Aberrant expression of Arpin in human breast cancer and its clinical significance

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Abstract

Arpin (Arp2/3 complex inhibitor), a novel protein found in 2013, plays a pivotal role in cell motility and migration. However, the precise role of Arpin in cancer is unclear. This study investigated the expression of Arpin in breast cancer and evaluated its correlation with the characteristics of clinical pathology and prognosis of breast cancer patients. Immunohistochemistry (IHC) for Arpin protein was performed on formalin-fixed, paraffin-embedded 176 breast cancer tissues and 43 normal breast tissues while qRT-PCR for Arpin mRNA with 104 paired tumour and paratumoural tissues from breast cancer patients respectively. The association of Arpin expression with clinical pathological features and survival was assessed in a retrospective cohort analysis of patients. The results showed that the expression of Arpin protein in cancer tissues was lower compared to that in normal breast and the expression of Arpin mRNA was also lower in cancer tissues than that in the matched paratumoural tissues. Among the 176 breast cancer patients, the lower expression of Arpin was significantly associated with advanced tumour, nodes and metastasis system stage, and the reduced Arpin expression was strongly associated with axillary lymph node metastasis using univariate and multivariate logistic regression analysis \( \text{odds ratio: 3.242; 95\% confidence interval (CI): 1.526, 6.888; } P < 0.05 \). Furthermore, Arpin expression was an independent risk factor for recurrence-free survival \( \text{HR: 0.373; 95\% CI: 0.171, 0.813; } P < 0.05 \). As Arpin expression was first examined in human breast cancer tissues with qRT-PCR and IHC, our results suggest that Arpin downregulation may contribute to the initiation and development of breast cancer metastasis. Therefore, as a potential predictive marker, Arpin deserves future studies.

Keywords: arpin ● breast cancer ● prognosis

Introduction

Breast cancer is the most common malignancy in females with an increasing annual incidence and it is usually accompanied by local invasion and distant metastasis, which are the main causative factors for cancer-related death [1, 2]. Clinical outcome of breast cancer patients is widely variable, due to the genetic alterations of breast cancer which are increasingly recognized as critical determinants of tumour initiation, development, local invasion and distal metastasis [3, 4]. More and more studies have shown that the metastatic capability of cancer is empowered by the molecular changes which may exist in the early stage of tumourigenesis [5]. Therefore, elucidation of these factors and the pattern of their expression may help to better understand the progression of breast cancer, and help to predict the clinical outcome of breast cancer patients. Furthermore, the capacity to catalogue the genetic alterations associated with breast cancer may facilitate advances in its treatment.

Lamellipodia is a major tool for cancer cells to explore their extraenvironment and to form new adhesion contacts with the extracellular matrix for motility and spreading. The common process of the formation of lamellipodia is driven by spatially and temporally regulated actin polymerization at the leading edge [6]. And actin polymerization is regulated by a very important molecule—Arp2/3 complex—which is considered to be a key regulator of cell motility [7], and was reported to be involved in the development and migration of some cancers such as pancreatic cancer, gastric cancer, colorectal cancer, breast cancer and so on [8–11]. Lamellipodia drives the motion of the cell through changing actin cytoskeletons such as assembling new actin or retracting the protrusive actin structure. Arp2/3 complex is a major controller of actin assembly and initiator of new actin filament in lamellipodia [12]. By binding actin filaments to the side of an existing filament and initiating branch formation, Arp2/3 complex accomplishes its mission of nucleating actin filaments [13].
Arpin is a newly found Arp2/3 complex inhibitor reported in Nature, 2013. Arpin is encoded by the gene C15orf38 and its protein is encoded by a single gene containing 220 residues and is structured with the exception of its highly mobile C-terminal end, which contains the putative binding site of Arp2/3 complex [14], which has been reported to be closely related to the development and migration of cancer cells for its key role in the filopodia initiation [15, 16]. Arpin and its acidic motif could compete with nucleation promoting factors for Arp2/3 binding and thereby inhibit the activation of the Arp2/3 complex [14, 17].

Furthermore, Arpin is involved in the regulation of the motility process in a variety of cells, including Dictyostelium discoideum amoeba, keratocytes and human breast cancer cell line MDA-MB-231 [14]. Arpin-depleted MDA-MB-231 cells can invade a larger territory than the control cells that contain Arpin in two or three dimensions [14]. However, little is known about whether the expression of Arpin is altered in human breast cancer tissues and how this expression is correlated with the clinicopathological characteristics and prognosis of the patients.

In this study, we employed qRT-PCR and immunohistochemistry (IHC) to examine the expressions of Arpin gene and protein in breast cancer tissues, and assessed its correlation with clinicopathological and prognostic variables of patients with breast cancer. We observed a markedly low expression level of Arpin in cancer tissues of patients and postulated that low expression level of Arpin may serve as a risk factor that is predictive of poor prognosis in patients with breast cancer.

Materials and methods

Patients and sample collection

Formalin-fixed, paraffin-embedded specimens (43 normal breast tissues versus 176 breast cancer tissues) for IHC assay were collected from breast patients who underwent surgical resection between January 2009 and June 2009 at the Affiliated Hospital of Qingdao University. Also, 104 paired tumour tissue and paratumour tissue samples (3–5 cm from the border of the tumour) for qRT-PCR assay came from curative resection specimens of breast cancer patients (January–July 2014) at the same hospital. Samples were histologically diagnosed for primary adenocarcinoma or normal tissue by haematoxylin and eosin staining. None of the patients had received radiation therapy or chemotherapy before the surgery. The following data about the patients were retrieved from the archives of our hospital. The median age (minimum, maximum) of all 280 cancer patients whose tissues were used for the IHC (176 cancer patients) and qRT-PCR (104 cancer patients) assays was 51 years old. The TNM stages of specimens for IHC assay were 72 (40.9%) in TNM stage I, 52 (29.5%) in stage II and 52 (29.5%) in stage III respectively. And 14 (8.0%), 68 (38.6%) and 94 (53.4%) cases were assessed as G1, G2 and G3 according to histological grade. The TNM stages of specimens for qRT-PCR assays were 34 (32.7%) in TNM stage I, 32 (30.8%) in stage II and 38 (36.5%) in stage III respectively. And 12 (11.5%), 48 (46.2%) and 44 (42.3%) cases were assessed as G1, G2 and G3 according to histological grade. The 43 normal breast tissues were from benign patients. The characteristics of the patients are shown in Tables 1 and 2.

Immunohistochemistry

Arpin IHC staining was performed on 4 μm-thick sections of 10% formalin-fixed, paraffin-embedded tissue. Briefly, sections were boiled in a pressure cooker for 3 min. and placed in 3% hydrogen peroxide (H₂O₂) for 10 min. to inhibit endogenous peroxide activity. The sections were then washed with buffer and incubated with goat polyclonal antibody specific to Arpin (sc-242049; Santa Cruz Biotechnology Inc., Bergheimer, Germany, final dilution 1:25) at 4°C for 24 hrs, followed again by a buffer wash thrice. Biotinylated anti-goat immunoglobulin was used as the second antibody. Subsequently, the sections were incubated with avidin-biotin-conjugated peroxidase. Finally, the sections were washed and stained with 3,3-diaminobenzidine tetrahydrochloride and hydrogen peroxide, and a brown pigment was obtained after counterstaining with haematoxylin.

Immunohistochemical analysis and scoring

The tumour cells in which the cytoplasm was stained dark brown under light microscopy were considered positive. For the quantification of Arpin expression, both the staining intensity and the percentage of stained cells were evaluated. Cells without staining were scored as 0 points, weak staining intensity, 1 point, moderate staining intensity, 2 points, and strong staining intensity, 3 points. The percentage of stained tumour cells 0% was 0 points, less than 25%, 1 point, 25–50%, 2 points, or more than 50%, 3 points. The final score for Arpin expression was the sum of the above two kinds of scores. A staining score from 0 to 3 points was considered as low expression and a score more than 3 points was considered as high expression [19].

Quantitative RT-PCR

Primer design and synthesis Primer Express software (Applied Biosystems, Foster, CA, USA) was used to design primers specific to the
Table 1 Association analysis of IHC staining for Arpin versus clinicopathological factors of breast cancer

| Variable                  | Number of patients (n = 176) | Arpin expression | P-value |
|---------------------------|-----------------------------|-------------------|---------|
|                           | High (n = 56)               | Low (n = 120)     |         |
| Age                       |                             |                   |         |
| >50                       | 79                          | 26                | 53      | 0.779 |
| <50                       | 97                          | 30                | 67      |       |
| Lymph node metastasis     |                             |                   |         |
| Yes                       | 88                          | 16                | 72      | <0.001|
| No                        | 88                          | 40                | 48      |       |
| TNM stage                 |                             |                   |         |
| I                         | 72                          | 32                | 40      | 0.003 |
| II                        | 52                          | 16                | 36      |       |
| III                       | 52                          | 8                 | 44      |       |
| Grade                     |                             |                   |         |
| 1                         | 14                          | 2                 | 12      | 0.065 |
| 2                         | 68                          | 28                | 40      |       |
| 3                         | 94                          | 26                | 68      |       |
| OR                        |                             |                   |         |
| Positive                  | 122                         | 34                | 88      | 0.091 |
| Negative                  | 54                          | 22                | 32      |       |
| PR                        |                             |                   |         |
| Positive                  | 132                         | 36                | 96      | 0.025 |
| Negative                  | 44                          | 20                | 24      |       |
| Her-2                     |                             |                   |         |
| Positive                  | 26                          | 6                 | 20      | 0.3   |
| Negative                  | 150                         | 50                | 100     |       |
| Ki-67                     |                             |                   |         |
| Positive                  | 66                          | 20                | 46      | 0.738 |
| Negative                  | 110                         | 36                | 74      |       |
| Diameter                  |                             |                   |         |
| ≥2                        | 52                          | 12                | 40      | 0.107 |
| <2                        | 124                         | 44                | 80      |       |

Ki-67 ≤ 20%: Negative. Ki-67 > 20%: Positive.

Table 1: Association analysis of IHC staining for Arpin versus clinicopathological factors of breast cancer.

- **Age:**
  - >50: 79 (High: 26, Low: 53) with P-value 0.779.
  - <50: 97 (High: 30, Low: 67) with P-value 0.779.

- **Lymph node metastasis:**
  - Yes: 88 (High: 16, Low: 72) with P-value <0.001.
  - No: 88 (High: 40, Low: 48) with P-value 6.976.

- **TNM stage:**
  - I: 72 (High: 32, Low: 40) with P-value 0.003.
  - II: 52 (High: 16, Low: 36) with P-value 0.003.
  - III: 52 (High: 8, Low: 44) with P-value 0.099.

- **Grade:**
  - 1: 14 (High: 2, Low: 12) with P-value 0.065.
  - 2: 68 (High: 28, Low: 40) with P-value 0.065.
  - 3: 94 (High: 26, Low: 68) with P-value 0.065.

- **OR:**
  - Positive: 122 (High: 34, Low: 88) with P-value 0.091.
  - Negative: 54 (High: 22, Low: 32) with P-value 0.091.

- **PR:**
  - Positive: 132 (High: 36, Low: 96) with P-value 0.025.
  - Negative: 44 (High: 20, Low: 24) with P-value 0.025.

- **Her-2:**
  - Positive: 26 (High: 6, Low: 20) with P-value 0.3.
  - Negative: 150 (High: 50, Low: 100) with P-value 0.3.

- **Ki-67:**
  - Positive: 66 (High: 20, Low: 46) with P-value 0.738.
  - Negative: 110 (High: 36, Low: 74) with P-value 0.738.

- **Diameter:**
  - ≥2: 52 (High: 12, Low: 40) with P-value 0.107.
  - <2: 124 (High: 44, Low: 80) with P-value 0.107.

The association between the protein and clinicopathological parameters was analysed using the chi-square test. The association between Arpin protein expression and axillary lymph node metastasis was assessed by Pearson correlation analysis. Survival curves for RFS were drawn by the Kaplan–Meier estimate, and comparisons between the survival curves were conducted using log-rank tests. A Cox proportional hazards regression model was constructed to identify factors that were independently associated with RFS and to evaluate the independent impact of Arpin expression on RFS. Statistical analysis was performed using SPSS 16.0 software (SPSS, Chicago, IL, USA). All tests were two-tailed, and P < 0.05 was considered to be statistically significant.
Results

Expression of Arpin protein is decreased in breast cancer tissues

In order to know the specificity of the Arpin antibody (sc-242049; Santa Cruz Biotechnology Inc.), the antibody was first used to detect the endogenous expression of Arpin by Western blot assay (final dilution 1: 200) in paired tumour tissue samples, paratumour tissue samples and in breast normal cell line Hs578bst depleted or not of Arpin. The result showed that Arpin was significantly decreased in tumour samples compared with paratumoural normal tissues, and Arpin was inhibited greatly in Arpin-depleted Hs578bst (Fig. S1). Although the endogenous Arpin protein is not very highly expressed in tissue samples, especially in cancer tissue samples, the results showed a clear major band at the right position of 25 kD. So, the Arpin antibody could recognize Arpin protein specifically.

From IHC assay, the results showed that the expression of Arpin protein was higher in normal tissues than that in cancer tissues (Fig. 1, Table 3). Whereas low and high expression could also be detected in a few normal breast tissues and breast cancer tissues, respectively, due to the heterogeneity of breast tumours (Fig. S2).

Expression of Arpin mRNA is decreased in breast cancer tissues

Primer specificity and amplification efficiency

To determine the levels of Arpin mRNA transcripts, total RNA was extracted from individual samples following reverse transcription into cDNA. As shown in the results, the DNA products were observed with Arpin (138 bp) and GAPDH (146 bp), respectively, suggesting the high specificity of the primers (Fig. S3). Further sequence analysis revealed that the PCR products were Arpin and GAPDH DNA fragments (Fig. S4). These preliminary results provided a reasonable basis for quantitative characterization of Arpin and GAPDH mRNA transcripts in the tissue samples. Amplification curves of serially diluted cDNA samples exhibited a standard S shape, suggesting good amplification efficiency and a linear relationship. For the Arpin gene, the log value of each cDNA dilution was plotted versus ΔCT, indicating that it had amplification efficiencies similar to GAPDH, justifying application of the 2−ΔΔCT method for relative quantification [21].

Low levels of Arpin mRNA transcripts in breast cancer tissues

To acquire insights into the role of Arpin in breast tumourigenesis, 104 pairs of fresh breast cancer tissues and their matched paratumour breast tissues were used to assess Arpin mRNA expression using qRT-PCR. The results showed that the Arpin mRNA expression level was significantly lower in breast cancer tissues compared with that of the matched paratumour tissues (P < 0.05). The median fold change in breast cancer tissues was only 0.27 of

Table 2 Clinical characters of the enrolled cancer patients for qRT-PCR

| Variable                  | Number of cancer patients (n = 104) |
|---------------------------|------------------------------------|
| Age                       |                                    |
| >50                       | 60                                 |
| ≤50                       | 44                                 |
| Lymph node metastasis     |                                    |
| Yes                       | 64                                 |
| No                        | 40                                 |
| TNM stage                 |                                    |
| I                         | 34                                 |
| II                        | 32                                 |
| III                       | 38                                 |
| Grade                     |                                    |
| 1                         | 14                                 |
| 2                         | 46                                 |
| 3                         | 44                                 |
| PR                        |                                    |
| Positive                  | 52                                 |
| Negative                  | 52                                 |
| PR                        |                                    |
| Positive                  | 36                                 |
| Negative                  | 68                                 |
| Her-2                     |                                    |
| Positive                  | 22                                 |
| Negative                  | 82                                 |
| Ki-67                     |                                    |
| Positive                  | 28                                 |
| Negative                  | 76                                 |
| Diameter                  |                                    |
| ≥2                        | 50                                 |
| <2                        | 54                                 |

Ki-67 ≤ 20%; Negative. Ki-67 > 20%; Positive.

TNM: tumour, nodes, metastasis system; OR: oestrogen receptor; PR: progesterone receptor.
that in the matched paratumoural tissues \((P < 0.05)\) (Fig. 2). So, the expression level of Arpin mRNA was significantly decreased in breast cancer tissues.

### The relationship between clinicopathological characteristics and Arpin expression

Correlations between Arpin expression and clinicopathological characteristics are shown in Figure 3, Tables 1 and 2. In the 104 samples for qRT-PCR, Arpin mRNA expression was significantly different between patients with and without lymph node metastasis \((P < 0.05)\) (Fig. 3 and Table 2). Analysing the IHC results from 176 tumour samples showed that Arpin protein expression was lower in stage III than in stage I or II. Low Arpin protein expression was significantly associated with advanced TNM stage (III versus I, \(P < 0.05\), Table 4). The chi-square analysis of the 176 samples for IHC showed that the protein expression level of Arpin in tumour tissues was significantly cor-

| Table 3 | IHC staining for Arpin in breast cancer and normal breast tissues |
|---------|---------------------------------------------------------------|
|         | No. of patients | Arpin expression |             |          |
|         |                 | High \((n = 87)\) | Low \((n = 132)\) | \(P\)    |
| Normal  | 43              | 31               | 12            | 0.001    |
| Cancer  | 176             | 56               | 120           |          |

**Fig. 1** Representative photomicrographs of Arpin immunohistochemical staining. **A** indicates the high expression in normal breast tissue and **B** indicates low expression in breast carcinoma. Original magnification \(\times 200\).

**Fig. 2** Relative expression levels of Arpin in breast cancer tissues and paratumoural tissue \((^*P < 0.05\)\).

**Fig. 3** Relative expression levels of Arpin in LNM and LNM-free breast cancers \(((***P < 0.05)\). LNM: lymph node metastasis.

| Table 4 | Association analysis of expression of Arpin protein versus TNM stage of breast cancer |
|---------|----------------------------------------------------------------------------------|
| TNM stage | No. of patients | Arpin expression | \(P\)       |
|          |                 | High \((n = 56)\) | Low \((n = 120)\) |
| I        | 72              | 32               | 40            | 0.138*   |
| II       | 52              | 16               | 36            | 0.102†   |
| III      | 52              | 8                | 44            | 0.001‡   |

*Stage I versus II.
†Stage II versus III.
‡Stage I versus III.
related with lymph node metastasis (P < 0.05), PR (P < 0.05) and
TNM stage (P < 0.05) (Table 1). Also, patients with positive PR
expression or lymph node metastasis tended to show low Arpin
expression. In contrast, there was no significant association between
Arpin expression and other clinicopathological features such as
patient age, tumour size, histologic type, expression of OR, Her-2 and
Ki-67 as well.

Impact of Arpin low expression on axillary lymph
node metastases in breast cancer

Among the 176 patients for IHC, 88 were free from lymph node
metastasis and 40 showed high expression of Arpin. Of the 88
patients with axillary lymph node metastasis, 72 showed low expres-
sion of Arpin. The sensitivity and specificity of low-expression Arpin
for lymph node metastases were 81.8% (72/88) and 45.5% (40/88)
respectively. Univariate and multivariate logistic regression with
covariate adjustment were performed to evaluate the association
between reduced Arpin expression and axillary lymph node metasta-
sis. As the status of the axillary lymph node metastasis is included in
the TNM stage, the TNM stage was not included in the analysis. We
first performed univariate analysis of traditional clinicopathological
variables and Arpin expression to identify the variables associated
with axillary lymph node metastasis. The presence of lymph node
metastasis was notably associated with tumour size (P < 0.05), PR
status (P < 0.05), Her-2 status (P < 0.05) and Arpin expression
(P < 0.05). All variables significantly associated with axillary lymph
node metastasis in the univariate analysis were included in a multi-
variate logistic regression model to find the best determinants or pre-
dictors of axillary lymph node metastasis. Results of the univariate
and multivariate analyses are presented in Table 5. Low Arpin expres-
sion could be an independent predictor of axillary lymph node metas-
tasis (Arpin: odds ratio: 3.242; 95% confidence interval: 1.526, 6.888,
P < 0.05).

Correlation of Arpin expression and RFS in
breast cancer patients

To validate the clinical utility of Arpin expression, we evaluated
the prognostic power of Arpin protein for 5 year RFS in 176
breast cancer specimens which were used for the IHC assay men-
tioned previously. The survival analysis revealed that patients with
low Arpin expression showed a worse prognosis for RFS com-
pared to those with high Arpin expression (Fig. 4, P < 0.05). To
analyse the relevance of Arpin expression and clinicopathological
features with RFS, a univariate Cox regression analysis was per-
formed wherein factors associated with RFS included TNM stage
(stage I and II versus stage III), lymph node status, tumour diam-
eter, Arpin expression, OR status, PR status and HER-2 status
(Table 6). In addition to Arpin expression (P < 0.05), tumour
diameter (P < 0.05), lymph node status (P < 0.05), and TNM
stage (P < 0.05) significantly affected the RFS (Table 6). Multivari-
ate Cox regression analysis indicated that low-expression Arpin
(P < 0.05) and tumour diameter (P < 0.05) were independent
prognostic factors related to RFS.

Discussion

Metastatic diseases which have been reported to be controlled by
specific genetic events contribute to the vast majority of cancer-
related deaths [23]. The cascade of metastasis from the primary
tumour to a distant site is a complicated process which contains
acquisition of many intermediate phenotypes. The metastatic cas-

date involved in the development and migration of some cancers such as pancre-
catic cancer, gastric cancer, colorectal cancer, breast cancer and so
on [8–11]. Thus, the regulation mechanism of the Arp2/3 complex
has been extensively studied and many factors were found to pro-
mote the function of the Arp2/3 complex [25]. However, the nega-
tive regulation mechanism of the Arp2/3 complex is rarely studied
and is still unclear.

Dang et al. [14] identified a new protein, Arpin, which was a nega-
tive regulator of the Arp2/3 complex. Arpin was utilized to modulate
actin nucleation activity at the leading edge of the lamellipodium to
steer the cell via inhibition of lamellipodia formation. Their experi-
ments proved that Arpin indeed binds to the Arp2/3 complex and the
levels of Arpin are inversely correlated with the speed and persis-
tence of lamellipodia in the breast human cell line MDA-MB-231. However,
the expression of Arpin in human breast cancer tissues is still
unknown.

In order to investigate whether the level of Arpin is altered in
breast cancer, we first compared the expression of Arpin protein
in breast cancer tissues and normal tissues using the formalin-
fixed, paraffin-embedded tissue from breast cancer surgical resec-
tion specimens. The results showed that Arpin protein was signifi-
cantly less expressed in tumour samples than that in paired
paratumoural normal tissues. The endogenous Arpin in breast nor-
cal cell line Hs578bst was inhibited greatly in Arpin-depleted
Hs578bst. So the Arpin antibody could recognize the endogenous
Arpin protein specifically. Furthermore, to gain insights into the
role of Arpin in breast tumorigenesis, we assessed Arpin mRNA
expression in 104 pairs of breast cancer tissues and their
matched paratumourous breast tissues using qRT-PCR. The Arpin
mRNA expression level was also significantly lower in breast can-

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Table 5: Risk factors for 176 cancer patients with axillary lymph node metastasis (univariate and multivariate logistic regression analysis adjusted by age)

| Variables | Univariate analysis | | Multivariate analysis | |
|-----------|---------------------| |----------------------| |
|           | B | SE | OR (95% CI) | p | B | SE | OR (95% CI) | p |
| Age (years) | | | | | | | | |
| ≥50       | 1.00 (reference) | | 1.00 (reference) | | | | | |
| <50       | 0.009 | 0.391 | 1.009 (0.469, 2.172) | 0.981 | 0.269 | 0.371 | 1.308 (0.633, 2.704) | 0.469 |
| Tumour size (cm) | | | | | | | | |
| ≥2       | 1.00 (reference) | | 1.00 (reference) | | | | | |
| <2       | -1.544 | 0.470 | 0.213 (0.085, 0.536) | 0.001 | -1.205 | 0.419 | 0.300 (0.132, 0.682) | 0.004 |
| Grade | | | | | | | | |
| 1       | 1.00 (reference) | | 1.00 (reference) | | | | | |
| 2       | -0.471 | 0.673 | 0.624 (0.167, 2.334) | 0.484 | | | | |
| 3       | 0.551 | 0.690 | 1.735 (0.449, 6.708) | 0.425 | | | | |
| OR | | | | | | | | |
| Positive | 1.00 (reference) | | 1.00 (reference) | | | | | |
| Negative | -0.025 | 0.720 | 0.975 (0.238, 3.999) | 0.972 | | | | |
| PR | | | | | | | | |
| Positive | 1.00 (reference) | | 1.00 (reference) | | | | | |
| Negative | -1.248 | 0.632 | 0.287 (0.083, 0.991) | 0.048 | -1.447 | 0.484 | 0.235 (0.091, 0.607) | 0.003 |
| Her-2 | | | | | | | | |
| Positive | 1.00 (reference) | | 1.00 (reference) | | | | | |
| Negative | -3.613 | 0.818 | 0.027 (0.005, 0.134) | <0.001 | -3.317 | 0.742 | 0.036 (0.008, 0.155) | <0.001 |
| Ki-67 | | | | | | | | |
| Positive | 1.00 (reference) | | 1.00 (reference) | | | | | |
| Negative | 0.554 | 0.486 | 1.740 (0.671, 4.512) | 0.255 | | | | |
| Arpin expression | | | | | | | | |
| High | 1.00 (reference) | | 1.00 (reference) | | | | | |
| Low | 1.486 | 0.430 | 4.420 (1.947, 10.262) | 0.001 | 1.176 | 0.385 | 3.242 (1.526, 6.888) | 0.002 |

Ki-67 ≤ 20%: Negative.
Ki-67 > 20%: Positive.
OR: oestrogen receptor; PR: progesterone receptor.

cancer tissues compared with that of the matched paratumour tissues. So, the decreased Arpin level in breast cancer may lead to the acquisition of invasion and metastasis ability. We then accessed the correlation of Arpin expression and tumour staging. The results of IHC showed that low Arpin expression was significantly associated with advanced TNM stage. As TNM stage was defined by tumour size, regional lymph node and metastasis, we further analysed the correlation of Arpin expression and tumour size and the correlation of Arpin expression and regional lymph node. We found that there was no significant association between Arpin and tumour size. However, low Arpin expression was significantly associated with more axillary lymph node metastases. Such
a result may explain that some axillary lymph node metastasis occurs when the tumour size is small.

More importantly, the present study found that the prognosis for RFS was significantly worse in breast cancer patients with low Arpin expression than in those with high Arpin expression. The recurrent cases of breast cancer generally include the regional recurrence and distant relapse. And the distant relapse cases usually occupy the majority of recurrent cases according to the rough statistics database of the Center of Diagnosis and Treatment of Breast Disease of our hospital. As reported previously, patients with lymph node involvement have a worse post-relapse outcome and the number of lymph node metastases has significant correlation with the distant relapse [26, 27]. Consistently, our study demonstrated that the expression of Arpin is an independent predictor of axillary lymph node metastasis and RFS. We presumed that Arpin probably played a role in breast cancer metastasis and such ability may suggest that breast cancer is apt to metastasis and occurrence of distant relapse. However, the exact molecular events leading to cancer metastasis have not yet been well elucidated and further research is still needed.

A limitation of our study is the limited number of cases and our findings should be further confirmed by large sample studies. As the number of death events in our enrolled cases is smaller compared to recurrent events, we only analysed the correlation between Arpin and RFS. Further studies are underway. To further clarify the role of Arpin as a prognostic biomarker in breast cancer, we will evaluate the association between Arpin expression and clinical outcome in a prospective cohort study. There are several positive regulators of the Arp2/3 complex, such as Wiskott-Aldrich syndrome protein (WASP), neural WASP, WASP family verprolin-homologous protein (WAVE; also known as SCAR) and WASP and SCAR homologue (WASH) complex [25]. The interactions between Arpin and these proteins are still unclear. Further study is needed to determine the possible interactions between Arpin and positive regulators of the Arp2/3 complex.

### Conclusion

Our study provides evidence that Arpin expression is associated with the clinical characters and outcome in breast cancer patients. Its decrease predicts a poor outcome. Arpin may be an important biomarker for predicting unfavourable biological behaviour such as axillary lymph node metastasis and recurrence. To our knowledge, the present study is the first report conducted to determine the

| Variable | Univariate analysis | Multivariate analysis |
|----------|---------------------|----------------------|
|          | HR  | 95% CI | P-value | HR  | 95% CI | P-value |
| Age      | 0.112 | 0.668—1.875 | 0.669 | 1.968 | 0.969—3.997 | 0.361 |
| LN       | 2.984 | 1.694—5.256 | <0.001 | 0.373 | 0.171—0.813 | 0.013 |
| TNM stage (I + II versus III) | 0.275 | 0.130—0.580 | 0.001 | 0.275 | 0.130—0.580 | 0.001 |
| OR expression | 1.492 | 0.818—2.723 | 0.192 | 0.373 | 0.171—0.813 | 0.013 |
| PR expression | 1.726 | 0.914—3.258 | 0.092 | 0.373 | 0.171—0.813 | 0.013 |
| HER-2 expression | 0.677 | 0.291—1.576 | 0.365 | 0.373 | 0.171—0.813 | 0.013 |
| Ki-67 expression | 0.728 | 0.420—1.259 | 0.256 | 0.373 | 0.171—0.813 | 0.013 |
| Diameter | 3.769 | 2.241—6.339 | <0.001 | 3.096 | 1.749—5.480 | <0.001 |
| Arpin | 0.275 | 0.130—0.580 | 0.001 | 0.373 | 0.171—0.813 | 0.013 |

CI: confidence interval; RFS: recurrence-free survival.

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value of Arpin in patients with breast cancer, although the results need to be confirmed in a further follow-up and with larger samples. Arpin may be used as a biomarker that could provide important tumour progress information and even a possible target for breast cancer therapy.

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Conflicts of interest

The authors confirm that they have no conflicts of interest.

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Supporting information

Additional Supporting Information may be found in the online version of this article:

Figure S1 Arpin expression in normal breast cells and in paired tumour and paratumoural normal tissues.

Figure S2 Representative photomicrographs of Arpin immunohistochemical staining.

Figure S3 Gel electrophoresis of PCR products. 1, 100–600 bp markers; 2–3, PCR products of Arpin; 4–5, PCR products of GAPDH.

Figure S4 Part of sequence chromatograms of Arpin and GAPDH PCR products.