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.[1] 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.[2]
  → 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.[3]
  - **miR-34a** is a tumor suppressive miRNA that has been found to be downregulated in various human cancers, including osteosarcoma. Due to the highly conserved nature of miRNAs, similar antitumorigenic effects have been identified in canine OSA cells when treated with a human, genetically engineered miR-34a prodrug, increasing tumor cell apoptosis and decreasing proliferation, migration and invasion.[4]
- **Proteomics preliminary research**
  - In a quantitative proteomics project, multiple proteins critical to the folate pathway – gamma-glutamyl hydrolase (GGH), thymidylate synthetase (TS), methylenetetrahydrofolate dehydrogenases (MTHFD1 and MTHFD2), and serine hydroxy methyltransferase 1 (SHMTI) – were downregulated by miR-34a.
  - Folic acid metabolism is essential for purine nucleotide synthesis, and consequently, cell survival.[5]
- **Hypotheses**
  - 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 GGH, 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 to analyze the differential expression of proteins involved in folic acid metabolism – namely GGH, MTHFD1, MTHFD2, SHMT1, and TS.
- RNA isolation, cDNA synthesis, and qPCR to determine intracellular levels of mature miR-34a.

Aim 2: Elucidate the anti-tumor effect of miR-34a with methotrexate
- OS cell lines were plated in 96-well plates and after 24 hours, treated with a range of concentrations of miR-34a prodrug, methotrexate, and both for 48 hours.
- Cellular proliferation was then evaluated using a resazurin-based fluorometric assay.
- For all treatment strategies, we will determine synergistic, additive or antagonistic effects using the Chou-Talalay method with CompuSyn software.
- Analyses performed used GraphPad Prism.

Results

Proteomics Study

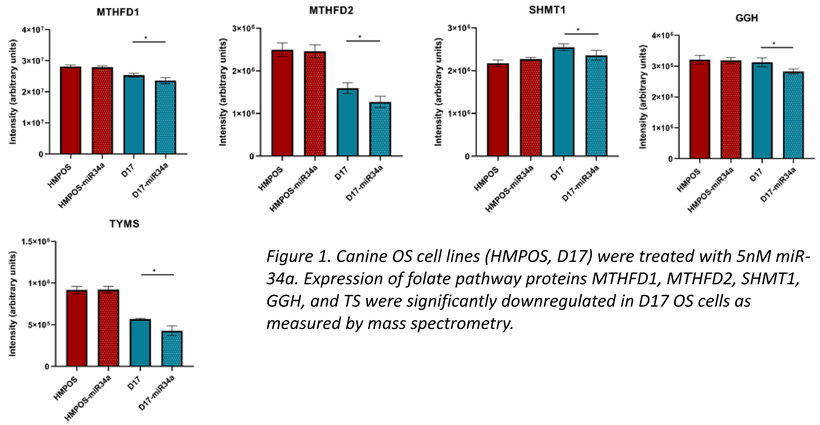

Figure 1. Canine OS cell lines (HMPOS, D17) were treated with 5nM miR-34a. Expression of folate pathway proteins MTHFD1, MTHFD2, SHMT1, GGH, and TS were significantly downregulated in D17 OS cells as measured by mass spectrometry.

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

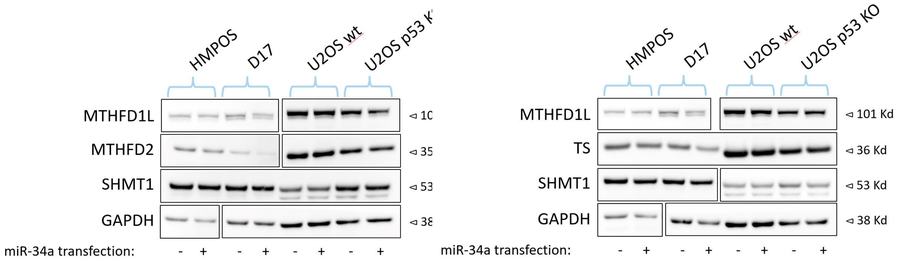

Figure 2. Western blot analysis of canine (HMPOS, D17) and human (U2OS WT, U2OS p53KO) OS cell lines untreated and treated with 5nM mi-R34a prodrug revealed minimal to undetectable changes in GGH, MTHFD1, MTHFD2, SHMT1, and TS.

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

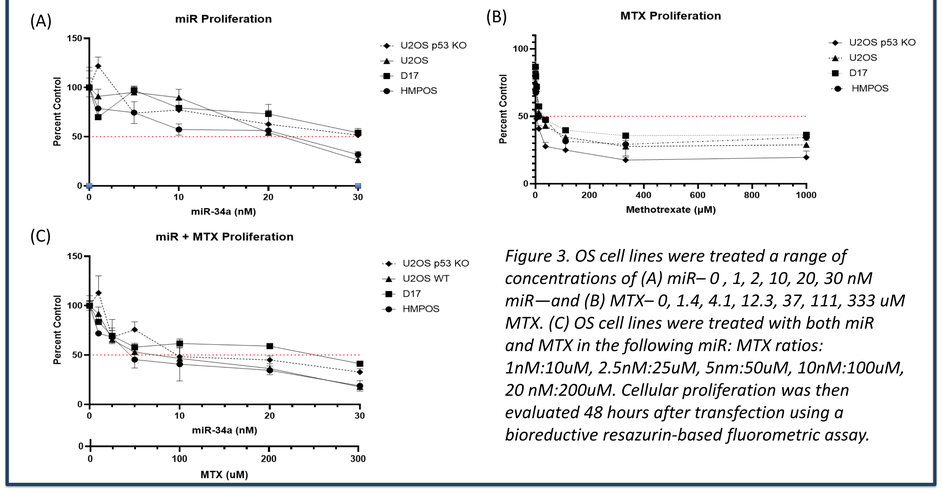

Figure 3. OS cell lines were treated a range of concentrations of (A) miR– 0 , 1, 2, 10, 20, 30 nM miR—and (B) MTX– 0, 1.4, 4.1, 12.3, 37, 111, 333 uM MTX. (C) OS cell lines were treated with both miR and MTX in the following miR: MTX ratios: 1nM:10uM, 2.5nM:25uM, 5nm:50uM, 10nM:100uM, 20 nM:200uM. Cellular proliferation was then evaluated 48 hours after transfection using a bioreductive resazurin-based fluorometric assay.

Conclusions

Aim 1: Validate the functional role of miR-34a in modulating the folate pathway
- miR-34a has an antitumorigenic 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 transfecting 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 and MTX than miR alone. Cells treated with MTX alone appear to have reduced cell viability compared to cells treated with both MTX and miR.

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 for levels of mature miR-34a.
3. CompuSyn 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 caspace 3/7 assay) with miR, MTX, and both drugs combined.

References

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