Differential expression of microRNAs in bovine papillomavirus type 1 transformed equine cells

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Abstract

Bovine papillomavirus (BPV) types 1 and 2 play an important role in the pathogenesis of equine sarcoids (ES), the most common cutaneous tumour affecting horses. MicroRNAs (miRNAs), small non-coding RNAs that regulate essential biological and cellular processes, have been found dysregulated in a wide range of tumours. The aim of this study was to identify miRNAs associated with ES. Differential expression of miRNAs was assessed in control equine fibroblasts (EqPalFs) and EqPalFs transformed with the BPV-1 genome (S6-2 cells). Using a commercially available miRNA microarray, 492 mature miRNAs were interrogated. In total, 206 mature miRNAs were differentially expressed in EqPalFs compared with S6-2 cells. Aberrant expression of these miRNAs in S6-2 cells can be attributed to the presence of BPV-1 genomes. Furthermore, we confirm the presence of 124 miRNAs previously computationally predicted in the horse. Our data supports the involvement of miRNAs in the pathogenesis of ES.

Introduction

Bovine papillomaviruses (BPVs) are non-enveloped double-stranded DNA epitheliotropic viruses that give rise to papillomas, fibropapillomas and/or bladder cancer in cattle.[1 – 4] It is also well established that BPVs (type 1 and less commonly type 2) are involved in the pathogenesis of equine sarcoid tumours (ES).[5 – 7] Sarcoids are the most common cutaneous tumours in horses and they are described as locally invasive fibroblastic neoplasia of equine skin.[8,9] Sarcoids can present in a range of clinical forms from mild to malignant and currently therapeutic options are limited. [9,10] In sarcoids, the viral DNA is detected primarily in fibroblasts[11 – 14] although BPV-1 genomes can be detected within the epidermis, suggesting a more complex aetiopathogenesis.[15] Whilst infection with BPV-1/-2 has been implicated as an essential factor in sarcoid development, the mechanisms by which BPV infection contributes to tumorigenesis remain unclear. The BPV-1/-2 genome encodes several early (E1, E2, E5, E6 and E7) and two late genes, L1 and L2, which code for capsid proteins. BPV-1/-2 E5 and E6 are considered the major oncogenes in ES.[16] E5 induces tumorigenic transformation by strongly and specifically binding to its cellular target, platelet-derived growth factor-β receptor (PDGF β -R) tyrosine kinase in a ligand-independent manner.[17] Activation of PDGFβ-R of the dermal fibroblasts results in mitogenesis in nearby epithelial cells,[18] contributing to the pseudoepitheliomatous hyperplasia that is typically observed in BPV-infected lesions. BPV-1/-2 E6 degrades bovine p53[19,20] and presence of BPV genomes in sarcoids induces mis-localization of p53 by an unknown mechanism.[21]

In this study, we describe the differences in microRNA (miRNA) expression that may lead to BPV-1 induced tumorigenesis. MicroRNAs (miRNAs) are 18–23 nucleotide, single-stranded RNA molecules that regulate gene expression at the post-transcriptional level[22] and are expressed in all metazoan eukaryotes. Through imperfect base-pairing to target mRNA, most commonly in the 3′-untranslated region (UTR), miRNAs inhibit mRNA translation by causing deadenylation and/or degradation of the target mRNA.[23] miRNAs participate in the regulation of the cell cycle, proliferation/differentiation, apoptosis and senescence, and dysfunctional expression of miRNAs has been linked to

malignancies.[24–30] Depending on the nature of their targets, miRNAs can function as oncogenes (oncomiRs) or tumour suppressors.[31]

Numerous studies show upregulation of oncogenic miRNAs and downregulation of tumour-suppressive miRNAs in human papillomavirus (HPV) cervical cancer biopsies and in cervical cancer lines in comparison with controls.[32 – 38] In HPV-induced cancer, numerous miRNAs have been identified as oncomiRs. Similarly, miRNAs including let-7 family, miR-7, miR-17-5p, miR-125, miR-218 and miR-433 have been reported to function as tumour suppressors.[39,40] In HPV-induced cancers, the expression of E6 and E7 has been linked to aberrant expression of miRNAs leading to alteration of p53 and pRB pathways and associated with carcinogenic transformation.[33,39,41]

In vitro changes to the equine fibroblast transcriptome induced by BPV-1 infection have been investigated previously by microarray expression pro ling.[42] The results demonstrated altered expression of genes involved in a range of processes including immunity and inflammation, cell adhesion, motility as well as cell cycle/cell proliferation and apoptosis.[42] To gain a better understanding of the molecular pathogenesis associated with BPV-1-induced tumorigenesis in sarcoids, the aim of this study was to identify cellular miRNAs that are differentially expressed in BPV-1-transformed equine fibroblasts. This was achieved by assessing differences in miRNA expression in normal equine fibroblasts (EqPalFs) compared with equine fibroblasts transformed with BPV-1 genomes, S6-2 cells. Our data identify for the first time, cellular miRNAs associated with BPV-1 transformation of equine cells.

Materials and methods

Equine fibroblast cell culture

Equine primary fibroblasts and BPV-1 trans- formed cells (S6-2) are well characterized cell lines that have been described previously.[21] All cells were maintained in culture in a 37°C humidified atmosphere with 5% CO_2, in culture media containing High Glucose Dulbecco's Modified Eagles medium with Pyruvate (DMEM-GlutaMAX™) (Gibco®, Life Technologies, Paisley, UK), 10% of heat inactivated foetal bovine serum (Gibco®),

penicillin – streptomycin (10 000 U mL−1) (Gibco®) and Fungizone® Antimycotic (Gibco®). Cells were routinely tested for mycoplasma and were consistently negative. Local ethical approval was granted to conduct this study.

miRNA microarray

Total RNA was isolated from EqPalFs and S6-2 cells at low passage (<10) using Trizol® Reagent (Life Technologies) as per the manufacture's advice, vortexed and kept at −80∘C until further use. Quality of RNA was assessed using a 2100 Bioanalyzer (Agilent Technologies, Stockport, UK). All samples had an RNA integrity number (RIN value) of 10.0. A sample of 750ng of total RNA from the cell lines was fluorescently labelled, using the miRCURY LNA™ microRNA Hi-Power Labelling Kit, Hy3TM/Hy5TM (Exiqon, Vedbaek, Denmark) following the manufacturer's instructions. The query samples (EqPalFs and S6-2 cells) were labelled using Hy3 whereas the reference sample (a pooled sample of RNA from both cell lines) was dyed using Hy5. Samples were run on a miRCURY LNA™ microRNA Array 7th Gen (Exiqon). This array comprises 2164 probes that contain human and viral miRNA sequences together with predicted equine microRNA sequences derived from miRBase 19.0.[43]

The slides were scanned using the Agilent G2565BA Microarray Scanner System (Agilent Technologies) and the image analysis was carried out using the ImaGene® 9 miRCURY LNA™ microRNA Array Analysis So ware (Exiqon). Pre-processing of the data included background subtraction (Normexp with o set value 10) and normalization using the global lowess regression algorithm. Moderated *t*-statistics were applied to determine differential expression of microRNAs between EqPalFs and S6-2 cells. Following this, the Benjamini and Hochberg multiple testing adjustment method was applied to the *P* values. *P* values of 0.05 or lower were considered to be statistically significant. Calculations were performed with R/Bioconductor using the limma package.

Validation of miRNA expressionby quantitative real-time PCR (qRT-PCR)

A total of 11 miRNAs were initially selected for validation of the microarray results based on fold-change (±2-fold), signal intensity (7.5–14.5) and biological significance (i.e. having previously been reported to be dysregulated in HPV-related disease and/or cancer). miRNAs were quantified using SYBR green; however, due to problems with non-specific amplification and low efficiency for some primer sets, a panel of miRNAs were examined using Taqman qRT-PCR.

Total RNA from each cell line was treated with DNase (DNA-free™ Kit, Life Technologies) and quantified with Qubit™ RNA Assay Kits (Invitrogen, Life Technologies). Using 10 – 300 ng of total RNA, cDNA was synthesized using the Mir-XTM-miRNA First-strand synthesis kit (Takara Bio Europe, St Germaine en Laye, France) or stem-loop primers from TaqMan® MicroRNA Reverse Transcription Kit (Life Technologies). Primers used for amplification were supplied as part of the kits with the exception of the 5′ primers for SYBR green and these are listed in Table 1. Quantification of miRNAs was performed with SYBR® qRT-PCR technology (Takara Bio Europe, St Germaine en Laye, France) or TaqMan MicroRNA Assays (Life Technologies) following the manufacturer's advice. The protocol was optimized by decreasing the amount of cDNA per reaction to 1μL, decreasing the concentration of the primers to 5 pmol per reaction for the SYBR green kit and increasing the amount of cDNA per reaction to 2 μL for the Taqman kit. Each amplification reaction was performed in triplicate and the average Ct value was used for subsequent analysis. Expression of each miRNA was normalized using U6 and differential expression was calculated using the ΔΔCt method.[44 – 46]

Table 1. List of 5' primer sequences used for qRT-PCR validation of microRNAs

Annotation	SYBR green 5' primer	miRBase accession
eca-miR-106a/17	CAAAGTGCTTACAGTGCAGGTAG	MIMAT0013194
eca-miR-127 (hsa-miR-127-3p)	TCGGATCCGTCTGAGCTTGGCT	MIMAT0013126
eca-miR-132 (hsa-miR-132-3p)	TAACAGTCTACAGCCATGGTCG	MIMAT0013021
eca-miR-143	TGAGATGAAGCACTGTAGCTC	MIMAT0013063
eca-miR-15b	TAGCAGCACATCATGGTTTACA	MIMAT0012954
eca-miR-181a-5p	AACATTCAACGCTGTCGGTGAGT	MIMAT0013178
eca-miR-23b	ATCACATTGCCAGGGATTACC	MIMAT0013113
eca-miR-409-3p	GAATGTTGCTCGGTGAACCCCT	MIMAT0013151
hsa-let-7b-5p	TGAGGTAGTAGGTTGTGTGGTT	MIMAT0000063
hsa-miR-4732-5p	TGTAGAGCAGGGAGCAGGAAGCT	MIMAT0019855
hsa-miR-663a	AGGCGGGGCGCCGCGGGACCGC	MIMAT0003326

Results

In this study we used microarray analysis to identify miRNAs that are differentially expressed in BPV-1 transformed EqPalF cells (S6-2 cells) compared with control parental EqPalF cells. The microarray comprised human and viral miRNA sequences as well as computationally predicted equine miRNA sequences. A total of 402 unique short RNA sequences were identified as potential miRNAs in equine fibroblasts, of which 124 sequences match the previously predicted miRNAs in the horse,[47] confirming that these miRNAs are expressed within the horse genome. The list of 124 miRNAs is presented as Table S1, Supporting Information.

Of the 2164 miRNA probes on the microarray, 492 had a signal above the background thresh- old level in both cell lines. Of these, 22 were repeated sequences, 1 repeated annotation, 12 small nucleolar RNAs, 9 synthetic microRNAs and 27 non-papilloma viral sequences. Furthermore, 19 false positives were removed from the analysis, as these probes can display atypical signal patterns (Exiqon).

Applying moderated *t*-statistics with a multiple test correction, 206 microRNA candidates were identified to be differentially expressed between S6-2 cells and EqPalF cells ($P < 0.05$). Over two thirds (144 miRNAs, 70%) of the differentially expressed miRNAs were

downregulated in the S6-2 cells and 62 (30%) were upregulated (Fig. 1). The top 10 upregulated and downregulated miRNA candidates on the basis of fold change are listed in Table 2. All raw and normalized miRNA expression data are available from the NCBI's Gene Expression Omnibus[48] and are accessible through GEO series accession number GSE73937 (http://www.ncbi. nlm.nih.gov/geo/query/acc.cgi?acc = GSE73937).

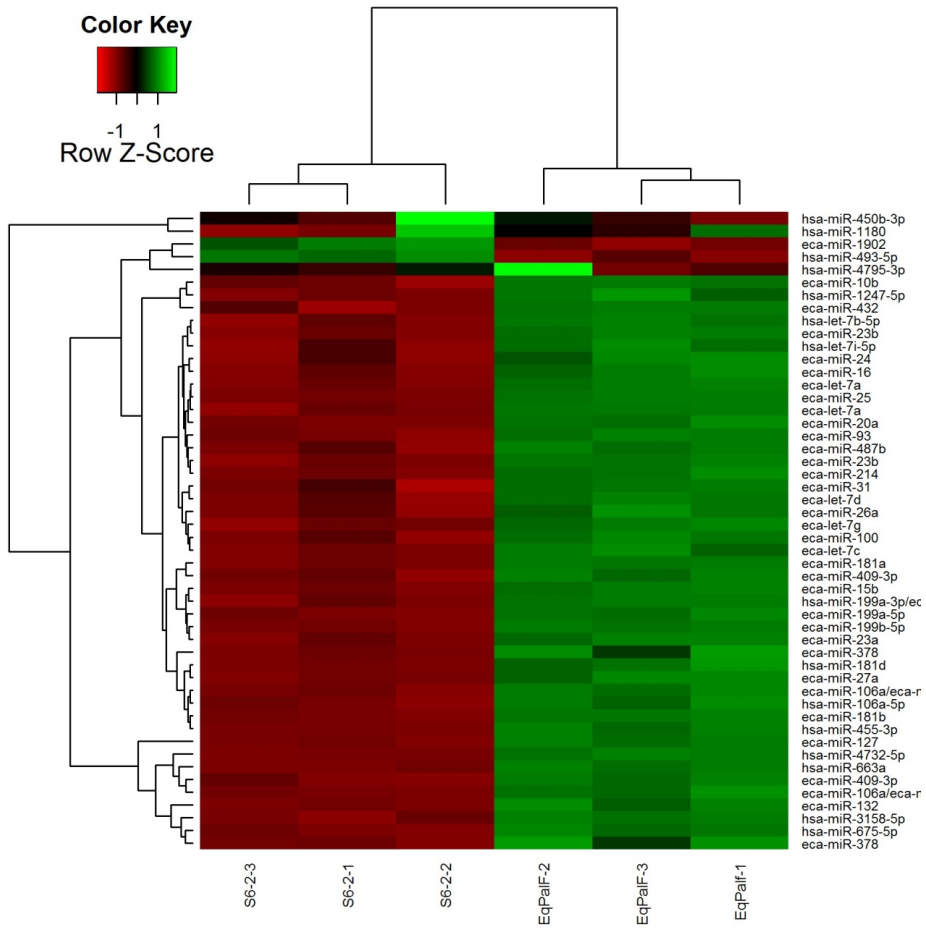

Figure 1. Heat map representing differences in expression top 50 microRNAs. S-62-1 to 3 are the three replicates of BPV transformed equine fibroblasts, EqPalF-1 to 3 are the three replicates of equine primary fibroblasts. The clustering was performed on all samples using the complete-linkage method together with the Euclidean distance measure, and on the top 50 miRNA with highest standard deviation. The normalized log ratio values have been used for the analysis. Red represents lower expression and green represents higher expression of microRNAs of the samples compared to the control channel of the microarray (a pooled sample of both cell lines).

Table 2. Top 10 down and 10 upregulated microRNAs in S6-2 cells (*in vitro* BPV-1 transformed equine primary fibroblasts) compared to EqPalFs (equine primary fibroblasts) ranked on fold change

Annotation	Fold change	RNA sequence probe	Adjusted P value
eca-miR-127	-4.35	UCGGAUCCGUCUGAGCUUGGCU	1.34×10^{-8}
hsa-miR-4732-5p	-2.94	UGUAGAGCAGGGAGCAGGAAGCU	1.34×10^{-8}
eca-miR-132	-2.78	UAACAGUCUACAGCCAUGGUCG	2.79×10^{-7}
eca-miR-181a	-2.38	AACAUUCAACGCUGUCGGUGAGU	3.65×10^{-8}
eca-miR-409-3p	-2.27	GAAUGUUGCUCGGUGAACCCCU	5.36×10^{-7}
hsa-miR-663a	-2.17	AGGCGGGGCGCCGCGGGACCGC	9.81×10^{-8}
hsa-miR-3158-5p	-2.04	CCUGCAGAGAGGAAGCCCUUC	2.79×10^{-7}
eca-miR-15b	-2.00	UAGCAGCACAUCAUGGUUUACA	2.20×10^{-7}
eca-miR-106a/ eca-miR-17	-1.96	UCAAAUGCUCAGACUCCUGUGGU	3.70×10^{-7}
eca-miR-199a-3p/ eca-miR-199b-3p	-1.96	GGUCCAGAGGGGAGAUAGGUUC	5.02×10^{-7}
eca-miR-1902	+1.52	AGAGGUGCAGUAGGCAUGACUU	1.1×10^{-5}
hsa-miR-493-5p	+1.48	UUGUACAUGGUAGGCUUUCAUU	8.20×10^{-6}
hsa-miR-4484	+1.42	AAAAGGCGGGAGAAGCCCCA	1.28×10^{-5}
hsa-miR-1285-3p	+1.41	UCUGGGCAACAAAGUGAGACCU	1.54×10^{-5}
hsa-miR-3121-5p	+1.40	ACUUUCCUCACUCCCGUGAAGU	8.04×10^{-6}
hsa-miR-639	+1.38	AUCGCUGCGGUUGCGAGCGCUGU	1.70×10^{-4}
eca-miR-193b	+1.36	AACUGGCCCACAAAGUCCCGCU	1.46×10^{-5}
hsa-miR-4742-3p	+1.32	UCUGUAUUCUCCUUUGCCUGCAG	6.63×10^{-5}
eca-miR-1248	+1.29	UCCUUCUUGUAUAAGCACUGUGCUAAA	3.94×10^{-5}
eca-miR-99a	+1.27	AACCCGUAGAUCCGAUCUUGUG	5.15×10^{-5}

To validate the S6-2 versus EqPalFs microarray results, 11 differentially expressed miRNAs were assessed by qRT-PCR using RNA prepared from three biological replicates of the same cell lines. The miRNAs were chosen based on the following thresholds advised by Exiqon: absolute fold-change of ≥2, microarray signal intensity between 7.5 and 14.5 and biological significance, i.e. previously shown to be implicated in HPV-associated cancer. Real-time quantification showed that 6 of the 11 microRNAs tested showed significantly higher expression in EqPalF than S6-2 cells, as expected (let-7b, miR-17, miR-23b, miR-132, miR-143 and miR-181a) (Fig. 2), confirming the results from the microarray. The Pearson's correlation coefficient between microarray and qPCR data was 0.68 (Fig. 3). One miRNA (miR-663a) showed no significant difference, whilst two miRNAs had an inverse expression pattern to the microarray data (miR-15b and miR-127). Two miRNAs (miR-409 and miR-4732) could not be quantified due to low efficiency and/or non-specific amplification.

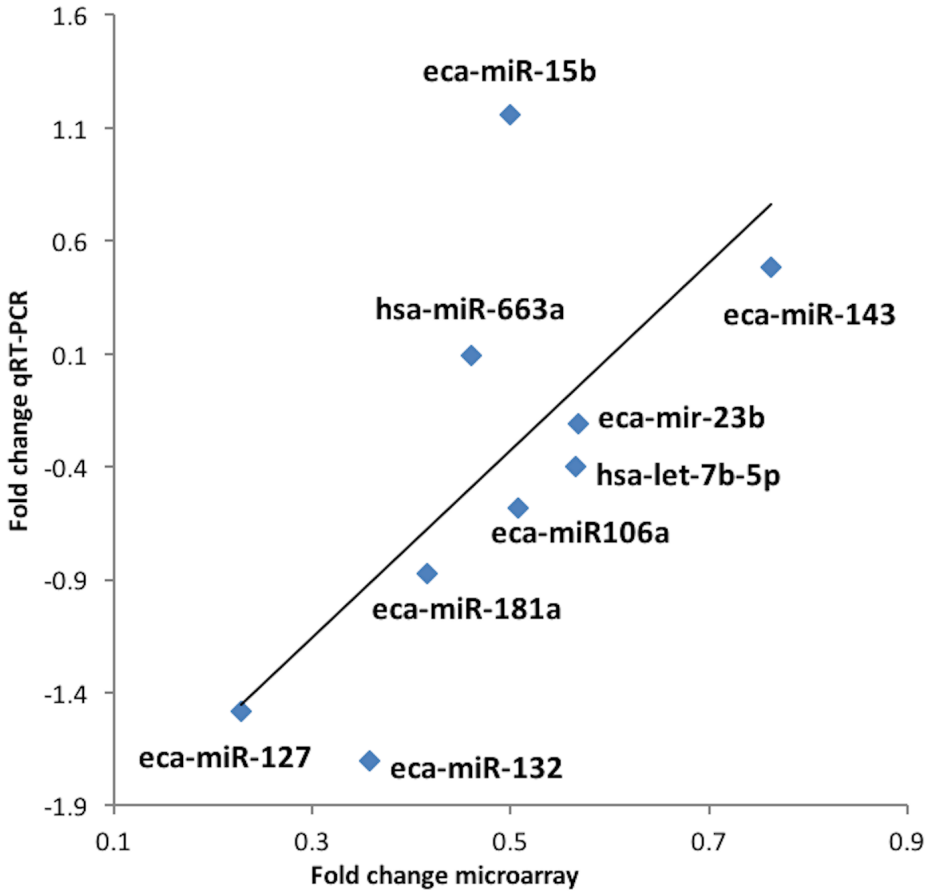

Figure 3. Correlation between results of microarray and qRT-PCR. The differential expression of miRNAs in control equine fibroblasts (EqPalF) and BPV-1 transformed equine fibroblasts (S6-2 cells) was calculated by microarray (X axis, S6-2/EqPalF ratio) and qRT-PCR (y axis, log2 fold-change of S6-2 vs EqPalF).

Discussion

Research and interest in the role of microRNAs in virus–host interactions has increased in recent years.[49 – 51] Viruses are able to remodel cellular miRNA expression networks impacting on the pathogenesis of virally induced cancers.[26,28,52–54] To date, there are few studies on equine miRNAs and, to the best of our knowledge, no studies have investigated the impact of viral infection on miRNA expression in equine cells.

MicroRNAs are highly conserved across species55 and phylogenetic conservation of the mature sequence was utilized in the first report on equine miRNAs. Using an integrated comparative genomic approach to the human genome and other animal genomes, a total of 354 mature sequences of miRNA candidates were computationally predicted in the horse.[47] In our study, we were able to confirm that 124 of these predicted miRNAs are

indeed expressed within the equine genome.

In addition, we show that 206 microRNA candidates are differentially expressed in BPV-1 trans- formed equine cells compared with control equine cells. Pathway analysis to group the candidate miRNAs on the basis of functional processes was attempted; however, due to the limited transcriptomic dataset available[42] and the low number of microRNAs present in the Ingenuity Pathway Analysis database, this method could not provide information on the pathways and networks involved (http://www.ingenuity.com/products/ipa).

An interesting observation in our study was that most of the differentially expressed miRNAs (70%) were downregulated in transformed cells, several of which have also been shown to be dysregulated in HPV-induced cancers (Table 3).[27–29,34,53,54,59]

Table 3. List of 25 miRNAs found dysregulated in this study and in HPV induced cancers

miRNA	Source
let-7d	Zheng and Wang, 2011[26]; Honnegger et al. 2015[56]
miR-100	Wang et al., 2014[54]
miR-10b	Witten et al., 2010[57]; Li et al., 2011[27]
miR-125b	Witten et al., 2010[57]; Li et al., 2011[27]; Nuovo et al. 2010[58]
miR-127	Li et al., 2011[27]; Zheng and Wang, 2011[28]
miR-133a	Zheng and Wang, 2011[28]
miR-143	Zheng and Wang, 2011[28]; Lajer et al., 2012[34]
miR-145	Lajer et al., 2012[34]
miR-155	Wald et al., 2011[29]
miR-15a	Zheng and Wang, 2011[28]; Lajer et al., 2012[34]
miR-181a	Wald et al., 2011[29]; Wang et al., 2014[54]
miR-181b	Wald et al., 2011[29]
miR-183	Wang et al., 2014[54]
miR-191*	Zheng and Wang, 2011[28]
miR-221	Wald et al., 2011[29]
miR-222	Wald et al., 2011[29]
miR-23b	Au Yang et al., 2011[26]
miR-24	Wang et al., 2014[54]
miR-27a	Wang et al., 2014[54]
miR-29a	Li et al., 2011[27]; Zheng and Wang, 2011[28]
miR-29c	Wang et al., 2014[54]
miR-30b	Zheng and Wang, 2011[28]
miR-31	Wang et al., 2014[54]
miR-378 (422b)	Zheng and Wang, 2011[28]
miR-455-3p	Zheng and Wang, 2011[28]; Wang et al., 2014[54]

We successfully validated differential expression of 6 of 11 selected microRNAs (let-7b, miR-17, miR-23b, miR-132, miR-143, miR-181a) by quantitative RT-PCR. For two of the selected miRNAs we were unable to optimize the PCR reactions. miR-663a was shown to be significantly down- regulated by microarray analysis (fold change of –2.17); however, qPCR showed no difference in expression. Similarly, we were unable to validate the results for miR-15b and miR-127, which showed an inverse relationship by quantitative PCR. Whilst it has been shown that there is generally an excellent correlation between different total RNA extraction methods, there are a few miRNAs whose levels do not correlate between microarray and qPCR measurements.[60]

In our study we observed that let-7b was significantly downregulated by BPV-1 expression. In most human cancers, let-7b is considered to function as a tumour suppressor, inhibiting cancer cell proliferation and tumour growth.[61 – 63] miRNAs from the let-7 family reduce cancer cell proliferation and tumour development by controlling the expression of a number of oncogenes, such as *RAS* and *MYC*.64 Let-7b has been shown to be downregulated in a range of cancers including HPV-induced cancers.[62] Because let-7b can function as a tumour suppressor, the downregulation of let-7b expression by BPV-1 may contribute to the tumoral transformation of equine cells.

Our study showed that miR-17 expression was also significantly downregulated in S6-2 cells. miR-17 has been reported to promote cell proliferation, to reduce apoptosis and to participate in the immune response to HPV-induced cervical cancer.[64] Zheng and Wang[28] and Li et al.[59] have shown that miR-17 is upregulated in HPV 16 cervical tissues when compared with normal cervix. Our observed downregulation suggests that miR-17 can be modulated differently by distinct papillomaviruses.

Analysis of miR-23b by qPCR demonstrated that this miRNA is also downregulated. miR-23b has been shown to be involved in a wide range of cellular processes[65,66] and aberrant expression of miR-23b has been reported in many types of cancers.[67 – 69] Zhang et al.[70] showed that miR-23b suppressed invasion and metastasis both *in vitro* and *in vivo*. HPV-16 E6 protein has been reported to downregulate expression of miR-23b via expression of urokinase type plasminogen activator (uPA).[26] We have previously shown

that BPV-1 oncogene E5 and E6 induced invasion of equine cells[42] and the downregulation of miR-23b by viral oncogenes may represent a pathway by which this occurs. miR-23 has been predicted to be encoded within a chromosomal region linked to the development of sarcoids.[71]

e results from microarray and qRT-PCR show that miR-132 is significantly downregulated in S6-2 cells compared with EqPalF cells. This miRNA has been shown to be upregulated in HPV cervical cancer[72] and gastric cancer.[73] However, in other types of cancer such as breast cancer, osteosarcoma and pancreatic cancer[74-76] miR-132 has been found to be downregulated.

miR-143 is downregulated by BPV-1. miR-143 has been shown to be dysregulated by HPV-16 leading to an inability to stop proliferation in cervical cancer via the p53 pathway and it has been claimed to be an anti-tumoral miRNA.[28,34] It is a miRNA that has consistently been shown to be downregulated in HPV-induced cancer.

Wald et al.[29] showed that the presence of the oncogene HPV-16 E6 and E7 results in downregulation of miR-181a in human cells. Roles attributed to this miRNA are inhibition of virus infection and replication in porcine reproductive and respiratory syndrome virus,[77] activation of anti-inflammatory pathways,[78] a tumour-suppressor role30 and as a prognostic biomarker in leukaemia patients[79] and non-small cell lung cancer patients[25] is miRNA has been predicted to be encoded in a chromosomal region that has been linked to the development of sarcoids in Warmblood horses.[71]

In summary, this is the first report of equine miRNAs that are differentially regulated by BPV-1. Future work using high-throughput sequencing will be necessary to verify the equine miRNA sequences identified here; however, this study should provide a basis for further studies investigating detailed roles of miRNAs in BPV-1-induced transformation and in the pathogenesis of ES tumours.

Acknowledgements

This work was kindly funded by the Horserace Bet- ting Levy Board and the James Herriot Scholarship Fund. e authors would like to thank Prof. M Saveria Campo for her critical review of the manuscript and helpful comments.

Conflict of interest

The authors have declared no conflicting interests.

Supporting Information

Additional Supporting Information may be found in the online version of this article:

Table S1. A total of 124 confirmed microRNA sequences in equine fibroblasts previously predicted by Zhou et al.[47]

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