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Expression of autophagy-related proteins ATG5 and FIP200 predicts favorable disease-free survival in patients with breast cancer

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ABSTRACT

Autophagy is a self-digesting process that is primarily responsible for the removal and recycling of long-lived proteins and damaged organelles to maintain the homeostasis of the cell. Recent studies have indicated dual roles for autophagy in cancer: suppression of tumor progression and promotion of survival. In this study, we sought to investigate the prognostic value of two autophagy-related proteins, autophagy-related gene 5 (ATG5) and FAK family kinase-interacting protein of 200 kDa (FIP200), in patients with operable breast cancer. More specifically, the expression of ATG5 and FIP200 was evaluated by immunohistochemistry (IHC) in surgical specimens collected from 200 patients who were diagnosed with histologically proven invasive ductal breast cancer. A stepwise Cox multivariate analysis was then performed to construct a risk prediction model. In this retrospective cohort study, both ATG5 (HR = 0.465, 95% CI 0.247–0.872, $P = 0.017$) and FIP200 (HR = 0.521, 95% CI 0.278–0.979, $P = 0.043$) correlated with prolonged disease-free survival (DFS). In a receiver operating characteristic (ROC) analysis, the addition of ATG5 and FIP200 expression led to a significantly improved area under the time-dependent ROC curve (AUC) at 3 years (0.748 versus 0.680, $P < 0.001$) and 5 years (0.756 versus 0.699, $P < 0.001$). Collectively, our findings established the prognostic significance of ATG5 and FIP200 in patients with breast cancer.

1. Introduction

Adjuvant systemic therapy has been proven to be an effective and comprehensive approach to reducing the risk of recurrence and mortality in patients with operable breast cancer. The selection of a regimen should be individualized based on the risk recurrence category. Accordingly, numerous risk models for the development of breast cancer have been created, incorporating clinical factors with or without biomarker assays [1,2]. However, no risk prediction models are currently widely used in clinical practice. Thus, the identification of novel biomarkers that can provide additional valuable risk assessment of personalized treatment modalities is urgent.

In recent years, studies of autophagy, a conserved cellular catabolic process characterized by the self-digestion of organelles,

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have provided insight into the adaptation process for cells, which either allows tolerance of adverse conditions or triggers cell suicide mechanisms [3]. Emerging evidence regarding autophagy in cancer suggests a double-edged sword: autophagy can enable tumor cells to survive stress, including hypoxia, starvation, chemotherapy and radiotherapy, or can be tumor suppressive through the elimination of oncogenic protein substrates, toxic unfolded proteins and damaged organelles [4]. Recent studies have suggested that autophagy participates in broad crosstalk with multiple pathways that determine cell fate, including apoptosis [5].

We have previously reported the prognostic significance of the autophagy marker LC3B in neoadjuvant chemotherapy (NCT)-treated breast cancer [6]. However, in NCT-naïve specimens, the prognostic value of LC3B is confined to luminal A breast cancer [7], which prompted us to evaluate the survival correlations of other autophagy-related proteins. Autophagy involves the spatially and temporally coordinated activation of a number of molecules, among which both autophagy-related gene 5 (ATG5) and FAK family kinase-interacting protein of 200 kDa (FIP200) are key players; the deletion of either gene leads to an autophagy deficiency [8,9]. A preclinical study has shown that forced expression of ATG5 sensitizes tumor cells to anticancer drug treatment, whereas silencing ATG5 results in chemotherapy resistance [10]. However, the prognostic value of ATG5 in breast cancer has not been studied. FIP200, also known as RB1CC1, is an important component of the ULK1-FIP200-ATG13-ATG101 complex, which is functionally coupled to the negative autophagy regulator mTOR complex 1 (mTORC1) and which initiates autophagy. In particular, FIP200 inhibits G_1-S phase progression, proliferation and clonogenic survival in breast cancer cells [11]. Previous studies have demonstrated that the abnormal RB1CC1/RB1/p53 pathway is associated with poor long-term prognosis in breast cancer [12] and have revealed the prognostic significance of FIP200 in prostate cancer [13] and salivary gland cancer [14].

Our current knowledge about ATG5 and FIP200 has been largely derived from *in vitro* studies, and thus, the *in vivo* relevance of these proteins needs more rigorous investigation. In the present study, we investigated the prognostic value of ATG5 and FIP200 using the expression profiles of the two markers in 200 specimens from stage I to III breast cancer. We then developed a risk model for the development of recurrence and metastases in patients following breast cancer surgical resection, and we assessed the relationships between biomarkers, clinical patient characteristics, and disease-free survival (DFS). Our results demonstrated that ATG5 expression and FIP200 expression are strongly associated with DFS and could serve as novel prognostic markers in breast cancer.

2. Materials and methods

2.1. Breast cancer specimens

In this study, we collected clinical data and surgical specimens from 200 female patients who were diagnosed with stage I to III primary breast cancer at the Department of Breast Surgery in Fudan University Shanghai Cancer Center (FDUSCC, Shanghai, China) between August 2001 and March 2006. All patients in this cohort were histologically confirmed as having invasive ductal carcinoma and either underwent a mastectomy and axillary lymph node dissection or breast conservation surgery. DFS was calculated from the date of surgery to the date of disease relapse at a local, regional or distant site. Patients with a study end date or who were lost to follow-up were considered as censored. This study was approved by the Ethics Committee of FDUSCC, and written informed consent was provided by all patients.

2.2. Tissue microarray construction

The tissue microarray (TMA) was constructed as described before [15].

2.3. Immunohistochemistry

TMAs were subjected to immunohistochemical staining for the ATG5 and FIP200 proteins using a 2-step protocol (GTVision™ III). ATG5 was detected using the rabbit anti-ATG5 polyclonal antibody 10181-2-AP (1:50; Proteintech Group), and FIP200 was detected using the rabbit anti-FIP200 polyclonal antibody 16172-1-AP (1:100; Proteintech Group). The negative controls consisted of phosphate-buffered saline (PBS) instead of primary antibodies. Positive controls were established according to the instructions provided with the antibodies. Detailed methods for the immunohistochemistry (IHC) are presented in the Supplementary Materials.

2.4. Evaluation of immunostaining for ATG5 and FIP200

As in our previous study [15,16], the expression of ATG5 was semi-quantitatively classified according to the staining index (SI; range 0–9), which was calculated by multiplying the staining intensity by the proportion score. The staining intensities were classified according to four grades (0 denoting negative; 1, weak; 2, moderate; and 3, strong), and the proportion score was graded as the percentage of cells that were stained (1 denoting 0 to < 10% of cells; 2, between 10 and 50% of cells; and 3, staining of >50% of cells). In this study, SI $\geq$ 5 was defined as ATG5-positive staining, whereas SI < 5 was defined as negative staining. Additionally, as described by Ikebuchi et al.[17], the grades of FIP200 expression were classified into 3 categories: cytoplasm-/nuclei- (Grade I), cytoplasm+/nuclei- (Grade II), or cytoplasm + or -/nuclei+ (Grade III). Grade III FIP200 expression was defined as positive, whereas Grade I or II FIP200 expression was negative. The scores were evaluated independently by two experienced pathologists who were blinded to all clinical data on a case-by-case basis, and the score used in all subsequent analyses was the average across the available scores.

2.5. Statistical analysis

The Pearson χ^2 test was used to compare qualitative variables, and Fisher's exact test was performed when necessary. We constructed prognostic models for DFS events using univariate and multivariate Cox analyses. Risk scores and Time-dependent receiver operating characteristic (ROC) curves were computed as described before [18]. $P < 0.05$ was considered to be statistically significant. All statistical analyses were completed using R 3.1.2 (R Development Core Team, Vienna, Austria).

3. Results

3.1. Expression patterns of ATG5 and FIP200 in breast cancer

The clinicopathological characteristics of the 200 participants, who had an average age of 52.2 years (SD 9.7, median 51.5, range 29–85), are presented in Table 1. After a median follow-up time of 7.8 years, 51 of the 200 cases have experienced recurrence or death. To investigate the clinical implications of ATG5 and FIP200 in breast cancer, we performed an immunohistochemical examination of the cohort (Fig. 1). As shown in Table 1, positive ATG5 staining was observed in 81 (40.5%) of the 200 cases according to the scoring criteria described in Materials and methods section. Moreover,

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