Extracellular Visfatin-Promoted Malignant Behavior in Breast Cancer Is Mediated Through c-Abl and STAT3 Activation

Amos C. Hung1, Steven Lo2, Ming-Feng Hou3, Yi-Chen Lee1,4, Chun-Hao Tsai1,5, Yuan-Yin Chen1, Wangta Liu3, Yu-Han Su1, Yi-Hsuan Lo1, Chie-Hong Wang1, Shiou-Chen Wu7, Ya-Ching Hsieh8, Stephen Chu-Sung Hu9,10, Ming-Hong Tai11, Yun-Ming Wang7, and Shyng-Shiou F. Yuan1,5,12,13

Abstract

Purpose: Visfatin is an adipocytokine involved in cellular metabolism, inflammation, and cancer. This study investigated the roles of extracellular visfatin in breast cancer, and explored underlying mechanisms in clinical and experimental settings.

Experimental Design: Associations of serum visfatin with clinicopathologic characteristics and patient survival were assessed with Cox regression models and Kaplan–Meier analyses. Effects of extracellular visfatin on cultured breast cancer cells were examined, followed by in vivo investigation of tumor growth and metastasis in xenograft animal models. Imatinib and Stattic were used to inhibit c-Abl and STAT3 activation, respectively.

Results: Breast cancer patients with high serum visfatin levels were associated with advanced tumor stage, increased tumor size and lymph node metastasis, and poor survival. Elevated phosphorylation of c-Abl and STAT3 in breast tumor tissues were correlated with high serum visfatin levels in patients. Visfatin-promoted in vitro cell viability and metastatic capability were suppressed by imatinib (c-Abl inhibitor) and Stattic (STAT3 inhibitor). Increased in vivo cell invasiveness was observed in zebrafish xenografted with visfatin-pretreated breast cancer cells. Tumor growth and lung metastasis occurred in visfatin-administered mice xenografted with breast cancer cells. Tail vein–injected mice with visfatin-pretreated breast cancer cells showed increased lung metastasis, which was suppressed by imatinib.

Conclusions: Serum visfatin levels in breast cancer patients reveal potential prognostic values, and our findings that visfatin promoted breast cancer through activation of c-Abl and STAT3 may provide an important molecular basis for future design of targeted therapies that take into account different serum visfatin levels in breast cancer. Clin Cancer Res; 1–13. ©2016 AACR.

Introduction

Breast cancer is one of the most common malignancies in women worldwide, with multiple risk factors identified, including differential expression of adipocytokines (1, 2). Visfatin, also known as nicotinamide phosphoribosyltransferase (NAMPT) or pre-B-cell colony-enhancing factor (PBEF), is a 52 kDa adipocytokine discovered both intracellularly and extracellularly (3). Intracellular visfatin functions as a rate-limiting enzyme in the biosynthesis of nicotinamide adenine dinucleotide (NAD). When released outside of cells, visfatin exhibits dual roles in enzyme-like activity on extracellular NAD formation as well as cytokine-like activity through a putative receptor–mediated pathway (4). Both intracellular and extracellular visfatin have been shown to be involved in the tumor development (5).

We previously reported that high intracellular expression of visfatin in breast tumor tissues was associated with poor patient survival (6). In recent years, the correlation of serum visfatin level with breast cancer behavior has started to emerge from different cohort studies (7–11). However, the mechanisms linking biologic functions of extracellular visfatin to its clinical
Visfatin is known to have a number of important biologic activities, including nicotinamide adenine dinucleotide (NAD) biosynthesis, cellular metabolism, inflammatory response, and cancer progression. Although previous studies have suggested a role for serum visfatin as a biomarker in breast cancer, the molecular mechanisms linking biologic functions of extracellular visfatin to its clinical significance remain unidentified. In this study, we report that activation of c-Abl and STAT3 is involved in extracellular visfatin–promoted breast cancer via clinical and experimental investigations. Moreover, the results of our in vitro and in vivo studies show that imatinib, a tyrosine kinase inhibitor currently used for clinical treatment of multiple cancers but not for breast cancer yet, inhibits extracellular visfatin–induced malignant behavior. This further raises a possibility that future design of clinical trials for evaluation of therapeutic interventions, such as that for imatinib, may benefit from stratification of breast cancer patients according to different serum visfatin levels.

Materials and Methods

Patient samples

Two hundred and fifty-eight female patients with pathologically confirmed invasive ductal carcinoma of the breast were included in this study. Sera and breast tumor tissues were collected from surgically treated patients at Kaohsiung Medical University Hospital (KMUH), Taiwan, from 2001 to 2011. After surgery, the patients were administered adjuvant radiotherapy, chemotherapy, or hormone therapy based on the clinical practice guidelines of breast cancer (12). Hormone therapy was administered to patients whose tumor sections were estrogen receptor (ER) positive. This study was approved by the Institutional Review Board of KMUH, and informed consent was obtained from each patient. Histologic type and grading of the primary tumor were assessed according to the World Health Organization (WHO) classification (13) and the modified Bloom–Richardson grading scheme (14), respectively, and staging was evaluated according to the American Joint Committee on Cancer (AJCC) TNM staging system (15). The status of ER, progesterone receptor (PR), and HER2 was determined by immunohistochemical staining.

Enzyme immunoassay

Serum visfatin levels for breast cancer patients (n = 258) and age-matched normal female participants (n = 100) were measured in duplicate by a human visfatin–specific enzyme immunoassay kit (Phoenix Pharmaceuticals) according to the manufacturer’s instructions.

Tissue microarray

Tumor tissue samples of the patients were obtained from formalin-fixed and paraffin-embedded tissue blocks for the construction of tissue microarray by an Alphelys BoostArrayer device (Plaisir) as described previously (6). Five-micrometer sections from the tissue microarray were obtained using a microtome and immunohistochemically stained for determination of protein expression.

Immunohistochemistry

The procedure of immunohistochemical staining was described previously (6). The primary antibodies used for immunohistochemistry in this study included the mouse monoclonal anti-ER, anti-PR, and anti-HER2 antibodies from DAKO (Glostrup), rabbit polyclonal anti–phospho-c-Abl (Y393/412) and anti–vimentin antibodies from GeneTex (Irvine), rabbit monoclonal anti–phospho-STAT3 (Y705) antibody from Cell Signaling Technology, and mouse monoclonal anti–cytokertatin 18 antibody from Leica. Images of immunohistochemically stained sections were captured by a Nikon Eclipse E600 microscope. For the scoring of protein expression in breast tumor tissues, the staining of phospho-c-Abl (Y393/412) and phospho-STAT3 (Y705) was stratified into quartiles (0, undetectable; 1, low; 2, intermediate; 3, high) on the basis of intensity as described previously (16). For the analysis of tissues from mice, the staining of phospho-c-Abl (Y393/412), phospho-STAT3 (Y705), cytokertatin 18, and vimentin was scored by the method of histochemical score (H-score), which was calculated as the product of percentage of stained cells and intensity of staining (17).

Cell culture

Human breast cancer cell lines MDA-MB-231, MCF7, and T-47D were purchased from the Bioresource Collection and Research Center of Taiwan with authentication for genotypes and phenotypes of the cells. All three cell lines were cultured in DMEM (Life Technologies) supplemented with 10% FBS (Biological Industries), 100 U/mL penicillin (Biological Industries), 100 μg/mL streptomycin (Biological Industries), and 2.5 μg/mL amphotericin B (Biological Industries) at 37°C in a humidified 5% CO2 incubator. The MDA-MB-231 cells stably expressing luciferase (MDA-MB-231-Luc) were kindly provided by Prof. Wen-Chun Hung, National Health Research Institutes, Taiwan.

XTT cell viability assay

Viable cells were determined by the tetrazolium-based XTT colorimetric assay (Sigma-Aldrich) according to the manufacturer’s instructions. MDA-MB-231, MCF7, and T-47D cells were treated with different concentrations of visfatin (0–100 ng/mL) for 72 hours before the XTT assay. For experiments involving imatinib (c-Abl inhibitor; ref. 18) and Statitic (STAT3 inhibitor; ref. 19), MDA-MB-231 cells were pretreated with imatinib (0 or 10 μmol/L) and Statitic (0 or 1 μmol/L) for 1 hour, followed by visfatin (0–100 ng/mL) treatment for 72 hours.

Immunoblotting

The procedures of protein extraction and immunoblotting were performed as described previously (20). MDA-MB-231 and MCF7 cells were treated with different concentrations of visfatin (0–100 ng/mL) for 2 hours before the immunoblotting analysis. The primary antibodies used for immunoblotting in this study included the rabbit polyclonal anti–phospho-c-Abl (Y393/412), anti–c-Abl, anti–STAT3, anti–IJK2 antibodies and rabbit monoclonal anti–phospho-IJK2 (Y1007/1008) antibody from GeneTex,
with 8- and quantified by Image Lab software (Bio-Rad Laboratories).

**In vitro cell migration and invasion assays**

*In vitro* cell migration was determined using Transwell inserts with 8-μm pores (Corning). MDA-MB-231 cells were pretreated with imatinib (0 or 10 μM) and Stat6 (0 or 1 μM) for 1 hour, followed by visfatin (0 or 100 ng/mL) pretreatment for 24 hours. The cells were then trypsinized and resuspended in serum-free DMEM, and added to the Transwell inserts (3 × 10^4 cells/insert) assembled on a 24-well plate, in which the wells contained DMEM with 10% FBS. After 24 hours of incubation, the cells on the underside of the membrane were stained with 0.5 g/L crystal violet (Sigma-Aldrich). The cells on the underside of the membrane were imaged by an Olympus SZX10 microscope, and quantification of the total area that the cells occupied was processed with the NIS-Elements software (Laboratory Imaging).

*In vitro* cell invasion was determined using Transwell inserts with 8-μm pores coated with Matrigel (Corning), and the procedure was performed as described previously (21), with the same protocol of cell treatment as described above.

**Gelatin zymography**

The procedure of gelatin zymography for secreted matrix metalloproteinases-2/9 was described previously (21). MDA-MB-231 cells were pretreated with imatinib (0 or 10 μM) and Stat6 (0 or 1 μM) for 1 hour, followed by visfatin (0 or 100 ng/mL) treatment for 48 hours. The supernatants from each experimental condition were collected for gelatin zymographic analysis.

**In vivo cell invasiveness in zebrafish**

The experiments using zebrafish in this study were approved by the Institutional Animal Care and Use Committee of Kaohsiung Medical University. Embryos of zebrafish (strain fli1:EGFP from the Taiwan Zebrafish Core Facility; http://tzcf-hdmrc.org/) were generated by natural pairwise mating (22), and the procedure of xenograft was based on a previous report (23). In brief, MDA-MB-231 cells pretreated with visfatin (0 or 100 ng/mL) for 24 hours were labeled with fluorescent probe DiI (Life Technologies), and implanted into the perivitelline cavity of 2-day-old zebrafish embryos through microinjection. After confirmation of the localized DiI-labeled cell mass at the injection site, the zebrafish were transferred to fresh water and maintained at 32.5°C for 48 hours, and cell invasion was determined by visualizing dissemination of the DiI-labeled cells from the injection site under a Nikon Eclipse Ti-S microscope.

**Soft agar colony formation assay**

The procedure of anchorage-independent colony formation in soft agar was described previously (21). MDA-MB-231 and MCF7 cells grown in the soft agar were treated with visfatin (0–100 ng/mL three times a week) for 3 weeks.

**In vivo orthotopic tumor growth and distant metastasis in mice**

Six-week-old female immunodeficient Foxn1nu/Foxn1nu mice (nude mice) were obtained from the National Laboratory Animal Center of Taiwan (http://www.nlac.org.tw/). All of the mice experiments in this study were approved by the Institutional Animal Care and Use Committee of Kaohsiung Medical University. MDA-MB-231 cells (2.5 × 10^6 cells) resuspended in 100 μL of normal saline were injected into the fourth mammary fat pads of mice. One week after implantation, the mice were randomly divided into two groups (n = 10/group), followed by intraperitoneal administration of visfatin (0.5 mg/kg) or normal saline three times a week. The tumor size was measured weekly and calculated by the formula of (width^2 × length)/2. After 8 weeks, all mice were sacrificed and the orthotopic tumors were collected for tumor weight measurement and immunohistochemical analysis. The lung tissues were examined for distant metastasis, which was determined by immunohistochemical analysis for the expression of cytokeratin 18 and vimentin, two tumor markers expressed in MDA-MB-231 cells (24).

**In vivo bioluminescent imaging of metastasis in mice**

The luciferase-expressing MDA-MB-231 (MDA-MB-231-Luc) cells were pretreated with imatinib (0 or 10 μM) for 1 hour, followed by visfatin (0 or 100 ng/mL) pretreatment for 24 hours before intravenous injection of the cells (1 × 10^6 cells in 100 μL of normal saline) into the lateral tail vein of nude mice (n = 3–4/group). The mice injected with the cells pretreated with imatinib alone, or the mice injected with the cells pretreated with imatinib followed by visfatin pretreatment, were intraperitoneally administered imatinib (50 mg/kg daily; ref. 25). On day 7 after tail vein injection of the cells, the mice were analyzed for the presence of bioluminescent signals by Xenogen IVIS Spectrum in vivo imaging system (Caliper Life Sciences). The mice were anesthetized with isofurane (Baxter) using a XGI-8 Gas Anesthesia System (Caliper Life Sciences) followed by intraperitoneal injection of D-luciferin (150 mg/kg; PerkinElmer) for the detection of luciferase expression. The optical images were acquired and analyzed by Xenogen Living Image software (Caliper Life Sciences).

**Statistical analysis**

All statistical analyses were performed using the SPSS 14.0 statistical package (SPSS). The cutoff point for high and low serum visfatin level was determined by the receiver operating characteristic (ROC) curve. The associations between visfatin levels and clinicopathologic characteristics were analyzed by the χ^2 test. Survival curves were generated using the Kaplan–Meier estimates and analyzed by the log-rank test. Univariate and multivariable analyses were performed using the Cox proportional hazards regression models for evaluation of the association between survival and clinicopathologic characteristics. The association between visfatin levels and disease recurrence was analyzed by the Fisher’s exact test or the χ^2 test. The Student t test was used for comparison between two groups. All results were considered statistically significant if P was less than 0.05 calculated with an appropriate two-sided statistical test.

**Results**

**Clinical association of serum visfatin level with breast cancer progression**

The levels of serum visfatin in breast cancer patients (n = 258) and normal female participants as controls (n = 100) were assessed by enzyme immunoassay. As shown in Fig. 1A, the serum visfatin levels were significantly increased in breast...
cancer patients ($P < 0.001$). In addition, the serum visfatin normalized for individual body mass index (BMI) was significantly higher in breast cancer patients ($P < 0.001$), despite that the significantly higher BMI was observed in the patients ($P < 0.001$; Fig. 1A).

The association of patient survival with serum visfatin levels and their correlation with ER status were analyzed by Kaplan-Meier survival curves, for a follow-up period up to 120 months (median = 48 months). The patients with high levels of serum visfatin had significantly poorer disease-free survival.

Figure 1.
Comparison of serum visfatin levels between breast cancer patients and normal participants, and association of serum visfatin levels with patient survival in breast cancer. A, serum visfatin (left) was measured by enzyme immunoassay. BMI (middle) was measured and used for normalization of serum visfatin (serum visfatin/BMI; right). Averaged serum visfatin in normal female participants and breast cancer patients were 1.47 (serum visfatin/BMI; right). Averaged serum visfatin in normal female participants and breast cancer patients were 32.20 (ng/mL) respectively. Averaged BMI in normal female participants and breast cancer patients were 21.97 and 0.78 and 1.77 ± 0.65 (ng/mL)/(kg/m²), respectively. The data were presented by box plots, where the lower boundary and top boundary of the box indicated the 25th percentile and 75th percentile, respectively. The line within the box marked the median, and the bars below and above the box indicated the 10th and 90th percentiles. $P$ values were determined by two-sided Student's $t$ test. Control indicated normal female participants ($n = 100$). Breast cancer indicated breast cancer patients ($n = 258$). B to E, patient survival was analyzed by Kaplan-Meier curves. The HRs and 95% confidence intervals (CI) were calculated using a Cox regression model. B, disease-free survival was analyzed according to serum visfatin levels in breast cancer patients. C, OS was analyzed according to serum visfatin levels in breast cancer patients. D, disease-free survival was analyzed according to combined serum visfatin levels and ER status in breast cancer patients. E, OS was analyzed according to combined serum visfatin levels and ER status in breast cancer patients. $P$ values were determined by two-sided log-rank test.
We further examined the combined association of serum visfatin levels and ER status with patient survival. The patients with both high serum visfatin and ER-negative (ER−) status revealed the worst disease-free survival (P < 0.001; Fig. 1D). Similar results for the combined association of serum visfatin levels and ER status with OS were observed (P = 0.001; Fig. 1E).

We also analyzed the association of serum visfatin levels with clinicopathologic characteristics in breast cancer patients. High serum visfatin levels were significantly associated with certain clinicopathologic variables, including tumor stage (P = 0.001), BMI (P = 0.023), tumor size (P = 0.001), lymph node (LN) metastasis (P = 0.003), and HER2 status (P = 0.013; Table 1). To evaluate the association of clinicopathologic characteristics and serum visfatin levels as independent variables with patient survival, univariate and multivariable Cox regression analyses were employed. In the univariate analysis, tumor size (e.g., P = 0.002 for >5 vs. <2 cm), LN metastasis (P < 0.001), hormone therapy (P = 0.001), and serum visfatin (P = 0.001) were significantly associated with OS (Table 2). In the adjusted multivariable analysis, LN metastasis (P = 0.009), hormone therapy (P = 0.017), and serum visfatin (P = 0.020) were significantly associated with OS (Table 2). Similar results were obtained for disease-free survival on univariate and multivariable analyses (Supplementary Table S1).

We further studied the association of serum visfatin levels with adjuvant therapies for patient survival by Kaplan–Meier curve analysis, and found that differential association only occurred in the hormone therapy (HT) treatment group (P = 0.027; Supplementary Fig. S1F). Serum visfatin levels had no effect on prognosis of OS in the other treatment groups (Supplementary Fig. S1A–S1E). Similar outcomes of the differential association of serum visfatin levels with HT treatment were observed for disease-free survival (Supplementary Fig. S2) and disease recurrence (Supplementary Table S2).

Effect of visfatin on breast cancer cell viability and signaling

Next, the biologic effects of visfatin on breast cancer cells were explored both in vitro and in vivo. The assessment of cell viability by XTT assay showed a significant increase of viable cells in breast cancer cells treated with recombinant human visfatin (Peprotech), including MDA-MB-231 (e.g., P = 0.001 for visfatin 100 vs. 0 ng/mL), SKBR-3 cells (e.g., P = 0.001 for visfatin 100 vs. 0 ng/mL), T-47D (e.g., P = 0.001 for visfatin 100 vs. 0 ng/mL; Fig. 2A), and SKBR-3 cells (e.g., P = 0.002 for visfatin 100 vs. 0 ng/mL; Supplementary Fig. S3A).

Activation of the cellular Abelson tyrosine kinase (c-Abl), also known as ABL1 and signal transducer and activator of transcription 3 (STAT3) may act coordinately on tumor growth and metastasis (26, 27). However, the roles of c-Abl and STAT3 in extracellular visfatin-mediated breast cancer have not been reported before. We found that treatment of MDA-MB-231 cells with visfatin resulted in increased levels of phosphorylated c-Abl at tyrosine-393/412 (Y393/412) residues (Fig. 2B). In addition, the levels of phosphorylated STAT3 at tyrosine-705 (Y705) residue and phosphorylated Janus kinase 2 (JAK2), a STAT3 activator (28), at tyrosine-1007/1008 (Y1007/1008) residues were increased in the cells treated with visfatin (Fig. 2B). Similar results were observed in MCF7 (Fig. 2B) and SKBR-3 cells (Supplementary Fig. S3B).

We further investigated the association of phosphorylation levels of c-Abl and STAT3 in breast tumor tissues with serum visfatin levels from patients. The protein expression in tumor tissues was examined by immunohistochemistry and scored into quartiles (0, 1, 2, and 3) as described previously (16). The high expression group (scores 2 and 3; n = 74) compared with the low expression group (scores 0 and 1; n = 26) of phosphorylated c-Abl at Y393/412 in the tumor tissues was significantly associated with increased serum visfatin levels determined from the corresponding patients (P = 0.002; Fig. 2C). Furthermore, the high expression group (scores 2 and 3; n = 50) compared with the low expression group (scores 0 and 1; n = 47) of phosphorylated STAT3 at Y705 in the tumor tissues was significantly associated with increased serum visfatin levels from the corresponding patients (P < 0.001; Fig. 2D).

Although phosphorylation of STAT3 may occur via activation of c-Abl in cells exposed to various cytokines and growth factors (26, 27), it remains unclear whether visfatin-mediated STAT3 activation relies on c-Abl activity. We observed that the visfatin-induced phosphorylation of STAT3 at Y705 was decreased in the presence of imatinib, an inhibitor of c-Abl kinase activity and clinical treatment of multiple cancers (Supplementary Fig. S3C; refs. 18, 29). Knockdown of the c-Abl protein expression

Table 1. Clinicopathologic characteristics of breast cancer patients and the association with serum visfatin

| Variable                  | Patient, n (%) | Low n (%) | High n (%) | P
|---------------------------|----------------|-----------|------------|---
| Total no.                 | 258 (100)      | 82 (31.8) | 176 (68.2) | -
| Stageb                   | 92 (35.7)      | 34 (41.5) | 58 (33.0)  | <0.001
| II                       | 118 (45.7)     | 44 (35.7) | 74 (42.0)  | -
| III and IV               | 48 (18.6)      | 4 (4.9)   | 44 (25.0)  | -
| Gradec                   | 34 (13.2)      | 13 (39.4) | 21 (61.9)  | 0.687
| 1                        | 156 (60.5)     | 48 (30.8) | 108 (61.4) | -
| 3                        | 68 (26.3)      | 23 (34.3) | 45 (67.7)  | -
| Age, y                   | 138 (53.5)     | 48 (35.8) | 90 (51.1)  | 0.267
| ≤50                      | 120 (46.5)     | 34 (41.5) | 86 (48.9)  | -
| >50                      | 153 (59.3)     | 57 (39.3) | 96 (54.5)  | 0.023
| BMI (kg/m²)              | 105 (40.7)     | 25 (30.5) | 80 (45.5)  | -
| <24                      | 103 (39.9)     | 43 (32.4) | 60 (34.1)  | <0.001
| ≥24                      | 119 (46.1)     | 36 (43.9) | 83 (47.2)  | -
| Tumor size (cm)          | 36 (14.0)      | 3 (3.7)   | 33 (18.8)  | -
| <2                       | 172 (66.7)     | 65 (75.9) | 107 (60.8) | 0.003
| ≥2                       | 86 (33.3)      | 17 (20.7) | 69 (39.2)  | -
| LN metastasis            | 169 (65.5)     | 65 (68.3) | 113 (68.2)| -
| 0–1                      | 118 (45.7)     | 32 (39.0) | 86 (48.9)  | 0.140
| ≥2                      | 140 (54.3)     | 50 (61.0) | 90 (51.1)  | -
| ER status                | 167 (64.7)     | 62 (75.6) | 105 (59.7) | 0.013
| Negative                 | 91 (35.3)      | 20 (24.4) | 71 (40.3)  | -

Abbreviations: LN, lymph node; -, not applicable.

aThe cutoff value of low (<33.75 ng/mL) and high (≥33.75 ng/mL) serum visfatin was determined by the ROC curve.
bStaging was based on the AJCC TNM staging system (15).
cGrading was based on the modified Bloom-Richardson grading scheme (14).
dP values were calculated by two-sided X² test.
in breast cancer cells also resulted in reduced levels of phosphorylated STAT3 at Y705 following visfatin treatment (Supplementary Fig. S3D). To further investigate the roles of c-Abl and STAT3 activation in visfatin-mediated cell viability, MDA-MB-231 cells were treated with visfatin in combination with imatinib and Stattic, an inhibitor of STAT3 (19). The results of XTT assay indicated that the visfatin-increased cell viability was significantly suppressed in cells pretreated with imatinib or Stattic (Fig. 3B). The results of Matrigel invasion assay indicated that MDA-MB-231 cells (P = 0.003) showed a significantly increased degree of cell invasion compared with the control cells without visfatin pretreatment (P = 0.035).

An important event in tumor metastasis is the degradation of extracellular matrix (ECM) and basement membranes through secreted matrix metalloproteinases (MMP), in particular MMP-2 and MMP-9 (30). We examined the levels of secreted MMP-2 and MMP-9 by gelatin zymography and found that after 48 hours of visfatin treatment, the two MMPs in the supernatant of MDA-MB-231 cells were significantly increased compared with control cells (P = 0.001 for MMP-2; P = 0.001 for MMP-9), and the visfatin-induced MMPs secretions were significantly decreased in the presence of imatinib (P = 0.018 for MMP-2; P = 0.024 for MMP-9; Fig. 3C). In addition, the visfatin-induced MMPs secretions in the supernatant of MDA-MB-231 cells were significantly reduced in the presence of Stattic (P = 0.002 for visfatin vs. Stattic + visfatin in MMP-2; P = 0.003 for visfatin vs. Stattic + visfatin in MMP-9; Fig. 3D).

We further evaluated whether the in vivo invasiveness of breast cancer cells could be altered by visfatin in a xenograft zebrafish model (23). As shown in Fig. 3E, MDA-MB-231 cells pretreated with visfatin for 24 hours before microinjection of the cells into the perivitelline cavity of zebrafish showed a significantly increased degree of cell invasion compared with the control cells without visfatin pretreatment (P = 0.035).

## Table 2. Association between clinicopathologic characteristics of breast cancer patients and OS

<table>
<thead>
<tr>
<th>Variable</th>
<th>Univariate* HR (95% CI)</th>
<th>Univariate* P</th>
<th>Multivariable* HR (95% CI)</th>
<th>Multivariable* P</th>
</tr>
</thead>
<tbody>
<tr>
<td>Grade&lt;sup&gt;b&lt;/sup&gt;</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>1 (referent)</td>
<td>1.00</td>
<td>0.419</td>
<td></td>
<td></td>
</tr>
<tr>
<td>2</td>
<td>1.56 (0.53–4.55)</td>
<td>0.112</td>
<td></td>
<td></td>
</tr>
<tr>
<td>3</td>
<td>2.47 (0.81–7.53)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Age, y ≤50</td>
<td>1.00 (referent)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>&gt;50</td>
<td>1.49 (0.81–2.73)</td>
<td>0.197</td>
<td></td>
<td></td>
</tr>
<tr>
<td>BMI (kg/m&lt;sup&gt;2&lt;/sup&gt;) &lt;24</td>
<td>1.00 (referent)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>≥24</td>
<td>1.14 (0.60–2.14)</td>
<td>0.690</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Tumor size (cm) ≤2</td>
<td>1.00 (referent)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>&gt;2</td>
<td>2.47 (1.05–5.80)</td>
<td>0.039</td>
<td>1.00 (referent)</td>
<td>0.245</td>
</tr>
<tr>
<td>LN metastasis 0–1</td>
<td>1.00 (referent)</td>
<td>0.002</td>
<td>2.13 (0.79–5.74)</td>
<td>0.136</td>
</tr>
<tr>
<td>≥2</td>
<td>3.34 (1.75–6.37)</td>
<td>&lt;0.001</td>
<td>2.43 (1.25–4.72)</td>
<td>0.009</td>
</tr>
<tr>
<td>HER2 status</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Negative</td>
<td>1.00 (referent)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Positive</td>
<td>1.14 (0.60–2.17)</td>
<td>0.699</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Radiotherapy</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>No</td>
<td>1.00 (referent)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Yes</td>
<td>1.11 (0.60–2.03)</td>
<td>0.744</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Chemotherapy</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>No</td>
<td>1.00 (referent)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Yes</td>
<td>0.89 (0.41–1.93)</td>
<td>0.774</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Hormone therapy</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>No</td>
<td>1.00 (referent)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Yes</td>
<td>0.36 (0.20–0.66)</td>
<td>0.001</td>
<td>0.46 (0.24–0.87)</td>
<td>0.017</td>
</tr>
<tr>
<td>Serum visfatin&lt;sup&gt;c&lt;/sup&gt;</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Low</td>
<td>1.00 (referent)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>High</td>
<td>5.67 (2.01–15.98)</td>
<td>0.001</td>
<td>3.55 (1.22–10.36)</td>
<td>0.020</td>
</tr>
</tbody>
</table>

Abbreviations: LN, lymph node; –, not applicable.

*Univariate and multivariable analyses were performed by Cox regression models. Variables with P values larger than 0.10 in the univariate analysis were excluded from multivariable analysis.

<sup>b</sup>Grading was based on the modified Bloom–Richardson grading scheme (14).

<sup>c</sup>Low and high serum visfatin were determined as described in Table 1.

<sup>d</sup>Two-sided P values were calculated by a univariable Cox proportional hazards regression model.

<sup>e</sup>Two-sided P values were calculated by a multivariable Cox proportional hazards regression model.
Effect of visfatin on anchorage-independent growth in vitro

The effect of visfatin on in vitro anchorage-independent growth was examined by soft agar colony formation assay. MDA-MB-231 cells treated with visfatin showed significantly increased colony formation compared with the control cells without visfatin treatment (e.g., \( P = 0.005 \) for visfatin 100 vs. 0 ng/mL; Fig. 4A). Although less capable of forming anchorage-independent growth than MDA-MB-231 cells (31), MCF7 cells treated with visfatin also showed significantly increased colony formation compared with the control cells without visfatin treatment (\( P = 0.002 \) for visfatin 100 vs. 0 ng/mL; Fig. 4A).

Anoikis, a specific form of cell death induced by cell detachment from ECM, is one of the mechanisms for avoiding metastatic colonization via prevention of anchorage-independent growth of cancer cells (32). We found that MDA-MB-231 cells treated with
Figure 3.
Effect of visfatin on breast cancer cell migration and invasion. A, migratory ability of MDA-MB-231 cells pretreated with imatinib (0 or 10 µmol/L) and Stattic (0 or 1 µmol/L) for 1 hour followed by visfatin (0 or 100 ng/mL) pretreatment for 24 hours was assessed by Transwell migration assay; n = 3. B, invasive ability of MDA-MB-231 cells pretreated with imatinib (0 or 10 µmol/L) and Stattic (0 or 1 µmol/L) for 1 hour followed by visfatin (0 or 100 ng/mL) pretreatment for 24 hours was assessed by Transwell invasion assay; n = 3; Scale bar, 20 µm. (Continued on the following page.)
visfatin revealed a significant reduction of anoikis cell death \((P = 0.006; \text{Supplementary Fig. S4A})\).

**Effect of visfatin on tumor growth and metastasis in mice**

To further confirm the association of visfatin with tumor formation and metastasis in vivo, we used an orthotopic xenograft model in nude mice, in which MDA-MB-231 cells were injected into the mammary fat pads of female mice, followed by intraperitoneal administration of visfatin (0.5 mg/kg) or normal saline as vehicle control three times a week. As shown in Fig. 4B, the mice administered with visfatin showed a significant increase in orthotopic tumor growth compared with the control mice \((P = 0.042\); tumor volume at week 8). All mice were sacrificed after 8 weeks of treatment, and the weight of orthotopic tumors from the mice administered with visfatin was found to be significantly higher than those from the control mice \((P = 0.003; \text{Fig. 4C})\).

The phosphorylated levels of c-AbI and STAT3 in the orthotopic tumors were examined after the mice were sacrificed. Immunohistochemical analysis revealed that the expression of phosphorylated c-AbI at Y393/412 in the orthotopic tumors from visfatin-treated mice was significantly higher than that from the control mice \((P = 0.002; \text{Fig. 4D})\). There was also a significant increase of phosphorylated STAT3 at Y705 in the orthotopic tumors from the visfatin-treated mice compared with the control mice \((P = 0.011; \text{Fig. 4E})\). We further investigated for the occurrence of distinct lung metastasis by immunohistochemical analysis. Two tumor marker proteins expressed in MDA-MB-231 cells, cytokeratin 18 (CK18) and vimentin (24), were significantly increased in the lung tissues of mice treated with visfatin compared with those from the control mice \((P < 0.001\) for CK18; \(P = 0.009\) for vimentin; \text{Fig. 4F and G}, respectively). The body weight of the mice during the course of treatment was not significantly different in each group \((\text{Supplementary Fig. S5A})\), neither were the levels of alanine aminotransferase (ALT), aspartate aminotransferase (AST), blood urea nitrogen (BUN), and creatinine for liver and renal functions of the mice \((\text{Supplementary Fig. S5B})\).

The role of c-AbI activation in visfatin-mediated metastasis was further studied by *in vivo* bioluminescent imaging using the intravenous tail vein injection model in nude mice. The cell viability of luciferase-expressing MDA-MB-231 (MDA-MB-231-Luc) cells was not significantly different in each group before the tail vein injection \((\text{Supplementary Fig. S4B})\). After 7 days post-injection, the mice injected with the visfatin-pretreated MDA-MB-231-Luc cells revealed a significantly higher bioluminescent signal in lung tissues than the mice injected with control MDA-MB-231-Luc cells \((P = 0.048)\), whereas the mice injected with the cells pretreated with imatinib followed by visfatin pretreatment had a significantly reduced bioluminescent signal in lung tissues \((P = 0.046; \text{Fig. 4H})\).

**Discussion**

**Serum visfatin as a biomarker for clinical evaluation in breast cancer**

Serum visfatin has previously been shown to be associated with breast cancer progression \((7–11)\). Yet, its prognostic value remains insufficiently assessed, except for Li and colleagues \((10)\) who reported that high serum visfatin level was associated with poorer survival of breast cancer patients. Although a similarly unfavorable outcome for the survival of the patients with high level of serum visfatin was observed in the present study, our data further analyzed important clinical factors that have not been previously elucidated. For instance, we reported that the patients with both high serum visfatin level and ER-negative status had the worst survival compared with the other groups of patients. Moreover, the ER-negative patients with high serum visfatin level without receiving hormone therapy (HT+) had a poorer survival and more recurrent incidence than the ER-positive patients with high serum visfatin level receiving hormone therapy (HT+), whereas there was no significant difference between HT+ and HT+ treatment groups of the patients with low serum visfatin. Together, the data suggest that different serum visfatin levels may have clinical relevance as a prognostic tool in breast cancer. Although the mechanisms underlying the relationship between serum visfatin levels and the patient response to hormone therapy are not clear at now, we propose that it may be linked through an indirect regulation in adipocytes, whose ER activation can lead to upregulation of visfatin expression \((33, 34)\). As adipocytes have been known the major source to release visfatin \((35, 36)\), the patients with anti-ER treatment may cause the reduction of visfatin release from adipocytes into circulation, which in turn alleviates accumulation of serum visfatin that may promote breast cancer progression. However, more experiments will be required to examine this hypothesis.

In this study, the level of serum visfatin in breast cancer patients \((40.87 \pm 13.86 \text{ng/mL})\) was observed to be higher than normal female participants \((32.20 \pm 17.42 \text{ng/mL})\). Analysis of clinicopathologic parameters in our study revealed that high level of serum visfatin \((cutoff at 33.75 \text{ng/mL})\) was associated with tumor stage, BMI, tumor size, LN metastasis, and HER2 status \((\text{Table 1})\). Among these clinicopathologic parameters, tumor stage, and LN metastasis were commonly found to be associated with higher serum visfatin level in this study and others \((7, 9, 11)\), suggesting a potentially unfavorable role of serum visfatin in breast cancer. It was noted that in our clinicopathologic analyses, status of HER2 protein expression in breast tumor tissues was positively correlated with serum visfatin level in patients. We further analyzed the
Figure 4. Effect of visfatin on breast tumor growth and metastasis. A, in vitro anchorage-independent growth of MDA-MB-231 and MCF7 cells treated with visfatin (0, 50, and 100 ng/mL) was determined by soft agar colony formation assay after 3 weeks in culture; n = 3; scale bar, 200 μm. B, in vivo breast tumor growth was assessed by the orthotopic xenograft model in nude mice injected with MDA-MB-231 cells to the 4th mammary fat pads. The mice were intraperitoneally administered visfatin (0.5 mg/kg) or normal saline as control three times a week (n = 10/group). Tumor volumes were measured weekly and calculated with the formula of (width ² × length)/2 for 8 weeks. C, after 8 weeks as described in B, all mice were sacrificed and the orthotopic tumors were collected for tumor weight measurement. The inset showed five representative orthotopic tumors for each group dissected from the mice; scale bar, 10 mm. D to G, after sacrifice of the mice, the orthotopic tumors were collected for protein expression of phospho-c-Abl (D) and phospho-STAT3 (E), and the lung tissues were collected for metastatic assessment for protein expression of cytokeratin 18 (CK18; F) and vimentin (G) by immunohistochemical analysis with H-score; scale bar, 100 μm. H, in vivo metastasis of luciferase-expressing MDA-MB-231-Luc (MDA-MB-231-Luc) cells pretreated with imatinib (0 or 10 μmol/L) for 1 hour followed by imatinib (0 or 10 μmol/L) combined visfatin (0 or 100 ng/mL) pretreatment for 24 hours was intravenously injected into nude mice via the lateral tail vein (n = 3–4 per group). The mice containing MDA-MB-231-Luc cells pretreated with imatinib alone, or with imatinib followed by visfatin pretreatment, were intraperitoneally injected with imatinib (50 mg/kg daily). After 7 days of tail vein injection, the mice were anesthetized and intraperitoneally injected with D-luciferin (150 mg/kg) for detection of bioluminescence by IVIS Spectrum in vivo imaging system. The data were presented as mean ± SD; *P < 0.05; **P < 0.01; ***P < 0.001; two-sided Student t test.
Characterization of the biologic effect of visfatin on breast cancer cells.

Our in vitro data showed that extracellular visfatin promoted breast cancer cell proliferation, migration, invasion, and MMP2/9 secretion. In addition, our in vivo data using zebrafish and nude mice as xenograft models further confirmed the promoting effect of extracellular visfatin on breast tumor growth and metastasis. Notably, we provided the first evidence for the involvement of c-Abl and STAT3, two important oncogenes (38–40), in extracellular visfatin-promoted breast cancer. Although the intracellular reactive oxygen species (ROS) was reportedly to be upregulated by visfatin stimulation in some cell types (41–43), it was not detectable in our current treatment with visfatin in breast cancer cells (Supplementary Fig. S3E and S3F). Other mechanisms reported in extracellular visfatin–mediated breast cancer growth and metastatic potential included cyclin D1 and cdk2 (44), Nf-xb and Notch1 (45), or PI3K/Akt and TGFβ (46). As the identity of “visfatin receptor” is not known to date, it limits our understanding for the molecular mechanisms of extracellular visfatin. Nevertheless, our current results raised a possibility that the putative receptor for visfatin could belong to the receptor family that transduces signals via c-Abl and STAT3 such as EGF, platelet-derived growth factor (PDGF), insulin receptor, and insulin-like growth factor (IGF) receptors (Supplementary Fig. S6D; refs. 26, 38, 39, 47). Whether there is a direct interaction between c-Abl and the putative receptor activated by visfatin, or a direct interaction between c-Abl and STAT3 after visfatin stimulation, remains to be determined.

Disclosing patients according to serum visfatin levels for potential targeted adjuvant therapy

Previous clinical trials have shown no clinical benefit from imatinib in breast cancer patients (48, 49). However, this may be due to a selection issue, as patients have been comprehensively included without stratifying those that may benefit most from imatinib treatment. Until now, there has been no clinical marker that may allow such stratification. Our animal studies showed that the lung metastasis of breast cancer cells pretreated with visfatin via tail vein xenograft in nude mice was largely suppressed by imatinib. Equally as important, there was no significant effect on metastasis with imatinib treatment in the group without exposure to visfatin, further providing a strong argument for stratification of treatment with imatinib according to extracellular levels of visfatin. The potential clinical importance of a targeted anti-metastatic agent in breast cancer patients, using an already widely available but unused drug in breast cancer, cannot be underestimated. We would suggest that future clinical trials on the efficacy of imatinib may stratify patients according to visfatin levels and select those with pre-metastatic, high serum visfatin breast cancer. In addition, this may advantageously offer alternative therapeutic options in the triple-negative breast cancer patients.

Study limitations

There are a few limitations to this study, including the lack of a standard definition of high or low serum visfatin level and size and geographic localization of study population. Therefore, care must be taken in interpreting clinical analyses from this study and others. In addition, although we showed the effects of extracellular visfatin on breast cancer cells in both in vitro and in vivo experiments that mainly used MDA-MB-231 cells for metastasis and animal studies, it may not fully represent heterogeneous human breast cancer behaviors in clinical settings. It should also be noted that imatinib is known to be a tyrosine kinase inhibitor for c-Abl, c-Kit, PDGFR, and DDR (50).

Conclusions

In conclusion, our clinical data suggested that high level of serum visfatin was associated with malignant breast cancer behavior, and the level of serum visfatin could offer a means for prognosis in breast cancer. Moreover, the results of our in vitro and in vivo experiments revealed that extracellular visfatin promoted breast cancer cell growth and metastatic ability via activation of c-Abl and STAT3. These are several key implications resulting from this study. First, stratification of breast cancer patients by serum visfatin levels may help identify patients that would benefit from inhibition of downstream effectors of visfatin signaling. Second, categorization of the patients according to the ER status may provide information for risk assessment of the survival of patients with different levels of serum visfatin. Third, our study is the first to suggest that the use of imatinib may have therapeutic potential in the treatment of breast cancer for the group of patients with higher levels of serum visfatin.

Further studies, including discovery of the receptor for visfatin, development of specific inhibitors for extracellular visfatin, and clinical trials stratifying patients for treatment according to different serum visfatin levels, will be potentially important to the future design of targeted therapies in consideration of different serum visfatin levels in breast cancer.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

Authors’ Contributions

Conception and design: A.C. Hung, C.-H. Tsai, Y.-M. Wang, S.-S.F. Yuan
Acquisition of data (provided animals, acquired and managed patients, provided facilities, etc.): A.C. Hung, M.-F. Hou, Y.-C. Lee, C.-H. Tsai, Y.-Y. Chen, W. Liu, Y.-H. Su
Analysis and interpretation of data (e.g., statistical analysis, biostatistics, computational analysis): A.C. Hung, S. Lo, Y.-C. Lee, C.-H. Tsai, Y.-H. Lo, S.-C. Wu, S.-C.-S. Hu, S.-S.F. Yuan
Writing, review, and/or revision of the manuscript: A.C. Hung, S. Lo, Y.-C. Hsieh, S.-C.-S. Hu, M.-H. Tai, Y.-M. Wang, S.-S.F. Yuan

www.aacrjournals.org
Clin Cancer Res; 2016
OF11

Downloaded from clincancerres.aacrjournals.org on October 2, 2017. © 2016 American Association for Cancer Research.
Hung et al.

Administrative, technical, or material support (i.e., reporting or organizing data, constructing databases): A.C. Hung, C.-H. Tsai, W. Liu, Y.-H. Lo, C.-H. Wang, S.-F. Yuan

Study supervision: M.-F. Hou, Y.-M. Wang

Grant Support

This work was supported by grants from Kaohsiung Medical University Hospital (KM1H1102-2T07 and KM1H1102-2R25), Kaohsiung Medical University (aim for the top journals grant, KMU-DT103010 and KMU-TP103D18), National Health Research Institutes (NHRI-EX100-9829BI and NHRI-EX104-10212B), and Ministry of Health and Welfare (MOHW103-TD-B-111-05) of Taiwan.

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 November 6, 2015; revised March 14, 2016; accepted March 22, 2016; published OnlineFirst April 1, 2016.

References

34. Singletary SE, Allred C, Ashley P, Bas}
Role of Extracellular Visfatin in Breast Cancer


Extracellular Visfatin-Promoted Malignant Behavior in Breast Cancer Is Mediated Through c-Abl and STAT3 Activation

Amos C. Hung, Steven Lo, Ming-Feng Hou, et al.

Clin Cancer Res  Published OnlineFirst April 1, 2016.

Updated version Access the most recent version of this article at: doi:10.1158/1078-0432.CCR-15-2704

Supplementary Material Access the most recent supplemental material at: http://clincancerres.aacrjournals.org/content/suppl/2016/04/01/1078-0432.CCR-15-2704.DC1

E-mail alerts Sign up to receive free email-alerts related to this article or journal.

Reprints and Subscriptions To order reprints of this article or to subscribe to the journal, contact the AACR Publications Department at pubs@aacr.org.

Permissions To request permission to re-use all or part of this article, contact the AACR Publications Department at permissions@aacr.org.