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MiRNA-21, miRNA-10b, and miRNA-34a expression in canine mammary gland neoplasms

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Abstract

The expression of miRNA-21, miRNA-10b, and miRNA-34a in malignant and benign tumours and non-neoplastic lesions in canine mammary gland, using real-time PCR with TaqMan probes was determined. The expression in normal tissues was compared to neoplastic and non-neoplastic lesions using one-way ANOVA test. Significant changes in miRNA expression in neoplastic tissues, as compared to normal ones, were demonstrated. In all neoplastic tissues, the miRNA-21 expression increased while in non-neoplastic lesions slightly decreased in comparison to normal ones. MiRNA-10b expression in malignant and benign tumours increased in comparison to normal tissues and non-neoplastic lesions. MiRNA-34a expression profile in neoplastic and non-neoplastic tissues differed from other examined miRNAs (miRNA-21 and miRNA-10b). In all samples miRNA-34a expression level decreased in comparison to normal tissues.

Keywords: dog, mammary gland tumours, miRNA, real-time PCR.

Introduction

MicroRNAs (miRNAs) are short (21-25 nt), highly conserved, non-coding sequences of ssRNA acting in organisms as regulatory molecules. The miRNAs-encoding genes are located in different sites in the genome, within noncoding and protein-coding sequences (in introns and exons). Bioinformatic analysis suggests that there are approximately 1000 miRNA genes in a mammalian genome and miRNAs are estimated to regulate approximately 30% of all genes (7). The primary miRNA (pri-miRNA) is formed as a result of transcription, conducted by the RNA polymerase II. Pri-miRNA is about 1000 nt transcript and contains numerous hairpin structures. Afterwards, the transcript undergoes further steps of maturation. In the first stage, the pri-miRNA transcript is processed by the nuclear enzyme Drosha and forms (70-100 nt) miRNA precursor (pre-miRNA). These structures are transported from the nucleus to the cytoplasm *via* Exportin 5. In the cytoplasm, pre-miRNA undergoes further processing by enzyme Dicer, which cleaves pre-miRNAs, generating the mature double-stranded miRNAs (21-25 nt). One of the two strands of miRNA is degraded, while the other is bound to the Argonaute protein (Ago) and forms a miRNA-induced silencing complex (miRISC). The mature miRNA is transported to the target mRNA. The miRNA, on the basis of sequence complementarity, binds to a suitable region in the 3'-UTR of the mRNA (25). The miRNA requires a perfect match to mRNA of only 2-7 nt (seed sequence) (12). The mRNA degradation or translation inhibition is the result of this binding. One miRNA molecule can bind to hundreds of mRNAs and inactivate them. In some cases, several miRNAs can target one mRNA.

Considering the fact that the miRNA target sequences are mRNAs encoding proteins involved in normal cell growth, proliferation, differentiation or apoptosis, abnormal expression of miRNA may cause serious consequences for the organism. Abnormal

regulation of cell cycle can lead to excessive cell proliferation or apoptosis inhibition (29). A natural consequence of such disorder is an uncontrolled growth of tissue, which can lead to cancer development. Depending on mRNA which is inhibited, miRNAs can function as oncogenes or tumour suppressors (30). Some miRNAs also play an important role in the metastases formation (pro-metastatic miRNA) (18) or its "prevention" (anti-metastatic miRNA) (19).

Until now, a large part of miRNA was carefully examined in humans. In recent years, particular emphasis was placed on the miRNAs expression profiles occurring in many human cancers, for instance leukemia (3), lung cancer (15), prostate cancer (17), but mainly focused on breast cancer (14).

Changes in miRNA expression were observed in all tumour types. Volina *et al.* (23) have found that miR-17-5p, miR-20a, miR-21, miR-92, miR-106a, and miR-155 are strongly associated with the development of many tumours (23). The role of miRNAs in breast cancer initiation, progression, and metastases was also observed. It has been revealed that miR-206, miR-17-5p, miR125a, miR-125b, miR-200 family, let-7 family, miR-34a, and miR-31 act as tumour suppressors in breast cancer (21, 28). The miR-21, miR-155, and miR-10b have been established as oncogenic and metastasis-promoting miRNAs in breast cancer (10, 26). Considering the fact that miRNAs are highly conserved across multiple species similar changes in miRNA expression may occur in normal and canine tumour tissues.

The aim of the study was to determine the expression of miRNA-21, miRNA-10b, and miRNA-34a in malignant, benign, and non-neoplastic lesions in canine mammary gland using real-time PCR with TaqMan probes. The hypothesis assumed significant changes in the expression of selected miRNAs in mammary gland pathological lesions as compared to normal tissues.

This study may enable to understand the influence of altered miRNAs in canine mammary gland cancer better and may allow identification of miRNA biomarkers for the diagnosis and prediction of the disease.

Material and Methods

Samples of neoplastic and non-neoplastic tissues were collected during therapeutic mastectomy, as well as from healthy euthanized dogs (control samples). Table 1 presents breed and age of the dogs examined.

Each sample was divided into two parts. One fragment, intended for histopathological examination, was fixed in 10% paraformaldehyde. The second, for a molecular biology study, was placed in RNAlater (Ambion Inc., USA).

In the first stage of the study, collected tissues were analysed histopathologically to determine the nature of the lesion, as well as to exclude any changes in the control tissues. Histopathological analysis was performed using sections stained with haematoxylin and eosin (HE).

Total RNA isolation and reverse transcription. Total RNA was isolated from all samples using the miRVana miRNA Isolation Kit (Ambion) following the manufacturer's protocol. The samples were evaluated for quality and quantity using the NanoVue® Spectrophotometer (Life Technologies). TaqMan® MicroRNA Assays (hsa-miR-21, mmu-miR-10b, and hsa-miR-34a) were used to reverse transcribe three mature miRNA sequences to cDNA (Applied Biosystems, USA). Each 15μL reverse transcription reaction contained: 5 μL of total RNA diluted to the appropriate concentration (10 ng per reaction); 1 × TaqMan MicroRNA RT primer; 0.25 U of RNase inhibitor; 1 × RT buffer; 3.33 U of MultiScribe™ reverse transcriptase, and 1 mM of dNTPs.

Table 1. Breed and age of dogs examined and type of lesions observed in the mammary glands

Sample	Breed	Age (years)	Lesions	Neoplasm type
T1	Dachshund	15	Tubulopapillare carcinoma	Malignant
T2	Bavarian Mountain	9.5	Carcinoma intraductale	Malignant
T3	Rottweiler	8	Lipoosteosarcoma	Malignant
T4	Polish Tatra Sheepdog	8	Complex adenoma	Benign
T5	Dachshund	15	Tubulopapillary carcinoma	Malignant
T6	Mix breed	7	Complex adenoma	Benign
T7	Pointer	8	Complex adenoma	Benign
T8	Poodle	9	Ductal hyperplasia	Non-neoplastic lesions
T9	Mix breed	6	Complex carcinoma	Malignant
T10	Boxer	12	Osteosarcoma	Malignant
T11	Pointer	8	Carcinoma simplex fibromatosum	Malignant
T12	Irish Setter	10	Lobular hyperplasia	Non-neoplastic lesions
T13	Mix breed	5	Adenosis	Non-neoplastic lesions
C1	Beagle	2.5	Normal	Normal
C2	Polish Tatra Sheepdog	3	Normal	Normal
C3	Dachshund	2	Normal	Normal

T - tumour, C - control,

The reverse transcription reactions were performed in a Biometra Thermocycler (Biometra T Gradient Professional Basic) for 30 min at 16°C, 30 min at 42°C, 5 min at 85°C, and then held at 4°C for 10 min. All samples were frozen at -80°C.

Real-time PCR. The reverse transcription products were diluted 1:15. The 20 µL of real-time reaction contained: 1.33 µL of diluted RT product, 1 × TaqMan Universal PCR Master Mix (No AmpErase® UNG), and 1 × TaqMan Assay, which contained the forward and reverse primers as well as the TaqMan probe. Each reaction was run in triplicate on a 96-well plate. Reactions containing no reverse transcription products served as a negative control. Reactions were incubated in 7500 Fast Real Time PCR System, (Applied Biosystems) for 10 min at 95°C, followed by 40 cycles of 95°C for 15 s, and 60°C for 1 min. After completion of the qPCR, the threshold value was automatically configured above the baseline displayed in the amplification plot. Relative quantification of gene expression was evaluated by utilising the comparative critical threshold (C_T). The C_T values for each mature miRNA reaction were subtracted from the respective C_T value of the RNU6B control, resulting in the ΔC_T value and then $\Delta\Delta C_T$ value was calculated. Fold changes were then generated for each mature miRNA by calculating $2^{-\Delta\Delta Ct}$. Expression levels of normal samples were compared to neoplastic samples using one-way ANOVA test.

Results

The results of histopathological examination are presented in Table 1. The data revealed various types of neoplasms and non-neoplastic lesions in the examined mammary gland samples.

The analysis of miRNA-21, miRNA-10b, and miRNA-34a demonstrated significant changes in miRNA expression in normal *versus* neoplasm tissues. In all neoplastic tissues, miR-21 expression increased 3-fold in sarcoma and benign tumours while in carcinoma increased 5-fold, relatively to U6. In non-neoplastic lesions, miRNA-21 expression slightly decreased (Fig. 1).

The expression of miRNA-10b in carcinoma, sarcoma, and benign tumours increased in comparison to normal and non-neoplastic lesions. Moreover, miRNA-10b expression level was similar in all neoplasms (Fig. 2).

The profile of miR-34a expression in neoplastic and non-neoplactic tissues differed from other examined miRNAs (miRNA-21 and miRNA-10b). In all studied samples, miRNA-34a expression level decreased in comparison to normal tissue. The miRNA-34a expression decreased 2.5-fold in carcinoma, 5-fold in sarcoma, benign and non-neoplastic lesions, relatively to U6 (Fig. 3).

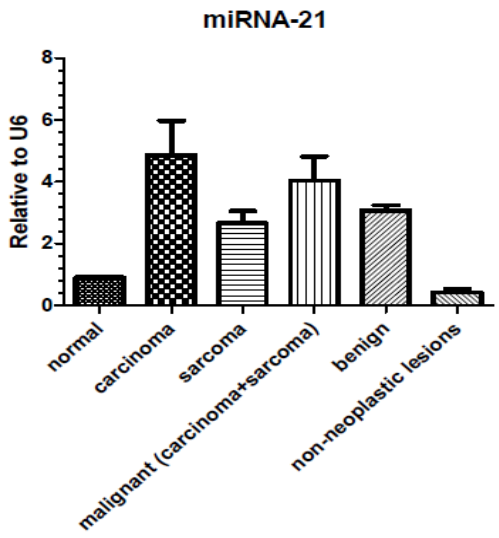


Fig. 1. miRNA-21 expression level (real-time PCR) in normal, non-neoplastic, and neoplastic lesions in the mammary gland samples

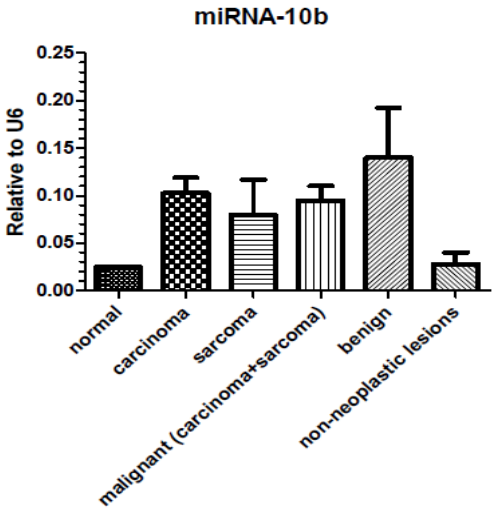


Fig. 2. miRNA-10b expression level (real-time PCR) from normal, non-neoplastic, and neoplastic lesions in the mammary gland samples

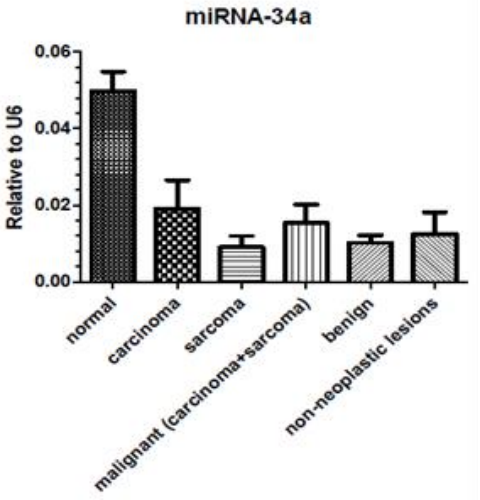


Fig. 3. miRNA-34a expression level (real-time PCR) in normal, non-neoplastic, and neoplastic lesions in the mammary gland samples

Discussion

Numerous studies conducted in humans have shown that characteristic expression profiles of miRNA can be found in tumours (9). There is also evidence that the changes in the expression of specific miRNAs contribute to cancer invasiveness (10), metastases (24), recurrence, (16), and resistance to chemotherapy (11).

Since miRNAs are highly conserved across multiple species, similar changes in miRNA expression in normal and pathologically changed tissues may occur in dogs as in humans. In available literature there are very few data regarding the expression of miRNAs in the dog. There is information about miR-17-92 expression in normal canine tissue (1), a comparison of miRNA-145 expression in melanoma of humans and dogs (13), and research on the changes in miRNA expression in canine lymphoblastic leukemia cell lines (20), or pathologically-changed bladder (22). The most recent data has also shown an altered miRNA expression in normal canine retina in comparison to retinal degeneration. The results showed up-regulation of anti-apoptotic and down-regulation of pro-apoptotic miRNAs (6). Furthermore, Fenger *et al.* (5) demonstrated that unique miRNA expression profiles correlated with biological behaviour of primary canine mast cell tumours (MCTs) and that miR-9 expression was increased in biologically high grade canine MCTs and malignant cell lines compared to biologically low grade tumours. Not much is known about miRNAs expression in mammary gland cancer in this species. Boggs *et al.* (2) conducted a study on changes in miRNA expression in this type of cancer. Using real-time PCR the authors demonstrated that the level of miRNAs expression (let-7f, miR-15a, miR-16, miR-17-5p, miR-21, miR-29b, miR-125b, miR-145, miR-155, and miR-181) in canine mammary gland cancer varied in a similar manner to the breast cancers in humans. This research was conducted on a small number of animals (n = 6) and only two types of mammary gland cancer - tubullar papillary carcinoma and ductal carcinoma were examined. Our study confirmed that miRNA-21 expression increased in mammary gland neoplasms. Another study of miRNA expression in canine mammary gland neoplasms was conducted by Charlotte von Deetzen *et al.* (4). This study was performed on ten simple mammary adenomas, ten simple carcinomas without evidence of tumour cells in the regional lymph node, and ten simple carcinomas with regional lymph node metastases, as well as normal mammary gland tissues of ten dogs. All neoplastic tissues differed in their miRNA-210 expression levels from normal gland. While metastatic cells differed in their expression of miRNA-29b, miRNA-101, miRNA-125a, miRNA-143, and miRNA-145 from the primary tumours, the comparison of miRNA expression in primary tumours of different malignancy failed to reveal significant differences, except for a significant downregulation of miRNA-125a in metastasising carcinomas when compared to adenomas. The miRNA-21 expression in non-metastasising and metastasising carcinoma increased in comparison to normal tissues 3.18-fold and 5.05-fold, respectively. Our study also revealed a 5-fold increase in miRNA-21 expression in carcinoma samples.

Overexpression of miR-21 in human breast cancer is associated with advanced clinical stage, lymph node metastasis, and potential poor prognosis (26). However, our study revealed that even in benign tumours, miRNA-21 expression significantly increased. Only in normal and non-neoplastic lesions its level remained low.

Recent data has also demonstrated that miRNA-34a inhibits proliferation, invasion, and tumour metastasis by directly targeting Fra-1, Bcl2 and SIRT1 (7, 27). Low miR-34a expression may suggest high proliferation and invasion of neoplasms used in our study.

There is lot of evidence that miRNA-10b is overexpressed in advanced and metastatic breast cancer (10). In our study, the expression level of miR-10b in neoplasms increased, while in non-neoplastic lesions remained low. It can be an additional evidence that miR-10b increases only in advanced neoplasms.

Our study demonstrated that miR-21, miR-10b, and miR-34a expression in normal and tumour tissues of dogs varied in the same manner as in human breast cancer (7, 10, 26). Based on available data of human miRNA expression profiles, researchers are able to specify the types of breast cancer, as well as anticipate their further development (for instance: metastasis, chemiotherapy resistance). Similarly, miRNA expression profiles in the dog could be a useful tool in the diagnosis of mammary gland tumours. Furthermore, the dog can be used as a model for the study of all types of breast cancers in humans.

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