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Abstract

Hemangiosarcoma is a highly metastatic and lethal cancer of blood vessel-forming cells that commonly spreads to the brain in both humans and dogs. Dysregulations in Phosphatase and Tensin Homolog (PTEN) have been identified in various types of cancers, including hemangiosarcoma. MicroRNAs (miRNAs) are short noncoding single-stranded RNA molecules that play a crucial role in regulating gene expression. Some miRNAs can function as oncogenes or tumor suppressors, influencing important processes in cancer, such as angiogenesis. This study aimed to investigate whether miRNAs targeting PTEN were disrupted in canine hemangiosarcoma and its corresponding brain metastases (BM). The expression levels of miRNA-10b, miRNA-19b, miRNA-21, miRNA-141 and miRNA-494 were assessed in samples of primary canine cardiac hemangiosarcomas and their matched BM. Furthermore, the miRNA profile of the tumors was compared to samples of adjacent non-cancerous tissue and healthy control tissues.

In primary cardiac hemangiosarcoma, miRNA-10b showed a significant increase in expression, while miRNA-494 and miRNA-141 exhibited downregulation. Moreover, the overexpression of miRNA-10b was retained in metastatic brain lesions. Healthy tissues demonstrated significantly different expression patterns compared to cancerous tissues. In particular, the expression of miRNA-10b was nearly undetectable in both control brain tissue and perimetastatic cerebral tissue. These findings can provide a rationale for the development of miRNA-based therapeutic strategies, aimed at selectively treating hemangiosarcoma.

Keywords: brain metastases, dog, hemangiosarcoma, miRNA, PTEN

Introduction

In dogs, hemangiosarcoma is a common malignant and highly metastatic cancer of blood vessel-forming cells. It shares similarities with its human counterpart in terms of biological behavior, treatment response, and prognosis.[1-3] Metastases occur early in the disease process, with up to 80% of dogs presenting with evident metastatic disease.[4] Notably, in dogs, hemangiosarcoma is one of the tumors that most often metastasize to the brain.[5,6] Brain metastases (BM) present a challenging treatment scenario due to the limited permeability of the blood-brain barrier to systemic therapies and the advanced stage of the disease.[7,8] Historically, the prognosis for patients with BM, both in human and veterinary oncology, has been extremely poor.[9] As a result, there is an unmet need to develop strategies for preventing BM in both human and veterinary oncology patients.

The exact pathogenesis of canine hemangiosarcoma remains largely unknown, and it often remains unidentified in most cases. However, there are established factors that contribute to its development, including genetic predisposition, environmental factors, and the inactivation of tumor suppressor genes, such as Phosphatase and Tensin Homolog (PTEN).[10] Recent research has demonstrated that the PTEN pathway is frequently disrupted in canine hemangiosarcoma, either through inactivating mutations or epigenetic downregulation.[11-14]

MicroRNAs (miRNAs) are small RNA molecules, typically 18-25 nucleotides in length, that are highly conserved and noncoding. They play a crucial role in gene expression regulation following transcription by specifically binding to complementary target messenger RNAs (mRNAs).[15,16] MiRNAs are involved in the regulation of various cellular processes, including cell differentiation, angiogenesis, proliferation and apoptosis.[15,16] Depending on the target mRNAs they interact with, miRNAs can function as either oncogenes or tumor suppressors, and there is substantial evidence

supporting a causal relationship between dysregulation of miRNAs and the development of cancer.[15,17]

Among the hundreds of miRNAs implicated in cancer development, a significant contribution to its pathogenesis is attributed to those that inhibit the expression of PTEN, including miRNA-494, miRNA-141, miRNA-21, miRNA-10b, and miRNA-19b.[18-23]

Considering this, the aforementioned miRNAs targeting PTEN were chosen for examination in canine cardiac hemangiosarcoma and matched BM. To accomplish this, the miRNA profile of the tumors was compared to samples of adjacent non-cancerous tissue and healthy control tissues.

Materials and methods

Histopathology

Formalin-fixed and paraffin-embedded (FFPE) post-mortem samples of primary cardiac hemangiosarcoma and matched BM from companion dogs were retrieved from the tissue bank of the (*masked for review*).

FFPE post-mortem samples of right cardiac auricle (n=6) and brain (n=6) from dogs deceased from tumor-unrelated causes were included as controls.

Ethical approval was not required since the study was conducted on archived FFPE tissue blocks collected for diagnostic purposes.

Four-millimeter cores of each primary tumor (PT), BM, perimetastatic brain (periBM) and control samples were punched out manually from donor blocks by use of commercially available skin biopsy punches and transferred to blank recipient paraffin blocks (one for each sample). The resulting blocks were placed in a conventional tissue embedding machine and re-embedded to completely integrate the donor tissue cores into the surrounding paraffin.

For PT and BM, the cores were chosen based on prior microscopic examination of the areas of interest in HE-stained sections. The first section of the new blocks was histologically evaluated to confirm the presence of neoplastic cells. For periBM, the perimetastatic tissue was observed histologically,

and the non-neoplastic area of interest was marked. Subsequently, the corresponding area of the tissue block was sampled with a punch and reincluded in a new block. The first section of the new block was examined histologically to exclude the accidental sampling of tumor cells.

RNA extraction

Twenty sections from each block were cut in a microtome at 10 µm and placed in 1.5 ml tubes for RNA extraction.

Peri-tumoral cardiac tissue was not analyzed, since hemangiosarcomas mostly occupied the whole right cardiac auricle, leaving no healthy tissue around.

Total RNA enriched by miRNA was extracted using the miRNeasy FFPE kit (Cat. 217504, Qiagen, Hilden, Germany) following the manufacturer's instructions. We performed a first step of deparaffinization using xylene, followed by ethanol washing and proteinase K incubation. The total RNA enriched by miRNA was then extracted using the dedicated columns and the elution step was performed in RNase-free water.

Representative samples of the extracted RNA (BM, n = 7; periBM, n = 6; PT, n = 6; CH, n = 4; CB, n = 4) was used to perform miRNA sequencing (miRNAseq) to obtain quality controls on miRNA integrity and reliability in terms of phred score, identifying the overall quality, and the trimmed reads' length, to evaluate the integrity of miRNAs. Sequencing quality analysis were performed at Qiagen company, using the Illumina technology specific for small RNA sequence (15 – 55 nt).

Reverse transcription

For the reverse transcription step the miRCURY LNA RT kit (Cat. 339340, Qiagen) was used following the manufacturer's instruction. Briefly, according to the concentration and total quantity of extracted RNA, 5–10 µl of each sample were diluted to reach a concentration of 5 ng/µl. For each reaction, 2 µl (10 ng total) of RNA were used in the reverse transcription mix, which also included the miRCURY RT enzyme (1 µl of the 10x solution), the miRCURY RT Reaction buffer for SYBR

green (2 µl of the 5x solution), the synthetic RNA spike-ins used as RT positive control template (0.5 µl) and 4.5 µl of RNase-free water, for a total volume of 10 µl per reaction. No-RT sample was added, using the same reaction mix without the RT enzyme.

Samples were incubated for 60 minutes at 42°C for the reverse transcription reaction and for 5 minutes at 95°C for enzyme inactivation, using the thermocycler Byometra (Bio-rad, Hercules, CA, USA), then stored at 4°C.

miRNA expression analysis

The miRCURY LNA SYBR Green PCR kit (Cat. 339346, Qiagen) was used for miRNA qPCR analysis. The following specific miRCURY LNA PCR Assay (Cat. 339306, Qiagen) were used to analyze specific miRNA expression: miRNA-10b (YP00205499 – rno-miR-10b-5p), miRNA-19b (YP02105441 – cfa-miR-19b), miRNA-21 (YP00204230 – has-miR-21-5p), miRNA-141 (YP02110018 – cfa-miR-141) and miRNA-494 (YP00204579 – has-miR-494-3p). The U6 snRNA was used as normalizer RNA (YP02119464, v2). Moreover, as positive RT control, the UniSP6 assay (YP00203954) was used to amplify the synthetic RNA employed during the RT step for each reaction. Following the manufacturer's instructions, all samples were diluted 1:60, adding 590 µl of RNase-free water to the 10 µl of the RT sample. Each reaction was composed by the miRCURY SYBR® Green Master Mix (5µl of the 2x solution), 1 µl of the specific PCR assay, 1 µl of the RNase-free water, and 3 µl of the diluted RT sample, for a total reaction volume of 10 µl.

The reactions were performed using the CFX96 machine (Bio-rad) with the following cycles: 2 minutes at 95°C (PCR initial heat activation), 2-step cycling (40 cycles) of 10 seconds at 95°C (denaturation) and of 60 seconds at 56°C (annealing/extension), followed by the melting curve analysis (60 – 95°C). Fluorescence data collection was performed during the annealing/extension step.

Quality control

Before proceeding with the qPCR data elaboration for the relative quantification of miRNA expression, we conducted a series of quality controls to ensure a reliable analysis, particularly due to the potential degradation induced by formalin fixation.

First, we assessed the RNA extraction yield using a spectrophotometer (Nanodrop 2000, Thermo-fisher scientific, Waltham, MA, USA) and concurrently quantified the 260/280 nm ratio for each sample. The data is included in Supplementary Table S1. The extracted RNA exhibited high concentrations (>50 ng/µl) in 23 out of 32 samples (≃72%) and low concentrations (<10 ng/µl) in only 1 sample (≃ 3%). Additionally, all analyzed samples displayed a 260/280 nm ratio exceeding the minimum threshold for high purity (>1.7).

The subsequent qPCR experiments validated the consistency of Ct values for each analyzed miRNA within their respective experimental groups. As outlined in the methods section, considering the specified number of samples per group, we conducted a total of 32 runs (in technical duplicates) for each of the 5 miRNAs of interest, resulting in a grand total of 160 runs. We categorized these runs based on their Ct values, defining "expressed" as $Ct \leq 35$, "low-expressed" as $Ct = 36 – 38$, and "not-expressed" as $Ct > 38$. Out of the 160 runs, the majority (104) were classified as "expressed" miRNAs, while 44 were labeled as "low-expressed," and only 12 fell under the "not-expressed" category. Among the five miRNAs (miRNA-10b, miRNA-19b, miRNA-21, miRNA-141, and miRNA-494), only miRNA-141 and miRNA-494 exhibited a "low-expression" profile, with only 2 samples in each miRNA group being marked as "not expressed" (Supplementary Table S2).

In the quality miRNAseq control, all samples exhibited a phred value ranging from 35 to 40. Phred levels are typically categorized as poor (0 – 20), medium (20 – 28), and good (>28). Therefore, all samples demonstrated high quality. This was further affirmed by the distribution of trimmed reads' lengths, which exhibited a distinct peak around 21 nt for all samples, a value characteristic of typical miRNA length.

Based on the combined assessment of RNA quantification data, Ct values and miRNAseq analysis obtained from all samples, we deemed the miRNA-qPCR analysis to be reliable, allowing us to proceed with the relative expression quantification.

Data analysis

The Geneglobe online software (geneglobe.qiagen.com) was used to analyze the entire experimental dataset in accordance with the quality controls provided by the kit. It was also utilized for the processing of miRNA expression compared to healthy tissues (Figure 2).

The $2^{(-\Delta\Delta Ct)}$ method was used for the calculation of gene expression in all other analyses (Figure 3 and 4). After the first normalization on the normalizer U6 snRNA (ΔCt), a second normalization was performed on the reference group, depending on the analysis. The reference group is specified in each figure legend. The average, minimum, and maximum Ct values for each group and each miRNA are reported in Supplementary Table S2.

For miRNA-10b expression in brain-derived tissues, two different analyses were performed: i) as for the other graphs, $2^{(-\Delta\Delta Ct)}$ was calculated using the average ΔCt values for each group (Figure 4A; see Supplementary Table S3 for raw data and elaboration); ii) each BM sample was normalized to its periBM control derived from the same animal (Figure 4B; see Supplementary Table S4 for raw data and elaboration).

The differential expression of the evaluated miRNAs was analyzed on ΔCt with One Way ANOVA, followed by Tuckey's post-test, or Student's T test. Statistical analysis was performed with Prism v. 5.0 (GraphPad, San Diego, CA, USA). Significance was set at $P<0.05$.

PTEN immunohistochemistry

Automated immunohistochemistry (IHC) was performed on the Discovery ULTRA system (Roche, Ventana Medical Systems Inc., Tucson, AZ, USA), using the primary antibody anti-PTEN (mouse monoclonal, clone A2B1, diluted 1:50, Santa Cruz Biotechnologies, Santa Cruz, CA, USA, cod. SC-

7974), which has previously been described to cross-react with canine tissues.[24] Briefly, 3-µm-thick sections were mounted onto superfrost plus slides, deparaffinized in aqueous-based detergent solution (Discovery Wash, Ventana) and underwent heat induced antigen retrieval (CC2, pH 6.0 32 min, 91°C). The primary antibody (dilution 1:10) was incubated for 1 h at room temperature (RT). The OmniMap anti-Mouse HRP (Roche) secondary antibody was incubated for 16 min at RT and the chromoMap DAB (Roche) was used as chromogen. After detection, sections were counterstained with hematoxylin (Hematoxylin II, Ventana), dehydrated and mounted with Eukitt (Kaltek, Padova, Italy). Positive controls from canine tissues (kidney and prostate) and sections with omission of the primary antibodies (negative controls) were included in each run.

Cell Line Validation Statement

No cell lines were used in the current study.

Results

Clinical, histologic and immunohistochemical data

Seven cases of canine cardiac hemangiosarcoma with BM were included in the analysis. There were 4 intact males and 3 spayed females. Median age was 10 years (range, 6-13). Breeds included one each of English setter, German Shepherd, Boxer, Akita, Cane Corso, Maremma Shepherd and mixed (Table 1).

Only one dog underwent therapy: after the removal of the cardiac tumor, chemotherapy was administered, followed by an experimental treatment. Survival time for this dog was 394 days. All the other dogs were euthanized upon diagnosis.

At necropsy, all dogs had multiorgan metastases, with the primary tumor being located in the right cardiac atrium or auricle. The heart has been hypothesized as the primary site because the other metastatic organs showed widespread lesions.

The primary tumor was sampled during post-mortem examination in 6 dogs. In one case the primary cardiac tumor had been surgically removed 13 months prior at a different veterinary clinic, and the paraffin block could not be obtained.

BM were sampled during post-mortem examination in all 7 dogs; they were mostly located within the cortex, in the parietal, frontal or temporal lobes, and consisted of 5 to 20 round multifocal hemorrhagic foci, 0.1-2 cm in diameter (Fig. 1A). Histologically, neoplastic cells were spindle-shaped, variably pleomorphic, arranged in irregular channels filled with erythrocytes and associated with hemorrhages (Fig. 1B).

miRNA analysis

In the first subset of samples, we analyzed miRNA expression in control tissues using the GeneGlobe online software for calculating the fold difference (FD) and conducting statistical analysis. We utilized the preset options for miRNA analysis through the miRCURY LNA PCR Assay. When comparing the expression of the five target miRNAs in control hearts and brains, the scatter plot and the clustergram revealed that all miRNAs were more expressed in the heart (Fig. 2A). However, only the expression of miRNA-10b resulted significantly different ($P=0.004$), with a FD of more than 300 times (Fig. 2B).

When comparing miRNA expression between cardiac hemangiosarcomas and control hearts, miRNA-10b, miRNA-141 and miRNA-494 were significantly dysregulated. MiRNA-10b was significantly overexpressed, with a mean FoC of 4.2 compared with control cardiac tissues ($P=0.031$; Fig. 3A). MiRNA-141 and miRNA-494 were significantly underexpressed, with a mean FoC of 0.05 ($P<0.001$; Fig. 3B) and 0.15 ($P=0.02$; Fig. 3C), respectively. No significant differences were observed in the expression levels of the remaining miRNAs.

Overall, miRNA-10b in BM was significantly overexpressed, with a mean FoC of 11.6 and 7.7 compared with periBM ($P=0.025$) and control brains ($P=0.013$), respectively (Fig. 4A). The

expression levels of miRNA-10b in periBM and control brains were similar and extremely low, requiring a mean number of amplification cycles of 38 and 39, respectively.

The expression levels of miRNA-10b were significantly lower in BM compared with the primary tumor ($P<0.001$), with a mean FD of 0.009. The individual ΔCt values utilized for the miRNA-10b analysis, along with the corresponding elaboration used to generate the FD values shown in Fig. 4A, are provided in Supplementary Table S3.

Normalizing the expression of miRNA-10b in BM to the periBM of the same animal, an upregulation was observed in 4 dogs (57%), ranging from 17.12 to 174.85 folds (Fig. 4B). However, there was no correlation in the expression of miRNA-10b between BM and PT. The individual ΔCt values used for the miRNA-10b single-sample normalization analysis, along with the corresponding elaboration used to generate the graph shown in Fig. 4B, are included in Supplementary Table S4.

In BM, miRNA-494 was downregulated compared with normal brain, although not statistically significant ($P=0.074$).

No other differentially expressed miRNAs were observed in BM compared with periBM or normal brains.

PTEN immunohistochemistry

PTEN immunohistochemical expression was assessed subsequent to miRNA analysis in 6 out of 6 PT and in 3 out of 7 BM due to insufficient tissue availability. Overall, moderate to strong nuclear and cytoplasmic positivity was observed in 2 out of 6 (33.3%) PT and 1 out of 3 (33.3%) BM (Figs. 1C and 1D). Considering the two PTEN-positive PTs, the positivity was confirmed in the corresponding BM in one case, while in the other case, unfortunately, there was not enough brain tissue available to conduct the analysis. The PTEN-positive BM also exhibited low miRNA-10b expression, whereas moderate to high miRNA-10b expression was detected in the 2 PTEN-positive PT (Table 1).

Discussion

Considering the significant role of PTEN in canine hemangiosarcoma, we selected 5 miRNAs that are known to be involved in its regulation. The aim was to investigate whether these miRNAs exhibited dysregulation in both primary and metastatic brain tissues, and to compare their expression with that in normal control tissues.

Among the miRNAs analyzed in this study, miRNA-10b, miRNA-141 and miRNA-494 demonstrated significant dysregulation in primary cardiac hemangiosarcoma. Furthermore, miRNA-10b exhibited sustained overexpression in metastatic brain lesions. MiRNA-10b showed elevated expression levels in both PT and matched BM, while miRNA-494 and miRNA-141 displayed reduced expression in primary cardiac hemangiosarcoma.

The findings of this study are consistent with previous research in human patients. The upregulation of miRNA-10b and dysregulation of miRNA-141 and miRNA-494 have been shown to function by inhibiting the tumor suppressor PTEN.[20,22,25] Loss of PTEN function is commonly observed in various cancers, leading to the accumulation of its activated downstream effectors, such as AKT, a Ser/Thr kinase associated with proliferation, migration, and growth.[22,26]

MiRNA-10b has been studied to the greatest degree in human breast cancer, and its overexpression has been associated with increased invasive potential both in vitro and in vivo.[22] To date, more than 100 studies have been carried out on miRNA-10b across 18 human cancer types, demonstrating its pleiotropic effects as a driver of tumor invasiveness and metastasis.[22]

In various types of human cancers, the expression of miRNA-494 and miRNA-141 has yielded conflicting results, suggesting tissue specificity. For instance, miRNA-494 is upregulated in hepatocellular, colorectal and non-small-cell lung carcinomas, while it is downregulated in gastric, prostatic, esophageal, mammary, pancreatic carcinomas, as well as in chondrosarcomas.[27-32] This dual

283 role also applies to miRNA-141, as observed in various human cancers, where some show
284 overexpression while others exhibit downregulation.[33]
285 In dogs, preliminary evidence of the dysregulation of miRNA-10b and miRNA-141 has been
286 provided in mammary cancer and intestinal T-cell lymphomas, respectively.[34,35] MiRNA-494
287 dysregulation has been reported in serum between dogs with splenic masses (namely
288 hemangiosarcoma or hematoma), however, conversely to the present study, an upregulation was
289 identified.[36]
290 The present study identified that miRNA-494 and miRNA-141 were downregulated in cardiac
291 hemangiosarcomas. It is conceivable that, similar to human medicine, in dogs, the function of
292 miRNA-494 is dependent on tumor type and tissue type. This could potentially account for the
293 variation in expression observed between the spleen, as previously reported, and the brain, as outlined
294 in this study.
295 Up to 30% of canine and human patients with hemangiosarcoma develop BM, with the highest
296 frequency observed in tumors originating from cardiac tissue.[5,6,37,38] Unlike metastases in other
297 anatomic locations, BM are subjected to the unique biology of the brain and its microenvironment.
298 This creates significant selective pressure on cancer cells, leading to adaptations that confer resistance
299 to current cancer therapeutics.[8,9]
300 Previous studies have shown that the manipulation of specific miRNA alterations using miRNA
301 mimics or antagomirs can restore the abnormal signaling downstream pathways, offering a promising
302 therapeutic approach for cancer treatment.[39] However, the therapeutic use of miRNAs has been
303 hindered by safety concerns, particularly regarding off-target biological effects.[40,41] Indeed, due to
304 the wide range of genes they regulate, each individual miRNA can have numerous targets, potentially
305 leading to unintended changes.[40] As of now, no miRNA-based drug candidates have advanced to
306 phase 3 clinical trials in humans.
307 In the current study, the expression profiles in healthy tissues differed significantly from those
308 observed in cancerous tissues. Specifically, the expression of miRNA-10b was nearly undetectable

in both normal brain tissue and perimetastatic cerebral tissue. The subtly altered miRNA profiles in periBM compared to control brains may be attributed to isolated tumor cells that went undetected during histologic examination, resulting in brain tissue dilution. Alternatively, it may be dependent on phenomena related to the tumor microenvironment.

Manipulating the miRNA profile observed in hemangiosarcoma could potentially involve inhibiting miRNA-10b if it is overexpressed, and mimicking miRNA-141 and miRNA-494 if they are lost. This approach may enable targeted attacks on cancer cells while minimizing toxicity to healthy tissues. However, further studies are needed to investigate the miRNA expression profile in other canine tissues to identify any potential toxicity. Also, our results demonstrate the complexity of the topic, particularly referring to tissue-specificity, and add intricacy to potential therapeutic aspects, but they help shed light on the function of these miRNAs.

The semi-quantitative approach used in this study to analyze miRNA expression has certain limitations, particularly in accurately quantifying low-expressed genes, necessitating two different normalization steps. Therefore, a quantitative real-time PCR analysis encompassing the entire dysregulation of the miRNome in canine hemangiosarcoma is warranted to better understand the involvement of miRNAs in the development of this tumor.

Furthermore, there are several challenges that contribute to the complexity of the issue. While miRNA-10b demonstrated overall overexpression in BM compared to control healthy brains and perimetastatic cerebral tissues, 3 dogs did not exhibit dysregulation. This finding is not unexpected, as miRNA expression is a dynamic process that undergoes temporal patterns. Some miRNAs are strongly induced or silenced by various biological stimuli, including chemotherapy, while others may be consistently over- or under-expressed during development and potentially disease progression.[42,43] Additionally, miRNA degradation may be regulated by disease-unrelated changes, which can affect miRNA turnover rates and consequently impact the interpretation of phenotypic changes.[44]

Of note, one of the BM showing no miRNA-10b dysregulation had immunohistochemically detectable PTEN, suggesting a potential negative regulatory role for miRNA-10b in PTEN expression, whereas in the remaining 2 cases, PTEN expression could not be assessed. This finding is not unexpected, as the over- or underexpression of miRNAs is thought to result in down- or upregulation of the protein product of the target genes, thereby affecting tumorigenesis. However, since we were not able to analyze PTEN gene expression and mutational status, a definitive conclusion cannot be drawn, and it remains to be determined if the observed miRNA alterations are truly tumor drivers.

Finally, all cases, except for one, were euthanized at the time of diagnosis. Therefore, it was not possible to correlate outcome data with the miRNA profile.

In conclusion, the dysregulation of multiple miRNAs targeting PTEN was observed in canine cardiac hemangiosarcoma and matched BM. Upon demonstration of their effects on tumor progression, these findings may provide a basis for developing a therapeutic strategy that selectively targets neoplastic disease. Using dogs with naturally occurring hemangiosarcoma as a preclinical model for drug testing may hold promise in advancing towards a pharmaceutical breakthrough for human treatment as well.

Data availability

The datasets generated during and/or analyzed during the current study are available from the corresponding author on reasonable request.

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Figure legends

Figure 1. A) Dog, brain metastasis of hemangiosarcoma. B) Histologic preparation of the metastatic lesion seen in A); hematoxylin and eosin, 20x magnification. C) Primary cardiac tumor showing intense PTEN positivity; hematoxylin counterstain, 20x magnification. D) Brain metastasis with positive PTEN expression; hematoxylin counterstain, 10x magnification.

Figure 2. miRNA-10b expression in control tissues and expression regulation in brain metastasis and cardiac hemangiosarcoma (CA). **A)** Scatter plot analysis of the target miRNAs (miRNA-10b, miRNA-141, miRNA-494, miRNA-21, and miRNA-19b) in healthy control heart tissues (CH) compared to healthy control brain tissues (CB). A clustergram is included in the figure, representing the magnitude of expression of the different miRNA in the whole samples. Colors represent the magnitude of expression (green: minimum, red: maximum) of the same miRNA within all the samples. **B)** Volcano plot representation of the Fold of Difference (FD) considering the statistical significance of $P < 0.05$. A table is included with all the average values of the FD of CH compared to CB. * $P \leq 0.05$; ** $P \leq 0.01$; *** $P \leq 0.001$.

Figure 3. Graph representation of the semiquantitiative analysis of miRNA-10b **(A)**, miRNA-141 **(B)** and miRNA-494 **(C)** expression in cardiac hemangiosarcoma (primary tumor, PT) normalized on control heart tissue (CH). Bars represent mean + SEM. Asterisks represent significant differences between groups. * $P \leq 0.05$; ** $P \leq 0.01$; *** $P \leq 0.001$.

Figure 4. A) Graph representation of the semiquantitative analysis of the miRNA-10b expression in brain metastasis (BM) and brain perimetastatic tissue (periBM) normalized on control brain tissue (CB). Bars represent mean + SEM. Asterisks represent significant differences between groups. **B)** Graph representation of the semiquantitative analysis of miRNA-10b expression in each BM compared to periBM of the same subject. * $P \leq 0.05$; ** $P \leq 0.01$; *** $P \leq 0.001$.