Microarray analysis of canine papillomavirus lesions

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Abstract: Papillomaviren verursachen eine breite Palette von Krankheitsbildern bei Mensch und Tier, welche von asymptomatischen Infektionen, gutartigen Warzen, pigmentierten Plaques, in situ Karzinomen bis zu invasiven Plattenepithelkarzinomen reichen. Bei Hunden sind die gesamten genomischen Sequenzen von dreizehn verschiedenen Papillomaviren beschrieben, die mit unterschiedlichen klinischen Erscheinungsbildern verbunden sind. Allerdings können weder die phylogenetische Klassifizierung eines Hundepapillomavirus (CPV) noch das Vorhandensein von unterschiedlichen Genregionen genau den Verlauf einer Infektion auf individueller Ebene vorhersagen. In der vorliegenden Studie führten wir eine Microarray-Analyse von CPV assoziierten Läsionen (CPV1, CPV3, CPV5) durch. Die Expression von sechsundneunzig Genen war signifikant mindestens 4-fach rauf oder runter reguliert. Weiter haben wir die RNA Levels von KLK8, MCM5, ORC1 und PAX6, die als Biomarker in der Humanmedizin diskutiert werden in Papillomavirus Läsionen untersucht. Wir konnten durch qRT-PCR die erhöhte Expression dieser Gene bestätigen. Papillomaviruses induce a wide range of clinical conditions in humans and animals, ranging from asymptomatic infection, benign warts, pigmented plaques to in situ carcinoma and invasive squamous cell carcinomas. In dogs, entire genomic sequences of thirteen different papillomaviruses are described so far and that appear to be associated with different clinical presentations. However, neither the phylogenetic classification of a canine papillomavirus (CPV) nor the presence of distinct motifs on the genome can unanimously predict the fate of an infection on the individual level. In the present study we performed a microarray analysis of CPV associated lesions (CPV1, CPV3, CPV5). Ninety-six genes were at least 4 fold up or down-regulated on a significant level. We further investigated in CPV lesions, the RNA levels of KLK8, MCM5, ORC1, and PAX6, which are discussed as biomarkers in the human medicine. We could confirm the up regulation of these genes by qRT PCR.

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Arbeit unter wissenschaftlicher Betreuung von
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Microarray analysis of canine papillomavirus lesions

Inaugural- Dissertation

zur Erlangung der Doktorwürde der
Vetsuisse-Fakultät Universität Zürich

vorgelegt von

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Zürich 2013

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Abstract

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In the present study we performed a microarray analysis of CPV associated lesions (CPV1, CPV3, CPV5). Ninety-six genes were at least 4 fold up or down-regulated on a significant level. We further investigated in CPV lesions, the RNA levels of KLK8, MCM5, ORC1, and PAX6, which are discussed as biomarkers in the human medicine. We could confirm the up regulation of these genes by qRT PCR.

Zusammenfassung

Papillomaviren verursachen eine breite Palette von Krankheitsbildern bei Mensch und Tier, welche von asymptomatischen Infektionen, gutartigen Warzen, pigmentierten Plaques, in situ Karzinomen bis zu invasiven Plattenepithelkarzinomen reichen. Bei Hunden sind die gesamten genomischen Sequenzen von dreizehn verschiedenen Papillomaviren beschrieben, die mit unterschiedlichen klinischen Erscheinungsbildern verbunden sind. Allerdings können weder die phylogenetische Klassifizierung eines Hundepapillomavirus (CPV) noch das Vorhandensein von unterschiedlichen Genregionen genau den Verlauf einer Infektion auf individueller Ebene vorhersagen. In der vorliegenden Studie führten wir eine Microarray-Analyse von CPV assoziierten Läsionen (CPV1, CPV3, CPV5) durch. Die Expression von sechsundneunzig Genen war signifikant mindestens 4-fach rauf oder runter reguliert. Weiter haben wir die RNA Levels von KLK8, MCM5, ORC1 und PAX6, die als Biomarker in der Humanmedizin diskutiert werden in Papillomavirus Läsionen untersucht. Wir konnten durch qRT-PCR die erhöhte Expression dieser Gene bestätigen.

Microarray analysis of canine papillomavirus lesions

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Submitted to the *American Journal of Veterinary Research*

Presented in abstract form at the 2011 ECVD/ESVD annual congress, Brussels, Belgium, Septembre 2011

This study was funded by Clinomics, Vetsuisse Faculty, Switzerland and Krebsliga, Switzerland.

The authors thank their colleagues from the Small Animal Department (Vetsiusse Faculty, University of Zurich, Switzerland) for help in sampling, the members of the Functional Genomic Center (ETH/University of Zurich, Switzerland), especially Jelena Kühn Georgijev, Tanguy le Carrour, and Hubert Rehrauer, for technical support, and the colleagues from the Institute of Virology (Vetsiusse Faculty, University of Zurich, Switzerland) for technical assistance.

Abbreviations:

bp	Base pair
cDNA	Complementary deoxyribonucleic acid
CPV	Canine papillomavirus
cRNA	Complementary ribonucleic acid
DNA	Deoxyribonucleic acid
FGCZ	Functional Genomic Center Zurich
GAPDH	Glyceraldehyde 3-phosphate dehydrogenase
KLK8	Kallikrein related peptidase 8
MCM5	Minichromosome maintenance protein 5
mRNA	Messenger ribonucleic acid
MUSTN1	Musculoskeletal, embryonic nuclear protein 1
ORC1	Origin of recognition complex 1
PAX6	Paired box gene 6
PVs	Papillomaviruses
qRT PCR	Quantitative real time polymerase chain reaction
RNA	Ribonucleic acid
RT	Reverse transcription

Abstract

Objective – To detect genetic biomarkers for outcome of papillomavirus infections of dogs.

Animals – 3 healthy adult dogs, 5 CPV affected dogs.

Procedures - In the present study we performed a microarray analysis of CPV associated lesions (CPV1, CPV3, CPV5).

Results - Ninety-six genes were at least 4 fold up or down-regulated on a significant level. We further investigated the RNA levels of KLK8, MCM5, ORC1, and PAX6, which are discussed as biomarkers in the human medicine. We could confirm the up regulation of these genes by qRT PCR.

Conclusions and Clinical Relevance - Furthermore, enrichment analysis were performed and revealed pronounced dysregulation of genes whose products being part of the cell cycle regulation.

Introduction

Papillomaviruses (PVs) are associated with a wide range of clinical conditions, varying from mild benign symptoms to cancer, but also with asymptomatic infections.[1] To date, the genomic sequences of more than a dozen different canine PVs (CPVs) have been uncovered and partially characterized.[2–16] Their phylogenetical classification correlates at least partially with the observed clinical conditions. For example, it appears that the classical benign oral papillomatosis is induced almost exclusively by a PV belonging to the Lambda Genus (CPV1) while pigmented plaques have thus far only been found associated with PVs of the Chi-Genus (CPVs 3,4,5,8,9,10,11,12,14). However, the infected host is likely to

contribute as much as the virus itself to a potentially malignant outcome of these infections. Yet, host responses towards the various CPVs have not yet been studied to great detail. Since PVs generally do not replicate in cell cultures, it is difficult to study the details of these infections in the context of experimental inoculations. Therefore, we set out to carry out such studies by making use of material obtained from rare clinical cases, which could be traced back to one of those viruses. Specifically, we analysed by microarray CPV1-, CPV3-, and CPV5-associated lesions as well as normal skin from clinically healthy dogs. First, viral mRNA expression and second, the patterns of cellular mRNA expression patterns were addressed. As a result, we were able to describe for the first time individual cases of the host response in lesions associated to these three CPVs. By comparing the various patterns, we detected sets of genes that may be suitable to distinguish the three viruses as well as patterns that are common to either pairs of the viruses or even to all three types of CPVs. Accordingly, we came up with a set of three genes, whose expression levels may be able to provide clinical markers for malignancy.

Material and Methods

Cases

Biopsy samples were collected from dogs infected by CPV1, CPV3 or-, CPV5 as well as from healthy dogs. Healthy dogs were defined as clinically free of any skin lesions and negative in PV-PCR (see below). Samples infected by CPV1 derived from mucous membrane and skin warts collected in three dogs. CPV3 samples were taken from one dog with viral plaques and virus-harbouring squamous cell carcinomas. CPV5 samples were taken from one dog with viral plaques and virus-

harbouring squamous cell carcinoma in situ. More details about these clinical cases have been provided elsewhere.[5, 8]

Sampling

Biopsy samples (0.5g – 2g) of lesional or clinically healthy tissue were collected and were immediately stored at -80°C until further processing. Three control samples were taken from freshly euthanized dogs with macroscopic healthy skin and were stored in an RNA storage buffer [a] according to the manufacturer's protocol. The absence of CPV DNA was confirmed by PCR using primer combinations FAP59/FAP64, CP4/CP5 and canPV/FAP64 (Table 1).[11,17,18]

RNA and DNA Extractions

A half pinhead sized piece of tissue was homogenized in liquid nitrogen using mortar and pestle. Total RNA was then extracted from the homogenate with a commercial RNA extraction kit [b] according to the manufacturer`s instructions. Extracted RNA was eluted in 50 µl RNase free water and to get rid of putative remnant DNA additionally digested using 1µl recombinant, RNase-free DNase I [c]. After incubation for 15 minutes at 37° C the DNase was heat-inactivated at 75° C for 10 minutes. The RNA was stored at -80° C after the concentration was measured [d]. DNA was extracted from a half pinhead sized piece of healthy skin using a commercial extraction kit [e] according the manufacturer`s instructions. The DNA was eluted in 200 µl and the concentration was measured.

RNA and DNA amplification

Reverse transcription. The RNA was converted to cDNA with reverse transcriptase [f] using random primers according to the manufacturers instructions.

PCR: PCR was performed with a ready PCR mix [g]. Primers are shown in Table 1. PCR was implemented in a total volume of 25 µl, containing 12µl from the PCR mix, 8

µl water, 2 µl of each forward and reverse primer (10µM each), with 1µl extracted DNA or cDNA as template. In case of all PV-type-specific primer pairs, the PCR program started with a denaturation for 3 min at 94°C, followed by 40 cycles of 30 s at 94°C, 30 s at 50°C and 30 s at 72°C. The PCR program for canPVf/FAP64 primers started with a denaturation step for 5 min at 94°C, followed by 45 cycles of 60 s at 94°C, 60 s at 50°C, 60 s at 72°C and a final elongation for 5 min at 72°C. The PCR program for CP4/CP5 and FAP59/FAP64 primers started with a denaturation step for 3 min at 94°C, followed by 40 cycles of 30 s at 94°C, 30 s at 42°C and 30 s at 72°C. PCR products were loaded onto 1% agarose gels containing ethidiumbromid and analyzed after 45 minutes in an electric field of 5 V cm^{-1} in TAE buffer.

Agilent GE Microarray Experiment Description

The custom arrays were designed [h] based upon 42034 probe sets of *Canis lupus familiaris* Agilent standard array (44k x 4 chip format). Additional groups of probesets were created by tiling the sequences of CPV1, CPV3, CPV5 and GFP every 30bp, what in the case of Agilent 60bp probe results in double coverage of every fragment of the viral genome. Consequently all changes in expression may be traced with the precision down to 30bp (Agilent custom design ID:028307).

The quality of the isolated RNA was determined [i]. Only samples with a 260 nm/280 nm ratio between 1.8–2.1 and a 28S/18S ratio within 1.5–2 were further processed. Total RNA samples (100ng) were reverse-transcribed into double-stranded cDNA in presence of RNA poly-A controls [j]. The double-stranded cDNAs were in vitro transcribed in presence of Cy3-labelled nucleotides using a commercial kit [k]. The Cy3- cRNA was purified using a commercial kit [l] and its quality and quantity was determined. Only cRNA samples with a total cRNA yield higher than 2 µg and a dye incorporation rate between 8 pmol/ µg and 20 pmol/ µg were considered for hybridization.

Cy3-labeled cRNA samples (1.65 µg) were mixed with a Agilent Blocking Solution, subsequently randomly fragmented to 100-200 bps at 65°C with Fragmentation Buffer, and resuspended in Hybridization Buffer using a commercial kit [m].
Target cRNA Samples (100µl) were hybridized to the *Canis familiaris* Agilent standard array for 17h at 65°C. Arrays were then washed using Agilent GE Wash Buffers 1 and 2 according to the manufacturer instructions [n]. Fluorescent intensity emitted by the labeled target was measured [o] and raw data processing was performed [p]. Quality control measures were considered before performing the statistical analysis. These included, inspection of the array hybridization pattern (absence of scratches, bubbles, areas of non-hybridization), proper grid alignment, performance of the spike-in controls (linear dynamic range between 5 orders of magnitude) and number of green feature non uniformity outliers (below 100 for all samples)
Initial experimental design included three replicates of CPV1, CPV3, CPV5 and control samples, allowing the margin of safety for the statistical power of the analyses. Thus 3 chips in the 44k x4 format were needed. After scanning, the quality control was performed, including scatterplots of all the arrays against the mean of their treatment groups, expression value histograms for arrays, correlation plots of samples, hierarchical clustering of samples (Pearson correlation as the similarity measure, Ward distance to cluster) and heatmaps of top probes. A pairwise comparison from CPV1, CPV3 and CPV5 versus control was done by t-test and fold change. Afterwards a one-way ANOVA with F-test was applied. All the quality controls described above and initial analyses have been performed using lab management system of the FGCZ [q]. Further analyses of the viral genome expression has been performed by creating separate datasets for the custom probes, setting

them in order as in the original genome and checking the differences between treatments and plotting them according to the viral genome coordinates.

Affected pathways

Genes that were identified to be up- or down-regulated by the microarray experiments were subsequently screened. Basically, all genes with at least a four-fold up- or down-regulation in expression compared to the controls were checked to determine if they might be relevant during CPV infections. Furthermore, it was evaluated whether these genes belong to known cellular pathway maps, process networks, and GO processes. For this purpose, the canine annotation was transformed into the human annotation. The 13489 transformed annotated genes were filtered by 1.74 ($2^{0.8}$)-fold up- or down-regulation and P-Values of 0.1 and then used [r] to identify enriched pattern in the data sets.

Quantitative RT-PCR

Genes that were identified to be up- or down-regulated by the microarray experiments were subsequently screened [s] for their relevance in causing cancer. All genes with at least a four-fold up- or down-regulation in expression compared to the controls were checked. Some potential tumor markers were identified. Consequently a quantitative RT-PCR (qRT-PCR) was applied to estimate the expression level of the cellular transcripts of MCM5, ORCI, PAX6, KLK8 and GAPDH. The primers (Table 1) were designed [t]. The amplimers of RT-PCR using the newly designed primers were cloned into a plasmid [u] according to the manufacturers instructions. Sensitivity and specificity of the primers were evaluated by standard curves on serial dilutions of the cloned templates. (Figure 1) The quantitative PCR was performed using a real time PCR detection system [v] according to the manufacturers instructions. In brief, qRT PCR was implemented as duplicates in a total volume of 20 µl, containing 10 µl detection mix [w], 0.6 µl Primer forward, 0.6 µl Primer reverse, 3.8

µl sterile water and 5 µl template. A two step protocol was applied. The following cycles were run:(i) 7 min at 95°C, (ii) 41 cycles of 10s at 95°C and 30s at 60°C, (iii) 95°C for 1 min and (iv) 55°C for 1 min. Increase setpoint temperature after cycle 2 by 0.5°C. A relative quantification was performed with the host gene GAPDH compared to healthy skin with the following formula: $\Delta\Delta Ct = (Ct_{sample} - Ct_{GAPDH\_sample}) - (Ct_{mock} - Ct_{GAPDH\_mock})$ Calculations of statistic significance between the gene expression of particular samples were performed [x] using t-test.

Results

Microarray analysis

Biopsy samples from CPV-positive and CPV-negative tissues (see cases in M+M) were used to evaluate by Microarray analysis (1) the relative RNA expression of CPV1, CPV3, and CPV5 as well as (2) the corresponding status of host gene expression. To estimate the viral RNA transcription, tiling probes representing in an overlapping manner the entire genomes of CPV1, CPV3, and CPV5 were included in the arrays as custom probes. The expression profiles along the viral genomes are shown in Figure 2. Indeed, signals corresponding to these probes, were elevated in virus infected tissue samples, whereas the signals of the healthy skin samples remained on background levels. Interestingly, the signal levels peaked in the proximity of the predicted polyadenylation signals on the viral genomes, suggesting that both early and late viral gene expression took place in the infected tissue samples. Based on these results, we conclude that the viruses were active in the affected tissues and not merely present.

The expression of the host genes was not measured along the entire RNA molecules but on the basis of a few representative sequences that had been previously

optimized for that purpose. Of note, the expression levels of several cellular transcripts were altered in virus-infected tissue compared to healthy skin. Furthermore, differences were detected between the different virus infected samples. In CPV1 infected samples 21 genes were at least four fold up- and 15 genes down-regulated compared to control samples. Of these genes, 18 were only up- regulated in CPV1 infected samples (Table 2; Figure 3a). Samples of the CPV3 infected dog showed an at least four fold higher expression of 13 genes and a lower expression of 17 genes compared to the control samples whereby 10 genes were up-regulated and 9 down-regulated only in CPV3 samples (Table 3; Figure 3a). Samples of the CPV5 infected dog showed the highest number of at least four fold up- or down-regulated genes. Thirty-one genes were up and 16 genes down-regulated with 28 of these genes beeing up- and 12 genes down-regulated only in CPV5 samples (Table 4; Figure 3a). Carbonic anhydrase II was up-regulated in both, CPV1 and CPV5 and diacylglycerol lipase beta in both, CPV3 and CPV5 (Table 5; Figure 3a). In case of CPV1 and CPV3 samples, 4 identical genes were down and one was up regulated (Table 5a). Only one gene (KLK8) was up- and 4 genes (MUSTN1, CCRL1, VIT, MT3) down-regulated in all samples infected with one of the three examined viruses (Table 5d; Figure 3a).

Enrichment analysis of the genes was conducted in order to identify pathway maps, process networks, and GO processes affected by viral infections (Table 6). Genes relevant in cell cycle regulation, DNA repair, and proteolysis were enriched in the individual data sets. Interestingly, the enrichment was more pronounced in samples from CPV5 than from CPV3 and only marginally from CPV1. Between 40% and more than 60% of genes assigned to the most enriched pathway maps, especially those associated with cell cycle regulation and DNA repair were differently expressed by CPV5 infection, but only 10% to 45% by CPV3 and 5% to 15% by CPV1 infection.

The genes assigned to different process networks were 30% to 50% differently expressed in CPV5 infected tissue but only 5% to 20% in CPV3 and around 5% in CPV1 infected tissue. A similar percentage of genes assigned to GO processes were enriched for the virus-infected tissue. Of note, the p-values for the CPV5 sample enriched genes were highly significant (10E-6 to 10E-44) and most significant (10E-1 to 10E-30) for CPV3 sample enriched genes. These results suggest that cell cycle regulation and DNA repair are deeply affected by CPV5 infection and markedly affected by CPV3 infection: On the contrary, CPV1 infection does not seem to markedly affect these pathways.

qRT PCR for potential diagnostic markers

Among the up-regulated genes in the samples were KLK8, MCM5, ORC1, and PAX6. These might serve as potential biomarker in dogs like, according to the literature, in humans. The kallikrein related peptidase 8 (KLK8) is a serine protease and is expressed in the extracellular matrix. The minichromosome maintenance protein 5 (MCM5) and the origin of recognition complex 1 protein (ORC1) play a role in the cell cycle. The paired box gene 6 (PAX6) product is a transcription factor in embryogenesis.

In order to confirm these up-regulations real time quantitative RT-PCR assay (qRT-PCR) was developed. The similar slopes (Figure 1) indicate similar efficiencies of the qPCR, which allows the use of the $\Delta\Delta$Ct method calculation of relative expression to GAPDH as internal control. The newly developed qRT-PCR was used for evaluation of the expression levels of the marker genes. KLK8 was up - regulated (between 23 and 26 times) in all virus infected samples to a significant level compared to the control samples (Table 7). MCM5 was only significantly up-regulated in CPV3 infected samples (7 times). In CPV5 infected samples MCM5 was up-regulated too (2.9 times), but not to a significant level (Table 7). The ORC1 gene expression was

highly significant up-regulated in CPV3 (6.8 times) and CPV5 (3.2 times) infected samples, while there was no difference between CPV1 and control samples. The expression of the PAX gene was also highly increased in CPV3 samples (34.4) and somewhat higher in CPV5 samples (3.4) compared to control samples. In contrast the PAX6 gene did not differ from the controls in samples from CPV1 infected dogs. (Table 7). In conclusion, the qRT-PCRs confirmed the micro array results.

Discussion

In this study, we have evaluated in a comparative manner the relative transcription levels of three CPVs in infected tissues obtained from natural infections in patients and analyzed the corresponding host gene expression patterns. Due to the scarcity of cases and corresponding rarity of fresh material, we can only describe the data found in a few individual cases. However, in the absence of viruses that can be grown in cell culture, this still may provide a set of reference data, which certainly should be challenged once experimental models become available. Several genes were found to be up- or down-regulated in canine skin affected by CPV1, CPV3 or CPV5 infection (Tables 2 to 5, 7). According the microarray results three genes, which were only up- regulated in CPV3 samples (MCM5, ORC1L, PAX6) were identified (Figure 2). MCM5 plays an essential role in the cell cycle, as it is responsible for the generation of a prereplicative complex during G1 phase. MCM5 protein was previously identified as a potential biomarker for prostate cancer and genito-urinary tract cancer, that can be detected in the urine.[19,20] Furthermore, it has been discussed[21], as a marker for the diagnosis of cervical dysplasia, as it increases proportionally with the severity of the lesions. MCM5 is indirectly up-regulated by the human PV (HPV) E7 Protein. The (HPV) E7 protein binds the retinoblastoma protein

(pRb), it leads to a releasing of inhibition of E2F and this leads to an up-regulation of MCM5.[21] CPV2 E7 is required for degradation of pRb.[22] In other studies, the MCM5 has been described as a diagnostic marker for malignancy in pancreaticobiliary disease and oesophageal cancer by detection in bile or gastric aspirates.[23,24] The ORC1 is involved in the initiation of DNA replication. In a gene expression study of HPV 16 E6 transgenic mice, the ORC1 transcription was up-regulated.[25] It was also shown that in HPV positive head and neck cancers the ORC1 was highly up-regulated compared to HPV negative ones.[26] It might be, that the CPV3 and CPV5 are also responsible for the up-regulation of ORC1 in the dog samples included here. Maybe the ORC1 could be used as a malignancy marker in CPV affected dogs.

The PAX6 is a member of the PAX gene family which encode a set of transcription factors in embryogenesis.[27] Transgenic mice expressing PAX6, cystic adenoma of the pancreas was induced.[28] Enhanced PAX6 expression is associated with carcinomas in the breast and pancreas.[28,29] Interestingly in contrast, PAX6 is also discussed as a marker for a favorable outcome in gliomas and that PAX 6 may have a tumor suppression function in gliomas.[30,31] In our cases the PAX6 gene is up-regulated in CPV3 and CPV5 samples which are rather associated with malignant tumors.

In the samples analyzed here one gene was found to be up-regulated in CPV1, as well as CPV3 and CPV5 infected skin (Table 5d; Figure 3a) This gene, encoding for the Kallikrein related peptidase 8 (KLK8) a serine protease which is responsible for the degradation of fibronectin in the extracellular matrix. It is down-regulated in breast cancer but up-regulated in cervical, colon and ovarian cancer. Therefore, KLK8 and other KLK are discussed as a biomarker for the outcome and detection of various tumors in human medicine.[32–35] KLK8 is also discussed as an early biomarker for the

detection of endometrial carcinomas, as low grade endometrial tumors are associated with a higher expression of KLK8 than high grade ones and normal tissue.[33] KLK8 was found to be down-regulated in HPV positive head and neck cancers compared to HPV negative ones and normal tissue.[36] It might be that the KLK8 is influenced by PV infection. As KLK8 was up-regulated in all our CPV infected samples, we suggest that it might serve as a potential marker for CPV infection in an already existing skin tumor.

In order to reveal which groups of genes were affected by CPV infections, gene enrichment analysis was conducted. These analyses clearly demonstrate that a set of genes, which is involved in the cell cycle regulation, is significant differently expressed in CPV5 infected tissue, less significant in CPV3 and only marginally significant in CPV1 infected tissue compared to control tissue. However, due to the low number of samples covered in this study, a sound biological conclusion could not be drawn. It may be worthwhile to test the potential usefulness of these markers in future clinical studies.

In summary, we have identified three potential markers, KLK8, MCM5 and ORC1 of cancer transformation in CPV infected tissues. Further studies are warranted to analyze more samples and to check whether the expression of the same or similar genes is also modified in further affected dogs.

Footnotes

a. RNAlater, Roche, Rotkreuz, Switzerland.
b. NucleoSpin® Total RNA Isolation kit, MACHERY-NAGEL, Düren, Germany.
c. RNase free DNase, Roche, Rotkreuz, Switzerland.

d. Nanodrop 1000 Photospectrometer, NanoDrop Technologies, Wilmington, DE.

e. QIAamp® DNA Mini Kit, QIAGEN, Germantown, MD.

f. Reverse Transcription (RT) System, Promega, Madison, WI.

g. REDTaq® ReadyMix™ PCR Reaction Mix with $MgCl_2$, SIGMA-ALDRICH, Buchs, Switzerland.

h. Agilent eArray software (https://earray.chem.agilent.com/earray/)

i. Bioanalyzer 2100, Agilent, Basel, Switzerland.

j. RNA Spike-In Kit, One-Color, Agilent, Basel, Switzerland.

k. Low Input Quick Amp Labeling Kit, one-color, Agilent, Basel, Switzerland.

l. RNeasy mini kit, QIAGEN, Germantown, MD.

m. Gene Expression Hybridization Kit, Agilent, Basel, Switzerland.

n. One-Color Microarray-Based Gene Expression Analysis Manual.

o. Agilent Microarray Scanner, Agilent, Basel, Switzerland.

p. Agilent Scan Control and the Agilent Feature Extraction Software Version 10

q. based on R/BioConductor scripts included in the bFabric of FGCZ

r. GeneGO (http://www.genego.com)

s. Wikigenes (http://www.wikigenes.org/)

t. NCBI Primer blast software (http://www.ncbi.nlm.nih.gov/tools/primer-blast/)

u. pCR 2.1-TOPO plasmid, Life Technologies, Zug, Switzerland.

v. iCycler, BIO RAD, Cressier, Switzerland.

w. IQ™ SYBR Green Supermix, BIO RAD, Cressier, Switzerland.

x. STATISTICA, *StatSoft*, Tulsa, OK.

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Figure legends

Figure 1: Standard curves of qPCR Serial dilutions of cloned cDNA were used in qPCR assay. Equation and R^2 values from linear regression analyses are shown.

Figure 2: Viral gene expression in tissue samples. Signal levels of CPV tilling probes representing CPV1 (a), CPV3 (b), and CPV5 (c) in infected tissue samples and control samples are shown with red lines and black lines, respectively. Polyadenylation signals and early and late gene regions are indicated.

Figure 3: Highly significant at least four fold up and down-regulated genes in samples infected with CPV1, CPV3 and CPV5. (a) total amount of up and down-regulated genes in one, two or all three virus affected samples; (b) three possible indicator genes for CPV3 diagnostics (MCM5, ORC1L, PAX6) and one possible indicator gene for CPV infections in general (KLK8).

Tables

Table 1: Primerlist

Primer name	Primer forward 5'-3'	Primer reverse 5´-3
Used for conventional PCR		
CPV1 E1	aga ctg agg atg gtg gtt gg	ggc gtt aag cta atg ctt gc
CPV1 E2	gca ctg gtc att gct cag aa	ccc agg tgt caa act cat cc
CPV1 E6	ttt gct tga tct gtc gct tg	gtt ctt tgt ccg cct cac tc
CPV1 E7	cgc aac cct ttt gga tat tg	cta gcc gcc atg gtc aag
CPV3 E1	agg gag gac aat agg cag gt	ggt tag acg ctc gca tta gc
CPV3 E2	tgc agg aca cca gct atg ag	tgt ggt gct ggt gac aga tt
CPV3 E6	gag tgt ctc tgt gcg cga ta	cac cgc cct ctc act ttt t
CPV3 E7	ttg gga aag acg cta ctt tga	ctc cat ggt caa agc ggt ag
CPV5 E1	agg gag gac aat agg cag gt	taa atg gcc tgg tca act cc
CPV5 E2	ctg tca gat cag gtc cag ca	tcc gtg aaa tac acg tgg tg
CPV5 E6	atg gag cct tgc agg tct ta	gcc ctc tga cct ttt tga at
CPV5 E7	ttg gga aag acg cta ctt tga	aga ccg gac agc aga atg at
canPVf / FAP64 CPV L1[6]	ctt cct gaw cct aay mak ttt gc	ccw ata tcw vhc atn tcn cca tc
FAP59/64 HPV L1[17]	taa cwg tig gic ayc cwt att	ccw ata tcw vhc atn tcn cca tc
CP4 / CP5 HPV E1	atg gta car tgg gca twt ga	gag gyt gca acc aaa amt grc t
Used for quantitative PCR		
ORC1L	aac ggc att tgc tgg gac gga	gcc caa agc tac tac ctg cac c
PAX6	gac aga cac agc cct cac aa	tgc agg agt acg agg agg tc
Dog GAPDH	tct ggc aaa gtg gat att gtc gcc	ct cag cct tga ctg tgc cgt g
MCM5	gct gtc gca aca cgc tta gt	cca cac act tgc act tgt ca
KLK8	gga agc ctg ggc agg aca ct g	agc cgg acg ccc tgg aac aaa g

Table 2: High significant (p<0.01) at least four fold up or down regulated gene expression in only CPV1 affected tissue samples compared to the control.

Identifier	log2 Ratio_CPV1 (fdr_CPV1)	PrimaryAccession	GeneSymbol	GeneName	Description
Up regulated genes					
A_11_P106901	3,434 (0,5339)	ENSCAFT00000036982			
A_11_P058156	3,281 (0,5339)	ENSCAFT00000025757	SERPINE2	serpin peptidase inhibitor clade E	cell adhesion
A_11_P084851	2,983 (0,5339)	ENSCAFT00000004629	AMTN	amelotin	Ameloblast-specific Protein
A_11_P159703	2,605 (0,5339)	TC53629			TTLL9 protein partial (29%)
A_11_P0000018450	2,394 (0,5733)	DN874207			Dog eye cornea
A_11_P0000021406	2,354 (0,5369)	ENSCAFT00000013192	HK2	hexokinase 2	Glycogen synthesis
A_11_P0000033381	2,353 (0,5339)	ENSCAFT00000020046	KCNK2	potassium channel subfamily K member 2	ionic channel
A_11_P0000022037	2,331 (0,5369)	XM_854937	SLC6A11	solute carrier family 6 (neurotransmitter transporter GABA)	neurotransmitter
A_11_P0000023510	2,317 (0,5339)	TC67847			Myosin Va variant (Fragment) partial (18%)
A_11_P132936	2,283 (0,5572)	DR105897			Canine cardiovascular system
A_11_P089701	2,252 (0,5575)	ENSCAFT00000003039	ALDH1L2	aldehyde dehydrogenase 1 family member L2	Probable 10-formyltetrahydrofolate dehydrogenase ALDH1L2
A_11_P0000018162	2,191 (0,5339)	DN872189			Dog eye cornea
A_11_P0000015832	2,15 (0,5339)	DN427137			Canis familiaris cDNA clone CLN10816113
A_11_P102006	2,134 (0,5339)	ENSCAFT00000008863	PFKP	phosphofructokinase platelet	glycolysis
A_11_P159778	2,132 (0,5629)	TC53683			Homer homolog 3 (Drosophila) partial (30%)
A_11_P0000029381	2,108 (0,5339)	ENSCAFT00000007795	PCDH8	protocadherin 8	Cell adhesion
A_11_P0000033238	2,03 (0,5339)	ENSCAFT00000031460			
A_11_P0000026847	2,029 (0,5572)	ENSCAFT00000004939	GGCT	gamma-glutamyl cyclotransferase	glutation metabolism
Down regulated genes					
A_11_P0000023399	-2,194 (0,5563)	ENSCAFT00000021079	TCEA3	transcription elongation factor A (SII) 3	Transcription elongation factor
A_11_P128391	-3,005 (0,5572)	XM_535721	ABI3BP	ABI family member 3 (NESH) binding protein	ABI gene family
A_11_P0000019920	-2,517 (0,5572)	NM_001003202	TJP3	tight junction protein 3 (zona occludens 3)	Tight junction
A_11_P0000021517	-2,516 (0,5339)	DN273224			Canis familiaris cDNA clone LIB30321_003_B03 mRNA sequence
A_11_P0000016997	-2,246 (0,5572)	XM_548082	LOC490959	similar to WNK lysine deficient protein kinase 4	Kinase
A_11_P103441	-2,242 (0,5572)	ENSCAFT00000021079	TCEA3	transcription elongation factor A (SII) 3	Transcription elongation factor
A_11_P0000023757	-2,235 (0,5339)	ENSCAFT00000015999	CMBL	carboxymethylenebutenolidase homolog (Pseudomonas)	Bioactivating hydrolase

Table 3: High significant (p<0.01) at least four fold up or down regulated gene expression in only CPV3 affected tissue samples compared to the control.

Identifier	log2 Ratio_CPV3 (fdr_CPV3)	PrimaryAccession	GeneSymbol	GeneName	Description
Up regulated genes					
A_11_P181438	2,772 (0,2961)	NM_001097544	PAX6	paired box 6	Transcriptionfactor
A_11_P0000027732	2,552 (0,3265)	NM_001097544	PAX6	paired box 6	Transcriptionfactor
A_11_P0000015553	2,509 (0,2261)	DN409432			Canis familiaris cDNA clone CLN9365865
A_11_P0000022344	2,454 (0,02144)	TC69055			Mitochondrial ribosomal protein L16
A_11_P089591	2,367 (0,2043)	ENSCAFT00000002672	MCM5	minichromosome maintenance complex component 5	DNA replication licensing factor, cell cycle deregulation
A_11_P085321	2,272 (0,2342)	ENSCAFT00000006021	ORC1L	origin recognition complex subunit 1-like	Replications factor
A_11_P090876	2,056 (0,2603)	ENSCAFT00000001525	KIFC1	kinesin family member C1	cell cycle: chromoseme movement
A_11_P0000024589	2,027 (0,1961)	ENSCAFT00000031608			DNA replication factor Cdt1
A_11_P0000031692	2,022 (0,2979)	ENSCAFT00000037553	LOC488258	similar to Histone H1.2 (H1d)	Histone protein
A_11_P0000016334	2,021 (0,276)	CX987798			Canis familiaris testis cDNA Canis familiaris cDNA mRNA
Down regulated genes					
A_11_P0000020888	-2,019 (0,3283)	ENSCAFT00000003739	TECRL	trans-2 3-enoyl-CoA reductase-like	Steroid 5-alpha-reductase 2-like 2 protein
A_11_P114966	-3,375 (0,2261)	ENSCAFT00000026677	GDPD2	glycerophosphodiester phosphodiesterase domain containing 2	Glycerophosphodiester phosphodiesterase
A_11_P0000025698	-3,178 (0,276)	ENSCAFT00000026677	GDPD2	glycerophosphodiester phosphodiesterase domain containing 2	Glycerophosphodiester phosphodiesterase
A_11_P146113	-2,768 (0,3358)	XM_843618	LOC607732	similar to 60S ribosomal protein L37a	Ribosomal protein
A_11_P0000015771	-2,37 (0,2261)	DN425546			Canis familiaris cDNA clone CLN10814030
A_11_P0000020109	-2,172 (0,1835)	NM_001005258	CCL21	chemokine (C-C motif) ligand 21	Chemokine ligand
A_11_P125231	-2,159 (0,1127)	XM_848166	LOC474960	similar to CG32333-PA isoform A	
A_11_P091981	-2,151 (0,3283)	ENSCAFT00000005805			Popeye domain-containing protein 3
A_11_P0000020548	-2,098 (0,2404)	ENSCAFT00000003030	NUDT2	nudix (nucleoside diphosphate linked moiety X)-type motif 2	

Table 4: High significant (p<0.01) at least four fold up or down regulated gene expression in only CPV5 affected tissue samples compared to the control.

Identifier	log2 Ratio_CPV5 (fdr_CPV5)	PrimaryAccession	GeneSymbol	GeneName	Description
Up regulated genes					
A_11_P0000027041	3,81 (0,2453)	ENSCAFT00000010061	TMCC3	transmembrane and coiled-coil domain family 3	transmembran protein
A_11_P050936	2,821 (0,3334)	NM_001114666	ARX	aristaless related homeobox	mental retardation
A_11_P0000013614	2,796 (0,2421)	CO719715			muscle Canis familiaris cDNA 3'
A_11_P000006555	2,646 (0,2936)	CO585953			brain Canis familiaris cDNA
A_11_P052151	2,533 (0,3357)	ENSCAFT00000035299	LOC610532	similar to Olfactory receptor 7A5	Olfactory receptor
A_11_P096371	2,505 (0,1946)	ENSCAFT00000024923	DAGLB	diacylglycerol lipase beta	lipase
A_11_P060766	2,469 (0,3575)	ENSCAFT00000035314			
A_11_P000008021	2,42 (0,2729)	CO600368			testis Canis familiaris
A_11_P0000027777	2,374 (0,3071)	XM_540616	APLNR	apelin receptor	apelin receptor on cell surface
A_11_P000005291	2,355 (0,2453)	CF411798			
A_11_P139526	2,311 (0,2438)	XM_536395	LOC479252	similar to Vinculin (Metavinculin)	adherens junctions
A_11_P133981	2,292 (0,3606)	CX990747			Canis familiaris testis cDNA
A_11_P000005041	2,27 (0,3396)	CF410968			
A_11_P0000031271	2,253 (0,2756)	ENSCAFT00000014087	HUNK	hormonally up-regulated Neu-associated kinase	Serine/threonine-protein kinase
A_11_P085301	2,252 (0,2421)	ENSCAFT00000005958	SHCBP1	SHC SH2-domain binding protein 1	
A_11_P137786	2,245 (0,3357)	CF410611			
A_11_P0000025585	2,211 (0,3396)	AF082505			Canis familiaris T cell receptor beta
A_11_P102756	2,2 (0,245)	ENSCAFT00000013773			Coiled-coil domain-containing protein
A_11_P000006860	2,16 (0,245)	CO589594			brain Canis familiaris cDNA
A_11_P0000017582	2,092 (0,3575)	DN866257			Dog eye lens
A_11_P0000013085	2,088 (0,3575)	CO709729			muscle Canis familiaris cDNA
A_11_P0000022158	2,086 (0,3637)	ENSCAFT00000023729	LOC476669	similar to Gamma-interferon inducible lysosomal thiol reductase precursor	
A_11_P0000017598	2,079 (0,2198)	DN866460			Dog eye lens
A_11_P054881	2,073 (0,3782)	X66173			Na K-ATPase
A_11_P000003861	2,054 (0,245)	BU751593			Canine heart normalized cDNA
A_11_P121531	2,051 (0,2851)	XM_537223	LOC480101	similar to SET and MYND domain containing 3	
A_11_P0000029906	2,05 (0,2343)	ENSCAFT00000039833	STMN4	stathmin-like 4	
A_11_P0000013414	2,007 (0,2453)	CO716705			muscle Canis familiaris cDNA
Down regulated genes					
A_11_P0000015591	-2,002 (0,3396)	XM_843905	TMEM52	transmembrane protein 52	transmembrane protein
A_11_P149068	-3,018 (0,3723)	ENSCAFT00000003358	PDK4	pyruvate dehydrogenase kinase isozyme 4	Citric acid cycle
A_11_P0000040538	-2,797 (0,3491)	XM_849378	ASB2	ankyrin repeat and SOCS box-containing 2	Cytocrin suppressor
A_11_P072941	-2,639 (0,3124)	ENSCAFT00000038852			PDZ domain-containing protein
A_11_P214793	-2,633 (0,2453)	XM_844878	JPH1	junctophilin 1	junctional complex
A_11_P180453	-2,541 (0,2219)	NM_001003332	PLN	phospholamban	heart muscle
A_11_P222388	-2,535 (0,3575)	XM_853354	CAP2	CAP adenylate cyclase-associated protein 2 (yeast)	
A_11_P119121	-2,329 (0,2453)	XM_844878	JPH1	junctophilin 1	junctional complex
A_11_P0000014907	-2,212 (0,3676)	DN371046			Canis familiaris cDNA clone
A_11_P157188	-2,137 (0,2219)	TC52285			Alpha/beta hydrolase
A_11_P0000033661	-2,128 (0,3575)	ENSCAFT00000018909	FITM1	fat storage-inducing transmembrane protein 1	Fat storage
A_11_P0000015569	-2,027 (0,2219)	DN411291			Canis familiaris cDNA clone CLN10728147

Table 5: High significant (p<0.01) at least four fold up or down regulated gene expression in two viruses affected tissues compared to the control. (a): Up or down regulated genes in CPV1 and CPV3 compared to the control; (b): Up or down regulated genes in CPV1 and CPV5 compared to the control; (c): Up or down regulated genes in CPV3 and CPV5 compared to the control; (d): Up or down regulated genes in all CPV1, CPV3 and CPV5 compared to the control.

(a)

Identifier	log2 Ratio_CPV1 (fdr_CPV1)	log2 Ratio_CPV3 (fdr_CPV3)	PrimaryAccession	GeneSymbol	GeneName	Description
Up regulated genes						
A_11_P0000040430	2,605 (0,5339)	2,376 (0,2948)	TC56305			NHP2 non-histone chromosome protein 2-like 1, partial (28%)
Down regulated genes						
A_11_P050111	-2,763 (0,5369)	-2,453 (0,3063)	NM_001103217	CGN	cingulin	tight junction
A_11_P106551	-2,673 (0,5572)	-2,326 (0,06731)	ENSCAFT00000025298	ALOX12	arachidonate 12-lipoxygenase	angiogenese
A_11_P050151	-2,172 (0,5339)	-2,312 (0,2579)	NM_001003332	PLN	phospholamban	calcium pump of cardiac sarcoplasmic reticulum
A_11_P108291	-2,042 (0,5339)	-2,069 (0,2243)	ENSCAFT00000032309			cytosceleton

(b)

Identifier	log2 Ratio_CPV1 (fdr_CPV1)	log2 Ratio_CPV5 (fdr_CPV5)	PrimaryAccession	GeneSymbol	GeneName	Description
Up regulated genes						
A_11_P0000023190	3,787 (0,5364)	2,882 (0,391)	NM_001145170	CA2	carbonic anhydrase II	Metalloenzyme

(c)

Identifier	log2 Ratio_CPV3 (fdr_CPV3)	log2 Ratio_CPV5 (fdr_CPV5)	PrimaryAccession	GeneSymbol	GeneName	Description
Up regulated genes						
A_11_P170638	2,063 (0,06834)	2,751 (0,01015)	XM_856253	DAGLB	diacylglycerol lipase beta	Axonal growth

(d)

Identifier	log2 Ratio_CPV1 (fdr_CPV1)	log2 Ratio_CPV3 (fdr_CPV3)	log2 Ratio_CPV5 (fdr_CPV5)	PrimaryAccession	GeneSymbol	GeneName	Description
Up regulated genes							
	4.918 (0.5339)	4.865 (0.2644)	4.816 (0.3357)	ENSCAFT00000004630	KLK8	kallikrein-related peptidase 8	Kallikrein-9 Precursor, Peptidase
Down regulated genes							
	-2.385 (0.00746)	-2.153 (0.000432)	-2.58 (0.003915)	CN005540	MT3	metallothionein 3	Brain - Cerebellum Library
	-2.667 (0.002943)	-2.349 (0.002191)	-2.052 (0.006194)	XM_540147	VIT	vitrin	PREDICTED: Canis familiaris similar to vitrin transcript
	-2.903 (0.007522)	-2.016 (0.005241)	-2.506 (0.003035)	ENSCAFT00000010325	CCRL1	chemokine (C-C motif) receptor-like 1	C-C chemokine receptor type
	-3.319 (0.007177)	-3.985 (0.0009268)	-4.972 (0.001332)	ENSCAFT00000039761	MUSTN1	musculoskeletal embryonic nuclear protein 1	Musculoskeletal embryonic nuclear protein

Table 6: Enrichment analysis of differently expressed genes performed by using GeneGO. For enriched pathway maps, process networks and GO processes, the total number of assigned genes and the percentage of the differently expressed genes in the virus infected tissues are listed. The p-value are given in brackets.

Enrichment by Pathway Maps	Total	%CPV1 (p-value)	%CPV3 (p-value)	%CPV5 (p-value)
Cell cycle_The metaphase checkpoint	36	5.6 (9.01E-02)	30.6 (2.66E-11)	63.9 (1.96E-15)
Cell cycle_Role of APC in cell cycle regulation	32	6.3 (7.35E-02)	31.3 (1.72E-10)	62.5 (2.57E-13)
Cell cycle_Spindle assembly and chromosome separation	33	15.2 (8.59E-05)	36.4 (2.91E-13)	48.5 (1.08E-08)
Cell cycle_Chromosome condensation in prometaphase	21	4.8 (2.57E-01)	47.6 (1.12E-12)	66.7 (3.07E-10)
Cell cycle_Sister chromatid cohesion	22	4.5 (2.67E-01)	31.8 (9.21E-08)	59.1 (1.16E-08)
DNA damage_ATM/ATR regulation of G1/S checkpoint	32	6.3 (7.35E-02)	15.6 (2.83E-04)	43.8 (4.53E-07)
DNA damage_ATM / ATR regulation of G2 / M checkpoint	26	3.8 (3.07E-01)	11.5 (1.21E-02)	46.2 (1.52E-06)
DNA damage_Nucleotide excision repair	36	2.8 (3.99E-01)	8.3 (2.89E-02)	38.9 (2.53E-06)
Cell cycle_Role of SCF complex in cell cycle regulation	29	3.4 (3.36E-01)	13.8 (1.92E-03)	41.4 (6.21E-06)
Enrichment by Process Networks				
Cell cycle_Core	115	4.3 (1.24E-01)	20.9 (2.97E-14)	53.0 (1.39E-19)
Cell cycle_S phase	149	3.4 (2.57E-01)	20.8 (4.16E-18)	47.7 (3.74E-19)
Cell cycle_Mitosis	179	4.5 (5.34E-02)	15.1 (2.66E-12)	39.1 (2.01E-13)
Cytoskeleton_Spindle microtubules	109	6.4 (1.24E-02)	19.3 (6.82E-12)	40.4 (2.01E-09)
Cell cycle_G2-M	206	4.4 (4.72E-02)	12.6 (4.14E-10)	33.5 (1.10E-09)
DNA damage_Checkpoint	124	4.0 (1.56E-01)	11.3 (2.00E-05)	37.9 (6.52E-09)
Cell cycle_G1-S	163	5.5 (1.25E-02)	12.3 (7.94E-08)	33.1 (1.11E-07)
DNA damage_DBS repair	116	4.3 (1.28E-01)	4.3 (2.74E-01)	33.6 (4.32E-06)
DNA damage_BER-NER repair	110	4.5 (1.08E-01)	12.7 (4.92E-06)	33.6 (7.49E-06)
Proteolysis_Ubiquitin-proteasomal proteolysis	166	4.2 (8.70E-02)	6.0 (2.86E-02)	30.1 (7.56E-06)
Enrichment by GO Processes				
mitotic cell cycle	771	3.6 (2.45E-03)	9.9 (3.87E-30)	35.8 (1.58E-44)
interphase of mitotic cell cycle	452	4.0 (5.62E-03)	8.6 (6.94E-14)	34.1 (1.21E-22)

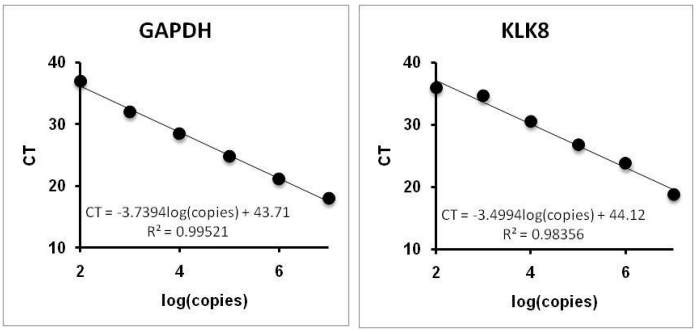
Table7: Relative expression (qRT-PCR) of the cellular transcripts of KLK8, MCM5, ORC1 and PAX compared to the GAPDH expression in CPV1, CPV3 and CPV5 affected tissue samples and their statistical significance (p value).

	KLK8		MCM5		ORC1		PAX	
virus	**increased**	**p <**	**increased**	**p <**	**increased**	**p <**	**increased**	**p <**
CPV1	25,5 ± 25,6	0,02	1,8 ± 0,8	0,51	1,7 ± 1,1	0,66	1,9 ± 2,4	0,88
CPV3	23,0 ± 13,3	0,01	7,1 ±0,9	0,05	6,8 ±1,6	0,01	34,4 ± 26,1	0,01
CPV5	23,3 ± 7,9	0,01	2,9 ± 1,6	0,32	3,2 ± 1,1	0,01	3,4 ± 3,1	0,07

Figures

Figure 1:

(a)



(b)

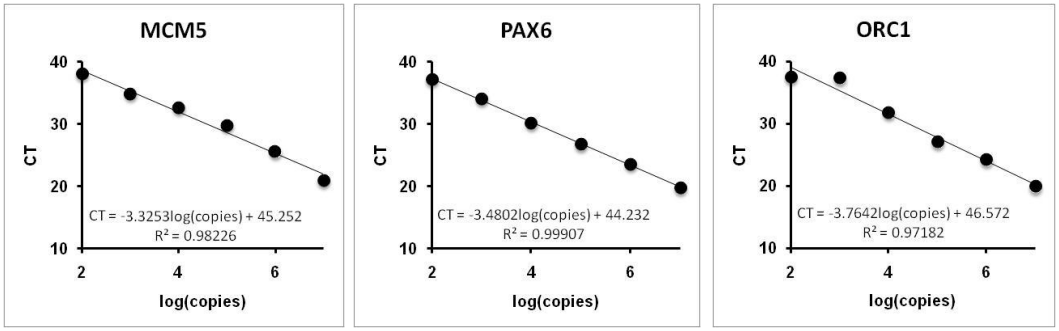


Figure 2:

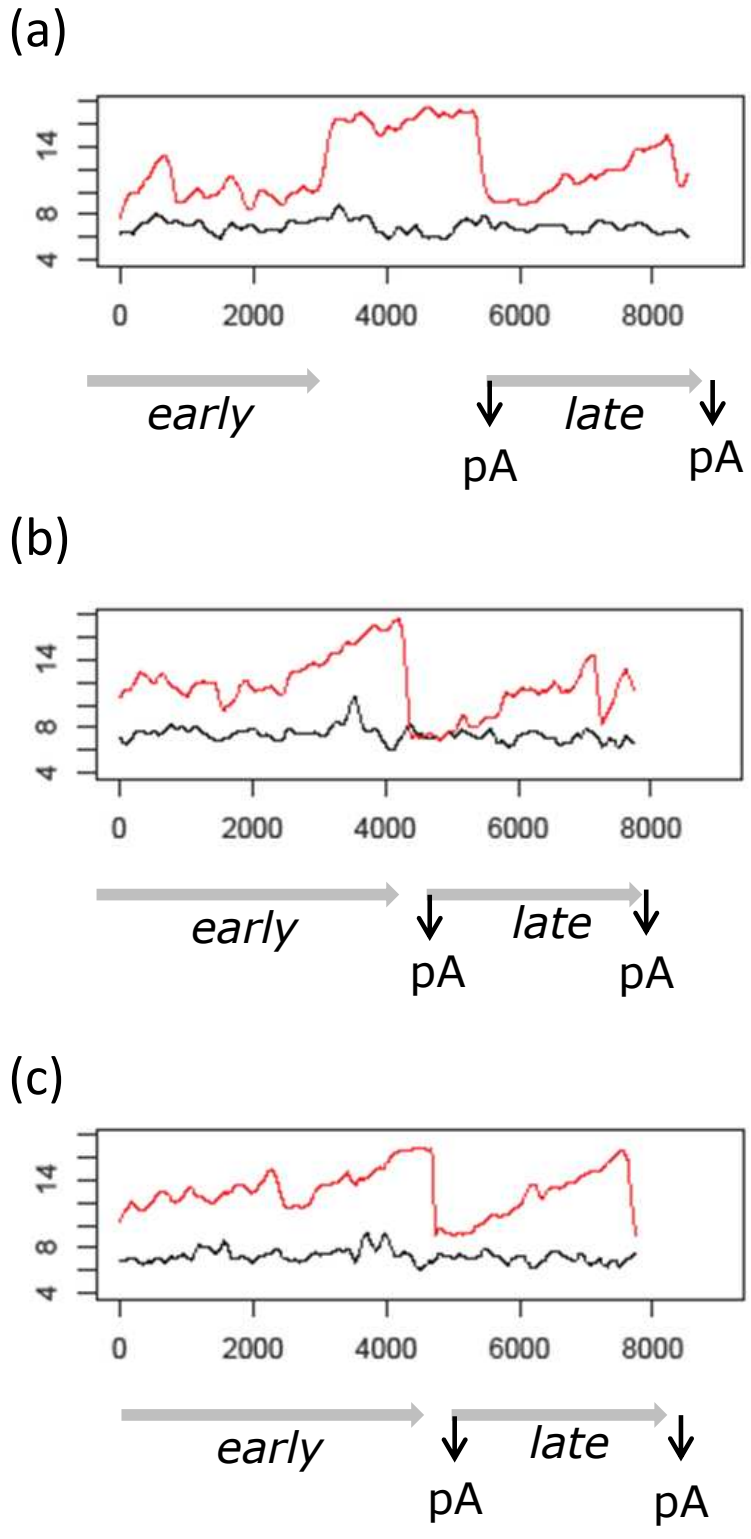
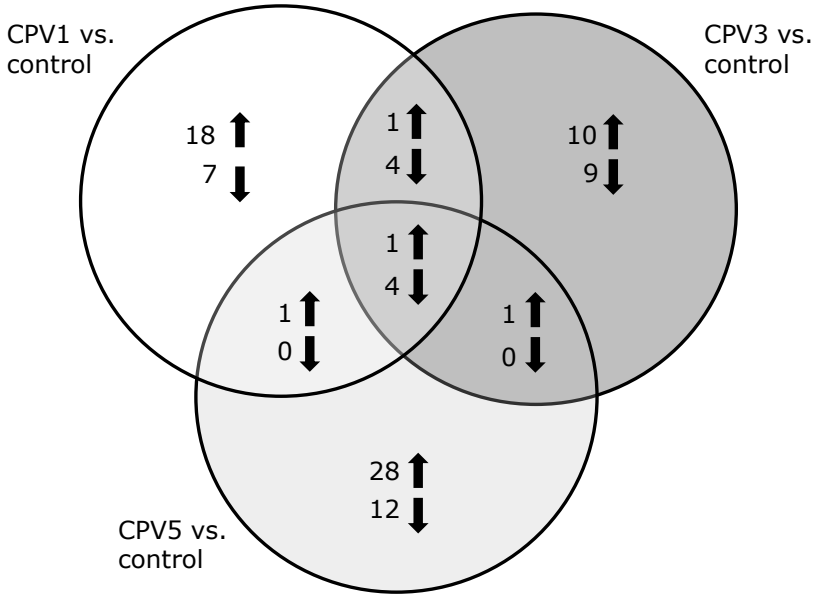
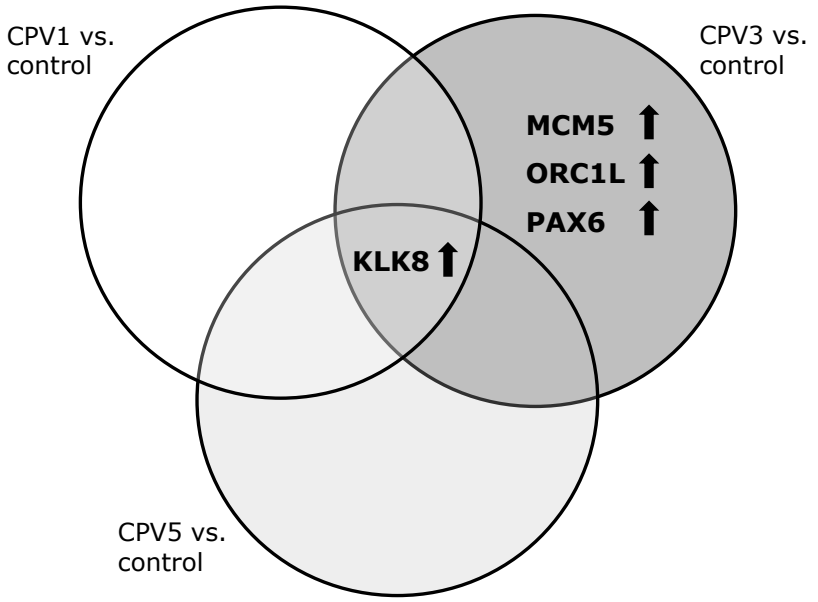

(a)
4
8
14
0
2000
4000
6000
8000
early
late
pA
pA
(b)
4
8
14
0
2000
4000
6000
8000
early
late
pA
pA
(c)
4
8
14
0
2000
4000
6000
8000
early
late
pA
pA

Figure 3:

(a)


CPV1 vs. control
CPV3 vs. control
CPV5 vs. control
18
7
1
4
10
9
1
4
1
0
1
0
28
12

(b)


CPV1 vs. control
CPV3 vs. control
CPV5 vs. control
MCM5
ORC1L
PAX6
KLK8

Curriculum Vitae

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