行政院國家科學委員會專題研究計畫 成果報告

微型核糖核酸矩陣分析探討女性乳癌癌化進程之遺傳標記
(第 3 年)
研究成果報告(完整版)

計 畫 類 別 ：個別型
計 畫 編 號 ：NSC 98-2314-B-040-009-MY3
執 行 期 間 ：100 年 08 月 01 日至 101 年 07 月 31 日
執 行 單 位 ：中山醫學大學生化暨生物科技研究所

計 畫 主 持 人：鄭鈞文

計畫參與人員：碩士級-專任助理人員：陳承佑
碩士班研究生-兼任助理人員：翁崇智
博士班研究生-兼任助理人員：張佳瑋

報 告 附 件 ：出席國際會議研究心得報告及發表論文

公 開 資 訊 ：本計畫可公開查詢

中 華 民 國 101 年 10 月 30 日

中 文 摘 要 ： 臨床發現，癌症復發和癌細胞發生淋巴或者是組織和器官的腫瘤轉移，是導致乳癌患者預後惡化的關鍵。近年來的文獻指出，特定微型核糖核酸分子(microRNAs，miRNAs)在癌細胞的發展進程中扮演著重要的角色。然而，攸關 miRNA 做為評估乳癌病患預後生物標記的研究報告，至今尚未有完整的被探討。有鑑於此，本研究計畫中，我們期望能找出特有的 miRNA 遺傳標記，來解釋國人婦女乳癌進程發展的原因。首先，我們以雷射捕獲微擷取技術將癌組織從乳癌患者的乳部組織分離，以 cyclin D1 和 beta-catenin 過度表現為基準，結合乳癌淋巴轉移的臨床資料為依據，以微型核糖核酸微矩陣(miRNA-microarray)和生物資訊統計分析。經由基因預測比對後，發現 26 個 miRNAs 在癌組織中表現量降低(down-regulated expression)和乳癌淋巴轉移相關。我們利用基因比對、分子選殖及核酸定序，發現間質細胞蛋白 vimentin (Vim)基因之 3‘-UTR 的核酸序列是 miR-30a 作用的標的；再以點突變實驗證實 Vim 3‘-UTR 位置上的核甘酸 GTTTAC 是 miR-3a 的結合序列。將 miR-30a 轉殖到乳癌細胞 Hs578T 和 MDA-MB-231，也發現乳癌細胞株 vimentin 蛋白的表現量有明顯的降低。再者，探討 miR-30a 的抑癌功能，我們發現當誘導 miR-30a 的表現時，會明顯抑制乳癌細胞侵襲和移動力。最後，臨床檢體上，收集了 221 乳管侵襲癌的病例組織中，發現癌組織中 miR-30a 低度表現和乳癌預後，包括：乳癌分期、淋巴轉移和五年復發率和存活率等預後因子有顯著的關連。總結而言，藉由 miR-30a 和乳癌預後的研究發現不僅可以對乳癌進程發展提供關鍵訊息，也進一步了解到 miR-30a 基因表現和抑制乳癌細胞侵襲能力的分子機制；在未來更可以提供對乳癌新的治療策略。

中文關鍵詞： 乳癌, 微型核糖核酸-微矩陣分析, MiR-30a, Vimentin, 預後, 上皮-間質細胞轉換

英 文 摘 要 ： Tumor recurrence and metastasis result in an unfavorable prognosis for cancer patients. Recent studies have suggested that specific microRNAs (miRNAs) may play important roles in the development of cancer cells.However, prognostic markers and the outcome prediction of the miRNA signature in breast cancer patients have not been comprehensively assessed. The aim of this study was to identify miRNA biomarkers relating to clinicopathological features and outcome of breast cancer. A miRNA microarray analysis was performed on breast tumors of

different lymph node metastasis status and with different progression signatures, indicated by overexpression of cyclin D1 and b-catenin genes, to identify miRNAs showing a significant difference in expression. The functional interaction between the candidate miRNA, miR-30a, and the target gene, Vim, which codes for vimentin, a protein involved assay, western blotting, and migration and invasion assays. The association between the decreased miR-30a levels and breast cancer progression was examined in a survival analysis. miR-30a negatively regulated vimentin expression by binding to the 30-untranslated region of Vim. Overexpression of miR-30a suppressed the migration and invasiveness phenotypes of breast cancer cell lines. Moreover, reduced tumor expression of miR-30a in breast cancer patients was associated with an unfavorable outcome, including late tumor stage, lymph nodemetastasis, and worse progression (mortality and recurrence) ($p<0.05$). In conclusion, these findings suggest a role for miR-30a in inhibiting breast tumor invasiveness and metastasis. The finding that miR-30a downmodulates vimentin expression might provide a therapeutic target for the treatment of breast cancer.

英文關鍵詞： Breast cancer, MicroRNA-microarray, MiR-30a, Vimentin, Prognosis, EMT

計畫成果自評

本研究計畫主題是以分子遺傳層面探討台灣地區婦女乳癌預後之生物遺傳標記。實驗內容是探討婦女乳癌進程中，攸關乳癌淋巴侵襲轉移的微型核糖核酸標記。在細胞模式試驗中，我們實驗室成功地建立起乳癌淋巴轉移之miRNA為矩陣剖面分析。進一步透過電腦生物資訊比對，以分子選殖建立miRNA群組，建構miRNA轉殖細胞株，進行表基因體和標的基因調控及其功能性分析。我們發現miR-30a結合到Vim基因3'-UTR來逆調控vimentin蛋白的表現。而文獻指出vimentin蛋白過度表現是與癌細胞轉移作用有關。因此，在乳癌細胞株藉由miR-30a持續的表達，則可以抑制vimentin蛋白的表現，並降低乳癌細胞進行EMT。在臨床檢體的研究方面，我們同中研院生醫所沈志陽教授和國防醫學大學俞志誠教授合作，建立台灣地區婦女乳癌組織資料庫。我們以雷射顯微擷取技術分離癌組織和相鄰之正常組織，最後，經由定量qRT-PCR驗證乳癌病灶組織miR-30a基因低度表現程度和臨床分期、淋巴轉移和預後存活評估，在統計上有顯著的關聯。相關的研究成果已發表於SCI期刊(Ann Surg Oncol 2012; Aug, in press; Breast Cancer Res Treat 2012; 134:1081-93, 如附件)，在癌症領域具相當水準，且國內的乳癌研究也尚未有相關的內容曾被發表，其研究專題清楚且已建立獨立的研究領域。藉由探討微型核糖核酸對乳癌進程發展的分子機轉，研究發現除了能建立起國人婦女乳癌臨床預後評估之生物標記資料庫；更展望以此研究基礎轉譯於臨床醫師對研症患者的個人化醫療評估分析，於未來發展有效的乳癌基因標靶治療策略。

Ann Surg Oncol
DOI 10.1245/s10434-012-2541-x

Annals of
SURGICAL ONCOLOGY
OFFICIAL JOURNAL OF THE SOCIETY OF SURGICAL ONCOLOGY

ORIGINAL ARTICLE – BREAST ONCOLOGY

Prognostic Significance of *cyclin D1*, *β-catenin*, and *MTA1* in Patients with Invasive Ductal Carcinoma of the Breast

Chun-Wen Cheng, PhD[1,2,3], Yu-Fan Liu, PhD[4], Jyh-Cherng Yu, MD[5], Hsiao-Wei Wang, MSc[1,3], Shian-Ling Ding, PhD[6], Chia-Ni Hsiung, MSc[3], Huan-Ming Hsu, MD[5], Jia-Ching Shieh, PhD[4], Pei-Ei Wu, MSc[3], and Chen-Yang Shen, PhD[3,7]

[1]Institute of Biochemistry and Biotechnology, Chung Shan Medical University, Taichung, Taiwan; [2]Clinical Laboratory, Chung Shan Medical University Hospital, Taichung, Taiwan; [3]Institute of Biomedical Sciences, Academia Sinica, Taipei, Taiwan; [4]Department of Biomedical Sciences, Chung Shan Medical University, Taichung, Taiwan; [5]Department of Surgery, Tri-Service General Hospital, National Defense Medical Center, Taipei, Taiwan; [6]Department of Nursing, Kang-Ning Junior College of Medical Care and Management, Taipei, Taiwan; [7]Institute of Environmental Science, China Medical University, Taichung, Taiwan

ABSTRACT

Background. To investigate markers for predicting breast cancer progression, we performed a candidate gene-based study that assessed expression change of three genes, *cyclin D1, β-catenin*, and *metastasis-associated protein-1* (*MTA1*), involving in aggressive phenotypes of cancerous cells, namely hyperproliferation, epithelial-mesenchymal transition, and global transcriptional regulation.

Methods. Specimens were from 150 enrolled female patients, with invasive ductal carcinoma, followed up for more than 10 years. mRNA expression of *cyclin D1*, *β-catenin*, and *MTA1* in cancerous and noncancerous cells microdissected from the primary tumor site was determined by quantitative real-time PCR. The relationship between mRNA expression levels of the genes and clinicopathologic features was assessed by statistical analysis. Disease-free and overall survival (DFS and OS) were analyzed by Kaplan–Meier analysis with log-rank test and a multivariate Cox regression model.

Results. *Cyclin D1* was shown to be overexpressed in late-stage breast cancer (stage III/IV). Breast cancer with lymph node metastasis (LNM) showed significantly higher frequency of overexpressed *cyclin D1*, *β-catenin*, and *MTA1* ($P < 0.05$). Patients carrying greater numbers of overexpressed genes had joint effects on increased risk in tumors of advanced stages ($P_{trend} = 0.03$) and LNM ($P_{trend} < 0.01$). In the LNM-negative group, patients whose tumors with greater number of *cyclin D1*, *β-catenin*, and *MTA1* overexpressions were associated with poorer clinical outcomes, with hazard ratio of 14.79 for OS ($P = 0.015$) and 7.54 for DFS ($P = 0.015$) using multivariate Cox regression analysis during the 10-year follow-up.

Conclusions. Higher expression of *cyclin D1*, *β-catenin*, and *MTA1* mRNAs in breast cancers may prove effective in predicting unfavorable outcomes of breast cancer.

In clinical practice, although cancer patients ultimately develop metastasis if not properly treated, not all patients manifest the same course of cancer progression. Identification of the specific patient subgroup with higher metastatic potential has become a critical issue in preventing mortality. To this end, recent gene-expression profiling studies have defined four main molecular classes of breast cancer: basal-like cancer, luminal-A cancer, luminal-B cancer, and HER2/neu (HER2)-positive cancer; basal-like breast cancers have the worst clinical outcome.[1,2] This grouping based on molecular profiling corresponds to that known estrogen receptor (ER), progesterone-receptor (PR), and HER2 expression statuses,

Electronic supplementary material The online version of this article (doi:10.1245/s10434-012-2541-x) contains supplementary material, which is available to authorized users.

First Received: 31 October 2011

C.-W. Cheng, PhD
e-mail: cwcheng@csmu.edu.tw

C.-Y. Shen, PhD
e-mail: bmcys@ibms.sinica.edu.tw

Published online: 03 August 2012

with ER-negative/PR-negative/HER2-negative tumors (namely "triple-negative" tumors) corresponding to basal-like breast cancers and the subgroup that usually has a poor clinical outcome.[3] Although these major subtypes of breast cancer have been considered as practical variables used to predict cancer patient survival, different patterns of gene expression analysis stratified by these subtypes used in prognostic evaluation remain a clinically challenging issue for breast cancer patients.[4–6]

Wnt signaling pathway is known to be important in many biological processes, such as embryonic development, stem cell growth, and tumor cell survival. Of this signaling pathway, beta-catenin (β-catenin), a cytoplasmic component of the cell-adherent protein family, takes part in the cytoskeleton to membrane via interacting to E-cadherin.[7,8] Alternatively, nuclear β-catenin binds to Tcf/lymphoid enhancer factor (Lef), and then activates transcription of cyclin D1, a major effector that promotes G1 process during cell cycle, and its upregulation in the mammary gland has been shown to induce mammary hyperplasia.[9] Metastasis-associated protein-1 (*MTA1*) is a transcriptional regulator that was identified to be a component of the vertebrate deacetylase complex and involved in nucleosome remodeling and deacetylation (NuRD).[10,11] It has been reported that dysregulation of MTA1 in mammary epithelium causes increased cell proliferation, hyper-branched ductal structure formation, and results in inappropriate mammary gland development and tumorigenesis in the *MTA1* transgenic virgin mice.[12] Besides, by examination of mammary gland of the *MTA1* transgenic mice, cyclin D1 and β-catenin were found to be overexpressed and correlated with mammary hyperplasia and adenocarcinoma.[12] However, knowledge among studies about whether mRNA levels of these three genes in surgically resected breast cancer tissues were correlated with unfavorable outcomes remains underestimated. Therefore, the present candidate gene approach-based study focused on determining mRNA transcripts of *cyclin D1*, *β-catenin*, and *MTA1* in primary breast tumors to examine roles of these genes to predict prognostic significance. By using quantitative real-time PCR (qRT-PCR), we compared expression levels for these genes in tumor tissues and adjacent nontumor tissues from female patients with invasive ductal carcinoma (IDC) of the breast. To overcome the problem of contamination with normal tissue due to the infiltrating nature of invasive breast tumors and to obtain pure tumor cells to provide the homogenous material required for reliable analysis, laser capture microdissection (LCM) was used. Based on this improved approach, this study aimed at providing a more reliable and more mechanistically reasonable gene expression signature that may aid in the prediction of breast cancer progression.

MATERIALS AND METHODS

Study Patients and Tissues

The present study is part of an ongoing cooperative study aimed at identifying markers for the evaluation of breast cancer progression in Taiwan, where breast cancer is characterized by low incidence, early tumor onset, reproductive hormone dependency, and novel genomic alterations.[13–16] This study was approved by the Ethics Committee of the Institutional Review Board of the Tri-Service General Hospital, Taipei, Taiwan, and informed consent was obtained from each participant before specimen acquisition. The tumors were classified according to the sixth edition of the AJCC Cancer Staging Manual.[17] The lymph node metastasis (LNM) status of each patient was determined by the presence of tumor cells in lymph nodes on immunohistochemistry staining of at least ten lymph node dissections collected from each tumor lesion of the patient. None of the patients received neoadjuvant treatment before primary surgery, thus avoiding any effects on gene expression.

Laser Capture Microdissection

To ensure that the tissue samples assayed contained more than 95 % tumor cells, LCM was performed on routinely immunostained slides using a PixCell laser capture microscope (Arcturus Engineering, Inc., CA) as described previously.[18–20] The dehydrated tissue section was overlaid with a thermoplastic film mounted on an optically transparent cap and visually selected areas were bound to the membrane by short, low-energy laser pulses, resulting in focal melting of the polymer. On average, each LCM shot performed to obtain homogenous cells for the multigenic quantitative real-time polymerase chain reaction (qRT-PCR) analysis provided 2,500–3,000 tumor cells. The cells were immersed in 50–100 µl of digestion buffer (10 mM Tris–HCl (pH 8.0), 1 mM EDTA, 400 µg/ml proteinase K, and 1 % Tween 20) and digested at 55 °C overnight. Then, the proteinase K was heat-inactivated and the extract was used for RNA isolation. Paired adjacent noncancerous cells for each tumor were collected from the same slide using the same procedure and subjected to RNA isolation.

Quantitative RT-PCR Analysis

Total RNA was extracted from tumor and the nontumor breast tissue for each patient using the PicoPur RNA isolation kit (Arcturus, USA). Reverse transcription PCR and cDNA synthesis with the use of β-actin as a positive control was performed according to the previous studies.[21,22] The sequences for probes and primer sets used in

detecting expression levels of *cyclin D1* (Hs0027 7039_m1), *β-catenin* (Hs00170025_m1), and *MTA1* (Hs00183042_m1) genes were performed using TaqMan gene expression assays (Applied Biosystems, Foster City, CA) on tumor and nontumor tissues based on normalization against *β*-actin. All reactions were done in triplicate, and the relative expression of the gene transcript, i.e., the Ct value, was calculated. Representation of the differential expression of a target gene in cancerous and noncancerous tissues was differentiated using the comparative C_T method.[21,23] The value $2^{-\Delta\Delta Ct}$ ($-\Delta\Delta Ct$; the target gene/*β*-actin ratio in tumors divided by this ratio in the nontumor tissues) was used to measure the relative expression of the individual gene in the microdissected cells from cancerous and noncancerous tissues. All quantitative comparisons in the "Results" section that show normalized ΔC_T values are plotted using box-whiskers plots stratified by clinicopathological parameters.

Immunohistochemistry

In immunohistochemical studies, fresh-frozen tissue sections were immersed with 3 % hydrogen peroxide in phosphate-buffered saline (PBS) to quench endogenous peroxidase activity of tissues. The tissue slide was reacted with a monoclonal antibody against human *cyclin D1*, *β-catenin*, and *MTA1* (dilution at 1:100; Santa Cruz Biotechnology, Inc., CA) in a moister chamber for 60 min at 37 °C. After that, the sections were washed in PBS and then treated with an appropriate horseradish peroxidase-conjugated second antibody for 30 min. The signal of the tissue slide was detected using the SuperPicture^{TM} polymer IHC detection kit and diaminobenzidine (DAB) as substrate for improvement of greater signal sensitivity (Invitrogen Crop., Carlsbad, CA). The assessment of IHC staining was quantified by the combination of coverage area and intensity. Tumor tissue area for protein staining was estimated with the following criteria: coverage of tumor cells defined as none detectable or faint staining, negative; 1, ≤10 %; 2, 10–50 %; 3, >50 %. A three-level scoring for the staining intensity of tumors cells as none/weak; 1, moderate; 2, strong expression. A scoring index (products of immunoreactive intensity and proportion score) was used to calculate tumors of immunostained tumor tissues and the value with a cutoff more than four were defined as an event of overexpression of the protein.

Information about Cancer Progression

This study was dependent on a reliable follow-up system established and maintained by ourselves to trace actively and regularly the progression of our patients.[24] This system obtained information on clinicopathological features and recurrence/survival status for the patients enrolled. Recurrent breast tumors were subjected to pathological confirmation to exclude the possibility of second primary tumors. Additionally, we confirmed the cause of death from the death certificates; patients whose deaths were clearly documented to be due to breast cancer were considered to have died of breast cancer, whereas other causes of deaths were considered censored events.

Statistical Analysis

We examined whether there was a correlation between expression levels of *cyclin D1*, *β-catenin*, and *MTA1* genes, individually or jointly, in primary cancer tissue and the clinicopathological features of the tumor. Because the goal was to evaluate the usefulness of these three genes as prognostic biomarkers for IDC, the estimated areas under the curves (AUC) for assessing the accuracy of predictions were calculated based on receiver operating characteristic (ROC) curves, which an optimal diagnostic cutoff point was determined to differentiate overexpression of individual genes. Based on these cutoffs, quantitative measurements of the expression levels of individual genes were converted into binary measurements, which were used to estimate a correlation between gene overexpression and clinicopathological features. Furthermore, we examined whether overexpression of these genes could be prospectively associated with breast cancer progression using the 10-year disease-free survival (DFS) and OS as outcomes of interest. DFS was measured as the time from surgery to recurrence/mortality or the end of the study, and OS was defined as the time from surgery to death or the end of the last follow-up for this patient. Survival curves were plotted by using the Kaplan–Meier method, and the Cox regression model was used to compute hazard ratios. The significance of these associations was assessed using the two-sided Chi-squared test, Fisher's exact test, log-rank test, and Mann-Whitney *U* test. Statistical significance was determined as a $P < 0.05$.

RESULTS

Clinicopathological Features of IDC patients

A group of 150 female patients with histologically proven IDC who had been followed up for more than 10 years was recruited for this study. The mean patient age was 50.6 (range 25–87) years. The clinical and pathological features of these patients are listed in Table 1. In the subgroup analyses, no heterogeneity in the effect of the cancer subjects was seen across the strata of age and hormone receptor status, including ER, PR, and HER2, which

TABLE 1 Tumor characteristics of female patients with invasive ductal breast carcinoma

Characteristics[a]	*N* (%)
Age (mean ± SD and range)	50.6 ± 11.6 (25–87 yrs)
Tumor size (mm)	
<30	115 (76.7)
≥30	35 (23.3)
Tumor grade	
I	36 (24)
II	70 (46.7)
III	44 (29.3)
Tumor stage	
I	59 (39.3)
II	64 (42.7)
III	22 (14.7)
IV	5 (3.3)
LNM	
Negative (N0)	91 (60.7)
Positive (N1/N2)	59 (39.3)
ER	
Positive	66 (45.5)
Negative	79 (54.5)
PR	
Positive	63 (42.9)
Negative	84 (57.1)
HER2	
Positive	80 (54.8)
Negative	66 (45.2)

ER, PR, or HER2 status was classed as negative when fewer than 30 % of cells were stained and as positive with more than 30 % staining

LNM Lymph node metastasis, *ER* estrogen receptor, *PR* progesterone receptor, *HER2* HER2/neu

[a] Tumor classification was referred to sixth edition of the AJCC Cancer Staging Manual (2006)

are similar to those reported in other breast cancer clinics in Taiwan.[24–26]

Relationship Between Expression of Cyclin D1, β-catenin, and MTA1 Genes in Primary Breast Tumors and Clinicopathological Features

To obtain a homogenous population of cells to precisely measure the mRNA expression level of the target gene, pure cancer cells and paired adjacent noncancerous cells from individual tumors were obtained by LCM (an example of LCM-treated is shown in Supplementary Fig. 1), and comparative quantification of mRNA levels for these genes performed for the individual patients. Expression levels of *cyclin D1*, *β-catenin*, and *MTA1* detected in primary tumor tissues were significantly higher than those in the adjacent noncancerous tissues, correlating with aggressive tumor phenotypes (advanced stages or LNM); however, nonsignificant association between expression levels of these three genes and tumor size, grade or status of ER, PR and HER2 was found (Fig. 1; Supplementary Table 1). Stepwise, using ROC curves based on the expression levels of *cyclin D1*, *β-catenin*, and *MTA1* in individual tumors, we determined the cutoff for defining "overexpression" of these three genes as 2.00 ($-\Delta\Delta Ct = 1.00$). According to this cutoff, the AUC values for these genes were found to be significantly lower in low-stage (stage I/II) tumors than those in high-stage (stage III/IV) tumors. Using the same cutoff, AUC values for these genes were also significantly lower in LNM-negative tumors than those in LNM-positive ones (Supplementary Table 2). Therefore, in the following analysis, we used the cutoff of twofold to define tumors with status of "overexpression" to investigate correlations between gene expression and clinicopathological features. Based on quantitative measurements, the frequencies of overexpression of *cyclin D1* genes were significantly higher in advanced-stage tumors (stage III and IV; $P < 0.05$). Again, under the same categorization in the multivariate logistic regression analysis, patients with increased mRNA levels of individual genes in primary cancerous sites were found to be associated with LNM-positive tumors (all $P < 0.05$; Table 2).

Overexpression of Cyclin D1, β-catenin, and MTA1 Genes in Primary Breast Tumors as Predictors of Advanced Stage and LNM in Breast Cancer

Given that *cyclin D1*, *β-catenin*, and *MTA1* are associated with different mechanisms, all of which are required for malignant progression of tumors, the prediction was that any defects due to overexpression of these genes involved in different mechanisms would act jointly, leading to worse clinicopathological features, and it was mechanistically reasonable to examine associations between the combined effect of genes and advanced tumor stage or LNM. However, considering that those subjects had all normal expression levels of *cyclin D1*, *β-catenin*, and *MTA1* genes simultaneously, there was a relatively small percentage in cancer patients of advanced stage (4.4 %) or with LNM-positive (8.6 %), we thus classified our subjects into three groups, those who carried no more than one (≦1) and those with at least two (2 or 3) overexpressed of these three genes, and such a definition can provide statistical

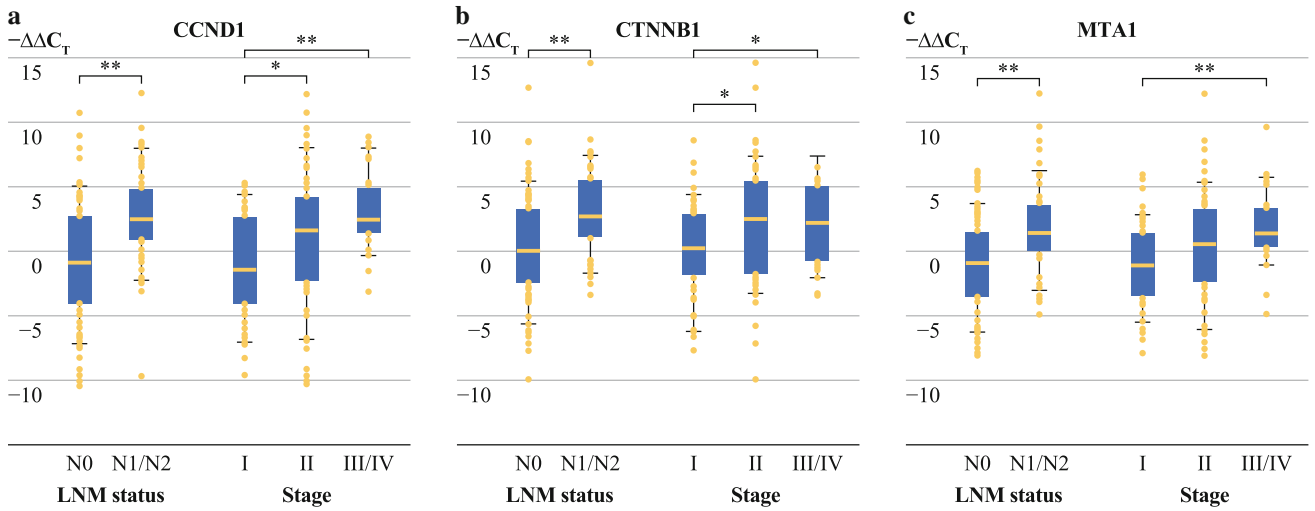


FIG. 1 mRNA expression level of each gene in IDC of different tumor stages or LNM status. The expression level of **a** cyclin D1 (*CCND1*), **b** β-catenin (*CTNNB1*), and **c** MTA1 (*MTA1*) based on RNA extracted from LCM-captured cancerous and adjacent noncancerous cells was measured by qRT-PCR. Differences were examined using the Mann-Whitney *U* test. * and ** indicate *p* values of <0.05 and <0.01, respectively. Each *dot* represents a case, the *line within the box marks* the median, and the *box* depicts the borders of the 25 and 75 % quartiles. The *error bars above and below the box* indicate the 90 and 10 % quartiles

TABLE 2 Relationship between overexpression of individual gene and clinicopathological parameters of IDC patients

Clinicopathological features	Gene overexpression					
	Cyclin D1	OR (95 % CI)	*β-catenin*	OR (95 % CI)	*MTA1*	OR (95 % CI)
Tumor size (mm)						
<30	56/115 (48.7 %)	1.00 (Ref)	65/114 (57.0 %)	1.00 (Ref)	44/115 (38.3 %)	1.00 (Ref)
≥30	19/35 (54.3 %)	1.25 (0.59–2.67)	22/35 (62.9 %)	1.28 (0.59–2.78)	16/35 (45.7 %)	1.36 (0.59–3.12)
Tumor grade						
I	20/36 (55.5 %)	1.00 (Ref)	19/35 (54.3 %)	1.00 (Ref)	15/36 (41.7 %)	1.00 (Ref)
II	32/70 (45.7 %)	0.67 (0.31–1.63)	41/70 (58.6 %)	1.19 (0.52–2.92)	24/70 (34.3 %)	0.73 (0.32–1.81)
III	23/44 (52.3 %)	0.89 (0.36–2.33)	27/44 (61.4 %)	1.34 (0.54–3.63)	21/44 (47.7 %)	1.28 (0.53–3.42)
Tumor stage						
I/II	54/123 (43.9 %)	1.00 (Ref)	67/122 (54.9 %)	1.00 (Ref)	45/123 (36.6 %)	1.00 (Ref)
III/IV	21/27 (77.8 %)	4.26 (1.60–11.34)*	20/27 (74.1 %)	2.35 (0.86–5.64)	15/27 (55.6 %)	2.17 (0.93–4.95)
LNM						
N0	31/91 (34.1 %)	1.00 (Ref)	40/91 (44.0 %)	1.00 (Ref)	28/91 (30.8 %)	1.00 (Ref)
N1/N2	44/59 (74.6 %)	5.42 (2.56–11.40)**	47/58 (81.0 %)	5.16 (2.36–11.03)**	32/59 (54.2 %)	2.64 (1.32–5.27)**

LNM Lymph node metastasis, *OR* odds ratio, *CI* confidence interval, *Ref* reference group, *Gene overexpression* the expression status of the individual genes was defined by comparing target gene expression in cancerous cells and adjacent noncancerous cells captured from the primary tumor site of the same patient and was categorized as "overexpression" by using the cutoff value determined from the AUC (cutoff; $-\Delta\Delta Ct \geq 1$ vs. <1)

* $P < 0.05$; ** $P < 0.01$

power to address the question regarding joint effects of *cyclin D1*, *β-catenin*, and *MTA1* overexpression on prognostic assessment of breast cancer. Using patients with no more than one overexpressed of these three genes served as the reference group, we found that the odds ratios (ORs) for overexpression of gene interactions were significant, in which the joint effects of overexpression of all three genes on tumor progression, with the ORs of 3.29 (95 % confidence interval (CI) = 1.26–8.54) and 6.64 (95 % CI = 2.87–15.36) for tumors of advanced-stage and LNM

($P < 0.05$), respectively (Table 3). Notably, there was a trend toward a significantly elevated risk of the joint effect on phenotypic manifestation of invasiveness (OR = 1.82; 95% CI = 1.12–2.96) and metastasis (OR = 2.57; 95 % CI = 1.71–3.95) as patients carrying greater numbers of overexpressed genes ($P_{trend} < 0.05$; Table 3).

Correlations Between Overexpression of Cyclin D1, β-catenin, and MTA1 and Poor Clinical Outcomes

The correlations between *cyclin D1*, *β-catenin*, and *MTA1* overexpression, individually or jointly, and poor clinical features prompted us to examine different expression status of these genes to predict breast cancer progression by comparing the survival (10-year DFS and OS) of patients. There were trends toward a decreased OS (Fig. 2, left panels) and DFS (Fig. 2, right panels) in patients overexpressing *cyclin D1, β-catenin,* or *MTA1* either individually (Fig. 2a–f) or jointly (Fig. 2g and h). Compared with those patients with no more than one overexpression of these three genes (≤1), the presence of great numbers of overexpressed genes (≥2) in primary tumors predicted a tendency toward poor rates of OS and DFS for all patients. Furthermore, across the strata of subgroup of tumor node, only the LNM-negative group with a higher number of overexpressed genes in primary tumors demonstrated a significant influence on OS (log-rank, $P = 0.005$) and DFS (log-rank, $P = 0.007$) using the Kaplan–Meier method with survive Bonferroni correction ($P < 0.014$; Fig. 2). Moreover, a multivariate Cox regression analysis that unraveled a correlation between synergistic effects of multiple genes and unfavorable outcomes was shown to be significant in patients of LNM-negative group, with HR of 14.79 for OS (95 % CI = 1.673–130.73; $P = 0.015$) and of 7.54 for DFS (95 % CI = 1.483–38.37, $P = 0.015$) during the 10-year follow-up period (Table 4).

DISCUSSION

Metastasis is the main cause of mortality in cancer patients and depends on the acquisition of genetic or epigenetic alterations by primary tumor cells, allowing them to surmount physical boundaries, disseminate, and colonize a distant organ.[27,28] In animal models, 0.01 % or fewer of cancer cells entering the circulation develop into metastases, suggesting that primary tumors consist of heterogeneous populations of cells, only among which accumulate essential abnormalities can result in a metastatic phenotype.[29] We have shown that overexpression of *cyclin D1*, *β-catenin*, and *MTA1* in breast cancer cells at the primary tumor site was significantly associated with poor clinical features of breast cancer (LNM and advanced stage) and a worse outcome. However, like many studies, investigating biomarkers for prediction of cancer progression have relied on a retrospective design in which a prospective design is required to confirm the clinical utility of tumor biomarkers. In this study, though the patients were not recruited prospectively as a randomized trial, we defined overexpressed genes by individually comparing transcriptional levels of target genes between the microdissected cells from tumor and nontumor parts and then tested whether overexpression of *cyclin D1*, *β-catenin*, and *MTA1* in tumor cells was associated with the clinical feature of breast cancer. Under the risk estimation of multiple tests in prediction of breast cancer prognoses, one needs to be careful when specifying the proportion of IDC cases in different subgroups because only sufficient sample size in the design for logistic regression with binary

TABLE 3 Correlation between the number of overexpressed genes (*cyclin D1, β-catenin,* and *MTA1*) and tumor stage or lymph node metastasis in breast cancer

Variable	Clinical feature					
No. of overexpressed genes	Stage			Lymph node metastasis		
	I/II *n* (%)	III/IV *n* (%)	OR (95 % CI)	N0 *n* (%)	N1/N2 *n* (%)	OR (95 % CI)
0–1	66 (88)	9 (12)	1.00 (Ref)	59 (78.7)	16 (21.3)	1.00 (Ref)
2	27 (84.4)	5 (15.6)	1.36 (0.42–4.43)	17 (53.1)	15 (46.9)	3.25 (1.34–7.9)*
3	29 (69)	13 (31)	3.29 (1.26–8.55)*	15 (35.7)	27 (64.3)	6.64 (2.87–15.36)**
Additive model of gene overexpression			1.82 (1.12–2.96)			2.57 (1.71–3.95)
			$P_{trend} = 0.016$			$P_{trend} < 0.001$

ORs and 95 % CIs were estimated in a logistic regression model, in which a group of dummy variables was used to represent different groups of patients showing different numbers of overexpressed genes. Overexpression of the *cyclin D1*, *β-catenin*, and *MTA-1* genes was defined as described in the "Results" section

Ref Reference group, *OR* odds ratio; *CI* confidence interval

* $P < 0.05$; ** $P < 0.01$

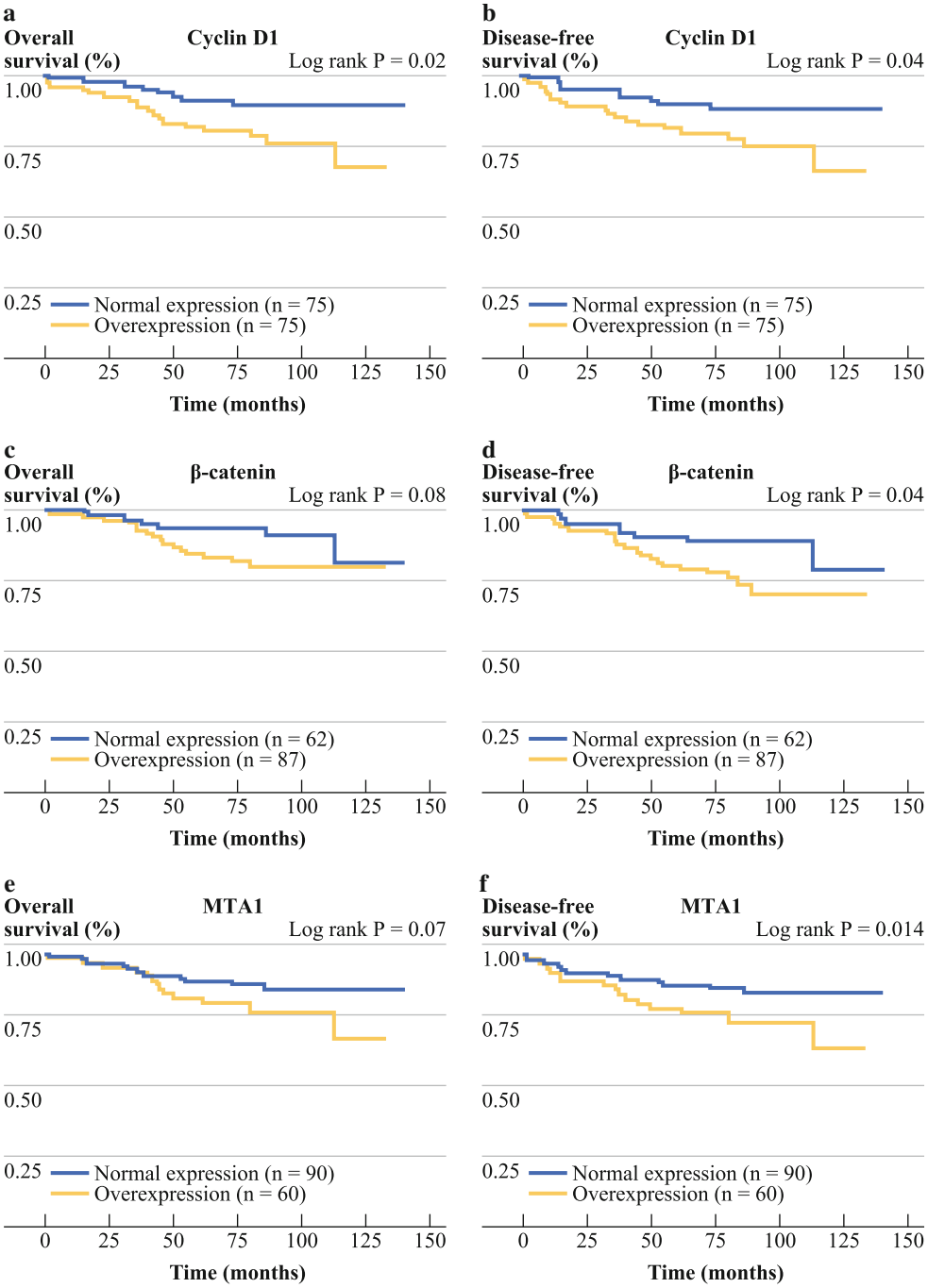
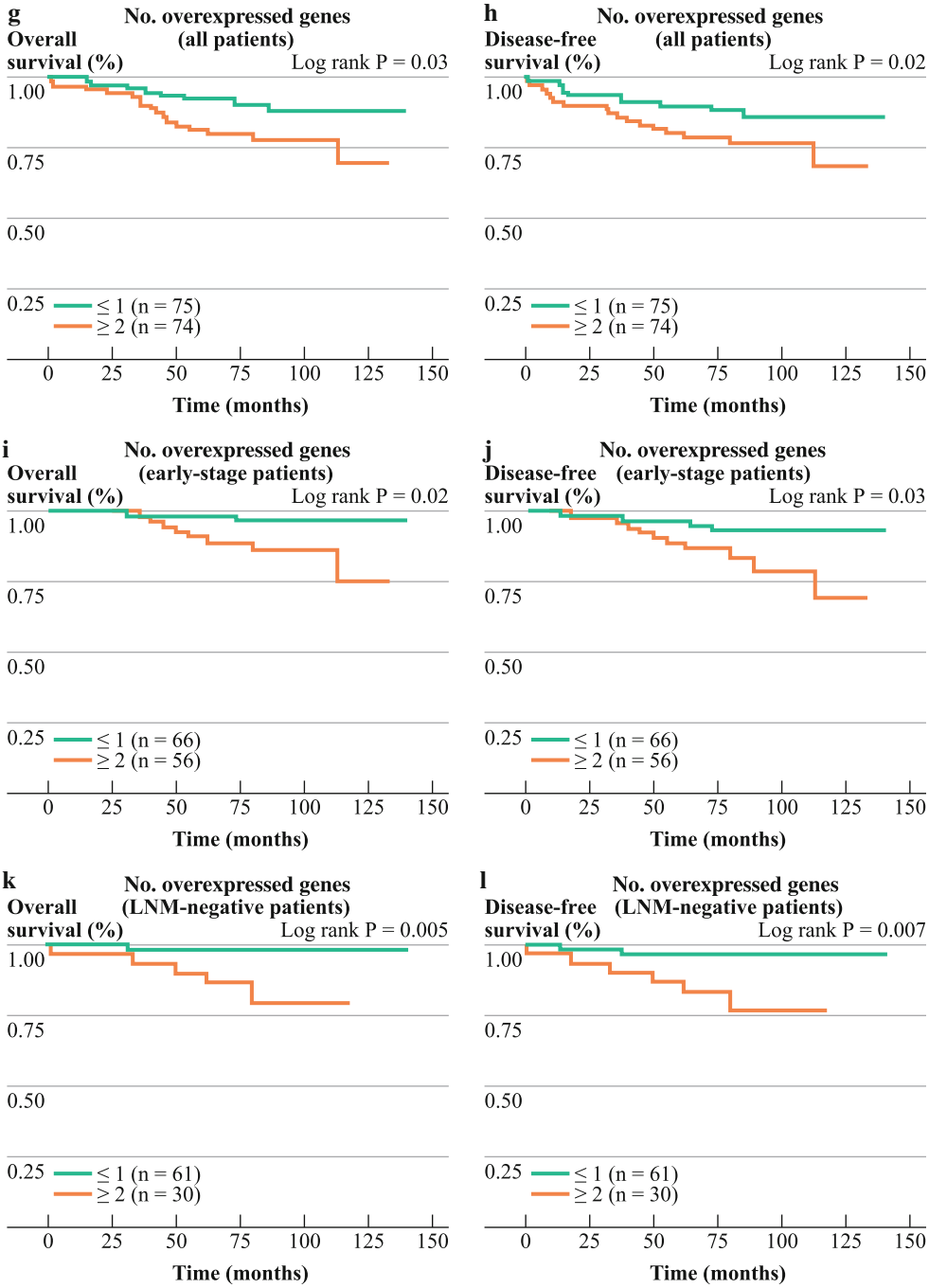


FIG. 2 Prognostic evaluation of increased mRNA expression levels of *cyclin D1*, *β-catenin*, or *MTA1* genes in IDC patients. Kaplan–Meier statistical analyses examining the association between OS (**a**, **c**, **e**, **g**, **i**, **k**) or DFS (**b**, **d**, **f**, **h**, **j**, **l**) and the expression of *cyclin D1*, *β-catenin*, or *MTA1* (*top three panels*) or the number of overexpressed genes in all patients (**a**–**h**) or in the subgroups of early-stage (stage I/II) (**i**, **j**) and LNM-negative (**k**, **l**) patients

interaction can prove the finding being positive.[30] As a result, the power calculation of meaningful enrollment for our tissue sample with logistic regression was determined, which was shown to be greater than 0.8 (power = 0.98), illustrated by using the Wald test, confirming a good practice in achieving different association studies.

Assessments of the transformation of malignant phenotype, prognoses, and therapeutic design of cancers that differ from patients are known to depend on the nature of biological heterogeneity of tumors and involve many genetic factors. Although a growing body of evidence indicates that epigenetic aberrances of *cyclin D1*, *β-catenin*, and *MTA1* genes may serve as biomarkers, the majority of those reports have detected single gene dose in prediction breast cancer progression.[31–34] Besides, it has been indicated that genetic signatures of breast cancer prognoses are varied by different subgroups of aggressive phenotypes.[35–38] We, herein, individually detected the elevated levels of *cyclin D1*, *β-catenin*, and *MTA1* genes in tumor tissues, and the significant associations between

FIG. 2 continued



overexpression of these three genes and poorer prognoses of breast cancer were similar to previous reported among different ethnic populations with different types of cancer.[39–43] Furthermore, the synergistic effects of epigenetic variants on clinical outcome of IDC were tested. Interestingly, of LNM-negative tumors, overexpression of tumors *cyclin D1*, *β-catenin*, and *MTA1* in tumors was shown to be associated with decreased survival rates using the Kaplan–Meier method through survive Bonferroni correction. In the mean time, a significantly elevated hazard ratio for DFS of 10-year follow-up was seen in multivariate Cox regression analysis considering other clinical parameters (Table 4). To the best of our knowledge, this is the first study to demonstrate that overexpression of *cyclin D1*, *β-catenin*, and *MTA1* can be considered as a uniquely epigenetic event linking to aggressive features, and poorer DFS and OS in LNM-negative group of breast cancer. Replicated datasets based on larger cohorts among different ethnic populations are necessary to determine the validity of these associations in the future.

TABLE 4 Cox regression model analyses of the prognostic covariates regarding OS and DFS in IDC patients of LNM-negative subgroup

Univariate regression analysis	OS		DFS	
Covariates	HR (95 % CI)	*P* value	HR (95 % CI)	*P* value
Age	1.044 (0.984–1.107)	0.154	1.023 (0.969–1.079)	0.417
Tumor size (≥30 mm)	5.625 (1.028–28.92)	0.046	2.805 (0.7–11.23)	0.145
Histology grade (I vs. II/III)	0.832 (0.152–4.544)	0.835	2.831 (0.348–23.02)	0.33
ER (positive vs. negative)	1.595 (0.292–8.715)	0.59	1.329 (0.317–5.563)	0.697
PR (positive vs. negative)	0.795 (0.16–3.94)	0.779	0.8 (0.2–3.201)	0.753
HER2 (positive vs. negative)	2.785 (0.51–15.21)	0.237	2.308 (0.551–9.661)	0.252
No. of overexpressed genes (≤ 1 vs. ≥ 2)	10.2 (1.187–87.71)	0.034	6.08 (1.223–30.21)	0.027
Multivariate regression analysis	OS		DFS	
Covariates	HR (95 % CI)	*P* value	HR (95 % CI)	*P* value
Tumor size (≥30 mm)	8.499 (1.53–47.22)	0.014	3.847 (0.945–15.66)	0.06
No. of overexpressed genes (≤1 vs. ≥2)	14.79 (1.673–130.73)	0.015	7.542 (1.483–38.37)	0.015

DFS disease-free survival, *OS* overall survival, *HR* hazard ratio

By immunological analyses, it has been shown that overexpression of *cyclin D1*, *β-catenin*, and *MTA1* correlates tumors of aggressive phenotypes in IDC patients.[44–46] In this study, the differences in expression of those three genes being sensitively detected between tumor and normal cells at mRNA level were validated at protein level in which elevated expressions of *cyclin D1, β-catenin*, and *MTA1* proteins were observed in IHC results ($n = 5$; Supplementary Fig. 2). It is noteworthy that the strength of this cohort of IDC patients is that the length of follow-up was more than 10 years. Besides, a standardized protocol of qRT-PCR analysis that increases the sensitivity of prognostic evaluation by comparing differences in expression levels of the genes in microdissected tumors ensures the validity of the three biomarkers being representable in breast cancer. In addition, the elevated *MTA1* level in mammary glands from the *MTA1* transgenic virgin mice has been shown to hyperactivate the Wnt signaling pathway, which leads to nuclear *β-catenin* accumulation and activate the transcription of downstream *cyclin D1* overexpression.[12,47] Thus, the nominally significant relationship that synergistic interactions of overexpressed *cyclin D1*, *β-catenin*, and *MTA1* overexpressed are associated with poor clinical outcome can apparently be translated into clinical relevance in our cohort. Moreover, to comprehensively understand the mechanism underlying Wnt signaling related to EMT that drives progression of breast cancer, consecutive studies to determine transcriptional levels of those mesenchymal markers, including *Six1*, *Snail 1*, *Slug*, *Twist*, and *Vimentin* genes, are required.[48–50]

To explore applicability of prognostic assessment by using the expression signature of multiple genes, *cyclin D1*, *β-catenin*, and *MTA1*, our data provide valid epigenetic information for classifying individual patients in advance so that they may receive the best treatment to benefit significant survival. Admittedly, the trade-off of the extended period of follow-up was relatively limited sample size in our study. Hence, a prospectively planned approach of the genetic signature with larger samples would make a more precise prediction of breast cancer prognosis. In conclusion, based on epigenetic changes of *cyclin D1*, *β-catenin*, and *MTA1* genes, our study will offer potential help for clinicians to subgroup patients, particularly in LNM-negative patients, to determine more adequate therapeutic protocols of breast cancer.

ACKNOWLEDGMENT We sincerely appreciate Ms. Show-Lin Yang for her assistance in organizing our study specimens. This work was supported by research grants NSC 98-2314-B-040-009-MY3 from the National Science Council, Taipei, Taiwan, ROC.

CONFLICT OF INTEREST None

REFERENCES

1. O'Shaughnessy JA. Molecular signatures predict outcomes of breast cancer. *N Engl J Med*. 2006;355:615–7.
2. Sotiriou C, Pusztai L. Gene-expression signatures in breast cancer. *N Engl J Med*. 2009;360:790–800.
3. Rakha EA, Reis-Filho JS, Ellis IO. Basal-like breast cancer: a critical review. *J Clin Oncol*. 2008;26:2568–81.
4. Andre F, Mazouni C, Liedtke C, et al. HER2 expression and efficacy of preoperative paclitaxel/FAC chemotherapy in breast cancer. *Breast Cancer Res Treat*. 2008;108:183–90.
5. Ellis MJ, Dixon M, Dowsett M, Nagarajan R, Mardis E. A luminal breast cancer genome atlas: progress and barriers. *J Steroid Biochem Mol Biol*. 2007;106:125–9.
6. Staaf J, Ringner M, Vallon-Christersson J, et al. Identification of subtypes in human epidermal growth factor receptor 2—positive breast cancer reveals a gene signature prognostic of outcome. *J Clin Oncol*. 2010;28:1813–20.

7. Brembeck FH, Rosario M, Birchmeier W. Balancing cell adhesion and Wnt signaling, the key role of beta-catenin. *Curr Opin Genet Dev*. 2006;16:51–9.
8. Gordon MD, Nusse R. Wnt signaling: multiple pathways, multiple receptors, and multiple transcription factors. *J Biol Chem*. 2006;281:22429–33.
9. Hatsell S, Rowlands T, Hiremath M, Cowin P. Beta-catenin and Tcfs in mammary development and cancer. *J Mammary Gland Biol Neoplasia*. 2003;8:145–58.
10. Nicolson GL, Nawa A, Toh Y, Taniguchi S, Nishimori K, Moustafa A. Tumor metastasis-associated human MTA1 gene and its MTA1 protein product: role in epithelial cancer cell invasion, proliferation and nuclear regulation. *Clin Exp Metastasis*. 2003;20:19–24.
11. Singh RR, Kumar R. MTA family of transcriptional metaregulators in mammary gland morphogenesis and breast cancer. *J Mammary Gland Biol Neoplasia*. 2007;12:115–25.
12. Bagheri-Yarmand R, Talukder AH, Wang RA, Vadlamudi RK, Kumar R. Metastasis-associated protein 1 deregulation causes inappropriate mammary gland development and tumorigenesis. *Development*. 2004;131:3469–79.
13. Yang PS, Yang TL, Liu CL, Wu CW, Shen CY. A case-control study of breast cancer in Taiwan—a low-incidence area. *Br J Cancer*. 1997;75:752–6.
14. Lo YL, Yu JC, Huang CS, et al. Allelic loss of the BRCA1 and BRCA2 genes and other regions on 17q and 13q in breast cancer among women from Taiwan (area of low incidence but early onset). *Int J Cancer*. 1998;79:580–7.
15. Cheng TC, Chen ST, Huang CS, et al. Breast cancer risk associated with genotype polymorphism of the catechol estrogen-metabolizing genes: a multigenic study on cancer susceptibility. *Int J Cancer*. 2005;113:345–53.
16. Shen CY, Yu JC, Lo YL, et al. Genome-wide search for loss of heterozygosity using laser capture microdissected tissue of breast carcinoma: an implication for mutator phenotype and breast cancer pathogenesis. *Cancer Res*. 2000;60:3884–92.
17. Singletary SE, Connolly JL. Breast cancer staging: working with the sixth edition of the AJCC cancer staging manual. *CA Cancer J Clin*. 2006;56:37-47; quiz 50–31.
18. Lo YL, Shen CY. Laser capture microdissection in carcinoma analysis. *Methods Enzymol*. 2002;356:137–44.
19. Petroff BK, Phillips TA, Kimler BF, Fabian CJ. Detection of biomarker gene expression by real-time polymerase chain reaction using amplified ribonucleic acids from formalin-fixed random periareolar fine needle aspirates of human breast tissue. *Anal Quant Cytol Histol*. 2006;28:297–302.
20. Yang C, Trent S, Ionescu-Tiba V, Lan L, Shioda T, Sgroi D, Schmidt EV. Identification of cyclin D1- and estrogen-regulated genes contributing to breast carcinogenesis and progression. *Cancer Res*. 2006;66:11649–58.
21. Cheng CW, Yu JC, Wang HW, et al. The clinical implications of MMP-11 and CK-20 expression in human breast cancer. *Clin Chim Acta*. 2010;411:234–41.
22. Huang CS, Shen CY, Wang HW, Wu PE, Cheng CW. Increased expression of SRp40 affecting CD44 splicing is associated with the clinical outcome of lymph node metastasis in human breast cancer. *Clin Chim Acta*. 2007;384:69–74.
23. Pfaffl MW. A new mathematical model for relative quantification in real-time RT-PCR. *Nucleic Acids Res*. 2001;29:e45.
24. Yu JC, Ding SL, Chang CH, et al. Genetic susceptibility to the development and progression of breast cancer associated with polymorphism of cell cycle and ubiquitin ligase genes. *Carcinogenesis*. 2009;30:1562–70.
25. Ding SL, Yu JC, Chen ST, et al. Diverse associations between ESR1 polymorphism and breast cancer development and progression. *Clin Cancer Res*. 2010;16:3473–84.
26. Lin CH, Liau JY, Lu YS, et al. Molecular subtypes of breast cancer emerging in young women in Taiwan: evidence for more than just westernization as a reason for the disease in Asia. *Cancer Epidemiol Biomarkers Prev*. 2009;18:1807–14.
27. Chiang AC, Massague J. Molecular basis of metastasis. *N Engl J Med*. 2008;359:2814–23.
28. Hunter KW, Crawford NP, Alsarraj J. Mechanisms of metastasis. *Breast Cancer Res*. 2008;(10 Suppl 1):S2.
29. Chambers AF, Groom AC, MacDonald IC. Dissemination and growth of cancer cells in metastatic sites. *Nat Rev Cancer*. 2002; 2:563–72.
30. Demidenko E. Sample size determination for logistic regression revisited. *Stat Med*. 2007;26:3385–97.
31. Lundgren K, Brown M, Pineda S, et al. Effects of cyclin D1 gene amplification and protein expression on time to recurrence in postmenopausal breast cancer patients treated with anastrozole or tamoxifen: a TransATAC study. *Breast Cancer Res*. 2012; 14:R57.
32. Martin MD, Hilsenbeck SG, Mohsin SK, et al. Breast tumors that overexpress nuclear metastasis-associated 1 (MTA1) protein have high recurrence risks but enhanced responses to systemic therapies. *Breast Cancer Res Treat*. 2006;95:7–12.
33. Roy PG, Pratt N, Purdie CA, Baker L, Ashfield A, Quinlan P, Thompson AM. High CCND1 amplification identifies a group of poor prognosis women with estrogen receptor positive breast cancer. *Int J Cancer*. 2009;127:355–60.
34. Verghese ET, Shenoy H, Cookson VJ, et al. Epithelial-mesenchymal interactions in breast cancer: evidence for a role of nuclear localized beta-catenin in carcinoma-associated fibroblasts. *Histopathology*. 2011;59:609–18.
35. King TD, Suto MJ, Li Y. The Wnt/beta-catenin signaling pathway: a potential therapeutic target in the treatment of triple negative breast cancer. *J Cell Biochem*. 2011;113:13–8.
36. Mavaddat N, Dunning AM, Ponder BA, Easton DF, Pharoah PD. Common genetic variation in candidate genes and susceptibility to subtypes of breast cancer. *Cancer Epidemiol Biomarkers Prev*. 2009;18:255–9.
37. Mukherjee N, Bhattacharya N, Alam N, Roy A, Roychoudhury S, Panda CK. Subtype-specific alterations of the Wnt signaling pathway in breast cancer: clinical and prognostic significance. *Cancer Sci*. 2011;103:210–20.
38. Tobin NP, Sims AH, Lundgren KL, Lehn S, Landberg G. Cyclin D1, Id1 and EMT in breast cancer. *BMC Cancer*. 2011;11:417.
39. Balasenthil S, Broaddus RR, Kumar R. Expression of metastasis-associated protein 1 (MTA1) in benign endometrium and endometrial adenocarcinomas. *Hum Pathol*. 2006;37:656–61.
40. Chen CH, Shen J, Lee WJ, Chow SN. Overexpression of cyclin D1 and c-Myc gene products in human primary epithelial ovarian cancer. *Int J Gynecol Cancer*. 2005;15:878–83.
41. Cheng H, Liang H, Qin Y, Liu Y. Nuclear beta-catenin overexpression in metastatic sentinel lymph node is associated with synchronous liver metastasis in colorectal cancer. *Diagn Pathol*. 2011;6:109.
42. Huang SF, Cheng SD, Chuang WY, Chen IH, Liao CT, Wang HM, Hsieh LL. Cyclin D1 overexpression and poor clinical outcomes in Taiwanese oral cavity squamous cell carcinoma. *World J Surg Oncol*. 2012;10:40.
43. Prisco MG, Zannoni GF, De Stefano I, et al. Prognostic role of metastasis tumor antigen 1 in patients with ovarian cancer: a clinical study. *Hum Pathol*. 2011;43:282–8.
44. Liu T, Niu Y, Feng Y, Niu R, Yu Y, Lv A, Yang Y. Methylation of CpG islands of p16(INK4a) and cyclinD1 overexpression associated with progression of intraductal proliferative lesions of the breast. *Hum Pathol*. 2008;39:1637–46.
45. Prasad CP, Mirza S, Sharma G, Prashad R, DattaGupta S, Rath G, Ralhan R. Epigenetic alterations of CDH1 and APC genes:

relationship with activation of Wnt/beta-catenin pathway in invasive ductal carcinoma of breast. *Life Sci*. 2008;83:318–25.
46. Mahoney MG, Simpson A, Jost M, et al. Metastasis-associated protein (MTA)1 enhances migration, invasion, and anchorage-independent survival of immortalized human keratinocytes. *Oncogene*. 2002;21:2161–70.
47. Kumar R, Balasenthil S, Manavathi B, Rayala SK, Pakala SB. Metastasis-associated protein 1 and its short form variant stimulates Wnt1 transcription through promoting its derepression from Six3 corepressor. *Cancer Res*. 2010;70:6649–58.
48. Kallergi G, Papadaki MA, Politaki E, Mavroudis D, Georgoulias V, Agelaki S. Epithelial to mesenchymal transition markers expressed in circulating tumour cells of early and metastatic breast cancer patients. *Breast Cancer Res*. 2011;13:R59.
49. Toh Y, Nicolson GL. The role of the MTA family and their encoded proteins in human cancers: molecular functions and clinical implications. *Clin Exp Metastasis*. 2009;26:215–27.
50. Micalizzi DS, Farabaugh SM, Ford HL. Epithelial-mesenchymal transition in cancer: parallels between normal development and tumor progression. *J Mammary Gland Biol Neoplasia*. 2010;15:117–34.

Breast Cancer Res Treat (2012) 134:1081–1093
DOI 10.1007/s10549-012-2034-4

PRECLINICAL STUDY

MicroRNA-30a inhibits cell migration and invasion by downregulating vimentin expression and is a potential prognostic marker in breast cancer

Chun-Wen Cheng · Hsiao-Wei Wang · Chia-Wei Chang · Hou-Wei Chu · Cheng-You Chen · Jyh-Cherng Yu · Jui-I Chao · Huei-Fang Liu · Shian-ling Ding · Chen-Yang Shen

Received: 13 December 2011 / Accepted: 14 March 2012 / Published online: 4 April 2012

Abstract Tumor recurrence and metastasis result in an unfavorable prognosis for cancer patients. Recent studies have suggested that specific microRNAs (miRNAs) may play important roles in the development of cancer cells. However, prognostic markers and the outcome prediction of the miRNA signature in breast cancer patients have not been comprehensively assessed. The aim of this study was to identify miRNA biomarkers relating to clinicopathological features and outcome of breast cancer. A miRNA microarray analysis was performed on breast tumors of different lymph node metastasis status and with different progression signatures, indicated by overexpression of *cyclin D1* and *β-catenin* genes, to identify miRNAs showing a significant difference in expression. The functional interaction between the candidate miRNA, miR-30a, and the target gene, *Vim*, which codes for vimentin, a protein involved in epithelial–mesenchymal transition, was examined using the luciferase reporter assay, western blotting, and migration and invasion assays. The association between the decreased miR-30a levels and breast cancer progression was examined in a survival analysis. miR-30a negatively regulated vimentin expression by binding to the 3′-untranslated region of *Vim*. Overexpression of miR-30a suppressed the migration and invasiveness phenotypes of breast cancer cell lines. Moreover, reduced tumor expression of miR-30a in breast cancer patients was associated with an unfavorable outcome, including late tumor stage, lymph node metastasis, and worse progression (mortality and recurrence) ($p < 0.05$). In conclusion, these findings suggest a role for miR-30a in inhibiting breast tumor invasiveness and metastasis. The finding that miR-30a downmodulates vimentin expression might provide a therapeutic target for the treatment of breast cancer.

Keywords Breast cancer · MicroRNA-microarray · MiR-30a · Vimentin · Prognosis

Abbreviations

IDC	Invasive ductal carcinoma
EMT	Epithelial–mesenchymal transition
miRNA	microRNA
3′UTR	3′-untranslated region
LNM	Lymph node metastasis
LCM	Laser capture microdissection
qRT-PCR	Quantitative real-time reverse transcription polymerase chain reaction

C.-W. Cheng (✉) · C.-W. Chang · C.-Y. Chen
Institute of Biochemistry and Biotechnology, Chung Shan Medical University, Taichung 40201, Taiwan
e-mail: cwcheng@csmu.edu.tw

C.-W. Cheng
Clinical Laboratory, Chung Shan Medical University Hospital, Taichung, Taiwan

C.-W. Cheng · H.-W. Wang · H.-W. Chu · C.-Y. Shen (✉)
Institute of Biomedical Sciences, Academia Sinica, Taipei 11529, Taiwan
e-mail: bmcys@ibms.sinica.edu.tw

J.-C. Yu
Department of Surgery, Tri-Service General Hospital, National Defense Medical Center, Taipei, Taiwan

J.-I. Chao · H.-F. Liu
Department of Biological Science and Technology, National Chiao Tung University, Hsinchu, Taiwan

S. Ding
Department of Nursing, Kang-Ning Junior College of Medical Care and Management, Taipei, Taiwan

C.-Y. Shen
Graduate Institute of Environmental Science, China Medical University, Taichung, Taiwan

DFS	Disease-free survival
OS	Overall survival
HR	Hazard ratio
OR	Odds ratio
95 % CI	95 % confidence interval

Introduction

In contrast to the total abolition of gene function by permanent mutation, the reversibility of epigenetic modification allows gene expression to be switched on and off and thus is suggested to provide selective advantage for clonal evolution during tumor progression [1, 2]. Tumor recurrence and metastasis result in an unfavorable prognosis for patients with cancer. Tumor cell metastasis is characterized by an unstable phenotypic heterogeneity, in which the phenotype fluctuates too frequently for the changes to be mediated exclusively by irreversible genetic alterations. During tumor metastasis, cancer cells undergo epithelial–mesenchymal transition (EMT) in which epithelial tumor cells in the primary cancer site are converted into aggressive and metastatic tumor cells. One of the main characteristics of EMT is the combination of loss of cell–cell contact, reduced E-cadherin expression, and enhanced expression of mesenchymal markers, such as vimentin [3–5]. The mesenchymal-like cells generated are transported to metastatic sites, where they may undergo mesenchymal–epithelial transition by regaining E-cadherin expression, allowing cell–cell adhesion and connecting adjacent cells to form new foci [6]. For this reason, epigenetic regulation appears to be more important than genetic level regulation in maintaining flexibility, and, in the case of E-cadherin, hypermethylation of the promoter region is seen in various human carcinomas [7–10]. In cancer metastasis, there is evidence for a significant contribution of epigenetic regulation of EMT by microRNAs (miRNAs) [11, 12], a novel class of short non-coding RNA molecules consisting of 19–25 nucleotides with the potential to inhibit gene expression by binding to complementary sequences in the 3′-untranslated region (UTR) of target mRNA transcripts. miRNAs are an emerging class of negative regulators, and deregulation of miRNAs in tumors affects the expression of oncogenes and/or tumor suppressor genes and leads to malignant transformation in human cancer [13–16]. However, knowledge of prognostic markers and the effectiveness of outcome prediction of the miRNA signature in breast cancer patients is limited.

We recently investigated a gene expression signature that is an important predictor of an unfavorable outcome in patients with invasive ductal carcinoma (IDC) and found that overexpression of the genes making up this expression signature, namely *cyclin D1* and *β-catenin*, is highly associated with tumor cells of an advanced stage, lymph node metastasis (LNM), and a decreased survival rate (manuscript submitted). To examine the mechanism responsible for the changes associated with this signature, the present study investigated whether and how deregulation of specific miRNAs associated with epigenetic aberrance was involved in driving the invasiveness and metastasis of tumor cells. The miRNA expression profile was then compared between breast tumors showing overexpression of *cyclin D1* and *β-catenin* and LNM and those with a normal signatures of both genes and no LNM. Among the miRNAs showing a significant difference in expression in tumor cells, particular attention was focused on miR-30a, as in silico prediction of target genes possibly regulated by this miRNA molecule resulted in the identification of the gene, *Vim*, coding for vimentin, a mesenchymal marker implicated in EMT during breast tumorigenesis [17–19]. We explored whether miR-30a regulated vimentin expression and showed that increased expression of miR-30a resulted in downregulation of vimentin expression and a subsequent decrease in the vimentin-mediated migration and invasiveness of breast cancer cells. Moreover, reduced levels of miR-30a expression in the primary breast tumor tissue were found to predict an unfavorable outcome, including advanced stage, LNM, and a decreased survival rate in IDC patients. These findings identify the role of miR-30a in breast cancer and shed light on a novel fundamental mechanism with clinical significance and translational implications.

Materials and methods

Study population

The present study is part of an ongoing cooperative study aimed at discovering markers for the evaluation of breast cancer progression in Taiwan, where breast cancer is characterized by low incidence [20], early tumor onset [21], reproductive hormone dependency [22, 23], and novel genomic alteration [23–26]. The study was approved by the Ethics Committees of the Institutional Review Boards of the Tri-Service General Hospital, Taipei, and the Chung Shan Medical University Hospital, Taichung, Taiwan. The enrolled female patients pathologically confirmed primary IDC of the breast were a subset of women randomly selected from the ongoing hospital-based breast cancer cohort collected in the Surgery Department of the Tri-Service General Hospital, collected between July 1997 and April 2006. Informed consent was obtained from each participant prior to specimen acquisition. The resected breast cancer tissues were immediately frozen in liquid nitrogen until analysis. Tumor grade in each patient was categorized as I, II, or III according to the Nottingham modification of the Scarff–Bloom–Richardson system, and the pathology of these tumors was classified according to

Table 1 Clinicopathological characteristics of the 221 female patients with IDC of the breast

Characteristics	N (%)
Age (mean ± SD)	50.6 ± 11.6 (range 23-87 yrs)
Survival (months, mean ± SD)	68.5 ± 28.9 (range 1-140 months)
Tumor size (mm)	
>20	95 (44.3)
≧20	123 (55.7)
ND	3
Grade	
I	34 (15.4)
II	98 (44.3)
III	89 (40.3)
Stage	
I	76 (34.4)
II	113 (51.1)
III	28 (12.7)
IV	4 (1.8)
Lymph node metastasis	
N0	118 (53.4)
N1	76 (34.4)
N2	27 (12.2)
Estrogen receptor	
Negative	117 (54.2)
Positive	99 (45.8)
ND	5
Progesterone receptor	
Negative	118 (54.6)
Positive	98 (45.4)
ND	5

ND non-detected

the sixth edition of the AJCC Cancer Staging Manual. None of the patients received neoadjuvant treatment before primary surgery, thus avoiding any effects on gene expression. The histological diagnosis of all specimens was reviewed by a certified pathologic physician, and the clinicopathological findings are summarized in Table 1.

Laser capture microdissection

To ensure that the tissue samples assayed consisted of >95 % pure breast tumor epithelial cells, laser capture microdissection (LCM) was performed on routinely immunostained slides using a PixCell laser capture microscope (Arcturus Engineering, Mountain View, CA, USA) as described previously [27, 28]. The dehydrated tissue section was overlaid with a thermoplastic film mounted on an optically transparent cap, and the visually selected areas (tumor cells) were bound to the membrane by short, low-energy laser pulses, resulting in focal melting of the polymer. On average, 2,500–3,000 LCM shots were performed on a single tumor to obtain sufficient tumor cells for comparative qRT-PCR analysis. The laser-captured tumor cells were immersed in 50–100 µl of digestion buffer (10 mM Tris–HCl, pH 8.0, 1 mM EDTA, 400 µg/ml of proteinase K, and 1 % Tween 20) and digested at 55 °C overnight. After digestion, the enzyme was heat-inactivated (95 °C for 10 min) and the extract used directly for RNA isolation.

RNA isolation

Total RNA was isolated from paired LCM-dissected tumor and non-tumor cells from each patient using an RNAqueous®-Micro Kit (Ambion Inc., Austin, TX, USA) and the yield of RNA determined by spectrophotometry at 260 nm. miRNAs from tumor and non-tumor cells were extracted from tissue sections using a mirVana miRNA isolation kit according to the manufacturer's instructions (Ambion Inc., Austin, TX, USA) and the RNA concentration in each sample quantified on a NanoDrop 1000 spectrophotometer (NanoDrop Technologies, Waltham, MA, USA).

miRNA microarray

A human miRNA microarray contained probes for 381 human miRNAs from TaqMan® Low Density Array Human MicroRNA Panel v1.0 (Applied Biosystems) and levels of mature miRNAs were analyzed on an Applied Biosystems 7900HT Fast Real-Time PCR System (Foster City, CA, USA). Expression level of each miRNA was measured using the same amount of template RNA in each well with small nuclear RNA U48 (RNU48) as an endogenous negative control and RNU6B as a positive control. Relative quantification of miRNA expression was performed using the 2-ddCt method. The threshold cycle, Ct was automatically calculated by the SDS2.2 software package (Applied Biosystems, Foster City, CA, USA). Ct values for all targets were determined using the automatic threshold in RQ Manager v1.1 analysis software.

Cell culture

The breast cancer cell lines Hs578T and MDA-MB-231 were cultured in DMEM (Life Technologies) containing 0.1 mM sodium pyruvate, 10 % fetal bovine serum (FBS), 2 mmol/l of L-glutamine, 100 IU/ml of penicillin, and 100 mg/ml of streptomycin (all from Biosource, Rockville, MD, USA) in a humidified 5 % CO_2 atmosphere at 37 °C. Transfection with different miRNAs and plasmid constructs was performed using DOTAP (Biontex, Laboratories, GmbH) and Turbofect™ (Fermentas, Germany) in

Table 2 Oligonucleotides used for the miRNA constructs and the generation of the *Vim* 3′UTR-mutants

Gene of interest	Primer sequences used for the miRNA constructs
hsa-miR-502	Forward: 5′-CCCAAGCTTCACAACATGGGACTT
	Reverse: 5′-CGGGATCCGCTCCATCTCATTGAA
hsa-miR-485	Forward: 5′-CCCAAGCTTGGGTGTATGTCACTCG
	Reverse: 5′-CGGGATCCCCAAGATTCAACTCCA
hsa-miR-519e	Forward: 5′-CCCAAGCTTAGGAACTGGAGATGGT
	Reverse: 5′-CGGGATCCTGTGGTGAAACTCCAT
hsa-miR-328	Forward: 5′-CCAAGCTTTCCATGAGCCTTCTTA
	Reverse: 5′-CGGAATTCTATTGCCCTACTACGC
hsa-miR-30a	Forward: 5′-CCAAGCTTATAAGTGAGCGCATTC
	Reverse: 5′-CGGAATTCGTGTTGGAGAACAGCA
Luc-Vim 3′UTR/Mut1	Forward: 5′-CATAATCTAGTCCCCAGAAAAATCTTGTGC
	Reverse: 5′-GCACAAGATTTTTCTGGGGACTAGATTATG
Luc-Vim 3′UTR/Mut2	Forward: 5′-CCTACAAGATTTAGAAAAAAGTCCCCAACATAATCTAGTTTACAG
	Reverse: 5′-CTGTAAACTAGATTATGTTGGAGAGCTTTTTTCTAAATCTTGTAGG
Luc-Vim' 3UTR/Mut12	Forward: 5′-TAGAAAAAAGTCCCCAACATAATCTAGTCCCCAG
	Reverse: 5′-CTGGGGACTAGATTATGTTGGGGACTTTTTTCTA

vitro transfection reagents according to the manufacturer's recommendations. Transfectants were cultured and selected for 2 weeks in the medium containing 3 μg/ml of puromycin.

Western blotting analysis

Cell extracts were prepared in ice-cold RIPA lysis buffer. After whole cell protein extracts were quantified by BCA protein assay, equivalent amounts of cell lysates were resolved by 8–12 % SDS polyacrylamide gel electrophoresis and transferred onto a polyvinylidene difluoride membrane, which was then blocked in 5 % non-fat milk in PBST and probed overnight at 4 °C with a monoclonal antibody against human vimentin (1:500, Santa Cruz Biotechnology). Anti-β-actin (Sigma-Aldrich Corp., St. Louis, MO, USA) was used to normalize for protein loading. The blot was incubated with an appropriate horseradish peroxidase conjugated second antibody and immunoreactive proteins visualized by the enhanced chemiluminescence assay (western blotting luminal reagent; Santa Cruz Biotechnology) and the band intensities quantified by densitometry (Digital Protein DNA Imagineware, Huntington Station, NY, USA).

Dual luciferase reporter assay

The 3′UTR sequence of the human vimentin gene (*Vim*) was cloned into plasmid pGL4.13 (Promega, Madison, WI), yielding the recombinant vector pGL4.13_1, containing the firefly luciferase open reading frame under the control of the SV40 promoter. Two miR-30a complementary sites with the sequence *GTTTAC* in the *Vim* 3′UTR were mutated singly or together to remove complementarity to miR-30a using a QuikChange II XL site-directed mutagenesis kit (Stratagene, La Jolla, CA, USA) with pGL4.13_1/*Vim*-WT as the template, and the mutants were named *Vim* 3′UTR/mut1, *Vim* 3′UTR/mut2, and *Vim* 3′UTR/mut12. The mutated nucleotides capitalized, and the sequences of the mismatch primers used to generate different *Vim* 3′UTR mutants are shown in Table 2. MDA-MB-231 cells were cotransfected with the reporter construct, the control or mutated *Vim* 3′UTR constructs, and pCDNA3/miR-30a, then the cells were lysed 24 h later, and the firefly luciferase/Renilla luciferase activity ratio of each sample measured in a dual-luciferase assay (Promega, Madison, WI, USA). The experiments were performed in triplicate. Data are presented as mean ± standard deviation (SD) for each transfection condition. Significance of comparisons was assessed by using the two-sided unpaired *t* test for means. A p value <0.05 is taken to indicate statistical significance.

Establishment of breast tumor cells stably expressing miR-30a

Using a lentivirus expression system (Thermo Fisher Scientific; Waltham, MA, USA) and the Trans-Lentiviral™ GIPZ Packaging System (Open Biosystems, Huntsville, AL, USA), breast cancer cells were transduced with plasmid pLemiR expressing primary-miR-30a (pri-miR-30a) transcripts under the control of the CMV promoter (Open Biosystems, Huntsville, AL, USA). TurboRed Fluorescent Protein and a puromycin-resistance selectable marker were

used to allow screening for non-transduced cells, and cell lines stably expressing miR-30a were established.

Invasion and migration assays

For the invasion assay, matrigel (Collaborative Biomedical Products, Bedford, MA, USA) was applied to 8-μm pore size polycarbonate membrane filters, and then cells (1.0×10^5) cultured in DMEM were seeded into the upper section of the Boyden chamber (Neuro Probe, Cabin John, MD, USA). The lower chamber contained the same medium plus 10 % FBS, and the chamber was incubated overnight at 37 °C, after which non-invading cells were removed from the interior of the insert using a cotton-tip applicator, and the invasive cells attached to the lower surface of the membrane were fixed with methanol and stained with Giemsa. Invading cells were quantified by counting five random high-powered fields using a Olympus Ckx41 light microscope. For the migration assay, the cells were seeded into the Boyden chamber on membrane filters that were not coated with matrigel and incubated for 16 h at 37 °C, then non-migrating cells were removed from the upper membrane surface, and the invading cells on the lower membrane surface quantified as described above.

Comparatively quantitative real-time PCR analysis

The LCM was performed on 221 breast cancer tissue slides, and the single-tube TaqMan miRNA assay (Applied Biosystems, Foster City, CA, USA) was used to detect and quantify the mature miRNA on an Applied Biosystems instruments. The level of expression of the miRNA biomarker was determined using the TaqMan real-time PCR assay and normalized to that for RNU6B. Triplicate qPCR experiments were performed on each breast carcinoma to determine the levels of the target mRNA in the isolated tumor and non-tumor cells. The comparative CT method (-ddCt) was used to estimate the relative expression (fold change) of the miR-30a transcript in the tumor and non-tumor cells in each case (2-ddCt, where $\mathrm{ddCt} = \mathrm{dCt}_{\text{miR-30a}} - \mathrm{dCt}_{\text{RNU6B}}$).

Statistical analysis

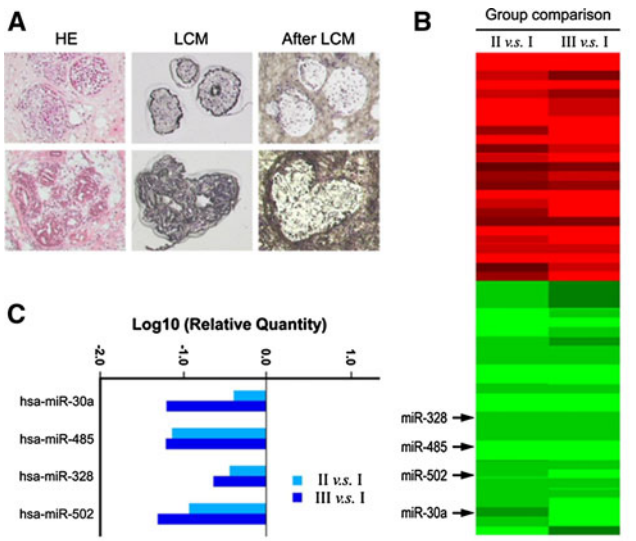
The Chi-squared test was used to examine whether there was an association between decreased miR-30a transcript levels in cancer tissue and the pathological features of tumor (stage and LNM status). The miR-30a transcript was quantified using the comparative CT method as described previously [29, 30]. To examine whether miR-30a could be used as a prognostic biomarker in breast cancer patients, Kaplan–Meier survival analysis (p value of the log-rank test) and Cox regression analysis [hazard ratio (HR) and 95 % confidence interval (95 % CI)] were used to explore the association between the 5-year recurrence-free/disease-free survival rate and miR-30a transcript levels in tumors of breast cancer patients. Recurrence-free survival was measured as the time from surgery to recurrence or the end of the study, while disease-free survival was defined as the time from surgery to recurrence or death or the end of the study.

Results

A miRNA expression signature in the primary tumor predicts its metastatic potential

To search for miRNAs that might play a role in breast cancer progression, we used miRNA chips to compare the miRNA expression profiles of three groups of patients with differential expression of the *cyclin D1* and *β-catenin* genes and different LNM status. We compared the profiles of five patients with Stage I/II cancer (LNM−) and normal expression of *cyclin D1* and *β-catenin* (group I), four tumors with Stage I/II (LNM−) and increased expression of *cyclin D1* and *β-catenin* (group II), and five tumors with Stage III/IV (LNM+) and increased expression of *cyclin D1* and *β-catenin* (group III). None of the fourteen patients had received neoadjuvant treatment before surgery, thus avoiding any confounding effect on gene expression. *Cyclin D1* and *β-catenin* make up an expression signature that is highly associated with poor pathological features and a worse clinical outcome (manuscript submitted). It should be noted that the determination of the miRNA expression pattern was based on cancerous cells microdissected from tumor tissues to ensure that the sample tested consisted of >95 % pure breast tumor epithelial cells (Fig. 1a), supporting the validity of our measurement. Using a cutoff of a greater than twofold difference in levels, we identified 52 miRNAs that were significantly deregulated in the primary tumor of patients with metastatic breast cancer than in those with no metastases, 25 of which were upregulated (red section of Fig. 1b) in group III or II compared with group I and 27 downregulated (green section of Fig. 1b). The five highly downregulated miRNAs were miR-502, miR-485, miR-328, miR-30a, and miR-519e that may affect vimentin expression due to their being predicted to bind 3′UTR of the *Vim* gene in an in silico analysis, and the results for the first four are shown in Fig. 1c. Those identified 52 miRNAs that were differentially expressed between the metastatic tumors and the non-metastatic samples are summarized in Fig. 1d.

To gain an insight into the functional consequences of differential miRNA expression, we used the miRNA target searching program TargetScan 5.1 (www.microrna.org) and a computational algorithm to explore whether the expression



D Differentially expressed miRNAs in the primary tumor in patients with metastatic or non-metastatic invasive ductal carcinoma of the breast

miRNA	Fold change (metastatic/non-metastatic)	miRNA	Fold change (metastatic/non-metastatic)
Upregulated		Downregulated	
hsa-miR-449	149.40	hsa-miR-329	0.01
hsa-miR-196b	66.10	hsa-miR-501	0.02
hsa-miR-187	53.09	hsa-miR-133b	0.03
hsa-let-7e	33.72	hsa-miR-213	0.03
hsa-miR-379	15.59	hsa-miR-217	0.03
hsa-miR-7	14.01	hsa-miR-422a	0.04
hsa-miR-199b	12.26	hsa-miR-491	0.06
hsa-miR-487b	8.57	hsa-miR-642	0.06
hsa-miR-503	7.80	hsa-miR-204	0.07
hsa-miR-449b	7.50	hsa-miR-485	0.07
hsa-miR-98	6.18	hsa-miR-502	0.12
hsa-miR-193b	6.12	hsa-miR-622	0.15
hsa-miR-194	5.85	hsa-miR-519e	0.16
hsa-miR-22	5.29	hsa-miR-489	0.17
hsa-miR-34a	5.28	hsa-miR-139	0.18
hsa-miR-221	4.65	hsa-miR-10a	0.22
hsa-miR-145	4.03	hsa-miR-324	0.27
hsa-miR-34c	3.94	hsa-miR-135b	0.31
hsa-miR-17	3.90	hsa-miR-218	0.32
hsa-miR-452	3.39	hsa-miR-345	0.32
hsa-miR-210	3.22	hsa-miR-380	0.34
hsa-miR-181c	2.67	hsa-miR-550	0.35
hsa-miR-594	2.57	hsa-miR-197	0.36
hsa-miR-149	2.44	hsa-miR-328	0.36
hsa-miR-335	2.29	hsa-miR-572	0.36
		hsa-miR-95	0.37
		hsa-miR-30a	0.40

Fig. 1 miR-30a expression is decreased in breast tumors with overexpression of *cyclin D1* and *β-catenin* or lymph node metastasis (LNM). **a** Illustration of the laser capture-microdissection technique. The *left*, *center*, and *right panels* show the tissue before laser capture-microdissection LCM (i.e., HE staining), the LCM-captured cells, and the tumor after LCM, respectively. **b** and **c** Total RNA was extracted from LCM tumor cells from low-stage (Stage I/II) breast tumors ($n = 5$) showing normal expression of *cyclin D1* and *β-catenin* and no LNM (group I); low-stage tumors ($n = 4$) showing increased expression of *cyclin D1* and *β-catenin*, but no LNM (group II); and high-stage (Stage III/IV) tumors ($n = 5$) showing increased expression of *cyclin D1* and *β-catenin* and LNM (Group III). RNAs from tumors in the same group were pooled and subjected to TaqMan-LDA miRNA microarray analysis. **b** shows a comparison of the results for group II and I (*left panel*) and for group III and I (*right panel*); 52 miRNAs showing a greater than twofold change in expression were associated with tumor stage and LNM, of which 25 were upregulated (*red*) and 27 downregulated (*green*) during tumor progression. **c** The five highly downregulated miRNAs were miR-328, miR-485, miR-502, miR-30a, and miR-519e; the quantitative data for the first four are shown. **d** 52 miRNAs that were differentially expressed between the metastatic tumors and the non-metastatic samples. (Color figure online)

of any EMT-associated genes was regulated by these five miRNAs, and the results suggested that expression of the *Vim* gene, encoding an intermediate filament normally expressed in cells of mesenchymal origin, might be associated with decreased expression of these miRNAs. Vimentin, an important EMT-associated marker, is involved in linking the cytoskeleton to the membrane, and deregulation of vimentin has been suggested to be associated with increased invasiveness or migration of cells [18, 31].

miR-30a directly targets the vimentin 3′UTR and downregulates vimentin expression

To test the in silico prediction that vimentin expression might be modulated by specific miRNAs, we measured vimentin levels by immunoblotting after transfection of the human breast cancer cell lines Hs578T and MDA-MB-231 with the precursors of the above five miRNAs predicted to affect vimentin expression or with vector alone (Fig. 2a). As shown in Fig. 2b, no significant difference in *Vim* mRNA levels was found in breast cancer cells transfected with these five miRNAs, whereas, in the same cells, vimentin protein expression was inhibited by more than 60 % by miR-30a; however, the other four miRNAs had only a minor, or a non-significant, effect (Fig. 2c, d).

Furthermore, to map putative interaction sites between vimentin 3′UTR and miR-30a, three algorithms, miRanda, PicTar, and TargetScan, were used to predict the mRNA targets for miR-30a, and two potential sites were identified within the 3′UTR of *Vim* in different species (Fig. 3a). To determine whether miR-30a directly targets *Vim* mRNA and the relative importance of these two sites in repressing

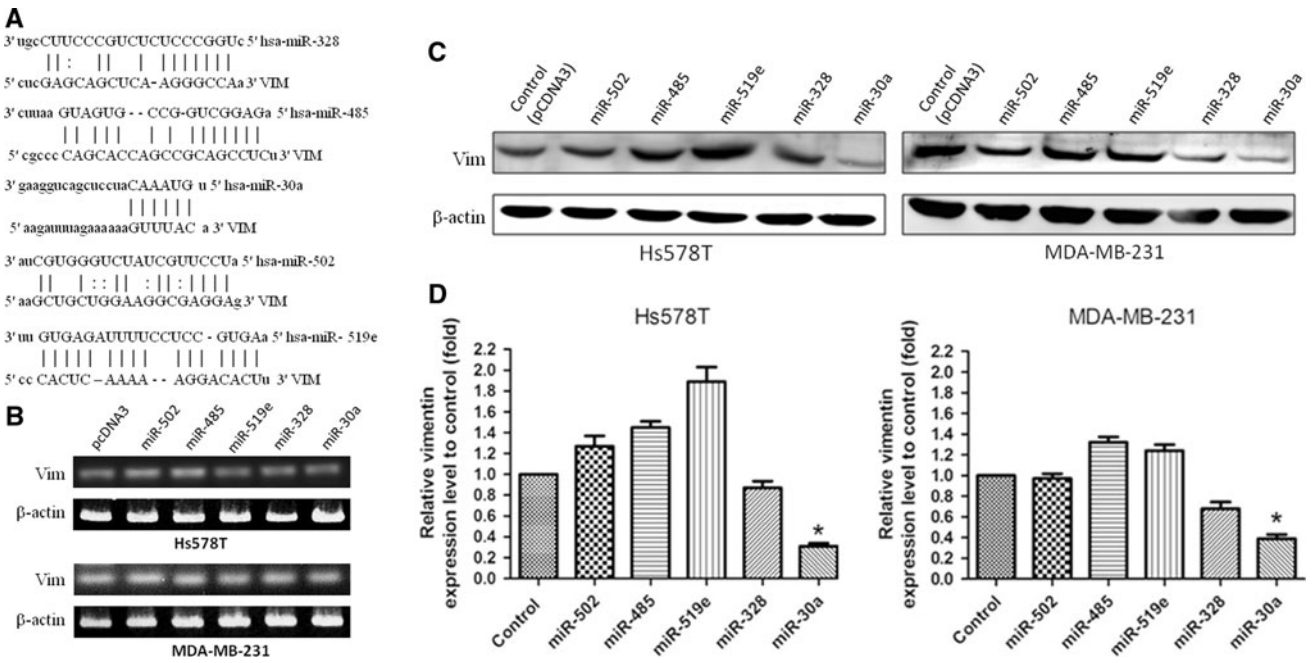


Fig. 2 miR-30a represses vimentin expression. **a** A computational algorithm was used to predict five miRNAs that might affect vimentin expression. **b–d** miRNAs that were predicted to impact vimentin expression were transfected into breast cancer cell lines Hs578T and MDA-MB-231; then, 24 h later, vimentin mRNA levels were measured by RT-PCR in (**b**) and vimentin protein levels by western blotting in (**c**) and compared to those in cells transfected with control vector. **d** Quantitative results for three independent experiments showing suppression of vimentin expression on western blots by overexpression of miR-30a in Hs578T cells (*left panel*) or MDA-MB-231 cells (*right panel*). $*p < 0.05$

vimentin expression, we inserted the full-length 3′UTR of *Vim* into the pGL4.13-luciferase reporter (*Vim* 3′UTR-luc) and examined the effect of miR-30a on the luciferase activity. In addition, we generated three 3′UTR mutants, *Vim* 3′UTR/Mut1-luc, and *Vim* 3′UTR/Mut2-luc with a mismatched version of the miR-30a complementary sequence within site 1 or 2, and *Vim* 3′UTR/Mut12-luc containing both mismatched sequences (Fig. 3b). We found that miR-30a significantly reduced the activity of the luciferase gene fused to the *Vim* 3′UTR by more than 45 %, supporting the notion that miR-30a can modulate vimentin expression by binding to its 3′UTR (Fig. 3c). In addition, a significant reduction (54 %) in luciferase activity was observed in the presence of pre-miR-30a using the reporter construct containing the *Vim* 3′UTR/Mut1 clone, but not the *Vim* 3′UTR/Mut2 or *Vim* 3′UTR/Mut12 clones. These results indicate that the region from 178 to 184 is the important site within the 3′UTR of the *Vim* gene that is required for miR-30a binding.

Overexpression of miR-30a suppresses the motility and invasiveness of breast cancer cells

Overexpression of vimentin has been associated with enhanced metastatic potential in breast cancer [32, 33]. To confirm the role of miR-30a in inhibiting breast cancer progression, we studied the effect of miR-30a on the migration and invasion of breast cancer cell lines. In two stable clones of Hs578T and MD-MBA-231 lentivirally transduced with miR-30a, we first confirmed that vimentin expression was inhibited by miR-30a (Fig. 4a), and then found that the migration (Fig. 4b) and invasiveness (Fig. 4c) of these cells were significantly inhibited compared with control oligonucleotide transfected cells. Moreover, decreased vimentin protein levels in the miR-30a-transduced breast cancer cells were restored by transfection of the Anti-miR™ miRNA inhibitor of miR-30a (anti-miR-30a) (Ambion, Inc), which also significantly enhanced cell migration (Fig. 4b, d) and invasion (Fig. 4c, d) compared with transfected cell lines with negative control. In parallel, we investigated a dramatic decrease in the migration, and invasion of breast cancer cells was detected following vimentin silencing (Fig. 4b–d), in which it was verified that the inhibitory effect of miR-30a on tumor motility was mediated by downregulation of vimentin.

Downregulation of miR-30a is associated with an unfavorable outcome in breast cancer

Since miR-30a suppresses tumor cell invasion and metastasis by downregulation of vimentin expression, we then measured miR-30a levels in IDCs. By using the comparative CT method (-ddCt), relative expression levels of the miR-30a

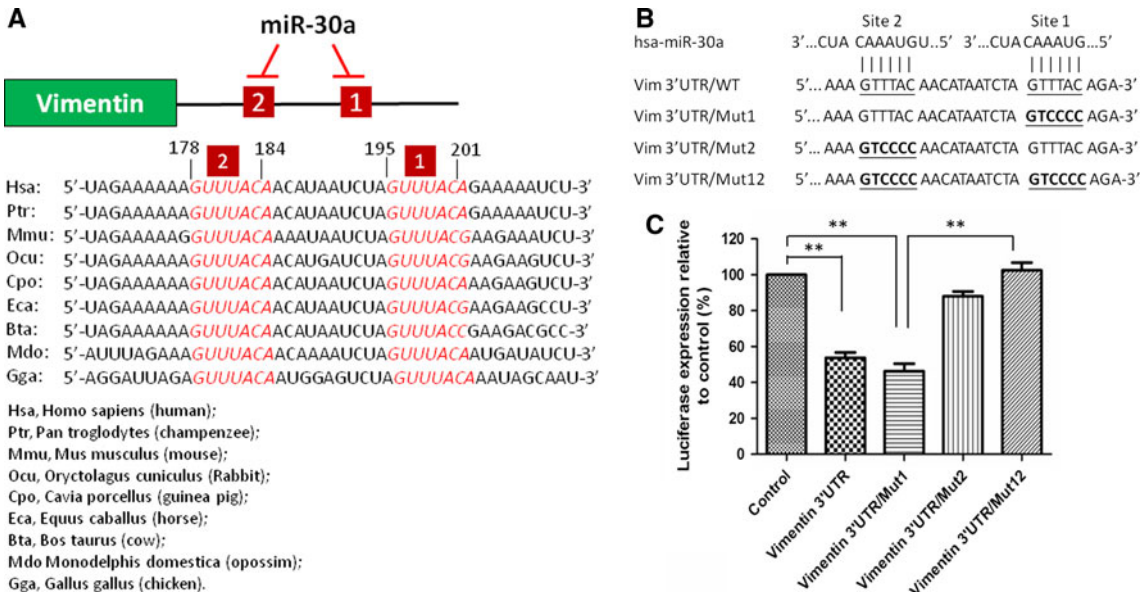


Fig. 3 miR-30a as a vimentin-targeting miRNA. **a** Sequence of the 3′UTR of vimentin mRNA showing the two predicted seed regions for the binding of miR-30a in different vertebrate species. **b** The sequences at sites 1 and 2 of the three mutants with a mismatch of the miR-30a complementary sequence at site 1 (*Vim* 3′UTR/Mut1) or site 2 (*Vim* 3′UTR/Mut2) or both (*Vim* 3′UTR/Mut12). **c** Constructs containing the reporter gene luciferase 3′-linked to the wild-type or a mutant 3′UTR of *Vim* (luc-*Vim* 3′UTR/WT) and miR-30a were cotransfected into MDA-MB-231 cells and the firefly luciferase activity of the reporter measured and normalized to that of the internal *Renilla* luciferase, The values are the mean ± SD for three independent experiments. $^{**}p < 0.01$

compared between tumor and non-tumor cells of 221 patients are shown in Fig. 5a. The results showed that the percentage of patients with reduced miR-30a levels, measured by a greater than twofold decrease in microdissected tumor cells compared to adjacent non-tumor breast cells, was significantly higher in patients with tumors of advanced stage or with LNM (Fig. 5b). We then examined whether decreased miR-30a levels were associated with an unfavorable outcome in IDC patients. In our cohort of breast cancer patients followed up for 5 years, there was a trend toward a decreased recurrence-free survival (Fig. 5c) and decreased disease-free survival (Fig. 5d) in patients with lower miR-30a levels in tumor cells compared with the corresponding non-tumor region. When stage and estrogen receptor status were taken into consideration in the Cox regression model, breast cancer patients with decreased miR-30a levels in the primary cancerous site had an increased HR for recurrence (HR = 3.96; Log-rank p value = 0.005) or recurrence plus death (OR = 1.94; Log-rank p value = 0.01) during the follow-up period. Our study confirms the clinical relevance of our experimental data on miR-30a in breast cancer.

Discussion

In the present study, to unravel the mechanisms by which miRNAs affect breast cancer progression, a combination of miRNA microarray analysis of tumor cells microdissected from the primary tumor site, three matching algorithms, and gene expression data was used to reveal associations between miRNAs and biological functions. Our findings in breast cell models showed that inhibition of the EMT-promoting migration of breast cancer was causally linked to downregulation of vimentin by miR-30a. Further, tumor cells of decreased miR-30a expression with increased metastatic activity was confirmed in breast cancer patients, in whom reduced expression of miR-30a was significantly associated with LNM, advanced stage, and decreased recurrence-free survival and disease-free survival.

Vimentin is required to maintain the architecture of the cytoplasm [34] and aberrant vimentin expression during EMT is suggested to be an essential element for epithelial plasticity and tumor cell metastasis [19, 33, 35]. EMT trans-differentiation processes involve the conversion of adherent epithelial cells into individual migratory cells, leading to changes in cell phenotype into more loose mesenchymal-like cells, and promoting local invasion and metastatic dissemination of tumor cells [33, 36]. miR-30a is involved in hepatobiliary, prefrontal cortex, and renal pronephros development during organogenesis in vertebrates [37–39]. Recently, elevated miR-30a expression has been suggested to inhibit motility of lung cancer cells by decreasing the expression of Snail, a transcriptional regulator that represses E-cadherin expression during MET [40]. In the present study, we showed that the suppressive effect of miR-30a on vimentin expression led to decreased

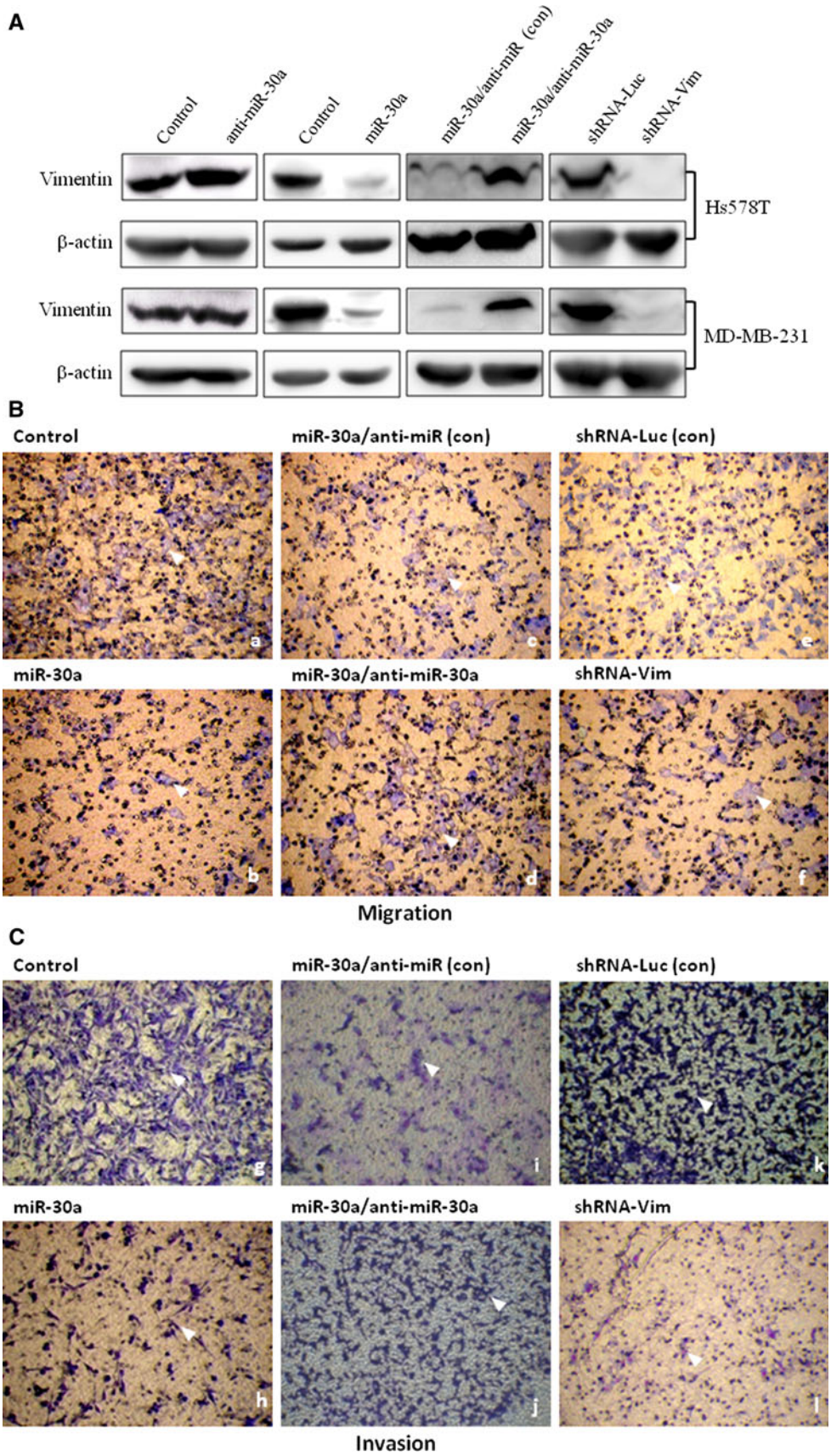


Fig. 4 Inhibitory effect of miR-30a on the migration and invasiveness of breast cancer cell lines. **a** Expression of miR-30a suppresses vimentin expression in the breast cancer cell lines Hs578T (*upper panel*) and MDA-MB-231 (*lower panel*), and this effect is overcome in cells treated with an inhibitor of miR-30a (anti-miR-30a). **b** and **c** Reduced vimentin levels caused by miR-30a are significantly associated with lower migration and invasiveness in Hs578T cells, and these effects are inhibited by introduction of anti-miR-30a. In addition, a dramatic decrease in the migration/invasion of breast cancer cells was detected following knockdown of *Vim* gene. Representative micrographs of migration/invasion filter membranes after crystal violet staining. **d** Quantitative analysis of migration/invasion. The values are the mean ± SD for three separate experiments. $^{**}p < 0.01$, $^{***}p < 0.001$

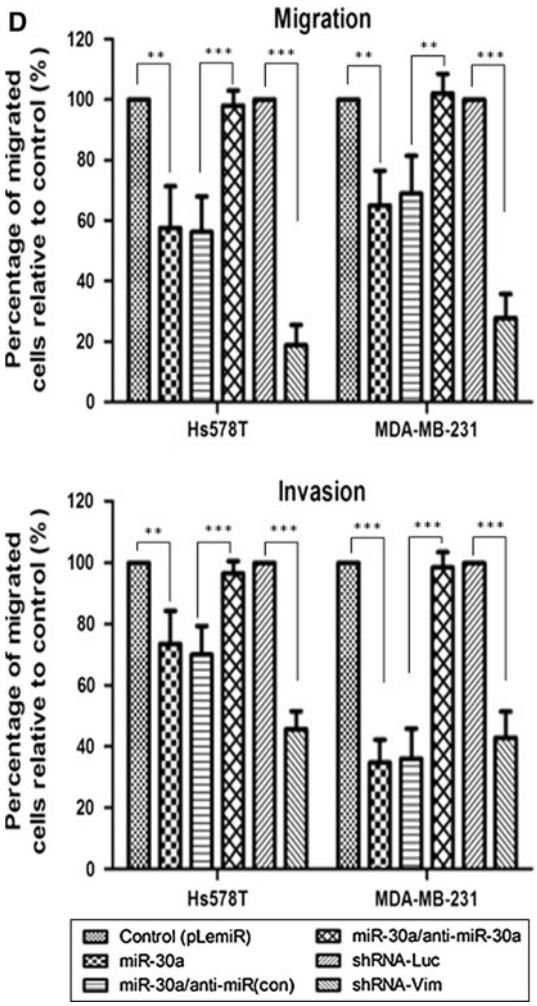


Fig. 4 continued

migration and invasiveness of breast cancer cells. Moreover, using the luciferase reporter assay, a significant increase in luciferase activity was seen in cells transfected with the mutated 3′UTR motif of *Vim* (Fig. 3c), confirming the binding of miR-30a to the vimentin gene.

In miRNA expression assays, the miR-30 family has been frequently found to be downregulated in diverse types of tumors [41, 42]. Interestingly, studies on the genetic loss of chromosome 6q13 hinted at a role for miR-30a in human breast cancer tumorigenesis [43, 44]. qRT-PCR results herein showed that reduced miR-30a mRNA levels in breast cancer cells microdissected from the primary tumor tissue were significantly associated with poor clinical features (advanced stage and LNM) and a worse outcome, consistent with the results of previous cancer cohort studies [45–47]. It is noteworthy that miRNAs bind to specific sequences of target mRNAs to eventually suppress protein translation. It has been reported that protein expression level of a gene is not uniquely modulated by one miRNA; alternatively, the identified miRNAs and their downstream target mRNAs may have different extents of less-than perfect complementarity, which allow one miRNA binding various mRNA transcripts [48, 49]. Similar to the results in vimentin silencing study in this study also, we found that introduction of miR-30a resulted in reduced vimentin expression and decreased migration and invasiveness in a breast tumor cell model. In addition, a good practice to achieve association study is to compare differences of miR-30a levels between paired laser capture microdissected tumor- and non-tumor cells to ensure the validity of this biomarker in reflecting of poor prognosis of breast cancer. More importantly, an investigation of miR-30a expression levels in a group of vimentin-ablated tissues with microdissecting treatment is the best solution to resolve the existence of specific correlation between levels of vimentin and miR-30a regarding in our female breast cancer cohort.

On the basis of databases for miRNA target prediction, members of the miR-30 family have been shown to share the same seed sequence. It has been suggested that downregulation of miR-30 family members decreases metastatic potential; using β-cell development of human fetal pancreatic islets as a model, miR-30d and miR-30a were shown to be involved in downregulating vimentin during EMT of primary pancreatic epithelial cells [50]. In addition, miR-30a and miR-30e share a common seed sequence in the *Snail* 3′UTR, and reduced expression of Snail is essential for maintaining epithelial-like cells, and, as a result, inhibits the invasiveness and metastasis of non-small cell lung cancers [40]. Recently, it was shown that transforming growth factor-β (TGF-β) acts as a positive regulator of EMT by stimulating normal mammary epithelial cells to adopt mesenchymal- and stem cell-like features and promoting invasion and metastasis [51, 52]. Expression of miR-30 family members is reduced during TGF-β-promoted tumor metastasis, in which increased expressions of invasion/metastasis-associated mesenchymal markers, including N-cadherin, Slug, Snail, and Twist, are seen [51]. Moreover, elevated miR-30c and miR-30a levels may help predict clinical benefit in patients with advanced breast cancer who receive tamoxifen therapy [53]. These results suggest that miR-30 family members may interact synergistically with each other in inhibiting tumor growth and regulating tumor progression. Given the importance of the miR-30 family in predicting progression and outcome of breast cancer, further investigations are required to extend this approach from a single miRNA to multiple miRNAs to understand the cross-talk among miR-30 members in etiological pathways, such as EMT-wide networks.

In conclusion, using microarray analysis of miRNAs expressed in breast cancers with different LNM status, the

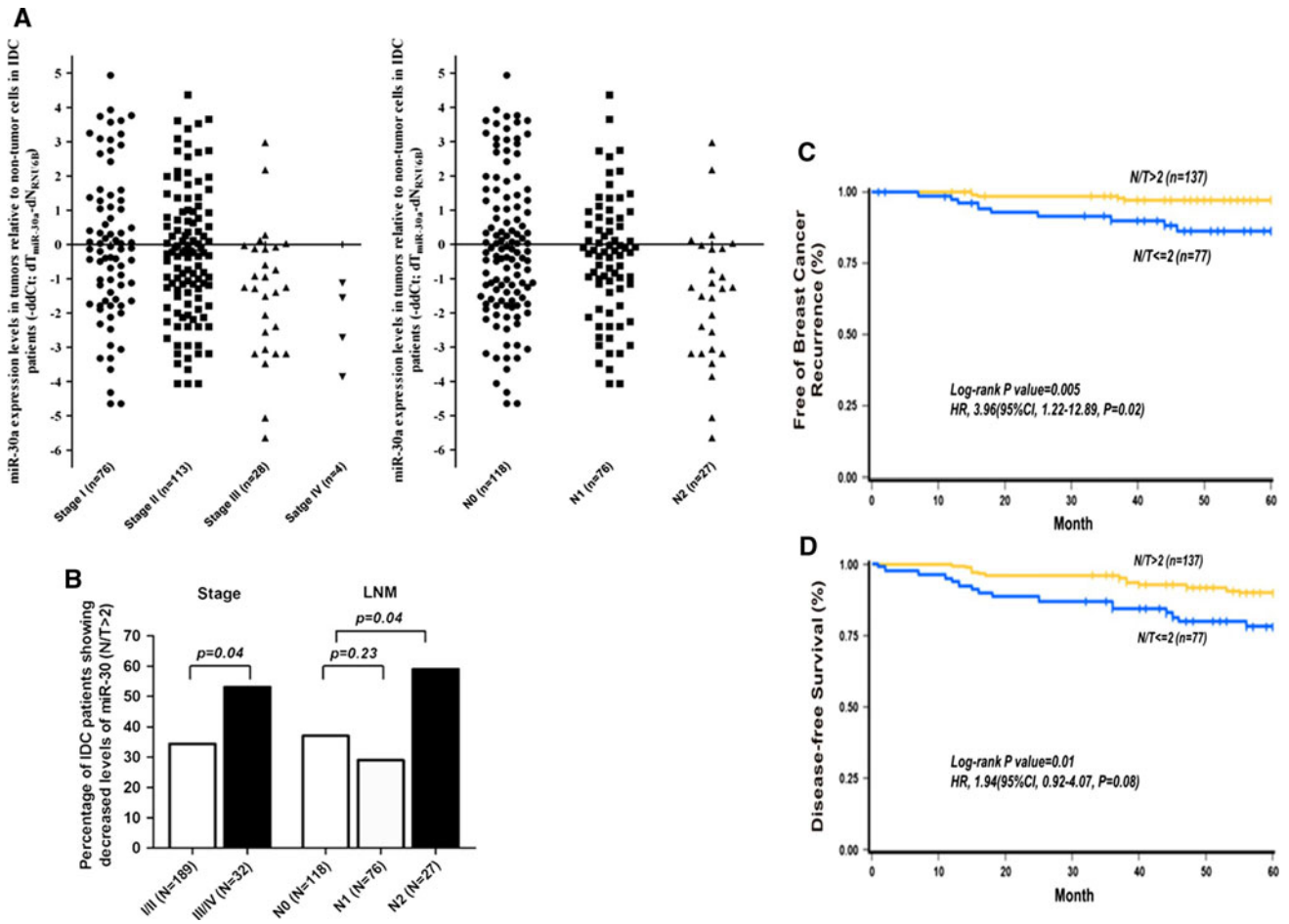


Fig. 5 Decreased expression of miR-30a is associated with poor clinical features and decreased survival rates. **a** Expression levels of miR-30a in tumor as compared with non-tumor in the different stages and LNM in patients with breast IDC. **b** Percentage of patients showing decreased miR-30a expression levels in tumors, defined as a twofold decrease in expression in the microdissected cancerous tissue (T) compared to adjacent non-cancerous breast epithelium (N), in tumors of different stages or different lymph node metastasis (LNM) status. **c** Kaplan–Meier analysis of the difference in 5-year breast cancer recurrence-free survival rate, and in **d** 5-year disease-free survival rate (i.e., breast cancer recurrence plus death) between tumors with high or low miR-30a levels. The hazard ratios (HRs) and 95 % confidence intervals (95 % CIs) shown in (**c**) and (**d**) were estimated using the Cox regression model adjusted for the effects of age, tumor stage, and estrogen receptor status

present study demonstrates, for the first time, a role of miR-30a in inhibiting breast tumor invasiveness and metastasis by directly targeting the vimentin gene. Significantly reduced levels of miR-30a were found in tumors compared with normal mammary ductal epithelium. Moreover, miR-30a expression was inversely correlated with prognosis in patients with IDC, supporting the notion that this miRNA may serve as a tumor biomarker for predicting the outcome of breast cancer. Finally, the finding that miR-30a inhibits vimentin expression may help in developing a potential therapeutic target for treating breast cancer.

Acknowledgments We sincerely appreciate Ms. Show-Lin Yang for her assistance in organizing our study specimens. This study was supported by research grant NSC 98-2314-B-040-009-MY3 from the National Science Council, Taipei, Taiwan.

Conflict of interest The authors declare that they have no conflict of interest.

References

1. Orlando FA, Brown KD (2009) Unraveling breast cancer heterogeneity through transcriptomic and epigenomic analysis. Ann Surg Oncol 16(8):2270–2279
2. Polyak K (2007) Breast cancer: origins and evolution. J Clin Invest 117(11):3155–3163
3. Guarino M, Rubino B, Ballabio G (2007) The role of epithelial–mesenchymal transition in cancer pathology. Pathology 39(3): 305–318
4. Micalizzi DS, Farabaugh SM, Ford HL (2010) Epithelial–mesenchymal transition in cancer: parallels between normal development and tumor progression. J Mammary Gland Biol Neoplasia 15(2):117–134

5. Trimboli AJ, Fukino K, de Bruin A, Wei G, Shen L, Tanner SM, Creasap N, Rosol TJ, Robinson ML, Eng C, Ostrowski MC, Leone G (2008) Direct evidence for epithelial–mesenchymal transitions in breast cancer. Cancer Res 68(3):937–945
6. Wells A, Yates C, Shepard CR (2008) E-cadherin as an indicator of mesenchymal to epithelial reverting transitions during the metastatic seeding of disseminated carcinomas. Clin Exp Metastasis 25(6):621–628
7. Marsit CJ, Posner MR, McClean MD, Kelsey KT (2008) Hypermethylation of E-cadherin is an independent predictor of improved survival in head and neck squamous cell carcinoma. Cancer 113(7):1566–1571
8. Prasad CP, Mirza S, Sharma G, Prashad R, DattaGupta S, Rath G, Ralhan R (2008) Epigenetic alterations of CDH1 and APC genes: relationship with activation of Wnt/beta-catenin pathway in invasive ductal carcinoma of breast. Life Sci 83(9–10):318–325
9. Yates DR, Rehman I, Abbod MF, Meuth M, Cross SS, Linkens DA, Hamdy FC, Catto JW (2007) Promoter hypermethylation identifies progression risk in bladder cancer. Clin Cancer Res 13(7):2046–2053
10. Graziano F, Humar B, Guilford P (2003) The role of the E-cadherin gene (CDH1) in diffuse gastric cancer susceptibility: from the laboratory to clinical practice. Ann Oncol 14(12):1705–1713
11. Braun J, Hoang-Vu C, Dralle H, Huttelmaier S (2010) Downregulation of microRNAs directs the EMT and invasive potential of anaplastic thyroid carcinomas. Oncogene 29(29):4237–4244
12. Vetter G, Saumet A, Moes M, Vallar L, Le Bechec A, Laurini C, Sabbah M, Arar K, Theillet C, Lecellier CH, Friederich E (2010) miR-661 expression in SNAI1-induced epithelial to mesenchymal transition contributes to breast cancer cell invasion by targeting Nectin-1 and StarD10 messengers. Oncogene 29(31): 4436–4448
13. Cho WC (2007) OncomiRs: the discovery and progress of microRNAs in cancers. Mol Cancer 6:60
14. Negrini M, Nicoloso MS, Calin GA (2009) MicroRNAs and cancer—new paradigms in molecular oncology. Curr Opin Cell Biol 21(3):470–479
15. Ortholan C, Puissegur MP, Ilie M, Barbry P, Mari B, Hofman P (2009) MicroRNAs and lung cancer: new oncogenes and tumor suppressors, new prognostic factors and potential therapeutic targets. Curr Med Chem 16(9):1047–1061
16. Shenouda SK, Alahari SK (2009) MicroRNA function in cancer: oncogene or a tumor suppressor? Cancer Metastasis Rev 28(3–4): 369–378
17. Iwatsuki M, Mimori K, Fukagawa T, Ishii H, Yokobori T, Sasako M, Baba H, Mori M (2010) The clinical significance of vimentin-expressing gastric cancer cells in bone marrow. Ann Surg Oncol 17(9):2526–2533
18. Mendez MG, Kojima S, Goldman RD (2010) Vimentin induces changes in cell shape, motility, and adhesion during the epithelial to mesenchymal transition. FASEB J 24(6):1838–1851
19. Usami Y, Satake S, Nakayama F, Matsumoto M, Ohnuma K, Komori T, Semba S, Ito A, Yokozaki H (2008) Snail-associated epithelial–mesenchymal transition promotes oesophageal squamous cell carcinoma motility and progression. J Pathol 215(3): 330–339
20. Yang PS, Yang TL, Liu CL, Wu CW, Shen CY (1997) A case-control study of breast cancer in Taiwan—a low-incidence area. Br J Cancer 75(5):752–756
21. Lo YL, Yu JC, Huang CS, Tseng SL, Chang TM, Chang KJ, Wu CW, Shen CY (1998) Allelic loss of the BRCA1 and BRCA2 genes and other regions on 17q and 13q in breast cancer among women from Taiwan (area of low incidence but early onset). Int J Cancer 79(6):580–587
22. Cheng TC, Chen ST, Huang CS, Fu YP, Yu JC, Cheng CW, Wu PE, Shen CY (2005) Breast cancer risk associated with genotype polymorphism of the catechol estrogen-metabolizing genes: a multigenic study on cancer susceptibility. Int J Cancer 113(3): 345–353
23. Ming-Shiean H, Yu JC, Wang HW, Chen ST, Hsiung CN, Ding SL, Wu PE, Shen CY, Cheng CW (2010) Synergistic effects of polymorphisms in DNA repair genes and endogenous estrogen exposure on female breast cancer risk. Ann Surg Oncol 17(3): 760–771
24. Ding SL, Sheu LF, Yu JC, Yang TL, Chen BF, Leu FJ, Shen CY (2004) Abnormality of the DNA double-strand-break checkpoint/repair genes, ATM, BRCA1 and TP53, in breast cancer is related to tumour grade. Br J Cancer 90(10):1995–2001
25. Hsu HM, Wang HC, Chen ST, Hsu GC, Shen CY, Yu JC (2007) Breast cancer risk is associated with the genes encoding the DNA double-strand break repair Mre11/Rad50/Nbs1 complex. Cancer Epidemiol Biomarkers Prev 16(10):2024–2032
26. Shen CY, Yu JC, Lo YL, Kuo CH, Yue CT, Jou YS, Huang CS, Lung JC, Wu CW (2000) Genome-wide search for loss of heterozygosity using laser capture microdissected tissue of breast carcinoma: an implication for mutator phenotype and breast cancer pathogenesis. Cancer Res 60(14):3884–3892
27. Lo YL, Shen CY (2002) Laser capture microdissection in carcinoma analysis. Methods Enzymol 356:137–144
28. Petroff BK, Phillips TA, Kimler BF, Fabian CJ (2006) Detection of biomarker gene expression by real-time polymerase chain reaction using amplified ribonucleic acids from formalin-fixed random periareolar fine needle aspirates of human breast tissue. Anal Quant Cytol Histol 28(5):297–302
29. Cheng CW, Yu JC, Wang HW, Huang CS, Shieh JC, Fu YP, Chang CW, Wu PE, Shen CY (2010) The clinical implications of MMP-11 and CK-20 expression in human breast cancer. Clin Chim Acta 411(3–4):234–241
30. Pfaffl MW (2001) A new mathematical model for relative quantification in real-time RT-PCR. Nucleic Acids Res 29(9): e45
31. McInroy L, Maatta A (2007) Down-regulation of vimentin expression inhibits carcinoma cell migration and adhesion. Biochem Biophys Res Commun 360(1):109–114
32. Satelli A, Li S (2011) Vimentin in cancer and its potential as a molecular target for cancer therapy. Cell Mol Life Sci 68(18): 3033–3046
33. Vuoriluoto K, Haugen H, Kiviluoto S, Mpindi JP, Nevo J, Gjerdrum C, Tiron C, Lorens JB, Ivaska J (2011) Vimentin regulates EMT induction by Slug and oncogenic H-Ras and migration by governing Axl expression in breast cancer. Oncogene 30(12):1436–1448
34. Franke WW, Grund C, Kuhn C, Jackson BW, Illmensee K (1982) Formation of cytoskeletal elements during mouse embryogenesis. III. Primary mesenchymal cells and the first appearance of vimentin filaments. Differentiation 23(1):43–59
35. Dutsch-Wicherek M, Lazar A, Tomaszewska R (2010) The potential role of MT and vimentin immunoreactivity in the remodeling of the microenvironment of parotid adenocarcinoma. Cancer Microenviron 4(1):105–113
36. Sarrio D, Palacios J, Hergueta-Redondo M, Gomez-Lopez G, Cano A, Moreno-Bueno G (2009) Functional characterization of E- and P-cadherin in invasive breast cancer cells. BMC Cancer 9:74
37. Mellios N, Huang HS, Grigorenko A, Rogaev E, Akbarian S (2008) A set of differentially expressed miRNAs, including miR-30a-5p, act as post-transcriptional inhibitors of BDNF in prefrontal cortex. Hum Mol Genet 17(19):3030–3042
38. Hand NJ, Master ZR, Eauclaire SF, Weinblatt DE, Matthews RP, Friedman JR (2009) The microRNA-30 family is required for vertebrate hepatobiliary development. Gastroenterology 136(3): 1081–1090

39. Agrawal R, Tran U, Wessely O (2009) The miR-30 miRNA family regulates Xenopus pronephros development and targets the transcription factor Xlim1/Lhx1. Development 136(23): 3927–3936
40. Kumarswamy R, Mudduluru G, Ceppi P, Muppala S, Kozlowski M, Niklinski J, Papotti M, Allgayer H (2012) MicroRNA-30a inhibits epithelial-to-mesenchymal transition by targeting Snai1 and is downregulated in non-small cell lung cancer. Int J Cancer 130(9):2044–2053
41. Izzotti A, Calin GA, Arrigo P, Steele VE, Croce CM, De Flora S (2009) Downregulation of microRNA expression in the lungs of rats exposed to cigarette smoke. FASEB J 23(3):806–812
42. Volinia S, Galasso M, Costinean S, Tagliavini L, Gamberoni G, Drusco A, Marchesini J, Mascellani N, Sana ME, Abu Jarour R, Desponts C, Teitell M, Baffa R, Aqeilan R, Iorio MV, Taccioli C, Garzon R, Di Leva G, Fabbri M, Catozzi M, Previati M, Ambs S, Palumbo T, Garofalo M, Veronese A, Bottoni A, Gasparini P, Harris CC, Visone R, Pekarsky Y, de la Chapelle A, Bloomston M, Dillhoff M, Rassenti LZ, Kipps TJ, Huebner K, Pichiorri F, Lenze D, Cairo S, Buendia MA, Pineau P, Dejean A, Zanesi N, Rossi S, Calin GA, Liu CG, Palatini J, Negrini M, Vecchione A, Rosenberg A, Croce CM (2010) Reprogramming of miRNA networks in cancer and leukemia. Genome Res 20(5):589–599
43. Chappell SA, Walsh T, Walker RA, Shaw JA (1997) Loss of heterozygosity at chromosome 6q in preinvasive and early invasive breast carcinomas. Br J Cancer 75(9):1324–1329
44. Noviello C, Courjal F, Theillet C (1996) Loss of heterozygosity on the long arm of chromosome 6 in breast cancer: possibly four regions of deletion. Clin Cancer Res 2(9):1601–1606
45. Heinzelmann J, Henning B, Sanjmyatav J, Posorski N, Steiner T, Wunderlich H, Gajda MR, Junker K (2011) Specific miRNA signatures are associated with metastasis and poor prognosis in clear cell renal cell carcinoma. World J Urol 29(3):367–373
46. Li X, Zhang Y, Ding J, Wu K, Fan D (2010) Survival prediction of gastric cancer by a seven-microRNA signature. Gut 59(5): 579–585
47. Tan X, Qin W, Zhang L, Hang J, Li B, Zhang C, Wan J, Zhou F, Shao K, Sun Y, Wu J, Zhang X, Qiu B, Li N, Shi S, Feng X, Zhao S, Wang Z, Zhao X, Chen Z, Mitchelson K, Cheng J, Guo Y, He J (2011) A 5-microRNA signature for lung squamous cell carcinoma diagnosis and hsa-miR-31 for prognosis. Clin Cancer Res 17(21):6802–6811
48. Brennecke J, Stark A, Russell RB, Cohen SM (2005) Principles of microRNA-target recognition. PLoS Biol 3(3):e85
49. Peter ME (2010) Targeting of mRNAs by multiple miRNAs: the next step. Oncogene 29(15):2161–2164
50. Ozcan S (2009) MiR-30 family and EMT in human fetal pancreatic islets. Islets 1(3):283–285
51. Kong W, Yang H, He L, Zhao JJ, Coppola D, Dalton WS, Cheng JQ (2008) MicroRNA-155 is regulated by the transforming growth factor beta/Smad pathway and contributes to epithelial cell plasticity by targeting RhoA. Mol Cell Biol 28(22): 6773–6784
52. Wendt MK, Allington TM, Schiemann WP (2009) Mechanisms of the epithelial–mesenchymal transition by TGF-beta. Future Oncol 5(8):1145–1168
53. Rodriguez-Gonzalez FG, Sieuwerts AM, Smid M, Look MP, Meijer-van Gelder ME, de Weerd V, Sleijfer S, Martens JW, Foekens JA (2011) MicroRNA-30c expression level is an independent predictor of clinical benefit of endocrine therapy in advanced estrogen receptor positive breast cancer. Breast Cancer Res Treat 127(1):43–51

出席國際學術會議心得報告

計畫編號	98-2314-B-040-009-MY3
計畫名稱	微型核糖核酸矩陣分析探討女性乳癌癌化進程之遺傳標記
出國人員姓名 服務機關及職稱	鄭鈞文 中山醫學大學生化暨生物科技研究所副教授
會議時間地點	March 31-April 4, 2012 ; Chicago, IL., United States
會議名稱	Annual Meeting 2012, American Association for Cancer Research, 2012.
發表論文題目	(1) MicroRNA-30a inhibits vimentin expression and as a prognostic marker in breast cancer (2) MicroRNA-190, regulated by estrogen receptor signaling, suppresses expression of the metastasis-promoting gene PAR-1 and is associated with breast cancer progression

一、參加會議經過

2012 年美國癌症醫學研究會研討會 (AACR Annual Meeting 2012, American Association for Cancer Research) 於 2012 年三月三十一日至四月四日，為期五天，在美國芝加哥市召開。本次大會有來自世界各癌症臨床醫學和學術機構、業界之專業研究人員，參與癌症基礎和臨床專科會議。參與本次癌症學術會議的人士超過一萬五千餘人；論文摘要發表共計有五千七百多篇，在五天的會議中分別以口頭發表、學術討論及論文海報張貼等方式同時進行。於年會開幕中，將 2012 傑出貢獻獎項頒發給 Bayard D. Clarkson 醫師，由於他在 chronic myelogenous leukemia (CML)的長期研究，建立起許多不同白血病的細胞模式，釐清正常細胞和癌的生長分化差異。另外，他也研發出多種癌症標靶治療，使其對傳統上化學藥物治療上無法作用的癌症幹細胞族群生長進入休止狀態。他同時是前 AACR 主席、主編、也是 AACR Prevention and Cure of Cancer 創始兼榮譽主席。因此，特將傑出研究獎頒給 Dr. Clarkson 以表彰他在癌症研究領域上的卓越貢獻。本屆 AACR 終身成就獎則是頒發給任教於費城 Fox Chase Cancer Center 的 Beatrice Mintz 教授。Mintz 教授專長於癌症生物學和遺傳學，她主要研究實驗轉殖基因鼠、表基因體遺傳學、胚胎細胞和癌細胞微環境改變。她發現許多癌症分子遺傳的致癌機轉，也指出 DNA 和蛋白質後轉譯修飾作用和癌症發生有著密切的關聯。尤其是以 DNA 轉殖技術發展惡性黑色素瘤轉殖鼠之建構，成功地表達黑色素瘤如何發生以致於腫瘤轉移的動物模式，首次解開皮下黑色素瘤的進程發展。Mintz 教授同時也是現今美國國家科學院院士，在癌症醫學研究榮獲無數的獎項，去年更獲頒 National Foundation of Cancer Research

(NFCR) Szent-Gyorgyi 的殊榮。本次大會頒給 Dr. Mintz 終生成就獎項，以彰顯他在癌症研究上的貢獻。另外，Alan D. D'Andrea 醫師發現特定 DNA 的受損影響到染色體的穩定性、細胞週期進行和癌症發生的關聯。針對 ATM mutated、Bloom syndrome 和 Fanconi anemia 引發急性白血病為研究主題，釐清 DNA 傷害修復蛋白功能異常影響到染色體遺傳物質的穩定性，諸如：ATM、BRCA1 和 FANC complex 等修復蛋白變異便是成為 Fanconi anemia 和癌症發生的病理成因，而獲頒 AACR-GHA Clowes Memorial 的獎項。另一方面，西北大學知名教授 Dr. Hendrix 以腫瘤細胞的塑性過度到轉移作用發生做了精闢的演講，以黑色素瘤為例，說明類內皮細胞到血管成型過程之癌細胞再塑現象，加以闡述為何以 endostatin 抑制內皮細胞血管新生作用下，黑色素瘤依然能夠發展成血管成型的關鍵，這些精闢的研究報告都是非常難能可貴的。而普林斯頓教授 Dr. Kang 也在特別演講中探討乳癌轉移過程中腫瘤基質環境的微變化現象。經由分子遺傳、動物模式結合遺傳體學研究，他發現到 JAGGED1 蛋白結合到骨細胞表面蛋白 Notch，刺激骨細胞蝕骨作用，讓該區域被破壞的骨細胞釋 TGF-b 腫瘤細胞刺激生長因子，而使得腫瘤細胞轉移至骨骼中。此過程中，也發現到 VCAM-1 高度表現於在轉移的腫瘤細胞。此外，Dr. Kang 以蛋白質體學的研究發現非轉譯核酸 miR-200c 會促使腫瘤細胞的侵襲和轉移。他在腫瘤細胞轉移機制的傑出研究，讓他在本次大會中，獲頒第三十二屆 AACR 傑出研究獎。

另外，在本次大會還有一項值得注意的議程是比較現今研究觀念和爭議中的研究(current concepts and controversies)的專題討論，分別就癌症臨床診斷、治療、預防和組織標的遺傳學研究；以不同的細胞組織特性和相異的遺傳角度切入，以釐清癌細胞的發展過程，探究個人化癌症治療的易感受性、抗藥性差異及其未來在癌症治療上的研發策略。這是個嶄新的主題，針對多種不同的癌症上的臨床預後標記說明荷爾蒙療法、EGFR 表現和細胞凋亡、酪胺酸激酶受體活化、MAPK、細胞分裂相關指標 CDKN2A、NF2 和 BAP1 等標記來評估必要性的治療（neoadjuvant therapy）的預測和術後評估，報告內容敘述深入淺出，可以提供給研究者不同的思維和體會。

會議舉行期間，邀請到現今世界各知名實驗室的主持人，就其專業研究領域給予講演、教育訓練課程和會議研討作廣泛的意見交流等。其研究內容涵蓋更為廣泛，包含有細胞生長訊息傳遞、細胞週期調控、細胞老化和死亡、基因修復機制、血管新生抑制作用、胞外基質和微環境變化、上皮細胞/間質細胞轉化和癌轉移機制之探討、蛋白質激酶標靶治療、微型核糖核酸表現和調控、非轉譯RNA和癌症發生研究的新發現、癌症幹細胞生物標記之探討、個人化癌症治療之新遺傳標記之研究、致癌基因體學、腫瘤侵襲和轉移機制、代謝遺傳學和癌症發生的關聯、腫瘤內分泌、基因體學/染色體醫學和癌症個人化臨床預後評估標記、藥物治療抗性，癌症疫苗研發、基因治療與預防等各項專題研究報告。針對各項研究主題皆有論文摘

要、小型研究會議、特別專題演講和專業進階會議之文獻和報告發表，能讓更多的研究人員了解癌症基礎和臨床研究成果，對於臨床標記和治療的體認，是值得讓國內學界來加以重視和發展的課題。

二、與會心得

本屆美國癌症醫學研究學會年會中，我們以探討 miR-30a 逆調節 vimentin 基因表現和乳癌進程發展為主題，透過雷射捕獲分析技術(laser capture microdissection)從病人癌組織和癌相鄰之正常組織分別擷取，再以定量 qRT-PCR 的表現來做為乳癌的分期關聯性分析。經過電腦基因比對分析後，發現 vimentin 3'UTR 為 miR-30a 的作用標地核酸序列。藉由共軛對焦顯微分析、螢光表達基因分析和乳癌細胞侵襲轉移實驗，證實 miR-30a 會抑制 vimentin 蛋白表現進而抑制乳癌細胞侵襲轉移的能力。進一步，分析 miR-30a 在乳癌組織表現程度高低，乳癌患者進程發展和預後有著密切的關聯，這些攸關乳癌預後的訊息和評估結果，將對台灣婦女乳癌的基礎研究有相當的貢獻。再者，我們也發現到 miR-190 會受到雌激素受體蛋白的調控，以逆調節腫瘤轉移促進基因 PAR-1 的表現，進而抑制乳癌細胞株侵襲轉移的能力。在我們研究中，藉由組織 miRNA 的表現差異，不但可以了解到基因被逆向調控於腫瘤抑制基因抑或是致癌基因活化對癌細胞的進程發展，加以應用研發這些發現，可以更有效率地判斷出與癌症生成或癌症標靶藥物治療的機制，這也極可能是未來癌症研究的重要議題。摘要發表期間，同各國學者就乳癌做廣泛的討論，交換研究心得，實獲益匪淺。現今，在我們的實驗室也陸續投諸許多的人力、心力和資源著手進行此方向的研究，冀望能夠透過對 miRNA 的研究，找出對台灣婦女乳癌發生更有意義的研究標記。

此次國內有許多研究學者參與美國癌症醫學研究學會年會，其中來自台大、陽明，北醫、國防、長庚、高雄醫大和高雄義守大學等各大專院校醫學院、中央研究院研究人員及各醫學研究中心之癌症研究人員。研究領域以臨床轉譯醫學、臨床組織、臨床治療、分子流行病學和臨床藥理等癌症醫學之相關研究課題。藉由會議舉行期間能夠與國內、外研究先進進行學術交流，尤其是針對亞洲地區，包括日、韓、新加坡、中國大陸、香港以及本國對於乳癌腫瘤生成和癌化發展成因、基因變異、癌症臨床標記之研究、腫瘤之基因治療和各種癌症致癌感受性危險相關因子之統計分析等各項專題，進行學術經驗的交流、分享彼此研究結果和心得。對於本研究室近年來致力於探討臨床組織檢體的基因體剖面研究、預後研究標記、術後存活率有更清楚的意見回饋。

三、會後建議：

在這次學術研討會中，本人發現國內、外的研究先進在癌症領域鑽研已相當深

入。他們除了在自己的專業研究室中指導研究生、博士後研究員，兼具該研究單位對外的合作機會，跨院校及國際合作的對象，拓展實驗室以外各項專業技術的進步。除此之外，更結合了臨床與基礎的研究交流，加速解決各項問題，這也是為什麼他們能夠走在學術尖端的理由。而透過參與此次國際性的研討會議，也深刻感受到亞洲各國在癌症學術領域的研究進步迅速，許多研究先進展現出強烈的企圖心。在國內的研究單位應可以朝著多方合作模式，更加能夠提昇本國在國際癌症研究的能量。目前我們實驗室已建構出 miRNA 基因庫，將部分 miRNA 的核酸分子片段感染至乳癌細胞株中，輔以基因比對、定性、表現差異和功能性分析，發現到數個基因標地為特定 miRNAs 所調控，這些研究成果將有助於未來在乳癌診斷及治療的發展。進一步，希望能夠透過動物模式和臨床檢體的分析研究，藉由癌症組織、早期癌變和臨床預後的研究觀點建立新的觀念來闡述癌症分期、發展，以致於在未來發展個人化臨床治療策略的轉譯醫學資訊。

Thursday, April 19, 2012

Re: AACR Annual Meeting 2012 in Chicago, Illinois
Temporary Abstract Number: 3641
Title: MicroRNA-30a inhibits vimentin expression and as a prognostic marker in breast cancer

Dear Dr. Chang:

Your above-referenced abstract has been scheduled for presentation in a Poster Session at the AACR Annual Meeting 2012 in Chicago, Illinois and will be published in the 2012 Proceedings of the American Association for Cancer Research. Presentation information pertaining to your abstract is below:

Session ID: Molecular and Cellular Biology 16
Session Date and Time: Monday Apr 2, 2012 8:00 AM - 12:00 PM
Location: Hall F, Poster Section 3

Permanent Abstract Number: 1094

Please refer to the printed Final Program [distributed onsite] or the online Annual Meeting Itinerary Planner [available in late February through the AACR Website at http://www.aacr.org] for the exact location of your presentation.

Instructions for Presenters in Poster Sessions can be found on the 2012 AACR Annual Meeting home page:
www.aacr.org/page28647.aspx#poster

AVAILABLE IN 2012! DISCOUNTED POSTER CREATION/PRINTING AND DELIVERY SERVICE
The AACR has selected Marathon Multimedia as our preferred poster printing service partner for the 2012 Annual Meeting. Presenters are encouraged to take advantage of this simple, convenient way to print their posters and pick them up on-site at the meeting in Chicago. Delivery is 100% guaranteed; avoid airport hassles and worries about shipping it yourself.

Free PowerPoint™ templates will be available to help you build your poster or you can simply upload your existing file for our high quality professional printing service. More information about the service—as well as a personalized login for the poster creation site—will be sent in mid-February.

Poster Session presenters at the AACR Annual Meeting must register for the full meeting at the rate appropriate to their membership status and obtain their own hotel accommodations. Registration and housing information are included below:

Advance Registration Deadline: February 6, 2012
Online Registration
http://www.aacr.org/page28434.aspx

Housing Deadline: February 15, 2012
Online Housing System
http://www.aacr.org/page28411.aspx

Travel Information
[Airline/Train Reservations; Ground Transportation;
Official Letters of Invitation for International Attendees]
http://www.aacr.org/page27126.aspx

For more information, visit the AACR Annual Meeting 2012 home page at:
http://www.aacr.org/page28521.aspx

Thank you for your participation in the AACR Annual Meeting 2012.

Sincerely,
Benjamin G. Neel, M.D., Ph.D.
Program Committee Chairperson

PLEASE NOTE: This document is your official notice of acceptance. No separate letter of acceptance will be mailed.
For all technical questions, please contact OASIS Helpdesk or call (217)398-1792. If you have policy related questions, please contact AACR at (215)440-9300 or (866)423-3965.

Leave OASIS Feedback

Print

Category: Molecular and Cellular Biology 5

Session Title: microRNA Expression Regulation: New Players, Old Tricks

#144 **MicroRNA-190, regulated by estrogen receptor signaling, suppresses expression of the metastasis-promoting gene *PAR-1* and is associated with breast cancer progression.** Hou-Wei Chu, Chun-Wen Cheng, Wen-Cheng Chou, Ling-Yueh Hu, Hsiao-Wei Wang, Chia-Ni Hsiung, Pei-Ei Wu, Huan-Ming Hsu, Jyh-Cherng Yu, Chen-Yang Shen. IBMS, Academia Sinica, Taipei, Taiwan.

Cancer metastasis contributes to mortality of breast cancer patients. The present study, based on the fundamental concept that dissociation of the extracellular matrix is the driving force for tumor metastasis, was performed to examine the hypothesis that microRNA-190 (miR-190) is important in regulating breast cancer metastasis by decreasing the expression of *protease-activated receptor-1* (*PAR-1*), a gene encoding a receptor for matrix metalloproteinase1 and thrombin that is associated with tumor metastasis. This hypothesis was initially suggested by the observations that the expression of a reporter gene could be regulated by a specific sequence in the 3' -untranslated region of PAR-1 and that miR-190 was complementary to this sequence. Support for our hypothesis came from the findings that (a) PAR-1 expression was directly inhibited by miR-190, (b) increased miR-190 expression suppressed cell migration and invasiveness, and (c) the level of miR-190 expression in primary breast carcinomas correlated with overall survival. Interestingly, we defined the promoter region of *miR-190* and noted that it contained half of an estrogen receptor (ER) response element, supporting the breast tumorigenic contribution of miR-190. This was further confirmed by the findings that miR-190 expression was activated by 17 β-estradiol and that the ER bound directly to this promoter and regulated miR-190 expression. The findings of the present study may explain why ER-positive patients usually have a favorable progression and how ER regulates cancer metastasis, i.e. ER signaling regulates miR-190 expression, thus causing suppression of PAR-1 expression, resulting in inhibition of breast cancer metastasis.

Citation Format
Chu H, Cheng C, Chou W, Hu L, Wang H, Hsiung C, Wu P, Hsu H, Yu J, Shen C. MicroRNA-190, regulated by estrogen receptor signaling, suppresses expression of the metastasis-promoting gene *PAR-1* and is associated with breast cancer progression [abstract]. Proceedings of the 103rd Annual Meeting of the American Association for Cancer Research; 2012 Mar 31-Apr 4; Chicago, Illinois. Philadelphia (PA): AACR; 2012. Abstract nr 144.

Print

AACR ANNUAL MEETING
March 31- April 4, 2012 • Chicago, IL

Category: Molecular and Cellular Biology 16

Session Title: microRNAs and Therapy: From Dream to Reality

#1094 MicroRNA-30a inhibits vimentin expression and as a prognostic marker in breast cancer. Chia-Wei Chang[1], Chun-Wen Cheng[2], Hsiao-Wei Wang[2], Cheng-You Chen[1], Hou-Wei Chu[2], Shian-ling Ding[3], Hui-Chun Wang[4], Jyh-Cherng Yu[5], Chen-Yang Shen[2]. [1]Institute of Biochemistry and Biotechnology, Chung Shan Medical University, Taichung, Taiwan; [2]Institute of Biomedical Sciences, Academia Sinica, Taipei, Taiwan; [3]Department of Nursing, Kang-Ning Junior College of Medical Care and Management, Taipei, Taiwan; [4]Graduate Institute of Natural Products, College of Pharmacy, Kaohsiung Medical University, Kaoshiung, Taiwan; [5]Department of Surgery, Tri-Service General Hospital, National Defense Medical Center, Taipei, Taiwan.

Recent studies have suggested a significant role of microRNAs (miRNAs) in the regulation of cancer development. This study examined whether they play a role in breast cancer progression.A miRNA microarray analysis was performed on laser-capture microdissected breast tumors of different lymph-node metastasis status showing different progression signatures, indicated by overexpression of cyclin D1 and β-catenin genes, to identify specific miRNAs that exhibit significant differences in expression. Functional interaction between the candidate miRNA (i.e. miR-30a) and the gene (i.e. *Vim* gene, coding for vimentin, a protein involved in epithelial-mesenchymal transition) possibly regulated by miR-30a was verified. We further examined whether decreased expression of miR-30a was associated with breast cancer progression.miR-30a negatively regulated vimentin expression, by binding to the 3' -untranslated region of *Vim*. Ectopic expression of miR-30a was found to suppress the migration and invasiveness phenotypes of breast cancer cell lines. More importantly, breast cancer patients with decreased miR-30a level in primary cancerous sites were found to be associated with poor clinical features (late tumor stage and lymph node metastasis) and worse progression, demonstrating an increased hazard ratio (HR) for recurrence or recurrence plus mortality during the follow-up period ($P<0.05$). These findings provide a support of clinical importance of miR-30a in mediating breast tumor progression. Identification of miR-30a-mediated regulation of vimentin might provide a promising therapeutic target in treating breast cancer.

Citation Format
Chang C, Cheng C, Wang H, Chen C, Chu H, Ding S, Wang H, Yu J, Shen C. MicroRNA-30a inhibits vimentin expression and as a prognostic marker in breast cancer [abstract]. Proceedings of the 103rd Annual Meeting of the American Association for Cancer Research; 2012 Mar 31-Apr 4; Chicago, Illinois. Philadelphia (PA): AACR; 2012. Abstract nr 1094.

國科會補助計畫衍生研發成果推廣資料表

日期:2012/10/28

國科會補助計畫	計畫名稱: 微型核糖核酸矩陣分析探討女性乳癌癌化進程之遺傳標記
	計畫主持人: 鄭鈞文
	計畫編號: 98-2314-B-040-009-MY3　　學門領域: 公共衛生及環境醫學

無研發成果推廣資料

98 年度專題研究計畫研究成果彙整表

計畫主持人：鄭鈞文	計畫編號：98-2314-B-040-009-MY3
計畫名稱：微型核糖核酸矩陣分析探討女性乳癌癌化進程之遺傳標記	

成果項目			量化			單位	備註（質化說明：如數個計畫共同成果、成果列為該期刊之封面故事...等）
			實際已達成數（被接受或已發表）	預期總達成數(含實際已達成數)	本計畫實際貢獻百分比		
國內	論文著作	期刊論文	0	0	100%	篇	
		研究報告/技術報告	0	0	100%		
		研討會論文	0	0	100%		
		專書	0	0	100%		
	專利	申請中件數	0	0	100%	件	
		已獲得件數	0	0	100%		
	技術移轉	件數	0	0	100%	件	
		權利金	0	0	100%	千元	
	參與計畫人力（本國籍）	碩士生	1	1	100%	人次	
		博士生	1	1	100%		
		博士後研究員	0	0	100%		
		專任助理	1	1	100%		
國外	論文著作	期刊論文	2	2	100%	篇	
		研究報告/技術報告	0	0	100%		
		研討會論文	2	2	100%		
		專書	0	0	100%	章/本	
	專利	申請中件數	0	0	100%	件	
		已獲得件數	0	0	100%		
	技術移轉	件數	0	0	100%	件	
		權利金	0	0	100%	千元	
	參與計畫人力（外國籍）	碩士生	0	0	100%	人次	
		博士生	0	0	100%		
		博士後研究員	0	0	100%		
		專任助理	0	0	100%		

其他成果 (無法以量化表達之成果如辦理學術活動、獲得獎項、重要國際合作、研究成果國際影響力及其他協助產業技術發展之具體效益事項等，請以文字敘述填列。)	無

	成果項目	量化	名稱或內容性質簡述
科教處計畫加填項目	測驗工具(含質性與量性)	0	
	課程/模組	0	
	電腦及網路系統或工具	0	
	教材	0	
	舉辦之活動/競賽	0	
	研討會/工作坊	0	
	電子報、網站	0	
	計畫成果推廣之參與（閱聽）人數	0	

國科會補助專題研究計畫成果報告自評表

請就研究內容與原計畫相符程度、達成預期目標情況、研究成果之學術或應用價值（簡要敘述成果所代表之意義、價值、影響或進一步發展之可能性）、是否適合在學術期刊發表或申請專利、主要發現或其他有關價值等，作一綜合評估。

1. 請就研究內容與原計畫相符程度、達成預期目標情況作一綜合評估

■達成目標

□未達成目標（請說明，以 100 字為限）

□實驗失敗

□因故實驗中斷

□其他原因

說明：

2. 研究成果在學術期刊發表或申請專利等情形：

論文：■已發表 □未發表之文稿 □撰寫中 □無

專利：□已獲得 □申請中 ■無

技轉：□已技轉 □洽談中 ■無

其他：(以 100 字為限)

本研究計畫的實驗目的以探討微型核糖核酸分子於乳癌侵襲轉移過程中所扮演的角色。針對微型核糖核酸對乳癌進程發展的分子機轉，其研究成果能建立起乳癌預後評估的微型核糖核酸生物標記，其研究成果已建立獨立的研究領域，期刊發表於 Ann Surg Oncol 2012, Aug, in press； Breast Cancer Res Treat 2012； 134:1081-93。

3. 請依學術成就、技術創新、社會影響等方面，評估研究成果之學術或應用價值（簡要敘述成果所代表之意義、價值、影響或進一步發展之可能性）（以 500 字為限）

根據本研究計畫的實驗設計，我們以雷射顯微擷取技術分離癌組織和相鄰之正常乳部上皮組織後，應用反轉錄聚合酶連鎖反應將 mRNA 加以進行轉錄。我們以 cyclin D1 和 beta-catenin 基因表現量為基準，分別進行 miRNA 的微矩陣(microarray)和生物資訊統計分析，將乳癌患者依據其 miRNA 基因表現量異常和臨床分期資料比較後加以分群。在研究 EST 資料庫中，藉由基因比對辨識法，歸納出有 53 個新的 miRNA 會影響到腫瘤的發展：有 27 個是在癌組織中表現量會增加(up-regulated expression)；相對地，有 26 個在癌組織中表現量會降低(down-regulated expression)。進一步，我們設計不同的 miRNA 轉譯調控 3'-UTR 的序列，利用聚合酶反應技術並加以分子選殖及核酸定序。我們發現當 Vimentin 的蛋白表現受到 miRNA 的抑制時，乳癌細胞侵襲和轉移的能力明顯的降低。更新的研究結果發現，癌細胞中 Vimentin 蛋白的表現量增加會拮抗 E-cad 的活化是受到 Snail 的影響，使上皮細胞過渡到間質細胞轉換，驅使癌細胞發生淋巴轉移。而我們的研究找到一個新的標的 miR-30a，它是直接對 Vimentin 蛋白的表現進行轉譯抑制(translational repression)，使得乳癌細胞的侵襲和轉移能力降低。在臨床檢體的分析上，我們也進一步證實 miR-30a 低度表現量關連於乳癌侵襲轉移和預後存活，這在過去的研究報告中是未曾被探討過的。因此，結合過去多年對乳癌標記的研究，能夠釐清 miR-30a 於抑制乳癌發

生和侵襲、轉移時所扮演的角色，而成為影響乳癌生成、進展、治療和預後的重要指標，這將有助於在未來發展成有效的乳癌治療策略的評估。