miR-218 Suppresses Nasopharyngeal Cancer Progression through Downregulation of Survivin and the SLIT2-ROBO1 Pathway

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Abstract
Nasopharyngeal carcinoma (NPC) is an Epstein–Barr virus–associated malignancy most common in East Asia and Africa. Here we report frequent downregulation of the microRNA miR-218 in primary NPC tissues and cell lines where it plays a critical role in NPC progression. Suppression of miR-218 was associated with epigenetic silencing of SLIT2 and SLIT3, ligands of ROBO receptors that have been previously implicated in tumor angiogenesis. Exogenous expression of miR-218 caused significant toxicity in NPC cells in vitro and delayed tumor growth in vivo. We used an integrated trimodality approach to identify targets of miR-218 in NPC, cervical, and breast cell lines. Direct interaction between miR-218 and the 3′-untranslated regions (UTR) of mRNAs encoding ROBO1, survivin (BIRC5), and connexin43 (GJA1) was validated in a luciferase-based transcription reporter assay. Mechanistic investigations revealed a negative feedback loop wherein miR-218 regulates NPC cell migration via the SLIT-ROBO pathway. Pleotropic effects of miR-218 on NPC survival and migration were rescued by enforced expression of miR-218–resistant, engineered isoforms of survivin and ROBO1, respectively. In clinical specimens of NPC (n = 71), ROBO1 overexpression was significantly associated with worse overall (P = 0.04, HR = 2.4) and nodal relapse-free survival (P = 0.008, HR = 6.0). Our findings define an integrative tumor suppressor function for miR-218 in NPC and further suggest that restoring miR-218 expression in NPC might be useful for its clinical management. Cancer Res; 71(6); 2381–91. ©2011 AACR.

Introduction
Gene expression is a complex cellular process that is tightly regulated at several levels to ensure proper gene dosage, hence maintenance of health in the organism. This process is dysregulated in human malignancies, leading to overexpression of tumor-promoting genes, and downregulation of tumor suppressor genes. Some of the altered regulatory mechanisms in tumor cells arise from genomic gains or losses, or aberrant protein translation (reviewed in refs. 1, 2). MicroRNAs (miR) are increasingly recognized to be key regulators of gene expression in several biological systems, including cancer (3, 4).

In silico analyses predict that approximately one third of the human genome is regulated by miRs, suggesting that several hundred or even thousands of transcripts could potentially be regulated by a single miR. Tumor cells exhibit abnormal miR expression profiles compared with their normal counterparts (5), suggesting possible role for miRs in tumor formation and disease progression. Using currently available analytic approaches, several miRs have been identified to mediate tumor progression via their respective gene targets (6, 7). There remains, however, a majority of miRs whose biological significance remains to be elucidated. miRs target identification is a complex undertaking given the inherent limitations of the currently available target prediction algorithms, which rely primarily on complementarities, thermodynamics, and inverse correlation analyses associated with high false discovery rates (8, 9). Using primary nasopharyngeal carcinoma (NPC) tissues and the C666-1 NPC cell line, we report underexpression of miR-218 in NPC and identified its many gene targets including BIRC5, ROBO1, and GJA1, which seem to play important roles in NPC biology. Epigenetic silencing of SLIT2 and SLIT3 promoters was discovered as an underlying mechanism by which miR-218 is suppressed in NPC. Reconstituting miR-218 expression in C666-1 cells led to significant toxicity in vitro and delayed tumor growth in vivo, underscoring the importance of miR-218 in NPC biology. Overexpression of miR-218 led to significant inhibition of NPC cell migration toward SLIT2.
in vitro, which was reversed by the expression of a miR-218–resistant ROBO1 open reading frame (ORF). Finally, ROBO1 overexpression was significantly associated with worse overall and nodal relapse-free survival in NPC.

Materials and Methods

Ethics statement
All tissue studies have received institutional Research Ethics Board approval. All animal experiments were conducted in accordance with the guidelines of the Animal Care Committee, University Health Network.

Quantification of miR-218 expression in NPC tissue
The clinical characteristics of the patients involved in this study are provided in Table 1. TaqMan Low Density Array (TLDA) miR expression profiling and single-well qRT-PCR were conducted in primary NPC tissues as previously described (10).

Cell culture and transfection experiments
The C666-1, CNE-1, HONE-1, C15, C17, HEK293, UT-SCC-8, UT-SCC-42a, and NOE (normal oral epithelial) cells were maintained as previously described (11–13). The C666-1 cell line was authenticated at the Centre for Applied Genomics (Hospital for Sick Children, Toronto, Ontario, Canada), using the AmpF/STR Identifiler PCR Amplification Kit (Applied Biosystems Inc.). miR precursors (Applied Biosystems Inc.) transfection was done as previously described (11).

Measurement of cell viability and apoptosis
Cell viability and cell-cycle analyses were conducted as previously described (11).

Cell migration
Cell migration was assessed using the 6-well BD Transwell migration system (BD Biosciences). On day 1, a total of 5 × 10^5 HEK293 cells were seeded in the lower chamber; 18 hours later, cells were transfected with 4 μg of control or hSLIT2 plasmid (14), using Lipofectamine 2000 (Invitrogen Inc.). Five hours later, the medium was changed to RPMI plus 1% FBS. On day 2, C666-1 cells were resuspended in RPMI plus 1% FBS and 3 × 10^5 cells were added to the upper chamber (2 ml). In some experiments as indicated, C666-1 cells were cotransfected with pre-miR (40 nmol/L) and plasmid (500 ng/24 wells), and 48 hours later, cells were counted and used in the migration experiments. Forty-eight hours later, inserts were fixed and stained using SIEMENS DIFF-QUICK stain set (Siemens Healthcare Diagnostics) and the number of migrating cells in ten 20× fields was quantified.

Immunoblotting
C666-1 cells were transfected with the indicated pre-miR and immunoblotting was conducted using anti-Survivin polyclonal antibody (1:1,000 dilution; Novus Biologicals), anti-ROBO1 polyclonal antibody (ab7279; 1:200 dilution; Abcam, Inc.), and anti-GAPDH (glyceraldehyde-3-phosphate dehydrogenase; antibody 1:12,500; Abcam) as described before (11).

Microarray and target prediction analysis
C666-1 cells were transfected with 40 nmol/L pre-miR-Ctrl or Pre-miR-218, and at 16 and 48 hours posttransfection, total RNA was extracted, hybridized onto the Affymetrix Human Genome U133 Plus 2.0 array, and analyzed as described before (15, 16). Predicted miR-218 gene target list was generated by compiling a nonredundant list of predicted targets using 6 commonly utilized miR target prediction algorithms (TargetScan, miRanda, PicTar, GenmiR++,

### Table 1. Patient and tumor characteristics

<table>
<thead>
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<td>N  = 71</td>
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</tr>
<tr>
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<td>4</td>
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<tr>
<td>Radiation alone</td>
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</table>
| Percent | 69% | 80%

Follow-up, y |    | 7.2 |
| Range | 0.7–14.2 | 0.7–14.2 |
| Stage |    |    |
| I | 4 | 6% |
| II | 25 | 35% |
| III | 25 | 35% |
| IV | 17 | 24% |
| Treatment |    |    |
| Concurrent chemoradiation | 10 | 14% |
| Neoadjuvant chemo + radiation | 4 | 6% |
| Radiation alone | 57 | 80% |
miRBase, and miRDB). Gene overlap was assessed using the gene list Venn diagram tool (17). Gene Ontology (GO) and pathway analyses on miR-218 targets were conducted using DAVID Bioinformatics Database functional-annotation tools (18, 19).

Luciferase assay
A fragment (150–200 bp) from the 3′-untranslated region (UTR) of BIRC5, ROBO1, and GJA1 containing the predicted binding sites for miR-218 (wild type or mutant) was individually amplified using the indicated primers in Supplementary Table S1 and then cloned at the SpeI and HindIII sites downstream of the firefly luciferase gene in the pMIR-REPORT vector (Applied Biosystems Inc.). Luciferase assay was subsequently conducted as previously described (11).

qRT-PCR and cloning
The expression levels of BIRC5, ROBO1, GJA1, LEF1, RGS1, CETN2, EGFR, HOX11, and LAMB3 in pre-miR-218–transfected cells were measured using the primers listed in Supplementary Table S1 by qRT-PCR. Cloning of BIRC5 ORF and ROBO1 (isoform b) ORF was conducted using the corresponding primers in Supplementary Table S1, the High Fidelity SuperScript III One-Step RT-PCR System, and pcDNA3.1 Directional TOPO Expression Kit (Invitrogen).

Expression of BIRC5 and ROBO1 was then validated using qRT-PCR.

5-Aza-2′-deoxycytidine treatment and quantitative CpG promoter methylation analysis
C666-1 cells were seeded overnight in a 12-well plate before adding 5-aza-2′-deoxycytidine (5-aza; Sigma Aldrich) at 1 μmol/L; fresh medium with 5-aza was added on day 3. Cells were harvested and RNA was extracted for qRT-PCR analysis on day 4. The CpG islands in the SLIT2 and SLIT3 promoter regions were identified using Methprimer (20, 21) and were analyzed for CpG methylation by using the EpiTYPER (SEQUENOM, Inc.; Supplementary Fig. S1) at the Analytical Genetics Technology Centre (AGTC), University Health Network, Toronto.

In vivo tumor growth experiments
Tumor formation experiments were conducted as described before (11).

Statistical analysis
Overall survival (OS) and nodal relapse-free survival were calculated using the Kaplan–Meier method. Differences between survival curves were analyzed by log-rank test. Cox proportional hazard regression model was applied to estimate the HR and 95% CI, using 2-sided tests. Pearson correlation was...
utilized to assess the correlation between miR-218 and ROBO1 expression. Statistical analyses were done using version 9.2 of the SAS system and User’s Guide (SAS Institute), presented using the GraphPad Prism software (GraphPad Software).

Results

**MiR-218 was downregulated in primary NPC tissues and cell lines, and its overexpression was lethal to tumor cells in vitro and in vivo**

miR expression profiling of 16 primary NPC samples revealed almost a 10-fold reduction in miR-218 expression observed in all NPC samples compared with normal NP tissue (Table 1A and Fig. 1A). Significant downregulation of miR-218 was also observed in the C666-1 (EBV positive), C15 (EBV positive), C17 (EBV positive), CNE-1 (EBV negative), and HONE-1 (EBV negative) NPC models compared with NOE cells (Fig. 1B). To assess the biological significance of miR-218 downregulation, C666-1 cells were transfected with pre-miR-Ctrl or pre-miR-218 and then assessed for cell viability. Transfection of C666-1 cells with 40 nmol/L pre-miR-218 led to significant increase in miR-218 expression (Supplementary Fig. S2). Interestingly, reconstituting miR-218 expression in C666-1 cells led to significant reduction in cell viability compared with transfection with control pre-miR (Fig. 1C, left), which was further reduced when cells were additionally exposed to ionizing radiation (IR), delivered 3 days posttransfection (Fig. 1C, right). Similar reduction in cell viability was observed for the human laryngeal UT-SCC-42a and UT-SCC-8 cells following transfection with pre-miR-218, whereas no significant cytotoxicity was observed for the NOE cells (Fig. 1D). To characterize the mode of cytotoxicity inflicted by restoration of miR-218 expression in C666-1 cells, cell-cycle analyses done at 3 days posttransfection have shown significant induction of apoptosis (sub-G0), reduction in G1, and an increase in S/G2M phases (Fig. 2A and B). To further confirm induction of apoptosis in the miR-218–transfected cells, caspase 3/7 activation was examined as a function of time in miR-218–transfected C666-1 cells (Fig. 2C). To further understand the biological significance of loss of miR-218 on NPC tumorigenesis in vivo, C666-1 cells were transfected with pre-miR-Ctrl or pre-miR-218 alone or combined with 4 Gy IR delivered on day 3 posttransfection, implanted into SCID mice 3 days later, and then monitored for tumor formation. MiR-218 expression in transfected cells was assessed by qRT-PCR prior to implantation (Supplementary Fig. S3). Significant reduction in tumor growth was observed in the pre-miR-218 or pre-miR-218 plus IR groups,
thereby corroborating the in vitro data of miR-218 cytotoxicity in a 3-dimensional xenograft model (Fig. 2D).

MiR-218 is a negative regulator of several genes involved in NPC pathogenesis

Given the profound downregulation of miR-218 in NPC, and the dramatic effects of miR-218 overexpression on C666-1 cells, we sought to determine the gene targets which might account for these observations. A novel 3-pronged approach was thus developed to address this challenge. First, a non-redundant list of predicted miR-218 gene targets was compiled from 6 miR target prediction databases (TargetScan, miRanda, PicTar, GenmiR++, miRBase, and miRDB), resulting in 1,577 potential mRNA targets (Fig. 3A). Second, C666-1
MiR-218 and the corresponding 3'-UTR regions by miR-218 (Fig. 3F, bottom), which was significantly abrogated when mutating the predicted miR-218 seed regions in the pMIR-REPORT UTR constructs, thereby validating the direct interaction between miR-218 and the corresponding 3'-UTRs. Concordantly, overexpressing BIRC5 and GJA1 3'-UTR (which includes miR-218-binding region) led to significant reduction in miR-218 expression in NOE cells (Supplementary Fig. S5). BIRC5 was the most notable miR-218 target given its documented prosurvival role in NPC. MiR-218 directly targets ROBO1, BIRC5, and GJA1 in NPC

Direct interaction between miR-218 and the 3'-UTR of ROBO1, BIRC5, and GJA1 was examined using the luciferase assay. Indeed, cotransfection experiments have shown direct regulation of these three 3'-UTR regions by miR-218 (Fig. 3F, bottom), which was significantly abrogated when mutating the predicted miR-218 seed regions in the pMIR-REPORT UTR constructs, thereby validating the direct interaction between miR-218 and the corresponding 3'-UTRs. Concordantly, overexpressing BIRC5 and GJA1 3'-UTR (which includes miR-218-binding region) led to significant reduction in miR-218 expression in NOE cells (Supplementary Fig. S5). BIRC5 was the most notable miR-218 target given its documented prosurvival role and the significant induction of cell death post-miR-218 transfection; hence, we sought to determine whether expressing miR-218-resistant BIRC5 (which lack the 3'-UTR) could rescue NPC cells from cell death upon miR-218 transfection. Data presented in Figure 3G clearly show significant rescue of C666-1 cells upon cotransfection with pre-miR-218 and pBIRC5 compared with cell transfected with control vector plus pre-miR-218 whereas transfection of C666-1 with pBIRC5 along with pre-miR-Ctrl had no significant effect on cell viability (data not shown).

Downregulation of miR-218 through epigenetic silencing of SLIT2 and SLIT3 promoters in NPC

The mechanism of miR-218 downregulation in NPC remained unclear, although several different mechanisms have been described to regulate miR expression in various biological systems (24). Hence, the Sanger miR database was interrogated for the chromosomal location of miR-218, which revealed 2 miR-218 primary transcripts (hsa-miR-218-1 and hsa-miR-218-2), which were embedded in the intronic regions of SLIT2 (4p15.31) and SLIT3 (5q35.1), respectively (Fig. 4A). Our previous microarray expression profiling indeed documented significant downregulation of SLIT3 expression in primary NPC samples (25). Hence, SLIT2 and SLIT3 expression was examined in C666-1 cells, showing significant underexpression of both transcripts, concordant with miR-218 downregulation (Fig. 4B). Downregulation of SLIT2 and SLIT3 through promoter hypermethylation has been reported in other human cancers (26, 27) but has not been described in NPC. Interestingly, 5-aza treatment, a potent inhibitor of DNA methylation, induced reexpression of SLIT2 and SLIT3 transcripts accompanied by significant induction of miR-218 reexpression (Fig. 4C, right). Subsequent quantitative analysis of SLIT2 and SLIT3 promoter methylation revealed approximately 70% and 90% methylation in C666-1 cells compared with approximately 15% and 45% in NOE cells, respectively (Fig. 4D). Significant SLIT2 promoter hypermethylation was also observed in the C15 and C17 NPC models (Fig. 4D), thereby confirming that epigenetic silencing of SLIT2 and SLIT3 promoters accounted for their suppressed expression in NPC.

ROBO1 expression was associated with lower overall and nodal relapse-free survival and mediated NPC cell migration in response to SLIT2 in vitro

Given the recent description of ROBO1 as a mediator of cell migration and invasion in the neural system (28, 29), and the fact that intracranial invasion is frequent clinical challenge in NPC, an independent cohort of 71 primary NPC samples were analyzed using qRT-PCR for miR-218 and ROBO1 expression, showing significant inverse relationship between miR-218 and ROBO1 expression (Fig. 5A), corroborating ROBO1 being relevant biological target for miR-218...
in NPC. The potential prognostic role for ROBO1 was assessed in this same cohort, showing that patients with lower than median ROBO1 expression experienced significantly higher 7-year-OS rate of 81% versus 63% for high-expressing ROBO1 patients (Fig. 5A; $P = 0.04$, HR = 2.4). Surprisingly, nodal relapse-free survival was also significantly more favorable for low ROBO1-expressing NPC patients of 95%, versus 67% for high ROBO1 expressors (Fig. 5B; $P = 0.008$, HR = 6.0). To acquire greater insight into the mechanism by which ROBO1 overexpression mediated NPC relapse, we evaluated the response of the C666-1 cells (which express high levels of ROBO1; data not shown) to SLIT2 (the ligand for ROBO1) in vitro, using a Transwell migration assay. The data presented in Figure 5D (left) clearly show significant increase in migration of C666-1 cells toward SLIT2 (the ligand for ROBO1) in vitro, using a Transwell migration assay. The data presented in Figure 5D (left) clearly show significant increase in migration of C666-1 cells toward SLIT2, thereby supporting the biological relevance of the ROBO1-SLIT2 pathway in mediating NPC cell migration. Conversely, overexpression of miR-218 led to significant inhibition of C666-1 migration toward SLIT2 (Fig. 5D, middle), which in turn was reversed by cotransfection with a miR-218-resistant ROBO1 expression plasmid (Fig. 5D, right).

**Discussion**

Deregulated gene expression is one of the major hallmarks of cancer cells, associated with a myriad of mechanisms leading to overexpression of tumor-promoting genes, or downregulation of tumor-suppressing genes, collectively driving tumor progression. We had previously documented that overexpression of the antiapoptotic protein BIRC5 was associated with poor clinical outcome in NPC (23), which has been similarly reported by other groups for different cancers (30). The mechanisms leading to overexpression of BIRC5 in NPC, however, remained unknown. Herein, we report for the first time that downregulation of miR-218 is a consequence of epigenetic silencing of SLIT2 and SLIT3 promoters, which, in turn, resulted in BIRC5 overexpression in NPC. Targeted depletion of BIRC5 significantly reduced viability and increased apoptosis (25) and delayed tumor growth in vivo (Supplementary Fig. S6), similar to the phenotypic changes observed in current study (Figs. 1C and 2A, B, and D). In fact, cotransfection with a miR-218-resistant BIRC5 expression plasmid led to significant rescue of the C666-1 cells posttransfection with pre-miR-218 (Fig. 3G). Taken together, our
data validated BIRC5 as a *bona fide* target for miR-218 in NPC and showed that the cytotoxicity caused by reexpressed miR-218 was mediated by the downregulation of BIRC5.

These molecular links were discovered through an integration of *in silico* and experimental approaches that was highly efficient in identifying several biologically and clinically relevant targets for miR-218. Although miRs are generally believed to regulate gene expression via translational repression, there is mounting evidence that miR–mRNA interactions oftentimes lead to mRNA destabilization and degradation, providing the scientific basis for our combinatorial strategy (31, 32). Using this method, approximately 40% of the experimentally determined miR-218 targets were indeed predicted by at least 1 miR target prediction algorithm (Fig. 3A and Supplementary Fig. S4). Thus, the advantage of this approach is the ability to exclude the vast majority (~91%) of false-positive target predictions, defined as mRNA targets that could not be experimentally validated. Interestingly, several of the identified targets were predicted by only 1 or 2 databases, underscoring the value of combining multiple databases to discover novel mRNA targets.

Search of the Sanger database identified the chromosomal locations of miR-218 transcripts within the intronic regions of *SLIT2* and *SLIT3*, reported to be underexpressed in several human malignancies and purported to function as tumor suppressors (26, 27, 33). In this study, we observed downregulation of the *SLIT2* and *SLIT3* transcripts in C666-1 cells, consistent with our own data documenting downregulation of *SLIT3* in primary NPC (25). Using 5-aza treatment and quantitative promoter methylation analyses, *SLIT2* and *SLIT3* promoter hypermethylation was documented to be the mechanism leading to their underexpression in NPC (Fig. 4C). Thus, our data provided novel insight into the importance of *SLIT2* and *SLIT3* in NPC, wherein their loss via promoter methylation drove tumorigenesis through downregulation of miR-218, whose target genes increased tumor proliferation, resisted apoptosis, and enhanced invasion. While treating NPC cells
with 5-aza led to substantial reexpression of SLIT2 and SLIT3. miR-218 was only slightly increased in (Fig. 4C). We have 2 plausible explanations for this observation. First, it is possible that the cells were unable to fully process the SLIT2 and SLIT3 transcripts to mature miR-218 due to significant toxicity of 5-aza treatment. Second, it is possible that miR-218 is subjected to another level of posttranscriptional miR-processing regulation, similar to regulation of the let-7/miR-98 family by Lin28 (34).

ROBO1 is another miR-218 target that was characterized in this study. It is a member of the Roundabout family of receptors involved in several different cellular processes (35). The interaction between ROBO1 and its SLIT receptors plays critical role in mediating axon guidance during neural development (28), recruitment of endothelial cells during angiogenesis (29), and mediating breast cancer cell migration (36). The observation that ROBO1 is direct target for miR-218, which is embedded within the SLIT2 and SLIT3 transcripts, suggests novel role for miR-218 in regulating ROBO-SLIT signaling in NPC through a negative feedback loop (Fig. 6), which would be consistent with a recent report documenting miR-218 expression (possibly through DNMT inhibitors) might provide potentially useful strategy for the future management of this disease.

Disclosure of Potential Conflicts of Interest

The authors declare that there are no conflicts of interest.

Acknowledgments

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