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Original Articles

The phosphorylation-specific association of STMN1 with GRP78 promotes breast cancer metastasis

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ABSTRACT

Metastasis is a major cause of death in patients with breast cancer. Stathmin1 (STMN1) is a phosphoprotein associated with cancer metastasis. It exhibits a complicated phosphorylation pattern in response to various extracellular signals, but its signaling mechanism is poorly understood. In this study, we report that phosphorylation of STMN1 at Ser25 and Ser38 is necessary to maintain cell migration capabilities and is associated with shorter disease-free survival (DFS) in breast cancer. In addition, we report that glucose-regulated protein of molecular mass 78 (GRP78) is a novel phospho-STMN1 binding protein upon STMN1 Ser25/Ser38 phosphorylation. This phosphorylation-dependent interaction is regulated by MEK kinase and is required for STMN1-GRP78 complex stability and STMN1-mediated migration. We also propose a prognostic model based on phospho-STMN1 and GRP78 to assess metastatic risk in breast cancer patients.

Introduction

Breast cancer is the most common malignancy and the leading cause of cancer-related morbidity and mortality among women worldwide [1]. Stathmin1 (STMN1), also known as Oncoprotein 18 (Op18), is a ubiquitously expressed, highly conserved, 18-kDa, cytosolic phosphoprotein that is up-regulated in several malignancies, including breast cancer [2]. Increased STMN1 protein expression and post-translational modification markedly enhances cancer cell proliferation and invasiveness [3,4], suggesting that STMN1 plays an important role in cancer progression. STMN1 is phosphorylated at four conserved serine residues (Ser16, Ser25, Ser38, and Ser63), and phosphorylation at either Ser16 or Ser63 strongly reduces or abolishes the ability of STMN1 to bind to and sequester soluble tubulin [5]. Phosphorylation at Ser38 is a proposed biomarker for tumor progression and poor prognosis [6].

STMN1 phosphorylation status differs based on the cellular and signaling context, and different protein kinases are known to regulate specific phosphorylation sites. STMN1 Ser16 can be phosphorylated by protein kinase C (PKC), P21-Activated Kinase 1 (PAK1), or Ca^{2+}/calmodulin-dependent kinase II/IV [4,7–9], whereas Ser25 and Ser38 are targeted by mitogen-activated protein kinases (MAPKs) and cyclin-dependent kinases (CDKs) [10,11]. Differential STMN1 phosphorylation generates different STMN1 phosphoisomers that contribute to the overall regulation of cell invasion and cancer metastasis. Whereas the role of phosphorylation at different serine residues has been explored in microtubule formation, proliferation, cell migration, and cancer invasion [5,12,13], the prognostic effect and clinical application of differential STMN1 phosphorylation has not been investigated in breast cancer.

Glucose-regulated protein of molecular mass 78 (GRP78) is an endoplasmic reticulum chaperone and heat shock protein [14]. GRP78 has anti-apoptotic functions and promotes tumor proliferation, survival, and metastasis. It confers resistance to a wide variety of antineoplastic therapies [15–18]. Previous studies have demonstrated that GRP78 expression is maintained at a low basal level in benign human breast lesions but is increased in breast cancers [19]. Elevated GRP78 protein expression has been reported in cancer cells that have metastasized to the lymph nodes and poor prognosis [20,21]. In multiple cancer-derived cell lines, GRP78 overexpression confers drug resistance, whereas GRP78 inhibition enhances cell sensitivity to chemotherapy [22–25]. As a cell surface protein, GRP78 has been shown to contribute to phosphatidylinositol 3-kinase (PI3K)/protein kinase B (AKT) pathway activation; conversely, a highly specific monoclonal antibody against GRP78 suppressed AKT activation [26,27]. Thus, GRP78 expression may serve as a biomarker for tumor progression and treatment response.

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In this study, we investigated the metastasis-specific phosphorylation states of STMN1 and their correlation with clinical outcomes in breast cancer patients. We identified a phosphorylation-dependent interaction between STMN1 and GRP78 and propose that the interaction between phospho-STMN1 and GRP78, which is regulated by mitogen-activated protein kinase kinase (MEK), plays an important role in breast cancer metastasis.

Materials and methods

Cell lines and constructs

MDA-MB-231 and HEK293T cells were obtained from the Shanghai Cell Bank Type Culture Collection Committee (CBTCCC, Shanghai, China). A highly metastatic variant of the parental MDA-MB-231 cell line, termed MDA-MB-231-HM, was established within the presented model system [28]. Culturing methods are described in the supplemental materials. Transient transfections were performed using Lipofectamine 2000 (Invitrogen, Carlsbad, CA, USA). Stable cells lines were generated by infection with a retrovirus containing various hemagglutinin (HA)- and Flag-tagged proteins, followed by selection with puromycin.

Plasmids, shRNAs, and antibodies

Human full-length STMN1 coding sequences (CDS) were cloned into HA- and Flag-tagged (HF)-pDEST-MSCV retroviral vectors for overexpression [29]. Singly (S16A, S25A, S38A, and S63A) and doubly (S16A/S63A and S25A/S38A) alanine-substituted STMN1 mutants were generated using a PCR technique [29]. Site-directed mutagenesis protocols and shRNA sequences for STMN1 and GRP78 are presented in the supplemental materials.

Phosphoprotein profiling by a phospho-specific protein microarray

We used a phospho-specific protein microarray (Full Moon Microsystems) containing 1318 antibodies against phosphorylation sites of 452 proteins for phosphorylation profiling.

The antibody array experiment was performed by Wayen Biotechnology (Shanghai, China) according to an established protocol. The fluorescence signal of each antibody was obtained from the fluorescence intensity of the antibody spot. A ratio computation was used to measure the extent of protein phosphorylation. The phosphorylation ratio was calculated as follows:

$$\text{phosphorylation ratio} = \textit{phospho value}/\textit{unphospho value}.$$

Immunoblotting analysis and antibodies

Whole cell lysates were generated with Pierce T-PER® Tissue Protein Extraction Reagent (Thermo Fisher Scientific Inc., Waltham, MA, USA) with complete ethylenediaminetetraacetic acid (EDTA)-free Protease Inhibitor Cocktail Tablets (F Hoffman-La Roche Ltd., Basel, Switzerland). Immunoblotting was performed using standard techniques. Each immunoblotting has implicated for at least three times. Densitometry analysis was performed using ImageJ software (National Institutes of Health, Bethesda, MD). Antibodies and their dilutions are presented in the supplemental materials.

Cell lysis and immunoprecipitation

Cells were lysed in NETN buffer (50 mM Tris-HCl, pH 8.0, 0.15 M NaCl, 1 mM EDTA, 0.5% Nonidet P-40) with protease inhibitors and protein phosphatase inhibitors, 1 mM NaF, and 1 mM Na_3VO_4. Immunoprecipitation (IP) experiments were performed in the same buffer with the appropriate antibodies and protein A/G-Sepharose beads (Santa Cruz Biotechnology) and incubated overnight at 4 °C. FLAG IP was performed using Flag (M2) beads (Sigma).

Kinase inhibitors and cell treatment

A panel of kinase inhibitors was used that specifically inhibit 20 different types of kinases from various pathways: C-Abl, PI3K, CDK, GSK3, STAT3, APC, Akt, ERK, Rac1, PKA, MEK, Aurora, Wnt, JNK, PKC, Raf, JAK, Src, P38 and ROCK. MDA-MB-231-HM cells were cultured in L15 medium containing 10% FBS to approximately 80% confluence and were then cultured in L15 medium without FBS for 12–16 h. Kinase inhibitors dissolved in dimethylsulfoxide (DMSO) were added to the L15 medium without FBS to a final concentration of 10 μM. MDA-MB-231-HM cells were treated for 2 h, at which time the medium was changed back to L15 with FBS for no more than 10 minutes. The cells were immediately lysed in Protein Extraction Reagent for immunoblotting analysis.

In vivo *mouse model*

All animal experiments were approved by the Animal Ethics committee. Nude mice from each group received a tail vein injection of 1×10^5 cells per week for three consecutive weeks (n = 8 for each group). The presence of lung metastases was determined using micro-computed tomography (CT) imaging and by hematoxylin and eosin (H&E) staining after 10 weeks.

Immunohistochemistry

Formalin-fixed, paraffin-embedded (FFPE) tissue samples from 310 patients with histologically confirmed stage I–III primary breast cancer who underwent mastectomy at Fudan University Shanghai Cancer Center were examined and were used to generate a tissue microarray (TMA). Patient characteristics of both cohorts are presented in Supplementary Table S1. Immunohistochemistry for STMN1, Ser16, Ser25, Ser38, Ser63 and GRP78 was conducted using a two-step protocol (GTVision™III). This study was approved by the independent ethical committee/institutional review board of Fudan University Shanghai Cancer Center (Shanghai Cancer Center Ethical Committee). All patients provided written informed consent before enrolling in the study.

Statistical analysis

The protein antibody array (phospho-specific protein microarray) was purchased from Full Moon Biosystems, Inc. (Sunnyvale, CA, USA) and processed according to the manufacturer's protocol. Data collected from three independent experiments were analyzed by analysis of variance (ANOVA) using MATLAB.

Disease-free survival (DFS) was defined as the interval from the initial surgery to clinically defined metastasis. Pearson's χ^2 or Fisher's exact tests were used to evaluate the significance of differences between covariates. The postoperative DFS probability was determined by the Kaplan–Meier method, and differences in survival between markers were estimated with 2-sided log-rank (Mantel–Cox) tests. The hazard ratios (HR) and confidence intervals (CI) at the 95% level were determined using Multivariate Cox regression analysis. Data were analyzed with SPSS (version 20.0; SPSS Inc.). All *P* values were two-sided, and a *P* value less than 0.05 was considered statistically significant.

For the predictive model, markers chosen as forward from the Cox analysis were used to construct multivariate Cox proportional hazards models. To estimate the utility of the prognostic model, the area under the receiver operating characteristic (ROC) curve for patient DFS was calculated. The time-dependent ROC curve was used to illustrate the relationship between sensitivity and the false-positive rate (1-specificity), and a cutoff risk score was determined. These statistical tests were conducted with R software, version 3.1.1.

Results

Identification of STMN1 phosphorylation sites in a highly metastatic cell line

We induced MDA-MB-231-HM invasiveness [30] *in vivo* by injecting them into nude mice (n = 8 for each group). CT imaging and histologic analysis confirmed that MDA-MB-231-HM cells had an increased ability to metastasize to the lungs compared to the parental MDA-MB-213 control. After two weeks, spontaneous pulmonary metastases developed in 100% of the mice, whereas the incidence of pulmonary metastasis in mice injected with the parental MDA-MB-231 cells was less than 50% (Fig. 1A).

We next used a phospho-specific protein microarray containing 1318 phosphoprotein antibodies to characterize the difference in phosphorylation patterns between MDA-MB-231 and MDA-MB-231-HM cells. More proteins were phosphorylated in MDA-MB-231-HM cells than MDA-MB-231 cells (Fig. 1B). We performed gene ontology (GO) and Kyoto Encyclopedia of Genes and Genomes (KEGG) analyses to determine the enriched pathways in the highly metastatic MDA-MB-231-HM cells (Fig. 1C). KEGG analysis revealed that the MAPK pathway was activated in MDA-MB-231-HM cells (Supplementary Fig. S1). We found that STMN1, a protein known to be highly expressed in multiple cancers and a downstream target of the MAPK pathway [31–33], exhibited a different phosphorylation pattern in MDA-MB-231-HM cells compared to control cells. STMN1 mRNA and protein levels were higher in multiple breast cancer-derived cell lines compared to normal mammary