Genomic Loss of miR-486 Regulates Tumor Progression and the OLFM4 Anti-apoptotic Factor in Gastric Cancer

Hue-Kian Oh1, Angie Lay-Keng Tan2, Kakoli Das2, Chia-Huey Ooi2, Nian-Tao Deng2, Iain BeeHuat Tan3, Emmanuel Beillard2, Julian Lee1, Kalpana Ramnarayanan1, Sun-Young Rha6, Nallasivam Palanisamy#, P. Mathijs Voorhoeve2, Patrick Tan1,2,4,5,*

1Cellular and Molecular Research, National Cancer Centre of Singapore, 11 Hospital Drive Singapore, 169610
2Cancer and Stem Cell Biology, Duke-NUS Graduate Medical School, 8 college road, Singapore 169857
3Division of Medical Oncology, National Cancer Centre of Singapore, 11 Hospital Drive Singapore, 169610
4Genome Institute of Singapore, 60 Biopolis Street Genome #02-01, Singapore, 138672
5Cancer Science Institute of Singapore, Yong Loo Lin School of Medicine, National University of Singapore, 5 Lower Kent Ridge Road, Singapore 119074
6Department of Internal Medicine, Yonsei Cancer Center, Yonsei University College of Medicine, 134 Shinchon-Dong, Seodaemun-Ku, Seoul, Korea 120-752
#Current Address : Michigan Center for Translational Pathology and Departments of Pathology, Ann Arbor, MI 48109

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*Corresponding author: Patrick Tan, Cancer and Stem Cell Biology, Duke-NUS Graduate Medical School, 8 college road, Singapore 169857. Phone: 65-65161783. Fax: 65-62212402; Email: gmstanp@duke-nus.edu.sg

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Abstract

**Purpose:** MicroRNAs (miRNAs) play pivotal oncogenic and tumor suppressor roles in several human cancers. We sought to discover novel tumor suppressor miRNAs in gastric cancer (GC).

**Experimental Design:** Using Agilent miRNA microarrays, we compared miRNA expression profiles of 40 primary gastric tumors and 40 gastric normal tissues, identifying miRNAs significantly downregulated in gastric tumors.

**Results:** Among the top 80 miRNAs differentially expressed between gastric tumors and normals (FDR<0.01), we identified hsa-miR-486 (miR-486) as a significantly downregulated miRNA in primary GCs and GC cell lines. Restoration of miR-486 expression in GC cell lines (YCC3, SCH and AGS) caused suppression of several pro-oncogenic traits, while conversely inhibiting miR-486 expression in YCC6 GC cells enhanced cellular proliferation. Array-CGH analysis of 106 primary GCs revealed genomic loss of the miR-486 locus in ~25-30% of GCs, including two tumors with focal genomic losses specifically deleting miR-486, consistent with miR-486 playing a tumor suppressive role. Bioinformatic analysis identified the secreted anti-apoptotic glycoprotein OLFM4 as a potential miR-486 target. Restoring miR-486 expression in GC cells decreased endogenous OLFM4 transcript and protein levels, and also inhibited expression of luciferase reporters containing a OLFM4 3’ untranslated region (UTR) with predicted miR-486 binding sites. Supporting the biological relevance of OLFM4 as a miR-486 target, proliferation in GC cells was also significantly reduced by OLFM4 silencing.

**Conclusions:** miR-486 may function as a novel tumor suppressor miRNA in GC. Its anti-oncogenic activity may involve the direct targeting and inhibition of OLFM4.

**Translational Relevance**

Gastric cancer (GC) is the second leading cause of global cancer mortality. In this study, we performed miRNA profiling of primary GCs and cell lines to identify hsa-miR-486-5p (miR-486) as a significantly downregulated miRNA in GC. Subsequent functional characterization revealed that miR-486 inactivation is required for the expression of several pro-oncogenic traits, and that this is likely mediated through miR-486 targeting the OLFM4 anti-apoptotic factor. miR-486 is located at
Chr 8p11, a region of frequent genomic loss in multiple cancers. Consistent with miR-486 playing a tumor suppressor role, we observed by array-CGH frequent genomic deletion of miR-486 in 20-30% of GCs. miR-486 may thus represent a novel tumor suppressor miRNAs in GC inactivated through genomic deletion. By understanding the mechanism and function of miR-486 as a tumor suppressor, it may be possible to develop miR-486 as a diagnostic or therapeutic agent in GC treatment.
Introduction

Gastric cancer (GC) is the second leading cause of global cancer mortality with a particularly high incidence in many Asian countries (1). Most GC patients are diagnosed with advanced stage disease and show extremely poor prognosis (2). The 5-year survival rate for patients with stage II disease ranges from 30-50%, falling to 10-25% for Stage III patients (3, 4). Disease recurrence is also common, with <20% of late stage patients surviving beyond five years (3, 4). Despite a global decline in incidence, GC remains a disease of outstanding morbidity and mortality. Achieving a better understanding of recurrent molecular aberrations associated with GC carcinogenesis might identify new diagnostic and treatment strategies for this disease.

Genetic factors implicated in GC development include somatic mutations in classical tumor suppressor genes and oncogenes (p53, beta-catenin), gene amplifications and deletions (eg c-Met and ERBB2), and epigenetic inactivation of CDKN2A (p16), CDH1 (E-cadherin), hMLH1, and RUNX3 (5, 6). Besides protein coding genes, recent evidence has demonstrated an important role for non-coding RNAs in human cancer and in particular microRNAs (miRNAs) (7). miRNAs are naturally occurring small RNA molecules of ~22-nucleotides (8) which base-pair with complementary sequences located in the 3’ untranslated regions (UTRs) of target genes, causing either target mRNA degradation or reduced protein translation (9,10). An individual miRNA can regulate a large number of target mRNAs (11), and conversely a single gene may be regulated by multiple independent miRNAs. The ability of miRNAs to affect multiple downstream target genes suggests that miRNAs may be particularly adept at broadly modulating the activity of multiple cellular pathways (9,10). In cancer, miRNAs can exert either oncogenic or tumor suppressive roles (12). Oncogenic miRNAs such as miR-21, mir-155, mir-17-92, and miR-
372/miR-373 are frequently upregulated in tumors and can negatively regulate tumor suppressor genes such as Bim, PTEN, and LATS2 (12-14). Conversely, tumor suppressive miRNAs, which are usually underexpressed in tumors, have been shown to inhibit oncogenes such as RAS, BCL2 and MYC (12). Some examples of tumor suppressor miRNAs include let-7, mir-15 and mir-16.

In GC, several miRNA profiling studies have been reported (15-16). However, most of these previous studies have largely focused on characterizing oncogenic miRNAs (eg miR-21, miR-27a and the miR106b-25 cluster) (17-19). In contrast, relatively few tumor suppressor miRNAs in GC are known, and while candidates such as miR-141, miR-143, miR-145, miR-9 and miR-218 have been proposed as possible tumor suppressor miRNAs, their functions and target genes have not been extensively explored (20-24). Here, we compared miRNA expression profiles of gastric tumors and matched normal tissues to identify hsa-miR-486-5p (miR-486) as a significantly downregulated miRNA in GC. Subsequent functional characterization revealed that miR-486 inactivation is required for the expression of several pro-oncogenic traits, which may involve miR-486 targeting the OLFM4 anti-apoptotic factor. Importantly, we observed frequent and focal genomic deletions of the miR-486 locus in 20-30% of GCs, consistent with miR-486 playing a tumor suppressor role. Finally, miR-486 downregulation has also been observed in several other human cancers (25), it is thus possible that miR-486 may exert important tumor suppressive functions in other cancer types besides GC.
Materials and Methods

Primary Gastric Cancer Samples and Cell Lines.

Primary gastric tumors and adjacent matched normal gastric tissues were obtained from the National Cancer Centre Singapore and the Singhealth Tissue Repository. Primary samples were collected with signed patient informed consent and with approval from institutional review boards. GC cell lines AGS, Kato III, SNU1, NCI-N87, and Hs746T were obtained from the American Type Culture Collection and AZ-521, TMK1, MKN1, MKN7, MKN45 cells were obtained from the Japanese Collection of Research Bioresources. SCH cells were a gift from Yoshiaki Ito (Cancer Sciences Institute of Singapore). YCC1, YCC3, YCC6, YCC7 cells were a gift from Sun-Young Rha (Yonsei Cancer Center, South Korea). All cell lines were tested and authenticated by the respective cell line bank (ATCC, JCRB) or originating institution (YCC) by several methods including DNA fingerprinting and/or cytogenetics. Prior to the commencement of this study, we independently re-authenticated the cell lines by comparing their genome-wide copy number (array-CGH) and mutational profiles to the published literature.

miRNA Expression Profiling

Total RNA was extracted from primary tissues and cell lines using the miRVana miRNA Isolation Kit (Ambion, Inc, Austin,TX,USA) according to the manufacturer’s instructions. RNA samples were hybridized to Agilent Human miRNA Microarrays (V2) representing 723 human and 76 human viral miRNAs, and scanned using an Agilent DNA Microarray Scanner (Model G2565BA). miRNA expression values were normalized against background signals using Feature Extraction Software.
(Agilent). The miRNA data was also subjected to a log 10 transformation followed by median centering across probes, prior to in-depth analysis. The miRNA expression data has been deposited into GEO under accession number GSE23739.

(The reviewer’s link is http://www.ncbi.nlm.nih.gov/geo/query/acc.cgi?token=lfonrsseimicalw&acc=GSE23739)

**Microarray Data Analysis**

Differentially expressed microRNAs were identified using the Significance Analysis of Microarrays (SAM) program in BRB-ArrayTools (http://linus.nci.nih.gov/~brb/tool.htm), using a False Discovery Rate (FDR) cutoff of < 0.01. Significance of Pearson correlations (R) between two N-element vectors were estimated from the Student t-distribution, against the null hypothesis that the observed value of $t = R/\sqrt{[(1–R^2)/(N–2)]}$ arises from a population in which the true correlation coefficient is zero. Of 146 differentially expressed miRNAs initially identified by SAM (FDR< 0.01), we focused on the top 40 miRNAs exhibiting the highest positive log fold-change (most upregulated) and the top 40 mirs with the lowest negative log fold-change (most downregulated). Among the 40 most significant downregulated miRNAs in tumors, we triaged 16 candidate tumor suppressor microRNAs meeting the additional criterion of detectible expression in an independent normal stomach sample from a healthy patient (Stratagene, La Jolla, CA), at levels greater than the median expression in GC cell lines.

**Real-time Quantitative Reverse Transcription-PCR (RT-qPCR).**

RT-qPCR was performed using a ABI7900HT Fast real-time PCR system (Applied Biosystems, Foster City, CA). TaqMan® Universal PCR Master Mix (Applied Biosystems), TaqMan® Reverse Transcription Kit and TaqMan® MicroRNA Assay
kits (Applied Biosystems) were used to measure \textit{miR-486} expression levels according to the manufacturer’s instructions. Each PCR was normalized against an \textit{RNU6B} internal control. All PCR reactions were performed in triplicate.

\textbf{Additional Methods}

Detailed methods on \textit{miR-486} expression and silencing, \textit{OLMF4} silencing and overexpression, cell proliferation and anchorage independent growth assays, cell migration and invasion assays, array-CGH assays and copy number analysis, miRNA target prediction, Western blotting, \textit{OLFM4} 3’UTR Luciferase Reporter Assays, and in-situ hybridization are reported in the Supplementary Data.
Results

Identification of Differentially Regulated microRNAs in Primary Gastric Tumors

To identify miRNAs exhibiting expression changes in GC, we profiled 40 gastric tumors and 40 non-cancerous gastric tissues on Agilent miRNA microarrays measuring ~800 miRNAs (723 human and 76 viral miRNAs). Gastric tumors are traditionally classified by histology into 2 major groups – intestinal (expanding, or differentiated), and diffuse (infiltrative or undifferentiated) (26). Our analysis covered both GC subtypes, since half of the tumors were intestinal-type GC (20 samples), while the other half were diffuse-type cancers. An initial unsupervised analysis where the gastric samples were clustered using miRNAs detectibly expressed in ≥25% of the samples (329 probes) resulted in an overall partitioning of gastric tumors away from normals, indicating the existence of pervasive miRNA expression differences between the two groups (Fig. S1). We focused our analysis on miRNAs exhibiting striking differences in expression between gastric normals and tumors. Using Significance Analysis of Microarrays (SAM), we identified 80 miRNAs that were significantly differentially expressed between the two groups (FDR<0.01) (Fig. 1A, Supplemental Table S1 provides a list of the top 80 miRNAs).

We divided the differentially regulated miRNAs into three distinct classes based on their global expression patterns. The first class of miRNAs exhibited high expression in tumors relative to normals - these may represent potential pro-oncogenic miRNAs contributing to GC development and progression. Supporting this idea, among the most highly expressed miRNAs in this class were miR-21, miR-27a and miR-17, three miRNAs that have been functionally confirmed to exert oncogenic
functions in multiple cancer types including GC (17,18). The re-identification of these known oncogenic miRNAs supports the biological validity of the miRNA microarray data. Interestingly, we also observed upregulation of hsa-miR-16 and hsa-miR-214 in GC, two miRNAs being previously reported to be down-regulated in prostate and cervical cancers (27,28). It is thus possible that certain miRNAs may exert either pro or anti-oncogenic functions that are dependent on tissue type.

The second class comprised miRNAs exhibiting downregulation in tumors relative to normals - such miRNAs might represent candidates for potential tumor-suppressor miRNAs. Given the relative lack of validated tumor suppressor miRNAs in GC compared to oncogenic miRNAs, we decided to focus on this list for further analysis. Using multiple filtering criteria (see Methods); we nominated a set of 16 candidate tumor-suppressor miRNAs (Fig. 1B). Providing confidence in our filtering criteria, among the 16 candidates we re-identified mir-375, a known tumor suppressor miRNA in GC (29). These miRNAs are further considered in the next section.

We also detected a third and smaller class of miRNAs that were differentially expressed between intestinal and diffuse-type GCs (Fig.1A, bottom panel). In general, these miRNAs (hsa-let7d*, hsa-miR-328, hsa-miR-32*, hsa-miR-1227, hsa-miR-206, hsa-miR-1229, hsa-miR-595 and hsa-miR-631) were largely downregulated in diffuse-type GC compared to intestinal-type GCs. Although not the primary focus on this study, these results raise the possibility that differences in miRNA expression may also exist between the two major histologic subtypes of GC.

Expression Patterns of miR-486, a Candidate Tumor Suppressor miRNA in GC

Hypothesizing that some of the 16 nominated miRNAs exhibiting decreased expression in GC might represent bona-fide GC tumor suppressors, we selected a
handful for functional validation. Here, we report our findings for the first of these candidates, hsa-miR-486-5p, hence after referred to as miR-486. miR-486 is located on chromosome 8p11 within intron 41 of the Ankyrin-1 (Ank1) gene (Fig. 2A). miR-486 is transcribed from an alternative promoter within intron 40 of the Ankyrin-1 gene (30), and has been previously shown to regulate PI3K signaling in muscle cells by targeting the PTEN gene (30). In contrast to its role in muscle development, the role of miR-486 in epithelial tumorigenesis is currently unclear.

Three observations suggest that miR-486 may play a potential tumor suppressive role in GC. First, besides exhibiting reduced expression in tumors compared to the normal tissues, miR-486 is expressed in a strikingly reciprocal pattern to the oncogenic miRNAs miR-17, miR-21 and miR-27a (Fig.2B, lower panel). Second, to validate the microarray results, we performed reverse-transcription quantitative PCR (RT-qPCR) to directly measure miR-486 expression levels in a cohort of 28 primary gastric cancer tissues and matched adjacent normal tissues. 80% of the tumors expressed decreased levels of miR-486 expression by at least two fold compared to matched normal tissues, confirming that miR-486 is expressed at significantly lower levels in GCs (p<0.05, paired t-test) (Fig. 2C). In-situ hybridization experiments on fixed gastric tissue sections confirmed miR-486 expression in normal gastric epithelial cells, but reduced or absent expression in gastric tumors (Fig. S2). Third, to further validate the reduced expression of miR-486 in GC cells, we investigated miR-486 expression in a panel of 15 GC cell lines. Similar to the primary tumors, fourteen out of fifteen GC cell lines underexpressed miR-486 while simultaneously overexpressing the mir-17 oncogenic miRNA (p=0.001; Fig. 2D). Taken collectively, these results suggest that miR-486 may play a tumor-suppressive role in GC.
**miR-486 Expression Inhibits Proliferation, Anchorage Independent Growth, Migration, and Invasion in GC Cells**

To investigate the functional significance of miR-486 downregulation in GC, we selected three GC cell lines (YCC3, AGS and SCH) for further studies as these three lines express low levels of miR-486 (Fig. 2D). Synthetic miR-486 precursor molecules were transfected into these cells lines to restore miR-486 expression, and restoration of miR-486 expression in these cells was confirmed by qRT-PCR (Fig. 3A). First, we compared the cell proliferation rates of control and miR-486 transfected cells at various time points. In all three cell lines, the growth of miR-486 transfected cells was significantly reduced compared to cells transfected with negative-control miRs (Fig. 3B). This result suggests that restoring miR-486 expression is sufficient to inhibit cellular proliferation in GC.

Second, to examine the importance of miR-486 in the tumorigenesis of GC cells, we performed anchorage-independent growth assays. Using a blasticidin selection protocol (see Methods), we generated stable pools of SCH, YCC3 and AGS cells expressing miR-486 or empty vector controls. SCH and YCC3 cells transfected with empty vector controls grew well in soft agar, forming distinct colonies (Fig. 3C). In contrast, SCH and YCC3 cells expressing miR-486 exhibited a dramatic reduction in the number of soft agar colonies (Fig. 3C), demonstrating transforming abilities less than 50% of the control cells. Similar data was obtained for AGS cells (Fig. 3C, right panel). These results suggest that miR-486 can suppress the tumorigenicity of GC cells in vitro.

Third, to assess the effect of miR-486 in GC migration and invasion, we tested AGS cells stably expressing miR-486 or empty vectors. AGS cells expressing vector
controls migrated robustly in Transwell assays (Fig. 3D, top), while AGS cells overexpressing miR-486 exhibited a significant reduction in migration capacity (p<0.05, about 2-fold). Similarly, in invasion assays, AGS cells overexpressing miR-486 exhibited a 2-fold reduced capacity for invasion compared to controls (p=0.05) (Fig. 3D, bottom).

Fourth, of the 15 GC cell lines tested in this study, we identified one line (YCC6) expressing above-average levels of miR-486 (Figure 2D). To investigate the cellular effects of silencing rather than overexpressing miR-486, we inhibited endogenous miR-486 expression in YCC6 cells by transfecting the cells with miR-486 inhibitors (anti-miR-486). Efficient inhibition of miR-486 expression was confirmed by qRT-PCR (Figure 3E). miR-486-suppressed YCC6 cells exhibited a modest but significant enhancement of cell proliferation compared to control transfected cells (p<0.05, Figure 3E). Taken collectively, these results indicate that restoring miR-486 expression is sufficient to suppress several pro-oncogenic traits in vitro, while conversely suppressing miR-486 expression is sufficient to enhance such traits, consistent with miR-486 playing a tumor suppressive role in GC.

**Frequent Genomic Loss of miR-486 in GC**

A preliminary transcriptomic analysis of GC cell lines treated with inhibitors of DNA methyltransferase (5′ aza) or histone deacetylation inhibitors (HDACs) failed to provide evidence of epigenetic silencing of miR-486 in GC (data not shown). Besides epigenetic silencing (31), genomic loss is another mechanism by which tumor suppressor miRNAs can be downregulated in cancer (32). Notably, miR-486 is located on chromosome 8p11, a frequent region of loss-of-heterozygosity in many cancers, including gastric cancer (33, 34). While ANKI, the gene within which miR-
lies, has been proposed as a potential tumor-suppressor gene in this region (34,35), there is in reality very little functional evidence supporting an anti-oncogenic role for ANKI. These findings raise the possibility that other genetic elements on 8p11 lying close to ANKI, such as miR-486, may represent important driver elements for the frequent genomic losses associated with this region in cancer.

To investigate if miR-486 might be genomically deleted in GC, we analyzed an in-house array-CGH database of 106 gastric tumors profiled on Agilent 244K microarrays. Strikingly, we obtained genomic loss of the miR-486 locus within a window of 500kb on chromosome 8 in 25-30% of GCs (Fig. 4A). In individual GCs, the size of the genomic deletion ranged from 100kb to 300 kb. Moreover, arguing against the possibility of another nearby gene driving these deletions, two tumors (GC990187 and GC200088) exhibited highly focal genomic regions specifically deleting the miR-486 locus (GC990187 - chr8:41,582,276-41,727,172 (135 kb); GC200088 - chr8:41,582,276-41,717,096 (145 kb)) (Fig. 4B and C), while retaining a portion of the ANKI gene. We also observed a genomic deletion at the miR-486 region in the GC cell line SCH (data not shown). These results indicate that the miR-486 genomic locus is frequently deleted in GCs, supporting the notion that miR-486 is a tumor suppressor miRNA.

The Anti-apoptotic Factor OLFM4 is a Direct miR-486 Target

To better understand the mechanisms underlying the tumor suppressive capacities of miR-486, we searched for candidate miR-486 target genes with potential pro-oncogenic functions. Using two miRNA target prediction programs (TargetScan 5.1 and miRanda v3.0), we identified 17 candidate miR-486 target genes commonly predicted by both programs (Fig. 5A). Among these 17 genes, two genes (TOBI and
ARID1A) have been previously shown to be associated with tumor suppressive functions (36,37). More importantly, we identified SP5 and OLFM4, as potential pro-oncogenic miR-486 target genes (38,39). Our attention was specifically drawn to OLFM4 (Olfactomedin-4/GW112), as OLFM4 has been previously reported to be highly expressed in gastric tumors and specifically gastric cancer cells compared to normal tissues (39-41). Both prediction programs identified one potential miR-486 binding site in the OLFM4 3’UTR.

Functionally, OLFM4 has also been shown to behave as an anti-apoptotic factor and to promote tumor growth and invasion (see Discussion) (42,43). Confirming previous reports, we found that OLFM4 was indeed highly expressed in GCs compared to gastric normals (p<0.001) (Fig. 5B). Also supporting recent findings, the high expression of OLFM4 in tumors was largely associated with intestinal-type GCs (Table 1) (40). No significant associations were observed between OLFM4 expression with tumor stage, grade, or patient survival (data not shown).

To investigate relationships between endogenous miR-486 and OLFM4 expression, we analyzed 11 intestinal-type primary GCs and matched non-malignant tissues for which both miR-486 and OLFM4 expression were available. miR-486 was significantly negatively correlated to OLFM4 expression (R=-0.619, p=0.0031) (Fig. 5C), consistent with miR-486 targeting OLFM4 in vivo. To directly assess the functional impact of miR-486 on OLFM4 expression, we transfected GC cells (YCC3 and AGS) with miR-486 precursors and measured endogenous OLFM4 expression levels. Restoration of miR-486 in YCC3 and AGS cells caused a decrease in OLFM4 protein and transcript levels, relative to control-transfected cells (Figure 5D). These results indicate that restoration of miR-486 in GC cells is sufficient to suppress OLMF4 expression.
We then performed luciferase reporter assays to demonstrate a direct functional role of the predicted miR-486 binding sites in the OLFM4 3’-UTR. Luciferase reporters were constructed containing either a wild-type OLFM4 3’-UTR sequence containing the miR-486 binding site (WT-UTR), or a mutated OLFM4 3’UTR where the miR-486 seed sequence binding sites were altered (MUT-UTR) (Fig. 5E). The WT-UTR and MUT-UTR luciferase reporter constructs were transfected into GC cells, along with miR-486 or negative control miRNAs. Luciferase expression of the WT-UTR reporter was significantly decreased compared to MUT-UTR (p<0.05) or vector-expressing cells in a miR-486-dependent manner, indicating that miR-486 is able to reduce the reporter activity of WT-UTR but not MUT-UTR (Fig. 5E). This result strongly indicates that miR-486 directly targets the OLFM4 3’UTR, resulting in the translation inhibition of OLFM4 protein.

**OLMF4 Expression Promotes GC cell proliferation and Inhibits the Anti-Oncogenic Effects of miR-486**

OLFM4 has been proposed to promote tumor growth by functioning as an anti-apoptotic protein (42,43) attenuating the apoptotic function of GRIM-19, a cell-death regulatory protein. To establish a functional role for OLFM4 in GC, we silenced OLFM4 in YCC3 and SCH cells, and conducted cell proliferation assays. OLFM4-silenced YCC3 and SCH cells exhibited significantly slower cellular proliferation compared to control-siRNA treated cells (Fig. 5F). This result suggests that OLFM4 activity may be required for GC development and progression. Interestingly, downregulation of OLFM4 by miR-486 also resulted in up-regulation of GRIM-19, the proposed target of OLFM4 (Fig. S3).
Finally, to establish if the anti-proliferative effects caused by *miR-486* restoration might depend, at least in part, on suppression of *OLFM4* activity, we performed rescue experiments where YCC3 cells stably expressing *miR-486* (Y4 cells) were transfected with *OLMF4* expressing constructs. We found that Y4 cells overexpressing OLMF4 exhibited a significant increase in cell proliferation compared to control Y4 cells, comparable to parental YCC3 cells (Fig. S4). These results suggest that the tumor-suppressive effects of *miR-486* are likely to be mediated, at least in part, through its effect on *OLFM4* activity. In summary, our data suggests that *miR-486* is a tumor suppressor in GC progression, that its down-regulation in GC by genomic deletion may facilitate tumor growth, in part by causing *OLFM4* upregulation.
Discussion

Recent evidence has convincingly shown an important role for miRNAs in many human cancers (7, 12). In GC, previous miRNA profiling studies have led to the collective identification of approximately ~80 miRNAs exhibiting dysregulated expression between tumors and normals. However, beyond their observed expression patterns, relatively few tumor suppressor miRNAs have been functionally explored in GC, notable exceptions being miR-375 (regulating PDK1 and 14-3-3ζ), and miR-141, whose downstream targets are still unclear (21,29). Thus, the true biological relevance of many miRNAs in GC requires further investigation. Here, we identified miR-486 as a candidate GC tumor suppressor. Restoration of miR-486 in multiple GC cell lines significantly reduced several pro-oncogenic traits, including cell proliferation, anchorage independent growth, and cell migration/invasion, while silencing of miR-486 in YCC6 cells enhanced proliferation. To our knowledge, our study is the first to functionally explore the role of miR-486 in cancer development and progression. However, it is worth noting that Navon et al. have recently reported that miR-486 is under-expressed in several other cancer types besides GC (25). Thus, the tumor suppressive role of miR-486 in cancer may not be limited to GC alone and our findings may have relevance to other cancer types.

The exact mechanisms causing miR-486 down-regulation in cancers deserve further study. In the current study, we found that about 25-30% of gastric tumors, exhibited a genomic loss of the chromosome 8p11 region where miR-486 is located. This frequency of 8p loss is comparable to previous array-based CGH studies of GC (44,45). Moreover, genomic deletions in miRNAs have been reported as a mechanism for miRNA downregulation, as shown for miR-101, miR-15a, and miR-16-1 (32,46). However, because 70% of GCs did not exhibit observable genomic loss of miR-486,
genomic deletions alone are unlikely to fully explain the pervasive downregulation of
miR-486 in GC. It is thus almost certain that other GC tumors must employ alternative
mechanisms to achieve miR-486 downregulation, such as for example epigenetic
silencing or transcriptional repression, as reported for miR-124 (47) or transcriptional
suppression of the miR-29a promoter by myc (48). In our study, we did not observe
evidence of miR-486 epigenetic regulation when GC cell lines were treated with either
inhibitors of DNA methyltransferanse or histone acetyltransferases, however these
experiments were far from exhaustive. More work is thus required to fully delineate
the spectrum of mechanisms responsible for miR-486 downregulation in GC.

Our study suggests that OLFM4 is likely a direct target gene of miR-486.
However, it is likely over-simplistic to expect that the anti-oncogenic effects of miR-
486 can be entirely explained by its ability to regulate a single gene alone, particularly
since previous studies investigating the cellular functions of miRNAs have shown that
a single miRNA can often regulate many genes and gene targets (11). Besides
OLF4M, our bio-informatic analysis we identified 16 other potential miR-486 target
genes, several of which may function in cancer (eg FBN1, HAT1, SP5, TOB1,
ARID1A and OLFM4), and miR-486 has also been shown to target PTEN in muscle
cells (30). Nevertheless, OLFM4 is likely to be a biologically relevant miR-486 target
in the context of GC. OLFM4 has been reported to be overexpressed in various
cancers including GC but also colon, breast and lung cancers (41), and has been
proposed as a potential serum biomarker of GC (39). Functionally, OLFM4 has been
shown to interact with GRIM19 (a cell-death regulatory protein), cadherins and
lectins, and OLFM4 has been shown to inhibit apoptosis and promote tumor growth
and invasion (41-43). However, a recent finding showed that OLFM4 has a pro-
apoptotic effect in myeloid leukemia cells (49). In our study, the biological relevance
of OLFM4 as a miR-486 target was supported by demonstrating that OLFM4 silencing can reduce GC cellular proliferation, and that OLMF4 overexpression can rescue the anti-oncogenic effects of miR-486. Interestingly, despite miR-486 being downregulated in both intestinal and diffuse type GCs, we found that OLFM4 overexpression was largely confined to intestinal-type GCs. It is possible that in diffuse type GCs OLFM4 might be targeted by additional miRNAs and not simply miR-486. Consistent with this notion, preliminary bioinformatic analysis suggests that the OLFM4 gene may be targeted by over 400 different miRs (data not shown). Thus, other miRNAs may also act to target OLFM4 in diffuse type GCs, while in intestinal-type GCs, miR-486 regulation of OLFM4 may exert a predominant role. In this regard, it is intriguing to note that OLFM4 has also been recently reported to be a robust marker of intestinal stem cells (50).

In conclusion, our data suggests that miR-486 may act as a novel tumor suppressor miRNA in GC, and that its down-regulation in gastric tumors, may be required for GC development and progression. Besides providing insights in basic aspects of GC biology, our findings may also have translational relevance as miRNAs have also been proposed to represent potential therapeutic candidates. By understanding the mechanism and function of miR-486 as a tumor suppressor, it may be eventually possible to develop miR-486 as a therapeutic agent in GC treatment.
References


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Figure Legends

Figure 1. Global identification of differentially expressed microRNAs between gastric normal tissues and tumors.

A) Identification of differentially expressed miRNAs. (top) Expression heatmap showing expression of 80 differentially expressed miRNAs between and 40 normal gastric tissues (grey bar) and 40 GCs (blue and yellow bars) (FDR < 0.01, using Significance Analysis of Microarrays, see methods). Bottom panel. Differential expression of miRNAs between intestinal- and diffuse-type GCs analyzed from a separate SAM run (FDR<0.01).

B) Heatmap showing differential expression of 16 candidate tumor suppressor miRNAs. Expression of miR-486 is highlighted in red type.
**Figure 2.** Expression of candidate tumor suppressor hsa-miR-486-5p in gastric tumors and cell lines.

A) Genomic location of miR-486. miR-486 is located on chromosome 8p11 within the *ANK1* gene, between exons 41 and 42. miR-486 is transcribed from the same strand from an alternative promoter located in intron 40 of the *ANK1* gene.

B) Reciprocal expression of miR-486 compared to three previously reported oncogenic miRNAs (mir-17, mir-21, and mir-27a) across gastric normal tissues and GCs.

C) Quantitative PCR (qPCR) analysis showing relative expression of miR-486 in 29 primary gastric cancer tissues compared to matched adjacent normal tissues. Quantifications were measured using TaqMan real-time PCR. Each column represents an individual tumor/normal pair. Fold-changes (tumor/normal) were transformed to log 2 values (y-axis). p-values denotes the significance of fold change observed.

D) Expression of miR-486 and mir-17 in primary gastric tissues (normals and tumors) and GC cell lines. The x-axis depicts primary GCs (first column, n=40, median), 15 GC cell lines, and primary normal gastric tissues (blue arrow and box, last column, n=40, median). Expression of miR-486 is in green while mir-17 is in pink (color legend). Error bars indicate respective standard deviations across tumors or normals. R denotes the Pearson correlation between the two microRNAs. p-values denoting the significance of the correlation coefficient R. Cell lines selected for functional analysis are highlighted in red (YCC3, AGS, SCH, YCC6).
**Figure 3.** *miR-486* expression modulates multiple pro-oncogenic traits.

A) Restoration of *miR-486* expression in GC cell lines. YCC3, SCH and AGS cells were transfected with control or *miR-486* precursors, qRT-PCR was performed to assess relative *miR-486* expression.

B) *miR-486* expression suppresses cellular proliferation in GC cell lines. *miR-486* transfected cell lines were assessed for cell proliferation at at 24, 48 and 72 hr post-transfection. Triplicate experiments were performed for each set. *, p<0.05, *t*-test; points, means; bars, SD.

C) *miR-486* expression suppresses anchorage-independent cell growth. The left panel shows a soft-agar colony formation assay reduction demonstrating reduced in colony numbers in SCH and YCC3 stably transfected with *miR-486* or empty vector control. Right panel shows the quantification of colonies observed. Triplicate experiments were performed for each cell lines. Columns, mean; bars, SD; *, p<0.05, *t*-test.

D) *miR-486* reduces motility and invasiveness of AGS cells. Top panel, migration of AGS cells stably expressing *miR-486* or vector controls, measured using a a Transwell migration assay, columns, mean; bars, SD; *, p<0.05. Bottom panel, invasion of AGS cells stably expressing *miR-486* or vector controls, measured using a Matrigel assay. Insert, phase contrast microscopy of the crystal-violet stained cells in Matrigel. Columns, mean; bars, SD; **, p=0.059.

E) Inhibition of *miR-486* expression promotes cellular proliferation in YCC6. Top panel, *miR-486* inhibitor and negative control inhibitor (anti-*miR-486* (red) and anti-miR-ctr (blue)) transfected cells were assessed for *miR-486* expression using qRT-PCR. Bottom panel, cell proliferation levels were assayed at 24 and 48 hr.
post-transfection. Triplicate experiments were performed for each set. *points*, means; *bars*, SD; significance for the difference in growth denoted by *, p<0.05.
**Figure 4.** Genomic Loss of miR-486 in primary GCs.

A) Recurrent genomic loss of miR-486. Red bars highlight the miR-486 locus. (top) Genomic location of miR-486 on Chr 8p11.21. (middle) Histogram showing frequency of genomic loss in this region across 106 primary GCs. 28% of GCs (30 tumors) are observed to exhibit loss of the miR-486 locus (lower) Genomic loss of the miR-486 locus in individual samples. Only samples with miR-486 loss are shown. The color gradient depicts the extent of copy number deletion. (bottom) Genome browser view of the Chr 8p11.21 region showing miR-486 and adjacent genes such as ANK1.

B and C) Focal deletion of miR-486 in two gastric tumor samples: 2000088 and 990187. The copy number log-ratio data is shown together with segments identified. The miR-486 locus (with focal deletion) is highlighted in red.
**Figure 5.** Direct regulation of Olfactomedin-4 (OLMF4) by *miR-486*.

A) *miR-486* target prediction using miRanda v3.0 and TargetScan 5.1 algorithms. 17 targets were common to both prediction programs (genes in box). *OLFM4* is highlighted in red.

B) *OLFM4* is highly expressed in primary gastric tumors compared to matched normal tissues (*p*<0.001).

C) OLMF4 and *miR-486* in intestinal-type primary GCs and matched normals. We observed a significant negative correlation of *miR-486* to *OLFM4* expression (*R*=-0.61852; *p*=0.003106) in 11 intestinal-type GCs and 7 matched normal tissues analyzed.

D) *OLFM4* protein levels are regulated by *miR-486*. Western blot analyses of *OLFM4* protein in cells transfected with *miR-486* or negative control mimics. *OLFM4* protein levels were lower in YCC3 cells expressing *miR-486* as compared to control-miR expressing cells. Similar reductions the *OLFM4* proteins were also observed in *miR-486* expressing AGS cells. Quantitative real-time PCR showed that *OLFM4* transcript is also reduced in cells transfected with *miR-486* as compared to controls. (* denotes the t-test for *OLFM4* expression in *miR-486* vs control cells)

E) *OLFM4* is a direct target of *miR-486*. The predicted *miR-486* target region found in the *OLFM4* mRNA 3′UTR was cloned downstream of luciferase in a pMIR-Report-luciferase reporter vector. Reporter constructs were co-transfected with *miR-486* and negative control mimic molecules into AGS cells. Luciferase reporter assays were normalized to β-galactosidase activities and experiments were performed in triplicates. Data were plotted after
normalized against the negative control miRNA mimics. Columns, mean; bars, SD; *, p<0.05.

F) Silencing of OLFM4 in GC cells YCC3 and SCH by siRNA reduces cell proliferation capacity. YCC3 and SCH cells were transfected with siRNAs against OLFM4 or scrambled controls (scr, negative control). Experiments were performed in triplicates. Y-axis denotes the absorbance at 490nm (cell proliferation) and x-axis is the assay time-points Points, mean; bars, SD; significance for the difference in growth is denoted by **, p<0.05.
Table 1. High *OLFM4* Expression is associated with intestinal-type gastric cancer.

<table>
<thead>
<tr>
<th>Lauren's Classification</th>
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<th>Diffuse</th>
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<td>42</td>
<td>8</td>
<td>87</td>
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<tr>
<td><em>OLFM4</em> high expression</td>
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<td>24</td>
<td>11</td>
<td>87</td>
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<tr>
<td>Total</td>
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<td>66</td>
<td>19</td>
<td>174</td>
</tr>
</tbody>
</table>

A Pearson chi-squared statistic was used to test the association of the expression of *OLFM4* with GC histological subtype (174 tumors). High *OLFM4* expression was positively associated with intestinal-type gastric cancer (Pearson chi-square test, p=0.019).
Genomic Loss of miR-486 Regulates Tumor Progression and the OLFM4 Anti-apoptotic Factor in Gastric Cancer

Hue-Kian Oh, Angie Lay-Keng Tan, Kakoli Das, et al.

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