22
	7	1.00	6.70±2.40	9.28±1.28	11.65±4.63	23.70±2.63	25.48±5.16	0.18
	8	1.00	5.63±3.11	8.80±8.56	26.71±5.74	41.27±1.68	54.67±4.40	0.09
	9	1.00	3.15±0.22	7.20±0.23	10.24±3.36	11.72±1.89	14.47±0.56	0.08

*MTT assay was performed as described in Materials and Methods. The value was converted to the relative change to that of day 1 and expressed as mean±standard deviation. *P* value was determined by *t*-test between 293 and the PERV-infected 293 clones. A *P* value of <0.05 was considered statistically significant.

activities were detected in the culture supernatant from PK-15 cells, but not from other cell clones. These results suggest that PERV derived from PK-15 can infect 293 human cells but hardly replicate and produce PERV effectively, which is a different result from other report (12). This difference may be due to different culture environment such as different passage numbers and cell line sources, or the presence of increased number of replication-defective PERV genomes in our cell line compared to that from others.

Since the growth rate measurement had limitation in cell counting with hemocytometer and the resultant seeding of cells in 96 wells was presumed to vary in each experiment, MTT results were expressed as the cell growth ratio of the relative changes compared to value obtained at day one (Table 2). The comparison of cell growth ratio in each clone with t-test showed that two clones had P value of <0.05. However, it cannot be said that the growth rate in each cell clone containing PERV genome is really different, because the original 293 cells consisted of heterogeneous cell population and already had many genetic abnormalities (Table 1). To verify the exact effect of PERV infection on cell growth, the normal cells should be included instead of the transformed or genetically altered cells.

Although infectious PERVs were not detected in cell clones, partial DNA sequences of PERV *pol* in cell clones were present and were of almost the same sequences as those of PK-15. This means that they could be produced in specific conditions such as immune suppression or stimulation by certain chemical agents (10, 13-15) if whole PERV genomes are present in clones. There also is a probability that a large portion of PERVs with defective genomes from PK-15 can infect human cells but not replicate effectively in human cells. Although our experiment showed that the presence of PERV genome in human cells did not have any effect on phenotypical growth rate, we cannot rule out the possibility that changes might have occur at genetic level. Further studies on PERV are required to analyse the infectivity and the reactivation in normal human cells, which would provide valuable information on establishing the harmfulness of PERV to human.

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