ORIGINAL ARTICLE

Distinct JNK/VEGFR signaling on angiogenesis of breast cancer-associated pleural fluid based on hormone receptor status

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Abstract
Malignant pleural effusion is a common complication in metastatic breast cancer (MBC); however, changes in the pleural microenvironment are poorly characterized, especially with respect to estrogen receptor status. Histologically, MBC presents with increased microvessels beneath the parietal and visceral pleura, indicating generalized angiogenic activity. Breast cancer-associated pleural fluid (BAPF) was collected and cultured with HUVECs to recapitulate the molecular changes in subpleural endothelial cells. The clinical progression of triple-negative breast cancer (TNBC) is much more aggressive than that of hormone receptor-positive breast cancer (HPBC). However, BAPF from HPBC (BAPF-HP) and TNBC (BAPF-TN) homogeneously induced endothelial proliferation, migration, and angiogenesis. In addition, BAPF elicited negligible changes in the protein marker of endothelial-mesenchymal transition. Both BAPF-HP and BAPF-TN exclusively upregulated JNK signaling among all MAPKs in HUVECs. By contrast, the response to the JNK inhibitor was insignificant in Transwell and tube formation assays of the HUVECs cultured with BAPF-TN. The distinct contribution of p-JNK to endothelial angiogenesis was consequently thought to be induced by BAPF-HP and BAPF-TN. Due to increased angiogenic factors in HUVECs cultured with BAPF, vascular endothelial growth factor receptor 2 (VEGFR2) inhibitor was applied accordingly. Responses to VEGFR2 blockade were observed in both BAPF-HP and BAPF-TN concerning endothelial migration and angiogenesis. In conclusion, the above results revealed microvesel formation in the pleura of MBC and the underlying activation of p-JNK/VEGFR2 signaling. Distinct responses to blocking p-JNK and VEGFR2 in HUVECs cultured with...
Breast cancer is the second-leading cause of MPE. Approximately 10% of patients with BC have MPE. Triple-negative breast cancer refers to the subtype that lacks expression of the ER, progesterone receptor, and human epidermal growth factor receptor 2. The incidence of MPE is significantly higher in patients with TNBC, and statistical analyses have indicated a poor prognosis of BC patients with MPE with a mean survival of less than 1 year. In addition, massive pleural fluid collection significantly compromises the daily activity of patients with BC, with symptoms including dyspnea and chest pain. In identifying potential targets for clinical intervention, there is a growing focus on the pleural microenvironment due to the abundance of both growth factors to promote tumor growth and vascular structures for cancer cell seeding. Recently, our group observed an increased number of microvessels in the subpleural layer of lung adenocarcinoma. Nevertheless, it remains unclear how the distribution pattern of subpleural vessels presents in breast carcinoma with pulmonary metastasis.

The pressure gradient between the pleural space and subpleural interstitium leads to the production of pleural fluids. Pleural effusions due to an imbalance in fluid production are common in congestive heart failure, infection, and malignancy. There is emerging evidence that inflammatory, mesothelial, and endothelial cells in the pleural microenvironment interact with cancer cells to propagate MPE. Multiple vasoactive factors, including VEGF, tumor necrosis factor, chemokine ligand 2, and osteopontin, have been identified in MPE and correlated with aberrant pleural vasculature in experimental animal models. However, no reports have described the impact of MPE derived from BC on vascular endothelial cells at the molecular level.

Extensive neovascularization has been widely observed in the tumor microenvironment of BC and is considered an integral component of distant metastasis. The angiogenic response of endothelial cells is modulated by BC cells through VEGFR1 and VEGFR2. In MBC, antiangiogenic agents have been investigated in clinical trials with controversial outcomes, especially with TNBC. Additionally, treatment with chemotherapy and antiangiogenic agents shows benefits in BC complicated with MPE. Based on our research regarding MAPF in lung cancer, the application of VEGFR2 inhibitors could reverse the propensity of BC cells for endothelial migration and angiogenesis. In MBC, the characteristics of pleural endothelial cells and druggable targets have not been investigated. Accordingly, the present study aimed to evaluate the angiogenic effects of BAPF on endothelial proliferation, migration, and angiogenesis. By collecting pleural fluid samples from patients with either HPBC or TNBC, we hope to provide an insight into the influence of the pleural microenvironment from vascular endothelial cells on BC and explore underlying signaling changes as future therapeutic targets.

### 2 | MATERIALS AND METHODS

#### 2.1 | Collection of pleural fluid samples and patient characteristics

The study was approved by the institutional review board of the Tri-Service General Hospital Research Ethics Committee. Under sonography-guided thoracentesis, pleural fluid samples were obtained from patients with MBC who provided written informed consent. Characteristics of the included patients, such as cancer staging, hormone receptor status, and treatment course, were extracted from their medical records. A total of 5 mL pleural fluid was collected from each patient. Fresh samples were immediately centrifuged at 1000 rpm for 15 minutes and filtered through a Millipore filter (0.22 μm) to obtain a cell-free specimen. All samples were stored at ~80°C until experimental use.

#### 2.2 | Hematoxylin-eosin staining

Pleural tissues were fixed in 10% formalin, embedded in paraffin, and sectioned at a thickness of 6 μm on a microtome. The paraffin sections were deparaffinized and stained with H&E in a standard manner to demonstrate the general tissue morphology.

#### 2.3 | Culture of primary endothelial cells

Human umbilical vein endothelial cells were purchased from the Bioresource Collection and Research Center (Taiwan) and cultured in endothelial cell medium (ScienCell Research Laboratories).

#### 2.4 | Drugs and reagents

Sunitinib and MTT were purchased from Sigma-Aldrich. SP600125, a JNK inhibitor, was dissolved in DMSO and purchased from Cayman Chemical.
2.5 | Cell survival assay

Human umbilical vein endothelial cells were plated at a density of $2 \times 10^4$ cells per well in a 96-well plate. Breast cancer-associated pleural fluid was then added to the culture medium for 24 hours. After the cells were washed with PBS (137 mmol/L NaCl, 2.7 mmol/L KCl, 1.5 mmol/L KH$_2$PO$_4$, and 8 mmol/L Na$_2$HPO$_4$; pH 7.4), 0.5 mg/mL MTT was added, and the plates were incubated for another 4 hours. The cells were then lysed with DMSO (Sigma). The absorbance at 590 nm was measured in each well.

2.6 | Wound healing and Transwell assays

Wound healing and Transwell assays were carried out to evaluate HUVEC mobility. After HUVECs reached 100% confluency, a scratch was created with a P200 pipette tip. The cells were cultured in the presence of 30% BAPF (v/v) for 6 hours and then photographed. Changes in the width of the wound area were analyzed using ImageJ.

For the Transwell assay, HUVECs were seeded in the upper chamber of a Transwell plate (Corning Costar) at a density of $2 \times 10^4$ cells per well. After the cells were treated with BAPF for 16 hours, those that migrated to the lower chamber were fixed with 10% formalin, washed with PBS, and stained with Coomassie brilliant blue G250 (Sigma). The migrated cells in five randomly selected fields from each membrane were examined; six independent experiments were carried out.

2.7 | Tube formation assay

The 96-well plates were prechilled, and 50 μL Matrigel was added to each well and incubated for 1 hour at 37°C. Then HUVECs were seeded at a density of $1 \times 10^4$ cells per milliliter of medium containing 30% BAPF (v/v). After a 12-hour incubation, tube formation was photographed, and the lengths of the capillary networks were quantified using ImageJ.

| TABLE 1 | Disease characteristics of breast cancer patients |
| --- | --- |
| **Sex** | Female 14 (100) |
| **Age, y** | ≥65 6 (43) |
| **ECOG performance status** | 0-2 4 (29) ≥3 10 (71) |
| **Disease stage at diagnosis (AJCC 8th)** | IV 7 (50) Hormone positive 9 (64) ER positive 9 PR positive 8 HER2 positive 6 Triple negative 5 (36) |
| **Presence of PE** | At diagnosis 6 (43) Following disease progression 8 (57) |
| **Microscopic malignant cell in PE** | Presence 13 (93) |

Data are shown as n or n (%). Abbreviations: AJCC, American Joint Committee on Cancer; ER, estrogen receptor; HER2, human epidermal growth factor receptor 2; PE, pleural effusion; PR, progesterone receptor.
2.8 | Immunofluorescence staining

Human umbilical vein endothelial cells were seeded on coverslips and incubated in the presence of BAPF for 24 hours. Cells were then rinsed with PBS and fixed with 10% formalin in PBS (pH 7.4). A blocking solution (5% milk in 0.1% Triton X-100) was applied to prevent nonspecific binding. Primary Ab against p-paxillin (BD Biosciences) in blocking buffer was incubated with the HUVECs at 4°C overnight. After the Ab was washed, the slides were incubated with phalloidin (for F-actin staining) and FITC-conjugated goat anti-mouse IgG (both from Sigma-Aldrich) for 1 hour. 4’-6-Diamidino-2-phenylindole was used for nuclear staining. Finally, the cells were mounted with mounting medium (Gel Mount Aqueous; Sigma) and photographed with a Nikon D1X digital camera (Carl Zeiss). The same protocol was carried out with a primary Ab targeting Ki-67 (Genetex) in place of the p-paxillin Ab to quantify proliferative activity.

2.9 | Western blot analysis

Human umbilical vein endothelial cells were rinsed once with PBS and lysed with buffer containing 0.15% Triton X-100, 10 mmol/L EGTA, 2 mmol/L magnesium chloride, 60 mmol/L piperazine-N,N’-bisis(2-ethanesulfonic acid), 25 mmol/L HEPES, 1 mmol/L sodium fluoride, 2.5 mmol/L sodium pyrophosphate, 1 mmol/L PMSF, 1 mmol/L sodium orthovanadate, 1 mmol/L β-glycerophosphate, 1 µg/mL leupeptin, 1 µg/mL pepstatin A, and 1 µg/mL aprotinin (pH 6.9). Forty micrograms of each sample was loaded in the wells of 10% SDS polyacrylamide gel, electrophoresed, and then transferred to a nitrocellulose membrane (Bio-Rad). The membranes were incubated with primary Abs in TBST (150 mmol/L sodium chloride, 50 mmol/L Tris base, and 0.1% Tween-20; pH 7.4) overnight at 4°C. The primary Abs included rabbit Abs specific for GAPDH, FAK, integrin β1, p-JNK, JNK, p-ERK, ERK, p-p38, p38, p-MLC, MLC, p-AKT, AKT, VE-cadherin, Snail, and VEGFR2 (Cell Signaling Technology), CD31.

**Figure 2** Increases in endothelial viability, motility, and angiogenesis induced by breast cancer-associated pleural fluid (BAPF). BAPF was obtained from breast cancer patients with complications related to massive pleural effusion who underwent sonography-guided thoracentesis. HUVECs were incubated with medium supplemented with BAPF from hormone receptor-positive breast cancer (BAPF-HP) or triple-negative breast cancer (BAPF-TN), or control medium (CTL) at the indicated times. A, MTT assay to measure cell viability after 24 h of treatment. B, Ki-67 immunostaining (green) was used to detect proliferative activity after 24 h of treatment. Nuclei were stained with DAPI (blue). Arrowheads indicate cells undergoing mitosis. C, HUVECs were seeded in the upper Transwell chamber. After an 18-h incubation in the presence or absence of BAPF, the number of cells in the lower chamber was stained and counted. Bar graphs show the quantification of the migration rate. D, Representative images show the tube formation ability of HUVECs cultured in the presence or absence of BAPF for 12 h. Arrows indicate tube thickness. Bar graphs indicate tube width. Values are expressed relative to those of the control groups. ****P < .0001 compared to the CTL group.
CD34, and VEGFR1 (Abcam), and mouse Abs specific for VEGF-A (Abcam), p-FAK, p-paxillin, paxillin, N-cadherin, and β-catenin (BD Biosciences). After the membranes were washed, the strips were incubated with a 1:5000 or 1:10 000 dilution of HRP-conjugated anti-rabbit or anti-mouse IgG (Cell Signaling Technology). Next, the blots were treated with an electrogenerated chemiluminescence substrate developing solution (Bio-Rad). Band densities were captured and quantified by densitometry using ImageJ. The control sample was set as 100%, and the test samples were expressed relative to their internal control.

2.10 | Statistical analysis

The collected data were expressed as the averages of at least triplicate samples and are presented as the mean ± SEM. P values were analyzed using Student’s t test, and a P value of less than .05 was considered to indicate statistical significance.

3 | RESULTS

3.1 | Observed abundance of microvessels in the subpleural layer of MBC

The pleural cavity consists of the inner visceral pleura and outer parietal pleura. The distribution of capillaries was assessed by H&E staining of the pleural tissue from MBCs (Figure 1). Both the parietal and visceral pleura presented with tumor cell invasion (Figure 1A, B). At 400x magnification, focal defoliation of mesothelial cells was observed, implying reactive changes in the pleura. Small blood vessels were abundant and scattered below the surface.
layer of the mesothelium, suggesting angiogenesis in the pleural microenvironment.

### 3.2 Propensity of BAPF to stimulate endothelial viability, motility, and angiogenesis through activation of focal adhesion proteins

Breast cancer-associated pleural fluid obtained from BC patients with MPE was cultured with HUVECs to investigate the angiogenic activity of the fluid. The clinical characteristics of the 14 MBC patients are summarized in Table 1. This study enrolled nine patients with HPBC and five patients with TNBC. Previous studies on BC have established distinct clinical courses and treatment algorithms for HPBC and TNBC. Accordingly, the following experiments analyzed the effects of BAPF obtained from both HPBC and TNBC. The MTT assay showed an estimated two-fold increase in endothelial viability of HUVECs treated with BAPF for 24 hours (Figure 2A). Immunostaining for the proliferative marker Ki-67 was enhanced in HUVECs cultured with BAPF for 24 hours compared to the control HUVECs; the arrows indicate endothelial cells undergoing mitosis (Figure 2B). There were no significant differences in endothelial viability between HPBC and TNBC. Along with increasing cell survival, BAPF could also stimulate HUVEC motility, as shown after 18 hours in the Transwell assays (Figure 2C). The tube formation assay was applied to mimic in vitro angiogenesis with the measurement of tube length, branchpoints, and thickness. After 12 hours of culture in the presence of BAPF, there were more viable endothelial cells in the view field that contributed to statistically significant increases in tube thickness compared with that in the control cells (Figure 2D). Comparatively, the changes in tube length and branch point were insignificant in the BAPF-treated cells (Figure S1). The ability to stimulate endothelial migration and angiogenesis was not significantly different between BAPF-HP and BAPF-TN.
Following the increased migration of cells cultured in BAPF, we further examined the expression of downstream proteins associated with migration. In HUVECs cultured with BAPF for 24 hours, the levels of both integrin β1 protein and phosphorylated components of focal adhesion complexes, including paxillin and MLC2, were upregulated (Figure 3A). Enhanced staining of p-paxillin and rearrangement of the cytoskeleton (F-actin) were observed within the lamellipodia protrusion of BAPF-cultured HUVECs (Figure 3B). In studies of tumor endothelial cells, the role of EndoMT has been associated with endothelial viability and motility, which are connected to cancer progression. The BAPF-cultured HUVECs showed increased viability and motility, whereas the expression of common EndoMT protein markers, such as VE-cadherin, N-cadherin, β-catenin, Snail, CD31, and CD34, did not differ between the control HUVECs and HUVECs treated with BAPF for 24 hours (Figure 3C). The above results suggest a distinct phenotype of HUVECs treated with BAPF from tumor endothelial cells. In conclusion, BAPF from both HPBC and TNBC patients initiated endothelial angiogenesis with increased cell viability and motility but no mesenchymal transformation.

3.3 Breast cancer-associated pleural fluid-induced upregulation of VEGFR1/VEGFR2/VEGF-A signaling and JNK phosphorylation

Vascular endothelial growth factor receptor 2 and its ligand VEGF are crucial in capillary tube formation. Compared with the control group, the BAPF-treated group showed significantly upregulated expression of the endothelial proteins VEGFR1, VEGFR2, and VEGF-A (Figure 4A). In the VEGFR2 signaling network relevant to angiogenesis, the phosphorylation of MAPK, PI3K/Akt, and PKC has been widely proposed in different models. In both the HPBC and TNBC subgroups, the levels of p-JNK but not of p-p38, p-ERK, p-Akt, or p-PKCα were significantly upregulated in cells cultured with BAPF (Figure 4B). Consistent with the phenotype of increased endothelial
angiogenesis, protein analysis revealed a general increase in angiogenic factors and sole increases in JNK signaling activation in cells treated with BAPF from HPBC and TNBC.

3.4 | Distinct response of p-JNK inhibitor in BAPF-induced endothelial motility and angiogenesis concerning hormone receptor status of patients with BC

Due to the increased JNK phosphorylation in HUVECs cultured with BAPF, SP, a p-JNK inhibitor, was used to evaluate its efficacy on BAPF-induced endothelial viability, motility, and angiogenesis. In the MTT assay, cotreatment with 10 μmol/L SP for 24 hours did not significantly suppress BAPF-induced endothelial viability in either the HPBC or TNBC subgroup (Figure 5A). In the Transwell and tube formation assays, JNK inhibition suppressed BAPF-induced endothelial migration and angiogenesis in the HPBC subgroup (Figure 5B, C).

Notably, the effect of BAPF derived from patients with TNBC was not compromised by cotreatment with SP (Figure 5B, C). Although increased p-JNK signaling was observed in the HPBC and TNBC subgroups, the above results suggest a differential contribution of JNK signaling in BAPF-induced HUVEC motility and angiogenesis related to hormone receptor status.

3.5 | Suppression of BAPF-induced endothelial viability, motility, and angiogenesis by VEGFR blockade in both HPBC and TNBC

Due to the upregulation of VEGFR1 and VEGFR2 protein in HUVECs cultured with BAPF, Sun was investigated as an inhibitor of VEGFR1 and VEGFR2 signaling in terms of endothelial viability, motility, and angiogenesis. When cultured with 10 μmol/L Sun for 24 hours, HUVECs showed a reduction in HPBC- and TNBC-derived BAPF by 25% and 10%, respectively (Figure 6A). Comparatively, the effect
of Sun on suppressing BAPF-induced endothelial proliferation was insignificant at 1 μmol/L (Figure S2A). In the Transwell and tube formation assays, the application of 10 μmol/L Sun significantly suppressed BAPF-induced endothelial motility and angiogenesis in the HPBC and TNBC subgroups (Figure 6B, C). At 1 μmol/L Sun, decreased endothelial motility was only observed in HUVECs treated with BAPF-HP (Figure S2B). These results suggest that Sun treatment could counteract the upregulation of endothelial angiogenesis by HPBC- and TNBC-derived BAPF (Figure 7).

4 | DISCUSSION

Our results highlight the first pathological description of increased subpleural capillaries in MBC. Previous studies regarding pleural effusion in MBC were mainly confined to poor prognosis and metastasis.23,24 To simulate the microvascular features of the pleural microenvironment of MBC, pleural fluid samples collected by thoracentesis were incubated with HUVECs to evaluate angiogenic activity. Compared with previous studies on MAPF in primary lung cancer, the present work interpreted BAPF as metastasis-related pleural effusion. Breast cancer-associated pleural fluid from a total of 14 patients with MBC ubiquitously induced proliferative, migratory, and angiogenic phenotypes in HUVECs. In the breast tumor microenvironment, tumor endothelial cells undergoing mesenchymal transition with increased N-cadherin and Snail signaling have been correlated with metastatic extravasation of tumor cells.25-27 In contrast, there were no significant changes in the expression levels of EndoMT protein markers in HUVECs cultured with BAPF. Despite their shared phenotype with tumor endothelial cells, subpleural endothelial cells were shown to exhibit differences in their molecular expression pattern, which provides a new understanding of these cells and hints at future therapeutic considerations.
Both HPBC and TNBC were found to possess distinct metastatic ability with specific signaling pathways. \(^{28,29}\) Despite the clinical heterogeneity in these two subtypes of BC, the differences between HUVECs cultured with BAPF-HP and BAPF-TN were insignificant in terms of proliferation, motility, and angiogenesis. Among all MAPKs, only p-JNK was upregulated in HUVECs cultured in either HPBC- or TNBC-derived BAPF. In MBC, p-JNK was found to promote tumor growth, chemotherapy resistance, and lung metastasis. \(^{30,31}\) Our findings of increased p-JNK levels in HUVECs cultured with BAPF implies a role of the subpleural endothelium in promoting tumor metastasis. In addition to increased JNK phosphorylation, p-p38 signaling was slightly downregulated in HUVECs cultured with BAPF. These findings have been observed in previous studies on colon cancer that the dominance of JNK signaling was negatively associated with p-p38 signaling concerning tumor angiogenesis. \(^{32}\) As for the insignificant change in p-ERK, there is speculation regarding the possible involvement of endogenous inhibitors, DUSPs, that specifically deactivate MAPK phosphorylation. \(^{33-35}\) However, the presence of DUSPs has not been examined in MPE and the expression pattern of 10 DUSPs in HUVECs remains to be determined. Whether endogenous activation of DUSPs could cause insignificant changes in p-ERK under BAPF culture in HUVECs requires further study. When the p-JNK inhibitor was applied, endothelial motility and angiogenesis were only suppressed in HUVECs cultured with BAPF-HP, but not BAPF-TN. However, targeting the JNK pathway has shown promising results in TNBC animal models. \(^{30,36}\) In BAPF-TN-mediated angiogenesis, less sensitivity to p-JNK inhibition in HUVECs cultured with BAPF-TN suggested the need to find a target other than JNK signaling.

Elevated expression of VEGFRs was identified in the tumor vasculature of BC, implying a potential benefit of antiangiogenic therapy. \(^{37}\) After HUVECs were cultured with BAPF, VEGFR1 and VEGFR2 protein expression were significantly upregulated. Targeting VEGFR1 and VEGFR2 signaling with 10 µmol/L Sun alleviated the increased endothelial viability, motility, and angiogenesis induced by HPBC- and TNBC-derived BAPF. In addition, 1 µmol/L Sun suppressed endothelial motility induced by BAPF-HP but not by BAPF-TN. Human umbilical vein endothelial cells cultured with BAPF-HP were more sensitive to VEGFR2 blockade than those cultured with BAPF-TN. Previous investigations of antiangiogenesis therapy have not shown definitive benefits for progression-free survival of MBC. \(^{19}\) but our results of a VEGFR2 inhibitor reducing the effects of BAPF-induced endothelial angiogenesis could be applied to develop a therapy for MBC complicated by MPE.

From pathological examinations of the visceral and parietal pleura, general microvessel formation was present in the subpleural layer of BC with lung metastasis. Both HPBC- and TNBC-derived BAPF possessed the ability to stimulate HUVEC viability, motility, and angiogenesis through upregulation of VEGFR2 and phosphorylated JNK. Only HUVECs cultured with BAPF-HP responded to the JNK inhibitor in terms of reducing endothelial motility and angiogenesis. The application of a VEGFR2 inhibitor efficiently reversed endothelial angiogenesis induced by HPBC- and TNBC-derived BAPF. The similarities and distinctions of HUVECs cultured with BAPF concerning the patients’ hormone receptor status could open the door for personalized therapy targeting VEGFR2 and p-JNK in the treatment of MBC.

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**CONFLICT OF INTEREST**

The authors declare that they have no conflict of interest.

**ETHICS APPROVAL AND CONSENT TO PARTICIPATE**

This study was approved by the institutional review board of the Tri-Service General Hospital Research Ethics Committee (No. B202005001). The samples were collected with informed consent.

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**SUPPORTING INFORMATION**

Additional supporting information may be found online in the Supporting Information section.

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