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RESEARCH ARTICLE

BMP-2 upregulates the AKT/mTOR pathway in breast cancer with microcalcification and indicates a poor prognosis

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Abstract

Background As a reliable biomarker of breast cancer, breast microcalcification has been reported to be correlated with poor prognosis. Bone morphogenetic protein 2 (BMP-2) plays an important role in microcalcification of breast cancer. Studies in other tissues have shown an association between BMP-2 and AKT/mTOR pathway, while their relationship in breast cancer still remains largely undetermined. To clarify the relationship of these three factors, we collected patients of invasive breast cancer with/without microcalcification and immunohistochemical examination was performed.

Method/patients A total of 272 patients with primary invasive breast cancer were selected from the First Hospital of China Medical University from January 2010 to January 2012. Immunohistochemical examination of the BMP-2, p-AKT and p-mTOR was performed on 4-μm tissue microarray (TMA) sections. Then, we analyzed the relationship of BMP-2, p-AKT, and p-mTOR and their correlation with disease-free survival (DFS) in breast cancer with/without microcalcification.

Results We found that breast cancer patients with microcalcification were correlated with HER-2 positive expression and poor prognosis. Immunohistochemical examination showed that the expressions of BMP-2 and p-mTOR were increased in breast cancer with microcalcification and the expressions of BMP-2, p-AKT, and p-mTOR were correlated with each other. Moreover, the high expressions of BMP-2, p-AKT, and p-mTOR were significantly correlated with poor prognosis.

Conclusions Based on the abovementioned findings, we hypothesized that the high expression of BMP-2 not only played a vital role in the formation of microcalcification, but also activated the AKT/mTOR pathway. Collectively, breast cancer patients with microcalcification were more likely to be resistant to targeted or endocrine therapy and be correlated with poor prognosis.

Keywords Microcalcification · Breast cancer · BMP-2 · AKT · mTOR · Prognosis

Introduction

Breast microcalcification (< 0.5 mm in diameter) is one of reliable biomarkers of breast cancer, especially in early non-palpable breast cancer. Mammography is now widely used in detection of microcalcification for breast cancer and the mortality of breast cancer is reduced approximately 20%, which is greatly attributed to the early diagnosis by mammography [1]. Several studies have reported that the microcalcification of breast cancer is a poor indicator of long-term clinical outcome [2–9], while the mechanism underlying microcalcification remains largely unexplored. Recent study shows that a subpopulation of breast cancer cells undergoes mesenchymal transition (EMT) and acquires osteoblastic characteristics during microcalcification [10]. Bone morphogenetic protein 2 (BMP-2) is a member of the TGF-β superfamily and it induces matrix mineralization in osteoblast-like cells [11]. Recent findings indicate that BMP-2 is upregulated in breast carcinoma with microcalcification compared with breast carcinoma without microcalcification [10]. Similarly, another study has observed that inoculation of the breast carcinoma cells overexpressing BMP-2 into the rat mammary results in breast tumors with microcalcification [12] and it has been also demonstrated that treatment with recombinant BMP-2 can also induce microcalcification in breast cancer tissue

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of rats [13]. Based on these findings, BMP-2 may play an important role in microcalcification of breast cancer.

The mammalian target of rapamycin (mTOR) is a highly conserved serine/threonine protein kinase, which is a downstream mediator in the PI3K/AKT signaling pathway [14–16]. When such a pathway is activated, AKT is phosphorylated into p-AKT and mTOR is phosphorylated into p-mTOR. The AKT/mTOR pathway has been demonstrated to regulate several cellular functions, including cell growth, survival, angiogenesis, as well as targeted or endocrine therapy resistance [17–20] and the PI3K/AKT/mTOR pathway is activated in multiple cancers, including breast cancer [21, 22]. Studies have indicated that BMP-2 can regulate chondrocyte maturation [23] and differentiation of osteoclasts [24, 25] through activating the PI3K/AKT pathway. Another two studies in gastric cancer have shown that BMP-2 can accelerate the motility and invasiveness of cancer cells via the activation of AKT [26, 27]. Therefore, BMP-2 may be an upstream regulator of AKT/mTOR pathway in breast cancer. However, it is still unclear whether AKT/mTOR pathway can be regulated by BMP-2 and there is any difference between the expression of BMP-2/AKT/mTOR in breast cancer with and without microcalcification. In the present study, we investigated the potential roles and relationship of BMP-2, p-AKT, and p-mTOR in breast cancer with microcalcification and without microcalcification.

Materials and methods

Patients and tissues

A total of 272 patients with primary invasive breast cancer was selected from the First Hospital of China Medical University from January 2010 to January 2012. Patients with invasive breast cancer of stage I to III who received preoperative mammography were selected in the present study according to the inclusion criteria. The exclusion criteria were set as follows: patients younger than 20 or older than 80 years; patients with distant metastasis at the time of diagnosis; patients with previous history of other malignant neoplasms, including breast cancer; patients who could not undergo radical surgery; and patients who had rare histologic subtype such as inflammatory breast carcinoma.

Patients were followed up for a median of 84 months after their initial cancer surgery. Relevant clinical and pathological parameters were described in Table 1. Archival formalin-fixed paraffin-embedded (FFPE) breast tissues were collected and prepared into tissue microarray (TMA). All of the carcinomas were histologically confirmed as invasive breast cancer according to the criteria of the World Health Organization and the molecular subtypes of breast carcinoma were identified.

This study was approved by the ethics committee of the First Affiliated Hospital (Shenyang, China) and written informed consent was obtained from each patient.

Immunohistochemical staining

Immunohistochemical examination was performed on 4-μm TMA sections. Briefly, following deparaffinization and rehydration, the endogenous peroxidase activity was blocked using 3% H_2O_2 (reagent A; UltraSensitive™ SP IHC kit; Maxim Biotech Inc., Fuzhou, China). Next, antigen retrieval was performed and normal serum (reagent B; UltraSensitive™ SP IHC kit; Maxim Biotech Inc.) was applied to the sections to block non-specific binding. Sections were then incubated at 4 °C overnight with the primary antibodies, including an anti-rabbit polyclonal antibody against BMP-2 (1:300, ab14933; Abcam, Cambridge, UK), an anti-rabbit polyclonal antibody against AKT (phospho T308) (1:300, ab38449; Abcam, Cambridge, UK) and an anti-rabbit polyclonal antibody against mTOR (phospho S2448) (1:500, ab131538; Abcam, Cambridge, UK). Subsequently, the sections were incubated with the secondary antibody (reagent C; UltraSensitive™ SP IHC kit; Maxim Biotech Inc.) for 15 min, followed by incubation with streptavidin-peroxidase (reagent D, UltraSensitiveTM SP IHC Kit, MXB, Fuzhou, China) and 3,3-diaminobenzidine (DAB) was used to stain the sections. Finally, sections were counterstained with hematoxylin and mounted. Sections incubated with normal rabbit serum (Dako, Carpinteria, CA, USA) served as negative controls. Sections of breast cancer tissue showing strong staining with the respective proteins during antibody optimization served as positive controls.

Evaluation of immunohistochemistry

The results of immunohistochemical staining were independently evaluated and scored by two pathologists in a blinded manner. Cases of disagreement were jointly reviewed to obtain a consensus score. The score was obtained from the average of ten distinct high-power fields (40× objective). The staining was considered positive when cytoplasmic and/or membranous staining was observed in the cancer cells and the staining was evaluated using a semi-quantitative scoring system considering both the extent and intensity. The proportion of stained cells was scored as 0 (no cells stained), 1 (1–10% of cells stained), 2 (11–50% of cells stained), 3 (51–80% of cells stained), or 4 (more than 80% of cells stained). Staining intensity was scored as 0 (negative), 1 (weak), 2 (moderate), or 3 (strong). These two parameters were then multiplied, resulting in an individual immunoreactivity score (IRS) ranging from 0 to 12 for every case.

Table 1 Clinical and pathological features of the patients

Characteristic	With microcalcification (%) $N=77$ (28.3)	Without microcalcification (%) $N=195$ (71.7)	χ^2 value	P value
Age			0.053	0.818
≤45	24 (31.2)	58 (29.7)		
>45	53 (68.8)	137 (70.3)		
Tumor size			9.629	0.022
T1	16 (20.8)	74 (37.9)		
T2	53 (68.8)	112 (57.4)		
T3	5 (6.5)	7 (3.6)		
T4	3 (3.9)	2 (1.0)		
Axillary metastasis			1.248	0.741
N0	35 (45.5)	102 (52.3)		
N1	22 (28.6)	46 (23.6)		
N2	14 (18.2)	31 (15.9)		
N3	6 (7.8)	16 (8.2)		
Hormonal receptor			1.015	0.314
Positive	48 (62.3)	134 (68.7)		
Negative	29 (37.7)	61 (31.3)		
Her-2			9.986	0.002
Positive	25 (32.5)	30 (15.4)		
Negative	52 (67.5)	165 (84.6)		
Surgery			0.185	0.667
Mastectomy	69 (89.6)	178 (91.3)		
Breast conserving	8 (10.4)	17 (8.7)		
Chemotherapy regimen			8.660	0.123
EC-T or TEC	20 (26)	53 (27.2)		
TP	19 (24.7)	36 (18.5)		
CEF	17 (22.1)	69 (35.4)		
EC	5 (6.5)	17 (8.7)		
CEF-T	15 (19.5)	17 (8.7)		
TC	1 (1.3)	3 (1.5)		
Follow up (month)	84	85		
Recurrence	18	23		

T docetaxel, *P* platinum, *E* epirubicin, *C* cyclophosphamide, F 5-fluorouracil

Statistical analysis

Statistical analyses were carried out using SPSS v 19.0. Mann–Whitney *U* test was performed to assess the independence between two independent samples without any distribution assumption. Pearson correlation coefficient revealed a relationship between two continuous variables. Receiver operating characteristic (ROC) curve analyses were used to select cutoff values (giving the highest combined sensitivity and specificity) to dichotomize the expression scores of BMP-2, p-AKT, and p-mTOR for the end point of disease-free survival (DFS). DFS was estimated using the Kaplan–Meier analyses and recorded from the date of surgery to the date of relapse or last follow-up date. The statistical significance of differential survival was assessed using the log-rank (score) test. Additionally, multivariate Cox regression analysis was performed by taking into account the expressions of BMP-2, p-AKT, p-mTOR, and HER-2, as well as axillary lymph node metastasis and microcalcification. All analyses were two sided and $P \leq 0.05$ was considered as statistically significant.

Results

Microcalcification is correlated with the high expression of HER-2 and poor DFS in patients with breast cancer

Among the 272 patients with breast cancer, existing microcalcification was found in 77 patients by preoperative mammography and Table 1 shows the pathologic outcome. We

found that 25 cases (32.5%) were HER-2 positive in patients with microcalcification and 30 cases (15.4%) were HER-2 positive in patients without microcalcification. HER-2 positivity was more likely to be associated with microcalcification ($\chi^2 = 9.986$, $P = 0.002$). We also found that there was a significant difference in tumor size between patients with microcalcification and without microcalcification. Patients with microcalcification were correlated with larger tumor size ($\chi^2 = 9.629$, $P = 0.022$) (Table 1).

Kaplan–Meier survival analyses were used to analyze the difference of DFS between patients with microcalcification and without microcalcification. Figure 1a reveals that patients with microcalcification were correlated with poor DFS ($\chi^2 = 5.002$, $P = 0.025$).

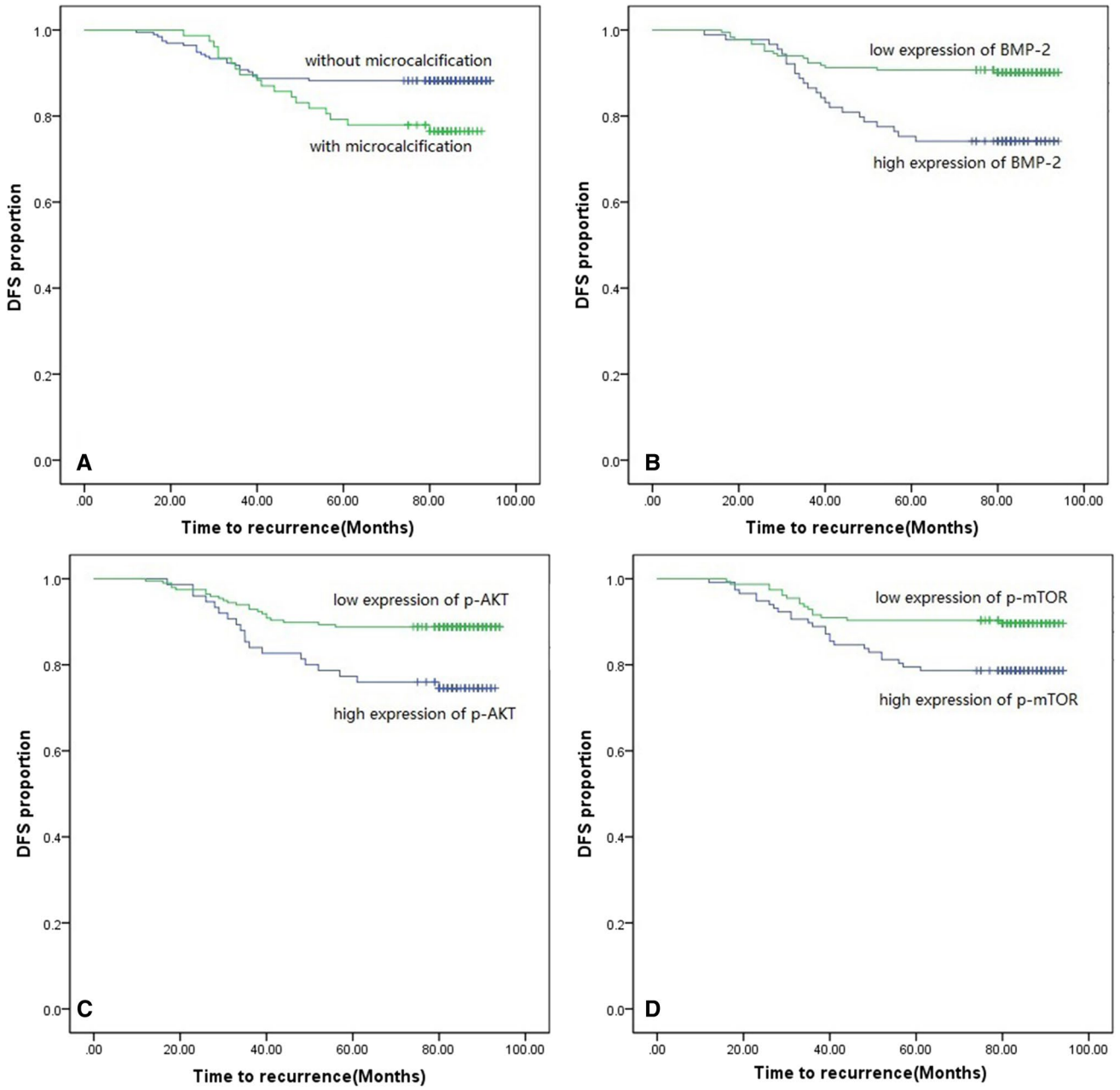

Fig. 1 Relationship between microcalcification or expression levels of BMP-2, p-AKT, and p-mTOR and patients' DFS **a** shows that patients with microcalcification were correlated with poor DFS ($P = 0.025$), **b–d** shows that the high expressions of BMP-2, p-AKT, and p-mTOR were significantly correlated with poor DFS ($P = 0.001$, 0.004, and 0.013, respectively)

The expression scores of BMP-2 and p-mTOR are significantly increased in breast cancer with microcalcification and correlated with each other

The staining of BMP-2 was found in cytoplasm, nucleus, and cell membrane and such positive staining was found in 74/77 cases (96.1%) and 175/195 cases (89.7%) of tissues with microcalcification and without microcalcification, respectively (Fig. 2a). The staining of p-AKT was detected in both nucleus and cytoplasm. The positive rate of p-AKT was 92.2% (71/77) and 88.7% (173/195) in tissues with microcalcification and without microcalcification, respectively (Fig. 2b). The p-mTOR was expressed in cytoplasm and nucleus, which was present in 72/77 cases (93.5%) and 173/195 cases (88.7%) in tissues with microcalcification and without microcalcification, respectively (Fig. 2c). Figure 3 illustrates the scores and their distributions.

The median expression scores of BMP-2, p-AKT, and p-mTOR were 8, 8, and 8 in tissues with microcalcification, respectively, which became 3, 6, and 6 in tissues without microcalcification, respectively. Mann–Whitney *U* test showed that there was a significant difference in the expressions of BMP-2 and p-mTOR between tissues with microcalcification and without microcalcification. BMP-2 and p-mTOR were significantly increased in tissues with microcalcification ($P = 0.000$ and $P = 0.026$, respectively) (Fig. 3a, c). The AKT expression was not significantly different in tissues with and without microcalcification ($P = 0.180$) (Fig. 3b).

We also examined the correlation of BMP-2, p-AKT, and p-mTOR and found that these three factors were significantly correlated with each other, indicating that BMP-2 might be a regulator of p-AKT and p-mTOR. The correlation coefficient and *P* value of BMP-2 and p-AKT, BMP-2 and p-mTOR, or p-AKT and p-mTOR were 0.177 and 0.003, 0.164 and 0.007, or 0.172 and 0.004, respectively (Fig. 3d).

High expressions of BMP-2, p-AKT, and p-mTOR are significantly correlated with poor prognosis

Kaplan–Meier survival analyses were performed to assess the differential survival with BMP-2, p-AKT, and p-mTOR. ROC curve analyses were used to dichotomize the expression scores into high and low expression groups. The cutoff values were obtained from the highest combined sensitivity and specificity at the end point of DFS and the cutoff values were selected as follows: BMP, 27, p-AKT, 8.5 and p-mTOR, 7. We found that the high expressions of BMP-2, p-AKT, and p-mTOR were significantly correlated with poor prognosis. ($P = 0.001$, 0.004, and 0.013, respectively) (Fig. 1b–d).

The univariate factor analysis of other clinicopathological features and DFS

Kaplan–Meier survival analyses were also performed to assess the correlations between differential survival and hormonal receptor, HER-2, age, tumor size, axillary metastasis, surgical method, or chemotherapy regimen. We found that HER-2 and axillary metastasis were the risk factors of the prognosis in breast cancer ($P = 0.018$ and 0.005, respectively). Other clinicopathological features had no significant correlation with prognosis.

Cox regression analysis

Finally, COX regression analysis was performed by taking into account the statistical significant variables in single factor analysis, including the expressions of BMP-2, p-AKT, p-mTOR, and HER-2, as well as axillary lymph node metastasis and microcalcification. We found that BMP-2, p-AKT, p-mTOR, HER-2, and axillary lymph node metastasis were the independent prognostic factors, with a hazard ratio of 0.454, 0.382, 0.483, 1.380, and 1.588, respectively and with a *P* value of 0.023, 0.003, 0.028, 0.007, and 0.002,

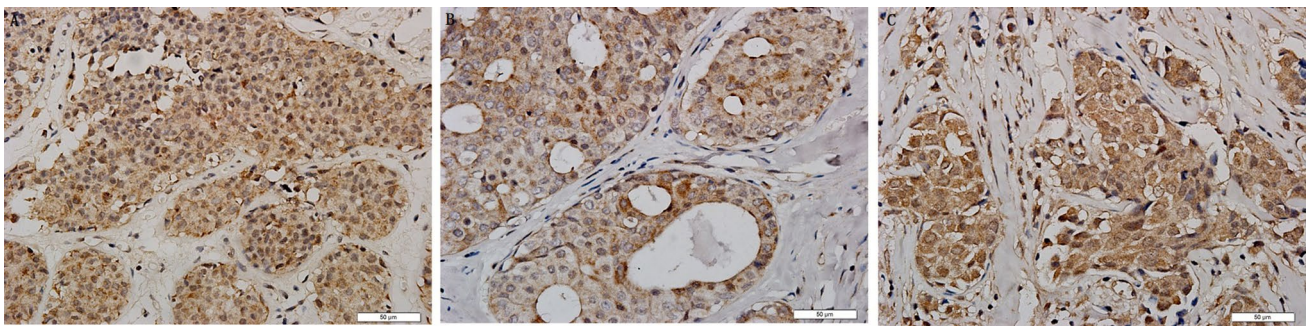

Fig. 2 Representative staining images of BMP-2, p-AKT, and p-mTOR in matched breast cancer tissues. **a–c** Shows the representative staining images of BMP-2, p-AKT, and p-mTOR, respectively

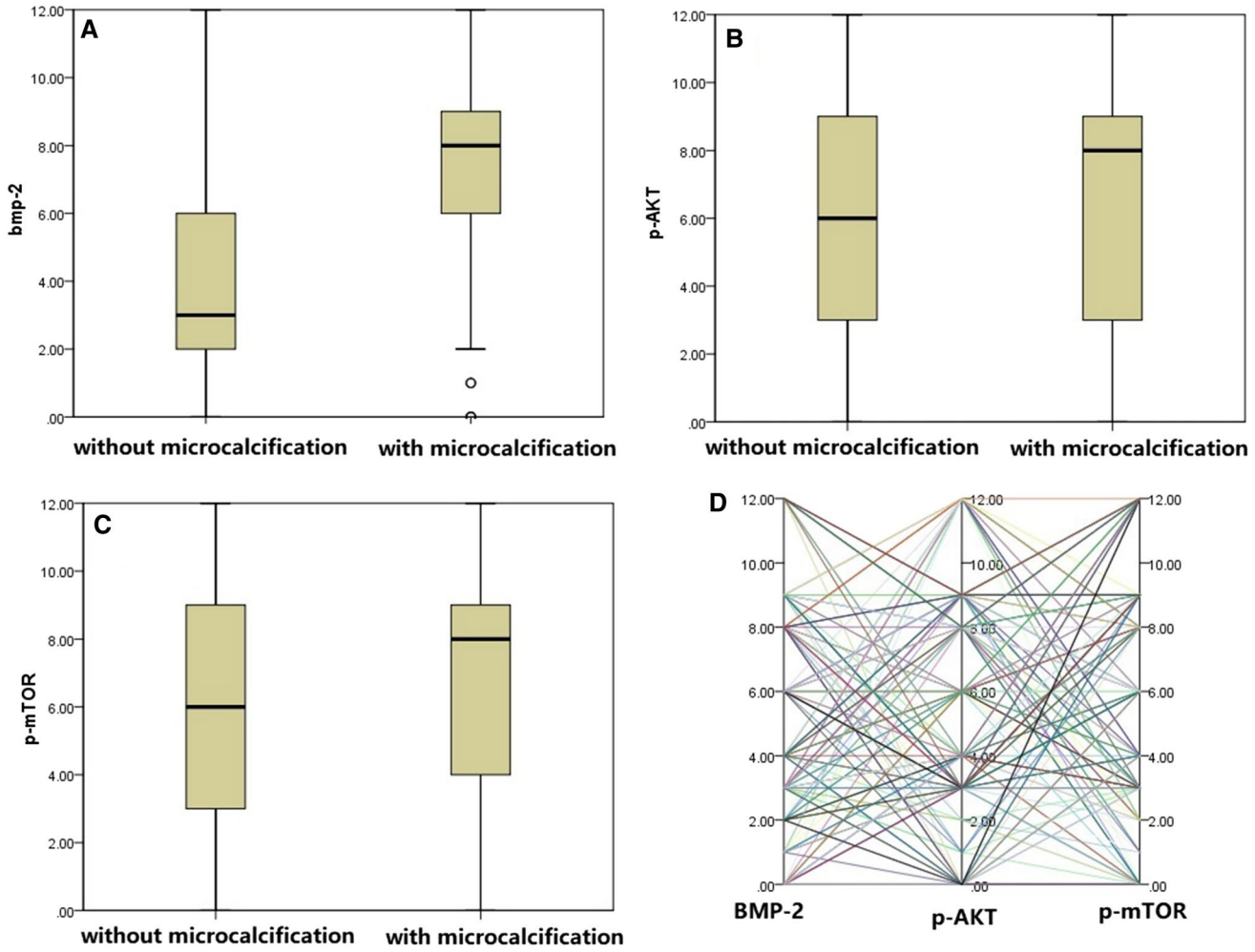

Fig. 3 The expression scores of BMP-2, p-AKT, p-mTOR and their distributions. **a–c** Shows that the expression scores' median values with interquartile ranges of BMP-2, p-AKT, and p-mTOR in breast cancer tissues with and without microcalcification. **d** Shows the distribution and correlation of BMP-2, p-AKT, and p-mTOR in breast cancer tissues

respectively. The microcalcification could not be regarded as an independent prognostic factor for breast cancer ($P = 0.881$).

Discussion

In the present study, we demonstrated that microcalcification was significantly correlated with poor prognosis in patients with invasive breast cancer ($P = 0.025$), which was consistent with the results of previous studies [2–9]. However, the formation of mammary microcalcification and its role in breast cancer remains largely unexplored. Previous study [28] has reported that cancers with calcification are more likely to have lymph node metastasis, while our study did not found significant correlation between microcalcification and lymph node metastasis. Moreover, we also found that microcalcification was correlated with HER-2 positivity, which was consistent with the previous studies [9, 29, 30]. It might be partly attributed to the correlation between microcalcification and poor prognosis.

Many studies have shown that BMP-2 may play an important role in the formation of microcalcification [10, 12, 13, 31]. On one hand, BMPs can increase the expression of transient receptor potential channel (TRPC), which may facilitate microcalcification by supplying Ca^{2+} [32]. On the other hand, they can induce EMT of cancer cells through the AKT pathway and Smad pathway [33–35], leading to transdifferentiation of EMT cells to osteoblast-like cells [10]. Previous studies have shown that BMP-2 can regulate the AKT pathway in osseous tissue [23–25] and gastric cancer [26, 27], while the relationship between BMP-2 and AKT still remains unknown in breast cancer. Furthermore, it is also unclear whether mTOR and AKT are regulated by BMP-2. To find the roles of these three factors in breast cancer with and without microcalcification and the relationship

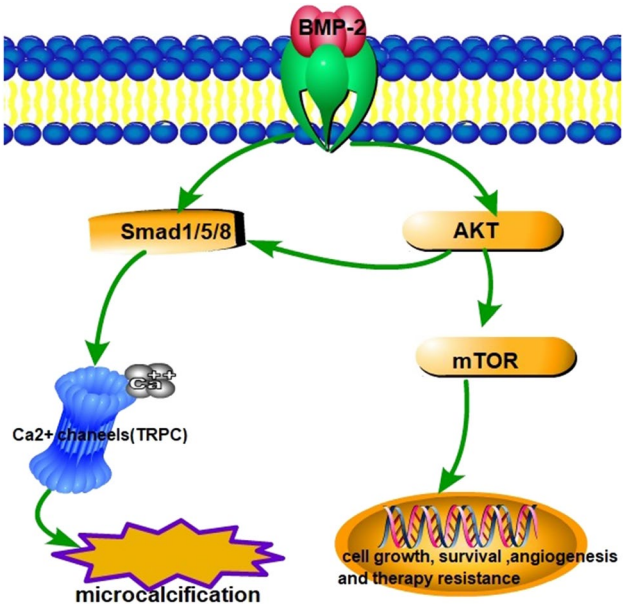

Fig. 4 Proposed molecular mechanism for BMP-2 in breast cancer tissues with microcalcification. On one hand, BMP-2 activated the Smad pathway and increased the expression of Ca^{2+} channel (TRPC), which might facilitate microcalcification by supplying Ca^{2+}. On the other hand, BMP-2 could upregulate the AKT/mTOR pathway, by which patients with microcalcification were more likely to resist targeted or endocrine therapy

among them, we performed the immunohistochemical staining to detect their expressions. We found that BMP-2 was significantly increased in tissues with microcalcification ($P=0.000$), which was consistent with the previous study [10]. We also demonstrated that the expression of p-mTOR was obviously increased ($P=0.026$) in tissues with microcalcification. As expected, these three factors also had a significant correlation with each other, indicating that the high expression of BMP-2 could upregulate the AKT/mTOR pathway and significantly increase the other two factors in breast cancer with microcalcification (Fig. 4). However, the AKT/mTOR pathway is regulated by multiple growth factors [17]. Therefore, further studies are required to clarify the deep relationship between BMP-2 and AKT/mTOR pathway.

Studies have shown that BMPs can promote invasion and migration of breast cancer cells [36–39] presumably by inducing EMT [33–35]. Recent literature has also indicated a positive association between serum BMP and cancer metastasis [40]. In our present study, we demonstrated that the high expression of BMP-2 was significantly associated with poor prognosis ($P=0.001$) and BMP-2 expression was significantly increased in tissues with microcalcification ($P=0.000$), which might partly explain the poor prognosis of breast cancer with microcalcification. The AKT/mTOR pathway has been shown to play a critical role in cell growth [17, 18]. Previous study has shown that mTOR is correlated with resistance to HER-2 therapies (trastuzumab) in breast cancer [19] and it has been demonstrated to be correlated with endocrine therapy resistance [20]. In our study, there was a significant association between the high expression of AKT/mTOR pathway and poor prognosis ($P=0.004$ and 0.013). We also found that the AKT/mTOR pathway was activated by BMP-2 in breast cancer with microcalcification as previously described. This finding indicated that breast cancer patients with microcalcification might be more likely to resist targeted or endocrine therapy due to the high expression of AKT/mTOR pathway. However, microcalcification could not be regarded as an independent prognostic factor of breast cancer in Cox regression analysis ($P=0.881$), which might be attributed to that there were some other mechanisms involved in the formation of microcalcification.

Conclusions

Collectively, microcalcification was a poor prognostic factor for breast cancer patients and BMP-2 might play an important role in the form of microcalcification. Moreover, BMP-2 was significantly increased in tissues with microcalcification, which could activate AKT/mTOR pathway at the same time. This finding might partly explain the correlation between microcalcification and a poor prognosis.

Compliance with ethical standards

Conflict of interest We declare that we do not have any commercial or associative interest that represents a conflict of interest in connection with the work submitted.

Ethical approval The experimental protocol was approved by the research ethics committee of First Affiliated Hospital, China Medical University.

Informed consent All patients involved in this study were aware of the study and signed informed consent forms.

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