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Sineoculis homeobox homolog 1 protein is associated with breast cancer progression and survival outcome

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ABSTRACT

Sineoculis homeobox homolog 1 (SIX1) is one of the transcription factors that act as master regulators of development and is frequently dysregulated in cancer. This study explores the roles of SIX1 in tumor progression and as a prognostic determinant of breast cancer. Breast cancer specimens from 262 patients were selected for analysis of SIX1 protein by immunohistochemistry (IHC). The localization of SIX1 protein was detected in MDA-MB468 breast cancer cells using immunofluorescence (IF) staining. The survival rates were calculated by the Kaplan–Meier method, and the relationship between prognostic factors and patient survival was also analyzed by the Cox proportional hazard models. SIX1 protein mainly showed cytoplasmic/perinuclear staining pattern in breast cancer using IHC in paraffin embedded breast cancer tissues and IF in MDA-MB468 cancer cells. The strongly positive rate of SIX1 protein was 61.8% (162/262) in breast cancer and 23.1% (12/52) in ductal carcinoma in situ (DCIS), which was significantly higher than adjacent normal breast tissues (6.7%, 3/45). SIX1 overexpression was positively correlated with clinical stage, lymph node metastasis, Her2 expression status, and disease-free survival (DFS) and 5-year overall survival (OS) rates of patients with breast cancer. Moreover, patients with late stage breast cancer and high SIX1 expression had poorer survival rates than those with low SIX1 expression. Further analysis using a Cox proportional hazard regression model revealed that high SIX1 expression emerged as a significant independent hazard factor for the DFS and OS rates of patients with breast cancers along with Her2 status and clinical stage. SIX1 may potentially be used as an independent biomarker for prognostic evaluation of breast cancer.

Introduction

Breast cancer is currently the most common malignant tumor in women, with increasing morbidity and mortality. Remarkable progress has been made in the fields of early diagnosis and adjuvant therapy (Al-Allak et al., 2012; Jiang et al., 2012). Unfortunately, there is often a widening gap between the available treatments developed from a better understanding of the biology of breast cancer, and the management of patients in clinical practice, in which the development and aggressiveness of breast cancer are not precisely known (Sperduti et al., 2013). Therefore, the biological and clinical features of breast cancer have prompted us to identify promising new biomarkers to close this gap.

SIX1 is a member of the subfamily of the SIX class of homeodomain-containing transcription factors, which share a lysine in the DNA-binding helix of the homeodomain (Oliver et al., 1995). Vertebrate SIX1 functions in the development of diverse organs including the brain, ear, eye, muscle and kidney (Laclef et al., 2003; Ozaki et al., 2004; Relaix et al., 1999; Xu et al., 2003; Zheng et al., 2003). During normal development, SIX1 stimulates the proliferation and survival of progenitor cells (Behbakht et al., 2007). Loss of function of SIX1 results in a reduction in size or absence of various organs, resulting from a decrease in proliferation and an increase in apoptosis (Li et al., 2003; Ozaki et al., 2004; Zheng et al., 2003). Notably, SIX1 overexpression has been strongly associated with aggressive, metastatic cancers and poor prognosis (Behbakht et al., 2007; Coletta et al., 2004; Yu et al., 2004). Moreover, alteration of SIX1 expression has been reported in human breast and Wilms' cancer as well as rhabdomyosarcomas, indicating its possible contribution in the tumorigenicity of different cancers (Ford et al., 1998; Li et al., 2002; Yu et al., 2004). However, the expression level of SIX1 in breast cancer and its correlation with the clinical outcome of the disease are unknown.

Here we performed immunohistochemical (IHC) staining of SIX1 protein in 262 breast cancer tissues, and found that SIX1 protein was frequently upregulated in breast cancer compared with the adjacent non-tumor tissues. SIX1 overexpression in breast cancer was associated

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with clinical stage, lymph node metastasis, and Her2 expression levels. Multivariate analysis revealed that SIX1 might be an independent biomarker for the prediction of breast cancer prognosis.

Materials and methods

Ethics statement

This research complied with the Helsinki Declaration and was approved by the Human Ethics Committee and the Research Ethics Committee of Yanbian University Medical College. Patients were informed that the resected specimens were stored by the hospital and potentially used for scientific research, and that their privacy would be maintained. Follow-up survival data were collected retrospectively through medical record analyses.

Clinical samples

A total of 262 breast cancer tissue samples were collected from Shanghai Outdo Biotech Co. Ltd. and Tumor Tissue Bank of Yanbian University Medical College. All samples were routinely fixed in 10% buffered formalin and embedded in paraffin blocks. The study protocol was approved by the institutional review board of Yanbian University Medical College. The pathological parameters, including age, histological grade, clinical stage, nodal metastasis and survival data, were carefully reviewed in all 262 breast cancers. The patients' ages ranged from 31 to 76 years with a mean age of 53.2 yrs. For grading of breast cancer, 103 cases were G1, 95 cases were G2, and 64 cases were G3. For staging of breast cancer, 160 cases were TNM stages 0–II, and 102 cases were TNM stages III–IV. TNM staging was assessed according to the staging system established by the American Joint Committee on Cancer (Li et al., 2003).

Patients with breast cancer received surgical treatment with a curative intent and did not receive adjuvant chemotherapy at the time of data collection. The study endpoint was defined as disease-specific survival. The mean follow-up time was 12.14 ± 4.98 months.

Immunofluorescence (IF) staining

Breast cancer MDA-MB468 cells were grown on coverslips to 70% confluence. The cells were fixed with 4% paraformaldehyde for 10 min, and after 24 h cells were permeabilized with 0.5% TritonX-100 for 10 min. Blocking was performed with 3% Albumin Bovine V (A8020, Solarbio, Beijing, China) for 1 h at room temperature. After washing with PBS, cells were incubated with anti-rabbit SIX1 (HPA001893, 1:100, Sigma-Aldrich, Sweden) at 4 °C overnight, followed by incubation with Alexa Fluor 488 Goat Anti-Rabbit IgG (H + C) (A11008, 1:1000, Invitrogen, USA) for 1 h at room temperature. After washing with PBS, the cells were counterstained with DAPI (C1006, Beyotime, Shanghai, China), and the coverslips were mounted with an Antifade Mounting Medium (P0126, Beyotime, Shanghai, China). IF signals were visualized and recorded with a Leica SP5II confocal microscope (Jin et al., 2012).

IHC staining

The Dako LSAB kit (Dako, Glostrup, Denmark) was used for immunohistochemistry. Serial 4 μm-thick tissue sections were prepared on silane-coated slides (Sigma, St. Louis, MO, USA), and deparaffinized, rehydrated and incubated with 3% H_2O_2 in methanol for 10 min at room temperature to eliminate endogenous peroxidase activity. The antigen was retrieved at 95 °C for 20 min by placing the slides in 10 mM sodium citrate buffer (pH 6.0). Slides were then incubated with the primary antibody, SIX1 (1:100, HPA001893, Sigma-Aldrich), at 4 °C overnight. After incubation at room temperature for 30 min with biotinylated secondary antibody, slides were incubated with streptavidin–peroxidase complex at room temperature for 30 min. Slides were immunostained with 3,3′-diaminobenzidine chromogen and then counterstained with Mayer's hematoxylin. Rabbit IgG isotope was used as the negative control. Positive tissue sections processed without the primary antibody were used as the negative control (Elzagheid et al., 2002; Jin et al., 2012).

Analysis of IHC results

All slides were scored independently by two investigators (Lin Z and Jin H) who were blind to all clinical data. Interpretation criteria were as previously described (Jin et al., 2012). The immunostaining for SIX1 was mainly semi-quantitatively scored as '−' (negative, no or less than 5% positive cells), '+' (5–25% positive cells), '++' (26–50% positive cells) and '+++' (more than 50% positive cells). Cytoplasmic and/or nuclear expression patterns were considered positive staining, and strongly positive indicated ++ and +++ positive cells. For survival data analysis, SIX1 expression levels were determined as high expression (++ and +++) or low expression (− and +).

Statistical analysis

Statistical analysis was performed using SPSS version 17.0 software for Windows (SPSS, Chicago, IL, USA). Correlations between SIX1 expression and clinicopathological characteristics were evaluated using Chi-square tests (χ^2) and Fisher's exact tests. Bivariate correlations between study variables were calculated by Spearman's rank correlation coefficients. Survival rates were calculated using the Kaplan–Meier method, and differences in survival curves were analyzed by log-rank tests. Univariate and multivariate survival analyses were performed

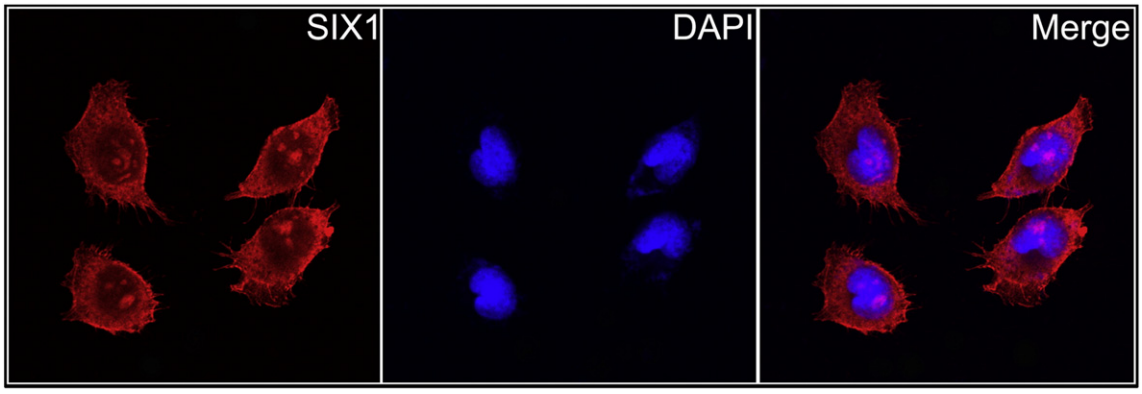

Fig. 1. Immunofluorescence staining for SIX1 protein in MDA-MB468 breast cancer cells. MDA-MB468 breast cancer cells were immunostained for SIX1 (red). Nuclei were visualized by DAPI staining. SIX1 protein is mainly located in the cytoplasm and nucleoli of MDA-MB468 cancer cells.