SHORT COMMUNICATION

miR-21-mediated tumor growth

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MicroRNAs (miRNAs) are ~22 nucleotide non-coding RNA molecules that regulate gene expression post-transcriptionally. Although aberrant expression of miRNAs in various human cancers suggests a role for miRNAs in tumorigenesis, it remains largely unclear as to whether knockdown of a specific miRNA affects tumor growth. In this study, we profiled miRNA expression in matched normal breast tissue and breast tumor tissues by TaqMan real-time polymerase chain reaction miRNA array methods. Consistent with previous findings, we found that *miR-21* was highly overexpressed in breast tumors compared to the matched normal breast tissues among 157 human miRNAs analysed. To better evaluate the role of *miR-21* in tumorigenesis, we transfected breast cancer MCF-7 cells with anti-miR-21 oligonucleotides and found that anti-miR-21 suppressed both cell growth *in vitro* and tumor growth in the xenograft mouse model. Furthermore, this anti-miR-21-mediated cell growth inhibition was associated with increased apoptosis and decreased cell proliferation, which could be in part owing to downregulation of the antiapoptotic Bcl-2 in anti-miR-21-treated tumor cells. Together, these results suggest that *miR-21* functions as an oncogene and modulates tumorigenesis through regulation of genes such as *bcl-2* and thus, it may serve as a novel therapeutic target.

Introduction

MicroRNAs (miRNAs) are a class of naturally occurring small non-coding RNAs that control gene expression by targeting mRNAs for translational repression or cleavage (Pillai, 2005; Zamore and Haley, 2005). It is predicted that miRNAs comprise 1–5% of animal genes (Berezikov *et al.*, 2005). miRNAs are transcribed as long primary transcripts in the nucleus and are subsequently cleaved to produce stem loop structured precursor molecules of ~70 nt in length (pre-miRNAs) by Drosha (Kim, 2005), which are then exported to the cytoplasm, where the RNase III enzyme Dicer further processes them into mature miRNAs (~22 nucleotides). Thus, miRNAs are related to short interfering RNAs, but they have distinct pathways (Bartel, 2004; Fitzgerald, 2005). Since the discovery of *lin-4* in *Caenorhabditis elegans* (Lee *et al.*, 1993; Wightman *et al.*, 1993), thousands of miRNAs have been identified to date in a variety of organisms (http://microrna.sanger.ac.uk/).

As a new layer of gene regulation mechanism, miRNAs have diverse functions, including the regulation of cellular differentiation, proliferation and apoptosis (Chen *et al.*, 2004; Croce and Calin, 2005). Thus, deregulation of miRNAs would alter the normal cell growth and development, leading to a variety of disorders including human cancer. For instance, about 65% of investigated patients suffering from B-cell chronic lymphocytic leukemia (CLL) have been reported to show a deletion located at chromosome 13q14 where the *miR-15* and *miR-16* genes are located and are under-represented in many B-CLL patients (Calin *et al.*, 2002). Of interest, miRNA-containing regions are often located at fragile sites or in repetitive genomic sequences (Calin *et al.*, 2004). Deregulation of other miRNAs has also been reported in different cancers (Michael *et al.*, 2003; Metzler *et al.*, 2004; Eis *et al.*, 2005), indicating that there is a direct correlation between aberrant expression of miRNAs and human malignancy. However, although miRNAs have been the object of extensive research in recent years, the molecular basis of miRNA-mediated gene regulation is not fully understood and their role in tumorigenesis remains largely to be determined yet.

In this study, we found that *miR-21* was overexpressed in breast tumor specimens, consistent with the previous report (Iorio *et al.*, 2005). Importantly, anti-miR-21 oligonucleotides suppress both cell growth *in vitro* and tumor growth *in vivo*, which is associated with increased apoptosis and downregulation of the antiapoptotic protein Bcl-2.

Results and discussion

miR-21 *is overexpressed in breast tumor tissues compared to matched normal breast tissues*
Previous studies have shown that several miRNAs are aberrantly expressed in various types of cancers by

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miRNA array or Northern blot (Calin *et al*., 2002, 2004; Michael *et al*., 2003; Metzler *et al*., 2004; Eis *et al*., 2005). In this study, we profiled miRNA expression in matched normal breast and breast tumor tissues by TaqMan real-time polymerase chain reaction (PCR) using a newly released miRNA array from ABI (Forest City, CA, USA). The array carries specific primer sets that allow for detection of 157 mature human miRNAs. This method uses stem–loop reverse transcription (RT) primers; it is specific for detection of mature miRNAs (Chen *et al*., 2005; Lao *et al*., 2006). Furthermore, this method is very sensitive and is able to analyse miRNA expression in a single cell (Tang *et al*., 2006). We used U6 RNA for normalization of expression in different samples. From a total of five pairs of matched advanced breast tumor tissue specimens, *miR-21* was the most abundantly expressed miRNA among all miRNAs in this array and moreover, the level of *miR-21* was much higher in the tumor tissues than in the matched normal tissues (Figure 1a). As one C_T (threshold cycle) unit is equivalent to ~ 2-fold difference (Chen *et al*., 2005), this conversion would result in over a five-fold increases in *miR-21* levels for tumor tissues compared to the matched normal tissues after normalization to U6 RNA (Figure 1b), consistent with the previous report (Iorio *et al*., 2005). Furthermore, using the individual *miR-21* primer set, we were able to confirm these results in more matched breast tumor samples (not shown).

Anti-*miR-21* inhibits cell growth *in vitro*

To test whether *miR-21* may function as an oncogene, we examined the effect of suppression of *miR-21* on breast tumor cell growth. Thus, we used anti-*miR-21* inhibitor as this approach has been successfully used to inhibit *miR-21* (Chan *et al*., 2005; Cheng *et al*., 2005). The anti-*miR-21* inhibitor is a sequence-specific and chemically modified oligonucleotide to specifically target
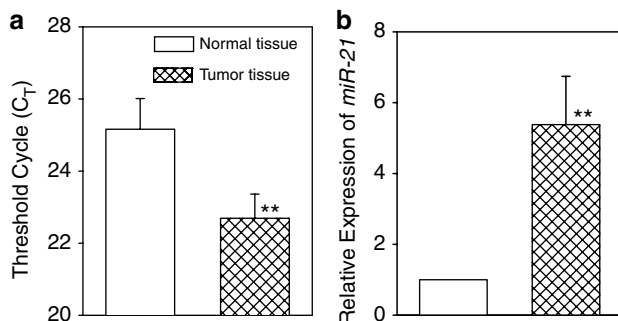

Figure 1 Expression of *miR-21* in matched normal and breast tumor tissues. Relative *miR-21* levels were determined by TaqMan miRNA assays (see Supplementary materials for detail), expressed as C_T (**a**) or fold change after normalization to U6 RNA (**b**). For RT reactions, 10 ng total RNA was used in each reaction (15 μl) and mixed with corresponding TaqMan miRNA assays RT primer (3 μl). The RT reaction was performed at the following conditions: 16°C for 30 min; 42°C for 30 min; 85°C for 5 min, and then hold on 4°C. After the RT reaction, the cDNA products were diluted at 15, 150 and 1500 $\times$, respectively, and 1.33 μl diluted cDNA was used for PCR reaction along with TaqMan primer (2 μl). The PCR reaction was carried out at 95°C for10 min, followed by 40 cycle of 95°C for 15 s and 60°C for 60 s. Values are means of five pairs of matched breast tumor samples $\pm$ s.e. **$P < 0.01$.

and knockdown *miR-21* molecule. TaqMan real-time PCR revealed that anti-*miR-21* significantly reduced *miR-21* level (Figure 2a), suggesting that anti-*miR-21* is efficiently introduced into the cells and knock down *miR-21*. This is probably due to the formation of highly stable complexes of *miR-21* with anti-*miR-21* that prevents miRNA detection by TaqMan real-time PCR. Of interest, we found that anti-*miR-21* reduced cell growth in a dose-dependent manner. At 50 nM, the growth inhibition by anti-*miR-21* reached about 25%, at day 3 after transfection (Figure 2b); this result was also in agreement with the previous report that *miR-21* inhibitors decrease human glioblastoma cell survival (Chan *et al*., 2005). To further assess the effect of anti-*miR-21* on cell growth, we treated the transfected cells with the anticancer drug topotecan (TPT) that is known to inhibit DNA topoisomerase I and cause DNA damage (Tanizawa *et al*., 1994). Anti-*miR-21*-mediated cell growth inhibition was increased up to 40% when the transfected cells were treated with 0.1 μM TPT (Figure 2c). Therefore, anti-*miR-21* can inhibit cell growth *in vitro*. These results also suggest that suppression of *miR-21* can sensitize tumor cells to anticancer agents.

Anti-*miR-21* inhibits tumor growth in the xenograft carcinoma mouse model

Although it has previously been shown that there is a direct correlation between aberrant expression of *miR-21* and breast cancer (Iorio *et al*., 2005), it is not clear whether suppression of *miR-21* alone will affect tumorigenesis. Therefore, we transiently transfected MCF-7 cells with anti-*miR-21* or the negative control, and then injected them into mammary pads of female nude mice. Of considerable interest, we found that tumors derived from MCF-7 cells transfected with anti-*miR-21* grew substantially slowly, compared to the negative control during the whole tumor growth period (Figure 3a). By day 28 when tumors were harvested, average weight for tumors derived from cells transfected with anti-*miR-21* was only about half of those derived from the cells transfected with the negative control (Figure 3b). Immunostaining with the anti-Ki-67 indicated that the reduced tumor growth is likely due to a lower proliferation caused by anti-*miR-21* because Ki-67 staining was much weaker for anti-*miR-21* than for the negative control (Figure 3c). These results strongly suggest that *miR-21* plays an important role in tumorigenesis. To test how long suppression of *miR-21* by anti-*miR-21* in tumors can sustain, we measured the miR-21 levels. We found that the suppression effect lasted up to 2 weeks (Supplementary materials), suggesting that the initial suppression of *miR-21* is sufficient to inhibit tumor growth.

Of interest, the inhibitory effect of anti-*miR-21* on tumor growth (Figure 3b) is greater ($\sim 50\%$), compared to its inhibitory effect on cell growth *in vitro* ($\sim 25\%$) (Figure 2b). Although the observation time for tumor growth is longer than *in vitro* cell growth inhibition assays, which could explain in part the difference, other
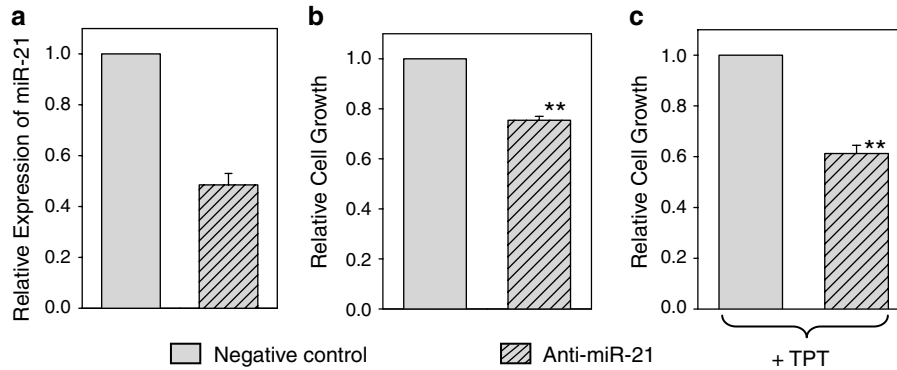
Figure 2 Inhibition of cell growth by anti-miR-21 oligonucleotide. (**a**) Suppression of *miR-21* expression by anti-miR-21 as detected by TaqMan real-time PCR. (**b**) Cell growth inhibition. MCF-7 cells were transiently transfected with the negative control or anti-miR-21 oligonucleotide at 50 nM and then were seeded in 96 well at 2500 cells/well. The cells were allowed to grow for 3 days before MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl-tetrazoliumbromide) assay, as described previously (Mo *et al.*, 2004). (**c**) Cell growth inhibition in the presence of the anticancer agent TPT. Cells were first transfected with 50 nM of negative control or anti-miR-21 and then treated with 0.1 μM of TPT for 3 days. Values in both (**b**) and (**c**) are means of three separated experiments ±s.e. **$P < 0.01$.
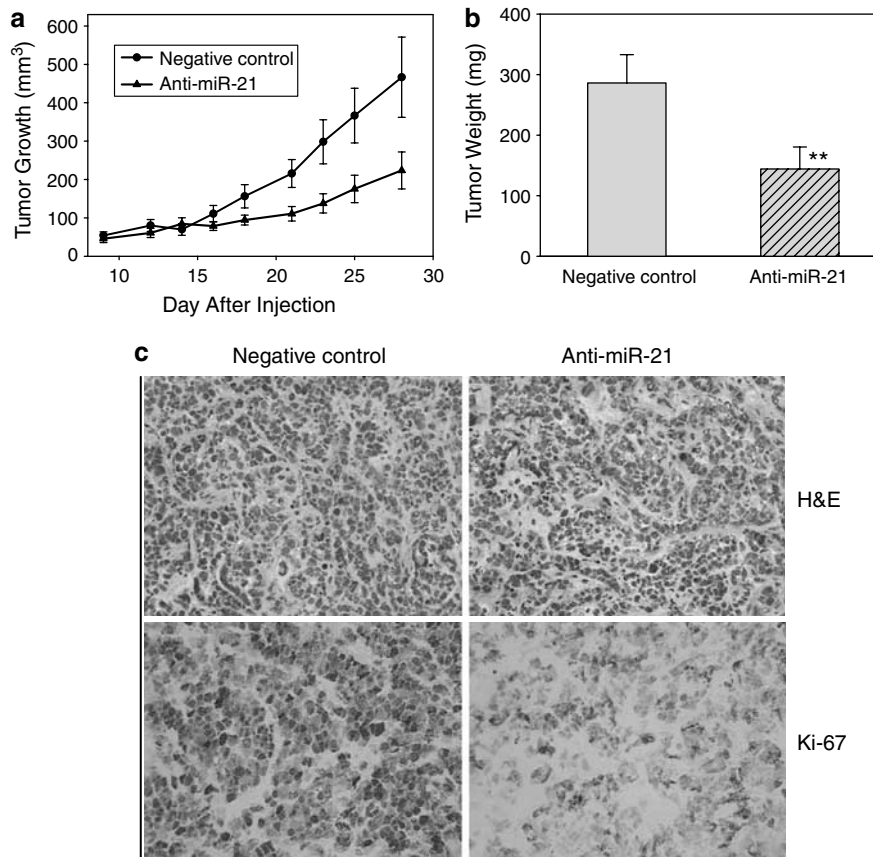

Figure 3 Suppression of tumor growth by anti-*miR-21* oligonucleotide. (**a**) Tumor growth curves measured after injection of MCF-7 cells transfected with either the negative control or anti-miR-21 oligonucleotides. The tumor volume was calculated using the formula volume $= D \times d^2 \times \pi/6$ (Zhang *et al.*, 2002), where D is the longer diameter, d is the shorter diameter. (**b**) Tumor weight. Values in (**a**) and (**b**) are means of tumor volume or weight±s.e. (negative control, $n = 14$; anti-miR-21, $n = 16$). **$P < 0.01$. (**c**) Tumors derived from anti-miR-21-transfected cells revealed a lower level of Ki-67 antigen than the negative control.

factors could also contribute to this difference. For instance, stress from the tumor microenvironment, such as hypoxia, may enhance the inhibitory effect of the anti-miR-21. This appears to be in agreement with the finding that other stresses, such as DNA damage caused by TPT, can increase the inhibitory effect mediated by
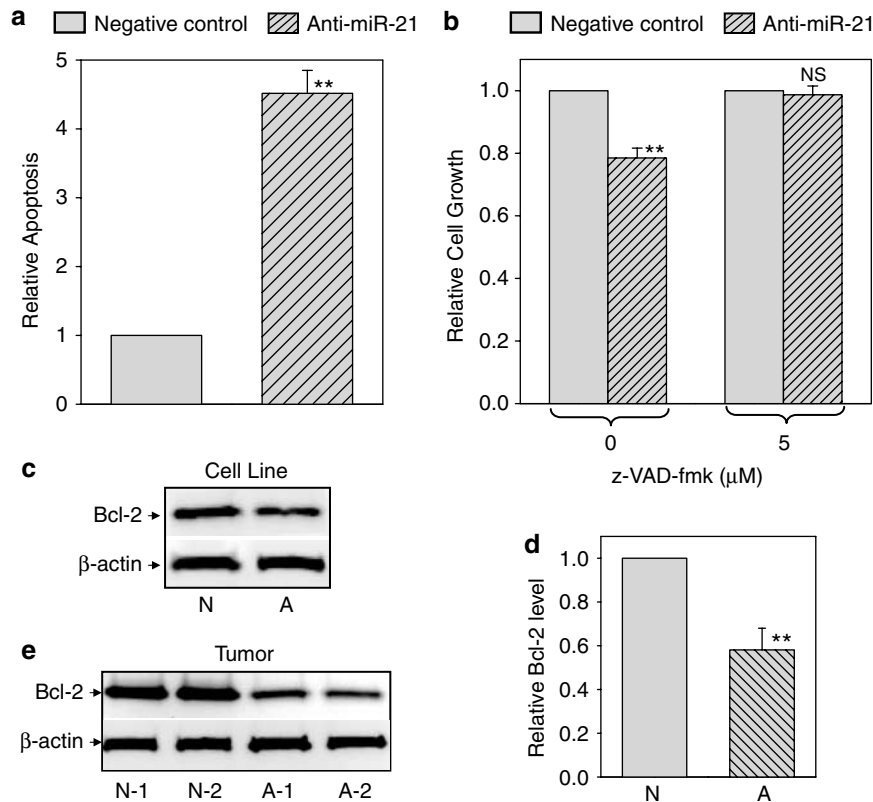
Figure 4 Anti-*miR-21*-induced apoptosis and downregulation of Bcl-2. (**a**) Detection of apoptosis in MCF-7 cells transfected with anti-miR-21 compared to the negative control using cell death detection ELISA^{plus} kit (Hoffmann-La Roche Ltd, Basel, Switzerland). (**b**) Suppression of anti-miR-21-induced growth inhibition by the general caspase inhibitor Z-VAD-fmk. MCF-7 cells were transfected with the negative control or anti-miR-21 as in Figure 2a and then the caspase inhibitor was added to the transfected cells 1 day after transfection. After 3 days later, cell growth inhibition was determined. (**c–e**) Expression of Bcl-2 protein in anti-miR-21 in MCF-7 cells (**c** and **d**) and tumors derived from MCF-7 cells transfected with the negative control or anti-miR-21 (**e**) as detected by Western blot. N-1 and N-2 are tumors 1 and 2 derived from the negative control-treated MCF-7 cells, respectively; A-1 and A-2 are tumors 1 and 2 derived from the anti-miR-21-treated MCF-7 cells, respectively. Values in (**a**), (**b**) and (**d**) are means of three separate experiments ±s.e. **$P < 0.01$. NS, not significant; N, negative control; A, anti-miR-21.

anti-miR-21 (Figure 2c). Alternatively, anti-miR-21 could also affect genes that are linked to other tumorigenesis factors, which might explain in part why more inhibition for anti-miR-21 was seen in tumors than cell growth *in vitro*.

Anti-miR-21 increases cell apoptosis which is associated with downregulation of bcl-2 *expression*
To dissect the molecular basis underlying this *miR-21*-associated alteration of tumor growth, we searched for potential *miR-21* targets using programs available (e.g., http://microrna.sanger.ac.uk/targets/v2/; http://genes.mit.edu/cgi-bin/targetscan_lookup2.pl?KEYWORD = miR-21) and tested several genes that are likely involved in tumorigenesis, such as FasL. However, they were not affected by anti-miR-21 (Supplementary materials). Thus, we tested whether anti-miR-21 suppresses cell growth by triggering apoptosis pathways as previous studies have suggested that *miR-21* regulates apoptosis pathways in tumor cells (Chan *et al*., 2005). Consistent with the previous report for glioblastoma cells (Chan *et al*., 2005), but contrary to the results in HeLa cells

(Cheng *et al*., 2005), we found that anti-miR-21 caused more apoptosis than the negative control in MCF-7 cells by a 4.5-fold (Figure 4a). To further determine the possible involvement of apoptosis in anti-miR-21-mediated growth inhibition, we treated transfected cells with the general caspase inhibitor Z-VAD-fmk. As shown in Figure 4b, Z-VAD-fmk was able to reverse the growth inhibition caused by anti-miR-21, suggesting that increased apoptosis in the anti-miR-21-treated MCF-7 cells is at least in part responsible for the observed growth inhibition. Furthermore, we detected a lower level of Bcl-2 protein in the anti-miR-21-transfected MCF-7 cells (Figure 4c and d) as well as tumors derived from the MCF-7 cells transfected with anti-miR-21 (Figure 4e). Given that suppression level of Bcl-2 *in vivo* (Figure 4e) is greater than that *in vitro* (Figure 4c), it is possible that tumor microenvironment may enhance downregulation of Bcl-2 in the anti-miR-21-treated tumors. We also tested other apoptosis-related proteins such as p53 and PUMA, and found no difference between the negative control and anti-miR-21 (Supplementary materials). Thus, the induction of apoptosis by

anti-miR-21 is possibly in part owing to downregulation of Bcl-2. We also examined *bcl-2* mRNA by RT–PCR and found that *bcl-2* mRNA was decreased in the anti-miR-21-treated cells (Supplementary materials), suggesting that *miR-21* may regulate *bcl-2* expression indirectly. Although we cannot exclude the possibility that anti-miR-21 may cause degradation of *bcl-2* mRNA, one possibility would be that anti-miR-21 suppresses expression of a gene(s) that negatively regulates *bcl-2* expression. Therefore, identification of direct *miR-21* targets may provide new insight into how *miR-21* controls expression of genes involved in apoptosis pathways including *bcl-2*.

In summary, we show that *miR-21* is overexpressed in breast tumor tissues and anti-miR-21 inhibits both cell growth *in vitro* and tumor growth *in vivo*. This is possibly owing to increased apoptosis associated with downregulation of *bcl-2* expression. As experiments with the xenograft carcinoma model indicate that one transient transfection with anti-miR-21 is sufficient to cause substantial inhibition of tumor growth, this raises the possibility that anti-miR-21 may have potential therapeutic value. Indeed, anti-miRNA oligonucleotides can stay a relatively long period of time in animals (Krutzfeldt *et al.*, 2005). Therefore, miRNAs, in particular *miR-21*, may serve as potential targets for cancer therapy.

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Supplementary Information accompanies the paper on the Oncogene website (http://www.nature.com/onc).