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TITLE:  Heparanase Mechanisms in Brain - metastatic Breast Cancer

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Heparanase (HPSE) is a potent pro-tumorigenic, pro-angiogenic, and pro-metastatic enzyme over-expressed in brain metastatic breast cancer (BMBC). However, regulation of the heparanase gene is poorly understood. We hypothesized that heparanase represents a potential target for the development of novel therapies for BMBC, whose gene expression and modalities can be regulated by microRNA. Using miRanda and RNAhybrid, we identified microRNA-1258 (miR-1258) to directly target HPSE and suppress BMBC. We demonstrated miR-1258 levels to inversely correlate with heparanase expression, enzymatic activity, and cancer cell metastatic propensities - lowest in highly aggressive BMBC cell variants, compared to either non-tumorigenic or non-metastatic human mammary epithelial cells. These findings were validated by analyses of miR-1258 and heparanase content in paired clinical tissues - normal mammary gland versus invasive ductal carcinoma, and primary breast cancer versus BMBC. Furthermore, we demonstrated that miR-1258 inhibits the expression and activity of heparanase in BMBC cells, and rescue experiments modulating heparanase blocked miR-1258 - mediated phenotypic effects. Finally, we showed that miR-1258 stably expressed in BMBC cells significantly inhibited heparanase, in vitro cell invasion, and suppressed experimental brain metastasis by 74%. These findings introduce a new concept that links microRNA mechanisms with brain metastatic breast cancer by downregulating HPSE, providing the groundwork for heparanase-based therapeutics in patients with brain metastases, BMBC in particular.

MicroRNA, Breast Cancer, Brain Metastasis
# Table of Contents

<table>
<thead>
<tr>
<th>Section</th>
<th>Page</th>
</tr>
</thead>
<tbody>
<tr>
<td>Introduction</td>
<td>4</td>
</tr>
<tr>
<td>Body</td>
<td>4</td>
</tr>
<tr>
<td>Key Research Accomplishments</td>
<td>9</td>
</tr>
<tr>
<td>Reportable Outcomes</td>
<td>9</td>
</tr>
<tr>
<td>Conclusion</td>
<td>10</td>
</tr>
<tr>
<td>References</td>
<td>10</td>
</tr>
</tbody>
</table>
Introduction

We demonstrated that levels of a specific microRNA, microRNA1258 or miR-1258, inversely correlate with the expression of an important glycosidase, named heparanase, its enzymatic activity, and cancer cell metastatic propensities with lowest expression in highly aggressive brain metastatic breast cancer (BMBC) cells. Second, we demonstrated that experimental brain metastasis are suppressed when miR-1258 is ectopically introduced into BMBC cells and these are applied to xenografts models.

Body

This represent the first annual report for the IDEA Award from CDMRP. We have completed specific aim 1 of the proposal and related sub-tasks per statement of work for the first year of this IDEA Award. These have been summarized below:

1a. Generate lentiviral vectors expressing miR-1258 and transduce parental (231P) and brain- metastatic BMBC cell lines (231BR1, -BR2, and -BR3) with miR-1258 lentivirus and corresponding control

1b. Determine the modulation of heparanase expression, activity in BMBC cells and respective non-brain metastatic counterparts

1c. Perform real-time PCR, Western blot analyses, and HPSE activity assays for transcripts and protein regulated by miR-1258

1d. Perform adhesion, migration, invasion assays in BMBC cells transduced with miR-1258 lentivirus or antisense control

1e. Carry-out experimental brain metastatic assays in animals (nu/nu mice) to demonstrate the in vivo miR-1258 physiological relevance by lentiviral deliver

1f. Detect miR-1258 by LNA-ISH in 50 formalin-fixed, paraffin-embedded paired BMBC clinical samples

1g. Complement LNA-ISH with IHC analyses to examine heparanase expression in same paired clinical samples Compare digital determinations of LNA-ISH and IHC reactivities and analyze them for statistical significance

1h. Preparation and submittal of a manuscript for dissemination of results in a peer - reviewed oncology journal.

As a compendium and background of our work, heparanase (HPSE) is a potent protumorigenic, pro-angiogenic, and pro-metastatic enzyme over-expressed in brain metastatic breast cancer (BMBC)(1-4). However, regulation of the heparanase gene and BMBC mechanisms are poorly understood. We hypothesized that heparanase represents a potential target for the development of novel therapies for BMBC, whose gene expression and modalities can be regulated by microRNA. Using miRanda and RNAhybrid, we identified microRNA-1258 (miR-1258) to directly target HPSE and suppress BMBC.
We have demonstrated that miR-1258 exerts its roles in BMBC cells by inhibiting heparanase expression and activity, and does so by directly targeting HPSE 3'-UTR (Figs. 1 and 2). To determine whether miR-1258 plays a role in breast cancer metastasis, notably to brain, six human breast cell lines were selected and examined for miR-1258 and heparanase expression. Their inverse correlation was demonstrated: the decrease of miR-1258 associated with an increase of HPSE content and correlated with metastatic abilities of these cell lines (Fig. 3A). Moreover, miR-1258 downregulated heparanase expression and activity (Figs. 2 and 4), inhibited cell invasion (Fig. 4A), and suppressed the formation of brain metastasis in xenografts by 74% (Figs. 4B-D).

These findings introduce new concepts that links microRNA mechanisms with brain metastatic breast cancer by downregulating HPSE, providing the groundwork for heparanase-based therapeutics in patients with brain metastases, BMBC in particular.

Figure 1. MiR-1258 directly targets heparanase. A., Alignment of miR-1258 with HPSE 3'-UTRs. Complementary sequences of miR-1258 to mammalian HPSE 3'-UTRs are shaded red. The underlined sequence (5'-CCU-3') in the human HPSE mRNA, and common to other mammalian heparanases, denotes a deletion in the construct carrying Luc-HPSE 3'-UTR-del. Seed sequences of miR-1258 are underlined. Hsa = human; Ptr = pan troglodytes; Mmu = mus musculus; Rno = rat. B. and C., Effect of miR-1258 (wild type and mutant) on HPSE 3'-UTR luciferase reporters. Constructs carrying Luc-HPSE 3'-UTR (B.) or Luc-HPSE 3'-UTR-del (C.) were transfected in 293T cells with indicated miRNAs. MiR-100 expressed in a lentiviral construct was used as an additional control for miR-1258 specificity. After transfections (24 hr), cells were harvested and luciferase activity assays were performed. Bars represent the mean and standard deviation of three independent experiments.
Figure 2. **MiR-1258 inhibits heparanase expression and activity in BMBC cells.** BMBC cells (231BR3 and 231BR1 for brevity) were transiently transfected with anti-miR-1258 and negative control (NC), or stably transduced with lentiviral constructs expressing wild type miR-1258 or scrambled miR (scrambled). After 48 hr, cell lysates were prepared and examined simultaneously using Western blotting and the TakaRa heparan sulfate degrading enzyme assay kit (4). HPSE protein and enzyme activity in 231BR1 and 231BR3 cells (A.) and (B.). β-actin was used as a loading control. Bars represent the mean and standard deviation of three independent experiments. C. Effect of miR-1258 - mediated inhibition of HPSE and heparanase - regulated proteins. Same procedures as (A.) and (B.), expression clones carrying HPSE or scrambled controls were transduced into 231BR3 cells. Cell lysates were then examined for protein expression using antibodies indicated. Three independent experiments were performed, and analyses are shown.
Figure 3. MIR-1258 inversely correlates with BMBC ability and HPSE expression

3A. MIR-1258 inversely correlates with metastatic ability and HPSE expression in human breast cells

3B. MIR-1258 and HPSE expression in paired human mammary gland/carcinoma

3C. MIR-1258 and HPSE expression in paired human breast cancer/BMBC
Figure 3. MiR-1258 levels inversely correlate with metastatic ability of human breast cell lines and patient tissues. **A.** miR-1258 analyses in breast cell lines with differing metastatic propensities. (Top) miR-1258 levels were examined in six breast cell lines by TaqMan RT-PCR and data shown by fold change compared to human mammary epithelial cells (HMEC). RNU44 was used as endogenous control. (Bottom) Differential HPSE expression in same cell lines was examined by Western blotting. Based on their tumorigenic and metastatic potential, cell lines were classified into three groups: 1) non-tumorigenic HMEC and MCF-10A; 2) tumorigenic but non-metastatic SUM-149 and SUM-225; 3) BMBC 231BR1 and 231BR3 cells. β-actin was used as control for equal loading. **B.** and **C.** miR-1258 analyses of paired (mammary gland versus invasive ductal carcinoma, or primary versus BMBC) patient tissues using locked nucleic acid in situ hybridization (LNA-ISH). Hybridized, complementary probes were detected by a catalyzed reporter deposition using biotinylated tyramide followed by colorimetric detection of biotin with an avidin-alkaline phosphatase conjugate (n = 13 pairs). (Left) Representative LNA-ISH images of miR-1258 expression of normal mammary gland, invasive ductal carcinoma, and brain metastatic breast cancer, and quantification of miR-1258 signal intensity. Graph depicts relative miR-1258 expression in indicated tissues. While high miR-1258 levels were detected in the epithelial component of normal mammary gland, HPSE IHC negativity was consistently detected. The small insert in the HPSE IHC panel of normal breast seen at the periphery of patient’s tumor indicates HPSE IHC staining of tumor demonstrating strong heparanase expression. Each group represents the average of total signals in examined fields. (Right) Heparanase expression (IHC) in paired cases of normal mammary gland and invasive ductal carcinoma, and primary and brain metastatic carcinoma was examined by IHC.
Figure 4. MiR-1258 downregulates heparanase, inhibits BMBC cell invasion, and suppresses brain metastasis. A., miR-1258 downregulates heparanase. (Left) Cells (SUM-149, 231BR3) were transfected with miR-1258, anti-miR-1258 (inhibitor), and negative controls (NC) followed by Western blotting to detect HPSE expression. (Right) miR-1258 inhibits BMBC cell invasion. Chemoinvasion analyses using Matrigel™ chambers were performed in parallel with Western blotting analyses for HPSE expression. Representative images of chamber inserts are shown. Cell invasive values were quantified (O.D. 560 nM) (n = 10). B., miR-1258 suppresses brain metastasis. 231BR3 cells stably expressing miR-1258 or scrambled-miR (scrambled) were injected intracardially into female nude mice (0.5 x 10⁶ cells per mouse, 30 mice per group), respectively. After 6 weeks, mice were sacrificed, lungs and brains harvested, and analyzed for metastatic tumors. (Top) Incidence, and mean number of brain metastases in each group. (Middle) hematoxylin and eosin (H&E) visualization of BMBC and its suppression by miR-1258 compared to scrambled. (Bottom) The combined data (n = 3 assays) are shown graphically with dots representing the number of brain metastases from each mouse; the box represents the 10th and 90th percentile; and the black line in each box is the mean for each group. C., Heparanase expression was examined by IHC in experimental BMBC, and representatives IHC and H&E sections are shown (n = 4). D. Rescue experiments showing that a modulation of HPSE expression blocked miR-1258 – mediated effects on BMBC in vitro cell invasion and in vivo brain metastasis formation. Shown are representative images visualizing brain metastases in animals (H & E staining; n = 10 mice per treatment group). Bars represent the mean and standard deviation of two independent experiments.

Key Research Accomplishments

The purpose of our studies was to investigate microRNA - mediated mechanisms that alter invasive and metastatic modalities of BMBC through the downregulation of heparanase. We provide evidence that:

- a specific microRNA - miR-1258 - targets the heparanase gene and has profound effects on heparanase gene expression and function of this molecule in BMBC cell invasion and metastatic profiles;

- the ectopic expression and activity of miR-1258 negatively regulates heparanase expression and its unique endoglycosidase, and results in the inhibition of BMBC cell invasion and onset of brain metastasis.

- the development of miRNA-based approaches regulating heparanase is critical and of potential therapeutic value.

Reportable outcomes

Conclusions

In summary, we have identified a link between miR-1258 and heparanase, which represents a novel mechanism of endogenous regulation of this molecule. Our findings indicate that miR-1258 is a suppressor of breast cancer cell invasion and brain metastasis by targeting heparanase and heparanase-mediated pathways. The discovery of miR-1258/HPSE mechanisms may represent an important advancement to understand heparanase action, and miR-1258 abilities to suppress brain metastasis can have profound implications for the development and application of heparanase-based therapeutics in brain metastasis in general, brain metastatic breast cancer in particular.

References


