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ORIGINAL ARTICLE

Identification of differentially expressed microRNAs in the skin of experimentally sensitized naturally affected atopic beagles by next-generation sequencing

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Abstract

Canine atopic dermatitis (AD) is a very common inflammatory skin disease, but limited data are available on the genetic characterization (somatic mutations, microarrays, and genome-wide association study (GWAS)) of skin lesions in affected dogs. microRNAs are good biomarkers in inflammatory and neoplastic diseases in people. The aim of this study was to evaluate microRNA expression in the skin of atopic beagles, before and after exposure to *Dermatophagoides farinae*. Four atopic and four unrelated age-matched healthy beagle dogs were enrolled. Total RNA was extracted from flash-frozen skin biopsies of healthy and atopic dogs. For the atopic dogs, skin biopsies were taken from non-lesional (day 0) and lesional skin (day 28 of weekly environmental challenge with *Dermatophagoides farinae*). Small RNA libraries were constructed and sequenced. The microRNA sequences were aligned to CanFam3.1 genome. Differential expressed microRNAs were selected on the basis of fold-change and statistical significance (fold-change ≥ 1.5 and $p \leq 0.05$ as thresholds. A total of 277 microRNAs were sequenced. One hundred and twenty-one differentially regulated microRNAs were identified between non-lesional and healthy skin. Among these, two were increased amount and 119 were decreased amount. A total of 45 differentially regulated microRNAs between lesional and healthy skin were identified, 44 were decreased amount and one was increased amount. Finally, only two increased amount microRNAs were present in lesional skin when compared with that of non-lesional skin. This is the first study in which dysregulation of microRNAs has been associated with lesional and non-lesional canine AD. Larger studies are needed to understand the role of microRNA in canine AD.

Keywords microRNA · Dog · Atopic Dermatitis · Skin

Rosanna Marsella and Paolo Ciaramella are senior authors on this work. Domenico Santoro and Antonio Di Loria equally contributed to this work.

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Introduction

Atopic dermatitis (AD) is a common pruritic, chronic, and inflammatory skin disease affecting people and dogs. In both species, an occurrence of around 10% has been reported (Marsella and De Benedetto 2017; Hillier and Griffin 2001). In the past decades, because of extreme immunological and structural similarities between human and canine AD, the recognition and use of the dog as spontaneous animal model of human AD has significantly increased (Marsella and De Benedetto 2017; Hillier and Griffin 2001; Santoro and Marsella 2014). In both people and dogs, the pathogenesis of AD is not completely elucidated. However, alterations of the skin barrier integrity and dysregulation of the cutaneous microbiota and of the immune system, paired with a genetic predisposition, play major roles in the pathogenesis of AD (Ledin et al. 2006; Tengvall et al. 2013; Pucheu-Haston et al. 2015a, b, c; Bizikova et al. 2015; Santoro et al. 2015; Tengvall et al. 2016; Ardesjö-Lundgren et al. 2017).

In human AD, complex genetic alterations have been identified (Bizikova et al. 2015). In particular, loss of function mutations in the filaggrin gene, R501X and 2282del4, represent the most important genetic alterations correlated with AD severity (Thyssen et al. 2012). More recently, single nucleotide polymorphisms (SNPs) in Toll-like receptor 2 and cytokine production have also been associated with AD in people, confirming the complex and multifactorial pathogenesis of AD (Salpietro et al. 2011; Oh et al. 2009).

In veterinary medicine, only few studies have shown an association between genetic alterations and AD in dogs. In 2010, Wood et al. (2010), investigating the presence of filaggrin mutations in several canine breeds worldwide, detected a filaggrin mutation only in Labrador retriever dogs living in the UK. These results were confirmed by another European study showing filaggrin protein abnormalities present in different canine breeds (Chervet et al. 2010). Furthermore, in a genome-wide association study (GWAS) (Wood et al. 2009) using a cohort of 242 atopic and 417 control dogs, multiple SNPs were identified. After meta-analysis, two specific SNPs, RS22114085 and RS23472497, were shown in all eight breeds tested, with an additional 12 SNPs identified only in some of the breeds (Wood et al. 2009). Unfortunately, filaggrin was not covered in the GWAS array used. Similar results were obtained from a more recent GWAS study on West Highland white terriers in the USA, showing mutations on genes associated with skin barrier function on CFA3 (Agler et al. 2019). However, once again, filaggrin was not present in the GWAS array (Agler et al. 2019). Altogether, these studies show the complexity of this inflammatory skin disease in both humans and dogs and how little it is known on the genetic abnormalities occurring in canine AD.

More recently, the identification of microRNA (miRNA) has significantly advanced the knowledge of the pathogenesis of many inflammatory and neoplastic disease in people (Rożalski et al. 2016; Dissanayake and Inoue 2016). microRNAs are abundant (more than 2500), short (22–25 nucleotides), single-stranded non-coding RNA molecules (Rebane and Akdis 2013). They are generated as short hairpin and processed in a linear form by a specific enzyme (dicer-1) (Rebane and Akdis 2013). microRNAs are silencing molecules, and they downregulate post-transcriptional gene expression via translational repression or mRNA degradation (Rebane and Akdis 2013). They may affect RNA expression in different ways; they cleave mRNA strands, destabilize mRNA shortening the poly(A) tail, and inhibit protein translation (Rebane and Akdis 2013). microRNA can function as messengers between cells or even organs; miRNAs have been described in cells and in body fluids (serum, plasma, urine, and saliva). Because miRNAs are an ancient component of the gene regulation system, they are highly conserved among species (Buza et al. 2014), and their alterations have been identified in neoplastic, inflammatory, and immunological disorders (Rebane and Akdis 2013). Because of their conserved structure, the ability to move between tissues and the strong correlation with pathological processes make miRNAs ideal biomarkers of physiological and pathological processes in humans and animals (Buza et al. 2014).

Recently, alteration of the expression of miRNAs has been identified in atopic humans (Rożalski et al. 2016; Dissanayake and Inoue 2016). In particular, three studies have analyzed the expression of miRNA in lesional and non-lesional skin of atopic patients and compared them with healthy or psoriatic skin (Sonkoly et al. 2007, 2010; Vennegaard et al. 2012). The results of these studies showed an upregulation of ≥ 10 miRNAs and a downregulation of about 30 miRNAs in atopic skin when compared with that of healthy controls. Interestingly, in one study, a partial overlap in altered miRNAs was seen between AD and psoriasis (Sonkoly et al. 2007). Among the miRNAs selected in AD patients, miR155 and miR146a were both upregulated in human AD, and they have attracted the most attention for their action on T helper cells and on both innate and adaptive immunity.

In dogs, very few studies have been published investigating the expression of circulating or tissue miRNAs in healthy (Ichii et al. 2014; Koenig et al. 2016) and diseased (infective respiratory (Zhao et al. 2014), or degenerative valvular disease (Hulanicka et al. 2014)) dogs. In particular, Koenig et al. (2016) have analyzed the tissue expression of miRNAs in healthy beagle dogs. In that study (Koenig et al. 2016), 16 tissues from five healthy male beagle dogs were analyzed, and a total of 106 tissue-specific enriched miRNAs were identified. However, no information was provided on the expression of cutaneous miRNAs. Thus, the goal of this study was to evaluate the expression of miRNAs in canine atopic (lesional and non-lesional) skin compared with healthy skin from breed- and age-matched dogs.

Materials and methods

Animals

Four atopic and four healthy, unrelated, age-matched beagle dogs were enrolled in the present study. The atopic dogs belong to a colony of naturally allergic dogs previously epicutaneously sensitized to *Dermatophagoides farinae* (DF). The colony has been used and validated in multiple, previously published studies as canine model of AD in dogs and people (Marsella and De Benedetto 2017; Hillier and Griffin 2001; Santoro and Marsella 2014).

Healthy, unrelated beagle dogs with no history of skin disease and pruritus were included in this study as breed- and age-matched controls. Healthy dogs were recruited either from the authors' institutions (Italy) for spaying and neutering ($n = 2$) or as part of a research colony of healthy beagles in the USA ($n = 2$). All procedures were approved by the Institutional Animals Care and Use Committee at the authors' institutions.

Housing

Atopic dogs were housed in a facility in individual temperature- and humidity-controlled cement runs (22–24 °C; relative humidity of 68–72%). The runs were washed daily with high temperature and high-pressure wash (water and bleach). Filters are changed routinely, and stuffed toys, carpets, soft bedding, or anything that could trap dust are not allowed in the runs. Walls of the runs and filters are checked monthly to ensure the absence of DF in the environment (MITE-T-Fast™ Allergen Detection System, Aveho Biosciences).

Chronic environmental challenge with DF

All atopic dogs were exposed to DF as previously described (White et al. 2018). Briefly, DF was prepared from a pure culture of natural DF mites (Greer Laboratories Inc., Lenoir, NC, USA) and mixed with filtered (0.2-μm syringe filter) phosphate buffer solution (pH = 7.2) to obtain a final concentration of 31 mg/ml. The solution was vortexed and rocked for 30 min prior to use. Dogs had 1.6 ml (total of 50 mg DF/exposure) of the solution smeared on the ventral aspect of their chest and abdominal area. All dogs were monitored for 30 min to ensure that the solution had sufficient contact time. The environmental challenge was performed every 7 days after the first skin biopsy (days 7, 14, 21, and 28) to keep the lesions active and mimic a chronic allergen exposure.

CADESI-03 scoring protocol

Clinical evaluation was performed using the previously validated CADESI-03 scoring system (Olivry et al. 2007). This is a validated clinical scoring system for assessment of canine AD disease severity in clinical trials and research studies. The maximal achievable CADESI-03 is 1240. All atopic dogs were blindly scored before and 6 and 24 h after each allergen exposure.

Skin sample collection

Two 8-mm skin biopsy punches, one from non-lesional (day-0) and one from lesional areas (day-28; erythematous maculopapular dermatitis), on the inguinal region were collected from all dogs following subcutaneous injection of 1 ml of buffered lidocaine (Lidocaine HCl 2%; Hospira Inc., Lake Forest, IL, USA). The skin biopsy specimens were immediately divided into quarters. Quarters were placed in 1.5-ml microfuge tubes and quickly frozen in liquid nitrogen and stored at − 80 °C until being processed for molecular analysis.

Sample collection and RNA isolation

Total RNA was isolated from skin biopsies as previously described (White et al. 2018). Briefly, one quarter of the 8-mm biopsy punch was homogenized using a PowerGen 125 homogenizer (Fisher Scientific, Pittsburgh, PA, USA) and then processed into RNA using the 5 PRIME PerfectPure™ RNA fibrous tissue kit (5 PRIME, Inc., Gaithersberg, MD, USA) according to manufacturer's protocols including the DNAse treatment. The RNA concentration, purity, and integrity were assessed by measuring the A260/A280 ratios using the Agilent 2200 TapeStation (Agilent Technologies Inc. Santa Clara, CA, USA) and the High-Sensitivity RNA ScreenTape assay (Agilent Technologies).

Library preparation analysis of miRNA sequences

The Illumina TruSeq Small RNA (Illumina San Diego, CA 92122 USA) sample preparation kit was used to generate the small RNA libraries starting from 1 μg of the total RNA sample. According to the manufacturer's instructions, miRNA libraries were prepared following the following steps: (1) 3′ adaptor ligation, (2) 5′ adaptor ligation, (3) cDNA synthesis, (4) PCR amplification, and (5) selection of size-specific ≈ 150 base pairs (bp) PCR amplicons (corresponding to the 22 nucleotides (nt) miRNAs). Next, the quality of each sequencing library was assessed using the Agilent 2200 TapeStation (Agilent Technologies). The small RNA of approximately 140–160 bp in size was excised from the gel, incubated overnight, and eluted using a spin column the next day. The obtained libraries were then validated using Agilent High-Sensitivity D1000 ScreenTape System (Agilent Technologies).

The libraries were adjusted to 10 nM before cluster generation. The samples were then denatured into single-stranded DNA molecules, captured on Illumina flow cells, amplified in

situ as clusters, and finally sequenced for 36 cycles on an Illumina HiSeq2500 sequencer (Illumina), according to the manufacturer's instructions. The HiSeq Rapid SBS Kit v2 (50 cycles) reagents following the manufacturer's protocol were used for sequencing.

Quantitative reverse transcriptase PCR biological validation

To verify the reliability of the NGS results, one hundredth of the cDNA of RNA extracted from three healthy and three lesional skin samples was amplified by quantitative reverse transcriptase PCR (qRT-PCR). Reactions were performed in triplicate and in a total volume of 20 µl. qRT-PCR reactions were performed using a Bio-Rad iQ™ 5 apparatus (Bio-Rad Laboratories) with the following conditions: initial denaturation step at 95 °C for 10 min, followed by 40 cycles of 10 s at 95 °C, and 1 min at 60 °C. Specificity of PCR products was checked by melting curve analysis. The same protocol was adopted to analyze miR-215-5p, miR-409-3p, and miR-187-3p expression profiles. Cel-miR-39 was used to normalize between samples in the analysis. The results were analyzed using the comparative C_T (cycle threshold) method, and the relative mRNA expression was determined using the C_T method comparing healthy and affected dogs.

Data analysis

Sequencing data were processed using the Illumina bcl2fastq software in order to demultiplex and generate Fastq files. The barcode look-up uses a maximum mismatch of 1 (MM = 1).

Quality control (QC) analysis was performed using the FastQC software, and single-end reads (raw reads) were filtered following several criteria: positive selection of reads with mean Phred quality > 30; reads with more than two nucleotides with QC lower than 30 and with uncalled bases were discarded. Cutadapt script was used to trim the 3′ adaptors (≥ 15 nt). Alignment was done using a computationally optimized Burrows-Wheeler transform (BWT) algorithm (COBWeB in StrandNGS Avadis Software), and sequences were mapped, allowing up to one mismatch, on CanFam3.1 small RNA-seq annotation (updates on 2016-10-07). Small RNA repository annotations were available from the Avadis NGS server and contain gene annotations for multiple small RNA species, such as miRNA precursor genes, tRNAs, snoRNAs, scRNAs, snRNAs, etc., according to miRBase version 21. In addition, the miRNA precursor genes were also annotated depending on the chromosomal locations of the mature miRNA sequences. miRNAs were considered expressed if at least 5 reads/sample were detected. Identification of differentially expressed miRNAs was performed with the Bioconductor DESeq package (Anders and Huber 2010).

miRNAs were considered differentially expressed if they presented a p value ≤ 0.05 and foldchange ≤ − 1.5 and ≥ 1.5. The Ingenuity Pathway Analysis (IPA, Ingenuity System Inc., Redwood City, CA) database was performed in order to generate an enrichment analysis. The IPA (Ingenuity System Inc., Redwood City, CA) database was interrogated to better characterize biological functions.

Results

The results of the clinical evaluation (CADESI-03 scores) are presented in the supplemental material (S1).

After RNA extraction, usable miRNA sequences were obtained from four dogs (day 0) and three dogs (day 28) after DF exposure and from four healthy dogs (control). One RNA sample obtained in day 28 was excluded because the RNA was too degraded for next-generation sequencing analysis.

The miRNA sequences were aligned to CanFam3.1 genome, and a total of 277 miRNAs were identified after sequence alignment. Differentially expressed miRNAs were selected based on the fold-change and statistical significance. In order to detect the differential miRNA expression between the different conditions, the miRNA expression profile of non-lesional atopic skin (day 0) was compared with that of healthy skin. This matching allowed the identification of 121 differentially regulated miRNAs; of those, two were increased amount (miR423a; miR193b) and 119 were decreased amount (Fig. 1 and S1). The comparison between lesional atopic skin (day 28) and healthy skin generated a total of 45 differentially regulated miRNAs; 44 were decreased amount and one was increased amount (miR409) (Fig. 1). The complete list of miRNAs is present in S1–2. Finally, the comparison between the miRNA profile of non-lesional atopic skin (day 0) and lesional atopic skin (day 28) revealed changes in only two transcripts (miR215 and miR409); both miRNA transcripts had increased levels after parasite exposure (S1–2).

In order to identify common expressed miRNAs between the different experimental conditions, the three generated lists were intersected with each other (Fig. 2 and S1–2). miRNAs were not shared among the three different sets (day 0 versus control; day 28 versus control; day 0 versus day 28). On the contrary, two miRNAs showed a significant upregulation when two sets were overlapped: miR409 (between day 0/day 28 and day 28/control) and miR215 (between day 0/day 28 and day 0/control). miR215 was significantly increased in amount in lesional atopic skin (day28) when compared with that in healthy control skin, whereas miR409 was only significantly increased in amount when lesional atopic skin (day 28) was compared with healthy control skin.

In order to gain insights into the biological implications of the data collected, the miRNA profiles were analyzed by the IPA. The differential expressed miRNAs fell into one major

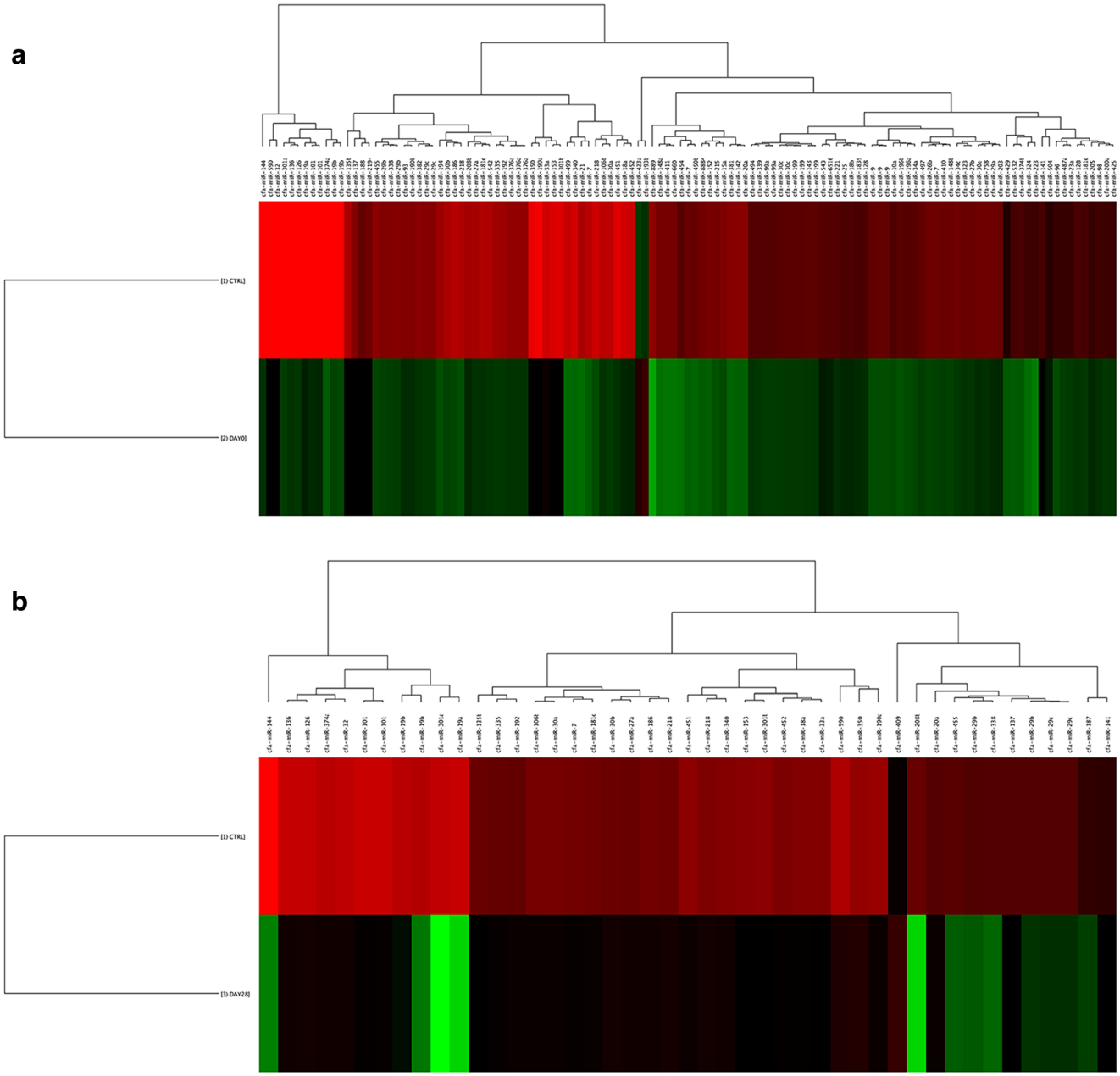

Fig. 1 **a** Heat map representing the differentially expressed miRNA detected in the atopic non-lesional (day 0) versus healthy (CTRL) skin. **b** Heat map representing the differentially expressed miRNAs detected in atopic lesional skin after exposure to *Dermatophagoides farinae* (day 28) versus healthy (CTRL) skin. RNA-seq data from pooled RNA (three samples/pool; two pools/group) and from corresponding individual samples of RNA (three samples/group). miR expression profile was defined based on a p value ≤ 0.05 and a FoldChange ≥ 1.5. Expression levels are shown as mean-centered log2 values. Red, upregulated genes; green, downregulated genes. The heat map scale extends from − 8 to + 8 in log2

biofunctions of "organism injury and abnormalities" enriched by 83, 17, and 4 miRNAs derived from day 0/control, day 28/control, and day 0/day 28 comparisons, respectively. Additional enriched biofunctions were "connective tissue disorders" (52, 20, and 2 miRNAs, respectively), "gastrointestinal disease" (72, 25, and 3 miRNAs), "inflammatory disease" (54, 18, and 2 miRNAs), and "inflammatory response" (41, 16, and 2 miRNAs) (Fig. 3 and S3).

Furthermore, in order to understand the biological relationship between differentially expressed miRNAs in the three experimental conditions, network analysis was performed. The resulted networks were algorithmically generated based on the miRNA connectivity and assigned an enrichment score. Only day 0/control comparison resulted in the networks output. Among these, network 1 included 25 transcripts resulting the best enrichment score. This network analysis revealed as

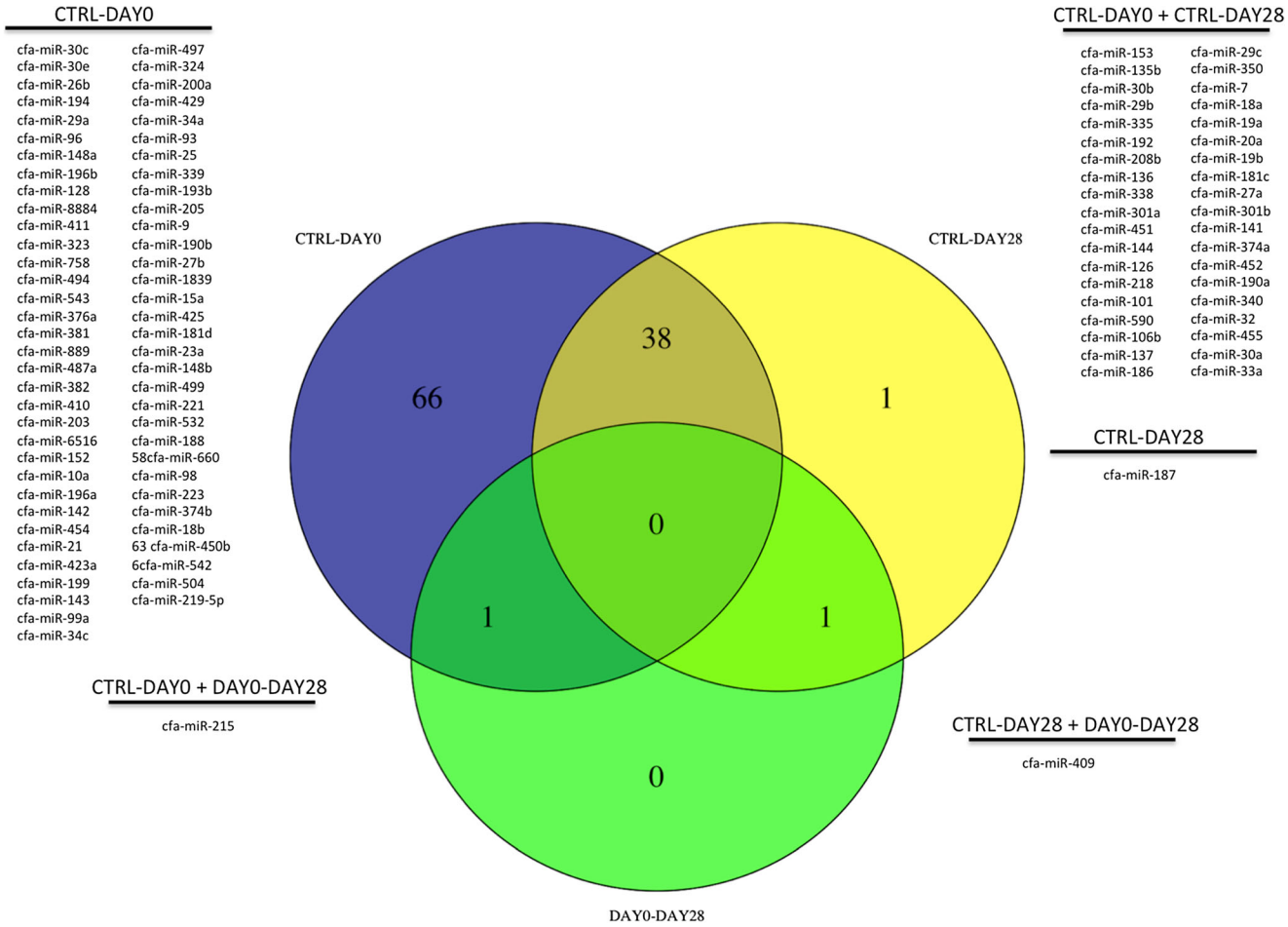

Fig. 2 Venn diagram representing the intersection between differentially expressed miRNA detected in the atopic non-lesional (day 0) versus healthy (CTRL) skin (CTRL-day 0); differentially expressed miRNA detected in the atopic lesional skin after exposure to *Dermatophagoides farina*e (day 28) versus healthy (CTRL) (CTRL-day 28); and differentially expressed miRNA detected in the skin of day 28 versus day 0 (day 0-day 28). In the figures is highlighting the three lists containing the overlapping differential expressed miRNA detected by miRNa seq analysis

the top representative nodes, the Erk1/2 kinase and NF-kB complex, as showed in Fig. 4 and S4.

For the biological validation, three samples extracted from lesional (day 28) canine skin and from healthy control skin were analyzed to verify the NGS results. On day 28, two of the three miRNAs selected (miR215-5p and miRNA409-3p) were significantly increased when compared with day 0, whereas, a significantly decreased expression of miR187-3p was seen on day28 compared with day 0. These results mimic the NSG results presented here validating the methodology and the results presented here (Fig. 5).

Discussion

This is the first study investigating the expression of miRNAs in the skin of atopic dogs, compared with healthy controls, before and after exposure to HDM. Two hundred seventy-seven miRNAs were identified and sequenced; of those, 168 transcripts were differentially expressed when the experimental groups were compared with each other. The vast majority of the miRNA isolated from lesional and non-lesional atopic skin were downregulated when compared with that of the healthy control skin. On the contrary, when the non-lesional skin was compared with lesional skin, only two transcripts (miR215 and miR409) were isolated and both were upregulated. Furthermore, 18 of the 168 transcripts altered in canine atopic skin have been shown to be altered in the skin of human atopic patients (Agler et al. 2019; Rożalski et al. 2016). However, contrary to human studies (Rożalski et al. 2016), miR155 and miR146a did not show significant differences of expression between healthy and atopic canine skin.

Among the alerted miRNAs, identified miR215 may play a significant role in AD. In fact, miR215 and miR192 have been shown to actively suppress interleukin (IL-17) receptors in people (Sun et al. 2018). In the present study, an increased expression of miR215 was seen between healthy and lesional skin as well as non-

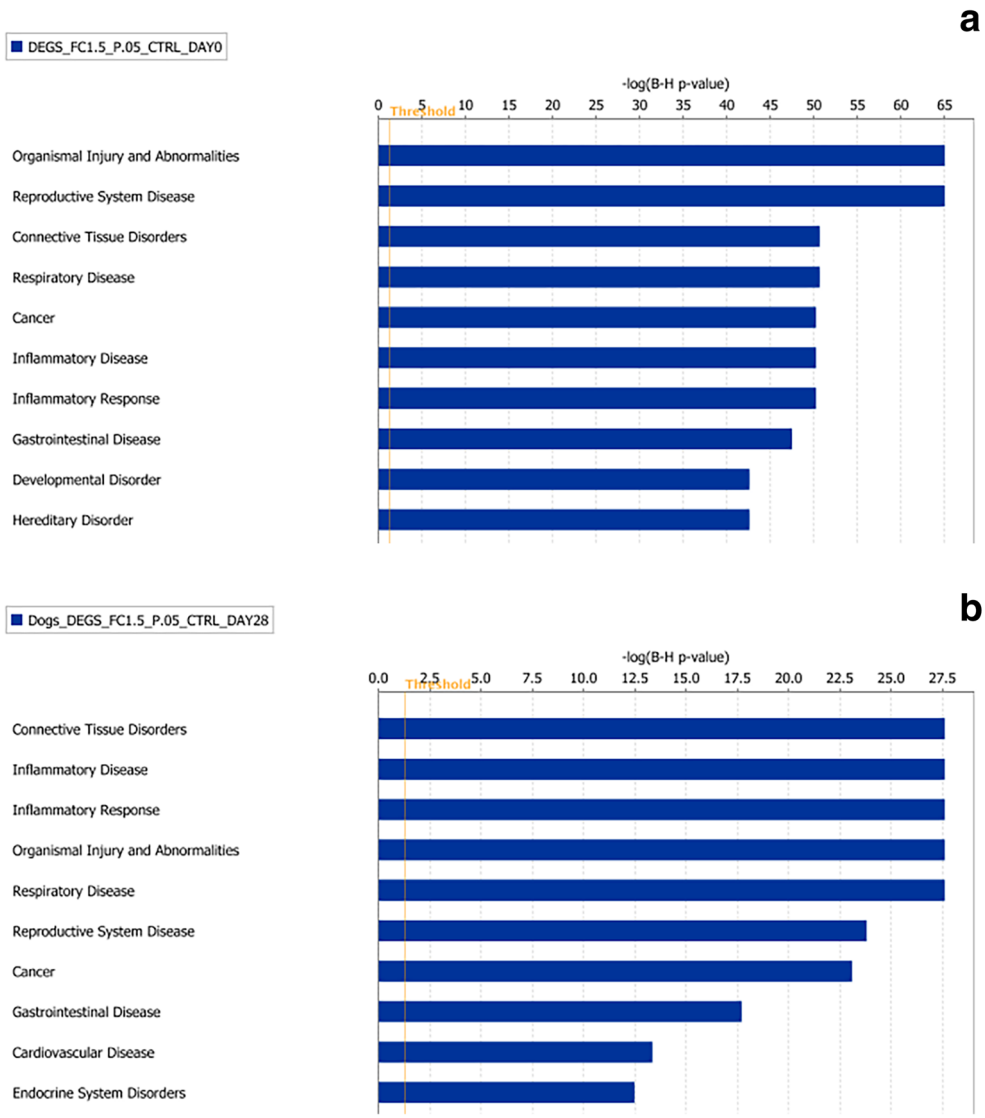

Fig. 3 **a** BioFunction enrichment analysis of differentially expressed miRNA detected in the atopic non-lesional (day 0) versus healthy (CTRL) skin (CTRL-day 0). **b** BioFunction enrichment analysis of differentially expressed miRNA detected in the atopic lesional skin after exposure to *Dermatophagoides farina*e (day 28) versus healthy (CTRL) (CTRL-day 28). The enriched BioFunction analysis was performed based on the Ingenuity Pathway Analysis (IPA) in order to better characterize biological functions and pathways. IPA uses Fisher's exact test to determine which BioFunctions are significantly associated with the miRNA of interest. In the figure are reported the top 10 enriched BioFunctions derived from IPA analyses according to the *p* value

lesional and lesional skin of atopic dogs suggesting an increased suppression of IL-17 receptor activation in canine atopic skin. Although the role of IL-17 has been shown to be relevant in some atopic phenotypes in humans, its role in canine AD is still unclear. The results of this study may suggest that miR215 acts as an anti-inflammatory molecule able to regulate the effects of IL-17 in lesional, but not in non-lesional atopic skin.

Another miRNA worth mentioning is the miR187. This molecule among others (miR106b, miR181, miR203, and miR223) is decreased in both canine and human atopic skin. Interestingly, miR187 has been associated with inflammatory response in human AD decreasing the expression of TGF-β (Zhang et al. 2015). In addition, miR187 influences cytoskeletal organization, cell adhesion, growth factor signaling, and hair follicle remodeling through the *Barx2* pathway (Roussel et al. 2015). In the present study, a decrease in miRNA187 was seen in lesional atopic skin when compared with that in healthy skin. These results could explain the significant decrease in TGF-β mRNA transcripts after chronic exposure to DF in atopic beagles shown in a previous study (White et al. 2018).

Other miRNAs of importance in AD are the miRNAs belonging to the miR200 family (200a, 200b, 429, 200c, 141). Such miRNAs have been strongly correlated with the integrity of cutaneous tight junctions in human skin. In particular, a recent study (Morlang et al. 2018) showed that when miR200s are suppressed/decreased, a prominent decrease in E-cadherin was observed. Such alteration may at least partially explain the presence of compromised thigh junctions in AD (Santoro et al. 2015; Kim et al. 2016). In the present study, a significant decrease in miR141, miR200a, and miR429 was observed in atopic compared with that in healthy control skin. Such results confirm the potential role that these molecules play on the well-known defects in the skin barrier function in AD (Santoro et al. 2015; White et al. 2018; Kim et al. 2016). Similar to tight junctions, adherent junctions are also

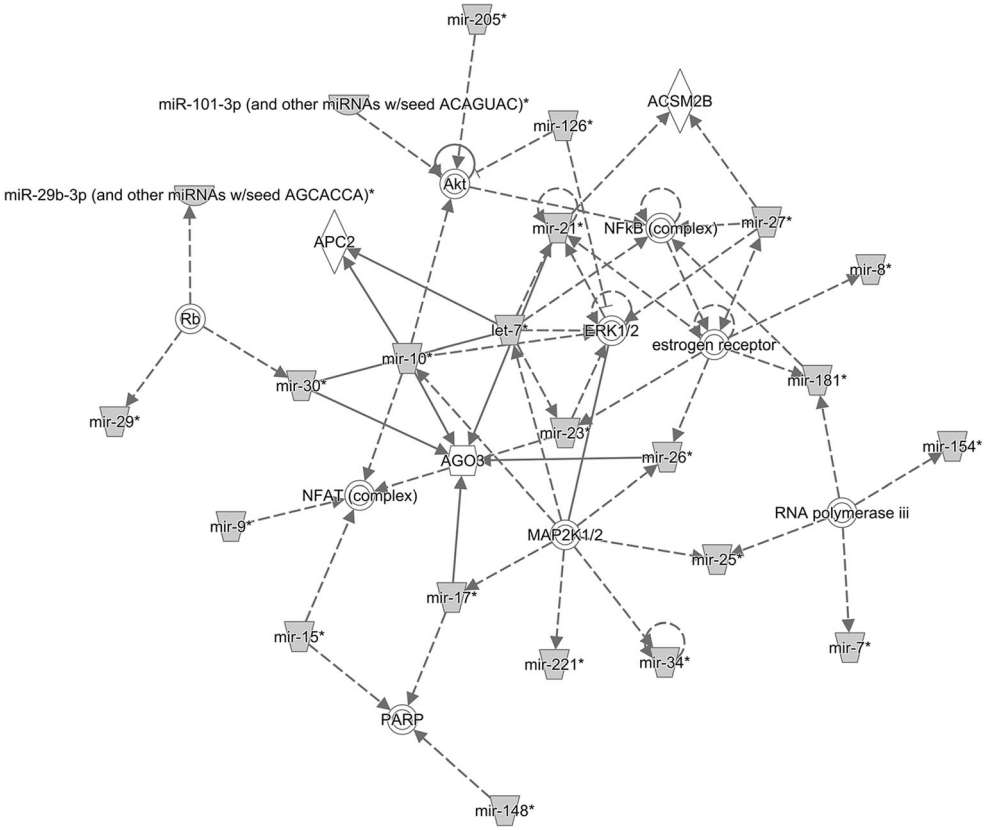

Fig. 4 Ingenuity Pathway Analysis (IPA). Network analysis of differentially expressed miRNA detected in the atopic non-lesional (day 0) versus healthy (CTRL) skin (CTRL-day 0) and in atopic lesional skin after exposure to *Dermatophagoides farinae* (day 28) versus healthy (CTRL) (CTRL-day 28). In the figure, we report that the network resulted in the CTRL versus day 0 (CTRL-day 0) comparison. The node indicates the gene or gene products that included the differential expressed miRNA with the best enrichment score

altered in AD. Interestingly, the overexpression of selected miRNAs (e.g., miR409), as reported in the present study, has been associated with a decrease in the activity of p120-catenin/catenin-δ1 (Wu et al. 2016), an important component of adherent junctions. Altogether, the increased activity of miR409 paired with the decreased expression of miR200s may, at least partially, explain the decreased activity of tight and adherent junctions in canine AD.

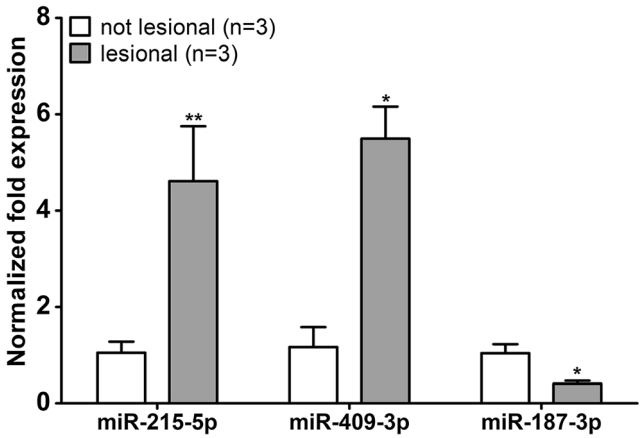

Fig. 5 Quantitative reverse transcriptase PCR analysis of miRNA levels in lesional atopic skin ($n = 3$) compared with that of healthy not lesional control skin ($n = 3$). Results, normalized to cel-miR-39-3p, are represented as fold expression. Bars represent standard error of the means. Variable were tested using the unpaired t test. Statistical significance is indicated at the top of the graph. $*p < 0.05$, $**p < 0.01$, $***p < 0.001$

As mentioned above, only few studies evaluating the tissue expression of miRNAs in dogs have been published (Ichii et al. 2014; Koenig et al. 2016). Recently, an abstract (Morlang et al. 2018) assessing the cutaneous expression of 18 selected miRNAs on formalin-fixed, paraffin-embedded skin samples collected from atopic, healthy, and non-atopic diseased (fungal infection, demodicosis, or mast cell tumor) privately owned dogs has been published. The authors reported a significant different expression of selected miRNAs between inflamed and healthy skin; however, this effect was independent from the atopic status. Although, a potential degradation of the miRNAs could have occurred because of the fixative, the authors concluded that the selected miRNAs might not be useful as biomarkers of AD in the evaluated breeds (Labrador and golden retrievers) (Morlang et al. 2018). Unfortunately, in the present study, a non-atopic control group was not available; thus, it is not possible to confirm or deny the hypothesis by Morlang et al. (Morlang et al. 2018). Nevertheless, the role of miRNAs in the regulation of the cutaneous immunological/inflammatory response is unquestionable.

In conclusion, the study of miRNAs and their role in cutaneous inflammatory diseases is at the early stages in both human and veterinary medicine. However, a potential role of such molecules in the pathogenies of inflammatory skin diseases, including AD, is clear. Further studies to better understand the

role of miRNAs in AD, and other inflammatory skin diseases are highly warranted, specifically using a larger number of dogs with multiple inflammatory skin diseases. Once the role of miRNAs will be clarified, it may lead to the potential identification of new treatments for AD in people and dogs specifically targeting these molecules (miRNA therapy).

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