SHORT COMMUNICATION

Barriers in systemic delivery and preclinical testing of synthetic microRNAs in animal models: an experimental study on miR-215-5p mimic

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Short title: Barriers in preclinical testing of synthetic microRNAs

Summary

Mus musculus is the most commonly used animal model in microRNA research; however, little is known about the endogenous miRNome of the animals used in the miRNA-targeting preclinical studies with the human xenografts. In the presented study, we evaluated the NOD/SCID gamma mouse model for the preclinical study of systemic miR-215-5p substitution with a semitelechelic poly[*N*-(2-hydroxypropyl)methacrylamide]-based carrier conjugated with miR-215-5p-mimic via a reductively degradable disulfide bond. Murine mmu-miR-215-5p and human hsa-miR-215-5p have a high homology of mature sequences with only one nucleotide substitution. Due to the high homology of hsa-miR-215-5p and mmu-hsa-miR-215-5p, a similar expression in human and NOD/SCID gamma mice was expected. Expression of mmu-miR-215 in murine organs did not indicate tissue-specific expression and was highly expressed in all examined tissues. All animals included in the study showed a significantly higher concentration of miR-215-5p in the blood plasma compared to human blood plasma, where miR-215-5p is on the verge of a reliable detection limit. However, circulating mmu-miR-215-5p did not enter the human xenograft tumors generated with colorectal cancer cell lines since the levels of miR-215-5p in control tumors remained notably lower compared to those originally transfected with miR-215-5p. Finally, the systemic administration of polymer-miR-215-5p-mimic conjugate to the tail vein did not increase miR-215-5p in NOD/SCID gamma mouse blood plasma, organs, and subcutaneous tumors. It was impossible to distinguish hsa-miR-215-5p and mmu-miR-215-5p in the murine blood and organs due to the high expression of endogenous mmu-miR-215-5p. In conclusion, the examination of endogenous tissue and circulating miRNome of an experimental animal model of choice might be necessary for future miRNA studies focused on the systemic delivery of miRNA-based drugs conducted in the animal models.

MicroRNA (miRNA) are highly conserved, small non-coding RNAs, 18–25 nucleotides in length. They act as post-transcriptional regulators of gene expression by either the post-transcriptional suppression of mRNA translation or induction of mRNA degradation (Lai 2002). MiRNAs expression is frequently either down-regulated or up-regulated in tumor tissue compared to healthy tissue (Ali Syeda et al. 2020). In the past decade, miRNAs were studied as potential biomarkers, therapeutic targets, or experimental drugs in various diseases, including cancer (Hanna, Hossain, and Kocerha 2019). The use of animal models in cancer research significantly contributed to discoveries and rapid advances in biomedical technologies. *Mus musculus* is a widely used animal model in miRNA studies; however, little is known about the role of miRNome of *Mus musculus* in miRNA-targeting preclinical studies. Only a handful of studies examined miRNA profiles of model organisms, including *mus musculus* (Takada et al. 2006; Pal and Kasinski 2017). Approximately 60% of mouse miRNA loci are conserved between mice and humans (Griffiths-Jones et al. 2007; Lagos-Quintana 2003). Despite the high degree of conservation of miRNAs across the species, remaining unconserved miRNAs can lead to significant obstacles in the studies focused on systemic delivery of miRNAs conducted in animal models. In the presented study, we evaluated NOD/SCID gamma mouse (NSG) as a model for the preclinical study of miR-215-5p substitution using a polymeric delivery system conjugated with miR-215-5p mimic.

MiR-215 is a small non-coding RNA acting as an oncogene or tumor suppressor in various malignant diseases. MiR-215-5p was found to have tumor suppressor effects in non-small cell lung cancer (Y. Yao et al. 2018; Hou et al. 2015), breast cancer (J. Yao et al. 2017; Gao, Zhu, and Zhu 2019), mesothelioma (Singh et al. 2019), hepatocellular carcinoma (Ren et al. 2017), and thyroid cancer (Han et al. 2019). MiR-215-5p has been confirmed to serve as a tumor suppressor in colorectal cancer (CRC) (Vychytilova-Faltejskova et al. 2017). Interestingly, the oncogenic effects of miR-215-5p were observed in high-grade glioma (Wei, Sun, and Li 2017) and gastric cancer (Chen et al. 2017; Li et al. 2016). MiR-215-5p was identified as a potential biomarker in several cancers, including CRC (Karaayvaz et al. 2011). However, the fact that miR-215-5p could serve as a potential therapeutic target or drug is far more interesting. Substitution of miR-215-5p in the CRC tumors might lead to a decrease of invasivity, metastatic potential, and inhibition of progression of the disease. However, the invention of the system for the targeted delivery of miR-215-5p and the appropriate animal model is necessary to uncover the full therapeutic potential of miR-215-5p in CRC. Unfortunately, mmu-miR-215-5p and human hsa-miR-215-5p have a high homology of mature sequences with only one nucleotide

substitution (Fig. 1A) exclude NSG mouse from the list of suitable animal models for such study.

Currently, there are no commercial miRNA delivery systems available, except liposomal formulations, which cannot ensure the targeted delivery of the cargo to the tumor. Our study used a semitelechelic linear polymer based on poly[*N*-(2-hydroxypropyl)methacrylamide] (PHPMA) with a terminal pyridyl disulfide group conjugated with a thiol group-terminated miR-215-5p mimic via disulfide bond formation (p(HPMA)n-S-S-miR-215-5p-mimic, Fig. 1B). Such a designed delivery system should provide miR-215-5p with higher stability in the blood, prolong its circulation half-life and increase its accumulation in the tumor cells due to enhanced permeability and retention effect (Golombek et al. 2018). The disulfide bond between the polymer chain and the miRNA should be cleaved due to the increased concentration of glutathione inside the cells, leading to the intracellular release of free miRNA. The sequence and chemical modification of miR-215-5p mimic was 5'-rArUrG rArCrC rUrArU rGrArA rUrUrG rArCrA rGrArC-3' and 5'-rUrCrU rGrUrC rArUrU rUrCrU rUrUrA rGrGrC rCrArA rUrA/3ThioMC3-D/-3' ("r" in the sequence means RNA).

In vivo studies were performed in NSG mice (obtained from The Jackson Laboratory, Bar Harbor, ME, USA) housed and monitored in an individually ventilated cage system (Techniplast, Buguggiate, Italy) with ad libitum access to water and feeding. Following national and EU animal welfare legislation, animal experiments were performed, and all procedures were approved by institutional (Masaryk University, Brno, Czech Republic) and national ethics committees. Identification number of project MSMT-9643/2017-3. Xenograft subcutaneous tumors were generated in thirty-five NSG mice in total using HCT-116, RKO, and HCT-15 cells. Mice were anesthetized by intraperitoneal injection of xylazine (100 mg/kg of weight)/ketamine (10 mg/kg of weight). The injected volume of cell suspension was 2.5 10^6 cells per 100 µL of PBS. Tumor growth and animal behavior were individually monitored during the experiment. For evaluation of miR-215-5p expression pre- and post-implantation, in total, eighteen mice were used for subcutaneous injection of HCT-116 (6 animals), RKO (6 animals), and HCT-15 cells (6 animals). Mice were sacrificed, and an autopsy was performed according to palpation tests - after 29 days (HCT-116), after 22 days (RKO), and after 15 days (HCT-15). Subcutaneous tumors were collected and stored at -80°C. Additionally, to examine miR-215-5p expression in organs, blood plasma and organs were collected from fifteen NSG mice. Moreover, two NU/NU mice and an individual of Rat Ratticus were included in the study, and their organs and blood plasma were collected to examine miR-215-5p expression level in

other rodent species. Lastly, pre-operative blood plasma samples of 8 patients planned for microdiscectomy for disc herniation in the Faculty Hospital Brno (Brno, Czech Republic) were used as controls. The patients signed an informed consent form. To evaluate the distribution of LS4 + miR-215-5p, the remaining seventeen NSG mice with HCT-116 subcutaneous tumors were used. One animal was excluded from the study due to small tumor size. After fourteen days, 217 µL of suspension of the LS4 + miR-215-5p (7mg/Kg, 5mg/Kg, 2,5mg/Kg, 1mg/Kg) conjugate was administered via tail vein to three animals in each group. As a control 217 µL Tris-EDTA (TE) buffer was administered to two animals, and 217 µL of suspension of LS4 (6,54g/L) in TE buffer was administered to two animals. Animals were sacrificed after 48h. Organs and blood plasma were collected, processed, and stored at -80°C. Collection of blood plasma was successful only in one animal in group LS4 + TE buffer. Total RNA enriched with a fraction of small RNAs from mice and human blood plasma samples were isolated using miRNeasy Serum/Plasma Kit (Qiagen, USA). Quantity and quality of RNA were measured using NanoDrop 2000 Spectrophotometer (Thermo Fisher Scientific, USA). Total RNA enriched with a fraction of small RNAs was isolated using mirVana miRNA Isolation Kit (Ambion, Austin, TX, USA) in the case of subcutaneous tumors and organs and tissues from mice. Direct-zol RNA Microprep Kit (Zymo Research, Irvine, CA, USA) was used to isolate RNA from cell lines according to the manufacturer's instructions. Quality and quantity of isolated RNA were measured spectrophotometrically using NanoDrop 2000c (Thermo Fisher Scientific, Waltham, MA, USA) and fluorometrically using Qubit 2.0 and Qubit BR RNA Assay Kit (Thermo Fisher Scientific). Isolated RNA was stored at -80°C until further processing. Expression levels of miR-215-5p were measured by a quantitative real-time polymerase chain reaction qRT-PCR. MiRNA reverse transcription was performed using 6,67 ng of total RNA, gene-specific primers (hsa-miR-215-5p; ID 000518, RNU48; ID 001006 (Applied Biosystems, Foster City, CA, USA)), and TaqMan™ MicroRNA Reverse Transcription Kit according to the TaqMan MicroRNA Assay protocol (Applied Biosystems, Foster City, CA, USA). MiRNA qRT-PCR was performed using TaqMan™ Universal Master Mix II, no UNG (Applied Biosystems, Foster City, CA, USA) according to the manufacturer's recommendations. In xenograft tumors, RNU48 was used as an endogenous control and miR-215-5p expression was normalized using formula Ct(miR-215-5p normalized expression)= $2^{-(\Delta Ct \text{ miR-215-5p duplicate}—\Delta Ct \text{ RNU48 duplicate})}$. MiR-215-5p measured in blood plasma and in murine tissues was normalized using the Ct-40 normalization approach with formula Ct(miR-215-5p normalized expression)= $2^{-(\Delta Ct \text{ miR-215-5p duplicate-40})}$ since RNU48 is not suitable endogenous control for murine samples. An unpaired parametric two-sided t-test was used to analyze

expression data. Analyses were done using GraphPad Prism 8 (GraphPad Software, San Diego, CA, USA). P-values of less than 0.05 were considered statistically significant.

Analyses of miR-215-5p expression levels in blood plasma showed surprising results. All rodents included in the study had a significantly higher concentration of miR-215-5p in the blood plasma compared to human blood plasma (Fig. 1C). In human blood plasma, miR-215-5p had relatively low expression in comparison to miR-215-5p expression in blood plasma of NSG mice log2(fold change over the Ct-40)=9,32; p=0,0233, in blood plasma of NU/NU mice log2(fold change over the Ct-40) =9,16; p<0,0001, and log2(fold change over the Ct-40) =9,63 in blood plasma of *Rattus norvegicus*. Despite the high sequence homology between hsa-miR-215-5p and mmu-miR-215-5p, functions of mmu-miR-215-5p and hsa-miR-215-5p will probably differ in both species. In our previous studies using CRC patients samples, we observed hsa-miR-215-5p to have high expression in colon epithelium and low expression in blood plasma. In the literature, miR-215-5p is usually reported in tissues like colon and small intestine, and its most notable role is the regulation of epithelial-mesenchymal transition (Vychytilova-Faltejskova et al. 2017). In the accessible resources, there is a lack of information on miR-215-5p expression in the mouse. On the other hand, several studies are showing the expression of miR-215 in *Rattus norvegicus.* Specific expression of miR-215 was described in rat ischemic brain and heart; moreover, a high number of miR-215 isomiRs was detected in rat intestine (Jeyaseelan, Lim, and Armugam 2008; Smith et al. 2016; Vacchi-Suzzi et al. 2012). Analysis of miR-215-5p expression in rodent organs showed high expression of miR-215-5p in all collected organs, especially in the liver and kidneys (Fig. 1D). However, the expression of miR-215-5p in the liver and kidneys was highly variable. Expression analyses of xenograft tumors using CRC cells stable transfected with miR-215-5p or empty vector as a control showed an inability of mmu-miR-215-5p to enter the xenograft tumor. CRC cell lines used for subcutaneous injection had similar expression of miR-215-5p as tumors of their origin (Fig. 1E). These results indicate the validity of NSG mice for *in vivo* experiments with stable transfected cell lines and NSG mice can be considered a suitable animal model for the study of miRNA influence on tumor growth and metastatic potential.

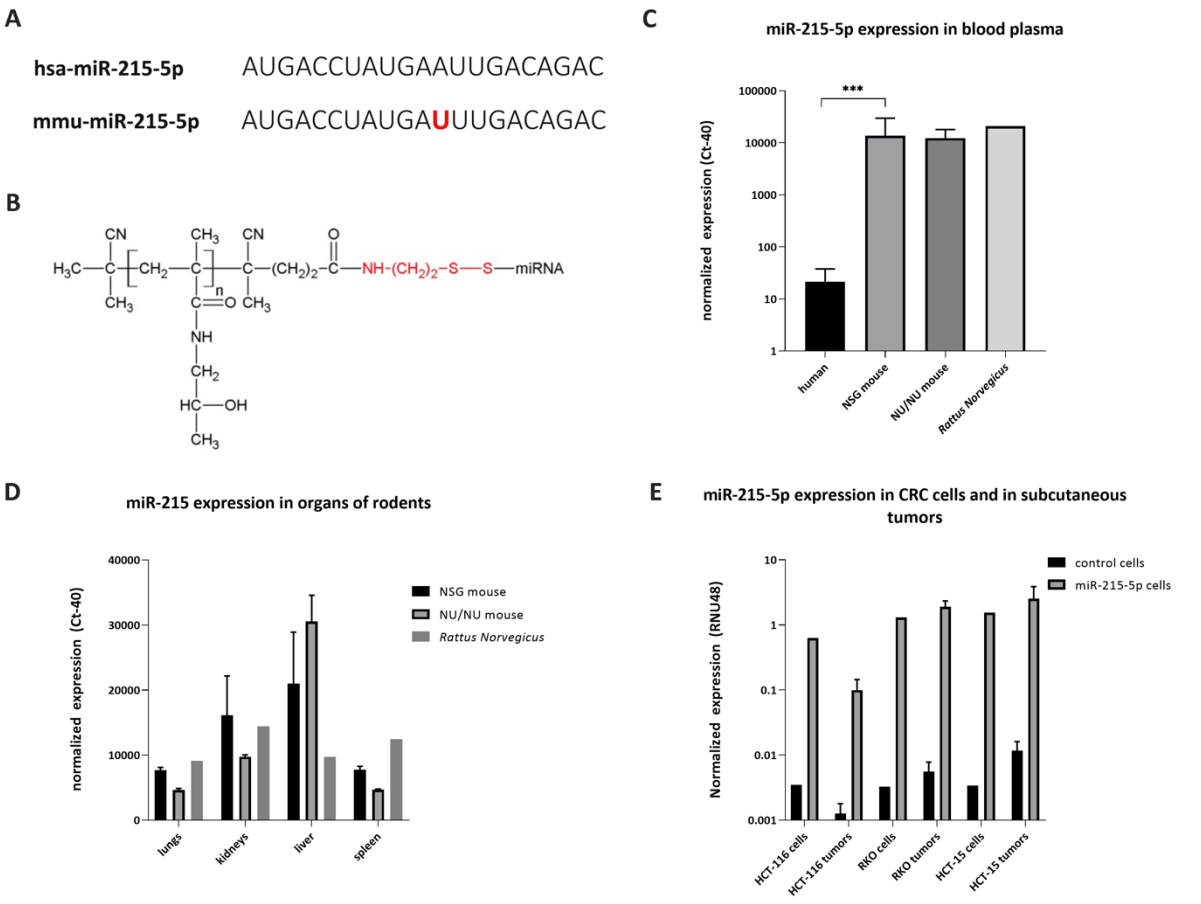

Figure 1: A Sequence homology of mature hsa-miR-215-5p and mmu-miR-215-5p **B** Semitelechelic linear homopolymer with a terminal pyridyl disulfide group conjugated with miR-215-5p mimic (miRNA-SH passenger strand). **C** Expression levels of miR-215-5p in the blood plasma of humans, NSG mice, NU/NU mice, and *Rattus norvegicus*. **D** Expression levels of miR-215-5p in organs of NSG mice, NU/NU mice, and *Rattus norvegicus*. **E** Expression levels of miR-215-5p in implanted cell lines (control, miR-215-5p transfected) and related xenograft tumors in NSG mice (control, miR-215-5p transfected).

Expression analyses of miR-215-5p in blood plasma and tissue specimens after systemic administration of LS4 + miR-215 did not reveal any significant increase in miR-215-5p levels independently on the concentration (Fig. 2A,2B). However, differences in expression were not probably detected due to high endogenous mmu-miR-215-5p expression levels. The dramatic difference in miR-215-5p expression between species and high level of sequence homology (one nucleotide substitution U>A) disabled the usage of species-specific miR-215-5p qPCR assays, and the hsa-miR-215-5p levels in murine blood plasma/tissue could not be accurately distinguished from mmu-miR-215-5p. Unfortunately, high expression of miR-215-5p in murine

blood plasma excludes *mus musculus* as a suitable animal model for studying the systemic administration of miR-215-5p based drugs.

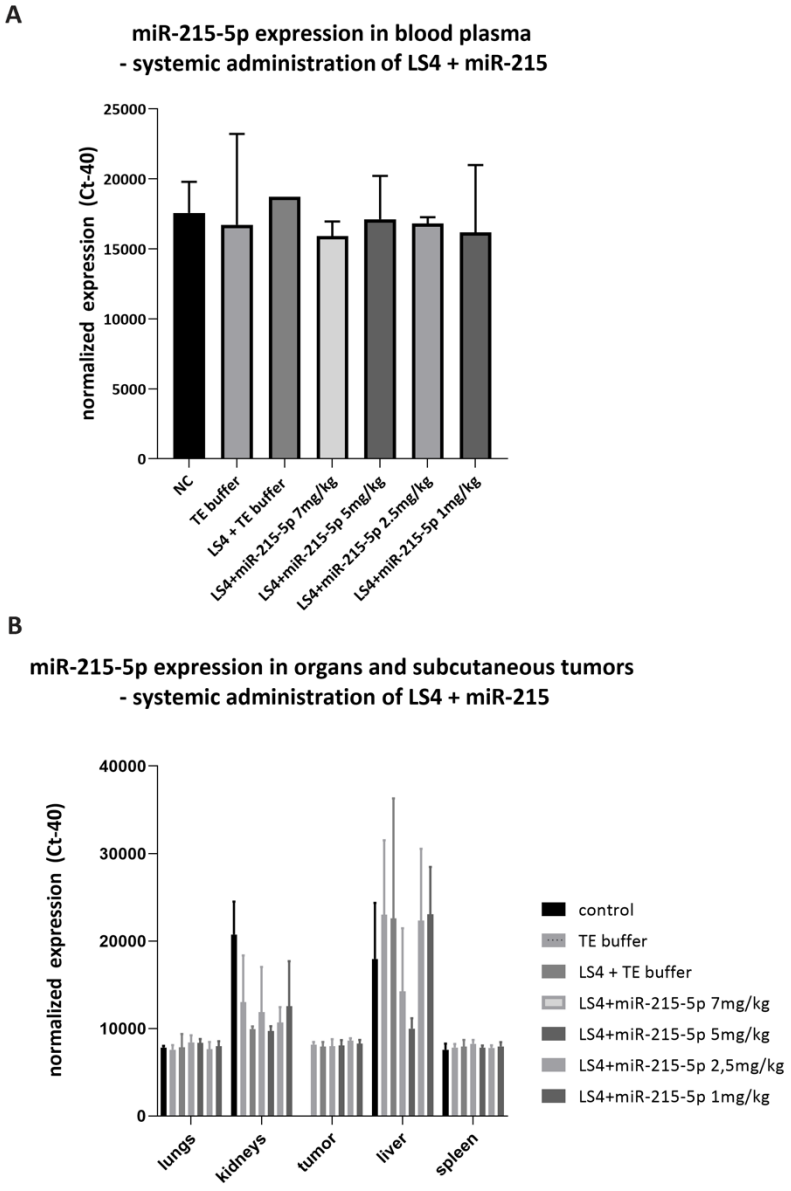

Figure 2: **A** Expression levels of miR-215-5p in NSG mice blood plasma after systemic administration of LS4+miR-215-5p conjugate. **B** Expression levels of miR-215-5p in NSG mice organs after systemic administration of LS4+miR-215-5p conjugate.

Considering these findings, it is highly probable that many more human miRNAs have significantly different function and expression levels in animal models and humans. However, little research was dedicated to comparing normal human and animal models' miRNA profiles.

Thus many miRNAs candidates in the preclinical animal studies might interfere with the endogenous miRNome of the animal. This issue might require use of transgenic animal models or fluorescent labeling of carrier-miRNA conjugates. In conclusion, the examination of endogenous tissue and circulating miRNome of an experimental animal model of choice might be necessary for future miRNA studies focused on the systemic delivery of miRNA based drugs conducted in animal models.

LIST OF ABBREVIATIONS

CRC – colorectal cancer

miRNA - microRNA

NSG – NOD/SCID gamma

PHPMA - poly[*N*-(2-hydroxypropyl)methacrylamide]

qRT-PCR – quantitative real-time polymerase chain reaction

TE buffer – Tris-EDTA buffer

DECLARATIONS

Ethics approval and consent to participate - Animal experiments were performed under national and EU animal welfare legislation, and all procedures were approved by institutional (Masaryk University, Brno) and national ethics committees. Project Solid Tumors and non/coding RNA 2017-march 2022.

Competing interests

The authors declare that they have no competing interests.

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