miR-21 induces myofibroblast differentiation and promotes the malignant progression of breast phyllodes tumors

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Abstract

Phyllodes tumors (PTs) of breast, even histologically diagnosed as benign, can recur locally and have metastatic potential. Histological markers only have limited value in predicting the clinical behavior of PTs. It remains unknown what drives the malignant progression of PTs. We found that the expression of myofibroblast markers, α-SMA, FAP and SDF-1, is progressively increased in the malignant progression of PTs. Microarray showed that miR-21 was one of the most significantly upregulated microRNAs in malignant PTs compared with benign PTs. 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 histological grading. Furthermore, miR-21 mimics promoted, while 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 PTs. 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 PT 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 PTs that is driven by increased miR-21.

Introduction

Phyllodes tumors (PTs) of the breast are typically large and fast growing tumors that accounts for up to 1% of all breast neoplasms(1). Although many PTs tend to
behave in a benign manner, the clinical outcome of PTs 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 PTs (2). However, even with wide surgical resection, local recurrence rate is still as high as 8%-36% (3). Furthermore, recurrent PTs showed a progression toward more malignant phenotype (4) with the acquisition of new genetic changes (5). It was reported that 22% of PTs that have undergone malignant transformation give rise to haematogenous metastasis (6). It remains unclear what drives malignant transformation of PTs and existing biological markers only have a limited value in predicting prognosis.

PTs, 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 PTs are regarded as having malignant potential, PTs 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 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 PTs (14). However, whether the fibroblasts-myofibroblasts transition (FMT) plays a role in the malignant transformation of PT and whether myofibroblast can be a prognostic marker of PTs are not known yet.

MicroRNAs (miRNAs) are non-coding RNAs that regulate gene expression and can be master regulators of many fundamental biological 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 myofibroblasts in the malignancy of PTs and investigated whether miRNA plays a role in FMT.

Materials and Methods

Patients and Tissue Samples

Breast phyllodes tumor samples were obtained from 268 female patients with 167 benign, 36 borderline and 65 malignant PTs in the Breast Tumor Center, SunYat-Sen Memorial Hospital, Sun Yat-Sen University, from January 2000 to June 2011. The patients were followed up for 8-148 months (median follow-up is 112 months). Pathological diagnosis, as well as mitoses and stromal overgrowth status, was
confirmed by two pathologists independently. Fresh PT 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, and in situ hybridization. All samples were collected with informed consent according to the internal review and ethics boards of SunYat-Sen Memorial Hospital.

In Situ Hybridization and data analysis

MiR-21 expression was examined by in situ hybridization (ISH) on the formalin-fixed and paraffin-embedded sections of breast PTs. Briefly, after dewaxing and rehydration, samples were digested with proteinase K, fixed again in 4% paraformaldehyde, hybridized with the 5’-digoxin-labeled LNATM-modified miR-21 probe (20nmol/L, 38102-01, Exiqon) at 51°C overnight, and then incubated overnight at 4°C with anti-digoxin mono-clonal antibody (1:1000, 11093274910, Roche Applied Science). After being stained with nitro blue 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 mono-clonal 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 based on 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 cut-off 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 PTs**

Normal breast stromal cells were extracted from the breast stroma of 4 samples obtained by reduction mammoplasty. Stromal cells from fibroadenoma and PTs (8 fibroadenoma, 8 benign and 8 malignant PTs) were isolated from tumors obtained by lumpectomies or mastectomies. Briefly, the samples were mechanically disaggregated and digested with collagenase type III (1 mg/ml; Boehringer Mannheim) and hyaluronidase (125 units/ml; Sigma) at 37°C with agitation for 12-18 hrs in Dulbecco’s Modified Eagle’s Medium (DMEM) with 10% fetal calf serum (FCS). The dissociated tissues were incubated, followed by the centrifugation at 250g for 5 min. The pellet was resuspended. For isolating primary cultured fibroblasts, isolated cells above were followed by differential sedimentation, plating, and growth in high serum media conditions which select for fibroblast growth. Fibroblast was then expanded and stored when cells underwent 2-3 population doublings (PDs) within total 8-10 days after tissue dissociation. We used fibroblasts passaged for up to 5 PDs for subsequent experiments, in order to minimize clonal selection and culture stress which 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 PTs stromal cells (1 × 10^7) mixed with matrigel in equal volume were inoculated into the mammary fat pads of six-weeks old female nude mice. When the xenografts were palpable (around 0.5 cm in diameter), Lipofectamine alone (5 μl) or with lin4/miR-21 minics (15 μg/injection) or lin4/miR-21 ASOs (20 μg/injection) was injected into the tumor twice a week. Tumor growth was evaluated by monitoring tumor volume (TV = length × width^2 × 0.5) every three days for eight 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. Cryosections (8 μm) of the harvested livers and lungs were stained with hematoxylin and eosin (HE) for histological assessment, RNA and protein were extracted for from the tumors for qRT-PCR and WB analysis.

**Statistics**

The in vitro data were depicted as mean ± S.D. of three independent experiments performed in triplicate. All statistical analyses were performed using SPSS 16.0 statistical software package (SPSS, Chicago, IL, USA). Student’s t test and one-way ANOVA was used to compare the markers of myofibroblasts and miR-21 expression levels between the PTs with different tumor grades, whereas chi-square test was used to analyze the relationship between α-SMA, miR-21 expression and clinicopathological 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 (1-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 PTs and is an independent prognostic marker for PT patients.

To investigate whether myofibroblast differentiation is associated with malignant progression of PTs, we examined the presence of myofibroblasts in 268 PT samples, including 167 benign, 36 borderline and 65 malignant PTs. 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 PT samples by immunohistochemistry (IHC). Similar to previous studies (20-22), the expression of Ki67 increased significantly with PTs 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 PTs (Fig. 1A).

To confirm the myofibroblast markers are over-expressed in the stromal cells of PTs, 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 PTs, with 8 cases per group. Using qRT-PCR, we found that
the mRNA levels of α-SMA, FAP and SDF-1 were progressively increased from normal breast tissue and fibroadenoma, to benign, borderline and malignant PTs (Fig. 1B). Immunofluorescent (IF) staining (Fig. 1C) and western blot (Supplementary Fig. S1A) also showed the similar results at the protein levels of α-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 PTs is significantly higher than that in benign and borderline PTs (Supplementary Fig. S1B). Together, these results indicate that myofibroblast differentiation is associated with the malignant progression of PTs.

Next, we tested whether the myofibroblast markers could be of prognostic value for PT patients. The 268 PT patients were followed up for 8-148 months (median follow-up is 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. Additionally, 31 cases were diagnosed with metastasis, with 3 in borderline group and 28 in malignant group.

The efficacy of α-SMA, FAP and SDF-1 to predict recurrence of PTs was calculated by receiver operating characteristic (ROC) curve, a commonly used tool to evaluate the value of diagnostic markers (23). The ROC curve analysis showed that α-SMA performed better in predicting the patients’ prognosis (recurrence/metastasis, area under curve (AUC)=0.89/0.94, 95% confidence interval (95% 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). Since AUC values higher than 0.8 are believed to
represent good discrimination (24), these results suggest that the levels of α-SMA could be used to predict the prognosis of PTs.

**miR-21 expression levels are up-regulated during malignant progression of PTs**

It has been reported that microRNAs (miRNAs) are important regulators of key biological processes including differentiation (18). To investigate whether miRNA plays a role in the myofibroblast differentiation of PTs, we used miRNA array to compare miRNA profiles between two benign and two malignant PTs. The criteria used to screen differentially expressed miRNAs between benign and malignant PTs were fold change > 3.0 or < 0.33, normalized data ≥ 1 in all samples, and the expression is consistently increased or decreased in both malignant PTs than two benign PTs. Among the 1285 human miRNAs in the array, 18 miRNAs were upregulated and 3 were downregulated in malignant PTs compared to benign PTs (Fig 2A). Among these differentially expressed miRNAs, three miRNAs including miR-21, miR-130b and miR-92a caught our attention because these microRNAs have been reported to modulate both differentiation and malignant progression of cancer (25-30).

The microarray results of miR-21, miR-30b and miR-92a were confirmed with quantitative RT-PCR (qRT-PCR) in stromal cells isolated from 8 cases each of benign and malignant PTs. 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 microRNAs may not play a
significant role in the malignant transformation of PTs. However, the expression of miR-21 was similar between normal breast tissue and fibroadenoma, but was significantly upregulated by 4.3-fold, 11.1 fold and 20.6-fold in benign, borderline and malignant PTs respectively (Fig. 2B, \( p < 0.001 \)). The progressive increase of miR-21 in malignant progression of PTs suggests that miR-21 may regulate the malignant progression of PTs and its myofibroblast differentiation.

To confirm the findings of miR-21 expression in more clinical samples, we examined miR-21 expression in the 268 paraffin-embedded PT specimen using miRNA locked nucleic acid \textit{in situ} hybridization (LNA-ISH). The results showed moderate or strong miR-21 staining in borderline or malignant PTs, compared with only minimal cytoplasmic staining of miR-21 in benign PTs (Fig. 2C).

To determine if miR-21 is expressed in the myofibroblasts, but not other fibroblasts in PTs, double staining of \( \alpha \)-SMA IHC and miR-21 LNA-ISH was done and the results demonstrated that miR-21 signals were primarily colocalized with \( \alpha \)-SMA signals (Fig. 2D). Furthermore, the percentage of miR-21\(^+\) cells was positively correlated with that of \( \alpha \)-SMA\(^+\) cells in the 268 PT 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 histological grading into benign, borderline and malignant PTs (recurrence/metastasis, AUC=0.67/0.91; 95% CI, 0.58-0.75/0.87-0.94), both miR-21 and \( \alpha \)-SMA performed better in predicting the recurrence of PT patients, 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 clinicopathological status of PTs (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 α-SMA and miR-21 was also more abundant in the PTs with local recurrence and distal metastasis \((p<0.001)\), Table 1). Further, Kaplan-Meier survival curve demonstrated that patients with low miR-21 expression (Staining index, SI≤4) have a longer OS and LRFS than those with high miR-21 expression \((p<0.001)\), Fig. 2G and Supplementary Fig. S2D). Importantly, multivariate Cox regression analyses demonstrated that miR-21 \((p=0.002)\), α-SMA \((p<0.001)\), stromal overgrowth \((p<0.001)\) and grade \((p=0.03)\) were independent prognostic predictors for LRFS. In addition, the analysis also showed that miR-21 \((p=0.017)\), α-SMA \((p=0.002)\), FAP \((p=0.002)\), stromal overgrowth \((p=0.003)\) and grade \((p=0.027)\) were independent prognostic predictors for OS (Table 2).

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

It was shown above that elevated miR-21 expression was primarily localized to α-SMA-expressing myofibroblast. To study whether miR-21 can induce the myofibroblast differentiation of stromal cells in PTs, we transfected the primary stromal cells from benign PTs with miR-21 mimics, or the cells from malignant PTs with miR-21 antisense oligonucleotides (ASO) to modulate their miR-21 expression.
miR-21 mimics, but not control Lin4 mimics, significantly increased the mRNA and protein levels of α-SMA, FAP and SDF-1 in the primary stromal cells from benign PTs (Fig. 3A and Supplementary Fig. S3A). On the other hand, miR-21 ASO, but not Lin4 ASO, markedly inhibited the expression of myofibroblast markers in the stromal cells from malignant PTs (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 PTs.

Since 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 PTs.

Previous studies reported that myofibroblasts in epithelial tumors have an increased proliferative activity and can 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 PTs. Cell viability and clonogenic assays showed that miR-21 mimics increased, while miR-21 ASO suppressed the growth of stromal cells (Fig. 3C and Supplementary Fig. S3D, E), indicating that miR-21 promote 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 (Fig. 3E and Supplementary Fig. S3G) malignant stromal cells \((p<0.01)\). 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 PTs.**

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’-UTR contain 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, while 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-E) 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 PTs.

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-D). 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 PTs 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 PTs 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 non-relevant lin4 mimics, significantly accelerated tumor growth of xenografts using stromal cells from benign PTs ($p<0.01$, 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 PTs ($p<0.01$, 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 (Supplementary Table S1). These data suggest that miR-21 play an important role in the malignant transformation of breast PTs.

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 $\alpha$-SMA, FAP and SDF-1 in the xenografts using qRT-PCR (Supplementary Fig. 6A), western blot (Fig. 5B) and immunohistochemistry (Fig. 5C). We also employed ISH to localize the miR-21 expression in the xenografts. Similar to the results obtained in vitro, injection of miR-21 enhanced, whereas miR-21 ASO attenuated, the expression of $\alpha$-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 PTs xenografts. Consistent with the
hematoxylin and eosin (H&E) staining results (Fig. 5D), miR-21 mimics significantly enhanced the liver metastasis of xenografts using stromal cells from benign PTs from 0% to 50%, whereas miR-21 ASOs markedly suppressed the liver metastasis of xenografts using stromal cells from malignant PTs from 60%-70% to 10% (Supplementary Table 1). Quantitative PCR also showed that human 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 histological examination, human HPRT mRNA 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 PT 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 PTs in breast. Further, α-SMA and miR-21 can serve as independent prognostic markers of PTs, with their predictive value better than histological classification.

It has been well established that the prognostic value of histological markers in PTs is not as good as that in cancer. Even benign PTs 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 PTs after surgery were 21% (111/540), 46% (18/39), and 65% (26/40) respectively (3). In our study, the local
recurrence rates are 11% (18/167), 25% (9/36) and 34% (22/65) for the patients with benign, borderline, and malignant PTs correspondingly. Importantly, the local recurrence rates for patients with low α-SMA or low miR-21 expression are 2.6% (4/156) or 2.6% (4/152), while the ones for patients with high α-SMA or high miR-21 expression are 40% (45/112) or 39% (45/116). 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 histological grade (AUC 0.67), suggesting that miR-21 and α-SMA could serve as novel molecular markers to predict the recurrence of PTs.

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 PTs and the recurring or metastasizing behavior of PTs is determined by the properties of fibroblasts. Here we show, for the first time, that myofibroblast is the major malignant component of PTs. Fibroblasts-myofibroblasts transition (FMT), driven by upregulated miR-21, underlies the malignant transformation of PTs. Inhibition of miR-21 reversed the FMT and decreased the malignancy of PTs, suggesting that miR-21 could be a therapeutic target in PTs.

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 grade 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 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 PTs, is not only important for the malignant progression of PTs, but also may be responsible for its poor response to chemotherapy and radiation therapy. Antagonizing miR-21 in PTs may re-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 PTs respectively.

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

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Figure Legends

Figure 1. Myofibroblast differentiation is associated with malignant progression of PTs and is an independent prognostic marker for PT patients. (A) Immunohistochemical (IHC) staining for α-SMA, FAP, SDF-1 and ki67 in paraffin-embedded normal breast tissue (Normal), fibroadenoma (FA) and breast phyllodes tumors (PT). (B) The mRNA level of α-SMA, FAP and SDF-1 determined by qRT-PCR in primary stromal cells of Normal, FA and breast PT. Bars indicate
RNA expression normalized to GAPDH (Mean ± SD). ***, \( p<0.001 \), **, \( p<0.01 \) and *, \( p<0.05 \) as compared with Normal. (C) Immunofluorescent staining of α-SMA and FAP in primary stromal cells of normal breast tissue, FA and breast PTs. (D-E) Receiver operator characteristic (ROC) curves were conducted to evaluate the value of α-SMA, FAP and SDF-1 for predicting the recurrence (D) and metastasis (E) of PTs.

Figure 2. miR-21 expression is up-regulated during malignant progression of PTs. (A) miRNA array analysis shows differentially expressed miRNAs in two cases each of benign PT and malignant PT. (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 (PT). Bars indicate RNA expression normalized to U6 (Mean ± SD). ***, \( p<0.001 \), **, \( p<0.01 \) and *, \( p<0.05 \) as compared with Normal. (C) In situ hybridization (ISH) of miR-21 in paraffin-embedded benign, borderline and malignant PTs. Scramble RNA was used as a negative control. (D) Representative images of colocalization of miR-21(green) and α-SMA (red) in the PT sample, with ISH staining for miR-21 and IHC staining for α-SMA. (E) Correlations between the percentage of miR-21\(^+\) cells and the percentage of α-SMA\(^+\) cells in breast PT samples. (F) Receiver operator characteristic (ROC) curves were conducted to evaluate the value of miR-21, α-SMA, and grade for predicting the recurrence of PTs. (G) Kaplan-Meier overall survival (OS) curve of patients with low (SI≤4) and high (SI>4) miR-21 staining.

Figure 3. miR-21 induces myofibroblast differentiation and promotes the proliferation and invasion of the stromal cells in PTs.
(A) The protein level of α-SMA, FAP and SDF-1 in benign PT stromal cells treated with mock transfection, or transfected with lin4 mimics (lin4) or miR-21 mimics (miR-21) and in malignant PTs stromal cells treated with mock transfection, or transfected with lin4 ASO or miR-21ASO.

(B) Collagen gel contraction was measured in stromal cells treated as in A. (C) Colony formation assays in stromal cells treated as in A. (D and E) Representative images of Boyden chamber assay for migrated (D) and invaded (E) stromal cells treated as in A.

Figure 4. Smad 7 and PTEN are targets of miR-21 in PTs.

(A) The protein level of smad7 and PTEN in benign PTs stromal cells treated with mock transfection, or transfected with lin4 mimics (lin4) or miR-21 mimics (miR-21) and in malignant PTs tromal cells treated with lin4 ASO transfection (NC), or transfected with mock or miR-21ASO.(B and C) The protein level of α302-SMA and FAP in the stromal cells of benign PTs with smad7 (B) and PTEN (C) knockdown by RNAi.(D) The protein level of α-SMA and FAP in untreated malignant PTs stromal cells, and in benign PTs stromal cells treated with mock transfection, or miR-21 mimics alone (miR-21), or co-transfection with empty vectors, or co-transfection with either or both of Smad7 and PTEN expressing vectors with their 3’UTRs mutated for miR-21 binding sites.(E) Representative images of Boyden chamber assay for migrated and invaded cells, the collagen gel contraction and colony formation, cells are treated as in D.

Figure 5. miR-21 induces myofibroblasts differentiation, accelerates tumor growth and
promotes metastasis of breast PTs xenografts.

(A) Tumors volume was measured in athymic nude mice that were inoculated into mammary fat pads with benign (B) and malignant (M) PTs stromal cells. When the xenografts were palpable, intratumor injection with lipofectamine alone (mock) or in combined with lin4 mimics (lin4) or miR-21 mimics (miR-21) for the benign PTs xenografts, or with lin4 ASO or miR-21 ASO for the malignant PTs xenografts, *, $P<0.05$ and **, $P<0.01$ as compared with mock;(B) The protein levels of $\alpha$-SMA, FAP and SDF-1 in the xenografts as described in A.(C) IHC staining for $\alpha$-SMA, FAP and SDF-1 and ki67, and ISH staining for miR-21 in the xenografts as described in A.(D) Representative images of H&E staining for xenografts as described in A showing the presence or absence of liver metastasis.(E) Expression of human HPRT mRNA relative to mouse 18S rRNA in the lungs and livers of mouse xenograts as described in A, **,$P<0.01$ as compared with lin4, ##, $P<0.01$ as compared with lin4 ASO.
Table 1. Correlations of α–SMA and miR-21 Expressing with Clinicopathological Status in 268 Cases of Patients with Breast PTs

<table>
<thead>
<tr>
<th>Characteristics</th>
<th>α–SMA</th>
<th></th>
<th>MiR-21</th>
<th></th>
</tr>
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<tbody>
<tr>
<td></td>
<td>SI≤4</td>
<td>SI&gt;4</td>
<td>SI≤4</td>
<td>SI&gt;4</td>
</tr>
<tr>
<td>Age (y)</td>
<td></td>
<td></td>
<td></td>
<td></td>
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<tr>
<td>&lt;40 (114)</td>
<td>69</td>
<td>45</td>
<td>0.508</td>
<td>67</td>
</tr>
<tr>
<td>≥40 (154)</td>
<td>87</td>
<td>67</td>
<td></td>
<td>85</td>
</tr>
<tr>
<td>Grade</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Benign (167)</td>
<td>123</td>
<td>44</td>
<td>&lt;0.001</td>
<td>120</td>
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<tr>
<td>Borderline (36)</td>
<td>15</td>
<td>21</td>
<td></td>
<td>14</td>
</tr>
<tr>
<td>Malignant (65)</td>
<td>18</td>
<td>47</td>
<td></td>
<td>18</td>
</tr>
<tr>
<td>Tumor size</td>
<td></td>
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<td></td>
<td></td>
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<tr>
<td>&lt;5 cm (166)</td>
<td>102</td>
<td>64</td>
<td>0.171</td>
<td>99</td>
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<tr>
<td>≥5 cm (102)</td>
<td>54</td>
<td>48</td>
<td></td>
<td>53</td>
</tr>
<tr>
<td>Local Recurrence</td>
<td></td>
<td></td>
<td></td>
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<tr>
<td>No (219)</td>
<td>152</td>
<td>67</td>
<td>&lt;0.001</td>
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<tr>
<td>Yes (49)</td>
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<td>45</td>
<td></td>
<td>4</td>
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<tr>
<td>Metastasis</td>
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<td></td>
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<tr>
<td>No (237)</td>
<td>155</td>
<td>82</td>
<td>&lt;0.001</td>
<td>149</td>
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<tr>
<td>Yes (31)</td>
<td>1</td>
<td>30</td>
<td></td>
<td>3</td>
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<tr>
<td>Mitoses</td>
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<td></td>
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<tr>
<td>&lt;5/HPF (173)</td>
<td>128</td>
<td>45</td>
<td>&lt;0.001</td>
<td>125</td>
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<tr>
<td>5–10/HPF (34)</td>
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<td>23</td>
<td></td>
<td>11</td>
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<tr>
<td>≥10/HPF (61)</td>
<td>17</td>
<td>44</td>
<td></td>
<td>16</td>
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<tr>
<td>Stomal overgrowth</td>
<td></td>
<td></td>
<td></td>
<td></td>
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<tr>
<td>Absence (182)</td>
<td>131</td>
<td>51</td>
<td>&lt;0.001</td>
<td>142</td>
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<tr>
<td>Present (86)</td>
<td>25</td>
<td>61</td>
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<td>10</td>
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Table 2. Multivariate Cox proportional hazard analysis of LRFS in 268 patients with breast PTs

<table>
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<tr>
<th>Variable</th>
<th>$x^2$</th>
<th>P value</th>
<th>HR</th>
<th>95% CI</th>
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<tr>
<td>Age</td>
<td>3.698</td>
<td>0.055</td>
<td>0.560</td>
<td>0.310-1.013</td>
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<tr>
<td>Size</td>
<td>0.485</td>
<td>0.486</td>
<td>1.242</td>
<td>0.675-2.285</td>
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<tr>
<td>Grade</td>
<td>4.718</td>
<td>0.030</td>
<td>0.605</td>
<td>0.393-0.931</td>
</tr>
<tr>
<td>Mitoses</td>
<td>0.116</td>
<td>0.734</td>
<td>1.152</td>
<td>0.511-2.598</td>
</tr>
<tr>
<td>Stromal overgrowth</td>
<td>16.085</td>
<td>&lt;0.001</td>
<td>5.742</td>
<td>2.518-13.091</td>
</tr>
<tr>
<td>$\alpha$-SMA</td>
<td>12.527</td>
<td>&lt;0.001</td>
<td>5.644</td>
<td>1.785-17.851</td>
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<tr>
<td>FAP</td>
<td>3.570</td>
<td>0.059</td>
<td>1.734</td>
<td>0.976-3.081</td>
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<tr>
<td>SDF-1</td>
<td>1.705</td>
<td>0.192</td>
<td>1.521</td>
<td>0.804-2.879</td>
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<tr>
<td>MiR-21</td>
<td>9.815</td>
<td>0.002</td>
<td>4.869</td>
<td>1.527-15.524</td>
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</tbody>
</table>

LRFS: local recurrence-free survival; PTs: Phyllodes tumors.
Figure 1

(A) Immunohistochemical staining of normal and fibrotic tissue in PT. HE, α-SMA, FAP, SDF-1, and Ki 67.

(B) Graph showing normalized α-SMA expression in normal, fibrotic, benign, borderline, and malignant PT.

(C) Immunofluorescence staining of normal and fibrotic tissue in PT. DAPI, α-SMA, FAP, and merged images.

(D) ROC curves showing α-SMA AUC 0.89, SDF-1 AUC 0.74, and FAP AUC 0.69.

(E) ROC curves showing α-SMA AUC 0.94, SDF-1 AUC 0.76, and FAP AUC 0.75.
Figure 2

A

mean Fold change Benign1 Benign2 Malignant1 Malignant2

6.428
5.662
5.241
5.021
4.492
4.391
4.213
4.067
3.879
3.823
3.782
3.692
3.604
3.591
3.389
3.300
3.269
3.226
3.229
0.317
0.229
0.194

-1.96 0 1.96

miR-21-5P
miR-130b-3p
miR-221-3p
miR-151a-3p
miR-134-3p
miR-23a-3p
miR-196a-3p
miR-767-5p
let-7c-5p
miR-93-5p
miR-519e-3p
miR-222-3p
miR-25-3p
miR-20a-5p
let-7f-5p
miR-186-5p
miR-451a
miR-127-3p
miR-149-3p
miR-498
miR-92a-3p

B

normalized miR-21

Normal FA Benign Borderline Malignant

* ** ***

C

Scramble PT Benign

Borderline Malignant

D

HE miR-21 \(\alpha\)-SMA Merge

E

\(r=0.817\) P<0.001

miR-21+ cell(\%)

\(\alpha\)-SMA cell(\%)

F

miR-21 AUC 0.92

\(\alpha\)-SMA AUC 0.89

grade AUC 0.67

Sensitivity

1-Specificity

G

OS(\%)

miR-21

SI\leq4

SI>4

P<0.001

months
Figure 4

(A) Benign vs. Malignant samples with mock, lin4, and miR-21 treatments.

(B) Expression of α-SMA, FAP, and GAPDH in NC, mock, si-smad7-2, si-smad7-3.

(C) Effect of si-PTEN-1 and si-PTEN-2 on α-SMA, FAP, and GAPDH expression.

(D) miR-21 effect on α-SMA, FAP, and GAPDH in mock, vector, smad7-mut, PTEN-mut, and smad7+ PTEN-mut samples.

(E) Migration, invasion, collagen, and colony formation assays under different conditions.

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miR-21 induces myofibroblast differentiation and promotes the malignant progression of breast phyllodes tumors

Chang Gong, Yan Nie, Shaohua Qu, et al.

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