Abstract. Mammalian sterile 20-like kinase 1 (Mst1) is a major inhibitor of cell proliferation, and is involved in apoptosis, oncogenesis and organ growth via its ubiquitously encoded serine threonine kinase. Previous studies have demonstrated that Mst1 has a tumor suppressor function in human breast cancer. Mst1 deletion or mutation is associated with tumorigenesis, whereas Mst1 overexpression leads to tumor cell apoptosis and decreases proliferation of tumor cells. Our previous study reported the tumor suppressive function of Mst1, and debated Mst1 as a prognostic factor in human breast cancer. In the present study, Mst1 levels were measured in the plasma of patients in order to elucidate their association with overall and disease-free survival. The results of the present study indicated that Mst1 is a strong prognostic and predictive factor in human breast cancer and a promising anticancer target.

Introduction

Breast cancer is the most common type of cancer in women worldwide and the second most common type of cancer overall (1-3). Despite advances being made in therapeutic and diagnostic study, only a fraction of treatment strategies are individualized, with treatment based not only on a careful risk assessment for each patient, but on specific clinicopathological features of the breast cancer they suffer from (4-6). There are a number of established predictive factors in breast cancer, the majority of which are also therapeutic targets [including estrogen receptor, progesterone receptor or human epidermal growth factor receptor 2 (Her2)] (7). Therapeutic approaches are primarily based on clinicopathological variables, including tumor size, lymph node stage, histological grade, type and lymphovascular invasion (8). However, there is a limited choice of prognostic factors, which are essential for decision-making, since these predict patient outcomes irrespective of treatment (9). Furthermore, breast cancer is one of the most heterogeneous diseases. There are a growing number of aged patients (>75 years) with breast cancer, and therefore there is an urgent requirement for personalized therapies in order to avoid over- or under-treatment (10-12). Multi-parameter gene expression analyses are evolving and progressively suggesting promising markers (13-15). Thus, it is important to study and propose methods for an effective and efficient quantification of specific gene expression status (16). At the same time, evidence of statistical association between the gene expression and overall survival (OS) and disease-free survival (DFS) are required for designing antitumor management strategies and prognosis evaluation (17,18).

Mammalian sterile 20-like kinase 1 (Mst1), alternatively termed serine threonine kinase 4 (STK4), has been known for decades, but regained significant attention when its role as a tumor suppression gene in various entities of human cancers was reported (19-22). Mst1 is a serine/threonine protein kinase, which builds a complex with Mst2. Mst1/Mst2 are activated by the phosphorylation of Thr^{183} and Thr^{180}, which leads to a
feedback stimulation that regulates oxidant levels through a number of mechanisms (23). One of them is the regulation of cellular redox state (23). This specific mechanism may represent a tumor suppressor function of Mst1/2 (23,24). Mst1/2 kinases also affect immune cell activation, proliferation, adhesion, migration, growth and apoptotic pathways, as they are essential for the Hippo signaling pathway (25). Loss of Mst1 results in hyper-proliferation and tumorigenesis (25). In addition, the Hippo pathway interacts with other signaling pathways, including Wnt and Notch pathways, known for their crucial roles in tumor pathogenesis (26,27). Finally, the pro-apoptotic function of Mst1 is also associated with pleckstrin homology-domain and leucine-rich repeat protein phosphatases (28). These two proteins synergize to achieve a greater potential of apoptosis (28). Phosphoinositide 3-kinase/protein kinase B acts as an inhibitor of Mst1 through phosphorylation of threonine 120 (29).

Our previous study, for the first time, presented data that supported the function of Mst1 as a prognostic factor in human breast cancer, using immunostaining as the Mst1-identification method (30). This study demonstrated that Mst1-positive patients had a significantly improved OS compared with Mst1-negative patients, and that Mst1 is an independent prognostic factor in breast cancer. In the present study, ELISA was performed to quantify Mst1 concentration in the serum of patients with breast cancer. The methodological concept facilitates a direct translation into the clinic, as it is easy, feasible, exact and less biased than immunohistological estimations of the amount of Mst1 in tumor cells. In addition, the sampling method is incomparably more attractive for the daily routine and comfort of patients. The present study demonstrated the prognostic significance of Mst1 expression for the rates of OS and DFS. The results of the present study revealed the tumor suppressive function of Mst1, and confirmed Mst1 as an independent prognostic factor in human breast cancer.

Materials and methods

Ethical approval. The present study was performed at the Central Laboratory and Department of Breast Surgery, Yangpu Hospital, Tongji University School of Medicine (Shanghai, China), and was approved by the local institutional review board. Written consent forms were collected from all patients involved in the present study. The ethics review board of Tongji University School of Medicine approved the study design.

Study population. Blood samples used in the present study were collected between January 2005 and December 2006 in the Department of Breast Surgery, Yangpu Hospital, Tongji University School of Medicine. In total, 98 women were included in the study, since they completed the entire period of follow-up. All blood samples were taken prior to any surgical interventions or antitumor treatment. Data of patients, including age, tumor size, tumor stage, histological grade, node status, histological type, molecular subtypes, hormone receptor status and Her2 status were obtained from the pathological reports. Table I describes the baseline demographics of the study population. The distribution of tumor grades and receptor status were representative. The majority of the patients presented with carcinoma of a ductal type with luminal subtype, grade 2. All patients, the median age was 52 and the age range of patients was 35-73, were Chinese females and were followed until mortality or the end of the follow-up period of 98 months.

Indirect ELISA detection of Mst1 in the plasma of patients. ELISA detection was used as an efficient and effective method in order to assess the expression level of Mst1 in plasma samples and associated them with the survival of patients. A total of 98 human plasma samples were assayed by ELISA using Mst1/STK4 (C-term) antibodies. The Mst1/STK4 purified protein was included in the assay system as a positive control for specificity and sensitivity, as well as to create a calibration curve. Each assay was repeated three times.

In brief, flat-bottom 96-well Costar plates were coated with 100 µl per well of rabbit polyclonal antibody specific for human Mst1/STK4 (cat. no. 3682; Cell Signaling Technology, Inc., Danvers, MA, USA) at a concentration of 1 µg/ml in carbonate buffer (15 mM Na2CO3, 35 mM NaHCO3, pH 9.6) as previously described (31). Following an overnight incubation at 4°C, the plates were washed three times with PBS-Tween-20 (PBST; 1.47 mmol/l KH2PO4, 8.10 mmol/l Na2HPO4, 136.89 mmol/l NaCl, 2.68 mmol/l KCl, 0.05% Tween 20), blocked with blocking buffer [1% bovine serum albumin (BSA; w/v; cat. no. A3858; Sigma-Aldrich; Merck KGaA, Darmstadt, Germany) in PBST] for 1 h at 37°C, followed by washing with PBST three times. Subsequently, the clinical plasma samples were diluted 5-fold (1:5) in sample diluent (1% BSA in PBST). Pre-diluted samples (100 µl) was added into micro ELISA plate wells. PBS served as a blank control. Following incubation for 2 h at 37°C, the wells were again washed and filled with 100 µl of a 1:10,000 dilution of horseradish peroxidase-conjugated goat anti-rabbit antibody (cat. no. 1662408EDU; Bio-Rad Laboratories, Inc., Hercules, CA, USA). Following incubation for 1 h at 37°C, plates were again washed with PBST, and wells were filled with 100 µl 3,3',5,5'-tetramethylbenzidine substrate solution and incubated for 15 min at room temperature in the dark. The reaction was stopped by adding 50 µl 2 mol/l H2SO4 per well. The optical density of each well was measured at a wavelength of 450 nm in the ELISA plate reader. Calibration curves were generated with log10 Mst1/STK4 purified protein concentrations plotted along the x-axis. If the detected values were higher than average, plasma samples were judged as positive.

Statistical analysis. Data were analyzed by SPSS standard version 20.0 (IBM SPSS, Armonk, NY, USA). The Kaplan-Meier method was used to estimate OS and DFS. A log rank test was used to compare the survival curves. Multivariate analysis was performed by Cox proportional hazards model. OS was calculated from the date of diagnosis to the date of mortality or the last follow-up. DFS was calculated from the date of surgery to the date of disease relapse. The differences between groups were analyzed using an unpaired two-tailed Student's t-test. All P-values were two-tailed. P<0.05 was considered to indicate a statistically significant difference.
Results

**Patient characteristics.** Characteristics of the 98 patients enrolled in the present study are summarized in Table I. No patients succumbed and no patients withdrew during the study period. The follow-up time was 98 months.

**Mst1 levels in patients with breast cancer.** A total of 98 human plasma samples were assayed by ELISA. The average Mst1 value of 98 human plasma samples was 1.8 µg/ml. Profiles of immunoglobulin IgG antibodies against human plasma Mst1 antigens were estimated by indirect ELISA (Fig. 1). The average concentration of 1.8 µg/ml was used to discriminate the status of Mst1-positive vs. Mst1-negative breast cancers.

The IgG level of Mst1-positive vs. Mst1-negative patients was significantly different (P<0.0001; t=9.167). In total, 85 Mst1-positive and 13 Mst1-negative patients with breast cancer were identified.

**Association of Mst1 levels with OS and DFS.** To evaluate the significance of Mst1 as a clinical prognostic factor in patients with breast cancer, the two groups of patients were followed up, and the associations between OS, DFS and Mst1 levels were investigated. Patients with positive expression of Mst1 had a significantly improved OS and DFS compared with patients with negative Mst1 expression (P<0.0001; Fig. 2A and B). Univariate Cox analysis indicated that Mst1 positivity had a significant difference in OS in patients with breast cancer (P=0.010). In multivariate Cox analysis, Mst1 positivity maintained significance as an independent prognostic factor in breast cancer (P=0.002; Table II).

**Associations between OS, DFS and clinicopathological features.** As expected, OS and DFS were significantly improved in patients with Her2-negative breast cancer (Fig. 2C, P=0.0438; Fig. 2D, P=0.0078), lymph node-negative breast cancer (Fig. 2E, P=0.0044; Fig. 2F, P=0.0379), stage 1 and 2 breast cancer (Fig. 2G, P<0.0001; Fig. 2H, P=0.0001) and tumor size <2 cm (Fig. 2I, P=0.0019; Fig. 2J, P=0.0121). Classification of grades and pathological types (ductal vs. all others) did not reveal a prognostic significance (Fig. 3A-D). In addition, no prognostic significance was observed in the comparison of molecular subtypes of breast cancer (luminal vs. others; Fig. 3E and F).

Discussion

Breast cancer is the most common type of non-cutaneous cancer and the leading cause of cancer-associated mortality among women (1-4). Along with demographical aging and cancer risk factors associated with modern lifestyle, female breast cancer incidence rates demonstrate a rising tendency (5). Screening techniques remain a...
crucial part of prevention and reduction of breast cancer mortality (1).

Diagnostic tumor markers are gaining increasing importance as prognostic and predictive factors (32-34). Current breast cancer markers, including hormone receptor status, tumor-node-metastasis grading are not sufficient, since breast cancer is complex, heterogeneous and alterable (35). Oncologists aim to identify high risk individuals, detect cancer at an early stage, predict outcome, monitor treatment and screen for disease recurrence. During tumor progression, metastasis and anticancer therapy, molecular changes result in various constellations of potential marker proteins (33,36,37). To date, comparatively few markers have been established (38). Therefore, it is crucial to identify easily detectable, non-invasive, novel biological markers with predictive power.

The results of the present study are promising for the use of Mst1 level as an outcome predictor in patients with breast cancer. Mst1 overexpression has been reported to inhibit the growth of human non-small cell lung cancer in vitro and in vivo, reduce intestinal stem cell proliferation and colonic tumorigenesis, inhibit cell proliferation and induce apoptosis.
Table II. Univariate and multivariate analysis of overall survival by the Cox proportional hazards model.

| Clinicopathological variables | Univariate analysis | Multivariate analysis |
|-------------------------------|---------------------|-----------------------|
|                               | HR (95% CI)         | P-value               | HR (95% CI)         | P-value               |
| Age                           | 0.999 (0.922-1.082) | 0.982                 | 0.696 (0.388-1.247) | 0.224                 |
| Tumor size                    | 15.987 (5.135-49.777)| 0.000                 | 0.079 (0.000-233.758)| 0.534                 |
| Tumor stage                   | 12.569 (3.514-44.961)| 0.000                 | 9.002x10^0 (0.000-5.364x10^34) | 0.801                 |
| Histological grade           | 0.750 (0.205-2.743) | 0.663                 | 0.081 (0.001-5.026) | 0.233                 |
| Lymph node status             | 6.811 (1.471-31.545) | 0.014                 | 7.377(0.581-93.673) | 0.123                 |
| Histological type             | 1.768 (0.659-4.740) | 0.257                 | 1.860 (0.185-18.743) | 0.598                 |
| Molecular subtypes            | 1.347 (0.822-2.207) | 0.237                 | 7.377 (0.581-93.673) | 0.239                 |
| ER/PR status                  | 0.757 (0.222-2.587) | 0.657                 | 0.002 (0.000-16.843) | 0.172                 |
| Her2 status                   | 2.187 (0.793-6.033) | 0.130                 | 5.215x10^0 (0.347-7.837x10^7) | 0.081                 |
| Mst1                          | 1.157 (1.065-1.257) | 0.010                 | 1.445(1.251-1.670) | 0.002                 |

HR, hazard ratio; CI, confidence interval; ER, estrogen receptor; PR, progesterone receptor; Her2, human epidermal growth factor receptor; Mst1, mammalian sterile 20-like kinase 1.

Figure 3. Kaplan-Meier survival analysis curves representing (A) overall and (B) disease free survival according to tumor grade, (C) overall and (D) disease free survival according to pathological type and (E) overall and (F) disease free survival according to subtype. Statistical significance is indicated.
of HepG2 cells, and induce cisplatin chemosensitivity in hepatocellular carcinoma (20). In colon cancer, nuclear Mst1 expression was associated with tumor grade and shortened survival time (39). Loss of cytoplasmic Mst1 expression is a marker of tumor progression in mismatch-repair-proficient as well as mismatch-repair-deficient colorectal cancers (39). Methylation of the Mst1 promoter is associated with a significantly decreased risk of tumor-associated mortality in patients with soft tissue sarcomas, while alterations of the Mst signaling pathway contribute to poor prognosis (40).

Mst1 is a member of the yeast Ste20-related kinase family and a component of the Ras association domain family member 1-large tumor suppressor kinase 1 tumor suppressor network (41,42). Although its physiological function remains to be fully established, it has been proposed as a tumor suppressor protein due to its association with cell proliferation and apoptosis (43). Mst1 is also involved in diverse biological processes, including cellular responses to oxidative stress and longevity (43). Deregulation of these fundamental developmental processes may lead to cancer. The molecular mechanisms are known in Drosophila, where the Hippo signaling pathway controls organ size by restricting mitosis and promoting cell death. In mammals, Mst1, a murine homolog of the Drosophila Hippo, contributes to size control of certain organs, but not all (44).

In the present study, the Mst1 levels of 98 patients with breast cancer with a follow-up period of 98 months were analyzed, and the association of Mst1 levels with survival and clinicopathological characteristics of patients were assessed. In contrast to our previous study (30), a more exact quantification method of ELISA was performed. Using this method obtained objective numeric values (Mst concentration) rather than biased immunohistochemistry-based observations on Mst1 amounts. Additionally, the plasma of patients was used as the detection material. This sampling is easier and more feasible than tumor tissues. To summarize, a novel, easy and effective way to assess Mst1 levels in patients with breast cancer was proposed, which may be further used to predict their prognosis and therapy response.

A cut off was established, and patients were divided into Mst1-positive and Mst1-negative groups. It was revealed that Mst1 positivity was significantly associated with OS, and Mst1-positive patients had an improved OS and DFS compared with Mst1-negative patients. Multivariate analysis also indicated that Mst1 positivity was an independent prognostic factor for breast cancer.

The present, long-term, follow-up study demonstrated that Mst1 expression has prognostic significance in patients with breast cancer and may present potential opportunities for breast cancer therapy in the future.

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