USP44<sup>+</sup> Cancer Stem Cell Subclones Contribute to Breast Cancer Aggressiveness by Promoting Vasculogenic Mimicry

Tieju Liu<sup>1,2</sup>, Baojun Sun<sup>1,2,3</sup>, Xiulan Zhao<sup>1,2</sup>, Yanlei Li<sup>1</sup>, Xueming Zhao<sup>1</sup>, Ying Liu<sup>1,2</sup>, Zhi Yao<sup>1</sup>, Qiang Gu<sup>1,2</sup>, Xueyi Dong<sup>1</sup>, Bing Shao<sup>1</sup>, Xian Lin<sup>1</sup>, Fang Liu<sup>1</sup>, and Jindan An<sup>1</sup>

Abstract

Vasculogenic mimicry (VM), a newly defined pattern of tumor blood supply, describes the functional plasticity of aggressive cancer cells that form vascular networks. In our previous study, breast cancer stem cells (CSC) were shown to potentially participate in VM formation. In this study, breast CSCs presented centrosome amplification (CA) phenotype and ubiquitin-specific protease 44 (USP44) upregulation. USP44 expression contributed to the establishment of bipolar spindles in breast CSCs with supernumerary centrosomes by localizing at pole-associated centrosomes. The bipolar spindle patterns of breast CSCs with CA, including planar-like and apico-basal-like, functioned differently during the VM process of CSCs. Moreover, the ability of transendothelial migration in VM-forming cells was increased. In vivo experiment results showed that CSC xenografts presented linearly patterned programmed cell necrosis, which provided a spatial foundation for VM formation as well as angiogenesis. Breast CSCs further showed increased levels of IL6 and IL8. However, USP44 silencing induced spindle multipolarity, abated VM, reduced transendothelial migration, and consequently decreased IL6 and IL8 levels in breast CSCs. Finally, USP44<sup>+</sup> CSC subclones (ALDH<sup>1<sup>+</sup></sup>/USP44<sup>+</sup>/IL6/<sup>+</sup>/IL8<sup>-</sup>) were identified in breast cancer specimens through consecutive sections scanning. The subclones were related not only to CA, but also to VM. Statistical analysis suggested that USP44<sup>+</sup> CSC subclones could be used as an independent prognostic biomarker of poor clinical outcomes in patients with breast cancer. Collectively, the identification of USP44<sup>+</sup> CSC subclones may contribute to the prediction of VM formation and aggressive behavior. This study provides novel insights into the therapy for advanced breast cancer.

Introduction

Cancers are heterogeneous and hierarchically organized entities. Although all tumor cells contain shared somatic mutations that reflect their clonal origin (“founding clone”), additional mutations in cell subpopulations may generate tumor subclones (1). Many cancer subclones are eliminated during therapy, but a few subclones may survive, resulting in cancer relapse and metastases. As clonal genetic events in metastases occur in the restricted subclone of primary medulloblastoma, only rare cells within the primary tumor can metastasize (2). Studies revealed that cancer stem cells (CSC) comprise the top of the tumor cell hierarchy and can be isolated from leukemias and revealed that cancer stem cells (CSC) comprise the top of the tumor cell hierarchy and can be isolated from leukemias and...
The role of USP44 in CSCs and cancer aggressiveness remains unclear.

**Materials and Methods**

**Cell and mammosphere culture**

MDA-MB-231, MCF-7, T47D, ZR-75-1, and HUVEC cells were cultured in RPMI1640 medium supplemented with 10% FBS (Invitrogen). MDA-MB-231 and MCF-7 cell lines were obtained from the ATCC in 2012 and authenticated using short tandem repeat (STR) analysis by Genewiz Inc. in 2014. STR analysis showed that the submitted samples were in good agreement with the reference cell lines. T47D and ZR-75-1 cells were provided by the Tumor Center, Chinese Academy of Medical Sciences (Beijing, China) in 2015. HUVEC cells were provided by KeyGEN BioTECH, and the karyotype was authenticated. The cells were passaged in our laboratory for less than 6 months after receipt.

For mammosphere formation, single-cell suspension at a density of 40,000 cells per milliliter was placed in DMEM/F-12 (1:1; Gibco) containing 2% B27 (Gibco), 0.5% EGF (Pepro Tech), and 0.5% basic fibroblast growth factor (Pepro Tech). The suspension was then seeded into 6-well plates (2.5 mL per plate) or 100 mm tissue culture dishes (10 mL per dish) coated with 1.2% poly 2-hydroxyethyl methacrylate (Sigma Aldrich). The cultures were fed weekly and passaged every 2 weeks.

**Lentiviral transduction**

The pEZ-Lv151 vector was used for gene transfer of MCF-7 cells to overexpress AURKA (GeneCopoeia), and the psiHIV-U6 vector (GeneCopoeia) was used for USP44 silencing in mammosphere-derived MCF-7 AURKA (MDMA) cells. The shRNA target sequences for USP44 was (5’-AACCTGGAACCTGAACTGTA-3’). Lentiviruses were produced by transient transfection of 293T cells, and the virus suspension was used to infect the target cells.

**Three-dimensional cultures**

The method was referenced to the literature (13, 16).

**Flow cytometry analysis**

A total of 1 × 10^6 cells were resuspended in 500 μL PBS and chilled on ice. A 5-mL tube containing 500 μL ice-cold 100% ethanol was prepared. The cold cell suspension was immediately pipetted into cold ethanol, mixed by forcing air bubbles through the suspension, and placed on ice for 15 minutes. After centrifugation, the liquid was carefully aspirated and then washed with PBS three times. The blocking buffer of 5% normal goat serum in PBS three times. The blocking buffer of 5% normal goat serum in PBS three times. The blocking buffer of 5% normal goat serum in PBS three times. The blocking buffer of 5% normal goat serum in PBS three times. The blocking buffer of 5% normal goat serum in PBS three times.

**Western blot analysis**

The methods were referenced to the literature (17), and the primary antibody (USP44, 1:500; and AURKA 1:2,000) was used.

**Measurement of angiogenic proteins with a human angiogenesis array kit**

The experiment was performed according to the manufacturer’s instructions (Ray Biotech).

**Xenograft**

The care and use of laboratory animals used in this study followed the guidelines established by Tianjin Medical University (Tianjin, China). Five-week-old non-ovariectomized female BALB/c nude mice were injected subcutaneously with 5 × 10^6 cells suspended in 100 μL of PBS. Tumor volume was monitored weekly using digital calipers and calculated using the following formula: \( V = \frac{1}{2} \times a \times b^2 \) (where \( a \) is the length and \( b \) is the width of tumor). After 4 weeks, mice were sacrificed, and xenograft tumors were processed for histology and immunohistochemical (IHC) analyses.

**Tissue specimens**

The procedures for tissue collection and analysis were approved by the Ethical Committee of Tianjin Medical University (Tianjin, China). All cases of breast surgical specimens, both formalin-fixed and paraffin-embedded, were anonymized after collection from the archival files of Pathology Department, Tianjin Medical University (Tianjin, China). Ninety-two cases of invasive ductal carcinoma from 1997 to 2005 were randomly selected. The pathologic diagnosis was counterchecked by two senior pathologists according to the 2003 WHO histological classification of breast tumors.

**Immunohistochemistry and histochemical double-staining methods**

Information on these staining methods may be referenced to the literature (13, 16). The primary antibodies of ALDH1 (LifeSpan BioSciences), USP44 (Abcam), vascular endothelial-cadherin (VE-cadherin, Abcam), IL6 (Abcam), IL8 (Abcam), endomucin (Abcam), and CD31 (Beijing Zhongshan Biotechnology Limited Company, China) were applied to the sections.

**Consecutive section scanning**

The C9600 NanoZoomer digital pathology scanner was used to scan consecutive sections of 92 breast cancer specimens at 40 × magnification.
Collecting mitotic cells
Mitotic cells of MCF-7, MDMA, and MDMA shUSP44 cells on 3D Matrigel in late stage of MDMA VM formation were selected by placing T75 tissue culture flasks on a shaker tray. The tray was placed in an incubator at 37°C and shaken for 30 seconds to 1 minute at 150 to 200 rpm. The medium containing mitotic cells was then collected in centrifuge tubes and placed on ice. The prewarmed medium was added to the flasks and incubated for 10 minutes. The above procedures were repeated to collect a sufficient number of mitotic cells.

Tumor cell transendothelial migration assay
Tumor cell transendothelial migration assay was performed using a modified Transwell chamber system, which is a two-chamber system with a porous membrane to provide an interface between the two chambers. Endothelial cells were cultured on top of the porous membrane coated with Matrigel. A total of 1 × 10^5 endothelial cells in 250 µL medium were added to each chamber. The endothelial cells formed a monolayer (the cells should be >95% confluent) after 48 hours. The mitotic cells of MCF-7, MDMA, and MDMA shUSP44 cells on 3D Matrigel were selected during the late stage of MDMA VM formation. A total of 2.5 × 10^5 mitotic cells in 250 µL medium were added above the endothelial monolayer. The invasion of tumor cells across the endothelium was determined by measuring the number of cells that migrated to the bottom chamber. Five random fields were counted under a light microscope after staining with 0.4% crystal violet.

Statistical analysis
Data analysis was performed with the SPSS16.0 software package. All P values were two-sided, and statistical significance was set at P = 0.05.

Results
Breast CSCs display CA phenotype
The centrosome-associated kinase AURKA was exogenously expressed in MCF-7, T47D, and ZR-75-1 cells to induce super-numerary centrosomes (Supplementary Figs. S1A and S2A). Based on the criteria of Moskvitzky and colleagues (18) with minor modifications, we considered cells containing one or two centrosomes as the normal centrosome phenotype and those containing more than two centrosomes as the CA phenotype. The presence of CA (Supplementary Figs. S1B and S2B) was observed in (10.8% ± 0.1%) MCF-7_AURKA, (10.1% ± 0.2%) T47D_AURKA, and (10.9% ± 0.2%) ZR-75-1_AURKA, whereas a regular number of centrosomes were observed in the parental MCF-7, T47D, and ZR-75-1 cells. Through flow cytometry analysis, the breast CSC marker ALDH1 displayed an increased expression in MCF-7_AURKA (15.6% ± 0.7%), T47D_AURKA (12.1% ± 0.4%), and ZR-75-1_AURKA (18.6% ± 0.6%) compared with MCF-7 (5.2% ± 0.2%), T47D (4.7% ± 0.2%), and ZR-75-1 cells (4.1% ± 0.1%) (Supplementary Figs. S1C, S1D, S2C, and S2D). Mammosphere culture has been widely used for breast CSC enrichment (19). The highest ALDH1 + population presented in MDMA (35.4% ± 0.8%) compared with MCF-7_AURKA and MCF-7, similarly in mammosphere-derived T47D_AURKA (MDTA; 17.7% ± 1.5%) and mammosphere-derived ZR-75-1_AURKA (MDZA) cells (30.4% ± 0.4%) (Supplementary Figs. S1C, S1D, S2C, and S2D). In accordance with the ALDH1 + subpopulation enrichment, the number of cells with CA was the highest in MDMA (38.3% ± 1.8%), similarly in MDTA (19.5% ± 1.0%) and MDZA (21.5% ± 0.8%) (Supplementary Figs. S1B, S1D, S2B, and S2D). These findings suggested that breast CSCs displayed the CA phenotype.

USP44 promotes breast CSCs with CA to form a bipolar spindle
In MCF-7 cells without CA, 98% of the cells (n = 390 cells) formed a bipolar spindle in the metaphase and both centrosomes were associated with spindle poles (Fig. 1A). Surprisingly, 96% MDMA (n = 400 cells) with extra centrosomes also formed a bipolar spindle in the metaphase. In addition to the two centrosomes that contributed to bipolar spindle establishment, we also observed centrosomes that were not associated with either spindle pole in MDMA cells (Fig. 1B). In the metaphase of MDMA cells, non–pole-associated centrosomes were usually not associated with the robust asters of microtubules. Hence, non–pole-associated centrosomes did not function during bipolar spindle formation. Similar results were observed in MDA-MB-231 cells with CA (Fig. 1C).

In accordance with increased AURKA level, upregulated USP44 expression was observed in MDMA, MDTA, MDZA, and MDA-MB-231 cells (Figs. 1A–D and Supplementary Fig. S2A). Importantly, in the metaphase and anaphase of MDMA and MDA-MB-231 cells, the positive USP44 signal was located in the pole--associated centrosomes (Fig. 1B and C). Although the USP44 punctate pattern was present in the adjacent area of non–pole-associated centrosomes (Fig. 1B and C), no colocalization was observed in non–pole-associated centrosomes and USP44 (Fig. 1B and C). Hence, in cells with CA, USP44 exhibited potential to promote bipolar spindles by localizing at pole-associated centrosomes.

USP44 + CSCs exhibit a distinct bipolar spindle phenotype that may contribute to VM
In accordance with our previous study (13), the current study showed that MDMA with the characteristics of CSCs formed tubular VM channels. However, the channels were undetected in MCF-7 cells (Fig. 2A). During the VM process, USP44 expression contributed to establish bipolar spindles in MDMA cells with CA (Fig. 2A–D). We observed two patterns of bipolar spindle. The first pattern was planar-like in epithelial tissues, and the polarity axis was aligned along the VM channel wall direction (Fig. 2B and C), parallel to the apical surfaces of the cells, thereby generating daughter cells located side by side. The second pattern was apico-basal–like pattern in the epithelia, in which the spindle axis was perpendicular to the VM channel wall (Fig. 2D). The planar-like pattern (15.7 ± 0.3, n = 20 mitotic figures) was frequently observed compared with the apico-basal–like pattern (4.3 ± 0.3, n = 20 mitotic figures) during the early stage (less than 3 days) of VM formation when few cells were present in the VM tubular structure (Fig. 2C and E). However, during the late stage of VM formation (more than 3 days), the amount of apico-basal–like pattern increased (7.7 ± 0.3, n = 20 mitotic figures) compared with that during the early stage (Fig. 2D and E). With apico-basal–like polarity establishment, one daughter cell would stay in the VM channel and the other daughter cell would be detached from the VM channel wall and flowed into the lumen of VM (Fig. 2A, black arrows).
After endothelial cells formed a monolayer (Fig. 2F) on the porous membrane coated with Matrigel in Transwell chamber, mitotic cells of MCF-7 and MDMA cells in the late stage of MDMA VM formation were collected and added above the endothelial monolayer. The results showed that the transendothelial migration ability of mitotic cells during the VM formation in MDMA significantly increased compared with that in MCF-7 cells (Fig. 2G). Hence, floating cells from the VM channels of MDMA cells could penetrate the endothelium and serve as the source of distant metastasis.

USP44 silencing in CSCs induces spindle multipolarity and VM formation inhibition in vitro

A stable USP44 knockdown (KD) in MDMA cells was generated using lentiviral shRNA (Fig. 2H). USP44 KD induced multipolar cell division that arrested in mitosis and succumbed to death. Approximately 4% of control MDMA contained multipolar spindles, whereas USP44 KD resulted in higher than 17% of the cells displaying multipolarity (Fig. 2I and J). Following USP44 KD and induction of multipolar spindles, VM channel formation was inhibited on the 3D Matrigel (Fig. 2K). The transendothelial migration ability was remarkably impaired, and the average number of penetrated cells in shUSP44 MDMA decreased by higher than 2-fold compared with that in MDMA cells (Fig. 2G). Meanwhile, the ALDH1+ subpopulation decreased in MDMA shUSP44 cells (Fig. 2L).

Breast CSC xenografts promote tumor growth and enhance VM, whereas USP44 silencing inhibits VM formation of CSCs in vivo

Following subcutaneous transplant of MCF-7, MCF-7 AURKA, MDMA, and MDMA USP44 KD cells into nude mice, xenografts in MDMA showed a significantly higher rate of tumor growth than the three other cell types and USP44 KD (P < 0.05) retarded tumor growth compared with nontarget MDMA in vitro (Fig. 3A). Endomucin/periodic acid–Schiff (PAS) double staining was performed to identify VM in xenografts (Fig. 3B). VM formation was negative in the MCF-7 group (0/10, 0%), a high percentage of VM presented in MCF-7 AURKA (3/10, 30%), and strikingly all MDMA xenografts harbored VM formation (10/10, 100%). However, the VM percentage considerably (P < 0.05) decreased in MDMA with USP44 KD (2/10, 20%; Fig. 3C).

In accordance with the results of in vitro 3D Matrigel culture, the part of the mouse blood vessel wall in MDMA xenografts was lined with endomucin-negative cancer cells. Some cancer cells had fallen away from the vessel wall and were floating in the blood (Fig. 3D). The cancer embolus present in blood vessel was also identified in MDMA xenografts (Fig. 3E). Similar to VM channels formation, VM marker vascular endothelial-cadherin (VE-cadherin) exhibited attenuated expression in USP44 KD xenografts (Fig. 3F) compared with that in MDMA controls (Fig. 3G).

Meantime, low USP44 expression was verified in USP44 KD xenografts (Fig. 3H), whereas USP44+ was present in MDMA controls (Fig. 3I). Further observation demonstrated that in MDMA xenografts, most population of USP44+ cancer cells was located in the neighboring area of necrosis (the hypoxic tumor area; Fig. 3J). Moreover, a specific form of tumor cell death, named as linearly patterned programmed cell necrosis.
LPPCN), which was identified in our previous work (20), was present within the USP44+ population. LPPCN distribution was similar to the blood vessel networks and anastomosed with the authentic blood vessel (Fig. 3K and L), thereby suggesting that this necrotic LPPCN cell death provided spatial foundation for VM formation and angiogenesis (Fig. 3M). However, in MDMA USP44 KD xenografts without VM formation, no LPPCN pattern was observed.

Figure 2.
The function of USP44+ CSCs in the VM process. A, VM formed by MDMA cells with USP44+ expression, whereas MCF-7 did not have the ability of VM formation (black arrows indicate cells detached from VM channel wall and flowed into the lumen of VM; magnification, 100×). B and C, the planar-like spindle pattern in the VM channel wall (white arrows indicate the bipolar spindle established by CSCs with CA; white double-headed arrows indicate the polarity axis aligned along the VM channel wall; magnification, 400×). D, the apico-basal-like pattern in the VM channel wall (red double-headed arrows indicate the polarity axis perpendicular to the VM channel wall; magnification, 600×). E, the counts of planar-like and apico-basal-like spindle during the early and late stages of the VM process. F, endothelial cells formed a monolayer on the porous membrane coated with Matrigel in Transwell chamber (magnification, 100×). G, considerable increase in the transendothelial migration ability of mitotic cells from VM formation of MDMA. Significant differences were observed in the number of penetrating cells in MCF-7, MDMA, and shUSP44 MDMA cells (P < 0.05; magnification, 100×). H, USP44 KD at the protein level was achieved by USP44 shRNA compared with the control. I, following USP44 KD, the percentage of cells with multipolar spindle formation increased in MDMA cells. J, cancer cells exhibited severely high-grade multipolarity (left). Right, multipolar cells of the same line succumbed to mitotic catastrophe (magnification, 600×). K and L, VM formation was inhibited, and the ALDH1+ subpopulation decreased in MDMA cells following USP44 KD.
USP44 silencing inhibits the secretion and expression of IL6 and IL8 in breast CSC xenografts.

To determine whether the enhanced VM formation ability in MDMA was related to the endogenous angiogenic factors, we performed human angiogenesis array using the cell culture supernatant from MDMA and MCF-7 cells on Matrigel. Higher levels of IL6 and IL8 were secreted in MDMA cells than that in MCF-7 cells (Supplementary Figs. S3A and S3B). Similar to in vitro results, the secreted IL6 and IL8 expression was higher in MDMA xenografts. Remarkably, following USP44 KD, the levels of IL6 and IL8 considerably decreased (Supplementary Figs. S3C–S2F).

Breast cancer specimens with USP44+ CSC subclones show VM and CA

The NanoZoomer digital pathology scanner was used to scan consecutive sections of 92 breast cancer specimens with ALDH1, USP44, IL6, and IL8 IHC staining at 40× magnification to identify CSC subclones. Figure 4A shows the representative overview of the entire specimen. As the scans were synchronized, when we move or zoom in one scan, the other open scan will also follow the same command. The scans clearly showed the distribution of ALDH1, USP44, IL6, and IL8 in successive sections of breast cancer tissues. ALDH1- and USP44-positive signals were located in the cancer cell cytoplasm (Fig. 4B). The IHC results of 92 breast cancer tissues showed a moderate to strong ALDH1 and USP44 expression in 42 (45.7%) and 40 (43.5%) cases, respectively. A correlation was observed between USP44 and ALDH1 expression ($r = 0.825; P = 5.12E−24, P < 0.05$, respectively). The same breast cancer subpopulation with ALDH1+ showed USP44+ by consecutive tissue section observation (Fig. 4B). IL6 and IL8 were found to be secreted and expressed in the surrounding area of USP44+ cells (Fig. 4C). In cancer patients, high levels of IL6 and IL8 were related to breast CSC self-renewal and tumor growth, as well as with poor patient outcome (21). Meanwhile, in preclinical models, IL6 promoted tumorigenesis, angiogenesis, and metastasis (22). Therefore, we identified the area containing at least 100 cancer cells with ALDH1+/USP44+/IL-6+/IL-8+ as the USP44+ CSC subclone characteristic. About 37 of 92 (40.2%) patients with breast cancer harbored USP44+ CSC subclones.
CD31/PAS double staining has been widely used to identify VM in tumor tissues (6, 13, 16, 17). The VM channels morphologically varied from large to small tubular structures, and the cells forming VM showed negative staining for CD31 and positive staining for cytokeratin (CK; Fig. 5A–D), indicating that these cells were cancer cells in nature. The VM channels were confined in areas in close proximity to CD31+ blood vessel (Fig. 5B), thereby suggesting that blood circulation insolated between VM and authentic blood vessel. Although the morphology of some VM channels was similar to the gland structure in breast cancer tissues, the following differences were observed between them: (i) PAS-positive substances deposited in the VM channel wall (Fig. 5B, green arrowhead), which mimicked the genuine blood vessel wall appearance (Fig. 5C, black arrowhead indicates genuine blood vessel, green arrowhead indicates PAS-positive substances in the BV wall). PAS-positive substances in VM were either continuous or intermittent, and no PAS-positive substances were deposited in the cancer glands wall (Fig. 5C, blue arrowhead) (ii) VM was identified by the presence of red blood cells in the vessels and the absence of necrosis and inflammatory cells infiltrating around the channels. Meanwhile, no erythrocyte was observed in the lumen of cancer glands and the cancer glands may be accompanied with necrosis and infiltrating inflammatory cells; (iii) strong staining in the cellular membrane for CK was detected in cancer gland cells (Fig. 5E), whereas cells forming VM showed weak positive staining for CK (Fig. 5D).

VM was detected in 24 (26.1%) of 92 breast cancer specimens. Further statistical analysis demonstrated that VM was positively associated with USP44+ CSC subclones in breast cancer specimens (P = 2.05E−6, P < 0.05). More patients with USP44+ CSC subclones displayed VM (51.4%, 19/37), whereas VM was present in only 9.1% (5/55) cases without USP44+ CSC subclones (Fig. 5E, Table 1). We also demonstrated that CSC subclones coincided with the VE-cadherin+ cancer cell fractions through successive tissue section observation (Fig. 4C). Our results showed that cancer cells with CA could also be found within the VM structures (Fig. 5F). CA (Fig. 5G and H) was present in 36 (39.1%) of 92 breast cancer specimens and had significant relationship to USP44+ CSC subclones (P = 1.45E−6, P < 0.05, Table 1).

USP44+ CSC subclones are an independent prognostic biomarker of poor clinical outcome

Analysis of clinical factors and their association with USP44+ CSC subclones was conducted through binary logistic regression (Table 1). The clinicopathologic data in patients with and without USP44+ CSC subclones were compared. Among all factors, nodal status, differentiation grade, tumor stage, and Her2 status significantly differed (P < 0.05) and the difference was independent of the other factors. Lymph node metastasis, higher grade and clinical stage, and positive Her2 status were correlated with USP44+ CSC subclones, whereas no correlation was found between USP44+ CSC subclones and patient age, tumor size, and ER/PR status.

Survival analysis through Kaplan–Meier method indicated that patients with breast cancer with USP44+ CSC subclones were significantly associated with poor overall survival (OS) and disease-free survival (DFS; Fig. 5I and J; P = 1.72E−12, OS; P < 0.05 and P = 1.57E−10, DFS; P < 0.05). The mean [95% confidence interval (CI)] OS and DFS periods were 70.298 (61.510–79.086) and 53.206 (45.624–60.788) months, respectively, for patients with USP44+ CSC subclones; meanwhile, for patients without USP44+ CSC subclones, the mean OS and DFS periods were 117.552 (109.561–125.544) months and 95.087 (86.446–103.728) months, respectively.
In multivariate analyses (including age, tumor stage, differentiation grade, tumor size, ER/PR/Her2 status, lymph node status, CA, and VM status, USP44⁺ CSC subclones), lymph node-positive and high Her2 expression were associated with poor OS, whereas lymph node-positive and younger age (<50 years) were associated with poor DFS (Supplementary Table S1). Importantly, USP44⁺ CSC subclones were an indicator of poor prognosis for OS (HR, 3.507; 95% CI, 1.692–7.272; P = 0.001), and DFS (HR,
In our previous study, we demonstrated that breast cancer cells with the characteristic of CSCs can promote VM formation (13). AURKA, as the centrosome-associated kinase, not only induced centrosome abnormalities, but also promoted the development of epithelial–mesenchymal transition and stemness of tumor cells (30). In accordance with previous studies, our current study found that AURKA overexpression induced extra centrosomes and stemness development in MCF-7 cells.

Table 1. Analysis of clinicopathologic parameters and their association with breast cancer harboring USP44+ CSC subclones.

<table>
<thead>
<tr>
<th>Variables</th>
<th>USP44+ CSC subclone</th>
<th>Positive (n = 37)</th>
<th>Negative (n = 55)</th>
<th>P</th>
</tr>
</thead>
<tbody>
<tr>
<td>Age, years</td>
<td>&lt;50</td>
<td>19</td>
<td>30</td>
<td>0.763</td>
</tr>
<tr>
<td></td>
<td>≥50</td>
<td>18</td>
<td>25</td>
<td></td>
</tr>
<tr>
<td>Nodal status</td>
<td>0</td>
<td>10</td>
<td>32</td>
<td>0.001*</td>
</tr>
<tr>
<td></td>
<td>1–3</td>
<td>11</td>
<td>15</td>
<td></td>
</tr>
<tr>
<td></td>
<td>4–7</td>
<td>16</td>
<td>8</td>
<td></td>
</tr>
<tr>
<td>Differentiation grade</td>
<td>I</td>
<td>4</td>
<td>18</td>
<td>0.0001*</td>
</tr>
<tr>
<td></td>
<td>II</td>
<td>10</td>
<td>24</td>
<td></td>
</tr>
<tr>
<td></td>
<td>III</td>
<td>16</td>
<td>13</td>
<td></td>
</tr>
<tr>
<td>Tumor size</td>
<td>T1: &lt;2 cm</td>
<td>10</td>
<td>14</td>
<td>0.142</td>
</tr>
<tr>
<td></td>
<td>T2: ≥2 cm</td>
<td>23</td>
<td>37</td>
<td></td>
</tr>
<tr>
<td></td>
<td>T3: &gt;5 cm</td>
<td>4</td>
<td>0</td>
<td></td>
</tr>
<tr>
<td>Tumor stage</td>
<td>I</td>
<td>8</td>
<td>16</td>
<td>0.005*</td>
</tr>
<tr>
<td></td>
<td>II</td>
<td>11</td>
<td>31</td>
<td></td>
</tr>
<tr>
<td></td>
<td>III</td>
<td>16</td>
<td>8</td>
<td></td>
</tr>
<tr>
<td></td>
<td>IV</td>
<td>2</td>
<td>0</td>
<td></td>
</tr>
<tr>
<td>ER</td>
<td>Positive</td>
<td>20</td>
<td>32</td>
<td>0.695</td>
</tr>
<tr>
<td></td>
<td>Negative</td>
<td>17</td>
<td>23</td>
<td></td>
</tr>
<tr>
<td>PR</td>
<td>Positive</td>
<td>19</td>
<td>32</td>
<td>0.518</td>
</tr>
<tr>
<td></td>
<td>Negative</td>
<td>18</td>
<td>23</td>
<td></td>
</tr>
<tr>
<td>Her2</td>
<td>0+/+</td>
<td>12</td>
<td>30</td>
<td>0.072*</td>
</tr>
<tr>
<td></td>
<td>++/++</td>
<td>10</td>
<td>15</td>
<td></td>
</tr>
<tr>
<td></td>
<td>+/+</td>
<td>15</td>
<td>10</td>
<td></td>
</tr>
<tr>
<td>CA</td>
<td>Positive</td>
<td>25</td>
<td>11</td>
<td>1.45E–6*</td>
</tr>
<tr>
<td></td>
<td>Negative</td>
<td>12</td>
<td>44</td>
<td></td>
</tr>
<tr>
<td>VM</td>
<td>Positive</td>
<td>19</td>
<td>5</td>
<td>2.05E – 6*</td>
</tr>
<tr>
<td></td>
<td>Negative</td>
<td>18</td>
<td>50</td>
<td></td>
</tr>
</tbody>
</table>

*Statistically significant (P < 0.05).

3.085; 95% CI, 1.723–5.524; P = 0.0002) as determined through multivariate analyses of Cox regression.

Discussion

The presence of VM was associated with high tumor grade, invasion and metastasis and short survival in malignant tumor specimens (5–9, 13, 16, 23–25), suggesting the functionally relevant advantage of VM to aggressive tumor cell phenotype. Our previous study (26) used mouse models bearing B16 and LiBr melanoma that were injected with activated carbons via the tail vein. A number of activated carbon granules were observed in the VM and endothelium-dependent vessels, and the results demonstrated the putative connection between VM and endothelium-dependent vessels. By using Doppler imaging of circulating microbeads, other studies elucidated the functions of VM channels and clarified the anticoagulant properties of tumor cells lining along the VM networks (25, 27). Consequently, VM can provide a functional perfusion pathway for rapidly growing tumors by transporting fluid from leaky vessels and/or connecting with endothelial-lined vasculature, as well as an escape route for metastasis (24, 28, 29).

www.aacrjournals.org

Mol Cancer Ther; 14(9) September 2015 2129
pattern was induced, and the VM formation was inhibited, thereby suggesting that the planar-like and apico-basal-like spindle patterns promoted by USP44 expression played key roles in VM and cancer metastasis.

Further xenograft experiments verified the in vitro 3D Matrigel culture results. All MDMA xenografts showed VM formation and increased VE-cadherin expression. Meanwhile, MDMA cells were incorporated into the tumor vasculature and cancer embolus which could promote distant metastasis present in MDMA xenografts. Interestingly, USP44+ CSCs were located in the hypoxic tumor area and a close spatial relationship was observed between USP44+ cells and LPPCN. Our previous study (20) proposed that under hypoxic conditions, some melanoma cells may undergo LPPCN, thus providing a spatial foundation for VM channel formation. Remarkably, LPPCN and VM formation were inhibited in the MDMA USP44 KD xenografts. Therefore, we speculated that breast CSCs with USP44+ may organize VM formation and promote tumor aggressiveness.

Tumors are heterogeneous and harbor different subclones. Tumor subclones possess unique phenotypic and/or functional properties, which may explain some aspects of a tumor and perhaps predict future potential for relapse or resistance to therapy (1). Based on the results of in vitro and in vivo experiments and through consecutive section scanning in human breast cancer tissues, we identified USP44+ CSC subclones in breast cancer specimens. USP44+ CSC subclones were not only correlated with CA, but also with VM. Moreover, the VM marker VE-cadherin was exclusively expressed by USP44+ CSC subclones and undetected in non-USP44+ CSC subclones. This finding suggested the possibility of vasculogenic switch in USP44+ CSC subclone cells. Importantly, USP44+ CSC subclones present an independent prognostic significance for both OS and DFS in patients with breast cancer.

Recent study identified USP44 as a negative regulator of H2B ubiquitination, whose downregulation during differentiation of embryonic stem cells (ESC) and embryonal carcinoma stem cells contributed to the increase in H2Bub1, thereby rendering USP44 as an ESCs-restricted H2Bub1 deubiquitinase. Given that H2Bub1 levels are strongly reduced in advanced human breast cancer, parathyroid tumors, and seminoma (44–46), excess USP44 in cancer is expected. Indeed high USP44 expression was recently reported in human T-cell leukemia (47). However, a study from Zhang and colleagues (37) found that mouse embryonic fibroblast lacking USP44 displayed abnormal spindle geometries and exhibited an increase in the rate of chromosome segregation errors and whole chromosome aneuploidy; subsequently, they found that aged USP44−/− mice had an approximately 5-fold increased incidence of spontaneously arising tumors, thus identifying USP44 as a tumor suppressor (37).

In the current study, we found that USP44 showed an upregulated expression in the breast CSC subpopulation, and USP44+ CSC subclones defined patients with breast cancer with poor prognosis. Therefore, USP44 may play dual roles in tumor development and progression. By promoting centrosome separation, preventing chromosome segregation errors, and inhibiting aneuploidy formation, USP44 exerts tumor suppressor effects on normal cells and early carcinomas. Hence, human tumors could arise because of USP44 gene deletion. As tumors develop and progress at advanced stage when tumor cells commonly harbor supernumerary centrosomes, the protective effects of USP44 are lost. Consequently, USP44 expression contributes to bipolar spindle establishment in the CSC subpopulation with CA and induces VM formation, thereby promoting cancer progression, invasion, and metastasis.

In conclusion, the identification of USP44+ CSC subclones may contribute to the prediction of VM formation and aggressive behavior in breast cancer. This study provides insights into the therapy for advanced breast cancer.

Disclosure of Potential Conflicts of Interest
No potential conflicts of interest were disclosed.

Authors’ Contributions
Conception and design: T. Liu, B. Sun
Development of methodology: T. Liu, Y. Li, Y. Liu, Q. Gu, X. Dong, X. Lin, F. Liu
Acquisition of data (provided animals, acquired and managed patients, provided facilities, etc.): B. Sun, X. Zhao, Z. Yao
Analysis and interpretation of data (e.g., statistical analysis, biostatistics, computational analysis): T. Liu, B. Sun, Z. Yao
Writing, review, and/or revision of the manuscript: T. Liu, B. Sun
Administrative, technical, or material support (i.e., reporting or organizing data, constructing databases): T. Liu
Study supervision: X. Zhao, J. An

Grant Support
This work was partly supported by grants from the Key Project of the National Natural Science Foundation of China (No. 81230050 to B. Sun), the National Natural Science Foundation of China (No. 81172046; to B. Sun; No. 81730091, to X. Lin, and No. 81201791, to T. Liu), and the National Basic Research Program of China (973 Program, No. 2010CB529403; to B. Sun). The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked advertisement in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

Received February 6, 2015; revised May 16, 2015; accepted June 10, 2015; published OnlineFirst July 31, 2015.

References
7. Luo F, Yang K, Liu RL, Meng C, Dang RF, Xu Y. Formation of vasculogenic mimicry in bone metastasis of prostate cancer: correlation with cell...