miR-21 Induces Myofibroblast Differentiation and Promotes the Malignant Progression of Breast Phyllodes Tumors

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Abstract
Phyllodes tumors of breast, even histologically diagnosed as benign, can recur locally and have metastatic potential. Histologic markers only have limited value in predicting the clinical behavior of phyllodes tumors. It remains unknown what drives the malignant progression of phyllodes tumors. We found that the expression of myofibroblast markers, α-smooth muscle actin (α-SMA), fibroblast activation protein (FAP), and stromal cell-derived factor-1 (SDF-1) is progressively increased in the malignant progression of phyllodes tumors. Microarray showed that miR-21 was one of the most significantly upregulated microRNAs in malignant phyllodes tumors compared with benign phyllodes tumors. In addition, increased miR-21 expression was primarily localized to α-SMA-positive myofibroblasts. More importantly, α-SMA and miR-21 are independent predictors of recurrence and metastasis, with their predictive value of recurrence better than histologic grading. Furthermore, miR-21 mimics promoted, whereas miR-21 antisense oligos inhibited, the expression of α-SMA, FAP, and SDF-1, as well as the proliferation and invasion of primary stromal cells of phyllodes tumors. The ability of miR-21 to induce myofibroblast differentiation was mediated by its regulation on Smad7 and PTEN, which regulate the migration and proliferation, respectively. In breast phyllodes tumor xenografts, miR-21 accelerated tumor growth, induced myofibroblast differentiation, and promoted metastasis. This study suggests an important role of myofibroblast differentiation in the malignant progression of phyllodes tumors that is driven by increased miR-21. Cancer Res; 74(16): 1–12. ©2014 AACR.

Introduction
Phyllodes tumors of breast are typically large and fast growing tumors that accounts for up to 1% of all breast neoplasms (1). Although many phyllodes tumors tend to behave in a benign manner, the clinical outcome of phyllodes tumors is hard to predict, with frequent local relapse and sometimes distant metastasis. Current approach to prevent recurrence and metastasis is surgical resection with wide margin, given that adjuvant chemotherapy or radiotherapy is not effective against phyllodes tumors (2). However, even with wide surgical resection, local recurrence rate is still as high as 8% to 36% (3). Furthermore, recurrent phyllodes tumors showed a progression toward more malignant phenotype (4) with the acquisition of new genetic changes (5). It was reported that 22% of phyllodes tumors that have undergone malignant transformation give rise to hematogenous metastasis (6). It remains unclear what drives malignant transformation of phyllodes tumors and existing biologic markers only have a limited value in predicting prognosis.

Phyllodes tumors, composed of an epithelial and a cellular stromal component, are fibroepithelial tumors that fall into the disease spectrum between fibroadenoma and fibrosarcoma (7). Although all forms of phyllodes tumors are regarded as having malignant potential, phyllodes tumors can be histologically classified as benign, borderline, or malignant on the basis of stromal cellularity, mitotic activity of stromal cells, stromal nuclear atypia, stromal overgrowth, and type of border (infiltrating or pushing). Their potentially recurring and metastasizing behavior is attributed to the characteristics of stromal cells, mainly fibroblasts.

Fibroblasts are highly heterogeneous, and those isolated from different sites reflect a substantial topographic diversity (8). A normal fibroblast can acquire an "activated" phenotype, which expresses α-smooth muscle actin (α-SMA) and is so named "myofibroblasts." Numerous growth factors, chemokines, and extracellular matrix (ECM)–degrading proteases...
have been shown to mediate the activation of fibroblasts (9). Myofibroblasts are found in the stroma of many cancers, including breast cancer, colorectal cancer, and melanoma (10–12). Myofibroblasts in epithelial cancers have an increased proliferative activity (9) and can promote cancer invasion (13). It was reported that myofibroblast differentiation also exists in the stromal cells of some phyllodes tumors (14). However, whether the fibroblasts–myofibroblasts transition (FMT) plays a role in the malignant transformation of phyllodes tumor and whether myofibroblast can be a prognostic marker of phylloides tumors is not known yet.

microRNAs (miRNA) are noncoding RNAs that regulate gene expression and can be master regulators of many fundamental biologic processes, including embryogenesis (15) and organ development (16). Our previous studies also showed that miRNAs play an important role in the differentiation of cancer stem cells and stromal cells (17, 18). In this study, we examined the role of miR-21 in the malignant transformation of phyllodes tumor and investigated whether miRNA plays a role in FMT.

Patients and Methods

Patients and tissue samples

Breast phyllodes tumor samples were obtained from 268 female patients with 167 benign, 36 borderline, and 65 malignant phyllodes tumors in the Breast Tumor Center, SunYat-Sen Memorial Hospital, Sun Yat-Sen University (Guangzhou, People’s Republic of China), from January 2000 to June 2011. The patients were followed up for 8 to 148 months (median follow-up, 112 months). Pathologic diagnosis, as well as mitoses and stromal overgrowth status, was confirmed by two pathologists independently. Fresh phyllodes tumor samples were obtained within 20 minutes after resection and were snap-frozen in liquid nitrogen for miRNA assay. The remaining tissues were fixed and embedded for studies of histology, immunohistochemistry (IHC), and in situ hybridization (ISH). All samples were collected with informed consent according to the Internal Review and Ethics Boards of SunYat-Sen Memorial Hospital.

ISH and data analysis

miR-21 expression was examined by ISH on the formalin-fixed and paraffin-embedded sections of breast phyllodes tumors. Briefly, after dewaxing and rehydration, samples were digested with proteinase K, fixed again in 4% paraformaldehyde, hybridized with the 5′-digoxin-labeled LNA TM-modified miR-21 probe (20 nmol/L, 38102–01; Exiqon) at 51°C overnight, and then incubated washed at 4°C with anti-digoxin monoclonal antibody (1:1,000; 1193274910; Roche Applied Science). After being stained with nitroblue tetrazolium/5-bromo–4-chloro–3-indolyl phosphate solution in the dark, the sections were mounted and evaluated. To determine the colocalization of miR-21 and α-SMA, FITC-conjugated anti-digoxin monoclonal antibody (1:200; 1120774191; Roche Applied Science) in combination with 594-conjugated goat antibodies against mouse (1:200; A11037; Invitrogen) were used in the hybridization assays. The sections were then examined by confocal microscopy. The staining scores were determined on the basis of both the intensity and proportion of positive cells in 10 random fields under ×400 magnification as described before (19). The staining index (SI) was calculated as follows: SI = staining intensity × proportion of positively stained cells. A SI score of 4 was used as a cutoff value based on the distribution of frequency of SI score for miR-21 expression and the expression levels of miR-21 were defined as high (SI > 4) or low (SI ≤ 4).

Separation and culture primary stromal cells from breast phyllodes tumors

Normal breast stromal cells were extracted from the breast stroma of four samples obtained by reduction mammoplasty. Stromal cells from fibroadenoma and phyllodes tumors (8 fibroadenoma, 8 benign, and 8 malignant phyllodes tumors) were isolated from tumors obtained by lpectomies or mastectomies. Briefly, the samples were mechanically disaggregated and digested with collagenase type III (1 mg/mL; Boehringer Mannheim) and hyaluronidase (125 U/mL; Sigma) at 37°C with agitation for 12 to 18 hours in Dulbecco’s Modified Eagle Medium with 10% fetal calf serum. The dissociated tissues were incubated, followed by the centrifugation at 250 × g for 5 minutes. The pellet was resuspended. For isolating primary cultured fibroblasts, isolated cells above were followed by differential sedimentation, platting, and growth in high serum media conditions that select for fibroblast growth. Fibroblast was then expanded and stored when cells underwent two to three population doublings within total 8 to 10 days after tissue dissociation. We used fibroblasts passaged for up to five population doublings for subsequent experiments, to minimize clonal selection and culture stress that could occur during extended tissue culture.

Animal experiment

All procedures of animal experiments were approved by the Animal Care and Use Committee of Sun Yat-Sen University and conformed to the legal mandates and national guidelines for the care and maintenance of laboratory animals. Breast phyllodes tumors stromal cells (1 × 10^5) mixed with Matrigel in equal volume were inoculated into the mammary fat pads of 6-week-old female nude mice. When the xenografts were palpable (around 0.5 cm in diameter), Lipoexantine alone (5 μL) or with lin4/miR-21 mimics (15 μg/injection) or lin4/miR-21 antisense oligonucleotides (ASO; 20 μg/injection) was injected into the tumor twice a week. Tumor growth was evaluated by monitoring tumor volume (TV = length × width^2 / 2) every 3 days for 8 weeks. The animals were sacrificed when the xenografts reached 1.5 cm in diameter. Tumor xenografts as well as the livers and lungs of mice were harvested for further evaluation. Paraffin sections (4 μm) of the harvested livers and lungs were stained with hematoxylin and eosin (H&E) for histologic assessment; RNA and protein were extracted from the tumors for qRT-PCR and Western blot analysis.

Statistical analysis

The in vitro data were depicted as mean ± SD of three independent experiments performed in triplicate. All statistical analyses were performed using SPSS 16.0 statistical software package (SPSS). Student t test and one-way ANOVA were used to compare the markers of myofibroblasts and miR-21...
expression levels between the phyllodes tumors with different tumor grades, whereas \( \chi^2 \) test was used to analyze the relationship between \( \alpha\)-SMA, miR-21 expression, and clinicopathologic status. Kaplan–Meier curves and log-rank test were used to compare the local recurrence-free survival (LRFS) and overall survival (OS) in different patient groups. Spearman order correlations were used to measure the association between different variables. Receiver operator characteristic (ROC) curves were constructed by plotting sensitivity versus specificity, and the areas under the curves (AUC) were calculated with the Hanley and McNeil method. In all cases, \( P<0.05 \) was considered statistically significant.

Results

**Myofibroblast differentiation is associated with malignant progression of phyllodes tumors and is an independent prognostic marker for phyllodes tumor patients**

To investigate whether myofibroblast differentiation is associated with malignant progression of phyllodes tumors, we examined the presence of myofibroblasts in 268 phyllodes tumor samples, including 167 benign, 36 borderline, and 65 malignant phyllodes tumors. Normal breast tissue and fibroadenoma were used as control. First, we tested the expression of Ki67 and myofibroblast markers, including \( \alpha\)-SMA, fibroblast activation protein (FAP), and stromal cell–derived factor-1 (SDF-1), in paraffin-embedded phyllodes tumor samples by IHC. Similar to previous studies (20–22), the expression of Ki67 increased significantly with phyllodes tumors grade (Fig. 1A). Interestingly, the expression of \( \alpha\)-SMA, FAP, and SDF-1 was also progressively increased from normal breast tissue and fibroadenoma to benign, borderline, and malignant phyllodes tumors (Fig. 1A).

To confirm the myofibroblast markers are overexpressed in the stromal cells of phyllodes tumors, we measured the mRNA and protein levels of \( \alpha\)-SMA, FAP, and SDF-1 in the primary stromal cells isolated from normal breast tissue, fibroadenoma, benign, borderline, and malignant phyllodes tumors with eight cases per group. Using qRT-PCR, we found that the mRNA levels of \( \alpha\)-SMA, FAP, and SDF-1 were progressively increased from normal breast tissue and fibroadenoma to benign, borderline, and malignant phyllodes tumors (Fig. 1B). Immunofluorescent (IF) staining (Fig. 1C) and Western blot analysis (Supplementary Fig. S1A) also showed the similar results at the protein levels of \( \alpha\)-SMA, FAP, SDF-1. ELISA assay, which measured the secretary SDF-1 protein in the medium of stromal cells, showed that the SDF-1 level in malignant phyllodes tumors is significantly higher than that in benign and borderline phyllodes tumors (Supplementary Fig. S1B). Together, these results indicate that myofibroblast differentiation is associated with the malignant progression of phyllodes tumors.

Next, we tested whether the myofibroblast markers could be of prognostic value for patients with phyllodes tumors. The 268 patients with phyllodes tumors were followed up for 8 to 148 months (median follow-up, 112 months). During the follow-up, 49 cases were diagnosed with recurrence, including 18 in benign group, 9 in borderline group, and 22 in malignant group. In addition, 31 cases were diagnosed with metastasis, with 3 in borderline group and 28 in malignant group.

The efficacy of \( \alpha\)-SMA, FAP, and SDF-1 to predict recurrence of phyllodes tumors was calculated by ROC curve, a commonly used tool to evaluate the value of diagnostic markers (23). The ROC curve analysis showed that \( \alpha\)-SMA performed better in predicting the patients’ prognosis [recurrence/metastasis, AUC, 0.89/0.94; 95% confidence interval (CI), 0.83–0.94/0.89–0.98] than FAP (recurrence/metastasis, AUC, 0.69/0.75; 95% CI, 0.60–0.78/0.66–0.85) and SDF-1 (recurrence/metastasis, AUC, 0.74/0.78; 95% CI, 0.66–0.82/0.70–0.87; Fig. 1D and E). Because AUC values higher than 0.8 are believed to represent good discrimination (24), these results suggest that the levels of \( \alpha\)-SMA could be used to predict the prognosis of phyllodes tumors.

**SDF-1 expression levels are upregulated during malignant progression of phyllodes tumors**

It has been reported that miRNAs are important regulators of key biologic processes, including differentiation (18). To investigate whether miRNA plays a role in the myofibroblast differentiation of phyllodes tumors, we used miRNA array to compare miRNA profiles between two benign and two malignant phyllodes tumors. The criteria used to screen differentially expressed miRNAs between benign and malignant phyllodes tumors were fold change \( >3.0 \) or \( <0.33 \), normalized data \( \geq 1 \) in all samples, and the expression is consistently increased or decreased in both malignant phyllodes tumors than two benign phyllodes tumors. Among the 1,285 human miRNAs in the array, 18 miRNAs were upregulated and three were downregulated in malignant phyllodes tumors compared with benign phyllodes tumors (Fig. 2A). Among these differentially expressed miRNAs, three miRNAs, including miR-21, miR-130b, and miR-92a, caught our attention because these miRNAs have been reported to modulate both differentiation and malignant progression of cancer (25–30).

The microarray results of miR-21, miR-130b, and miR-92a were confirmed with qRT-PCR in stromal cells isolated from eight cases each of benign and malignant phyllodes tumors. Nevertheless, the expression of miR-130b or miR-92a was also significantly upregulated (Supplementary Fig. S2A) or downregulated (Supplementary Fig. S2B) in the stromal cells of fibroadenoma, respectively. Because fibroadenoma is still a benign disease and has very low chance of becoming malignant (31), the results indicate that these two miRNAs may not play a significant role in the malignant transformation of phyllodes tumors. However, the expression of miR-21 was similar between normal breast tissue and fibroadenoma, but was significantly upregulated by 4.3-, 11.1-, and 20.6-fold in benign, borderline, and malignant phyllodes tumors, respectively (Fig. 2B; \( P<0.001 \)). The progressive increase of miR-21 in malignant progression of phyllodes tumors suggests that miR-21 may regulate the malignant progression of phyllodes tumors and its myofibroblast differentiation.

To confirm the findings of miR-21 expression in more clinical samples, we examined miR-21 expression in the 268...
parafin-embedded phyllodes tumor specimen using miRNA locked nucleic acid ISH (LNA-ISH). The results showed moderate or strong miR-21 staining in borderline or malignant phyllodes tumors, compared with only minimal cytoplasmic staining of miR-21 in benign phyllodes tumors (Fig. 2C).

To determine whether miR-21 is expressed in the myofibroblasts, but not other fibroblasts in phyllodes tumors, double staining of α-SMA IHC and miR-21 LNA-ISH was done and the results demonstrated that miR-21 signals were primarily colocalized with α-SMA signals (Fig. 2D). Furthermore, the percentage of miR-21+ cells was positively correlated with that of α-SMA+ cells in the 268 phyllodes tumor samples (Fig. 2E; \( r = 0.817; P < 0.001 \)).

The ROC curve analysis showed that miR-21 is a good marker to predict the patients’ prognosis (recurrence/metastasis, AUC, 0.92/0.87; 95% CI, 0.87–0.96/0.79–0.94). More importantly, compared with histologic grading into benign, borderline, and malignant phyllodes tumors (recurrence/metastasis, AUC, 0.67/0.91; 95% CI, 0.58–0.75/0.87–0.94), both miR-21 and α-SMA performed better in predicting the recurrence of patients with phyllodes tumors, although no difference was observed in their ability to predict metastasis (\( P > 0.05; \) Fig. 2F and Supplementary Fig. S2C).

We next analyzed the association of α-SMA and miR-21 expression with the clinicopathologic status of phyllodes tumors (Table 1). The expression of α-SMA and miR-21 increased with higher tumor grade, mitotic activity, and
stromal overgrowth \((P < 0.001)\), but was not associated with the age and size of tumor (Table 1). The expression of \(\alpha\)-SMA and miR-21 was also more abundant in the phyllodes tumors with local recurrence and distal metastasis \((P < 0.001; \alpha\)-SMA and \(P < 0.01;\) and \(P < 0.05\) as compared with normal. C, ISH of miR-21 in paraflin-embedded benign, borderline, and malignant phyllodes tumors. Scramble RNA was used as a negative control. D, representative images of colocalization of miR-21 (green) and \(\alpha\)-SMA (red) in the phyllodes tumor sample, with ISH staining for miR-21 and IHC staining for \(\alpha\)-SMA. E, correlations between the percentage of miR-21 \(^+\) cells and the percentage of \(\alpha\)-SMA \(^+\) cells in breast phyllodes tumor samples. F, ROC curves were conducted to evaluate the value of miR-21, \(\alpha\)-SMA and grade for predicting the recurrence of phyllodes tumors. G, Kaplan–Meier OS curve of patients with low \((SI \leq 4)\) and high \((SI > 4)\) miR-21 staining.

**miR-21 induces myofibroblast differentiation and promotes the proliferation and invasion of the stromal cells in phyllodes tumors**

It was shown above that elevated miR-21 expression was primarily localized to \(\alpha\)-SMA–expressing myofibroblast. To study whether miR-21 can induce the myofibroblast differentiation of stromal cells in phyllodes tumors, we transfected the primary stromal cells from benign phyllodes tumors with miR-21 mimics, or the cells from malignant phyllodes tumors with miR-21 ASOs to modulate their miR-21 expression. miR-21 mimics, but not control Lin4 mimics, significantly increased the mRNA and protein levels of \(\alpha\)-SMA, FAP, and SDF-1 in the primary stromal cells from benign phyllodes tumors (Fig. 3A and Supplementary Fig. S3A). On the other hand, miR-21 ASO, but not Lin4 ASO, markedly inhibited the expression of miR-21. miR-21 expression is upregulated during malignant progression of phyllodes tumors (PT). A, miRNA array analysis shows differentially expressed miRNAs in two cases each of benign phyllodes tumor and malignant phyllodes tumor. B, real-time qRT-PCR quantified the expression of miR-21 in the stromal cells of normal breast tissue (Normal), fibroadenoma (FA), and breast phyllodes tumors. Bars, RNA expression normalized to U6 (mean \pm SD). \(*\), \(P < 0.001; \#\#\), \(P < 0.01;\) and \(*\), \(P < 0.05\) as compared with normal. C, ISH of miR-21 in paraflin-embedded benign, borderline, and malignant phyllodes tumors. Scramble RNA was used as a negative control. D, representative images of colocalization of miR-21 (green) and \(\alpha\)-SMA (red) in the phyllodes tumor sample, with ISH staining for miR-21 and IHC staining for \(\alpha\)-SMA. E, correlations between the percentage of miR-21 \(^+\) cells and the percentage of \(\alpha\)-SMA \(^+\) cells in breast phyllodes tumor samples. F, ROC curves were conducted to evaluate the value of miR-21, \(\alpha\)-SMA and grade for predicting the recurrence of phyllodes tumors. G, Kaplan–Meier OS curve of patients with low \((SI \leq 4)\) and high \((SI > 4)\) miR-21 staining.
myofibroblast markers in the stromal cells from malignant phyllodes tumors (Fig. 3A and Supplementary Fig. S3A). Similar results were obtained for the SDF-1 levels in media determined by ELISA (Supplementary Fig. S3B). These results suggest that miR-21 induces the expression of myofibroblast markers in the stromal cells of phyllodes tumors.

Because myofibroblasts are known to have an increased ability to induce collagen gel contraction (30), collagen contraction assay was used to test whether miR-21-treated stromal cells have the function of myofibroblast. Indeed, we observed that benign stromal cells transfected with miR-21 mimics contracted collagen gels to a much greater extent than cells transfected with Lin4 mimics. In contrast, the contractile ability was significantly decreased in malignant stromal cells when transfected with miR-21 ASO (Fig. 3B and Supplementary Fig. S3C). Together, these findings suggest that miR-21 induce myofibroblast function in the stromal cells of phyllodes tumors.

Previous studies reported that myofibroblasts in epithelial tumors have an increased proliferative activity and can

### Table 1. Correlations of α-SMA and miR-21 expression with clinicopathologic status in 268 cases of patients with breast phyllodes tumors

<table>
<thead>
<tr>
<th>Characteristics</th>
<th>α-SMA</th>
<th>miR-21</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>SI ≤ 4</td>
<td>SI &gt; 4</td>
</tr>
<tr>
<td>Age, y</td>
<td></td>
<td></td>
</tr>
<tr>
<td>&lt;40 (114)</td>
<td>69</td>
<td>45</td>
</tr>
<tr>
<td>≥40 (154)</td>
<td>87</td>
<td>67</td>
</tr>
<tr>
<td>Grade</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Benign (167)</td>
<td>123</td>
<td>44</td>
</tr>
<tr>
<td>Borderline (36)</td>
<td>15</td>
<td>21</td>
</tr>
<tr>
<td>Malignant (65)</td>
<td>18</td>
<td>47</td>
</tr>
<tr>
<td>Tumor size, cm</td>
<td></td>
<td></td>
</tr>
<tr>
<td>&lt;5 (166)</td>
<td>102</td>
<td>64</td>
</tr>
<tr>
<td>≥5 (102)</td>
<td>54</td>
<td>48</td>
</tr>
<tr>
<td>Local recurrence</td>
<td></td>
<td></td>
</tr>
<tr>
<td>No (219)</td>
<td>152</td>
<td>67</td>
</tr>
<tr>
<td>Yes (49)</td>
<td>4</td>
<td>45</td>
</tr>
<tr>
<td>Metastasis</td>
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</tr>
<tr>
<td>No (237)</td>
<td>155</td>
<td>82</td>
</tr>
<tr>
<td>Yes (31)</td>
<td>1</td>
<td>30</td>
</tr>
<tr>
<td>Mitoses</td>
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<td></td>
</tr>
<tr>
<td>&lt;5/HPF (173)</td>
<td>128</td>
<td>45</td>
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<tr>
<td>5–10/HPF (34)</td>
<td>11</td>
<td>23</td>
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<tr>
<td>&gt;10/HPF (61)</td>
<td>17</td>
<td>44</td>
</tr>
<tr>
<td>Stomal overgrowth</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Absence (182)</td>
<td>131</td>
<td>51</td>
</tr>
<tr>
<td>Present (86)</td>
<td>25</td>
<td>61</td>
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### Table 2. Multivariate Cox proportional hazard analysis of LRFS in 268 patients with breast phyllodes tumors

<table>
<thead>
<tr>
<th>Variable</th>
<th>$\chi^2$</th>
<th>P</th>
<th>HR (95% CI)</th>
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<tr>
<td>Age</td>
<td>3.698</td>
<td>0.055</td>
<td>0.560 (0.310–1.013)</td>
</tr>
<tr>
<td>Size</td>
<td>0.485</td>
<td>0.486</td>
<td>1.242 (0.675–2.285)</td>
</tr>
<tr>
<td>Grade</td>
<td>4.718</td>
<td>0.030</td>
<td>0.605 (0.393–0.931)</td>
</tr>
<tr>
<td>Mitoses</td>
<td>0.116</td>
<td>0.734</td>
<td>1.152 (0.511–2.598)</td>
</tr>
<tr>
<td>Stromal overgrowth</td>
<td>16.085</td>
<td>&lt;0.001</td>
<td>5.742 (2.518–13.091)</td>
</tr>
<tr>
<td>α-SMA</td>
<td>12.527</td>
<td>&lt;0.001</td>
<td>5.644 (1.785–17.851)</td>
</tr>
<tr>
<td>FAP</td>
<td>3.570</td>
<td>0.059</td>
<td>1.734 (0.976–3.081)</td>
</tr>
<tr>
<td>SDF-1</td>
<td>1.705</td>
<td>0.192</td>
<td>1.521 (0.804–2.879)</td>
</tr>
<tr>
<td>miR-21</td>
<td>9.815</td>
<td>0.002</td>
<td>4.869 (1.527–15.524)</td>
</tr>
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</table>
promote cancer invasion and metastasis (9, 14). We then examined the effects of miR-21 on the proliferation, migration, and invasion of primary stromal cells from phyllodes tumors. Cell viability and clonogenic assays showed that miR-21 mimics increased, whereas miR-21 ASO suppressed the growth of stromal cells (Fig. 3C and Supplementary Fig. S3D and S3E), indicating that miR-21 promotes the proliferation of stromal cells. Boyden chamber assays also showed that miR-21 mimics, but not lin4 mimics, significantly increased the number of migrated (Fig. 3D and Supplementary Fig. S3F) and invaded benign stromal cells (Fig. 3E and Supplementary Fig. S3G; P < 0.01). In contrast, miR-21 ASO drastically decreased the number of migrated (Fig. 3D and Supplementary Fig. S3F) and invaded malignant stromal cells (Fig. 3E and Supplementary Fig. S3G; P < 0.01). These data suggest that miR-21 not only induces the myofibroblast differentiation of stromal cells, but also promotes their malignant properties, including proliferation and invasion.

Smad 7 and PTEN are targets of miR-21 in phyllodes tumors

To identify the targets for miR-21, we used miRNA Target Scan and miRBase databases to screen for genes that are targeted by miR-21. Smad7 and PTEN were identified to be potential miR-21 targets because their 3'-untranslated region (3'-UTR) contains sequences that are complementary to miR-21. To evaluate whether miR-21 targets the 3'-UTRs of Smad7 and PTEN, we used a luciferase reporter vector cloned with the 3'-UTR of Smad7 or PTEN. miR-21 mimics significantly decreased the luciferase activities of these reporters, while having no effect on the reporters cloned with mutated miR-21 binding sites (Supplementary Fig. S4A). In addition, the basal levels of Smad7 and PTEN protein were much higher in benign stromal cells than those in malignant cells. Furthermore, miR-21 mimics decreased, whereas miR-21 ASO increased, the protein levels of Smad7 and PTEN in stromal fibroblasts (Fig. 4A), suggesting that miR-21 directly regulates Smad7 and PTEN. To study the role of Smad7 and PTEN in myofibroblast differentiation, we knocked down the expression of Smad7 and PTEN (Supplementary Fig. S4B–S4E) and found that the protein levels of α-SMA or FAP were significantly increased after silencing Smad7 or PTEN, respectively (Fig. 4B and C), indicating that both Smad7 and PTEN are involved in the myofibroblast differentiation of stromal cells in phyllodes tumors.

We then restored the expression of Smad7 and PTEN with their expression vector containing mutated 3'-UTRs in the miR-21-treated benign stromal cells and measured the effect...
on the expression of myofibroblasts markers as well as the proliferation, migration, invasion, and collagen contraction activity. Interestingly, restoration of Smad7, but not PTEN, decreased the mRNA and protein levels of α-SMA (Fig. 4D and Supplementary Fig. S5A) and abrogated the miR-21-promoted effects on migration, invasion, and collagen gel contraction in benign stromal cells transfected with miR-21 mimics (Fig. 4E and Supplementary Fig. S5B–S5D). In contrast, restoring PTEN, but not Smad7, decreased the mRNA and protein level of FAP in benign cells (Fig. 4D and Supplementary Fig. S5A), and thus abrogated the miR-21-promoted effects on proliferation (Fig. 4E and Supplementary Fig. S5E). Collectively, these data suggest that miR-21 targets Smad7 to induce the expression of α-SMA and promotes the migration, invasion, and collagen gel contraction of the cells, while miR-21 also targets PTEN to induce the expression of FAP and enhance the proliferation of the cells.

miR-21 induces myofibroblasts differentiation, accelerates tumor growth, and promotes metastasis of breast phyllodes tumors xenografts

To investigate the role of miR-21 on tumor progression in vivo, athymic nude mice were inoculated with stromal cells from benign or malignant phyllodes tumors in their mammary fat pads. When the xenografts became palpable, miR-21 mimic or ASO was injected into the tumor twice a week. Injection of miR-21 mimics, but not nonrelevant lin4 mimics, significantly accelerated tumor growth of xenografts using stromal cells from benign phyllodes tumors (P < 0.01; Fig. 5A). On the other hand, injection of miR-21 ASO, but not lin4 ASO, markedly inhibited tumor growth of xenografts using stromal cells from malignant phyllodes tumors (P < 0.01; Fig. 5A). In agreement with the tumor growth, the tumor formation efficiency was also increased by miR-21 mimics in benign stromal cells and reduced by miR-21 ASO in malignant stromal cells.
miR-21 play an important role in the malignant transformation of breast phyllodes tumors. To further evaluate whether miR-21 regulate the expression of myofibroblast markers as well as cell proliferation in vivo, we examined the mRNA and protein levels of α-SMA, FAP, and SDF-1 in the xenografts using qRT-PCR (Supplementary Fig. S6A), Western blot analysis (Fig. 5B), and IHC (Fig. 5C). We also used ISH to localize the miR-21 expression in the xenografts. Similar to the results obtained in vitro, injection of miR-21 mimics enhanced, whereas miR-21 ASO attenuated, the expression of α-SMA, FAP, SDF-1, and Ki67 in the xenografts.

Increased migration and invasion are linked with metastasis. Thus, we evaluated whether miR-21 promotes the metastasis of breast phyllodes tumors xenografts. Consistent with the H&E staining results (Fig. 5D), miR-21 mimics significantly enhanced the liver metastasis of xenografts using stromal cells from benign phyllodes tumors from 0% to 50%, whereas miR-21 ASOs markedly suppressed the liver metastasis of xenografts using stromal cells from malignant phyllodes tumors from 60% to 70% to 10% (Supplementary Table S1). Quantitative PCR also showed that human hypoxanthine phosphoribosyltransferase (HPRT) mRNA in mouse liver was increased by 3.7-fold by miR-21 mimics, but was significantly decreased by miR-21 ASO.
(Fig. 5E). However, there was no significant lung metastasis observed in the mice according to histologic examination, human HPRT miRNA level (Fig. 5E), and wet weight of lung (Supplementary Fig. S6B). Together, these results indicate that miR-21 promotes myofibroblast differentiation, tumor formation as well as liver metastasis in breast phyllodes tumor xenografts.

Discussion

In this study, we have found that myofibroblast differentiation, driven by the upregulation of miR-21, is progressively increased during the malignant progression of phyllodes tumors in breast. Furthermore, α-SMA and miR-21 can serve as independent prognostic markers of phyllodes tumors, with their predictive value better than histologic classification.

It has been well established that the prognostic value of histologic markers in phyllodes tumors is not as good as that in cancer. Even benign phyllodes tumors can have malignant potential and a significant portion of them can recur locally. It was reported that the local recurrence rates of patients with benign, borderline, and malignant phyllodes tumors after surgery were 21% (111 of 540), 46% (18 of 39), and 65% (26 of 40), respectively (3). In our study, the local recurrence rates are 11% (18 of 167), 25% (9 of 36), and 34% (22 of 65) for the patients with benign, borderline, and malignant phyllodes tumors correspondingly. Importantly, the local recurrence rates for patients with low α-SMA or low miR-21 expression are 2.6% (4 of 156) or 2.6% (4 of 152), whereas the ones for patients with high α-SMA or high miR-21 expression are 40% (45 of 112) or 39% (45 of 116). Furthermore, the ROC curve analysis showed that both miR-21 (AUC, 0.92) and α-SMA (AUC, 0.89) performed better in predicting the recurrence than histologic grade (AUC, 0.67), suggesting that miR-21 and α-SMA could serve as novel molecular markers to predict the recurrence of phyllodes tumors.

Previous studies reported that stromal myofibroblasts are important promoters of tumor growth and progression in multiple cancer types, but myofibroblast is hardly reported to be a direct tumor-initiating component of tumors. Fibroblast is a major component of phyllodes tumors and the recurring or metastasizing behavior of phyllodes tumors is determined by the properties of fibroblasts. Here, we show, for the first time, that myofibroblast is the major malignant component of phyllodes tumors. FMT, driven by upregulated miR-21, underlies the malignant transformation of phyllodes tumors. Inhibition of miR-21 reversed the FMT and decreased the malignancy of phyllodes tumors, suggesting that miR-21 could be a therapeutic target in phyllodes tumors.

miR-21 has been reported to be an important oncomir in many types of cancer (32–34). Most of the studies so far focused on the increased expression of miR-21 in cancer cells or tumor mixture. However, it was reported that miR-21 expression was predominantly seen in cancer-associated fibroblast-like cells in breast cancer, with no difference in expression levels between low- and high-grade cancers (35). It was also shown that miR-21 expression in esophageal squamous-cell carcinoma was mainly localized in the cytoplasm of stromal cells adjacent to malignant cells (36) Thus, it is possible that myofibroblast transition, driven by high miR-21 in the stromal cells, could also be an important tumor-promoting event in many epithelial cancers. On the other hand, breast cancer cells can directly induce the expression of myofibroblast markers in fibroblasts (37), suggesting that both primary tumor and myofibroblasts can work together to promote tumorigenesis. Highly expressed miR-21 could be shuttled between cancer cells and fibroblasts in the form of secreted exosomes to play their function in respective cells.

Besides its tumor-promoting role, miR-21 is also implicated in drug resistance (38) as well as radiation resistance (39) of cancer cells. It is possible that high miR-21 in phyllodes tumors is not only important for the malignant progression of phyllodes tumors, but also may be responsible for its poor response to chemotherapy and radiotherapy. Antagonizing miR-21 in phyllodes tumors may sensitize tumors to chemotherapy or radiotherapy, in addition to the decreased malignancy.

PTEN is a well-established target of miR-21. Our study demonstrated that miR-21 inhibited PTEN expression, and thus induced FAP expression. FAP, a member of the serine protease family, has been shown to support tumor growth and proliferation (40). A recent study also showed that miR-21 induced lung fibrosis by targeting Smad7 and mediating pulmonary fibroblasts differentiation into myofibroblasts (30). Our results demonstrated that miR-21 induced FMT and α-SMA expression via the downregulation of Smad7. Together, our data indicate that miR-21 downregulates PTEN and Smad-7, which then upregulates the expression of FAP and α-SMA that are responsible for the enhanced proliferation and invasiveness of phyllodes tumors respectively.

In summary, our study suggests an important role of FMT in the malignant transformation of phyllodes tumors that are driven by increased miR-21. Inhibition of miR-21 may be a novel approach in treating phyllodes tumors. α-SMA and miR-21 are independent predictors of recurrence for phyllodes tumors.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

Authors’ Contributions

Conception and design: C. Gong, Y. Nie, E. Song, Q. Liu
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Acquisition of data (provided animals, acquired and managed patients, provided facilities, etc.): C. Gong, Y. Nie, E. Song, H. Yao
Analysis and interpretation of data (e.g., statistical analysis, biostatistics, computational analysis): C. Gong, Y. Nie, S. Qu, J.-Y. Liao, H. Yao, F. Su, Q. Liu
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Administrative, technical, or material support (i.e., reporting or organizing data, constructing databases): Y. Zeng, Q. Liu
Study supervision: E. Song, Q. Liu

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