RESEARCH ARTICLE

Effects of Multiple-target Anti-microRNA Antisense Oligodeoxyribonucleotides on Proliferation and Migration of Gastric Cancer Cells

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Abstract

Backgrounds: To investigate the inhibiting effects of multi-target anti-microRNA antisense oligonucleotide (MTg-AMOs) on proliferation and migration of human gastric cancer cells. Methods: Single anti-microRNA antisense oligonucleotides (AMOs) and MTg-AMOs for miR-221, 21, and 106a were designed and transfected into SGC7901, a gastric cancer cell line, to target the activity of these miRNAs. Their expression was analyzed using stem-loop RT-PCR and effects of MTg-AMOs on human gastric cancer cells were determined using the following two assay methods: CCK8 for cell proliferation and transwells for migration. Results: In the CCK-8 cell proliferation assay, 0.6 μmol/L was selected as the preferred concentration of MTg-AMOs and incubation time was 72 hours. Under these experimental conditions, MTg-AMOs demonstrated better suppression of the expression of miR-221, miR-106a, miR-21 in gastric cancer cells than that of single AMOs (P = 0.014, 0.024; 0.038, respectively). Migration activity was also clearly decreased as compared to those in randomized and blank control groups (28 ± 4 Vs 54 ± 3, P <0.01; 28 ± 4 Vs 59 ± 4, P < 0.01). Conclusions: MTg-AMOs can specifically inhibit the expression of multiple miRNAs, and effectively antagonize proliferation and migration of gastric cancer cells promoted by oncomirs.

Keywords: MicroRNAs - stomach neoplasms - oligonucleotides - antisense - cell proliferation - migration

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Introduction

MicroRNAs (miRNAs) are evolutionarily conserved, endogenous, small, non-coding RNA molecules of approximately 22~25 nucleotides. They are encoded in the genome and are generally transcribed by RNA polymerase II, exerting their effects by associating with a group of proteins termed as the 'RNA-induced silencing complex' (RISC). The primary role of RISC is to target mRNAs via imperfect sequence complementarities existing between the miRNA and 3'-untranslated region (3'-UTR) of target mRNAs. In many studies, it has been concluded that RISC leads to down-regulate gene expression through mRNA cleavage or translation inhibition (Denli et al., 2004; Gregory et al., 2004). It has been proved through research studies that miRNAs play crucial role in diverse biological processes, including cell apoptosis, differentiation, development, and signal transduction. An in-depth study of microRNAs in a variety of human tumors, indicates that some miRNAs may function as oncogenes or tumor suppressors (Li et al., 2009; Ferdin et al., 2010). Different types of tumors have different abnormal expression level of the miRNA, indicating that tumors have a tissue-specific miRNA expression profiling. In addition, miRNA expression profiling of tumors in digestive organs has been identified through signatures associated with diagnosis, staging, progression, prognosis and response to treatment (Schetter et al., 2008; Bartels et al., 2009; Wang et al., 2009).

Currently, in miRNA functional researches, anti-miRNA antisense oligonucleotides (AMOs) are widely used for inhibiting miRNAs. Recent studies supported that a modified AMO method in which multiple antisense units are designed into a single unit has the ability to inhibit multiple-target miRNAs (Lu et al., 2009). In this study, multi-target anti-microRNA antisense oligonucleotide (MTg-AMOs) was designed to inhibit the expression of miR-221, miR-106a, miR-21: there have been reported to be over-expressed in many human cancers, and they have been proved to have close relationship with human cancers in many studies. We have evaluated the effects of MTg-AMOs on proliferation and migration of gastric cancer cell in vitro. We hope that this will help us in elucidating the possible relation between miRNAs and tumor biological functions in gastric cancer, providing experimental basis for this relationship.

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Table 1. Primers for Real-time Stem-loop RT- PCR

Gene	Primers	Primer Sequence
Mir-221	RT primer (5'-3')	GTCGTATCCAGTGCAGGGTCCGAGGTATTCGCACTGGATACGACGAAACC
	Forward (5'-3')	CGCAGCTACATTGTCTGCTGG
	Reverse (5'-3')	GTGCAGGGTCCGAGGT
Mir-21	RT primer (5'-3')	GTCGTATCCAGTGCAGGGTCCGAGGTATTCGCACTGGATACGACTCAACA
	Forward (5'-3')	CGCTAGCTTATCAGACTGAT
	Reverse (5'-3')	GTGCAGGGTCCGAGGT
Mir-106a	RT primer (5'-3')	GTCGTATCCAGTGCAGGGTCCGAGGTATTCGCACTGGATACGACCTACCT
	Forward (5'-3')	CGCAAAAGTGCTTACAGTGCA
	Reverse (5'-3')	GTGCAGGGTCCGAGGT
U6 RNA	RT primer (5'-3')	CGTTCACGAATTTGCGTGTCAT
	Forward (5'-3')	GCTTCGGCAGCACATATACTAAAAT
	Reverse (5'-3')	CGCTTCACGAATTTGCGTGTCAT

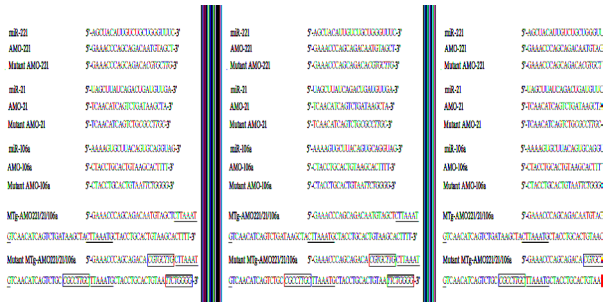

Figure 1. Sequences of the AMOs and MTg-AMOs. All AMOs and MTg-AMOs were phosphorothioate modified. In sequences of MTg-AMOs, an 8-nt linker (underlined letters) was inserted to connect the two adjacent antisense units. Box represents the different parts of base between randomized control group and experimental group

Materials and Methods

Cell culture

Human gastric cancer cell lines SGC-7901 was obtained from Shanghai Institute of Biochemistry and Cell Biology, Chinese Academy of Sciences (Shanghai, China). Cells were cultured in 6 well cell culture plates at a temperature of 37 °C in a humidified atmosphere of 5% CO_2 with RPMI-1640 Medium (Life Technologies, Grand Island, NY, USA) containing 10% fetal calf serum with 50 U/ml penicillin and 50 μg/ml streptomycin. Exponentially growing cells were used for experiments.

Synthesis of Primers for miRNAs and AMOs

According to the method described by Chen et al (Chen et al., 2005) for mature miRNA stem-loop-PCR, primers for miR-221, miR-21 and miR-106a were designed (Invitrogen Corporation Shanghai synthetic). AMOs of miR-221 (AMO-221), miR-106a (AMO-106a), miR-21 (AMO-21) and MTg-AMOs (MTg-AMO221/21/106a) were chemically synthesized respectively. At the same time, missense oligonucleotides (Mutant AMO-221, Mutant AMO-21, Mutant AMO-106a and Mutant MTg-AMO221/21/106a) were synthesized as randomized controls. (Invitrogen, Shanghai, China) (the sequences were shown in Table 1 and Figure 1)

Cell transfection

The AMOs including randomized controls were transfected into SGC-7901, a gastric mucosa cell line. Cells without transfection were used as the control. 30 pmol AMOs were diluted in 50 μl serum-free RPMI-1640 medium. 2 μl of the liposome Lipofectamine TM2000 were diluted in 50 μl RPMI-1640 medium by mixing the components gently and they were incubated at room temperature for 5 min. Then, the two agents were mixed gently and incubated at room temperature for 20 min. The resulting complexes were added into cells and shaken gently, and were incubated overnight at a temperature of 37 °C, and humidity of 5% CO_2. In the follow-up experiments, the medium was replaced with those containing 10% fetal calf serum and cultured for 48 h.

Cell proliferation assays using CCK8 kit

Cell proliferation assays were performed using CCK-8 kit (cell counting kit-8) (Dojido, Japan). According to the manufacture's instruction, 5~10 × 10^3 cells were seeded into 96-well culture plates. Adhesion was verified once (about 12 h later); the cells were incubated with MTg-AMOs at the following different concentration levels of 0, 0.2, 0.4, 0.6, 0.8 μmol/L for 24, 48 and 72 h respectively. At the endpoint, 20 μl CCK-8 (5 g/L) was added for further 4 h. Absorbance at 450 nm was measured using Victor3 micro-plate reader (Perkin-Elmer, Waltham, MA, USA).

Reverse transcriptase reactions

Reverse transcriptase reactions were carried out using RNA samples, 50 nM stem–loop RT primer, 1 × RT buffer (Biosystems), 0.25 mM each of dNTPs, 3.33 U/ml MultiScribe reverse transcriptase (Biosystems), and 0.25 U/ml RNase inhibitor (P/N: N8080119; Biosystems, CA, USA). The 7.5 μl of the reaction mixture was incubated in a PTC-200 Pletier Thermal Cycler in a 30- or 48-well plate for 30 min at 16 °C, 30 min at 42 °C, 5 min at 85 °C and then held at a constant temperature of 4 °C (Schetter et al., 2008).

Real time - polymerase chain reaction (Real time-PCR)

Real-time polymerase chain reaction (PCR) was performed using miScript SYBR Green PCR Kit (Qiagen, Shanghai, China). The 20 μl PCR mixture included 2 μl reverse transcription product,10 μl 2× QuantiTect SYBR Green PCR Master Mix, 2 μl 10× miScript Universal Primer, 2 μl 10× miScript Primer Assay, and 4 μl autoclaved distilled water. The reaction mixtures were incubated at a temperature of 95 °C for 10 min, followed by 40 amplification cycles of 94 °C for 15 s, 55 °C for 30 s, and 70 °C for 30 s. In each sample, a ΔCt (target-

Table 2. Real time PCR Data with miRNAs are Amplified in Gastric Cancer Cell Lines Transfected Using Experimental Group and Control Groups

	miR-221	U6 RNA	ΔCt	ΔΔCt	$2^{-\Delta\Delta Ct}$
AMO-221	25.66±0.42	20.64±0.24	5.02±0.19	1.49±0.19	0.36±0.05*
M-AMO-221	24.15±0.25	20.36±0.17	3.79±0.34	0.26±0.34	0.85±0.21**
Blank control	23.53±0.31	20.01±0.19	3.53±0.27	0±0.27	1.01±0.20
	miR-106a	U6 RNA	ΔCt	ΔΔCt	$2^{-\Delta\Delta Ct}$
AMO-106a	25.79±0.22	20.93±0.21	4.86±0.20	1.57±0.20	0.34±0.05*
M-AMO-106a	24.04±0.10	20.46±0.19	3.58±0.10	0.28±0.10	0.82±0.06**
Blank control	23.35±0.42	20.06±0.18	3.30±0.27	0±0.27	1.01±0.20
	miR-21	U6 RNA	ΔCt	ΔΔCt	$2^{-\Delta\Delta Ct}$
AMO-21	25.68±0.22	20.92±0.26	4.77±0.15	1.6±0.15	0.33±0.03*
M-AMO-21	23.44±0.25	20.25±0.36	3.18±0.40	0.02±0.40	1.01±0.26**
Blank control	23.43±0.21	20.26±0.26	3.17±0.46	0±0.46	1.04±0.36

	miR-221	miR-106a	miR-21	U6 RNA	$2^{-\Delta\Delta Ct221}$	$2^{-\Delta\Delta Ct106a}$	$2^{-\Delta\Delta Ct21}$
MTg	25.90±0.33	26.10±0.07	25.95±0.24	20.27±0.16	0.19±0.02	0.23±0.03	0.24±0.02*
M-MTg	23.63±0.43	23.54±0.29	23.85±0.28	20.22±0.18	0.91±0.27	1.31±0.25	0.99±0.07**
Blank control	23.37±0.39	23.85±0.24	23.76±0.27	20.15±0.13	1.01±0.18	1.00±0.09	1.02±0.26

*P<0.05; **P>0.05

reference) was calculated; it is equal to the difference between threshold cycles for miRNAs (target) and those for U6 RNA (reference). The fold-change for miRNAs was calculated by the $2^{-\Delta\Delta Ct}$ method. $2^{-\Delta\Delta Ct}$ values were calculated for each sample relative to the normal control for expression of miRNAs. Each sample was tested three times.

Cell migration assay

Cell migration assays were performed in transwell chambers having 8 μm pores (Chemicon, Temecula, CA, USA). The lower chambers of the transwell plates were filled with 500 μl medium containing 10% fetal bovine serum as a chemo-attractant. The cell suspension (300 μl) was then added to the upper chamber, and plates were incubated at 37 °C for 72 h. Cells migrating to the lower surface of the polycarbonate membrane were stained with Giemsa solution and quantified by counting 5 randomly selected microscope fields at × 200 magnification.

Statistical Analysis

Data were expressed as mean ± SD. The data of Real-time PCR were $2^{-\Delta\Delta Ct}$-transformed and analyzed using ANOVA; the results of CCK8 assay were analyzed using independent samples t-test and ANOVA. The data of transwell assay were analyzed using ANOVA. $P < 0.05$ was considered statistically significant.

Results

The selection of appropriate concentrations and incubation time for studying the effect of MTg-AMOs on gastric cancer cell proliferation

For deciding the appropriate concentration of MTg-AMOs and incubation time for the experiments in the latter part, MTg-AMOs were transfected to SGC-7901, a kind of gastric cancer cell strains, at different concentrations at different times. The proliferation rate of cells was evaluated using CCK-8 kit. The results indicated that

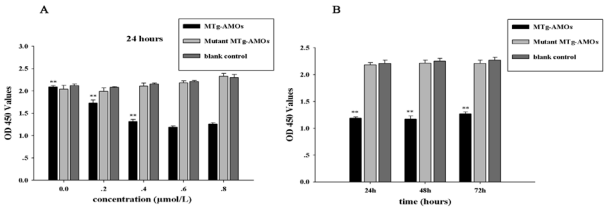

Figure 2. Effect of MTg-AMOs on the Proliferation of Gastric Cancer Cells in Different Concentrations and Incubation Times. (A)gastric cancer cells.were cultured with MTg-AMOs in different concentrations for 24 hours. *P<0.05; **P<0.01 were statistically significant differences compared to different concentration groups; (B) gastric cancer cells.were cultured with MTg-AMOs in different incubation times from 24 to 72 hours. the cell proliferation were evaluated by CCK-8 method as described in materials and methods. *P<0.05; **P<0.01 were statistically significant differences compared to control groups. Results are represented as mean plus or minus SD obtained from three independent experiments

proliferation rates of cells transfected with MTg-AMOs decreased significantly at concentrations in the range of 0.2-0.8 μmol/L after incubation, compared with that of blank and negative controls (Figure 2A, $P < 0.001$), which had similar proliferation rate in the experiment. Moreover, there was a dose-dependent inhibitory effect for it, implying that the inhibitory effects of MTg-AMOs increased according to the dose of MTg-AMOs added to the cells culture medium.

Furthermore, with the preferred concentration of MTg-AMOs used in above experiment (0.6 μmol/L), the cells were cultured and collected at different time intervals of 24, 48, 72 h respectively. The cell proliferation rates were analyzed using CCK-8 kit that were described previously. As shown in the result, the cells cultured for 72 h had the most significant effect (Figure 2B, $F = 4.007$, $P = 0.033$). The inhibitory effect of MTg-AMOs on the expression of miR-221, miR-106a, miR-21.

Under the preferred MTg-AMOs concentration and incubation times, we investigated the inhibitory effect of

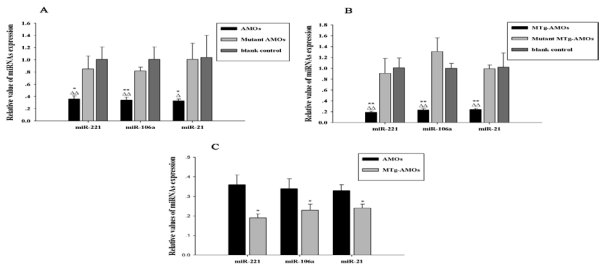

Figure 3. Expression of miRNAs in Gastric Cancer Cells Transfected by MTg-AMOs and AMOs. (A) expression of miRNAs in gastric cancer cells transfected by three single AMOs compared with blank control groups and negative control groups (transfected by Mutant AMOs). (B) expression of miRNAs in gastric cancer cells transfected by MTg-AMOs compared with blank control groups and negative control groups (transfected by Mutant MTg-AMOs). (C) expression of miRNAs in gastric cancer cells transfected by MTg-AMOs compared with those transfected by single AMOs. After transfected by the single AMOs and MTg-AMOs, the cells were collected and miRNAs were detected by real-time PCR. Results are represented as mean plus or minus SD obtained from the three independent experiments. *P < 0.05; **P < 0.01 were statistically significant differences between experimental groups and Mutant MTg-AMOs groups. control groups. $^{\Delta}$P < 0.05; $^{\Delta\Delta}$P < 0.01 were statistically significant differences between experimental groups and blank control groups

MTg-AMOs on the expression of miR-221, miR-106a, miR-21. Firstly, we studied the inhibitory effect of AMO-221 on miR-221. After incubating the cells with AMO-221 at a concentration of 0.6 μmol/L for 72 h, cells from experimental groups and control groups were collected; miRNAs were detected using quantitative PCR. The effect of AMO-106a, AMO-21, and MTg-AMOs on expression of miR-106a, miR-21 was determined in a similar manner. The results showed that AMO-221, AMO-106a, AMO-21 inhibited the expression of miR-221, miR-106a, miR-21 significantly compared with the blank and negative control groups (Figure 3 and Table 2, P= 0.003, 0.000, 0.015, respectively). We were quite surprised to find that MTg-AMOs not only had the inhibitory effect on miR-221, miR-106a, miR-21, but also had better suppression ability on the expression of miR-221, miR-106a, miR-21 compared to that of single AMOs. For example, miR-221 expression levels were 0.19 ± 0.02 in the experimental groups transfected by MTg-AMOs. This was significantly lower than 0.36 ± 0.05, which was the miR-221 expression level in the control group transfected by AMO-221 (P = 0.014). Moreover, similar results were also obtained in AMO-106a and AMO-21 groups (miR-106a expression were 0.34 ± 0.05 in AMO-106a group and 0.23 ± 0.03 in MTg-AMOs group, P = 0.024; miR-21 expression were 0.33 ± 0.03 in AMO-106a group and 0.24 ± 0.02 in MTg-AMOs group, P = 0.038). The data indicated that MTg-AMOs has better inhibitory effect on gastric cancer cells than single miRNA.

Effect of MTg-AMOs on the migration of gastric cancer cells

To examine the effect of MTg-AMOs on gastric cancer cell migration, transwell assay were used and the migration rate of cells was detected simultaneously in

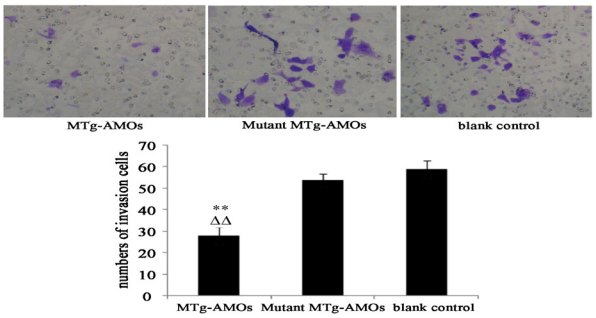

Figure 4. Cell Migration Measured Using Transwell Migration Assay. The gastric cancer cells transfected with MTg-AMOs were added to the upper chamber, and plates were incubated at 37 °C at 5%CO2 for 72 hours, and cells migration was evaluated by transwell migration assay. The result showed that the number of migrated cells were significantly less than those in randomized control group and blank control group. Results are represented as mean plus or minus SD obtained from the three independent experiments. * P < 0.05; **P < 0.01 were statistically significant differences between experimental groups and Mutant MTg-AMOs groups. control groups. Δ P < 0.05; ΔΔP < 0.01 were statistically significant differences between experimental groups and blank control groups

MTg-AMOs group, randomized control group, and blank control group. The result indicated that the migrated cells were 28 ± 4 per microscope fields at ×200 magnification in experimental groups, which was significantly less than those in randomized control group (54 ± 3) and blank control group (59 ± 4) (Figure 4, P <0.01). The result showed that the migration rate of gastric cancer cells transfected with MTg-AMOs (52.93 ± 9.66%) at the preferred concentration of 0.6μmol /L for 72 h, was significantly lower as compared to the control groups (7.52 ± 11.30%). It indicates that MTg-AMOs can effectively the migration activity of gastric cancer cells induced by cancer- related miRNAs.

Discussion

In recent years, expression of miRNAs has been reported to be expressed in different ways across a number of tumor types, and it probably contributes to carcinogenesis (Esquela-Kersher et al., 2006; Liu et al., 2007). The prognostic potential of miRNAs has been evaluated in several cancers, including lung cancer, neuroblastoma, pancreatic cancer and gastric cancer (Yanaihara et al., 2006; Chen et al., 2007; Lee et al., 2007; Xiao et al., 2009). These studies have provided new avenues for basic research, clinical diagnosis and treatment of cancers. A variety of appropriate technologies for miRNAs function has been developed, in which antisense technology was most commonly used to block miRNAs expression. Antisense oligodeoxyribonucleotides is a technology based on the principle of base complementary artificial complementary DNA and RNA for specific gene were transferred into cells to block gene transcription or translation (Wagner et al., 1994). In China and in several countries all over the world, many studies have proved that antisense oligonucleotide is an effective method to inhibit miRNAs expression (Krützfeldt et al., 2005). Presently, miRNAs antisense inhibition methods include

phosphorothioate modified antisense oligonucleotides, locked nucleic acid (LNA), and multi-target antisense oligonucleotide (MTg-AMOs) (Ørom et al., 2006; Weiler et al., 2006; Schetter et al., 2008).

In this study, MTg-AMOs was designed to simultaneously inhibit three miRNAs: miR-21, miR-106a and miR-221, which were shown to be involved in carcinogenesis. In CCK8 cell proliferation assay, it was found that MTg-AMOs can effectively inhibit gastric cancer cell proliferation in a much shorter duration of time, and its inhibitive efficiency can be sustained longer. When the concentration rose to 0.8μmol/L, its effects reached plateau. This could be attributed to the transiently transfected degradation of the nucleotide or cell proliferation and division of remnants. After single AMOs and MTg-AMOs were transfected into the gastric cells, it was revealed that MTg-AMOs can not only decrease the expression of miR-21, miR-106a and miR-221, but also become more efficient than single AMOs. The exact mechanism of this phenomenon is elucidated. It is possible that modulation of gene transcription or translation is a web procession, implying that every miRNA can modulate several genes, whereas a single gene could be modulated by many miRNAs. Conversely, many genes can modulate the expression of miRNA. So MTg-AMOs, designed to target several miRNAs, could be more effective to inhibit the expression of miRNAs than single AMOs. This study indicates that MTg-AMOs can not only induce a lasting inhibition for expression of specific miRNAs, but also decrease gastric cancer cells proliferation. Furthermore, the study for migration assay showed that MTg-AMOs can effectively antagonize the migration of gastric cancer cells.

In summary, MTg-AMOs can decrease the expression of several miRNAs, and this will be more beneficial in decreasing the proliferation and migration of gastric cancer cells. The use of antisense nucleic acid technology for oncomirs may reveal some startling facts on gene therapy of gastric cancer. In future, MTg-AMOs can not only be used as an ideal choice of clinical anti-tumor drugs, but can also serve as an important tool for exploring the functions of miRNAs in a number of human tumor types.

Acknowledgements

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References

Bartels CL and Tsongalis GJ (2009). MicroRNAs: novel biomarkers for human cancer. *Clin Chem*, **55**, 623-31.

Chen CF, Ridzon DA and Broomer AJ, et al (2005). Real- time quantification of microRNAs by stem-loop RT- PCR. *Nucleic Acids Res*, **33**, e179.

Chen Y, Stallings RL (2007). Differential patterns of microRNA expression in neuroblastoma are correlated with prognosis, differentiation, and apoptosis. *Cancer Res*, **67**, 976-83.

Denli AM, Tops BJ, Plasterk PH, et al (2004). Processing of primary microRNAs by the microprocessor complex. *Nature*, **432**, 231-5.

Esquela-Kerscher A and Slack FJ (2006). Oncomirs - microRNAs with a role in cancer. *Nat Rev Cancer*, **6**, 259-69.

Ferdin J, Kunej T and Calin GA (2010). Non-coding RNAs: Identification of Cancer-Associated microRNAs by Gene Profiling. *Technol Cancer Res Treat*, **9**, 123-38.

Gregory RI, Yan K, Amuthan G, et al (2004). The microprocessor complex mediates the genesis of microRNAs. *Nature*, **432**, 235-40.

Krützfeldt J, Rajewsky N, Braich R, et al (2005). Silencing of microRNAs in vivo with 'antagomirs'. *Nature*, **438**, 685-9.

Lee EJ, Gusev Y, Jiang J, et al (2007). Expression profiling identifies microRNA signature in pancreatic cancer. *Int J Cancer*, **120**, 1046-54.

Liu W, Mao SY and Zhu WY (2007). Impact of tiny miRNAs on cancers. *World J Gastroenterol*, **13**, 497-502.

Ørom UA, Kauppinen S, Lund AH (2006). LNA-modified oligonucleotides mediate specific inhibition of microRNA function. *Gene*, **372**, 137-41.

Schetter AJ, Leung SY, Sohn JJ, et al (2008). MicroRNA expression profiles associated with prognosis and therapeutic outcome in colon adenocarcinoma. *JAMA*, **299**, 425-36.

Li C, Feng Y, Coukos G, et al (2009). Therapeutic microRNA strategies in human cancer. *AAPS J*, **11**, 747-57.

Lu Y, Xiao J, Lin H, et al (2009). A single anti-microRNA antisense oligodeoxyribonucleotide (AMO) targeting multiple microRNAs offers an improved approach for microRNA interference. *Nucleic Acids Res*, **37**, e24.

Wagner RW (1994). Gene inhibition using antisense oligodeoxynucleotides. *Nature*, **372**, 333-5.

Wang V and Wu W (2009). MicroRNA-based therapeutics for cancer. *BioDrugs*, **23**, 15-23.

Weiler J, Hunziker J, Hall J (2006). Anti-miRNA-oligonucleotides (AMOs): ammunition to target miRNAs implicated in human disease. *Gene Ther*, **13**, 496-502.

Xiao B, Guo J, Miao Y, et al (2009). Detection of miR-106a in gastric carcinoma and its clinical significance. *Clin Chim Acta*, **400**, 97-102.

Yanaihara N, Caplen N, Bowman E, et al (2006). Unique microRNA molecular profiles in lung cancer diagnosis and prognosis. *Cancer Cell*, **9**, 189-98.