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miR-106B-25 Cluster expression: a comparative human and canine osteosarcoma study

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ABSTRACT

Background Osteosarcoma (OS) is the most common primary malignant bone tumour in dogs and human beings, characterised by similar genetic and clinical features. With the aim to define similarities and differences in the biological aspects involved in OS progression, a comparative study was performed to create a model to improve patient outcome.

Methods First, the expression of microRNAs (miRNAs) belonging to the cluster miR-106b-25 (miR-106b, miR-25 and miR-93-5p) in human and canine OS tissue was compared.

Results miR-25 and miR-106b presented a variable expression not significantly different from the corresponding normal bone, while miR-93-5p expression was increased in all OS specimens, with higher levels in the canine subset compared with human. Accordingly, its target p21 presented a weaker and less homogeneous immunostaining distribution in the canine group. Given the high expression of miR-93-5p in all OS specimens, the functional response of human 143B and canine DAN OS cells to miRNA inhibition was evaluated. Although p21 expression increased after miR-93-5p inhibition both at mRNA and protein level, a more significant cell response in terms of proliferation and apoptosis was seen in canine OS cells.

Conclusions In conclusion, canine OS tissue and cell line presented higher expression levels of miR-93-5p than human OS. In addition, the introduction of miR-93-5p inhibitor caused a cell response in 143B and DAN that differed for the more intense functional impact in the canine OS cell line.

BACKGROUND

Osteosarcoma (OS) is the most common tumour of bone in human beings and dogs. In human beings, it predominantly occurs in children and adolescents while it has a higher frequency in middle-aged to old large-breed dogs. Canine and human OS show clinical and molecular similarities and current treatment strategies are similar in both species. Adjuvant and neoadjuvant chemotherapy have significantly extended human long-term survival,[1] and adjuvant chemotherapy accompanied by amputation has prolonged dog survival.[2] However, the outcome of chemoresistant and/or metastatic patients remains unfavourable, highlighting the need for urgent novel treatment approaches.

Comparative studies that underline similarities and differences[3–6] between canine and human OS may be useful to create a model for a better understanding of the signalling pathways driving this disease[2 7] also representing targets for more effective molecular therapies.

MicroRNAs (miRNAs) are a class of small non-coding RNA molecules regulating gene expression by either upregulating or inhibiting target mRNAs.[8–10]

More than 50 per cent of all known miRNA genes are located in cancer-associated regions or in fragile sites of the genome, indicating that miRNAs could play an important role in oncogenesis.[11–13]

Previous studies demonstrated that down-expression of miR-1, miR-133b[14–16] and miR196a[17] in human and canine OS was associated to overexpression of the targets MET, MCL1 and ANXA1 involved in cell proliferation and invasion. By the evidence that miRNA dysregulation has a functional role in OS development, miRNA libraries from human OS cell lines were previously prepared.[18] The data demonstrated that all overexpressed miRNA are related to tumour development including miR-106b and miR-93-5p although p53wt OS cells responded to ectopic miR-93-5p overexpression more significantly increasing proliferation and invasion than cells lacking functional p53.

These results agree with data showing that miR-93-5p cooperates to inhibit p21, which is the real effector of p53 activity, through E2F1 regulation.[18 19] Concomitantly, knockdown of miR-93-5p induced upregulation of p21 and G1 cell-cycle arrest in OS cell lines.[20]

With the aim to define similarities and differences in the biological aspects involved

in OS progression, a comparative study was performed to create a model to improve patient outcome.

In this study, the authors first compared the expression of miRNAs belonging to the cluster miR-106b-25 (miR-106b, miR-25 and miR-93-5p) in human and canine OS. Then, given the increased mR-93 expression in both species, the functional response of 143B and DAN OS cells to miR-93-5p inhibition was evaluated in order to define their possible role in OS progression.

METHODS

Human OS population

Forty human high-grade OS diagnosed at the Rizzoli Orthopaedic Institute by bone expert pathologists were included in the study. All patients had complete clinical and histological data with paraffin-embedded material. In total, 23 were male and 17 female with a mean age of 26 years (from 8 to 57 years). Frozen samples of 21 of the 40 OS were stored at the Rizzoli BioBank and available for real-time PCR analysis. For all tumour specimens, the percentage of neoplastic cells in reference histological sections was ≥90 per cent. Normal bone tissue was used as control.

Canine OS population

The study was carried out on 22 paraffin-embedded and frozen tumour samples from large dogs with high-grade spontaneous osteoblastic OS provided from the University of Perugia. Histological classification was defined following the World Health Organization guidelines of histological classifications of bone and joint tumours in domestic animals.[21] Fourteen were male and eight female with a mean age of 8 years (from 1 year to 13 years). Comparable bone tissue from five large-sized dogs with no evidence of neoplastic disease was used as control.

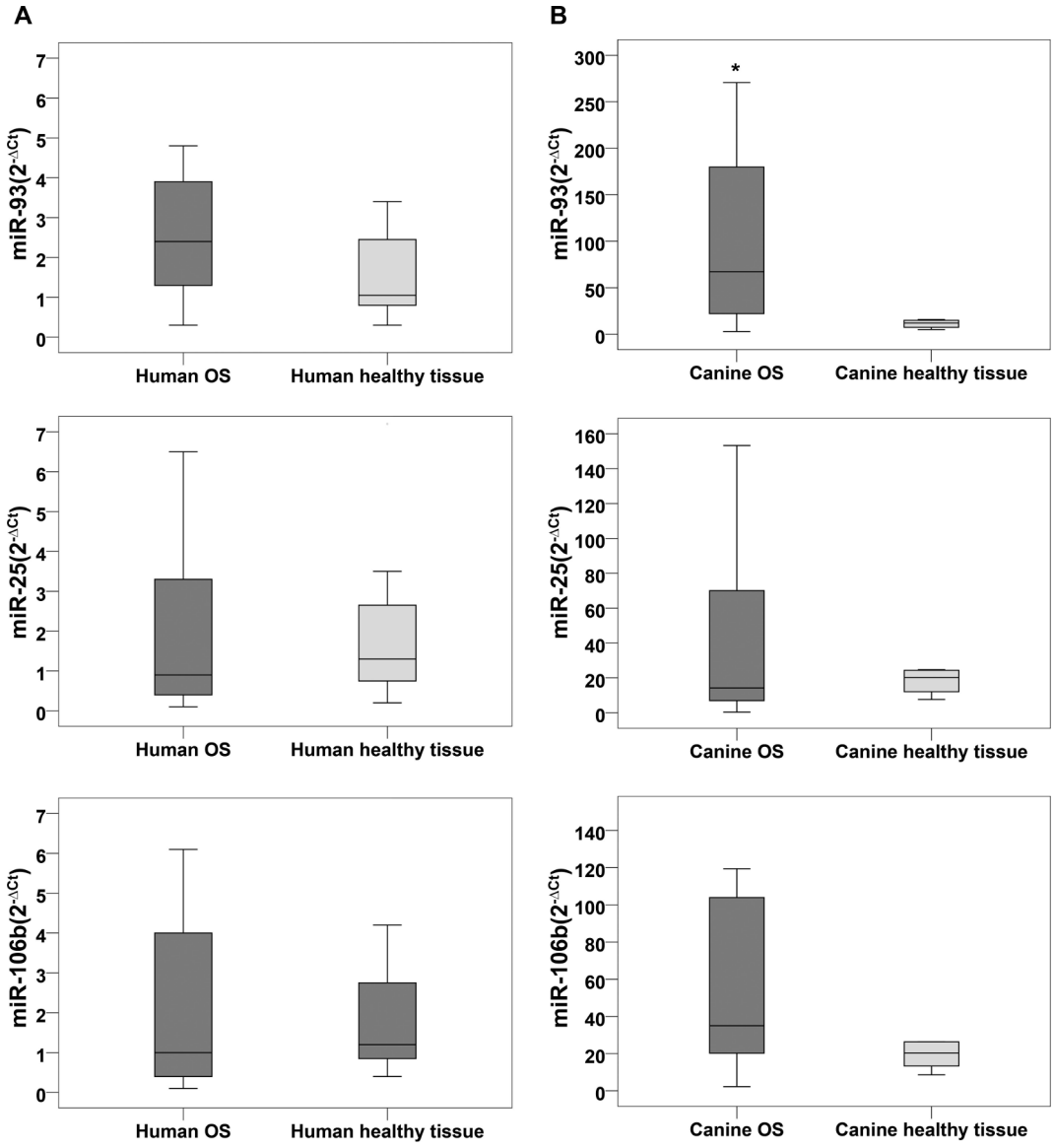

Figure 1 Distribution of $2^{-\Delta Ct}$ values of miR-93-5p, miR-25 and miR-106b in human and canine primary osteosarcoma (OS) and normal (healthy) tissue. Mann-Whitney test revealed a statistical difference for miR-93-5p in canine subgroup. *P<0.05.

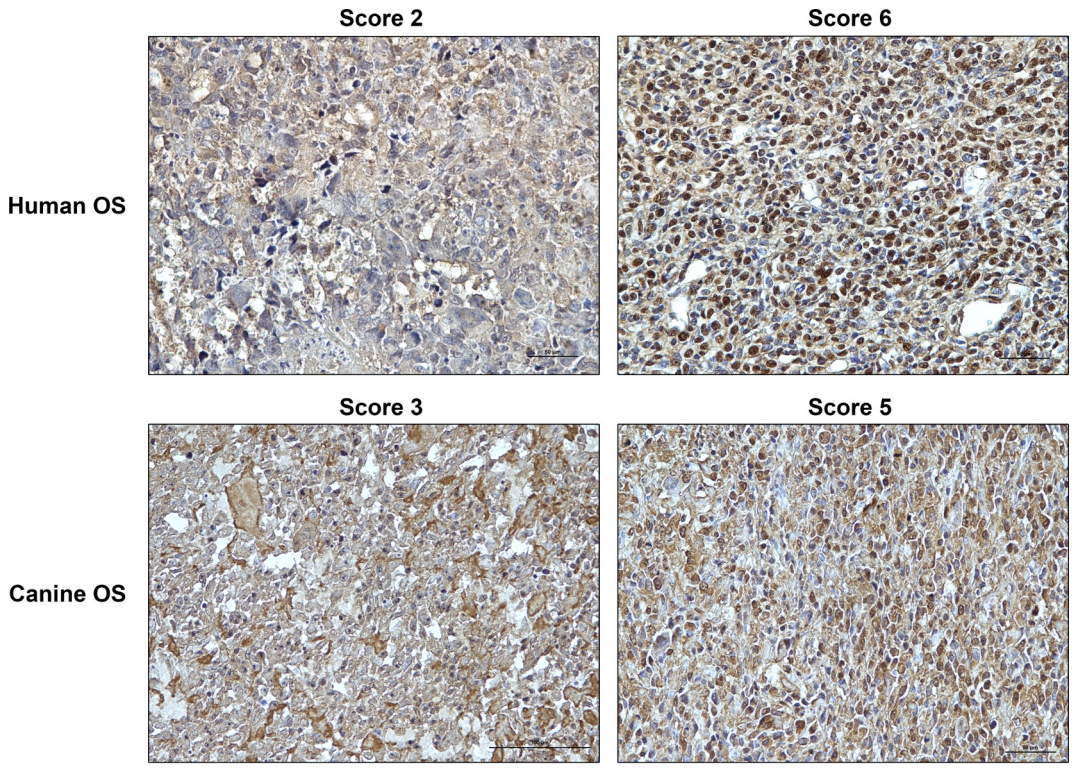

Figure 2 Representative immunostaining of p21 in human and canine osteosarcoma (OS). Human OS: p21 was variably expressed ranging from weak and focal (score 2) to strong and homogeneous (score 6). Canine OS: weak expression in <50% tumour cells (score 3); moderate expression in >50% tumour cells (score 5). In both subsets, a predominant nuclear localisation was seen in the tumour tissues with 5–6 score immunoreactivity.

OS cell lines

The human OS cell line 143B (ATCC, Manassas, VA, USA; no. CRL-11372) was cultured in Dulbecco's modified Eagle's medium supplemented with 10 per cent of fetal bovine serum (FBS), 100 U/ml penicillin, 100 mg/ml streptomycin and 1 mmol/l L-glutamine.

The canine OS cell line DAN (no. CRL-2130 ATCC), which derives D-17 canine osteogenic sarcoma cell line, was cultured in Eagle's minimal essential medium supplemented with 8 per cent of FBS, 0.4 mg/ml G418 and 2 mmol/l L-glutamine.

RNA extraction

Total RNA was extracted from OS frozen specimens, normal bone tissues, OS cell lines, hMSC and bMSC cells, following the manufacturer's instructions using TRizol reagent (Invitrogen, Carlsbad, CA, USA) and stored at −80°C.

miRNA expression analysis by real-time PCR miR-93-5p, miR-25-3p and miR-106b-5p have the same sequence in human beings and dogs (MirBase http://www.mirbase.org/index.html). Reverse transcription and real-time PCR were carried out on all tissues and cell line following TaqMan MicroRNA Assay Protocol (Applied Biosystems, Foster City, CA, USA) and the expression of miR-93-5p (miRNA assay no. 001090), miR-25-3p (miRNA assay no. 000403) and miR-106b-5p (miRNA assay no. 000442) were quantified using $2^{-\Delta Ct}$ comparative method (Applied Biosystems, User Bulletin no. 2 P/N 4303859) and normalised using RNU44 as endogenous reference (miRNA assay no. 001094) that is the most constant and reliable also in dogs.[17]

p21 protein expression by immunohistochemistry p21 protein immunostaining was evaluated on Tissue Macro Array (TMA) (3D Histech, Budapest, Hungary) performed in 40 human and 22 canine paraffin-embedded OS samples and in the corresponding normal tissues by spotting duplicated samples for each patient.

Antigen retrieval was performed by incubation in citrate buffer (pH 6.0) and maintained at a sub-boiling temperature for 10 minutes in microwave. Sections were incubated overnight with rabbit monoclonal anti-p21 (H-164 sc-756) (Santa Cruz Biotechnology, Santa Cruz, CA, USA) diluted 1:500 in PBS. Sections were then washed and incubated with the streptavidin–biotin peroxidase DAB detection system (Dako, Glostrup, Denmark), according to the manufacturer's protocol.

One thousand cells per lesion were evaluated, sample staining was scored for intensity (0, no visual staining; 1, weak; 2, moderate; 3, strong) and percentage of positive tumour cells (negative or minimally expressed (<10 per cent); 1, <25 per cent; 2, 25–49 per cent; 3, ≥50 per cent). Cut-off levels, determined by the score sum, were applied as 0 for negative cases, 2–4 for weak or moderate positivity in less than 50 per cent of tumour cells, 5–6 for a moderate positivity in at least 50 per cent of tumour cells

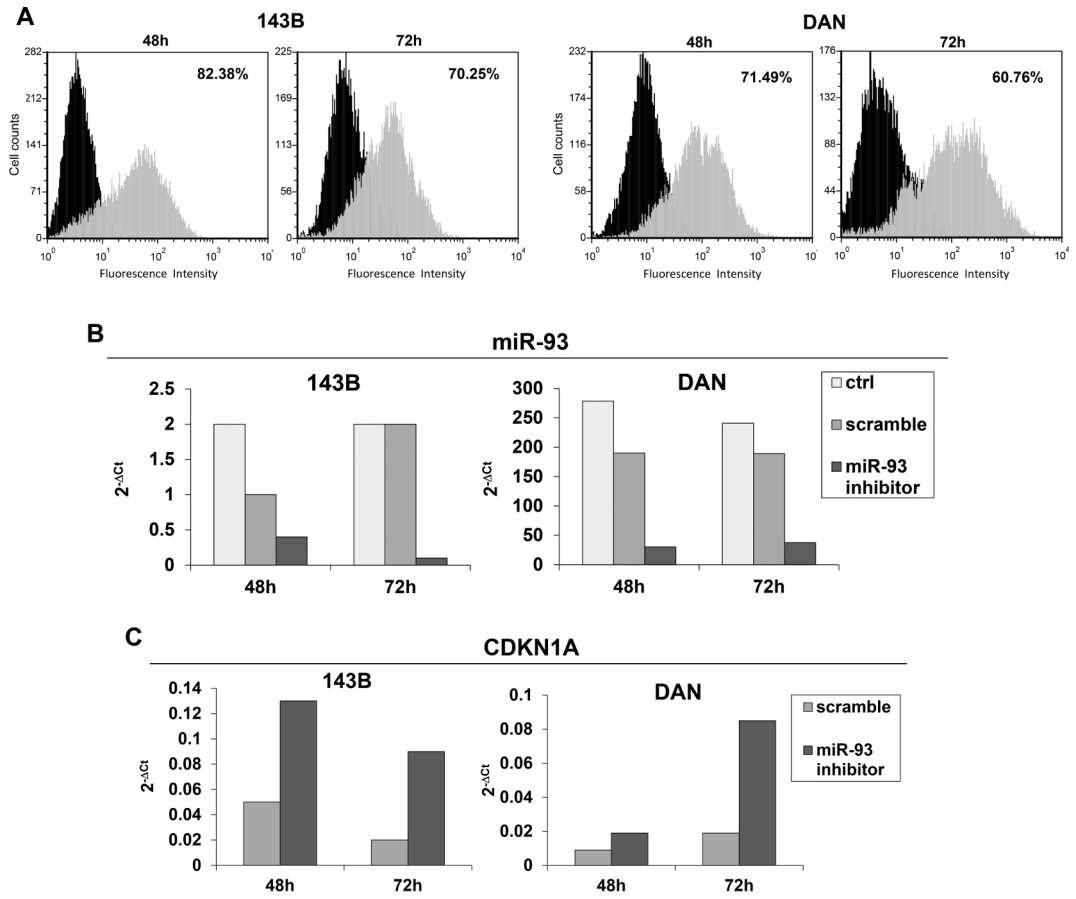

Figure 3 miR-93-5p inhibitor transfection efficiency. (A) Efficiency was measured at 48 hours and 72 hours following transfection by flow cytometry. miRNA control (scramble) in black and miR-93-5p inhibitor in grey. (B) Efficiency of transfection with miR-93-5p inhibitor in human 143B and canine DAN cell lines by RT-qPCR ($2^{-\Delta Ct}$ values). (C) p21 expression in 143B and DAN cells by RT-qPCR ($2^{-\Delta Ct}$ values). ctrl, non-transfected cells; miR-93-5p inhibitor, transfected cells; scramble, negative control.

or a strong positivity in at least 25 per cent of tumour cells.

Transfection of miR-93-5p-5p inhibitor molecule

OS cell lines were plated at 2.0×10^5 cells/well in six-well plates for each transfection in 2 ml of complete medium containing 10 per cent Fetal Calf Serum (FCS) for 24 hours. Transfections were performed using reagent Lipofectamine 2000 (Invitrogen). For each well, 200 nM of miR-93-5p-5p inhibitor (ID: MH10951; Ambion) and 200 nM miRNA inhibitor negative control (scramble) (code 4464084; Ambion) were transfected into tumour cells. Transfection efficiency was monitored after 48 hours and 72 hours by flow cytometry (FACSCalibur; BD, San Jose, CA, USA) using Cy3 dye-labelled anti-miR negative controls (code AM17011; Ambion) and by real-time PCR using ΔCt relative method.[22]

Cell growth assay

The number of adherent and viable cells was assessed microscopically using a Neubauer chamber, and viability was evaluated as the percentage of cells that excluded 0.2 per cent trypan blue. After 48 hours and 72 hours from transfection, cells were washed once with 1× Dulbecco's PBS, harvested by trypsinisation and cell number was determined by counting using trypan blue.

Apoptosis assay

Apoptotic cell death was analysed in non-transfected and transfected cells at 48 and 72 hours from transfection with the Annexin V-FITC apoptosis detection kit (MEBCYTO Apoptosis kit; MBL International, Woburn, MA, USA). Following the manufacturer's instructions, adherent cells were washed with PBS 1×, trypsinised, centrifuged and washed twice with PBS. Cells were then suspended in 500 µl of staining solution containing FITC-conjugated Annexin V antibody and propidium iodide (PI) and after 30 min of incubation on ice were analysed by flow cytometry using a FACSCalibur flow cytometer and CellQuest Software (BD Biosciences, San Jose, CA, USA).

Cell-cycle analysis

Flow cytometric analysis was performed to define cell-cycle distribution for transfected and non-transfected cells. After 72 hours from transfection, cells were harvested by trypsinisation and fixed with 70 per cent ethanol. Cells were stained for total DNA content with a solution containing 20 µg/ml PI. Cell-cycle distribution

was then analysed with a FACSCalibur flow cytometer (BD Biosciences).

CDKN1A (p21) gene expression by real-time PCR

Reverse transcription of mRNA from 143B and DAN non-transfected and transfected cells was carried out in 100 µl final volume from 400 ng total RNA using High Capacity cDNA Archive kit (Applied Biosystems) according to the manufacturer's instructions.

Real-time PCR was performed using Viia7 sequence detection system (Applied Biosystems) according to the manufacturer's protocol. *CDKN1A* gene was quantified by ΔCt method using TaqMan Expression Assays (Hs00355782_m1 for human and canine cell line) (Applied Biosystems) and normalised to a housekeeping ACTB (Hs99999903_m1 for human cell line and cf 03023880_g1 for canine cell line) (TaqMan Expression Assays; Applied Biosystems).

p21 protein expression by Western blot

According to standard procedures, 50 µg of protein extracts from cell lysates before and after 48 hours of transfection was prepared and analysed by 10 per cent SDS-PAGE. Western blot analysis was performed by using anti-p21 (H-164 sc-756) (Santa Cruz Biotechnology, Santa Cruz, CA, USA) diluted 1:1000. The signal was visualised by Immobilon Western Chemiluminiscent HRP substrate (Millipore, Billerica, MA, USA) and quantified by densitometric analysis using GS-800 imaging densitometer and Quantity One software (Bio-Rad, Hercules, CA, USA). A rabbit anti-actin antibody (Sigma Chemical, St. Louis, MO, USA) was used as reference protein.

Statistical analysis miRNA expression is presented as the $2^{-\Delta Ct}$ value for each sample. Values of median and 25th–75th percentile were calculated within the two subsets. A non-parametric Mann-Whitney U test was performed to compare miRNA median levels in the subsets. Student's *t* test was performed for in vitro experiments. All assays were performed in triplicate and for all tests P value ≤0.05 was considered statistically significant. All statistical analyses were performed using SPSS V.19.0.

RESULTS

miRNA expression in human and canine OS specimens

miR-93-5p, miR-25-3p and miR-106b-5p expression was evaluated in 21 human and 22 canine OS tissues. Corresponding normal bone tissue was used as control.

In human beings, miR-93-5p presented higher median $2^{-\Delta Ct}$ values in tumour tissue than in healthy bone (2.4; 1.15–4.20 and 1.05; 0.80–2.92, respectively; p=0.8) without reaching statistical significance. In contrast, miR-25 and miR-106b were slightly less expressed with median values respectively of 0.9 (0.35–3.5) and 1.0 (0.35–5.0) in OS and 1.3 (0.72–3.07) and 1.2 (0.82–3.32) in normal tissue (figure 1A).

A significant difference was seen for miR-93-5p expression between canine OS and normal bone tissue (67.2; 21.7–202.4 and 14.1; 7.42–58.8, respectively; p=0.04). No statistical significance was seen for miR-106b expression in canine tumours compared with controls (44.2; 19.7–157.7 v 20.3; 10.9–126.1, respectively). miR-25-3p was found less expressed than the control (14.2; 6.67–90.8 and 23.9; 12–101, respectively) (figure 1B).

p21 protein expression in human and canine OS specimens

p21 protein expression was evaluated by immunohistochemistry (IHC) in 22 canine and 40 human paraffin-embedded OS tissues (figure 2).

In human OS, p21 immunoreactivity was homogeneously distributed (>50 per cent of tumour cells) in the majority of cases with an intensity ranging from weak to moderate (score 4–5) and a predominantly nuclear localisation. In four cases, the expression was very weak and focal (score 2), while eight cases showed a strong positivity in at least 50 per cent of tumour cells (score 6).

In 14 of 22 canine OS, p21 showed a variable immunostaining from weak to moderate in less than 50 per cent of tumour cells (score 2–4). In two cases, p21 was moderately expressed in at least 50 per cent of tumour cells (score 5) with a localisation both at nuclear and cytoplasmatic level. Six cases were p21 negative.

Transfection of miR-93-5p inhibitor in human and canine OS cell lines

Human 143B and canine DAN cell lines presented miR-93-5p $2^{-\Delta Ct}$ levels respectively of 2.0 and 278.5 and were transfected with 200 nM of miRNA-93 inhibitor and 200 nM of negative control (scramble).

The transfection efficiency was 82.38 per cent and 70.25 per cent at 48 hours and 72 hours, respectively, for 143B cells, and 71.49 per cent and 60.76 per cent at 48 hours and 72 hours, respectively, for the DAN cells (figure 3A).

Real-time PCR analysis confirmed a decreased expression of miR-93-5p in the 143B-transfected cells (−60 per cent at 48 hours and −95 per cent at 72 hours with respect to scramble) as well as in the DAN-transfected cells (−84 per cent at 48 hours and −79.9 per cent at 72 hours) (figure 3B).

Accordingly, an increase of p21 target gene (*CDKN1A*) was seen in both cell lines (+61 per cent and +77.7 per cent in 143B-transfected cells and +52.6 per cent and +77.6 per cent at 72 hours in DAN-transfected cells) when compared with scramble (figure 3C).

Effect of miR-93-5p inhibitor in OS cell lines

Human 143B OS cells responded to transfection by decreasing cell proliferation up to 72.5 per cent of control at 72 hours (figure 4), with no changes in apoptotic fraction (figure 4). Concomitantly, a slight increase of G1 cells was seen (66.2 per cent and 59.9 per cent in control and scramble, respectively; 65.2 per cent in transfected cells) (figure 4).

Canine DAN responded to a greater extent significantly decreasing proliferation up to 37.3 per cent of control after 72 hours (figure 5) associated to a significant increase of apoptosis (32 per cent of apoptotic cells v 8 per cent and 19 per cent in control and scramble, respectively) (figure 5).

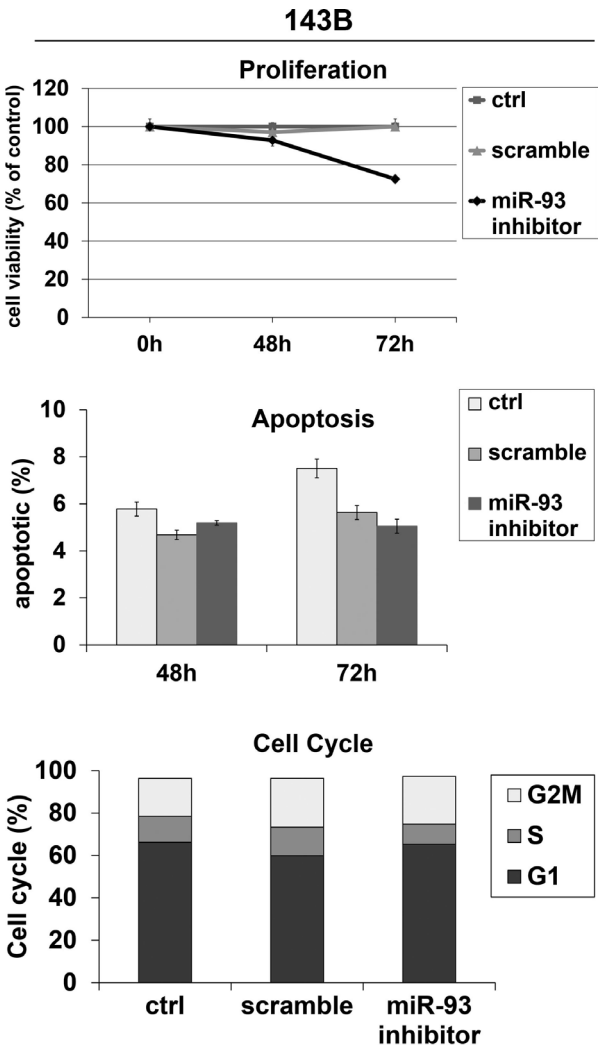

Figure 4 Effects of miR-93-5p inhibition in human 143B cell lines. miR-93-5p inhibition led to a slight decrease of cell viability at 72 hours of transfection. No changes in apoptotic fraction was seen. Cell-cycle modulation showed a slight increase of cells in G1 phase. ctrl, non-transfected cells; miR-93-5p inhibitor, transfected cells; scramble, negative control.

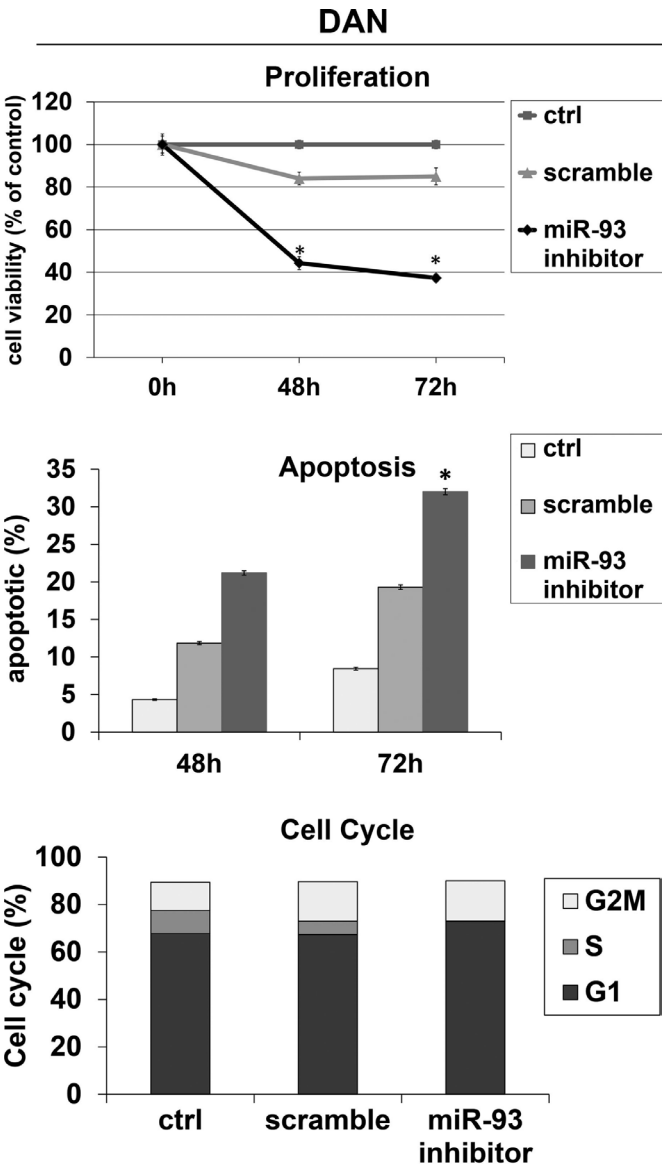

Figure 5 Effects of miR-93-5p inhibition in canine DAN cell line. Cell viability significantly decreased after 72 hours of transfection, associated with an increase in apoptotic cell percentage. Cell-cycle modulation showed an almost complete disappearance of S phase, associated to an increase of G1 phase. *P<0.05. ctrl, non-transfected cells; miR-93-5p inhibitor, transfected cells; scramble, negative control.

Accordingly, an increase of cells in G1 phase (from 67 per cent in control and scramble to 72.9 per cent in transfected cells) and an almost complete disappearance of S phase was seen after 72 hours of transfection (9.7 per cent and 5.6 per cent in control and scramble, respectively; 0.11 per cent in transfected cells) (figure 5).

By Western blot, increased levels of miR-93-5p target, p21, were seen after miR-93-5p inhibitor transfection in both 143B and DAN cells compared with control and scramble (figure 6).

CONCLUSION

Human and canine OS present similar clinical features and molecular abnormalities determined by aberrant gene expression and deregulation of p53 and RB pathways.[2 23–26] This may cause trouble in prognosis and therapy response. However, since dogs are genetically more homogeneous, a comparative study may lead to a better understanding of the biology of OS, improving disease management.[27 28] Previous results demonstrated that all miRNAs in the paralogous clusters miR-17-92, miR-106b-25 and miR-106a-92 were overexpressed in human OS cell lines.[29] The oncogenic role of the miR-106b-25 cluster (miR-106b, miR-93-5p and miR-25) in prostate cancer appears to be mediated by deregulation of focal adhesion and apoptotic pathways and it is associated with poor prognosis.[30] Recently, data showed increased expression of miR-93-5p in OS tissues,[18 20] but no data in canine OS have ever been reported.

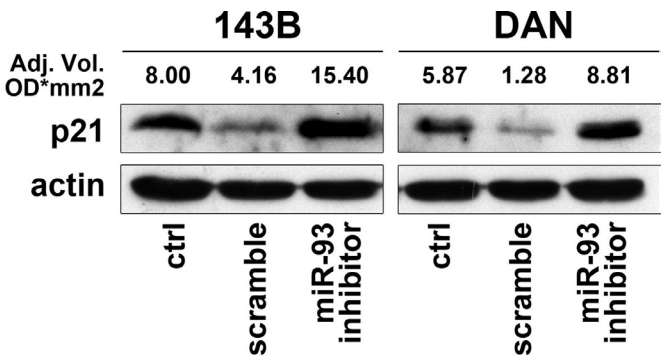

Figure 6 Representative detection of p21 protein expression in 143B and DAN. A higher level of p21 was observed in miR-93-5p inhibitor transfected cells when compared with non-transfected cells (ctrl) and scramble. Actin was used as reference protein.

In this study, the authors compared miRNA-106b-25 cluster expression in human and canine OS tissues and reported higher miRNA levels in the canine OS than in the human subset. In detail, miR-25 and miR-106b presented a variable expression in OS in both subsets, with similar median values as compared with the corresponding control, but with a wide distribution towards higher levels.

In contrast, miR-93-5p was overexpressed in all OS specimens, with higher levels in the canine OS subset compared with human. In dogs, this expression was significantly higher compared with corresponding normal bone.

This evidence together with previous analyses that established p21 as a target of miR-93-5p (http://www.targetscan.org, http://mirdb.org) programmes[19 20] led us to perform IHC of the p21 protein that is involved in multiple important functions.[31] In fact, previous data showed that miR-93-5p, via p21, enhances cell proliferation in nasopharyngeal carcinoma and inhibits apoptosis in hepatocellular carcinoma.[32]

Our results demonstrated that the expression of p21 was variable in terms of intensity and distribution within both subgroups with a predominant nuclear localisation in cases with moderate to strong and homogeneous immunoreactivity. This might be related to a more intense inhibitory activity on cell-cycle progression.[31]

When miR-93-5p inhibitor was introduced into 143B and DAN cell lines, a different functional response was obtained. In DAN, the significant reduction of proliferation rate was associated to an increase of p21 expression and apoptotic cells and an almost complete disappearance of the S phase fraction.

Similarly, in human beings, an increase in p21 expression was found both at gene and protein level after miR-93-5p inhibition, but slight changes in proliferation and cell-cycle modulation were evident. No influence on apoptotic rate was demonstrated.

Our data show that miR-93-5p is overexpressed in canine OS specimens and, to a lesser extent, in human samples when compared with healthy tissue, thus indicating a common end point in tumour development.

In conclusion, canine OS tissue and DAN cell line presented higher expression levels of miR-93-5p than human OS with a significant difference when compared with normal bone tissue. In addition, although p21 expression increased both at mRNA and protein level after miR-93-5p downregulation in both subsets, the introduction of miR-93-5p inhibitor caused a cell response in 143B and DAN that differed for the more intense functional impact in the DAN. Although further studies are needed to support these findings, the data suggest that the control of miR-93-5p on cell proliferation and malignant progression might be mediated by different growth signalling pathways depending on the tumour cell type[33–36] and species. A better characterisation of these species-correlated signalling pathways might be relevant for the identification of new therapeutic targets in OS.

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Ethics approval The study was approved by the Rizzoli Institute Ethic Committee (human OS population no. 0033276).

Provenance and peer review Not commissioned; externally peer reviewed.

Data availability statement All data relevant to the study are included in the article.

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