ABSTRACT

Development of improved RNA interference–based strategies is of utmost clinical importance. Although siRNA-mediated silencing of EphA2, an ovarian cancer oncogene, results in reduction of tumor growth, we present evidence that additional inhibition of EphA2 by a microRNA (miRNA) further “boosts” its antitumor effects. We identified miR-520d-3p as a tumor suppressor upstream of EphA2, whose expression correlated with favorable outcomes in two independent patient cohorts comprising 647 patients. Restoration of miR-520d-3p prominently decreased EphA2 protein levels, and suppressed tumor growth and migration/invasion both in vitro and in vivo. Dual inhibition of EphA2 in vivo using 1,2-dioleoyl-sn-glycero-3-phosphatidylcholine (DOPC) nanoliposomes loaded with miR-520d-3p and EphA2 siRNA showed synergistic antitumor efficiency and greater therapeutic efficacy than either monotherapy alone. This synergy is at least in part due to miR-520d-3p targeting EphB2, another Eph receptor. Our data emphasize the feasibility of combined miRNA–siRNA therapy, and will have broad implications for innovative gene silencing therapies for cancer and other diseases.

SIGNIFICANCE: This study addresses a new concept of RNA inhibition therapy by combining miRNA and siRNA in nanoliposomal particles to target oncogenic pathways altered in ovarian cancer. Combined targeting of the Eph pathway using EphA2-targeting siRNA and the tumor suppressor miR-520d-3p exhibits remarkable therapeutic synergy and enhanced tumor suppression in vitro and in vivo compared with either monotherapy alone. Cancer Discov; 3(11); 1–14. ©2013 AACR.

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**INTRODUCTION**

RNA interference (RNAi)-based therapeutics such as siRNA therapy are a novel approach that is currently under investigation to improve clinical trials and patient care for different cancers (1). However, early clinical trials testing siRNAs for cancer management have resulted in modest response and are yet to deliver on the full potential of this technology (2). One chief clinical concern with any targeted or siRNA-based therapy is their single-gene management approach, which confines them to the “one-drug–one-target” paradigm and renders them susceptible to resistance in due course. On the contrary, by the virtue of their ability to simultaneously target multiple protein-coding genes, microRNAs (miRNA, miR) have emerged to be promising novel intervention tools for cancer management (3). These small regulatory noncoding RNAs show widespread deregulation in many human cancers, and are thus associated with tumorigenesis and progression (4).

Epithelial ovarian cancer remains the most lethal form of gynecologic malignancy, with the 5-year survival rate for patients of less than 44% (5). In recent years, EphA2 has emerged as an important target for ovarian cancer therapy (6). EphA2, a member of the Eph-receptor family, is a receptor tyrosine kinase that has been shown to be oncogenic in several human malignancies, including ovarian cancer (6, 7), breast cancer (8), colorectal cancer (9, 10), glioblastoma (11), pancreatic cancer (12), esophageal cancer (13), lung cancer (14), melanoma (15), and prostate cancer (16), and promotes proliferation, migration, invasion, and metastasis (17–19). EphA2 is overexpressed in more than 75% of patients with ovarian cancer, and its expression has been linked to increased tumor growth and angiogenesis and poor clinical outcome (7, 20, 21). Consistent with these findings, it was shown that delivery of EphA2 siRNA to ovarian cancer tumors using neutral nanoliposomes potently inhibits EphA2 expression and suppresses tumor growth and prolongs survival in orthotopic mouse models of ovarian cancer, emphasizing that EphA2 is a strong oncogenic target for ovarian cancer therapy (22–24). However, given the incomplete target silencing with siRNA alone, we questioned whether the addition of a miRNA that targets the same pathway would further enhance the efficacy of EphA2 inhibition and tumor suppression.

On the basis of this, we hypothesized that the combination of miRNA- and siRNA-based treatment could afford improved dual inhibition of a target protein as well as concurrent modulation of other oncogenic members of the same pathway. In this study, we test the hypothesis that EphA2 siRNA will present greater antitumor potency when combined with a specific miRNA targeting the Eph pathway. For this purpose, we identified a clinically relevant miRNA, miR-520d-3p, that is an independent prognostic marker for patients with epithelial ovarian cancer using The Cancer Genome Atlas (TCGA) and The MD Anderson Cancer Center (MDACC; Houston, TX) datasets. We showed that dual targeting of EphA2 using EphA2 siRNA and miR-520d-3p exhibits synergistic inhibition of EphA2 and significantly augments tumor regression compared with either monotherapy alone. These findings provide proof-of-principle for the clinical application of a
previously unrecognized approach combining miRNA and siRNA therapy for targeting a common oncogenic pathway in ovarian cancer.

RESULTS

miR-520d-3p Is an Independent Prognostic Factor in Serous Ovarian Cancer

To detect novel miRNAs associated with clinical outcome, we used the data available at the beginning of our study from the 2009 TCGA (25) dataset for ovarian cancer, comprising 186 patients whose survival status was available (recorded as living, n = 92, or deceased, n = 94). Response to therapy was known for 118 of them, and was recorded as complete response (CR = 84), partial response (PR = 19), progressive disease (PROG = 13), or stable disease (n = 2). Using ANOVA, we identified 80 miRNAs that were significantly associated with longer overall survival (OS; when comparing alive vs. deceased) and 75 miRNAs that correlated with good response to therapy (when comparing CR vs. PROG). A total of 14 miRNAs were found to be common between the two lists (Supplementary Table S1). We also conducted additional univariate Cox regression analysis on the discovery cohort with miRNA expression levels as continuous variables (data not shown). Next, we used multiple miRNA target prediction programs (RNA22, TargetScan, miRanda, microT, and PicTar) to determine whether any of these 14 miRNAs were predicted to target EphA2, an important oncogenic target in ovarian cancer. Interestingly, we identified miR-520d-3p (also called miR-520d) to be predicted to target EphA2, as well as to statistically correlate with better survival and prognosis in patients with ovarian cancer (Fig. 1A; univariate and multivariate analysis in Table 1). High miR-520d-3p had an HR of 0.0218 (95% confidence interval = 0.00185–0.2563; Wald test P = 0.000234; Fig. 1A). Subjects with high miR-520d-3p expression (cutoff = 0.54) had a significantly longer survival time (median, 52 months) compared with patients with low miR-520d-3p expression (median, 39 months; P = 0.01; Fig. 1B). Instead, miR-520d-5p (also called miR-520d*), which is produced from the same precursor miRNA and is considerably less expressed in ovarian cancer cell lines (Supplementary Fig. S1), does not correlate with any of these clinical parameters and is also not predicted to target EphA2 (data
Therapeutic Synergy between miRNA and siRNA

...not shown), thus further supporting the biologic exclusivity and relevance of miR-520d-3p:EphA2 interaction.

To validate our findings, we confirmed miR-520d-3p as a favorable prognostic factor in an independent cohort of ovarian cancer samples collected from MDACC (n = 91; clinical data in Supplementary Table S2). The patients with high miR-520d-3p expression showed longer survival time (median, 54 months) as compared with those with low miR-520d-3p levels (median, 38 months; P = 0.038; Fig. 1C; Table 1). High miR-520d-3p expression was also a favorable predictor of progression-free survival (PFS) in these patient samples (P = 0.0016; Supplementary Fig. S2; Table 1). As expected, miR-520d-5p was not found to correlate with either OS or PFS in this dataset (data not shown). miR-520d-3p was also confirmed to be prognostic for patients with ovarian cancer in the updated 2012 TCGA dataset (n = 556, including the 186 patients initially analyzed and recorded as living, n = 265, or deceased, n = 291; P = 0.046; Fig. 1D). These findings suggest that miR-520d-3p is a favorable prognostic factor for ovarian cancer independent of other clinicopathologic parameters.

We further sought to determine whether combined expression of miR-520d-3p and EphA2 would serve as a better prognostic set for outcome of patients with ovarian cancer. In agreement with previous reports, EphA2 is differentially expressed in high-grade ovarian cancer (cutoff = 0.386; P = 0.0014; data not shown), and high EphA2 expression levels correlated with shorter OS (median survival of 41 months compared with 56.5 months in patients with low expression; P = 0.0002; Fig. 1E). However, combined expression of EphA2 and miR-520d-3p significantly improved the separation curves, and patients showing EphA2(high)/miR-520d-3p(low) had significantly shorter survival (median, 38.2 months) compared with those with EphA2(low)/miR-520d-3p(high) (median, 70.8 months; P = 0.00006; Fig. 1F). These findings further validate the importance of miR-520d-3p in ovarian cancer, which led us to investigate its specific cellular and biologic functions and its association with EphA2.

**EphA2 Is a Direct Functional Target of miR-520d-3p**

To determine whether EphA2 is indeed a direct target of miR-520d-3p, we first examined the correlation between miR-520d-3p and EphA2 mRNA expression in the 91-patient MDACC ovarian cancer patient dataset. We found a statistically significant inverse correlation between miR-520d-3p and EphA2 expression in these patient samples (P = 0.0016; Supplementary Fig. S2; Table 1). As expected, miR-520d-5p was not found to correlate with either OS or PFS in this dataset (data not shown). miR-520d-3p was also confirmed to be prognostic for patients with ovarian cancer in the updated 2012 TCGA dataset (n = 556, including the 186 patients initially analyzed and recorded as living, n = 265, or deceased, n = 291; P = 0.046; Fig. 1D). These findings suggest that miR-520d-3p is a favorable prognostic factor for ovarian cancer independent of other clinicopathologic parameters.

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**Table 1. Univariate and multivariate analysis of OS and PFS results of 556 patients from the TCGA dataset (a) and 91 patients with ovarian cancer from MDACC (b and c)—Data portal (https://tcga-data.nci.nih.gov/tcga)**

<table>
<thead>
<tr>
<th>Variable</th>
<th>a. OS (n=556)</th>
<th>Multivariate analysis</th>
<th>b. OS (n=91)</th>
<th>c. PFS (n=91)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Variable</td>
<td>Univariate analysis</td>
<td>HR (95% CI)</td>
<td>P</td>
<td>Multivariate analysis</td>
</tr>
<tr>
<td>EphA2 Low vs. high</td>
<td>0.62 (0.48–0.8)</td>
<td>0.0002</td>
<td>0.78 (0.7–1.01)</td>
<td>0.0575</td>
</tr>
<tr>
<td>EphB2 Low vs. high</td>
<td>0.69 (0.53–0.9)</td>
<td>0.0051</td>
<td>0.93 (0.73–1.2)</td>
<td>0.5972</td>
</tr>
<tr>
<td>miR-520d-3p Low vs. high</td>
<td>1.29 (1.02–1.63)</td>
<td>0.03</td>
<td>1.32 (1.03–1.69)</td>
<td>0.0297</td>
</tr>
<tr>
<td>Dicer Low vs. high</td>
<td>1.43 (1.11–1.82)</td>
<td>0.0032</td>
<td>0.94 (0.73–1.21)</td>
<td>0.6271</td>
</tr>
<tr>
<td>Cytoreduction operation (Residual disease vs.</td>
<td>0.94 (0.62–1.43)</td>
<td>0.77</td>
<td>0.92 (0.6–1.41)</td>
<td>0.6935</td>
</tr>
<tr>
<td>no residual disease)</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>miR-520d-3p Low vs. high</td>
<td>1.873 (1.026–3.420)</td>
<td>0.041</td>
<td>3.168 (1.654–6.066)</td>
<td>0.0005</td>
</tr>
<tr>
<td>Recurrence Positive vs. negative</td>
<td>29.967 (1.587–565.723)</td>
<td>0.023</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Dicer Low vs. high</td>
<td>2.518 (1.396–4.542)</td>
<td>0.002</td>
<td>2.551 (1.306–4.984)</td>
<td>0.0061</td>
</tr>
<tr>
<td>Drosha Low vs. high</td>
<td>2.594 (1.431–4.705)</td>
<td>0.002</td>
<td>2.471 (1.220–5.003)</td>
<td>0.0119</td>
</tr>
<tr>
<td>miR-520d-3p Low vs. high</td>
<td>2.183 (1.208–3.944)</td>
<td>0.01</td>
<td>2.871 (1.563–5.275)</td>
<td>0.0006</td>
</tr>
<tr>
<td>Cytoreduction operation Suboptimal vs. optimal</td>
<td>1.638 (0.864–3.102)</td>
<td>0.007</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Node metastasis Positive vs. negative</td>
<td>2.458 (1.091–5.0658)</td>
<td>0.03</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Dicer Low vs. high</td>
<td>2.396 (1.319–4.355)</td>
<td>0.004</td>
<td>3.224 (1.7444–5.959)</td>
<td>0.00018</td>
</tr>
<tr>
<td>Drosha Low vs. high</td>
<td>2.244 (1.241–4.060)</td>
<td>0.008</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

Abbreviations: PFS, progression-free survival; CI, confidence interval.
High EphA2 immunostaining in qRT-PCR analysis showing transient overexpression of miR-520d-3p in ES2 and SKOV3ip1 cells (top) results in downregulation of EphA2. Representative images of the immunostaining from 4 patients showing negative correlation in ovarian cancer tumors. Its expression is regulated by miR-520d-3p in ovarian cancer. EphA2 is a direct functional target of miR-520d-3p (Fig. 2G). Taken together, these data suggest that EphA2 is a direct functional target of miR-520d-3p and its expression is regulated by miR-520d-3p in ovarian cancer.

Figure 2. EphA2 is a direct and functional target of miR-520d-3p. A, scatter plot showing negative correlation between EphA2 mRNA (normalized to 18S) and miR-520d-3p (normalized to U6) in the MDACC patient set using Spearman’s correlation analysis (R = –0.248, P = 0.02). B, quantification of EphA2 and miR-520d-3p immunostaining from 4 patients showing negative correlation in ovarian cancer tumors. C, representative images of the immunostaining in B. D, qRT-PCR analysis showing transient overexpression of miR-520d-3p in ES2 and SKOV3ip1 cells (top) results in downregulation of EphA2 mRNA after 48 and 72 hours (bottom). E, immunoblotting of EphA2 and GAPDH in ES2 and SKOV3ip1 cells transfected with miR-520d-3p (100 or 200 nmol/L) or a scrambled control. F, representative diagram of the conserved binding site of miR-520d-3p in the 3′-untranslated region (3′-UTR) of EphA2 mRNA. G, luciferase activity of a reporter construct fused to wild-type or mutant EphA2 3′-UTR in ES2 and SKOV3ip1 cells with ectopic miR-520d-3p expression. Control, cells transfected with a scrambled miRNA control. Data are average of three independent experiments. Statistical significance was determined by unpaired, two-tailed Student t test. *, P ≤ 0.05; **, P ≤ 0.01; ***, P ≤ 0.001. Data are mean ± SD.

To study the in vitro and in vivo functions of miR-520d-3p, we stably overexpressed miR-520d-3p in SKOV3ip1 and HeyA8 (chosen because HeyA8 has been better characterized in vivo in ovarian cancer as compared with ES2) cells. We observed a marked reduction in EphA2 protein levels after miR-520d-3p overexpression (data not shown). On the basis of the miR-520d-3p and EphA2 expression levels, multiple clones from both cell line models were selected for carrying out further functional studies (Supplementary Fig. S4). Ectopic expression of miR-520d-3p, both transiently and stably, in SKOV3ip1 cells significantly decreased cell proliferation (Supplementary Fig. S5). To understand the role of miR-520d-3p in tumor progression, we studied the in vitro migratory and invasive capacity of miR-520d-3p-overexpressing clones. Tumor SKOV3ip1, two cell lines with low miR-520d-3p expression. Compared with the negative control, transient miR-520d-3p expression reduced EphA2 mRNA levels in ES2 cells (Fig. 2D), whereas EphA2 protein levels were decreased in both cell lines (Fig. 2E). Conversely, miR-520d-3p transfection did not influence EphA2 protein levels in SKOV3ip1 cells (Supplementary Fig. S3). Using multiple target prediction programs, we identified a conserved miR-520d-3p binding site in the 3′-untranslated region (3′-UTR) of EphA2 mRNA (Fig. 2F). In both ES2 and SKOV3ip1 cells, ectopic expression of miR-520d-3p significantly reduced the activity of a luciferase reporter fused to the wild-type EphA2 3′-UTR. Deletion mutations in the miR-520d-3p interacting seed region rescued the luciferase activity, thus confirming a direct interaction between the EphA2 3′-UTR and miR-520d-3p (Fig. 2G). Taken together, these data suggest that EphA2 is a direct functional target of miR-520d-3p and its expression is regulated by miR-520d-3p in ovarian cancer.
Therapeutic Synergy between miRNA and siRNA in SKOV3ip1-520d-M3 (decreased significantly in HeyA8-520d-M10, SKOV3ip1-E3 respectively; Fig. 3A). Similarly, cell invasion was significantly reduced in miR-520d-3p overexpressing stable clones (HeyA8-520d-M10, P < 0.001; SKOV3ip1-520d-M3, P < 0.001; and SKOV3ip1-520d-M10, P < 0.001) when compared with empty controls (HeyA8-E3 and SKOV3ip1-E3 respectively; Fig. 3A). Similarly, cell invasion decreased significantly in HeyA8-520d-M10 (P < 0.05), and in SKOV3ip1-520d-M3 (P < 0.001) and SKOV3ip1-520d-M10 (P < 0.001) when compared with empty clones (Fig. 3B). Thus, restoration of miR-520d-3p was able to substantially reduce cell migration and invasion in ovarian cancer models.

We next injected the empty and miR-520d-3p-overexpressing clones of both cell lines into the peritoneal cavity of a murine orthotopic metastasis model (1 × 10⁶ cells/mouse; n = 10 per group) that mimics the pattern of tumor spread in patients with advanced ovarian cancer (26–28). Mice were sacrificed and necropsied after 33 days for HeyA8 clones and 46 days for SKOV3ip1 clones. Increased expression of miR-520d-3p was confirmed by quantitative reverse transcription PCR (qRT-PCR) in 5 mice per group (Supplementary Fig. S6), and a corresponding decrease in EphA2 protein levels was observed in the same mice (Supplementary Fig. S6). In both models, mice bearing miR-520d-3p-overexpressing tumors had a significant reduction in aggregate intraperitoneal metastatic burden (HeyA8-520d-M10, P < 0.001; SKOV3ip1-520d-M3, P < 0.05; and SKOV3ip1-520d-M10, P < 0.05) as compared with empty vectors (Fig. 3C). This also corresponded with a significant decrease in intraperitoneal tumor nodules in these mice (HeyA8-520d-M10, P < 0.05 compared with E3; SKOV3ip1-520d-M3, P < 0.05; SKOV3ip1-520d-M10, P < 0.05 compared with E3, respectively; Fig. 3D). Because increased EphA2 expression has been associated with enhanced angiogenesis (20), we immunostained tissues with an endothelial marker (CD31 staining) to identify blood vessels in control or EphA2-transfected HeyA8 and SKOV3ip1 tumors (Fig. 3E). Quantification of migratory cells counted is shown at right. Experiment was repeated in duplicate at three independent times. Statistical significance was determined by unpaired, two-tailed Student t test when compared with empty clones for all analyses; * P ≤ 0.05; **, P ≤ 0.01; ***, P ≤ 0.001. Data are mean ± SD. N.S, not significant.
from empty and miRNA-overexpressing tumors for CD31, a marker of endothelial cells (20). As expected, microvessel density (MVD) decreased significantly in miR-520d-3p tumors when compared with empty tumors (HeyA8-520d-M10, P < 0.01, and SKOV3ip1-520d-M10, P < 0.001; Fig. 3E). Thus, restoration of miR-520d-3p effectively inhibits cell migration, invasion, and angiogenesis both in vitro and in vivo in ovarian cancer.

To assess whether this function of miR-520d-3p is mediated by EphA2 activity, we asked whether reintroduction of miR-520d-3p-resistant EphA2 could rescue the tumorigenic phenotype of ovarian cancer cell lines. Empty and miR-520d-3p–overexpressing HeyA8 stable clones (E3 and 520d-M10) were transiently transfected with EphA2-ORF plasmid, which lacks the 3′-UTR and hence miR-520d-3p binding sites. Immunoblot assays confirmed the overexpression of EphA2 in these cells (Fig. 3F). EphA2-transfected cells showed a significant increase in migration compared with untransfected controls (Fig. 3G). Furthermore, EphA2-transfected 520d-M10 stable cells showed no change in their migratory ability compared with EphA2-transfected Empty-E3 controls (Fig. 3G). These results suggest that activity of miR-520d-3p in ovarian cancer is dependent on EphA2 downregulation.

Synergistic Effect of Combined miR-520d-3p and EphA2 siRNA Therapy

Because EphA2 has been previously shown to be a targetable protein in ovarian cancer (6, 22–24), we sought to experimentally evaluate whether dual inhibition of EphA2 by siRNA and miRNA showed synergistic antitumor efficacy. For this purpose, we designed four different siRNAs targeting EphA2 and confirmed their ability to knockdown EphA2 (Supplementary Fig. S7). On the basis of their efficiency, si-EphA2-1 (currently under consideration for human clinical trials) and si-EphA2-2 (highest efficiency in EphA2 knockdown) were selected for further analysis. Combination of each siRNA with miR-520d-3p led to a remarkable reduction in EphA2 protein levels in both HeyA8 and SKOV3ip1 cells (Fig. 4A). Because the combination of si-EphA2-1 and miR-520d-3p showed the highest efficiency in EphA2 knockdown.

**Figure 4.** Combination of miR-520d-3p and EphA2 siRNA treatment shows enhanced EphA2 inhibition and antitumor efficacy in vitro. A, immunoblotting of EphA2 and GAPDH in HeyA8 and SKOV3ip1 cells after treatment with miR-520d-3p, different EphA2-targeting siRNAs, or a combination of both (1–6). B and C, representative images showing the effect of different combination treatments (1–4) on SKOV3ip1 and HeyA8 migration (B) and invasion (C) using Transwell migration assay (left). Absorbance was measured at 590 nm after 24 hours and the percentage migratory or percentage invasive cells were calculated compared with control treatment. A representative experiment is shown at right. The experiment was carried out in duplicate at three independent times. Statistical significance was determined by unpaired, two-tailed Student t test when compared with empty clones for all analyses. *, P ≤ 0.05; **, P ≤ 0.01; ***, P ≤ 0.001. Data are mean ± SD. NS, not significant.
Therapeutic Synergy between miRNA and siRNA

520d-3p-DOPC (200 μg siRNA/kg) and miR-520d-3p-DOPC (200 μg miRNA/kg) resulted in a modest increase in apoptosis compared with the control treatment (5- and 10-fold, respectively; \( P < 0.05 \)) for miR-520d-3p-DOPC and \( P < 0.01 \) for si-EphA2-1-DOPC), whereas the combination treatment resulted in substantially higher apoptosis compared with the control treatment (20-fold; \( P < 0.001 \)). A similar effect on tumor angiogenesis, proliferation, and apoptosis was also confirmed in the HeyA8 model (Fig. 5E–G and Supplementary Fig. S11). Detailed analysis of the data indicated that although combined treatment afforded improved tumor inhibition, its action was not synergistic, but additive. However, it is important to consider that even though the miR-520d-3p/si-EphA2-1 combination showed an additive effect in inhibiting \( \text{in vivo} \) tumor angiogenesis, proliferation, and apoptosis (Fig. 5B–G), it displayed a synergistic reduction in total tumor weight after dual therapy (Fig. 5A). Putting together the \( \text{in vitro} \) and \( \text{in vivo} \) combination therapy data, these results together confirm the improved therapeutic efficiency and antitumor activity of combined miRNA and siRNA therapy as compared with individual therapies in ovarian cancer.

miR-520d-3p Also Targets EphB2, Which Is an Independent Prognostic Marker for Ovarian Cancer

We hypothesized that the superior tumor suppression following combination treatment is possibly due to miR-520d-3p targeting multiple Eph pathway oncogenes in addition of EphA2. To identify these additional targets, we performed microarray gene expression with miRNA target prediction analyses to look for transcripts that are downregulated in stable clones of both cell lines and also predicted as miR-520d-3p targets. We identified three members of the Eph-receptor family: EphA2 (as expected by our previous results), EphA8, and EphB2 (Supplementary Fig. S12). Using luciferase reporter analysis, we confirmed that miR-520d-3p targets EphB2 at a conserved seed region in its 3'UTR (Fig. 6A). Consistent with the reporter assay, endogenous expression of EphB2 protein was reduced by ectopic miR-520d-3p overexpression in both ES2 and SKOV3ip1 cells (Fig. 6B), as well as in miR-520d-3p–overexpressing SKOV3ip1 stable clones (Fig. 6C). Although EphA8 was not confirmed as a direct target, it might still be a potential indirect target. Further analysis of EphB2 and miR-520d-3p levels by immunostaining revealed that ovarian cancer tumors exhibit an inverse expression pattern between miR-520d-3p and EphB2 levels. Tumors with high miR-520d-3p expression showed weak EphB2 staining, whereas tumors with low miR-520d-3p expression showed strong EphB2 staining (Fig. 6D), further confirming EphB2 as an additional target of miR-520d-3p.

control, MVD decreased significantly after miR-520d-3p-DOPC (\( P < 0.001 \)) as well as si-EphA2-1-DOPC (\( P < 0.001 \)) treatment. However, the combination of these treatments resulted in further reduction (\( P < 0.001 \)) in CD31-positive cells (Fig. 5B). To determine the effect on cell proliferation, Ki67 staining was assessed for all treatment groups. Although monotherapy with miR-520d-3p-DOPC and si-EphA2-1-DOPC individually decreased cell proliferation (\( P < 0.001 \) for both), combined treatment resulted in improved reduction (\( P < 0.001 \)) in tumor cell proliferation (Fig. 5C). We next assessed the degree of apoptosis for all treatments using the terminal deoxynucleotidyl transferase–mediated dUTP nick end labeling (TUNEL) assay (Fig. 5D). miR-520d-3p-DOPC or si-EphA2-1-DOPC treatment resulted in a modest increase in apoptosis compared with the control treatment (5- and 10-fold, respectively; \( P < 0.05 \)) for miR-520d-3p-DOPC and \( P < 0.01 \) for si-EphA2-1-DOPC), whereas the combination treatment resulted in substantially higher apoptosis compared with the control treatment (20-fold; \( P < 0.001 \)).

miR-520d-3p Also Targets EphB2, Which Is an Independent Prognostic Marker for Ovarian Cancer

We hypothesized that the superior tumor suppression following combination treatment is possibly due to miR-520d-3p targeting multiple Eph pathway oncogenes in addition of EphA2. To identify these additional targets, we performed microarray gene expression with miRNA target prediction analyses to look for transcripts that are downregulated in stable clones of both cell lines and also predicted as miR-520d-3p targets. We identified three members of the Eph-receptor family: EphA2 (as expected by our previous results), EphA8, and EphB2 (Supplementary Fig. S12). Using luciferase reporter analysis, we confirmed that miR-520d-3p targets EphB2 at a conserved seed region in its 3'UTR (Fig. 6A). Consistent with the reporter assay, endogenous expression of EphB2 protein was reduced by ectopic miR-520d-3p overexpression in both ES2 and SKOV3ip1 cells (Fig. 6B), as well as in miR-520d-3p–overexpressing SKOV3ip1 stable clones (Fig. 6C). Although EphA8 was not confirmed as a direct target, it might still be a potential indirect target. Further analysis of EphB2 and miR-520d-3p levels by immunostaining revealed that ovarian cancer tumors exhibit an inverse expression pattern between miR-520d-3p and EphB2 levels. Tumors with high miR-520d-3p expression showed weak EphB2 staining, whereas tumors with low miR-520d-3p expression showed strong EphB2 staining (Fig. 6D), further confirming EphB2 as an additional target of miR-520d-3p.
Figure 5. Cotreatment with miR-520d-3p and EphA2 siRNA shows potent synergy and improved therapeutic efficiency in vivo. A, total tumor weight after various combination treatments (1–4) of HeyA8 (left) and SKOV3ip1 (right) tumors. Bottom, calculation to show synergism as described in Methods. B–D, effect of combined miR-520d-3p + siEphA2-1 treatment on angiogenesis, proliferation, and apoptosis in SKOV3ip1 cells. Representative images of CD31 (B), Ki67 (C), and TUNEL (D) immunostaining following various combination treatments (1–4) are shown (images were acquired at x100). E–G, effect of combined miR-520d-3p + siEphA2-1 treatment on angiogenesis, proliferation, and apoptosis in HeyA8 cells. Representative images of CD31 (E), Ki67 (F), and TUNEL (G) immunostaining following various combination treatments (1–4) are shown (images were acquired at x100). Quantification of immunostaining in E–G are shown at right. Statistical significance was determined by unpaired, two-tailed Student t test when compared with empty clones for all analyses. *, P ≤ 0.05; **, P ≤ 0.01; ***, P ≤ 0.001. Data are mean ± SD.
Figure 6. EphB2 is a direct and functional target of miR-520d-3p, and a prognostic factor for patients with ovarian cancer. A, luciferase activity of a reporter construct fused to wild-type or mutant EphB2 3’-UTR in MCF7 cells with ectopic miR-520d-3p expression (top). Control, cells transfected with a scrambled control. Data are average of four independent experiments. Representative diagram of miR-520d-3p binding site on EphB2 mRNA (bottom). B, immunoblotting of EphB2 and GAPDH in ES2 and SKOV3ip1 cells transfected with miR-520d-3p or a scrambled control. C, immunoblotting of EphB2 and GAPDH in miR-520d-3p–overexpressing SKOV3ip1 stable clones. D, representative images of the immunostaining for EphA2 and miR-520d-3p from patients showing negative correlation in ovarian cancer tumors. E, immunoblotting of EphA2, EphB2, and GAPDH in SKOV3ip1 cells after various combination treatments (1–6). F, immunoblotting of EphB2 and GAPDH in SKOV3ip1 cells after treatment with miR-520d-3p or different EphA2-targeting siRNAs or a combination of both (1–6). G, Kaplan–Meier curves representing the percentage OS of 556 patients from the TCGA 2012 dataset based on EphB2 median expression (F), combined EphB2 and miR-520d-3p expression levels (G), or combined EphA2, EphB2, and miR-520d-3p expression levels (H). The colored numbers (red or blue) below the curves represent patients at risk at the specified time points. Statistical significance was determined by unpaired, two-tailed Student t test. *, P ≤ 0.05. Data are mean ± SD.

To investigate the functional significance of EphB2-miR-520d-3p interaction in ovarian cancer, we sought to determine whether EphB2 played a role in the tumor suppression observed after miR-520d-3p/si-EphA2-1 dual-targeting of EphA2. We discovered that EphB2 protein levels were maximally reduced in combined miR-520d-3p/si-EphA2-1-treated SKOV3ip1 cells (Fig. 6E). Although EphA2-targeting siRNAs (si-EphA2-1 and si-EphA2-2) alone did not affect EphB2 levels, combined treatment with miR-520d-3p and si-EphA2 greatly reduced EphB2 levels (Fig. 6F). On the basis of these data, we concluded that miR-520d-3p targets multiple genes in the Eph pathway, and when combined with standard single-target siRNA therapy, it contributed to superior tumor regression by potentially targeting Eph pathway signaling among other possible targets.

Further analyzing the TCGA dataset, we found that patients with EphB2-high tumors had significantly shorter OS (median, 41.1 months) than those with EphB2-low tumors (median, 55.2 months; P = 0.0051; Fig. 6G). Addition of miR-520d-3p expression status to EphB2 expression further improved the stratification of patient OS. Patients showing EphB2(high)/miR-520d-3p(low) had a significantly shorter survival and poorer prognosis (median, 38.2 months) compared with patients with EphB2(low)/miR-520d-3p(high) (median, 64 months; P = 0.0024; Fig. 6H). We next looked at combined EphA2, EphB2, and miR-520d-3p expression status in the TCGA dataset. Remarkably, this signature showed a further improved separation; patients having EphA2(low)/EphB2(low)/miR-520d-3p(high) survived significantly longer (median, 81.1 months) than patients with EphA2(high)/EphB2(high)/miR-520d-3p(low) (median, 38 months; P = 0.0009; Fig. 6I). These data suggest the potential use of EphA2/EphB2/miR-520d-3p gene expression in prognostic
stratification of patients with ovarian cancer and highlight the rationale and therapeutic potential of using miRNAs to target multiple oncogenic pathways simultaneously.

**DISCUSSION**

In this study, we exploited large-scale cancer genomic databases and bioinformatic approaches to discover novel therapeutic applications of RNAi in ovarian cancer. We report that inhibition of EphA2 by an EphA2-targeting siRNA and miR-520d-3p led to substantial augmentation in therapeutic efficiency and potently suppressed tumor progression and metastasis in vitro and in vivo. This is the first study depicting that dual inhibition of a specific oncogene by its single-targeting siRNA and a multitargeting miRNA can be used to obtain improved therapeutic efficiency. We named this the “boosting effect” of miRNAs, and the mechanism of this improved activity is partly explained by the ability of miR-520d-3p to transcriptionally repress two Eph-receptors, EphA2 and EphB2, both highly upregulated in cancers (21, 31). This could provide an important clinical advantage as targeting of a single Eph receptor might raise the possibility of mutual compensation by other Eph receptors due to their overlapping functional roles, several common downstream targets, and structural homology (32). Our data, however, do not rule out that miR-520d-3p might target additional genes involved in multiple cancer-associated pathways, and these targets might assist in its antitumor phenotype. In summary, the data collectively support application of combined miR-520d-3p/si-EphA2-1 therapy for clinical trials in patients with ovarian cancer.

Our experimental findings are also clinically relevant, as the principle presented in this article can be successfully applied to other tumor-suppressive miRNAs in different human cancers. This approach is useful for concurrent targeting of distinct molecular defects in canonical cancer-associated pathways. One potential complexity with this approach could be the apparent saturation of the RNA-induced silencing complex assembly essential for RNAi occurrence. Although no loss of efficiency was observed at the concentrations used in our study, further dose-response studies need to be carried out to determine the optimal pharmacologic therapeutic window for this combination.

We also identified that miR-520d-3p serves as an effective and independent predictor of outcome in ovarian cancer. Although one previous study reported the role of miR-520d-3p in nonobstructive azoospermia (33), our study is the first to show a role of miR-520d-3p in cancer. We identified that high miR-520d-3p expression is linked to better clinical outcome and longer overall and relapse-free survival. Although miR-520d-3p can serve as a useful prognostic marker in ovarian cancer, our study also highlights its tumor-suppressive function both in vitro and in vivo. We further showed that miR-520d-3p directly inhibits expression of EphA2 and EphB2, two key receptors of the Eph pathway. Constitutive activation of the Eph pathway by overexpression of Eph receptors has been linked to tumor aggressiveness in multiple human cancers, modulating the tumor microenvironment and contributing to tumor growth, invasiveness, angiogenesis, metastasis, and resistance (34). Thus, therapeutic restoration of miR-520d-3p expression or function using miRNA mimics could be a useful approach for ovarian cancer treatment.

Another key finding of this study is the identification of a novel gene-expression signature comprising EphA2(low)/EphB2(low)/miR-520d-3p(high) that can predict favorable ovarian cancer prognosis with powerful accuracy. High expression of EphA2 and EphB2 has been previously shown to be associated with clinical aggressiveness, shorter survival, and poorer prognosis in ovarian cancer (21, 31). Our study reveals that integration of these into a multigene signature markedly improved its prognostic power, and this signature, alone or in combination with other molecular markers, may improve outcome prediction and stratification of patients with ovarian cancer if prospectively validated.

Finally, although genomic approaches are identifying many new potential therapeutic targets, the targeted treatments currently available (e.g., small-molecule inhibitors or antibodies) are still impractical or impossible due to a number of factors, including large structure (e.g., proteins such as p130Cas), kinase-independent functions, and multiple structural domains with independent functions. Several small-molecule inhibitors lack specificity and can be associated with intolerable side effects. Similarly, although monoclonal antibodies have shown promise against specific targets such as VEGF, their use is limited to either ligands or surface receptors. The development of combined miR-520d-3p and EphA2-targeting siRNA therapy allows for therapeutic targeting of the Eph pathway and other proteins that would otherwise not be “druggable” in ovarian cancer. The findings reported in our study emphasize the development and application of novel RNAi-based therapeutics to improve the efficacy of targeted therapy and hold potential for improved management of patients with ovarian cancer.

**METHODS**

**Clinical Samples**

A total of 91 samples from patients with ovarian cancer used in this study were collected from the Gynecologic Oncology tumor bank at MDACC. All samples were collected according to the institutional policies and obtained following patient’s informed consent. Data were de-identified before any analyses using standard procedures. The clinicopathologic features of the ovarian cancer are detailed in Supplementary Table S1. The 2009 (n = 186) and 2012 (n = 556) TCGA datasets were downloaded from data portal at https://tcga-data.nci.nih.gov/tcga.

**Cell Culture**

All the epithelial ovarian cancer cell lines used in this study (HeyA8, SKOV3ip1, and ES2) were purchased from the American Type Culture Collection and cultured under the conditions specified by the manufacturer. All cell lines were validated by the Characterized Cell Line Core at The University of Texas MDMCC using short tandem repeat DNA fingerprinting.

**RNA Extraction and Real-Time RT-PCR**

Total RNA was isolated from tissues and cell lines as previously described (35) for both miRNA and EphA2 mRNA expression analyses. For quantification of miRNA levels, total RNA was reverse transcribed with miRNA-specific primers using the TaqMan MicroRNA Reverse Transcription Kit (Applied Biosystems), and then real-time PCR was
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conducted using the TaqMan MicroRNA Assay Kit (Applied Biosystems) according to the manufacturer's instructions. For EphA2 miRNA expression study, RNA was reverse transcribed using SuperScript III (Invitrogen) with random hexamers and real-time PCR analysis was conducted with iQ SYBR Green Supermix (Bio-Rad) using primer sequences as previously described (22) according to the manufacturer's protocol. 18S and U6 were used as normalizing controls for miRNA and miRNA quantification, respectively. The 2^−ΔΔCT method was used to calculate the relative abundance compared with empty controls.

**Plasmids and siRNAs**

The interaction sites for miR-520d-3p were predicted on EphA2 (NM_004431.5) and EphB2 (NM_004442, NM_017449) 3'-UTRs and the sequences were PCR amplified from human genomic DNA using primers: EphA2-F: 5'-CGTCTAGAGGCGCATGCGGATCTTTT-3', EphA2-R: 5'-CGTCTAGACACGCAGATTTGCTGC-3', EphB2-F: 5'-GGCGCAGAATACAGGAATA-3', and EphB2-R: 5'-GTCATTTCCCAAGGAGGTTCCTC-3'. PCR products were cloned into the XbaI site of pgl3-control vector (Promega), to obtain pGL3-EphA2-WT and pGL3-EphB2-WT luciferase constructs. Mutant constructs, pGL3-EphA2-mut and pGL3-EphB2-mut, were prepared by deleting the entire miR-520d-3p binding seed region in their 3'-UTRs using the QuickChange II Site-Directed Mutagenesis Kit (Stratagene). To establish stable cell lines with miR-520d-3p overexpression, we used the pcDNA3.1(+)-vector system (Invitrogen). We first amplified a human genomic span that contains miR-520d-3p by using the following primers: pF: 5'-TCTAGAGAAGAAGGTGAAACCC CGAAGGT-3', and pR: 5'-TCTAGACTCGAGCAAAACAGAACCCC ACCATC-3'. We then cloned this PCR product in pcDNA3.1(+) using EcoRI and XbaI restriction enzymes. The nonspecific, non-targeting siRNA used in this study is 5'-UUCUCCGAACGUGUCA CGU-3', whereas the EphA2-targeting siRNAs used are: si-EphA2-1: 5'-UGACAUGCCGAUCUCAUACG-3', si-EphA2-2: 5'-CCAUCAGAAG GCAGCAGA-3', si-EphA2-3: 5'-CGUAUCUUCAUCAGUGCCUA-3', and si-EphA2-4: 5'-CAGAGAACAGCCGGACUGGA-3'.

**Luciferase Reporter Assay**

Luciferase reporter assay to confirm miRNA targets was performed as previously described (35).

**Immunoblotting**

Immunoblotting staining of EphA2, EphB2, and glyceraldehyde-3-phosphate dehydrogenase (GAPDH) was carried out on cell lines and miRNA expression study, RNA was reverse transcribed using SuperScript III (Invitrogen) with random hexamers and real-time PCR analysis was conducted with iQ SYBR Green Supermix (Bio-Rad) using primer sequences as previously described (22) according to the manufacturer's protocol. 18S and U6 were used as normalizing controls for miRNA and miRNA quantification, respectively. The 2^−ΔΔCT method was used to calculate the relative abundance compared with empty controls.

**Immunohistochemistry**

Immunohistochemical staining of EphA2, Ki67, and CD31 was carried out on tumor samples from patients and from mice as described previously (22).

**In Situ Hybridization**

The formalin-fixed, paraffin-embedded tissue sections were first digested with 5 μg/mL proteinase K for 20 minutes at room temperature, and were then loaded onto the Ventana Discovery Ultra system (Ventana Medical Systems, Inc.) for in situ hybridization. The tissue slides were incubated with double-digoxigenin (DIG)-labeled LNA probe for miR-520d-3p (Exogen) for 2 hours at 55°C. The DIGs were detected with a polyclonal anti-DIG antibody and alkaline phosphatase-conjugated second antibody (Ventana) using nitroblue tetrazolium-5-bromo-4-chloro-3-indolyl phosphate (NBT-BCIP) as the substrate.

**In Vitro Transfections**

Transfections with miRNA, anti-miRNA, or siRNA were conducted as previously described (35). Briefly, cells were transfected with 100 or 200 nmol/L of specified RNA using Lipofectamine 2000 (Invitrogen) for the specified number of hours, and cells were then used for further analysis.

**In Vitro Migration and Invasion Assays**

Cell migration assay was conducted using 6.5-mm diameter Transwell chambers with 8.0-μm porous membrane (Corning Incorporated), and cell invasion assay was conducted using BioCat growth factor–reduced Matrigel invasion chambers (BD Bioscience) according to the protocol previously described (35) on parental untreated, empty-vector clones and miR-520d-3p-overexpressing clones of HeyA8 and SKOV3ip1 cells. Each experiment was carried out at least in triplicate, and repeated three times. For in vitro combination analysis, 24-well Transwell plates with 8.0-μm pore size chambers were coated with 0.1% gelatin (migration) or defined matrix (invasion) separating the upper and lower wells. HeyA8 or SKOV3ip1 cells were transfected with miRNA or siRNA. Twenty-four hours later, 6 × 10⁴ cells were resuspended in 100 μL of serum-free media and added to upper wells, and 5% serum-containing media was added to the lower wells. The culture system was incubated (migration, 6 hours; invasion, 24 hours) at 37°C. Membranes were fixed, stained, and counted (10 random fields under ×40) using light microscopy. Experiments were carried out in triplicate and in three independent experiments.

**Xenograft Models of Ovarian Cancer**

Female athymic nude mice (NCr-nu) were purchased from the National Cancer Institute, Frederick Cancer Research and Development Center (Frederick, MD) and maintained in specific pathogen-free conditions. The animals were cared for according to guidelines set forth by the American Association for Assessment and Accreditation of Laboratory Animal Care and the U.S. Public Health Service Policy on Human Care and Use of Laboratory Animals. All mouse studies were approved and supervised by the MDACC Institutional Animal Care and Use Committee. To produce orthotopic tumors, mice were injected into the peritoneal cavity with 1 × 10⁶ parental untreated, empty-vector clones or miR-520d-3p-overexpressing clones of HeyA8 and SKOV3ip1 cells (n = 10 mice/group). The cells were treated with trypsin, washed, and resuspended in Hank’s balanced salt solution ( Gibco) at a concentration of 5 × 10⁶ cells/mL. About 33 days for HeyA8 clones and 46 days for SKOV3ip1 clones after cell injection, all mice were sacrificed and necropsy was conducted. The individual tumor nodules were isolated from the supporting tissue and counted. The total tumor weight was also measured. Tissue samples were fixed in formalin for paraffin embedding, and frozen in optimal cutting temperature (OCT) media for preparation of frozen slides or snap-frozen for miRNA as described above.

**Liposomal siRNA and miRNA Preparation**

For in vivo delivery, siRNA and miRNA were incorporated into DOPC. DOPC and siRNA or miRNA were mixed in the presence of excess tertiary butyl alcohol at a ratio of 1:10 (w/w) siRNA/DOPC (22). Before in vivo administration, the preparation was hydrated with normal 0.9% saline (100 μL/mouse) for intravenous or intraperitoneal injection.

**In Vivo Treatment with miR520d-3p-DOPC and si-EphA2-1-DOPC**

miR520d-3p, control miRNA, EphA2 siRNA, and control siRNA were purchased from Sigma. These miRNAs and siRNAs were conjugated with DOPC as described above. The appropriate dosage for miRNA treatment was determined by conducting dose-response analysis consisting of three different doses (200, 400, and 600 μg/kg) of miR520d-3p and 200 μg/kg of control miRNA (data not shown). For in vivo combination analysis, female athymic nude mice (NCr-nu) were injected into the peritoneal cavity with 1 × 10⁵ HeyA8 or
SKOV3ip1 cells. Mice were divided into four groups (n = 10 per group): (i) Control miRNA + siRNA, (ii) miR-520d-3p-DOPC + control siRNA, (iii) Control miRNA + si-EphA2-1-DOPC, and (iv) miR-520d-3p-DOPC + si-EphA2-1-DOPC. One week after injection, each miRNA was administered three times weekly at 200 µg/kg body weight, and each siRNA was given twice weekly at 200 µg/kg body weight. Treatment was continued until control mice became moribund (33 days in HeyA8 cells and 46 days in SKOV3ip1 cells), and the last treatment was done 48 (HeyA8) and 24 hours (SKOV3ip1) before sacrificing them. At the time of sacrifice, mouse weight, tumor weight, number of nodules, and distribution of tumors were recorded.

Calculations for Synergism

Isobologram analysis was conducted using the Compusyn software program (CombioSyn, Inc.; ref. 36). Briefly, cells were treated with different concentrations of miR-520d-3p alone, si-EphA2-1 alone, or combination of miR-520d-3p + si-EphA2-1 in 1:1 ratio for 72 hours followed by MTT analysis to determine the percentage cell viability, and a CI was calculated using the Chou–Talalay method (37). A CI < 1.0 indicates synergism, a CI of 1 indicates additive activity, and a CI > 1.0 indicates antagonism. For single-dose experiments, the potential synergy between two antitumor agents was evaluated as described in references 29 and 30. Using this method, the fractional activity for miR-520d-3p alone, si-EphA2-1 alone, or combined treatment is calculated compared with the control, and the ratio of “Expected Fa” to “Observed Fa” is determined for the combination of the two agents. A ratio of more than 1 indicates a synergistic effect, and a ratio of less than 1 indicates less than additive effect.

TCGA Data Analyses

The input data were downloaded from the data portal at https://tcga-data.nci.nih.gov/tcga. Data have been imported on BRB-ArrayTools Version 3.7.2, and average values of the replicate spots of each miRNA were background subtracted, normalized, and subjected to further analysis. Normalization was conducted by using per chip median normalization method and the median array as referenced. Class comparison analysis using t test identified miRNAs that were differentially expressed (P < 0.001). Class prediction algorithms in BRB array tools were used to determine whether miRNA microarray expression patterns could accurately differentiate between classes (P < 0.001).

Statistical Analysis

Survival analyses were conducted in R (version 2.14.2) and SPSS 16.0. The patients were grouped into percentiles according to miRNA/miRNA expression. We checked for a relation with survival by choosing a cutoff to optimally split the samples into two groups. “Optimally” was defined as significant separation in OS or PFS using the best P values for both TCGA and MDACC datasets. The log-rank test was used to determine the association between miRNA/miRNA expression and OS and PFS, respectively. The Kaplan-Meier method was used to generate survival curves. We computed a significance level for each miRNA and clinicobiologic factor based on a univariate Cox proportional hazard regression model. For multivariate analysis, a full Cox proportional hazards model was fitted. The relationship between miRNA expression and experimental groups (transfection group vs. control) was assessed using Student t test. Data are represented as mean ± SD. Statistical analysis was conducted using SPSS 16.0. All tests were two-sided, and an effect was considered to be statistically significant at P < 0.05.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

Authors’ Contributions


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