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Research paper

Comprehensive characteristics of microRNA expression profile of equine sarcoids

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ABSTRACT

Equine sarcoids are the most common neoplasms occurring in horses. Despite frequent occurrence, they are still not well described at the molecular level. Thus, in the present study, we performed a comprehensive comparative analysis of sarcoid miRNAome profile to identify aberrantly expressed microRNAs, along with their structural variants, potentially useful as biomarkers and, in a wider perspective, broaden the knowledge about this tumor and underlying mechanisms. To this end, we conducted next generation sequencing and as a result we identified both known and potentially novel miRNAs. Differential expression analysis revealed the existence of almost one hundred miRNAs being over- or underexpressed in sarcoids in comparison to healthy tissue (p-adj<0.05), of which many are known for their involvement in processes crucial for neoplastic transformation. Among upregulated miRNAs there were those associated with decreased cell adhesion abilities as well as engaged in global protein production, while downregulation of some miRs i.a. increased cell expansion abilities. Moreover, we identified altered expression levels of miRNA variants (isomiRs) between the investigated tissues. Further analysis revealed that 5′ isomiRs comprise different seed sequences leading to target gene switching followed by activation of different biological pathways. Our results are the first which revealed the complexity of microRNA profiles in equine sarcoids and skin tissue, along with the dynamism of their growing in importance concomitants, namely isomiRs. They also showed miRNA molecules and biological pathways important from the sarcoid oncogenesis point of view.

1. Introduction

microRNAs, belonging to the class of small non-coding RNAs [1], have an ability to orchestrate gene expression through binding to gene transcripts. It further leads to translational repression, degradation [2] or in some cases up regulation of target gene expression [3].

Numerous miRNA-focused studies [4–7] showed their importance and engagement in the regulation of a wide range of vital biological processes such as development, metabolism or apoptosis [8,9]. Considering that these processes are essential for proper cell functioning, it is unsurprising that alterations of microRNA expression profiles were identified in the course of different diseases, such as cancer [10,11]. In the carcinogenesis process, miRNAs can function as oncogenes (e.g. miR-155) or tumor suppressors (e.g. miR-15a) [12]. A unique combination of oncogenic and tumor suppressor microRNA expression profiles constitutes tumor signature [8,12]. These signature microRNAs may serve as potential biomarkers for early diagnosis, staging, prognosis as well as response to treatment [12,13]. For example, serum levels of miR-21 were correlated with diagnosis and prognosis of colorectal cancer

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Despite a significant improvement in the field of identification of new miRNA molecules in recent years, the coverage of microRNA profiles of livestock species is still lacking. The horse can be a good example with its 690 records in miRBase Release 21.0 [15,16]. When it comes to characterization of horse miRNAome profile in pathological states, the data are even more scarce. Research conducted so far concerned osteochondrosis [17] and bovine papillomavirus type 1 transformed equine cells [18]. The latter studies were focused on a cell culture model of sarcoid tumor, which is the most common skin cancer occurring in horses. It is estimated that it may constitute as much as 40% of all diagnosed cancers [19–21]. Sarcoids are classified as locally malignant, benign fibroblastic tumors of fibrous tissue [22], differing in aggressiveness [23–25]. Despite the fact that they do not metastasize, they may recur if treatment is not carried out properly [26]. In sporadic cases, complete and constant remission is observed [27] but, on the other hand, protracted, unsuccessful treatment may lead to animal euthanasia. BPV (Bovine Papillomavirus) is believed to be the causative agent, however, the mechanism of infection and its oncogenic activity have not been fully understood yet [22,28,29].

Since horses are of great importance in livestock production, it is vital to acquire knowledge about mechanisms which orchestrate gene expression and may be disrupted in the course of carcinogenesis. Therefore, taking into account the fact that different types of cancers exhibit perturbed expression profiles of microRNAs which are involved in the oncogenesis process, we hypothesized that sarcoid cells are also characterized by altered expression of oncomiRs and their structural variants. We applied next generation sequencing along with a subsequent bioinformatical analysis. The obtained results will shed light on the horse miRNAome profile and its disruptions caused by neoplastic transformation to sarcoid. This will further facilitate understanding of microRNA functioning and mechanisms engaged in the oncogenesis process occurring in sarcoids.

2. Materials and methods

2.1. Small RNA library preparation

Tissue samples from equine sarcoids as well as healthy skin tissue from five horses were collected in Equine Clinic in Bern, Switzerland as well as in Horse Clinic Służewiec in Warsaw, Poland. The samples were provided from clinical patients treated for equine sarcoid disease in those clinics. Sample collection for this study was approved by the Animal Experimentation Ethics Committee of the Canton of Berne, Switzerland (BE 30/11; 11 April 2011). For the samples obtained in Horse Clinic Służewiec in Warsaw, Poland, Part 1, Division 1.2, Paragraph 1 of "Legislation for the protection of animals used for scientific or educational purposes" of Poland, stating that no Ethic Committee approval is needed when providing veterinary services, was applied. Moreover, all efforts were made to minimize suffering of animals. Immediately after excision, the samples were immersed in RNAlater Stabilization Solution (Thermo Fisher Scientific) and stored in −20 °C. Total RNA was isolated using Direct-zol RNA kit (Zymo Research) according to the manufacturer protocol. The RNA quality control was carried out with the use of 2200 TapeStation instrument (Agilent). Then, one μg of total RNA was used to construct libraries from each sample following the TruSeq Small RNA Sample Preparation Kit protocol (Illumina, protocol 15004197 Rev.F, February 2014). In brief, the samples were ligated with 3′ and 5′ adapters, respectively. Then, RNA-adapter ligation products were reverse transcribed and PCR amplified using one of 12 different indexed primers for each sample. Next, the libraries were gel-purified on Novex 6% TBE PAGE gel (Invitrogen). The quality of the obtained libraries was checked using 2200 TapeStation instrument (Agilent) whereas concentration was assessed using Qubit 2.0 Fluorometer (Life Technologies). Furthermore, the libraries were mixed with the PhiX control library (Illumina). Then, they were clustered on Illumina Flowcell_v3 in cBot cluster station and sequenced with the use of HiScanSQ (Illumina) according to the manufacturer protocol, applying 36 cycles.

2.2. Data analysis

The resulting reads were analyzed with the use of UEA sRNA Workbench V3.2. [30]. First, 3′ adapter sequences were trimmed off and then the obtained sequences were filtered using the following parameters: 17–35 nt in length, minimum abundance of at least 3 supporting reads, tRNA and rRNA sequences excluded from the data. Then, miRNA sequences were identified applying miRCat tool with default parameters for animals, except for: minimum abundance (3 or greater), minimum length (17 nt) and maximum length (25 nt). The obtained miRNAs were then checked for the concordance with the miRBase 21.0. Next, to profile miRNAs' abundance, miRProf tool was used applying the following parameters: minimum length of 17 nt, maximum length of 25 nt. The potentially new sequences were then checked for the presence of other sRNA species, such as snoRNAs, using RNAcentral database (Release 5) [31].

2.3. Differential expression analysis

To characterize differential expression (DE) of isomiRs, first, we prepared a library of reference sequences on the basis of miRBase 21.0. Then, we assigned a unique number to each isomiR of a miRNA and carried out the DE analysis using two algorithms applying different methodological approaches, namely: DESeq2 (a parametric method based on the negative binomial distribution) [32] and SAMSeq (a nonparametric method based on the Wilcoxon test) [33]. The paired tests were used for both approaches. For further analyses, only miRNAs showing statistically significant differences between the tested samples ($p \leq 0.05$ for both algorithms, corrected for multiple testing) were chosen to augment the likelihood of the estimation.

2.4. Pathway analysis

In order to identify target genes and pathways potentially modulated by the differentially expressed microRNAs, the mirPath DIANA Tools web application [34] was used with DIANA − TarBase v7.0 as a reference database of target genes.

When it comes to the analysis of pathways in which potentially new miRNA are engaged, first, we employed MR-microT software [35,36] to predict target genes for four chosen novel miRNAs. Then, the target genes were subjected to pathway analysis using KEGG Pathway Database [37].

2.5. IsomiRs' analysis

First, the analysis of alternative seed sequences was carried out. 5′ isomiRs of all miRNAs detected in the study were extracted from the obtained data, and then prediction of their target genes using TargetRank software was performed [38]. Pathway analysis was carried out with the use of KEGG Pathway Database [37]. Next, the analysis of modification types of isomiR sequences was conducted. Estimations of occurrences of particular nucleotides at both ends of isomiRs were carried out and the obtained results were then subjected to Chi-square test.