Regulation of folate metabolism by microRNA-34a identifies a potential combination therapy against canine osteosarcoma

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Background

- Osteosarcoma (OS) is an aggressive primary malignant bone tumor common in both humans and dogs. The standard of care, namely chemotherapy drugs such as doxorubicin and methotrexate (MTX), still yields a poor prognosis in canine OS patients, with less than 20% of dogs surviving more than 2 years after diagnosis.

- There is a clear need to identify alternate treatment strategies to improve clinical outcomes for OS patients.

- miRNAs are small, single-stranded, noncoding RNAs that can silence gene expression through inhibiting mRNA translation or inducing mRNA degradation.

- miR-34a is a tumor suppressive mRNA that has been found to be downregulated in various human cancers, including osteosarcoma. Due to the highly conserved nature of miRNAs, similar antitumorogenic effects have been identified in canine OS cells when treated with a human, genetically engineered miR-34a prodrug, increasing tumor cell apoptosis and decreasing proliferation, migration, and invasion.

- Proteomics preliminary research

  - In a quantitative proteomics project, multiple proteins critical to the folate pathway – gamma-glutamyl hydrolase (GSH), thymidylate synthase (TS), methylenetetrahydrofolate dehydrogenases (MTHFD1 and MTHFD2), and serine hydroxymethyltransferase 1 (SHMT1) – were downregulated by miR-34a.

  - Folic acid metabolism is essential for purine nucleotide synthesis, and consequently, cell survival.

- Hypothesis

  - miR-34a decreases human and canine OS cell survival through decreasing folic acid metabolism.

  - Synergistic anti-folate activity will be observed in OS cells treated with miR-34a and a routinely used anti-folate, MTX.

Aims

1. Validate the functional role of miR-34a in modulating the folate pathway

- Hypothesis: Treatment of canine and human OS cells with miR-34a will decrease expression of folate metabolism mediators GSH, MTHFD1, MTHFD2, SHMT1, and TS.

2. Elucidate the anti-tumor effect of miR-34a with methotrexate

- Hypothesis: Methotrexate combined with miR-34a will result in greater anti-tumor effects in OS cells compared to either treatment alone.

Methods

Aim 1: Validate the functional role of miR-34a in modulating the folate pathway

- Two canine (HMPOS, D17) and two human (U2OS WT, U2OS p53KO) OS cell lines were transfected with miR-34a prodrug (5 nM) according to the JetPrime protocol. Control cells were treated with LSA miR control vehicle.

- Western blot analysis of OS cells transfected with increased concentrations of miR-34a and/or MTX, and both for 48 hours.

- RNA isolation, cDNA synthesis, and qPCR to determine intracellular levels of mature miR-34a.

- Densitometry analysis of western blots to achieve a quantifiable change in protein expression. To analyze the proteomics project.

Aim 2: Elucidate the anti-tumor effect of miR-34a with methotrexate

- OS cell lines were treated with 5nM miR-34a with and without MTX.

- Plus an MTT assay (B) MTX-0, 1.4, 4.1, 12, 37, 111, 333 μM MTX. (C) OS cell lines were treated with both miR-34a and MTX in the following miR: MTX ratios: 10nM:200uM, 25nM:500uM, 50nM:1000uM, 20nM:200uM. Cellular proliferation was then evaluated after 48 hours after transfection using a bioreductive resaunin-based fluorometric assay.

Results

Proteomics Study

Figure 1. Canine OS cell lines (HMPOS, D17) were treated with 5nM miR-34a. Expression of folate pathway proteins MTHFD1, MTHFD2, SHMT1, GSH, and TS were significantly downregulated in D17 OS cells as measured by mass spectrometry.

Figure 2. Western blot analysis of canine (HMPOS, D17) and human (U2OS WT, U2OS p53KO) OS cell lines untreated and treated with 5nM miR-34a prodrug revealed minimal to undetectable changes in GSH, MTHFD1, MTHFD2, SHMT1, and TS.

Figure 3. OS cell lines were treated a range of concentrations of (A) miR-0, 1, 2, 10, 20, 30 nM miR—and (B) MTX-0, 1.4, 4.1, 12, 37, 111, 333 μM MTX. (C) OS cell lines were treated with both miR-34a and MTX in the following miR: MTX ratios: 1nM:10μM, 2nM:25μM, 5nM:50μM, 10nM:100μM, 20nM:200μM. Cellular proliferation was then evaluated 48 hours after transfection using a bioreductive resaunin-based fluorometric assay.

Conclusions

Aim 1: Validate the functional role of miR-34a in modulating the folate pathway

- miR-34a has an antitumorogenic effect through inhibition of the folate pathway, but this effect is not quantifiable on the protein expression level by semi-quantitative western blot.

- Western blot results neither confirm nor deny results from the previous quantitative proteomics project. Densitometry analysis of western blots revealed no statistically significant difference between cells transfected with miR-34a and the controls.

- Likely, this discrepancy is because western blot is less sensitive to changes in protein/gene expression than mass spectrometry. Consequently, the slight but significant changes observed in the proteomics project could not be visualized with western blot analysis.

- Alternatively, the miR prodrug could have been inefficient in entering and translocating the cells and achieving a high enough intracellular concentration to achieve a quantifiable change in protein expression. To analyze the intracellular miR concentrations, we will perform qPCR of mature miR-34a.

Aim 2: Elucidate the anti-tumor effect of miR-34a with and without methotrexate

- Proliferation assay results reveal a dose-dependent decrease in cell survival with miR-34a, MTX, and both drugs combined.

- There appears to be a greater decrease in cell viability in cells treated with miR-34a alone. Cells treated with MTX alone appear to have reduced cell viability compared to cells treated with both miR and MTX.

Future Directions

1. Western blot analysis of OS cells transfected with increased concentrations of miR (10 nM, 20 nM) based on proliferation assay results.

2. Real-time qRT-PCR of transfected cells to evaluate levels of mature miR-34a.

3. Comparison analysis of single compound and combination treated cells to allow for statistical determination of synergistic, additive or antagonistic activity of combined miR-34a and MTX.

4. Apoptosis assays (fluorometric activated caspase 3/7 assay) with miR, MTX, and both drugs combined.

References


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