Dysregulation of the Mitogen Granulin in Human Cancer through the miR-15/107 microRNA Gene Group

Wang-Xia Wang1, Natasha Kyprianou1, Xiaowei Wang2, and Peter T. Nelson1

Abstract

Granulin (GRN) is a potent mitogen and growth factor implicated in many human cancers, but its regulation is poorly understood. Recent findings indicate that GRN is regulated strongly by the microRNA miR-107, which functionally overlaps with miR-15, miR-16, and miR-195 due to a common 5′ sequence critical for target specificity. In this study, we queried whether miR-107 and paralogs regulated GRN in human cancers. In cultured cells, anti-argonaute RNA coimmunoprecipitation with downstream microarray analyses indicates that GRN mRNA is directly targeted by numerous miR-15/107 miRNAs. We further tested this association in human tumors. MiR-15 and miR-16 are known to be downregulated in chronic lymphocytic leukemia (CLL). Using pre-existing microarray datasets, we found that GRN expression is higher in CLL relative to nonneoplastic lymphocytes (P < 0.00001). By contrast, other prospective miR-15/miR-16 targets in the dataset (BCL-2 and cyclin D1) were not upregulated in CLL. Unlike in CLL, GRN was not upregulated in chronic myelogenous leukemia (CML) where miR-107 paralogs are not known to be dysregulated. Prior studies have shown that GRN is also upregulated, and miR-107 downregulated, in prostate carcinoma. Our results indicate that multiple members of the miR-107 gene group indeed repress GRN protein levels when transfected into prostate cancer cells. At least a dozen distinct types of cancer have the pattern of increased GRN and decreased miR-107 expression. These findings indicate for the first time that the mitogen and growth factor GRN is dysregulated via the miR-15/107 gene group in multiple human cancers, which may provide a potential common therapeutic target. Cancer Res;70(22); OF1–6. ©2010 AACR.

Introduction

Granulin (GRN) contributes to multiple human cancers. This gene product potentiates neoplastic transformation; stimulates tumor growth, metastases, and tissue invasion; inhibits antiapoptotic mechanisms; and adversely impacts therapeutic responses (1, 2). GRN is a pleiotropic but evolutionarily conserved protein that has been given multiple names, including progranulin (PGRN), acrogranin, gp88, proepithelin, PC cell–derived growth factor (PCDGF), and granulin-epithelin precursor (1). To avoid confusion, we refer here to GRN for the protein and GRN for the gene or mRNA.

Recent work has shown that GRN expression is suppressed posttranscriptionally by miR-107 (3), a member of microRNA (miRNA) gene group that also includes miR-15, miR-16, miR-103, miR-195, miR-424, miR-497, miR-503, and miR-646 (4). All of these miRNAs are moderately to highly expressed in many human tissues and share a common 5′ seed sequence, AGCAGC. The 5′ end sequence homology confers similar specificity in terms of targeting miRNAs for posttranscriptional decay and/or translational inhibition (4). Paralogous miRNAs, miR-15 and miR-16 genes, reside within the human chromosome 13q minimal deletion region that confers chronic lymphocytic leukemia (CLL) susceptibility (5). We hypothesized that because miR-15 and miR-16 are capable of strongly suppressing GRN, CLL lymphocytes may have increased expression of oncogenic GRN. We also note that other human cancers, including carcinoma of the prostate, show increased GRN expression with decreased miR-107 expression (6, 7). We sought to evaluate whether prior studies using gene expression methods and our own experiments with cultured cancer cell lines would substantiate the hypothesis that miR-107 dysregulation correlates with increased GRN expression in human cancers.

Materials and Methods

RNA coimmunoprecipitation with downstream microarray analyses

RNA coimmunoprecipitation with downstream microarray analysis (RIP-Chip) methods have been described in detail previously (8). Briefly, H4 cells (American Type Culture Collection), cultured under the vendor’s recommended conditions, were plated in 10-cm culture plates at a density of 2.5 × 10⁶/plate day before transfections. Cells were transfected with

Authors’ Affiliations: 1Department of Pathology and Division of Neuropathology, University of Kentucky Medical Center and Sanders-Brown Center on Aging, University of Kentucky, Lexington, Kentucky; and 2Department of Radiation Oncology, Washington University School of Medicine, St. Louis, Missouri

Corresponding Author: Peter T. Nelson, Department of Pathology, Division of Neuropathology, and the Sanders-Brown Center on Aging, Room 311, Sanders-Brown Center Building, 800 S. Limestone, University of Kentucky, Lexington, KY 40536-0230. Phone: 859-257-1412, ext. 254; Fax: 859-257-6054; E-mail: pnels2@email.uky.edu.

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25 nmol/L of “Pre-miRNA” (siRNA-like reagents from Ambion) referent to hsa-miR-103, hsa-miR-107, hsa-miR-15b* (antisense strand), hsa-miR-16, hsa-miR-195, hsa-miR-320, negative control miRNA 1 using siRMAX (Invitrogen), and mutated miRNAs, according to the manufacturer’s instructions. Statistical tests were performed using Student’s t-test with a P < 0.01 cutoff.

Analyses of NIH Gene Expression Omnibus data
Data from the Gene Expression Omnibus (GEO) database were assessed to allow us to infer whether GRN mRNA is decreased in cancers where miR-15/107 gene group members show increased expression. Information about the individual datasets used is provided below.

Tissue culture cell transfections with miR-15/107 gene group members and controls
Transfections were performed as noted above. Western blots were performed as previously described (8).

Results and Discussion
We previously performed the requisite experiments including reporter assays with recognition site mutation and miRNA inhibitor studies to show that miR-107 can target specifically GRN mRNA with resulting decreased GRN protein (3). Here we tested the hypothesis that this mechanism is relevant to human cancers. Specifically, we investigated whether downregulation of miR-15/107 gene group miRNAs (4) contributes to human cancers through increasing expression of GRN.

We first used a cell culture system to test multiple members of the miR-15/107 gene group directly, along with control miRNAs, to ascertain whether the miR-15/107 group miRNAs cause specific incorporation of GRN mRNA into the microribonucleoparticle (miRNP) that contains the argonaute (AGO) protein for mRNA targeting. We used our anti-AGO antibody and the RIP-Chip experimental design as described previously (8). In accordance with our previous findings for miR-107, we
observed incorporation of GRN mRNA into the AGO-miRNP following transfections with miR-103, miR-16, and miR-195, but not miR-15b* (an antisense-oriented control), miR-320, or a negative control miRNA. We also transfected the cells with nonphysiologic “mutant” miRNAs that are related to but distinct from miR-107, with changes in the 5′ portion (MUT1) or 3′ portion (MUT2) as shown in Fig. 2. Note that incorporation of GRN into the miRNP is abolished by MUT1 but not MUT2, which underscores the importance of the 5′ seed portion of the miRNA for target specificity. Because

Figure 2. Data from multiple laboratories, accessed via the NIH GEO database, show that GRN mRNA levels are elevated in cancers (CLL and prostate carcinoma) where miR-15/107 gene expression also is decreased. A, data from the Christian Stratowa laboratory used miRNA from mononuclear cells in blood samples followed by downstream Affymetrix human genome U95A or U95Av2 microarrays to test the differences in expression between normal (n = 11) and CLL (n = 100) cells (13). Note that GRN levels are dramatically increased in CLL cells (P < 0.00001 using Student’s t-test) whereas other putative miR-15/16 targets are not significantly increased in this study. B, the findings in CLL seem to be specific. Data assessing myelogenous cells from bone marrow or peripheral blood, from Lucy Crossman and colleagues’ (14) assessed bone marrow and peripheral blood samples from normal (n = 8) and CML (n = 9) patients using Affymetrix human genome U95Av2 microarrays. MiR-15/107 gene group member expressions are not decreased in CML (19, 20), whereas GRN levels are actually downregulated in CML cancer cells relative to controls. Results with peripheral blood and bone marrow are pooled. C, the increased GRN expression in prostate cancer result is in accordance with data from the Arul Chinnaiyan lab (16) using Affymetrix HG-U133 plus 2 microarrays. Here, metastatic prostate cancer cells express increased GRN. These data helped motivate further work on prostate cancer (CA) cells to show directly that miR-15/107 gene group members downregulated GRN.
BCL2 and CCND1 have been shown to be targets of miR-16, we evaluated them in the RIP-Chip assay and they showed a far lesser degree of enrichment in the miRNPs following miR-16 transfection relative to GRN (data not shown).

After showing that multiple miR-15/107 gene group paralogs can target GRN, we next analyzed publicly accessible data from the GEO database (9) to see if potentially oncogenic target mRNAs are upregulated in cancers with known miRNA downregulation. CLL is the best established example of a tumor with decreased miR-15a and miR-16 expression because these tandem genes reside in the CLL minimal deletion region (10). There have been putative target genes described as oncogenic through the miRNA pathways, i.e., miR-15 and miR-16 targets oncogenically derepressed after the miRNAs are deleted. These putative miR-15/16 targets are BCL-2 and cyclin D1 (11, 12). We used previously developed high-quality datasets from the GEO database showing mRNAs in normal mononuclear blood cells and CLL cells assessed by Christian Stratowa and colleagues (13) at the Department of Lead Discovery, Boehringer Ingelheim Austria (Fig. 1A). Based on these data we pursued the assessment of GRN and other putative miR-15/16 targets. Consistent with our hypothesis, levels of GRN mRNA in CLL cells were much higher than the normal lymphocytes (P < 0.0001). In comparison, the differences in gene expression between normal lymphocytes and CLL cells for other putative miR-15/16 target mRNAs were much more marginal (BCL-2) or nonexistent (Cyclin-D1).

To determine if this is a nonspecific effect in human leukemias, we subsequently evaluated data about chronic myelogenous leukemia (CML) from Dr. Lucy Crossman’s group at the Oregon Health and Science University Cancer Institute, Portland, OR (14), also available from the GEO database. MiR-15 and miR-16 have not been shown to be downregulated in CML. We found that, indeed, the level of GRN mRNA is actually higher in normal leukocytes than in CML cells. These data are compatible with the hypothesis that miR-15 and miR-16 downregulation in CLL contributes specifically to increased expression of the oncogene GRN.

There have been other human cancers where miR-15/107 gene group members have been shown to be decreased, and independently, GRN levels are found to be upregulated. These cancers are listed in Table 1. Note that these are only cancers where both of these findings have been described; in

<table>
<thead>
<tr>
<th>miR-15/107 group member downregulated? (methods)</th>
<th>miRNA downregulated in this cancer</th>
<th>Granulin upregulated? (methods)</th>
<th>References</th>
</tr>
</thead>
<tbody>
<tr>
<td>Carcinomas</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Bladder carcinoma</td>
<td>Yes (A)</td>
<td>miR-195</td>
<td>Yes (G)</td>
</tr>
<tr>
<td>Endometrial carcinoma</td>
<td>Yes (C)</td>
<td>miR-424</td>
<td>Yes (G)</td>
</tr>
<tr>
<td>Gastric carcinoma</td>
<td>Yes (A, D)</td>
<td>miR-195</td>
<td>Yes (F)</td>
</tr>
<tr>
<td>Head and neck carcinoma</td>
<td>Yes (A, B)</td>
<td>miR-16, -195</td>
<td>Yes (G)</td>
</tr>
<tr>
<td>Hepatocellular carcinoma</td>
<td>Yes (B, D)</td>
<td>miR-15b, -16</td>
<td>Yes (G)</td>
</tr>
<tr>
<td>Breast carcinoma</td>
<td>Yes (B)</td>
<td>miR-16, -497</td>
<td>Yes (G)</td>
</tr>
<tr>
<td>Non–small cell lung carcinoma</td>
<td>Yes (B)</td>
<td>Multiple</td>
<td>Yes (F)</td>
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<tr>
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<td>Yes (B)</td>
<td>miR-195</td>
<td>Yes (G)</td>
</tr>
<tr>
<td>Prostatic carcinoma</td>
<td>Yes (A)</td>
<td>miR-15, -16, -107</td>
<td>Yes (G)</td>
</tr>
<tr>
<td>Hematologic malignancies</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Chronic lymphocytic leukemia</td>
<td>Yes (C, D)</td>
<td>miR-15a, 16</td>
<td>Yes (E)</td>
</tr>
<tr>
<td>Multiple myeloma</td>
<td>Yes (A)</td>
<td>miR-15a</td>
<td>Yes (G)</td>
</tr>
</tbody>
</table>

NOTE: Studies in multiple subtypes of human neoplasms have observed miR-15/107 gene member downregulation and granulin upregulation. Both miR-15/107 genes and GRN have been found to be dysregulated in other tumor types, but shown here are the tumor types where both were independently verified. The miR-15/107 gene group comprises miR-15a, miR-15b, miR-16, miR-103, miR-107, miR-195, miR-424, miR-497, miR-503, and miR-646. The methods used for the various studies are indicated in parentheses. “Other” methods to detect downregulated miRNAs include Northern blots, specialized high-throughput methods, and in situ hybridization.

Methods to detect downregulated miRNA: A, quantitative PCR; B, microarray; C, both A and B; D, other.

Methods to detect upregulated granulin: E, mRNA level; F, protein level; G, both E and F.
the vast majority of human cancers, either GRN or the miRNAs’ levels have not been queried reliably.

To directly test whether multiple members of the miR-15/107 gene group can regulate GRN in cancer, we used human prostate cancer cells. We chose prostate cancer because independent laboratories have found that miR-15/107 gene group member expression is decreased in prostate cancer, and GRN plays a potent role in prostate tumorogenesis and malignancy (6, 7, 15). To confirm the upregulation of GRN in prostate cancer we used more data from the GEO database (Fig. 2C), testing mRNA from different prostate samples based on the data reported by Dr. Arul Chinnaiyan’s laboratory (16) at the University of Michigan. These data indeed show that GRN levels are increased in metastatic prostate cancer.

Finally, to directly test the hypothesis that GRN protein levels are regulated through members of the miR-15/107 gene group, we transfected the prostate cancer cell lines PC-3 and CW22 (17) with specific miRNAs. These data are shown in Fig. 3. Note that miR-107MUT2 (with the 5′ seed portion intact) inhibits GRN expression whereas the miR-107MUT2 miRNA (with the 3′ part of the miRNA intact) does not.

These data show collectively that multiple members of the miR-15/107 gene group can regulate GRN expression and that the GRN expression is exerted through the 5′ seed sequence of the miRNAs. However, this does not exclude the possibility that in other systems other portions of miRNAs can regulate GRN (or other miRNA targets) differentially. It is also unknown how the different miR-15/107 group members interact in vivo in terms of combinatorial effects. There is a fast-expanding research that has focused on the miR-15/107 group of genes and their effects on metabolism, cell cycle functions, and cell stress (4).

In conclusion, the present study supports the hypothesis that regulation of GRN through members of the miR-15/107 gene group may have an important oncogenic impact on multiple human cancers. This is a biological phenomenon with potential therapeutic implications. Theoretically, a therapeutic strategy that “replaces” miR-15/107 gene group expression would attenuate GRN expression and possibly decrease the malignant potential of tumors. We note that methods have been developed for delivering miR-16 systematically, which was helpful in reducing prostate cancer burden in a mouse model (18), and we hypothesize that this could have been accomplished through GRN. We hope that in the future this and other methods will be tried for human therapies that work through this novel mechanism.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

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