Liposomal Delivery of MicroRNA-7–Expressing Plasmid Overcomes Epidermal Growth Factor Receptor Tyrosine Kinase Inhibitor-Resistance in Lung Cancer Cells

Kammei Rai¹, Nagio Takigawa¹, Sachio Ito², Hiromi Kashihara¹, Eiki Ichihara¹, Tatsuji Yasuda³, Kenji Shimizu², Mitsune Tanimoto¹, and Katsuyuki Kiura¹

Abstract

Epidermal growth factor receptor (EGFR) tyrosine kinase inhibitors (TKI) have been strikingly effective in lung cancers harboring activating EGFR mutations. Unfortunately, the cancer cells eventually acquire resistance to EGFR-TKI. Approximately 50% of the acquired resistance involves a secondary T790M mutation. To overcome the resistance, we focused on EGFR suppression using microRNA-7 (miR-7), targeting multiple sites in the 3′-untranslated region of EGFR mRNA. Two EGFR-TKI–sensitive cell lines (PC-9 and H3255) and two EGFR-TKI–resistant cell lines harboring T790M (RPC-9 and H1975) were used. We constructed miR-7-2 containing miR-7–expressing plasmid. After transfection of the miR-7–expressing plasmid, using cationic liposomes, a quantitative PCR and dual luciferase assay were conducted to examine the efficacy. The antiproliferative effect was evaluated using a cell count assay and xenograft model. Protein expression was examined by Western blotting. The miR-7 expression level of the transfectants was approximately 30-fold higher, and the luciferase activity was ablated by 92%. miR-7 significantly inhibited cell growth not only in PC-9 and H3255 but also in RPC-9 and H1975. Expression of insulin receptor substrate-1 (IRS-1), RAF-1, and EGFR was suppressed in the four cell lines. Injection of the miR-7–expressing plasmid revealed marked tumor regression in a mouse xenograft model using RPC-9 and H1975. EGFR, RAF-1, and IRS-1 were suppressed in the residual tumors. These findings indicate promising therapeutic applications of miR-7–expressing plasmids against EGFR oncogene–addicted lung cancers including T790M resistance by liposomal delivery.

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Introduction

Epidermal growth factor receptor (EGFR) is overexpressed in more than 60% of human non–small cell lung cancer (NSCLC) samples (1). EGFR signaling is so essential for the initiation and progression of cancer that it has become the focus of molecular targeting therapy (2). EGFR tyrosine kinase inhibitors (EGFR-TKI) have a striking effect in NSCLC with activating EGFR mutations, and longer progression-free survival has been observed if patients are treated with EGFR-TKI compared with conventional chemotherapy (3–5). This efficacy was attributed to blocking EGFR oncogene addiction (6, 7).

Unfortunately, approximately 50% of patients who initially respond to EGFR-TKI acquired resistance to EGFR-TKI due to an additional EGFR T790M mutation in exon 20 (8). Because the T790M mutation restores the affinity of ATP to the ATP-binding site of mutated EGFR, the oncogene addiction by the EGFR pathway is presumed to be sustained in EGFR-TKI–resistant cells harboring the T790M mutation (9, 10). Thus, a method of suppressing the restored EGFR pathway is needed to overcome the resistance caused by the T790M mutation.

To develop a new approach for the suppression of EGFR, we focused on microRNA (miRNA), which is known to intrinsically suppress mRNA by pairing with the 3′-untranslated region (UTR) of the mRNA; the mRNA is finally translated into a target protein (11). Compared with conventional molecular targeting therapies, such as EGFR-TKI and anti-EGFR antibodies, miRNA is expected to be independent of unexpected conformational changes due to secondary mutations. In recent years, it has become apparent that miRNAs play crucial roles in carcinogenesis (12). Moreover, some miRNAs can work as tumor suppressors in vitro (13, 14). The induction of overexpression of such miRNAs may contribute to suppression of the target protein (15). Different from short interfering RNA, miRNAs are
expected to work as part of a network and could affect the components of the same pathway at multiple levels (14). Using the TargetScan software, miRNA-7 (miR-7) was mathematically predicted to effectively suppress EGFR in 3 different sites (16). Here, we show the antiproliferative effect of miR-7 against EGFR-addicted lung cancer cells both in vitro and in vivo.

Materials and Methods

Cell culture and reagents

PC-9, H3255, H1975, and A549 cells were derived from adenocarcinoma of the lung. PC-9 cells were obtained from Immuno-Biological Laboratories. H1975 and A549 cells were purchased from the American Type Culture Collection. H3255 cells were kindly gifted by Drs. N. Fujimoto and J.M. Kurie (MD Anderson Cancer Center, Houston, TX). Gefitinib-resistant RPC-9 cells were established using PC-9 by continuous exposure to gefitinib in our laboratory (17). PC-9 harboring EGFR delE746-A750 in exon 19 and H3255 cells harboring EGFR L858R in exon 21 were sensitive to EGFR-TKI. RPC-9 cells carrying delE746-A750 with T790M and H1975 carrying L858R with T790M were resistant to EGFR-TKI. A549 cells harbored wild-type EGFR. All cell lines were cultured at 37°C, 5% CO2 using RPMI 1640 (Sigma-Aldrich) medium supplemented with 10% heat-inactivated FBS and 100 U/mL penicillin-streptomycin.

Construction of miR-7–expressing plasmids

The pre-miR-7-2 site was cloned from DNA of RPC-9 cells with 50-bp flanking sequences by PCR. The forward primer was 5'-ATTGGATCCCTGACCTGGTGGC-GAGGGGA-3' and the reverse primer was 5'-TAAAGCTTACACGTGGAAGGATAGCCA-3'. After double digestion with BamHI and HindIII, the fraction was inserted into pSilencer 4.1-CMV neo (Ambion). All sequences were confirmed by direct sequencing.

Quantitative PCR of miR-7

Quantitative reverse transcription (RT)-PCR validated the miR-7 expression by the constructed plasmid, using TaqMan MicroRNA Assays (Applied Biosystems) following the manufacturer’s instructions. Briefly, RT reactions containing RNA samples (24 hours after transfection), looped primers, 1X buffer, reverse transcriptase, and RNase inhibitor were incubated for 30 minutes each at 16°C and at 42°C. Real-time PCR was carried out on an AB 7300 Real-Time PCR System (Applied Biosystems). The PCR program was 10 minutes at 95°C, 40 cycles of 15 seconds at 95°C, and 60 seconds at 60°C. U44 was adopted as an internal control. Data were collected from 4 independent experiments.

Cell count assay

Each cell line was cultured in a 10-cm plate and divided equally into a 6-well plate, then combined and divided into paired wells again. After 24 hours, they were transfected with miR-7–expressing plasmid or control scrambled miRNA-expressing plasmid following the manufacturer’s instructions (Hokkaido System Science). Total cells were macroscopically observed and counted using vital staining with 0.6% trypan blue 72 hours after incubation at 37°C.

Dual luciferase assay

The dual luciferase assay was conducted following the manufacturer’s instructions (Promega Dual-Luciferase Reporter Assay System; Promega Corporation). We constructed reporter plasmids by inserting full-length EGFR 3'UTR into pTK-hRG-Luci. The cells were cultured in a 6-well plate and transfected with either plasmids expressing miR-7 or control expressing scrambled miRNA, with reporter plasmid and pOA-hRG-Luci as internal controls. Fluorescence was measured by a Turner Designs Model TD-20/20 Luminometer (Turner Designs).

Injection of liposome-coated, miR-7–expressing plasmid into a mouse xenograft model

Female 7-week-old athymic mice were purchased from Charles River Laboratories Japan, Inc. All mice were provided with sterilized food and water and housed in a barrier facility under a 12/12-hour light/dark cycle. Cancer cells (2 × 106) were subcutaneously injected into the backs of the mice. At 1 week after injection, mice harboring tumors with approximately 5-mm longitudinal diameters were randomly assigned into 1 of 2 groups (5 mice per group) that received 3 μg/body of miR-7–expressing plasmid or control scrambled miRNA-expressing plasmid using in vivo optimized cationic liposome complexes by direct injection, as reported previously (Hokkaido System Science; refs. 18–20). Tumor volume (width2 × length/2) was determined twice per week until either the disappearance of the tumor or day 20.

Western blot analyses

Cells or frozen tissue were lysed in radioimmunoprecipitation assay buffer (1% Triton X-100, 0.1% SDS, 50 mmol/L Tris-HCl, pH 7.4, 150 mmol/L NaCl, 1 mmol/L EDTA, 1 mmol/L EGTA, 10 mmol/L β-glycerophosphate, 10 mmol/L NaF, and 1 mmol/L sodium orthovanadate containing protease inhibitor tablets; Roche Applied Sciences). Proteins were separated by electrophoresis on PAGE, transferred onto nitrocellulose membranes, and probed with specific antibodies, followed by detection with Enhanced Chemiluminescence Plus (GE Healthcare Biosciences). All antibodies were used following the manufacturer’s instructions (Cell Signaling Technology).

Apoptosis detection by Hoechst staining and Western blot analysis

Apoptosis was determined using the technique following previous reports (21–23). Briefly, all cells at 48 hours after the transfection were collected and resuspended in 100 μL of staining solution (70 μg/mL Hoechst 33342 and

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miR-7 is overexpressed by liposomal delivery of plasmids

Constructed plasmids containing miR-7-2 were transfected using cationic liposomes. After transfection, miR-7 expression by RPC-9 and H1975 cells was significantly increased by approximately 30-fold compared with each cell line transfected with control plasmids (P < 0.0000002 each). The mean expression ratios to control in RPC-9 and H1975 cells were 31.7 (95% CI: 30.1–33.4) and 27.7 (95% CI: 23.1–33.4), respectively (Fig. 1).

miR-7 transfection suppresses the EGFR expression by binding EGFR 3\'-UTR

miR-7 directly inhibits EGFR expression via its 3\'-UTR. Using the TargetScan software, we assessed the complementarity of miR-7 in silico to the EGFR 3\'-UTR and found 3 sites of seed matches (refs. 13, 16, 24; Fig. 2A). To assess miR-7 inhibition of EGFR, RPC-9 cells were transfected with the constructed plasmid expressing either miR-7 or control plasmid. We tested the inhibitory ability of the plasmid by transfection with miR-7 activity reporter, a synthesized plasmid containing the full length of EGFR 3\'-UTR combined with the luciferase gene. A dual luciferase assay showed aberrant inhibition of luciferase activity by 92%. The mean relative luciferase activities in control and miR-7 were 255.5 (95% CI: 223.5–287.6) and 19.8 (95% CI: 10.3–29.2), respectively (P < 0.001; Fig. 2B). This indicates highly effective inhibition by miR-7 against the full length of EGFR 3\'-UTR.

miR-7 has antiproliferative effects in vitro through the suppression of EGFR

To evaluate the efficacy of the constructed plasmid expressing miR-7, each cell line was transfected with cationic liposomes. The cell number was apparently reduced under microscopic observation. The numbers of transfected cell lines were significantly reduced 72 hours after transfection (Fig. 3). Cell numbers (x10⁴/mL) of control versus transfectants in PC-9, RPC-9, H3255, and H1975 cell lines, respectively, were 51.7 (95% CI: 38.9–64.4) versus 26.7 (95% CI: 18.3–35.0), 14.7 (95% CI: 11.8–17.6) versus 9.3 (95% CI: 8.0–10.7), 4.8 (95% CI: 3.3–6.3) versus 2.1 (95% CI: 1.3–3.0), and 3.9 (95% CI: 2.7–5.2) versus 1.2 (95% CI: 0.8–1.6). All P values for the differences between the control and the transfectants were less than 0.05.

We also transfected plasmids expressing miR-7 into A549 cells carrying wild-type EGFR and K-RAS mutations (25). There was no significant aberrant growth suppression in the cells (Supplementary Fig. S1). We suggest that the oncogene addiction of EGFR plays an important role in miR-7 efficacy.

miR-7 suppresses EGFR-AKT pathway activation

Because miR-7 was proven to effectively suppress the expression of mRNA of total EGFR, it can also suppress its downstream signals such as AKT (13, 26). Western blot analyses showed a direct inhibitory effect against total AKT.
miR-7 by miR-7, as well as the suppression of AKT phosphorylation (Fig. 4A).

miR-7 has multiple targeting effects, as predicted in silico

miR-7 was reported to suppress insulin receptor substrate-1 (IRS-1) and proto-oncogene serine/threonine protein kinase (RAF-1), as predicted in silico (27). The inhibitory effects of total IRS-1 and RAF-1 by miR-7 were shown to varying degrees in the 4 cell lines (Fig. 4B).

miR-7–expressing plasmid has antitumor effects against EGFR-TKI–resistant cell lines in vivo through multiple suppression of the EGFR-AKT and salvage pathway

The antitumor effect of plasmids expressing miR-7 was examined by in vivo liposomal delivery against mouse xenograft models. We selected a nonviral transfection method using cationic liposomes, which has been reported to effectively express plasmids in a mouse model of peritoneal dissemination (19). In the mouse xenograft models of RPC-9 cells, 60% of target tumors disappeared macroscopically, and tumor volume shrank significantly (Fig. 5A). Ratios (%) of tumor volume on day 18 to those on day 1 (control vs. miR-7) were 296.4 (95% CI: 190.8–402.0) versus 1.6 (95% CI: 0.008–3.1; P = 0.005), respectively (Fig. 5B). In addition, the mouse xenograft models of H1975 showed significant tumor volume reduction by the same treatment. Ratios (%) of tumor volumes on day 19 to those on day 1 (control vs. miR-7) were 786.5 (95% CI: 416.9–1,156.0) versus 154.0 (95% CI: 154.0–295.8; P = 0.049), respectively (Fig. 5C and D). Expression of EGFR, RAF-1, and IRS-1 in residual tumors was suppressed, even in the cells that survived after the treatment (Fig. 6).

miR-7 transfection induces apoptosis

Forty-eight hours of treatment of miR-7 resulted in the increase of apoptotic cells as well as necrotic cells in these 4 oncogene addicted cell lines (Supplementary Fig. S2). Percentage of apoptotic cell numbers of control versus transfectants in PC-9, RPC-9, H3255, and H1975 cell lines, respectively, were 0 (95% CI: 0–0) versus 11.2 (95% CI: 9.16–13.3), 1.30 (95% CI: 0.62–1.97) versus 22.1 (95% CI: 18.5–25.6), 0 (95% CI: 0–0) versus 9.67 (95% CI: 9.01–10.3), and 2.22 (95% CI: 1.85–4.26) versus 13.5 (95% CI: 11.4–15.6). All P values for the differences between the control and the transfectants were less than 0.05. The increase in the expression of cleaved PARP was observed even in the residual tumors in xenograft models (Fig. 6).

Discussion

To our knowledge, this is the first report showing that miR-7 caused a dramatic response in an EGFR-TKI–resistant lung cancer xenograft model. Regardless of T790M mutation status, using cationic liposomes to inhibit EGFR signaling by plasmids expressing miR-7 has great clinical significance, because it may overcome approximately 50% of acquired resistance to EGFR-TKIs in the clinic. In nature, miR-7 was thought to be strongly associated with both neural differentiation of embryo stem cells and the development of neural networks in embryos, although the mechanism remains to be clarified (28). The targets of miR-7 have been shown to be total EGFR (2 or 3 sites in the 3′-UTR of mRNA), IRS-1 (single site), and RAF-1 (2 sites; refs. 13, 26, 27), which have important roles in the activated EGFR pathway in various cancer cell lines (2, 29, 30). We confirmed the suppression of the expression of these proteins in this study. Thus, multiple targeting inhibitory effects by miR-7 might be advantageous to prevent cross-talk with EGFR signaling.

Although the efficacy of EGFR short interfering RNA or miR-7 against A549 cells by suppressing EGFR signaling has been reported, the efficacy may be limited because of escape in the signal transduction pathway, typically due to K-RAS activation (27, 31). In fact, miR-7 caused no significant growth inhibition in the A549 cells, even though moderate suppression of RAF downstream of K-RAS by miR-7 resulted in some degree of decreased survival. In contrast, miR-7 suppressed the growth of EGFR oncogene–addicted cells, regardless of the EGFR T790M mutation.
The let-7, one of the best-studied miRNA, was altered in human lung cancers. The reduced let-7 expression was significantly associated with shortened postoperative survival and overexpression of let-7 resulted in the inhibition of lung cancer cell growth (32). The let-7g was confirmed to work against K-RAS that has a crucial role in proliferation of lung cancer cells, especially in the cell lines with K-RAS mutations (33). Thus, we explored miRNA that could target EGFR directly and could suppress EGFR-addicted cancers. Two groups reported that miR-7 had antitumor effects in glioblastoma cell lines (13) and breast cancer cell lines (26), and that the effect depended primarily on the suppression of total EGFR. In contrast, Chou and colleagues showed that miR-7 had the possibility of promoting tumorigenesis in EGFR wild-type cell lines through Ets transcriptional repression factor 1 suppression (34); however, there was no valid analysis on whether ectopic miR-7 was in fact overexpressed by lentiviral introduction. Moreover, the adopted CL1-5 cells had TP53 mutations and were not driven by EGFR signaling (35, 36). They also suggested that miR-7 was concomitantly overexpressed in EGFR-expressing lung cancer patients (34). We examined intrinsic miR-7 expression in EGFR-addicted cells and EGFR-nonaddicted A549 cells and found that intrinsic miR-7 was significantly more overexpressed in EGFR-addicted cells than in A549 cells (Supplementary Fig. S3). Thus, we suspected that miR-7 was, at least, activated by EGFR in a positive feedback mechanism as suggested by Li and Carthew in their description of photoreceptor differentiation (37). However, in our experiments, ectopic miR-7 were obviously overexpressed (approximately 30-fold; Fig. 1), and under these conditions, miR-7 inhibited the cell growth of EGFR-addicted cell lines both in vitro and

Figure 3. Antiproliferative effect of transfection of plasmids expressing miR-7. Top, microscopic pictures of each cell line 72 hours after transfection. Bottom, the cell counts after transfection of miR-7-expressing plasmids or controls. Means (bars) and 95% CIs (error bars) are shown.
in vivo. Although there have been many problems in the realization of the multiple targeting effects of miRNA, targeting the crucial pathway by miRNA in selected cancers will be an Achilles’ heel, similar to molecular targeting therapy by EGFR-TKI (38). Our results of apoptosis assays indicated that this multiple targeting effect restored the apoptotic pathway that was previously caused by EGFR-TKI (7, 39, 40).

Just before the submission of our article, Saydam and colleagues reported that transfection of precursor miR-7 inhibited schwannoma cell growth by targeting EGFR, p21-activated kinase 1, and associated cdc42 kinase 1 oncogenes, as well as tumor suppressor function, in a xenograft model by evaluating the implantation of schwannoma cells. However, the adopted schwannoma cells (HEI-193) had not been shown to be under EGFR oncogene addiction. Moreover, they used miR-7 before implantation of the cancer cells but not after tumor formation (41). The evaluation of the degree of EGFR oncogene addiction in each malignant cell line is very important when we pursue the major role of the miR-7 in tumorigenesis or in tumor suppression, because miR-7 seems to have a strong correlation with EGFR regulation among its multiple targets. In our experiments, if the EGFR oncogene addiction was restored because of

![Figure 4](image-url)

Figure 4. Western blot analyses of the signal transduction pathway. β-Actin was used as a loading control. A, Western blot analyses of EGFR and phosphorylated protein kinase B (pAKT) protein as the main stream of oncogene addiction in PC-9, RPC-9, H3255, and H1975 cells 60 hours after transfection of plasmids expressing miR-7 or scrambled miRNA as a control. B, Western blot analyses of IRS-1 and RAF-1 as the salvage stream of oncogene addiction.

![Figure 5](image-url)

Figure 5. Xenograft mice treated with miR-7. Mean tumor volumes (bars) and 95% CIs (error bars) are shown. A, typical photographs of xenograft models (RPC-9). A plasmid expressing miR-7 (3 μg/body) was directly injected into tumors weekly in RPC-9 xenograft mice. Control mice were injected with control scrambled miRNA-expressing plasmids. B, growth curves of RPC-9 xenograft tumors. The relative percentage from original tumors was plotted. C, typical photographs of an H1975 xenograft model. D, growth curves of H1975 xenograft tumors.
Western blot analyses of the signal transduction pathway. Frozen tumors were obtained from RPC-9 and H1975 xenograft models at the end of the final tumor size evaluation. β-Actin was used as a loading control. EGFR, phosphorylated protein kinase B (AKT), IRS-1, RAF-1, and cleaved PARP were examined.

Figure 6. Western blot analyses of the signal transduction pathway. Frozen tumors were obtained from RPC-9 and H1975 xenograft models at the end of the final tumor size evaluation. β-Actin was used as a loading control. EGFR, phosphorylated protein kinase B (AKT), IRS-1, RAF-1, and cleaved PARP were examined.

T790M mutation, the antitumor efficacy of miR-7 was evident even in the EGFR-TKI-resistant models.

Transfection by liposomes seems to be safer than that by viral vectors, although some conditions should be optimized (42, 43). The adopted cationic liposome and the constructed plasmid appears to have high miRNA expression efficiency, compared with results in a previous report (44). To our knowledge, this is the first report to show a dramatic effect of miR-7 against EGFR-TKI-resistant lung cancer cells in vivo. It suggests that the delivery of plasmids expressing miR-7 by cationic liposomes has therapeutic potential for overcoming acquired resistance to EGFR-TKI and that a novel approach in RNA levels to overcome the resistance caused by secondary mutations in EGFR-addicted tumors might be warranted.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

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