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Diagnostic potential of three serum microRNAs as biomarkers for equine sarcoid disease in horses and donkeys

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Abstract

Background: MicroRNAs (miRNAs) are potential biomarkers for equine sarcoids (ES).

Objectives: To assess eca-miR-331, eca-miR-100, and eca-miR-1 as serum biomarkers for ES disease.

Animals: Sixty-eight ES cases (56 horses, 12 donkeys), 69 tumor-free controls (60 horses, 9 donkeys), and 20 horses with other skin tumors.

Methods: For this case-control study, expression of serum eca-miR-331, eca-miR-100, and eca-miR-1 in ES-affected equids was compared to tumor-free age-, sex-, and breed-matched control horses and donkeys with other skin tumors using reverse transcription quantitative PCR (polymerase chain reaction) for relative miRNA quantification. Biological, preanalytical, and clinical variable influences on miRNA expression were examined. Receiver operator characteristic (ROC) curve analyses were used to determine differences in miRNA expression between groups.

Results: The expression of eca-miR-100 was affected by age (P = .003) and expression of eca-miR-100 and eca-miR-1 were affected by hemolysis (both P < .001). Eca-miR-331 was unaffected by biological variation, hemolysis, ES type, and disease severity. Eca-miR-331 concentrations were higher in ES-affected compared to tumor-free controls (P = .002). The ROC curve analysis indicated an area under the curve of 0.65 (P = .002) with a sensitivity of 60%, specificity of 71%, and positive and negative likelihood ratios of 2.1 and 0.56, respectively, to diagnose ES. Eca-miR-331 expression did not discriminate between horses with ES and other skin tumors. Expression of eca-miR-100 and eca-miR-1 was not different between groups.

Conclusions and Clinical Importance: Serum eca-miR-331 expression is neither sensitive nor specific enough as a single ES biomarker. If combined with other miRNAs, it may be helpful for ES diagnosis.

KEYWORDS
circulating microRNA, eca-miR-331, equine tumor marker, transcriptomic biomarker

Abbreviations: AUC, area under the curve; BPV, bovine papillomavirus; CI, confidence interval; Cq, cycle quantification; DOR, diagnostic odd ratio; ES, equine sarcoids; HBV, hepatitis B virus; HPV, human papillomavirus; ISME, Swiss Institute of Equine Medicine; LR−, negative likelihood ratio; LR+, positive likelihood ratio; miRNA, microRNA; NGS, next generation sequencing; PCR, polymerase chain reaction; POP, post-test probability; PRP, pretest probability; ROC, receiver operating characteristic; RT-qPCR, reverse transcription quantitative PCR; SCC, squamous cell carcinoma.

1 | INTRODUCTION

Sarcoids are benign, but locally invasive, bovine papillomavirus (BPV)-associated fibroblastic tumors and represent the most common neoplastic condition in equids worldwide.[1-4] The gold standard diagnostic test for equine sarcoid (ES) disease is histopathology of tumor tissue specimens.[5] However, veterinarians often avoid biopsy because it may lead, as would any kind of trauma, to exacerbation of ES lesions.[6] Furthermore, except for an increased density of dermal fibroblasts, the histopathological features of ES are not consistent. Therefore, differentiation from other spindle cell tumors can be challenging, even with combined BPV-1 and -2 polymerase chain reaction (PCR) testing.[5,7] Thus, clinicians often rely on clinical diagnosis, which has satisfactory sensitivity and specificity of approximately 80%.[8] In challenging diagnostic cases and for less experienced veterinarians, the use of a clinical diagnostic protocol can improve the accuracy of diagnosis.[9] Additional attempts have been made to reach a definitive ES diagnosis by noninvasive means, such as detection of BPV-1 and -2 DNA from swabs or scrapings collected from the tumor surface.[10] However, this approach may lack sensitivity if minimal epidermal changes are present and if BPV loads are low. Specificity also may be decreased because BPV-1 and -2 DNA also may be present on the skin of ES-free horses.[11] Because neither invasive nor noninvasive methods for diagnosis of ES are universally valid, novel biomarkers are needed, ideally so-called liquid biopsies, which can be used as adjunctive diagnostic tools.

MicroRNAs (miRNAs) are small, noncoding RNA molecules involved in post-transcriptional regulation of the expression of almost two-thirds of all protein coding genes and may favor tumorigenesis at the tumor tissue expression (eg, in breast or colorectal cancer in humans).[12-14] MicroRNAs are present not only in cells, but also in a cell-free, circulating form. They are remarkably stable in diverse body fluids despite high RNase activity because they are bound to proteins and high-density lipoproteins or encapsulated in extracellular vesicles.[15] In cancer research in humans, circulating miRNAs are gaining increased attention as noninvasive diagnostic and prognostic biomarkers.[16] The differential expression of cellular miRNAs has been confirmed in ES tissues and BPV-transformed fibroblasts.[17-19] Furthermore, circulating whole blood and serum miRNA fingerprints have been proposed as potential prognostic and diagnostic biomarkers for ES disease.[20,21]

In an initial exploratory study using next generation sequencing (NGS), we found 9 miRNAs that were differentially expressed in the serum of ES-affected compared with control horses and proposed them as diagnostic biomarkers for fibroblastic and mixed-fibroblastic ES lesions.[21] The aim of the our study was to assess the potential of 3 out of the 9 candidate miRNAs as diagnostic biomarkers for ES disease in a larger and more diverse study cohort and using reverse transcription quantitative PCR (RT-qPCR). We hypothesized that miRNA expression would neither be influenced by biological variation among the tested individuals nor by the degree of hemolysis in serum, and that the candidate miRNAs would serve as unique biomarkers for ES disease.

2 | MATERIALS AND METHODS

2.1 | Study design and study cohort

For this retrospective case-control study, serum samples from skin tumor-bearing and tumor-free control horses and donkeys collected from February 2015 until December 2017 and stored in the bioarchives of the Swiss Institute of Equine Medicine (ISME) were included. The owners of all animals included in the study gave their informed consent that blood samples obtained by venipuncture for diagnostic or therapeutic purposes could also be used for research. Additional samples from cases and controls were collected during experiments approved by the Animal Experimentation Committee of the Canton of Bern, Switzerland (BE110/15 and BE 7/19). Individuals were assigned to the following 5 groups: ES-affected horses, ES-affected donkeys, horses with other skin tumors (melanoma, genital squamous cell carcinoma [SCC]), tumor-free control horses and tumor-free control donkeys). For all cases, age, sex, and breed were reported. Breeds were classified as follows: Warmblood, Franches-Montagnes, American Quarter Horse/Paint Horse, mixed breeds, pony breeds, Friesian, Arabian, Spanish breeds, Irish Cob/ Tinker, Rocky Mountain Horse, or Thoroughbred. Age categories were 1 to 5, 6 to 10, 11 to 15, 16 to 20, 21 to 25 and 26 to 30 years.

Inclusion criteria for cases with skin tumors were privately owned equids referred for the diagnosis and treatment of histologically confirmed (ES, genital SCC) or clinically or histologically diagnosed or both neoplastic conditions (melanoma). Exclusion criteria were equivocal histological or clinical diagnosis, incomplete phenotyping, or concurrent occurrence of other tumors. Skin tumors were classified according to their location and type. The ES lesions were categorized as occult, verrucous, fibroblastic, nodular, mixed fibroblastic, or mixed without fibroblastic component. In ES-affected equids, disease severity was scored as previously described,[22] with scores ranging from 3 (mild lesions) to 19 (severe lesions). Briefly, the following classification scheme was applied: score 3 to 8 = mild disease, score 9 to 14 = moderate disease, score 15 to 19 = severe disease. Melanoma cases were classified as melanocytic nevus/melanocytoma, dermal melanoma/melanocytosis, or melanosarcoma. Biological behavior was assessed histologically as benign, potentially malignant, malignant, or unknown. For genital SCC cases, the presence of metastases (none identified vs suspected local, local or distant metastases) was recorded.

The control group consisted of privately owned equids that were presented for non-neoplastic conditions, horses that accompanied them, and horses owned by the ISME. Cases only were included if they were free of skin tumors and other dermatological problems or neoplastic disease. Presenting complaints and diagnoses were recorded. Controls were selected by group matching to the variables species, breed, age, and sex. Matching was done to minimize biological variation when comparing ES-affected horses to tumor-free control horses and ES-affected donkeys to tumor-free control donkeys.[23] Cases and controls were not matched for comparison of ES-affected horses to horses with other skin tumors, because different breed

dispositions exist for ES disease compared to melanoma or genital SCC. Furthermore, ES disease often manifests earlier in life than do the other 2 skin tumors.[24-30]

2.2 | Sample collection and storage

Blood was collected from the jugular vein into serum tubes. For serum separation, blood was allowed to clot at room temperature for 30 minutes and then centrifuged at 1400*g* for 10 minutes. Serum was transferred into cryotubes (Sarstedt, Nümbrecht, Germany) and stored at −80°C in the ISME bioarchive before RNA extraction.

2.3 | Preanalytics

Ribonucleic acid[21,31] According to the manufacturer's instructions, extraction efficiency was monitored by addition of 5.6×10^8 copies of a synthetic spike-in control, cel-miR-39-3p (Ce_miR-39_1 miScript Primer Assay, Qiagen, Hombrechtikon, Switzerland), before RNA extraction. Hemolysis in serum samples was assessed by measurement of the hemolysis index using Cobas 6000 (Roche Diagnostics International AG, Rotkreuz, Switzerland) as previously recommended.[21] Samples with hemolysis index >15 were considered hemolyzed.

2.4 | Choice of candidate miRNAs

Three candidate miRNAs were selected from the panel of 9 differentially expressed serum miRNAs previously discovered in the initial exploratory study.[21] Eca-miR-486-5p was excluded from further analysis because it is highly abundant in equine plasma under physiological conditions.[32] The following 3 differentially expressed miRNAs were selected as candidate miRNAs for assessment of their potential as diagnostic biomarkers for ES disease: eca-miR-1, eca-miR-331, and eca-miR-100. Additionally, eca-miR-92a was used as an endogenous control because it is highly abundant in equine plasma.[32]

2.5 | Reverse transcription quantitative PCR

Information on all primers and probes included is depicted in Table S1. The extracted miRNAs were reverse transcribed using the TaqMan MicroRNA Reverse Transcription Kit (Fisher Scientific, Reinach, Switzerland) in combination with the individual miRNA specific stem-loop RT primer (TaqMan MicroRNA Assay, Fisher Scientific, Reinach, Switzerland). A modified multiplexed RT was prepared by pooling the stem-loop RT primers for the 3 miRNAs of interest (eca-miR-331, eca-miR-100, eca-miR1) and the endogenous and exogenous controls (eca-miR-92a, cel-miR-39-3p) as described elsewhere.[33] In a pilot experiment, this method was established using whole blood samples from 5 randomly assigned horses included in the study and then used as the standard method for the complete sample pool. All miRNA-specific RT primers were pooled and diluted in 1× Tris-EDTA (TE) buffer to obtain a final dilution of 0.05× each. Twelve microliters of this RT primer pool solution was added to the RT reaction mix containing 0.6 μL 100 mM dNTP, 6 μL enzyme (50 U/μL), 3 μL 10× RT buffer, 0.38 μL RNase inhibitor (20 U/μL), 6 μL of RNA eluate and 2 μL nuclease-free water, to reach a final volume of 30 μL. The RT reaction conditions were as follows: 30 minutes at 16°C for primer annealing, 30 minutes at 42°C for the extension phase and 5 minutes at 85°C to stop the reaction. The modified RT-qPCR reactions were performed the same day. The final volume was 25 μL and consisted of 2.5 μL cDNAs, 12.5 μL 1× TaqMan Universal PCR Master Mix, no AmpErase UNG (Fisher Scientific), 0.625 μL of 1× TaqMan MicroRNA Assay (Fisher Scientific), and 9.375 μL diethylpyrocarbonate water. All reactions were run in duplicate using a 7300 RT PCR System (Applied Biosystems, California) with 1 denaturing step at 95°C for 10 minutes, followed by 40 cycles consisting of denaturing at 95°C for 15 seconds, annealing and elongation at 60°C for 60 seconds, and a final inactivation step of 10 minutes at 99.9°C. Efficiencies of the RT-qPCRs were calculated using the standard curves generated with serial dilutions of the 5 tested miRNAs (Microsynth AG, Balgach, Switzerland). The RT-qPCR for all miRNAs had an efficiency of 99%.

For data normalization, we used the $2^{-\Delta\Delta Cq}$ (cycle quantification) method to measure the relative changes in miRNA expression (relative quantification).[34] The ΔCq is the Cq value of the candidate miRNA gene as well as of the endogenous control (eca-miR-92a) normalized to the exogenous control (cel-miR-39-3p), which allows correction for any technical error related to RNA extraction efficiency (ΔCq candidate miRNA gene = Cq candidate miRNA gene − Cq exogenous gene; ΔCq endogenous gene = Cq endogenous gene − Cq exogenous gene). The ΔΔCq is the ΔCq value of the candidate miRNA gene normalized to the ΔCq of the endogenous control (ΔΔCq candidate miRNA gene = ΔCq candidate miRNA gene − ΔCq endogenous gene). This calculation permits differentiation of true biological variation from experimentally induced artifacts mainly related to RNA input quantity. To obtain the relative amount of the expressed gene, the normalized value ΔΔCq was transformed to a negative value (−ΔΔCq), and then subjected to the exponent of the power of 2, because the Cq value is the negative log concentration of the gene and all calculations are in base 2 log.

2.6 | Statistics

The NCSS 12 software (NCSS, Kaysville, Utah) was used for statistical analyses. A chi-squared test was performed to determine the distribution of age, sex, and breed in all 5 groups of equids and of ES disease severity in the groups of ES-affected horses and donkeys. The miRNA expression data were not normally distributed and thus were

log-transformed. Influence of biological variation among equids, including species, breed, age, and sex, on the expression of the 3 candidate miRNAs was assessed using multivariable analysis of variance. Furthermore, in ES-affected equids, the effect of disease severity and type of ES tumors on expression of the candidate miRNA was investigated using 1-way analysis of variance. The same test was used to examine the effect of hemolysis and of health condition (ES-affected, tumor-free control, affected by other skin tumors) on expression of the candidate miRNAs. The miRNA candidates that were significantly influenced by the factor "health condition" then were compared among groups using the Mann-Whitney *U* test. If >2 groups were compared at a time, Bonferroni correction was manually performed by dividing the *P* value threshold of .05 by the number of multiple comparisons. For each candidate miRNA with a significant difference in expression among groups, an area under the curve (AUC) receiver operating characteristic (ROC) curve analysis was performed to calculate its specificity and sensitivity as a diagnostic serum biomarker for ES disease when compared to the diagnostic gold standard, which is histopathology of a tumor biopsy specimen. Additionally, the positive (LR+) and negative (LR−) likelihood ratios, as well as the diagnostic odd ratio (DOR), were evaluated. The post-test probability (POP) of using a specific miRNA to diagnose ES was calculated using the following formula: POP = PRP (pretest probability) × LR+/(1 − PRP + PRP × LR1).[35] The PRP was calculated as the sum of the number of true positive and true negative equids divided by the number of examined equids. On each occasion, *P* values ≤.05 were considered statistically significant.

3 | RESULTS

3.1 | Study cohort

In total, 123 equids with skin tumors and 90 tumor-free controls from the ISME bioarchive were assessed for eligibility. Of those, 88 horses and donkeys with skin tumors and 69 controls fulfilled the criteria for inclusion in the study. Reasons for exclusion from the study are depicted in Figure S1A for tumor-bearing and in Figure S1B for tumor-free control equids. Fifty-three ES-affected horses met the inclusion criteria and had a mean ± SD (range) age of 10.2 ± 5.6 (2-26) years. Of those, 3 horses were presented on 2 occasions (case numbers 1034/2112, 1831/1832, and 1899/2056), resulting in 56 ES cases. Furthermore, 12 ES-affected donkeys with an age of 8.3 ± 4.7 (3-15) years and 20 horses with other skin tumors (8 genital SCC and 12 melanoma) aged 18.4 ± 6.9 (3-27) years were included. Sixty tumor-free horses aged 12.0 ± 6.4 (2-29) years and 9 tumor-free donkeys aged 12.1 ± 3.9 (6-16) years were identified as tumor-free controls. Five control horses were clinic-owned, all other control horses and donkeys were privately owned. Age, breed, and sex distribution are shown in Tables 1-3. When comparing the groups of ES-affected horses to tumor-free control horses, no statistically significant differences regarding breed (*P* = .89), age (*P* = .68), and sex distribution (*P* = .84) were observed. In the groups of ES-affected donkeys vs tumor-free control donkeys, sex (*P* = 1) and age distribution (*P* = .05) did not differ significantly. As anticipated, ES-affected horses were younger than horses with melanoma or genital SCC (*P* < .001), but breed (*P* = .08) and sex distribution (*P* = .1) were not significantly different. Disease severity in ES-affected equids was as follows: 5 horses had mild, 19 horses and 5 donkeys had moderate, and 32 horses and 7 donkeys had severe disease manifestations. Equine sarcoid disease severity scores did not differ significantly between horses and donkeys (*P* = .53), with mean disease severity scores of 13.3 ± 3.3 (5-19) and 13.6 ± 2.6 (9-17), respectively. In the group of ES cases, 69.6% (39/56) had fibroblastic or mixed fibroblastic, 17.9% (10/56) mixed without fibroblastic component, 7.1% (4/56) only verrucous, and 5.4% (3/56) only nodular ES lesions. In the group of ES-affected donkeys, 10 donkeys had fibroblastic or mixed fibroblastic and 2 donkeys only nodular ES lesions. A more detailed classification of tumors and presenting complaints as well as diagnoses for tumor-free control equids are provided in Table S2.

TABLE 1 Age distribution in the study cohort

Age (years)	ES horses	Control horses	ES donkeys	Control donkeys	Horses with other skin tumors
1-5	(11/56) 19.6%	(8/60) 13.3%	(4/12)	(0/9)	(1/20) 5%
6-10	(26/56) 46.4%	(23/60) 38.3%	(3/12)	(3/9)	(1/20) 5%
11-15	(12/56) 21.4%	(13/60) 21.7%	(5/12)	(3/9)	(4/20) 20%
16-20	(3/56) 5.4%	(8/60) 13.3%	(0/12)	(3/9)	(6/20) 30%
21-25	(3/56) 5.4%	(6/60) 10%	(0/12)	(0/9)	(5/20) 25%
26-30	(1/56) 1.8%	(2/60) 3.5%	(0/12)	(0/9)	(3/20) 15%

Abbreviation: ES, equine sarcoid.

3.2 | Preanalytics

The RNA quantity was too low to be measured in 50 samples. In the remaining 107 samples, RNA quantity was 8.7 ± 6.5 (1-42.4) ng/μL (Table S2). In the 157 serum samples included in our study, the hemolysis score was 11.7 ± 14.3 (0-98) with 31 samples having a hemolysis score >15 and thus being considered hemolytic (Table S2).

TABLE 2 Horse breed distribution in the study cohort

Breed	ES horses	Control horses	Horses with other skin tumors
Warmblood	(20/56) 35.7%	(23/60) 38.3%	(5/20) 25%
Franches Montagnes	(7/56) 12.5%	(9/60) 15%	(0/20) 0%
American Quarter Horse/ Paint Horse	(6/56) 10.7%	(5/60) 8.3%	(0/20) 0%
Mixed breeds	(5/56) 8.9%	(2/60) 3.3%	(2/20) 10%
Pony breeds	(5/56) 8.9%	(7/60) 11.7%	(5/20) 25%
Friesian	(4/56) 7.1%	(4/60) 6.7%	(0/20) 0%
Arabian	(3/56) 5.4%	(3/60) 5%	(3/20) 15%
Spanish breeds	(2/56) 3.6%	(5/60) 8.3%	(4/20) 20%
Irish Cob/Tinker	(2/56) 3.6%	(1/60) 1.7%	(1/20) 5%
Rocky Mountain Horse	(1/56) 1.8%	(1/60) 1.7%	(0/20) 0%
Thoroughbred	(1/56) 1.8%	(0/60) 0%	(0/20) 0%

Abbreviation: ES, equine sarcoid.

3.3 | Reverse transcription quantitative PCR

The raw Cq values and the $2^{-\Delta\Delta Cq}$ values of the 3 candidate miRNAs in each serum sample included in our study are depicted in Tables S3 and S4. There was no effect of species, breed, age, and sex on expression of the examined miRNAs, except for eca-miR-100 which was influenced by the factor age (P = .03; Table 4). In individuals with ES, ES type and disease severity did not have significant effects on expression of the candidate miRNAs (Table 5). In contrast to expression of eca-miR-331, expression of eca-miR-1 and eca-miR-100 was influenced by hemolysis (P < .001 and P < .001, respectively; Table 4). Health condition affected expression of eca-miR-331 (P = .009), but not that of eca-miR-100 (P = .43) and eca-miR-1 (P = .57). Exclusion of hemolyzed samples did not significantly change the differences in miRNA expression among the different groups (eca-miR-331, P = .02; eca-miR-100, P = .26; eca-miR-1, P = .21). Because the factor "species" did not influence miRNA

TABLE 5 Effect of ES type and disease severity on relative miRNA expression in ES-affected equids

miRNA	ES type	ES disease severity
eca-miR-331	P = .35	P = .09
eca-miR-100	P = .51	P = .45
eca-mir-1	P = .23	P = .36

Abbreviations: ES, equine sarcoid; miRNA, microRNA.

TABLE 3 Sex distribution in the study cohort

Sex	ES horses	Control horses	ES donkeys	Control donkeys	Horses with other skin tumors
Mares	(26/56) 46.4%	(30/60) 50%	(6/12)	(5/9)	(7/20) 35%
Geldings	(26/56) 46.2%	(25/60) 41.7%	(6/12)	(4/9)	(12/20) 60%
Stallions	(4/56) 7.1%	(5/60) 8.3%	(0/12)	(0/9)	(1/20) 5%

Abbreviation: ES, equine sarcoid.

TABLE 4 Effect of species, breed, age, sex, and hemolysis on relative miRNA expression

miRNA	Species	Breed	Age	Sex	Hemolysis
eca-miR-331	P = .69	P = .28	P = .09	P = .25	P = .24
eca-miR-100	P = .44	P = .25	P = .03*	P = .84	P < .001*
eca-miR-1	P = .45	P = .92	P = .2	P = .8	P < .001*

Note: Significant P values are highlighted with an asterisk.
Abbreviation: miRNA, microRNA.

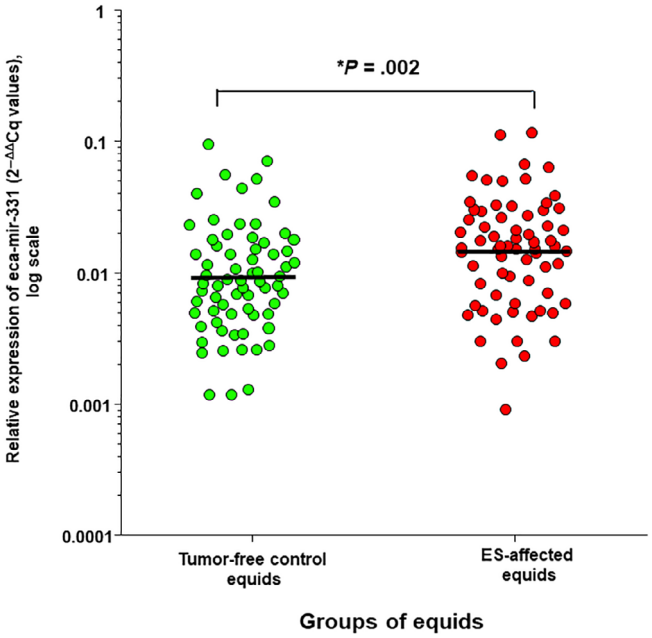

FIGURE 1 Dot plots depicting relative serum expression of eca-miR-331 in tumor-free control equids and equine sarcoid affected equids. The y-axis shows the $2^{-\Delta\Delta Cq}$ values of eca-miR-331 on a log scale. The horizontal lines represent the geometric mean. Asterisk represents the significant *P* value. Cq, cycle quantification

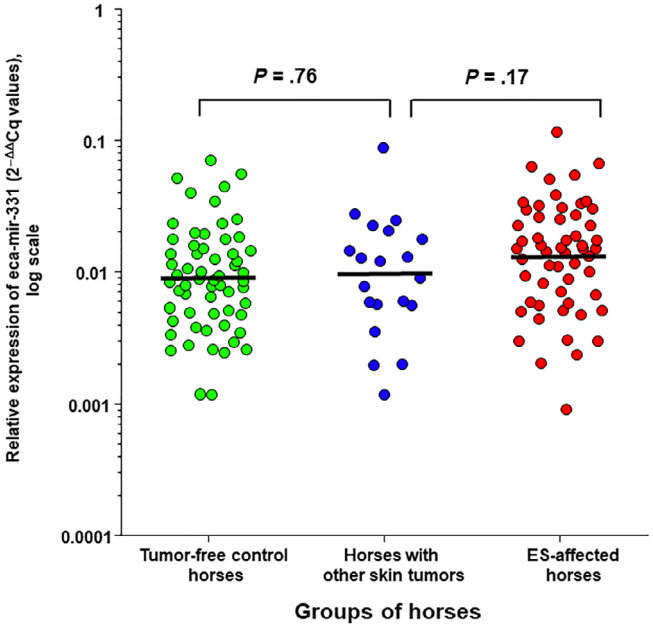

FIGURE 2 Dot plots depicting relative serum expression of eca-miR-331 in tumor-free control horses, horses with other skin tumors, and equine sarcoid affected horses. The y-axis shows the $2^{-\Delta\Delta Cq}$ values of eca-miR-331 on a log scale. The horizontal lines represent the geometric mean. Asterisk represents the significant *P* value. Cq, cycle quantification

expression, ES-affected horses and donkeys as well as tumor-free horses and donkeys were merged for comparison of expression of eca-miR-331 in ES-affected equids vs tumor-free control equids. In ES-affected equids (geometric mean = 0.014; 95% confidence interval [CI] = 0.011-0.018) compared to tumor-free control equids (geometric mean = 0.009; 95% CI = 0.007-0.011), significant upregulation of serum eca-miR-331 was observed (*P* = .002; Figure 1). The ROC curve analysis indicated AUC was 0.65 (*P* = .002; 95% CI = 0.54-0.73) with sensitivity of 60% (95% CI = 0.48-0.72) and specificity of 71% (95% CI = 0.6-0.81) for serum eca-miR-331 in discriminating ES-affected equids from tumor-free control equids (Figure S2). The LR+ and LR− for serum eca-miR-331 to diagnose ES were 2.1 (95% CI = 1.37-3.16) and 0.56 (95% CI = 0.4-0.78), respectively, with a DOR of 3.7 (95% CI = 1.83-7.58), PRP of 49.6%, and POP of 67%. Expression of eca-miR- 331 in serum of horses with other skin tumors (geometric mean = 0.009; 95% CI = 0.006-0.015) compared to tumor-free control horses (geometric mean = 0.009; 95% CI = 0.007-0.011) and compared to ES-affected horses (geometric mean = 0.013; 95% CI = 0.010-0.017) both were not significant (*P* = .76 and .17, respectively; Figure 2).

4 | DISCUSSION

Based on our findings, eca-miR-331 may serve as a serum biomarker to aid in the diagnosis of ES disease, but with only moderate specificity and sensitivity. Its expression was neither influenced by biological variation among individuals nor by the degree of hemolysis, which emphasizes its suitability as a robust biomarker in a diverse study population, using samples of variable quality. Eca-miR-100 and eca-miR-1 did not serve as discriminatory classifiers between ES cases and controls and additionally were influenced by hemolysis.

Biological variation among individuals strongly influences biomarker discovery and may even have a larger impact on the results of RT-PCR experiments than technical aspects.[36] In our study, hospital-based controls were chosen because they reflect the population from which cases are derived and are easier to recruit compared to population-based controls.[23] Particular attention was paid to match the groups of ES-affected horses and donkeys to tumor-free control horses and donkeys to minimize the effects of biological variation in the study cohort. Breed-related differences in miRNA expression have been described in horses, and age and sex have been found to influence miRNA fingerprints in humans.[37-40] In our study, the only miRNA the expression of which was influenced by biological variation among tested individuals (specifically, the factor age) was eca-miR-100. Species, breed, age, sex, ES type, and disease severity did not have effects on the expression of eca-miR-331 and eca-miR-1. Hence, both miRNAs may be considered potential biomarker candidates for any equid and any ES phenotype.

Hemolysis leads to leakage of red blood cell-derived miRNAs and thus changes circulating miRNA quantities in serum and plasma by up to 30-fold.[41] This effect substantially impairs interpretation of miRNA fingerprints in hemolyzed samples and must be taken into account as a confounding factor not only in studies of humans, but also equine

serum or plasma miRNA.[21,41-44] Hemolysis most commonly occurs in vitro as a complication of blood sample collection and further processing.[45] Additionally, persistent low-grade in vivo hemolysis may be present in horses exposed to daily active exercise.[44] Because horses are physiologically hyperbilirubinemic, we relied on absorbance measurements of oxyhemoglobin at high wavelengths, thus avoiding bilirubin interference as proposed previously.[21] In the initial exploratory NGS study, presence of hemolysis did not have any impact on differential expression of the 3 miRNAs selected as candidates for our study. Nonetheless, in the current RT-qPCR study using a larger sample size, the expression of eca-miR-100 and eca-miR-1 was found to be influenced by hemolysis, whereas the expression of eca-miR-331 was not. Serum and plasma miRNA studies in humans also have shown that amounts of some miRNAs, such as has-miR-451 or has-miR-16, are highly variable depending on the degree of hemolysis in the corresponding sample, whereas others such as has-miR-23a are largely unaffected by hemolysis.[43,45] In general, circulating miRNAs that are influenced by hemolysis should not be selected as biomarkers, because lysis of erythrocytes will introduce unpredictable changes to their serum and plasma quantities.[45] Thus, eca-miR-331 qualifies as a reliable serum biomarker candidate, but not eca-miR-100 and eca-miR-1.

Next generation sequencing is a very sensitive method for miRNA detection and relative quantification. Importantly, and unlike RT-qPCR, NGS allows for identification of novel miRNAs and hence is the most appropriate tool for miRNA biomarker discovery studies.[46] In contrast, RT-qPCR can only detect known miRNAs and therefore mainly is used in the validation phase, when possible candidate miRNAs have already been identified (as in our study). Compared to NGS, this method uses widely available and affordable equipment, and thus is the more useful modality for diagnostic purposes.[47] Reverse transcription-qPCR has high sensitivity for detection of miRNAs from samples with low RNA concentrations such as serum, as used in our study.[48] Even if sensitivity, specificity, and reproducibility of RT-qPCR generally are considered as good, it often is ignored that the RT is a highly variable and hence error-prone procedure,[49] that may introduce bias in miRNA output data.[46] Alternative methods that do not involve reverse transcription, such as miRNA enzyme immunoassays, are more robust and allow for more precise miRNA quantification.[46,50] Those procedures, however, are only suitable for samples with sufficient RNA quantity, such as whole blood and tissue, but not for serum.[46] Hence, RT-qPCR was considered the most appropriate method for miRNA detection and quantification in our study. The multiplex RT method, which to date had only been validated for human plasma samples,[33] enabled reverse transcription and preamplification of multiple equine serum miRNAs simultaneously, making it a convenient and cost-efficient procedure.

Based on our findings, only 1 of 3 tested candidate miRNAs holds promise as a diagnostic biomarker for ES disease. This result is best explained by the fact that the candidate miRNAs were selected based on results of the exploratory NGS study. Our study only used a small sample size,[21] resulting in limited statistical power.[51,52] In this context, the magnitude of a true effect might be overestimated and subsequently be lost or diminished in larger scale meta-analyses.[53] Furthermore, other currently unknown factors besides species, breed, age, sex, and preanalytical variables may influence circulating, cell-free miRNA expression. In humans, it has been shown that one-third of plasma miRNAs show diurnal variations and approximately 20% of serum, plasma, and specific white blood cell subpopulation miRNAs show random but significant, intraindividual variation over a 1-year period.[54,55] Time-dependent variation in miRNA expression was not assessed in our study. In future studies, blood samples for circulating miRNA analysis should be collected at multiple time points throughout the day and throughout the entire study period to assess if diurnal and long-term miRNA variability also occurs in equids and needs to be taken into account for interpretation of miRNA expression.

Eca-miR-331 was significantly upregulated in serum of ES-affected horses when compared to tumor-free control equids. Dysregulation of its human analogue, hsa-miR-331, has been reported in various types of cancer in humans, both in tumor tissue samples and in cell cultures. Hsa-miR-331 is mainly thought to act as a tumor suppressor,[56-63] but also may be oncogenic.[64,65] Moreover, hsa-miR-331 has been found to be differentially expressed in virus-induced malignancies. For example, in hepatitis B virus (HBV)-associated hepatocellular carcinoma, HBV was shown to promote expression of hsa-miR-331 and thus indirectly inhibit the expression of its target gene, the tumor suppressor gene ING5.[65] In human papilloma virus (HPV)-associated cervical cancer, upregulation of hsa-miR-331 was shown to suppress the expression of its target gene NRP2, which in turn led to downregulation of the main HPV oncoproteins E6 and E7 and thus to decreased cell proliferation, induction of cell cycle arrest and apoptosis.[66] In BPV associated ES disease, differential expression of eca-miR-331 so far has only been detected in the serum of ES-affected equids, but not in ES-derived tissues nor in BPV-transformed fibroblasts.[17-19,21] Whether or not miRNAs influence the expression of BPV oncoproteins in the tumor microenvironment, or in turn, if BPV influences cellular or circulating miRNA expression remains elusive.

In humans, serum or plasma hsa-miR-331 is considered a promising diagnostic and prognostic biomarker for hepatocellular carcinoma,[67,68] esophageal adenocarcinoma,[69] and for differentiation of local luminal vs metastatic breast cancer.[70] For specific diagnostic indications, hsa-miR-331 even may outperform established tumor markers.[68] In equids, however, serum eca-miR-331 increased the probability of correctly identifying an ES-affected equid from 50% to only 67%, as determined by calculations of PRP and POP, and its sensitivity and specificity for diagnosis of ES disease were inferior to clinical diagnosis. This outcome is not surprising because a single miRNA often is not specific enough for diagnosing a particular disease.[71] However, the combination of different candidate miRNAs in a biomarker panel may improve diagnostic value and differentiation among pathologies.[72]

We evaluated a selection of serum miRNAs that were proposed as diagnostic biomarkers for ES disease.[21] Serum is the most commonly used biofluid for circulating biomarker studies because the miRNA composition is stable for up to 10 years if stored at −80°C.[73,74] As stated previously, whole blood also may be used for

equine miRNA analysis. Whereas preanalytical sample handling is less error-prone, and the RNA and thus also miRNA content higher compared to serum, other factors, most importantly the degree of RNA degradation and changes in white blood cell counts, must be considered as possible confounding factors for whole blood miRNA expression.[21] Thus, use of either serum or whole blood for miRNA studies has specific advantages and disadvantages. Taking all of these considerations into account, in addition to testing more serum miRNAs as biomarkers for ES disease, further validation of whole blood miRNA biomarker candidates should be performed in a larger study cohort.[21]

Serum quantities of eca-miR-331 could only discriminate between ES-affected and tumor-free equids, but not between ES-affected equids and horses with other skin tumors. This result may have been because of limited statistical power, because a comparatively small group of horses with other skin tumors was used in our study. Even if eca-miR-331 is only a nonspecific skin tumor marker in equids, it still might be helpful as an adjunctive diagnostic tool in clinical settings. Because of their typical appearances, ES, melanoma, and SCC are rarely confused during clinical examination. However, nonneoplastic skin conditions such as eosinophilic granuloma, fly bite reactions, granulation tissue formation, or habronemiasis may be easily mistaken for ES lesions. Depending on level of expertise, clinical diagnosis of skin disorders resembling ES results in up to 30% false-positives and thus overdiagnosis of ES disease.[8] In this context, additional testing for serum eca-miR-331 potentially may help to corroborate or reject a clinical diagnosis of ES. To test this possibility, serum eca-miR-331 activity in horses and donkeys with ES-like lesions, but with histopathological exclusion of ES-disease, should be compared to results in equids with histopathologically confirmed ES disease.

To our knowledge, ours is the first study to examine miRNA expression in donkeys. Although the number of ES-affected donkeys and tumor-free control donkeys was limited, we deemed it important to include samples from donkeys, because ES disease is a highly prevalent condition in donkeys and additional noninvasive diagnostic tools are needed for them.[2,27,75] On the assumption that miRNA genes are highly conserved across species,[76] homologues of the equine candidate miRNAs that we examined in donkey serum samples should be renamed eas (*Equus asinus* asinus)-miR-331, eas-miR-100, and eas-miR-1. The expression of the 3 candidate miRNAs did not significantly differ between horses and donkeys. Thus, our study provides some of the first evidence that ES-affected donkeys might have miRNA fingerprints similar to those of ES-affected horses. This possibility may represent an important starting point for future miRNA research in donkeys.

In conclusion, eca-miR-331 may serve as a serum biomarker for ES disease in equids, but only if it can be combined with other discriminatory miRNAs in a panel. As a solitary measurement, serum eca-miR-331 expression is not sufficient for routine diagnostic testing in equine practice, because it lacks sufficient sensitivity and specificity. Nonetheless, a diagnostic miRNA panel may be a useful adjunctive diagnostic tool for challenging, atypical ES cases, particularly if combined with other noninvasive tests such as clinical examination and BPV PCR using a swab taken from the putative ES lesion.

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CONFLICT OF INTEREST DECLARATION

Authors declare no conflict of interest.

OFF-LABEL ANTIMICROBIAL DECLARATION

Authors declare no off-label use of antibiotics.

INSTITUTIONAL ANIMAL CARE AND USE COMMITTEE (IACUC) OR OTHER APPROVAL DECLARATION

All animals only participated after an informed consent was given by the owner for research use of blood samples from venipuncture performed for diagnostic or therapeutic reasons. Further samples from cases and controls were collected in the context of experiments approved by the Animal Experimentation Committee of the Canton of Bern, Switzerland (BE110/15 and BE7/19).

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SUPPORTING INFORMATION

Additional supporting information may be found online in the Supporting Information section at the end of this article.

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