Intraperitoneal Administration of Synthetic MicroRNA-214 Exhibits Tumor Suppression in an Intraperitoneal Dissemination Mouse Model of Canine Hemangiosarcoma

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Abstract

Canine hemangiosarcoma (HSA) has extremely poor prognosis, making it necessary to develop new systemic treatment methods. MicroRNA-214 (miR-214) is one of many microRNAs (miRNA) that can induce apoptosis in HSA cell lines. Synthetic miR-214 (miR-214/5AE), which showed higher cytotoxicity and greater nuclease resistance than mature miR-214, has been developed for clinical application. In this study, we evaluated the effects of miR-214/5AE on stage 2 HSA in a mouse model. Mice intraperitoneally administered with miR-214/5AE (5AE group) had significantly fewer intraperitoneal dissemination tumor foci (median number: 80 vs. 233; $p < 0.05$) and lighter median foci weight (0.26 g vs. 0.74 g; $p < 0.05$). There was an increase in p53 and cleaved caspase-3 expression in the 5AE group. The mice in this group also had a significantly lower proportion of Ki-67-positive cells than those in the non-specific miR group. Notably, there were no significant side effects observed. These results indicate that intraperitoneal administration of miR-214/5AE exhibits antitumor effects in the intraperitoneal dissemination mouse model of HSA by inducing apoptosis and suppressing cell proliferation. We strongly believe that miR-214/5AE could be a novel miRNA-based chemotherapeutic agent capable of improving the prognosis of HSA.

Introduction

Canine hemangiosarcoma (HSA) is a malignant tumor that affects various tissues and organs throughout the body (Hammer et al. 1996; Brown et al. 1985; Sorenmo et al. 1993). The most commonly affected anatomical sites are the spleen, right atrium, liver, and skin. HSA is an aggressive tumor with high rates of metastasis and local tissue invasion (Prymak et al. 1998). The peritoneum, liver, lung, muscle, and lymph nodes are the most common sites of metastasis (Brown et al. 1985). HSA is divided into three clinical stages: stage 1 involves the primary tumor only; stage 2 involves the rupture of primary tumor or lymph node metastasis; and stage 3 involves distant metastasis. The prognosis of HSA is extremely poor; the median survival time after post-operative chemotherapy is approximately 4 – 6 months (Sorenmo et al. 2004). In clinical practice, HSA is usually diagnosed at stage 2 or 3 because of its malignant biological behavior. The disease is frequently diagnosed in the later stages because many dogs with HSA may not show any clinical signs, and the signs that appear upon tumor rupture may not be noticeable to the owners and are often transient. The delay in diagnosis adds to the challenge in successfully treating this condition. Therefore, it is necessary to develop a new systemic treatment method.

MicroRNAs (miRNAs) are a class of small non-coding RNAs that regulate various biological processes. miRNA controls protein synthesis by binding to the 3' untranslated region of the target messenger RNA (mRNA). Previous studies have suggested that deregulation of miRNAs is involved in carcinogenesis and cancer progression (Croce 2009). In particular, microRNA-214 (miR-214) has been shown to be involved in controlling tumorigenesis and angiogenesis (Sharma et al. 2015; Van Mil et al. 2012). A previous study reported that miR-214 was downregulated in HSA cells and some clinical samples of HSA; it was also able to induce apoptosis in canine HSA cell lines (Heishima et al. 2015). Since miRNAs directly target

mRNA, they are highly specific. Furthermore, as miRNAs work in the cytoplasm, they have little effect on nuclear genomic DNA. Hence, miRNAs have been attracting attention as therapeutic targets in recent years, and miR-214 could be an effective therapeutic drug for HSA. However, *in vivo*, natural miRNAs are hydrolyzed immediately by nuclease. For effective therapeutic application, it is necessary to confer nuclease resistance to miRNAs. To enable the administration of miR-214 *in vivo*, a previous study developed a synthetic miR-214 (miR-214/5AE) and reported that it had higher cytotoxicity, nuclease resistance, and transfection rate than mature miR-214 *in vitro* (Yoshikawa et al. 2020). However, the effect of miR-214/5AE *in vivo* remains unknown.

In this study, we evaluated the effect of intraperitoneal injections of miR-214/5AE in an intraperitoneal dissemination mouse model of HSA. The purpose of this study was to verify the *in vivo* antitumor effect of miR-214/5AE and to guide its future clinical application.

Material And Methods

Cell lines and culture conditions

The HSA cell line, Re21 (Murai et al. 2012), was cultured in Dulbecco's Modified Eagle Medium (DMEM) with 10% fetal bovine serum (Biosera, Kansas City, MO, USA). Cells were grown and maintained at 37°C and 5% CO₂ in a cell incubator.

Cell line validation statement

The Re21 cell line used in this study has been established and validated in a previous study (Murai et al. 2012). The cell line was kindly provided by the manufacturer. The cell line was tested and found to be free of mycoplasma (unpublished data).

In vivo xenograft model

BALB/cSlc-nu/nu nude mice were obtained from Japan SLC (Shizuoka, Japan). The mice were inoculated with Re21 cells, at 4.0×10^6 cells per 300 μ L DMEM intraperitoneally, in the lower right abdomen. The subjects were sacrificed 33 days after the inoculation. Blood tests and evaluations of peritoneal dissemination, which include counting the number of foci and measuring the tumor weight, were performed, and the tissues were processed for histological examination. The protocol of animal experiments was reviewed and approved by the Ethics Review Board of the Joint Faculty of Veterinary Medicine of Gifu University (approval number: 2019 – 117).

miRNA administration

The study flow is shown in Fig. 1. Intraperitoneal injection of miRNA was performed on days 21 to 30 every 3 days for a total of four times. miR-214/5AE was developed using previously described methods (Yoshikawa et al.2020; Koizumi et al. 2018; Kano et al. 2018). In the 5AE group (n = 6), 2 nmol of miR-

214/5AE in 50 μ L of Opti-MEM was mixed with 10 μ L of Lipofectamine RNAiMAX (Invitrogen, Carlsbad, CA, USA), and the mixture was injected intraperitoneally. In the non-specific (ns) miR group (n = 6), 2 nmol of ns miRNA (5'-GUA GGA GUA GUG AAA GGC C-3') (Hokkaido System Science, Hokkaido, Japan) in 50 μ L of Opti-MEM was mixed with 10 μ L of Lipofectamine RNAiMAX, and the mixture was injected intraperitoneally. In the non-treat group (n = 5), the mice were not inoculated with either cells or miRNA.

Magnetic resonance imaging

To evaluate the intraperitoneal dissemination foci, magnetic resonance imaging (MRI) was performed on the day before (pre-injection) and the one after (post-injection) the injection of miRNA (Fig. 1). Images were acquired using a 3T MRI scanner (Ingenia 3.0T CX, Philips Japan, Tokyo, Japan) with a radiofrequency coil for the fingers (dStream Microscopy coil set, Philips Japan). Images acquired included T2-weighted images (echo time, 90; repetition time, 3000), T1-weighted images (echo time, 9; repetition time, 500), and fat-suppressed T2 images (echo time, 70; repetition time, 3195.49).

One-step reverse transcription polymerase chain reaction using canine-specific primers

Intraperitoneal dissemination foci, canine spleen tissue, and mouse spleen tissue were used for one-step reverse transcription polymerase chain reaction (RT-PCR). Total RNA was isolated from tissue using RNeasy Plus Universal Kits (QIAGEN, Hilden, Germany) according to the standard protocol. RT-PCR was performed using a one-step RT-PCR kit (QIAGEN). The PCR primer sequences were as follows: canine-specific CD31 – forward: 5'-GCACACAAGAGGCATGGTAAC-3', reverse: 5'-GAATGGAGCACACAGGTTT-3' and canine-specific β -actin – forward: 5'-ATTGAGCACGGCATCGTC-3', reverse: 5'-GTCACCGGAGTCCATCACG-3'.

Immunoblotting

The foci in the ns miR and 5AE groups were subjected to immunoblotting. Total protein was extracted from the tissue using 1% sodium dodecyl sulfate. Protein concentrations were measured using Bradford's protein assay (DC Protein Assay kit, Bio-Rad, Hercules, CA, USA). Protein samples (15 μ g/lane) were subjected to 10–20% sodium dodecyl sulfate-polyacrylamide gel electrophoresis and were transferred to 0.48 μ m polyvinylidene fluoride membranes (Immobilon-P Membrane, EMD Millipore, USA). After blocking of non-specific reactions for 1 hour with 2% ECL Prime Blocking Agent (RPN418, GE Healthcare, USA), the membrane was incubated overnight at 4°C with a given primary antibody. The primary antibodies used for immunoblotting were anti-cleaved caspase-3 rabbit monoclonal antibody (1:2000; clone 5A1E, Cat. #9664, Cell Signaling Technology, Danvers, MA, USA), anti-COP1 rabbit polyclonal antibody (1:2000; ab70889, Abcam), and anti-p53 mouse monoclonal antibody (1:1000; clone 80/p53, Cat. #610183, BD Biosciences, USA). The membranes were then washed and incubated for 1 hour at room temperature with secondary horseradish peroxidase-linked anti-mouse immunoglobulin G (IgG) or anti-rabbit IgG antibodies (1:1000; Cell Signaling Technology) and subsequently washed three times with 0.1% Tris buffered saline with Tween 20. The immunoblots were visualized using Immobilon Forte Western HRP Substrate (EMD Millipore). The loaded amount was verified by re-incubating the same membrane with

anti-glyceraldehyde 3-phosphate dehydrogenase rabbit monoclonal antibody (1:5000; #2118, Cell Signaling Technology) overnight at 4 °C. Each experiment was performed a minimum of three times, and similar results were obtained each time. Quantitative analysis of the bands was performed by using an imaging software (Image J, ver.1.52a, National Institutes of Health, Bethesda, MD, USA) which yielded densitometric values.

Immunohistochemical staining

The disseminated foci in the ns miR and 5AE groups were also subjected to immunohistochemical staining. Tissue sections (3–5 µm thick) of formalin-fixed, paraffin-embedded specimens were deparaffinized in Lemosol (Wako Pure Chemical Industries, Osaka, Japan), rehydrated in graded ethanol, immersed in Immunoactive (Matsunami Glass Industries, Ltd., Osaka, Japan), and heated for 15 min at 121°C in an autoclave. Endogenous peroxidase was blocked by incubation in 0.3% H₂O₂ in methanol for 30 min at room temperature. To prevent the binding of non-specific proteins to the primary antibody, the sections were treated with 2.5% normal horse serum (Wako Pure Chemical Industries) for 15 min at room temperature. Sections were incubated with primary antibody overnight at 4°C; for the negative control, phosphate-buffered saline (PBS) was used instead of the primary antibody. The primary antibody used for immunohistochemical staining was anti-Ki-67 mouse monoclonal antibody (1:50; Clone MIB-1, M7240, Dako, Glostrup, Denmark). The sections were then washed with PBS and incubated with the appropriate secondary antibody (ImmPRESS Universal Polymer Kit Peroxidase, Horse Anti-Mouse/Rabbit IgG, MP-7500, Vector Laboratories, Inc, CA, USA) at room temperature for 30 min. After three washes with PBS, binding was detected using 3,3'-diaminobenzidine hydrochloride (Liquid DAB + Substrate Chromogen System; Dako). Finally, the sections were washed in distilled water and counterstained with Mayer's hematoxylin. The percentage of Ki-67-positive cells in tumor tissue was calculated as the number of reddish brown-positive nuclei in a total of 1000 neoplastic cells from 10 fields.

Statistical analysis

The results are presented as the mean ± standard deviation. Mouse weight and blood test results were analyzed using the Kruskal–Wallis test. The number of foci, tumor weight, immunoblotting densitometric values, immunohistochemical staining results were analyzed using the Mann–Whitney U test. *P* values ≤ 0.05 were considered reflective of statistical significance. All statistical analyses were performed using EZR software, which is a graphical user interface for R and is a modified version of R commander (Kanda 2013).

Results

Intraperitoneal dissemination mouse model of HSA

To develop the stage 2 HSA mouse model, HSA cells were inoculated intraperitoneally into mice. Multiple foci (> 1–5 mm) were present intraperitoneally at the dissection. Histopathological examination suggested these foci were HSA (Fig. 2A). Furthermore, mice foci and canine spleen tissue were positive

for canine-specific CD31 and β -actin, whereas mice spleen tissue and negative control tissue were negative for these factors (Fig. 2B). These findings indicate that the mice intraperitoneal foci were derived from HSA.

Nodules were not clear on T2- and T1-weighted images, so fat-suppressed T2-weighted images were used in this study. Pre- and post-injection MR images showed multiple hyperintense nodules in both the ns miR and 5AE groups, but not in the non-treat group. In the pre-injection MR images, the lesions in the ns miR and administration groups were similar. However, post-injection MR images showed a clear difference in lesion size in each group (Fig. 3). Many foci were engrafted around the gastric pylorus, caudal liver, spleen, and genitals (Fig. 4). Hyperintensity nodules (arrowheads) were observed in the 5AE group (Fig. 4A, 4D) and the ns miR group (Fig. 4B, 4E) but not in the non-treat group (Fig. 4C).

Before miRNA administration, nodular shadows were confirmed in the 5AE group (Fig. 4A) and ns miR group (Fig. 4B) almost equally. However, after miRNA administration, nodular shadows were decreased in the 5AE group (Fig. 4D) compared to the control group (Fig. 4E).

Intraperitoneal administration of miR-214/5AE resulted in tumor growth suppression of mouse intraperitoneal dissemination foci

The number and weight of foci are shown in Fig. 5. The mean number of foci was significantly lower in the 5AE group than in the ns miR group (5AE: 80, range, 64–111; ns miR: 233, range, 138–312; $P < 0.05$) (Fig. 5A). Moreover, the mean weight of foci was significantly lower in the 5AE group than in the ns miR group (5AE: 0.26 g, range: 0.13–0.39 g; ns miR: 0.74 g, range, 0.2–1.37 g; $P < 0.05$) (Fig. 5B). These results imply that intraperitoneal administration of miR-214/5AE reduced the number and weight of foci.

miR-214/5AE induced apoptosis and exerted a growth suppressive effect in vivo

To determine the cytotoxic mechanism of miR-214/5AE, the proportion of cells expressing cleaved caspase-3, p53, COP1, and Ki-67 proteins were estimated through immunoblotting and immunohistochemical staining. The results of immunoblotting are summarized in Fig. 6. The expression of p53 and cleaved caspase-3 tended to be higher in the 5AE group than in the ns miR group. There was no significant difference in COP1 expression between the groups. The 5AE group had a significantly lower proportion of Ki-67-positive cells than did the ns miR group (5AE: $7.2\% \pm 1.0\%$; ns miR: $15.0\% \pm 3.5\%$; $P < 0.05$) (Fig. 7). These findings support the fact that miR-214/5AE induces apoptosis and exhibits a growth suppressive effect in intraperitoneal foci.

Adverse events of miR-214/5AE administration

The mean body weight in each group was not significantly different (Fig. 8). Blood test results are shown in Table 1. Marked kidney failure and hepatic failure were not observed in any of the subjects.

Discussion

In this study, we demonstrated that intraperitoneal administration of miR-214/5AE can induce apoptosis and growth suppression in an intraperitoneal dissemination mouse model of HSA. We also found that the administration of miR-214/5AE did not result in any adverse event.

Mature miRNAs are hydrolyzed immediately by nuclease *in vivo*. Thus, for effective therapeutic application, it is necessary to confer nuclease resistance to miRNAs. To enable the administration of miR-214 *in vivo*, a previous study developed synthetic miR-214/5AE, which showed higher cytotoxicity, nuclease resistance, and transfection rate than mature miR-214 *in vitro* (Yoshikawa et al. 2020). In the present study, we found that the administration of miR-214/5AE induced tumor suppression in mice intraperitoneal dissemination lesions. Previous reports have suggested that intraperitoneal administration of cisplatin or paclitaxel can have beneficial effects for progressive ovarian cancer (Moore et al. 1991; Olsen et al. 1994; Armstrong et al. 2006). Intraperitoneal administration of cisplatin has been reported to facilitate exposure of the intraperitoneal tumor to a higher concentration of the anticancer drug than intravenous systemic administration (Pretorius et al. 1981). Herein, we expected that intraperitoneally administered miRNA would be delivered into the tumor via two routes: endocytosis due to direct exposure to the tumor and hematogenous distribution through the capillaries. It has been demonstrated that the residual amount of miR-214/5AE decreases over time with prolonged exposure to RNase (Yoshikawa et al. 2020). Therefore, it is desirable that miRNAs are introduced into tumor cells immediately after administration. Since intraperitoneal administration can expose miR-214/5AE directly to tumor cells, the miRNAs are likely to be less susceptible to immediate degradation and can be introduced into the tumor more efficiently using this method.

To determine the cytotoxic mechanism of miR-214/5AE, we analyzed the expression of cleaved caspase-3, p53, COP1, and Ki-67. In the 5AE group, p53 and cleaved caspase-3 expression levels tended to be higher than those in the ns miR group, and the proportion of Ki-67-positive cells in the 5AE group was significantly lower than that in the ns miR group. The p53 pathway is known to be involved in both apoptosis induction and cell proliferation suppression (Heishima et al. 2015). A previous study reported that p53 was downregulated in HSA cells, and its expression was recovered by transfection of miR-214 into cells (Heishima et al. 2015). In this study, activation of p53 was observed, but the detailed mechanism has not been elucidated and needs to be investigated in the future. We hypothesized that the p53 pathway was activated by the same mechanism, inducing an antitumor effect. Moreover, cleaved caspase-3 protein expression was higher in the 5AE group than in the ns miR group. It was previously revealed that miR-214/5AE induces apoptosis in HSA cells (Yoshikawa et al. 2020). In our study, we found that miR-214/5AE induced apoptosis *in vivo*. Additionally, there were fewer Ki-67-positive cells in the 5AE group than in the ns miR group. Ki-67 is widely used as a marker of cell proliferation (Gerdes et al. 1984); hence, we considered that the administration of miR-214/5AE also suppressed tumor cell

proliferation in our mouse model. Taken together, these results indicate that miR-214/5AE has an antitumor effect on HSA by inducing apoptosis and suppressing cell proliferation.

In this study, we developed a mouse model for intraperitoneal dissemination of HSA. Similar intraperitoneal dissemination mouse models have been reported for stomach and ovarian cancers (Prantner et al. 2018; Miwa et al. 2019); however, it was difficult to evaluate the lesions *in vivo* in these models because they presented intraperitoneally. Herein, we succeeded in evaluating the intraperitoneal lesions using MRI. According to previous reports, HSA masses are observed as regions of high signal intensity on T2-weighted images and regions of low signal intensity on T1-weighted images when compared to the signal intensity of normal liver and spleen (Kim et al. 2015; Clifford et al. 2004; Kippenes et al. 1999). Our results are consistent with the findings of these previous reports; the fat-suppressed T2 images showed high signal enhancement of the tumor. Furthermore, the *in vivo* imaging findings and the macroscopic findings after dissection were in agreement, and the results strongly supported our hypotheses.

Adverse events due to the miR-214/5AE administration were evaluated using the body weight and blood test results. Our findings show no difference between the ns miR and 5AE groups. The blood test results showed an elevation in the white blood cell count and a reduction in the hematocrit value in the ns miR group compared to the non-treat group. These findings were consistent with the clinicopathological findings of HSA and may represent a tumor effect. In a prior study, Lipofectamine RNAiMAX (a cationic carrier) exhibited toxicity to the liver and kidney by non-specific binding with blood components (Hongtao et al. 2006; Hatanaka et al. 2010). Intraperitoneal administration is considered to have a risk of hepatic injury because miRNA absorbed from capillaries is transferred into the liver via the portal vein. However, no obvious hepatic injury was observed in this study. Although longer-term evaluation is required in the future, intraperitoneal administration of miR-214/5AE according to the protocol of this study is considered to be well tolerated without any significant side effects.

In contrast to previous *in vitro* reports, COP1 expression did not significantly differ between the 5AE and ns miR groups *in vivo*. Unfortunately, we were not able to clarify the cause of this problem in this study. Further investigation into the mechanism of the anti-tumor effect is necessary.

In conclusion, we showed that intraperitoneal administration of miR-214/5AE exerts an antitumor effect in an intraperitoneal dissemination mouse model of HSA by inducing apoptosis and suppressing cell proliferation. In addition, no significant side effects were observed. These results could form the basis for the development of a new treatment method using miR-214/5AE for spontaneous HSA cases. Further studies regarding its clinical application are needed to support these claims.

Declarations

Funding:

Not applicable.

Conflicts of interest:

Not applicable.

Code or data availability:

Not applicable.

Authors' contributions

Conception and Design: Ryutaro Yoshikawa, Yoshihito Ueno, Takashi Mori

Acquisition of Data: Ryutaro Yoshikawa, Atsushi Maeda, Hiroki Sakai, Shintaro Kimura, Tomohiro Sawadaishi, Satoru Kohgo, Kohei Yamada

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Final Approval of the Completed Article: Ryutaro Yoshikawa, Atsushi Maeda, Yoshihito Ueno, Hiroki Sakai, Shintaro Kimura, Tomohiro Sawadaishi, Satoru Kohgo, Kohei Yamada, Takashi Mori

Ethics approval:

The protocol of animal experiments was reviewed and approved by the Ethics Review Board of the Joint Faculty of Veterinary Medicine of Gifu University (approval number: 2019-117).

Consent to participate (include appropriate statements) :

Not applicable.

Consent for publication (include appropriate statements):

Not applicable.

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Table

Due to technical limitations, table 1 is only available as a download in the Supplemental Files section.

Figures

Figure 1

The study flow is shown in Fig. 1.

A

B

Figure 2

Histopathological examination suggested these foci were HSA (Fig. 2A). Furthermore, mice foci and canine spleen tissue were positive for canine-specific CD31 and β -actin, whereas mice spleen tissue and negative control tissue were negative for these factors (Fig. 2B).

Figure 3

Nodules were not clear on T2- and T1-weighted images, so fat-suppressed T2-weighted images were used in this study. Pre- and post-injection MR images showed multiple hyperintense nodules in both the ns miR and 5AE groups, but not in the non-treat group. In the pre-injection MR images, the lesions in the ns miR and administration groups were similar. However, post-injection MR images showed a clear difference in lesion size in each group (Fig. 3).

Figure 4

Many foci were engrafted around the gastric pylorus, caudal liver, spleen, and genitals (Fig. 4). Hyperintensity nodules (arrowheads) were observed in the 5AE group (Fig. 4A, 4D) and the ns miR group (Fig. 4B, 4E) but not in the non-treat group (Fig. 4C). Before miRNA administration, nodular shadows were confirmed in the 5AE group (Fig. 4A) and ns miR group (Fig. 4B) almost equally. However, after miRNA administration, nodular shadows were decreased in the 5AE group (Fig. 4D) compared to the control group (Fig. 4E).

Figure 5

The number and weight of foci are shown in Fig. 5. The mean number of foci was significantly lower in the 5AE group than in the ns miR group (5AE: 80, range, 64-111; ns miR: 233, range, 138-312; $P < 0.05$) (Fig 5A). Moreover, the mean weight of foci was significantly lower in the 5AE group than in the ns miR group (5AE: 0.26 g, range: 0.13-0.39 g; ns miR: 0.74 g, range, 0.2-1.37 g; $P < 0.05$) (Fig 5B). These results imply that intraperitoneal administration of miR-214/5AE reduced the number and weight of foci.

Figure 6

To determine the cytotoxic mechanism of miR-214/5AE, the proportion of cells expressing cleaved caspase-3, p53, COP1, and Ki-67 proteins were estimated through immunoblotting and immunohistochemical staining. The results of immunoblotting are summarized in Fig. 6

Figure 7

The 5AE group had a significantly lower proportion of Ki-67-positive cells than did the ns miR group (5AE: 7.2% ± 1.0%; ns miR: 15.0% ± 3.5%; P<0.05) (Fig. 7). These findings support the fact that miR-214/5AE induces apoptosis and exhibits a growth suppressive effect in intraperitoneal foci.

Figure 8

The mean body weight in each group was not significantly different (Fig 8).

Supplementary Files

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