RESEARCH ARTICLE

High Levels of Nucleolar Spindle-Associated Protein and Reduced Levels of BRCA1 Expression Predict Poor Prognosis in Triple-Negative Breast Cancer

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Abstract

Purpose

Nucleolar spindle-associated protein (NuSAP1) is an important mitosis-related protein, and aberrant NuSAP1 expression is associated with abnormal spindles and mitosis. This study investigated the prognostic value of NuSAP1 in breast cancer.

Methods

Two sets of tissue microarrays (TMAs) that included samples from 450 breast cancer patients were constructed, of which 250 patients were training set and the other 200 patients were validation set. Immunohistochemical staining was performed to determine the NuSAP1 levels. A Kaplan-Meier analysis was used to estimate the prognostic value of NuSAP1 in breast cancer. A stepwise Cox analysis was performed to construct a risk-prediction model for triple-negative breast cancer (TNBC). All statistical analysis was performed with SPSS software.

Results

There were 108 (43.5%) and 88 (44.0%) patients expressed NuSAP1 in the training set and validation set respectively. High levels of NuSAP1 expression were related to poor disease-free survival (DFS) in both training ($P = 0.028$) and validation ($P = 0.006$) cohorts, particularly in TNBC. With combination of two cohorts, both NuSAP1 (HR = 4.136, 95% CI: 1.956–8.747, $P < 0.001$) and BRCA1 (HR = 0.383, 95% CI: 0.160–0.915, $P = 0.031$) were independent prognostic indicators of DFS in TNBC. A receiver operating characteristic (ROC) analysis revealed that the combination of NuSAP1 and BRCA1 significantly improved the prognostic power compared with the traditional model (0.778 versus 0.612, $P < 0.001$).
Conclusions

Our study confirms the prognostic value of NuSAP1 in breast cancer. The combination of NuSAP1 and BRCA1 could improve the DFS prediction accuracy in TNBC.

Introduction

Breast cancer is the most common type of cancer in women worldwide, and approximately 1.2 million new cases and 465,000 deaths occur each year[1, 2]. Therefore, breast cancer is one of the most serious health problems for women. Early diagnosis and timely treatment are the most effective strategies for fighting breast cancer. However, an effective marker for breast cancer diagnosis or prognosis has not yet been identified. Increasing amounts of evidence indicate that cancers are often heterogeneous and that the response to treatment depends on the subtype of breast cancer[3, 4]. Treatment with the guidance of molecular subtypes is important. Triple-negative breast cancer (TNBC) is a subtype of breast cancer with estrogen receptor (ER) negative, progesterone receptor (PR) negative, and human epidermal growth factor receptor 2 (HER-2) negative. BRCA1 is responsible for DNA repair and has been closely related to breast cancer, particularly TNBC[5–7]. More recently, the androgen receptor (AR) has been identified as a new marker of a specific subtype of TNBC[8–10]. However, with high heterogeneity, treatment of TNBC has always been a challenge. Therefore, additional efforts should be expanded to identify new indicators of breast cancer prognosis, especially for TNBC.

During mitosis, accurate cell division is required for the generation of two genetically identical daughter cells. The entire process must be performed with high fidelity to ensure that the duplicated chromosomes are equally distributed, and this process requires the coordinated operation of numerous proteins. Nucleolar-spindle associated protein (NuSAP1) is a microtubule- and chromatin-binding protein that stabilizes microtubules to prevent depolymerization, maintains spindle integrity, and further cross-links spindles into aster-like structures, fibers and networks[11–14]. NuSAP1 is transported into the nucleolus by importins and localizes to the chromatin-proximal microtubules throughout metaphase and anaphase. NuSAP1 is essential for mitosis from the stages of spindle assembly to cytokinesis. The overexpression of NuSAP1 results in the profound bundling of spindle microtubules. In contrast, the depletion of NuSAP1 by RNA interference results in G2-M arrest, aberrant mitotic spindles, cytokinesis, reductions in spindle microtubules, and abnormal chromosome segregation. Consequently, the aberrant expression of NuSAP1 has been associated with defective embryogenesis and cancer.

NuSAP1 is overexpressed and related to poor prognosis in hepatic carcinomas[15]. NuSAP1 has also been related to lung adenocarcinoma, cervical cancer, melanoma, meningioma, pituitary adenoma, and prostate cancer[16–20]. In the setting of breast cancer, Dilek Colak et al. reported that NuSAP1 expression significantly differs between ductal carcinoma in situ (DCIS) and invasive ductal carcinoma (IDC)[21]. Therefore, NuSAP1 might be involved in tumorigenesis and progression. However, the NuSAP1 expression status of the subtypes of breast cancer remains unknown. The current study investigated the correlation between NuSAP1 expression and the prognosis of different subtypes of breast cancer, particularly TNBC.
Materials and Methods

Ethics Statement

The study has been approved by the Human Research Ethics Committee of Fudan University Shanghai Cancer Center, Shanghai, China. The approved number of ethics committee is 050432–4. Written informed consent was provided by all patients. All samples and medical data used in this study have been anonymized.

Patients and specimens

The present study included 450 patients who were diagnosed with stage I to III primary breast cancer from August 2001 to March 2006 according to histopathological analysis conducted at Fudan University Shanghai Cancer Center (FDUSCC). There were a training set and a validation set with 250 and 200 patients, respectively. All patients were subjected to either a mastectomy and axillary lymph node dissection or breast conservation surgery. The clinico-pathological information, including age, menopausal status, tumor size, lymph node status, grade, ER, progesterone receptor (PR), HER-2 status, and TNM stage, was collected and shown in Table 1. Patients were further classified into four subtypes according St Gallen International Breast Cancer Conference (2011) Expert Panel[22]. All patients were regularly followed, and the median follow-up time was 96 months.

Breast cancer tissue microarray construction

The breast cancer tissue samples used to construct the tissue microarrays (TMAs) were obtained before treatment, fixed in formalin and embedded in paraffin. Tumor regions were stained with hematoxylin and eosin (HE) to identify representative tumor regions from which two 1.0-mm tissue cores were retrieved and transferred into recipient array blocks using a tissue micro arrayer (UNITMA Instruments, Seoul, Korea). TMAs were composed of duplicate cores from different areas of the same tumor to compare staining patterns in our research. Two sets of TMAs were generated by the Department of Pathology of FDUSCC with 250 patients and 200 patients, respectively.

Immunohistochemistry

The TMAs were subjected to immunohistochemical staining for the NuSAP1 and BRCA1 proteins with a 2-step protocol (GTVisionTMIII). NuSAP1 was detected with a rabbit anti-NuSAP1 polyclonal antibody (Proteintech Group, Chicago, IL, USA), and BRCA1 was detected with mouse anti-BRCA1 (Santa Cruz Biotechnology, Dallas, Texas, USA). The TMAs were deparaffinized with xylene, gradually rehydrated in a gradient ethanol series and then rinsed with phosphate-buffered saline (PBS) prior to NuSAP1 or BRCA1 immunohistochemical staining. Antigen retrieval was performed by immersing the sections in 0.01 M Tris–sodium citrate (pH 6.0). After boiling at 121°C for 10 min, the sections were incubated with NuSAP1 or BRCA1 for 2 minutes. After blocking for 20 minutes, the slides were subjected to anti-NuSAP1 (1:200) or anti-BRCA1 (1:200) primary antibodies at 4°C overnight. HRP-conjugated secondary antibodies were used to detect the primary antibodies with subsequent colorimetric detection using 3, 3-diaminobenzidine (DAB). The TMAs were then counterstained with Gill hematoxylin and dehydrated in an ascending ethanol series before being cleared with xylene and mounted with a coverslip.
Table 1. Correlation between clinicopathologic variables and expression of NuSAP1.

| Variables                      | Training set | Validation set |  |
|--------------------------------|--------------|----------------|------------------|
|                                | Number of patients | NuSAP1 expression (%) | Positive n (%) | P<sup>a</sup> value | Number of patients | NuSAP1 expression (%) | Positive n (%) | P<sup>a</sup> value |
| Total                          | 248          | 140(56.5)      | 108(43.5)       | 0.275                | 200              | 112(56.0)           | 88(44.0)        | 0.053 |
| Age                            |              |                |                 |                      |                  |                   |                |                  |
| ≤ 50                           | 120          | 72(29.0)       | 48(19.3)        | 0.053                | 113              | 70(35.0)           | 43(21.5)        | 0.285 |
| > 50                           | 128          | 68(27.5)       | 60(24.2)        |                      | 87               | 42(21.0)           | 45(22.5)        | 0.944 |
| Menopausal status              |              |                |                 |                      |                  |                   |                |                  |
| Premenopause                   | 108          | 59(23.8)       | 49(19.8)        | 0.611                | 121              | 68(34.0)           | 53(26.5)        | 0.682 |
| Postmenopause                  | 137          | 81(32.7)       | 59(23.7)        |                      | 79               | 44(22.0)           | 35(17.5)        |                 |
| Tumor size                     |              |                |                 |                      |                  |                   |                |                  |
| ≤ 2 cm                         | 115          | 66(26.2)       | 50(20.2)        | 0.529                | 101              | 51(25.5)           | 50(25.0)        | 0.285 |
| > 2 cm, ≤ 5 cm                 | 119          | 65(26.2)       | 54(21.8)        |                      | 91               | 56(28.0)           | 35(17.5)        |                 |
| > 5 cm                         | 14           | 10(4.1)        | 4(1.5)          |                      | 8                | 5(2.5)             | 3(1.5)          |                 |
| Lymph node status              |              |                |                 |                      |                  |                   |                |                  |
| Negative                       | 151          | 85(34.3)       | 66(26.6)        | 0.949                | 99               | 54(27.0)           | 45(22.5)        | 0.682 |
| Positive                       | 97           | 55(22.2)       | 42(16.9)        |                      | 101              | 58(29.0)           | 43(21.5)        |                 |
| Grade                          |              |                |                 |                      |                  |                   |                |                  |
| 1                              | 5            | 3(1.2)         | 2(0.8)          | 0.627                | 2                | 0(0.0)             | 2(1.0)          | 0.158 |
| 2                              | 183          | 100(40.4)      | 83(33.5)        |                      | 142              | 77(38.5)           | 65(32.5)        |                 |
| 3                              | 60           | 37(14.9)       | 23(9.2)         |                      | 56               | 35(17.5)           | 21(10.5)        |                 |
| ER status                      |              |                |                 |                      |                  |                   |                |                  |
| Negative                       | 143          | 82(33.1)       | 61(24.6)        | 0.741                | 112              | 63(31.5)           | 49(24.5)        | 0.936 |
| Positive                       | 105          | 58(23.4)       | 47(18.9)        |                      | 88               | 49(24.5)           | 39(19.5)        |                 |
| PR status                      |              |                |                 |                      |                  |                   |                |                  |
| Negative                       | 185          | 106(42.7)      | 79(31.9)        | 0.710                | 113              | 66(33.0)           | 47(23.5)        | 0.434 |
| Positive                       | 63           | 34(13.8)       | 29(11.6)        |                      | 87               | 46(23.0)           | 41(20.5)        |                 |
| HER-2 status                   |              |                |                 |                      |                  |                   |                |                  |
| Negative                       | 148          | 86(34.7)       | 62(25.0)        | 0.522                | 100              | 53(26.5)           | 47(23.5)        | 0.393 |
| Positive                       | 100          | 54(21.8)       | 46(18.5)        |                      | 100              | 59(29.5)           | 41(20.5)        |                 |
| BRCA1 (IHC)                    |              |                |                 |                      |                  |                   |                |                  |
| Negative                       | 128          | 83(33.5)       | 45(18.1)        | 0.006                | 106              | 69(34.5)           | 37(18.5)        | 0.006 |
| Positive                       | 120          | 57(23.0)       | 63(25.4)        |                      | 94               | 43(21.5)           | 51(25.5)        |                 |
| TNM                            |              |                |                 |                      |                  |                   |                |                  |
| I                              | 74           | 42(16.9)       | 32(12.9)        | 0.924                | 65               | 33(16.5)           | 32(16.0)        | 0.514 |
| II                             | 133          | 76(30.5)       | 57(23.0)        |                      | 126              | 73(36.5)           | 53(26.5)        |                 |
| III                            | 41           | 22(8.9)        | 19(7.6)         |                      | 9                | 6(3.0)             | 3(1.5)          |                 |
| Subtype                        |              |                |                 |                      |                  |                   |                |                  |
| Luminal A                      | 48           | 29(11.7)       | 19(7.7)         | 0.063                | 50               | 29(14.5)           | 21(10.5)        | 0.169 |
| Luminal B                      | 57           | 29(11.7)       | 28(11.3)        |                      | 50               | 25(12.5)           | 25(12.5)        |                 |
| Her-2 overexpression           |              |                |                 |                      |                  |                   |                |                  |
| Triple-negative                | 100          | 57(23.0)       | 43(17.3)        | 0.006                | 50               | 24(12.0)           | 26(13.0)        | 0.169 |

Abbreviations: NuSAP1, Nucleolar spindle-associated protein; ER, estrogen receptor; PR, progesterone receptor; HER-2, human epidermal growth factor receptor 2; BRCA1, breast cancer type 1 susceptibility protein.

<sup>a</sup> <i>P</i> value was calculated using Pearson's χ<sup>2</sup>.

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Evaluation of the immunostaining for NuSAP1 and BRCA1

For each antibody, the TMAs were stained and semi-quantitatively scored according to a staining index (SI; range 0–9) with the following formula: SI = intensity × proportion scores. The staining intensities were classified into three grades (1: weak, 2: moderate, and 3: strong), and proportion scores were assigned based on the percentages of stained cells (0:0%, 1: < 10%, 2: 10–50%, and 3: 50–100%). For NuSAP1 and BRCA1, SIs ≥ 5 were considered positive staining, whereas SIs < 5 were defined as negative staining. Two experienced pathologists who were blinded to all clinical data conducted the scoring in parallel.

Statistical analysis

The associations between the clinicopathological parameters and NuSAP1 expression were evaluated with Pearson χ², and Fisher’s exact tests as appropriate. A Kaplan-Meier (KM) analysis and log-rank test were performed to determine the correlation between NuSAP1 expression and disease-free survival (DFS) and overall survival (OS). Univariate and multivariate analysis of the DFS were performed with Cox risk proportion models. P < 0.05 was considered to indicate significant differences. The statistical analysis was performed using SPSS (version 13.0; SPSS, Chicago, IL, USA).

Results

Clinicopathological characteristics and NuSAP1 expression in breast cancer patients

In training set, a total of 250 female breast cancer samples were collected, but two of the samples lacked follow-up data. Thus, the remaining 248 samples were included in the subsequent analysis. All patients were female and had been diagnosed with stages I to III primary breast cancer at a median age of 51 years. The ER, PR, and HER-2 statuses were collected, and the patients were classified into four subtypes, i.e., Luminal A, Luminal B, HER-2 overexpression, and TNBC. The ER, PR and HER-2 subtypes were defined based on immunohistochemistry (IHC) and fluorescence in situ hybridization (FISH) results. Of the patients, 19.4%, 23.0%, 17.3% and 40.3% were classified as Luminal A, Luminal B, HER-2 overexpression, and TNBC subtypes, respectively. To investigate the clinical function of NuSAP1 in breast cancer, its expression in the cohort was examined by immunohistochemistry (Fig 1a). As shown in Table 1, 108 (43.5%) of the samples expressed NuSAP1 protein and 140 (56.5%) samples did not in training set. Specifically, among the 108 NuSAP1-positive patients, the Luminal A, Luminal B, HER-2 overexpression and Triple-negative subgroups included 19 (7.7%), 28 (11.3%), 18 (7.2) and 43 (17.3) patients, respectively. NuSAP1 expression was not related to ER, PR or HER-2 status (Table 1). However, the association between the expression of NuSAP1 and breast cancer subtypes bordered on significant (P = 0.063). As early as 1995, Marilyn E. Thompson et al. reported that BRCA1 expression decreases during the progression of breast cancer[23]. BRCA1 has long been known to be associated with breast cancer and ovarian cancer[24]. Interestingly, the expression levels of NuSAP1 and BRCA1 were significantly correlated in our patient cohort (P = 0.006). Similar correlation was found in validation set (Table 1, P = 0.006).

Univariate and multivariate analysis of breast cancer

Univariate analysis was performed to evaluate the correlations between clinicopathological parameters and DFS, and several factors were significantly associated. As shown in Table 2, in training set, tumor size > 5 cm, positive lymph node status, grade 3 status, and positive
NuSAP1 expression were associated with a greater risk of recurrence and a lower DFS ($P = 0.016$). In contrast, PR expression was related to improved DFS for all patients ($P = 0.033$). Accordingly, a stepwise multivariate analysis that included age, menopausal status, lymph node status, PR, and NuSAP1 expression was conducted. Menopausal status, lymph node status, PR, and NuSAP1 were identified as significant prognostic factors for DFS (Table 3, $P < 0.05$). In validation set, we found NuSAP1 was significantly prognostic for DFS (Tables 2 and 3, $P < 0.05$).

NuSAP1 expression was associated with poor DFS in breast cancer, particularly in TNBC

To explore the prognostic value of NuSAP1 for DFS and OS of the breast cancer patients, a KM analysis of all patients was performed. As shown in Fig 2a, the expression of NuSAP1 was generally associated with a poor DFS in both training ($P = 0.028$) and validation cohort ($P = 0.006$). In all patients with combination of two cohorts, similar trends were found with $P < 0.001$; further analysis of the prognostic value of NuSAP1 in four subtypes of breast cancer revealed that NuSAP1 expression was significantly correlated with poor DFS in triple-negative subgroup (Fig 2b, $P < 0.001$). However, non-significant differences were observed in the other three subgroups (Fig 2b).

NuSAP1 and BRCA1 were associated with DFS in TNBC

In a study of various gene profiles that further classified TNBC into six subtypes with distinct characteristics, Brian D. Lehmann identified BRCA1 as an important molecular marker of TNBC; BRCA1 was included among the gene sets in their study[25]. In the current study, a KM analysis was performed to verify the prognostic values of BRCA1 in TNBC, and the expression of BRCA1 was related to improved DFS in all TNBC with combination of training and validation cohort patients (Fig 3a, $P = 0.024$). This finding was in agreement with that in general breast cancer[26]. Moreover, univariate and multivariate analysis were performed in the TNBC group (Tables 4 and 5). As shown in Table 4, tumor size $> 5$ cm, positive lymph node status, and positive NuSAP1 expression were significantly associated with worse DFS ($P < 0.05$).
Table 2. Univariate analysis for disease-free survival.

| Variables        | Training set | Validation set |
|------------------|--------------|----------------|
|                  | HR (95% CI)  | P<sup>a</sup> value | HR (95% CI)  | P<sup>a</sup> value |
| Age              |              |                |              |                |
| <50 years        | 1            |                | 1            |                |
| >50 years        | 0.965 (0.566–1.647) | 0.897 | 0.873 (0.463–1.645) | 0.674 |
| Menopausal status|              |                |              |                |
| Premenopause     | 1            |                | 1            |                |
| Postmenopause    | 1.582 (0.905–2.767) | 0.108 | 1.333 (0.708–2.512) | 0.374 |
| Tumor size       |              |                |              |                |
| <2 cm            | 1            |                | 1            |                |
| >2, 5≤cm         | 1.338 (0.752–2.378) | 0.322 | 1.002 (0.134–7.504) | 0.998 |
| >5 cm            | 4.817 (2.030–11.429) | 0.000 | 1.182 (0.158–8.845) | 0.870 |
| Lymph node status|              |                |              |                |
| Negative         | 1            |                | 1            |                |
| Positive         | 2.175 (1.273–3.716) | 0.004 | 1.400 (0.742–2.640) | 0.298 |
| Grade            |              |                |              |                |
| 1 or 2           | 1            |                | 1            |                |
| 3                | 1.756 (1.004–3.071) | 0.048 | 1.098 (0.535–2.255) | 0.799 |
| ER status        |              |                |              |                |
| Negative         | 1            |                | 1            |                |
| Positive         | 0.836 (0.483–1.446) | 0.522 | 0.535 (0.275–1.043) | 0.066 |
| PR status        |              |                |              |                |
| Negative         | 1            |                | 1            |                |
| Positive         | 0.421 (0.190–5.0933) | 0.033 | 0.695 (0.361–1.338) | 0.276 |
| HER-2 status     |              |                |              |                |
| Negative         | 1            |                | 1            |                |
| Positive         | 1.013 (0.590–1.737) | 0.964 | 1.493 (0.775–2.874) | 0.230 |
| NuSAP1           |              |                |              |                |
| Negative         | 1            |                | 1            |                |
| Positive         | 1.948 (1.132–3.354) | 0.016 | 2.458 (1.272–4.750) | 0.007 |

Abbreviations: NuSAP1, Nucleolar spindle-associated protein; ER, estrogen receptor; PR, progesterone receptor; HER-2, human epidermal growth factor receptor 2; BRCA1, breast cancer type 1 susceptibility protein.

P<sup>a</sup> value was calculated using Pearson's χ.  
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Table 3. Multivariate analysis for disease-free survival.

| Variables        | Training set | Validation set |
|------------------|--------------|----------------|
|                  | HR (95% CI)  | P<sup>a</sup> value | HR (95% CI)  | P<sup>a</sup> value |
| Age              | 0.586 (0.304–1.128) | 0.110 | 0.835 (0.371–1.877) | 0.662 |
| Menopausal status| 2.010 (1.011–3.995) | 0.046 | 1.464 (0.648–3.308) | 0.359 |
| Lymph node status| 2.232 (1.298–3.837) | 0.004 | 1.553 (0.802–3.005) | 0.191 |
| PR               | 0.380 (0.171–0.846) | 0.018 | 0.691 (0.351–1.361) | 0.285 |
| NuSAP1           | 2.102 (1.220–3.621) | 0.007 | 2.606 (1.338–5.076) | 0.005 |

Abbreviations: NuSAP1, Nucleolar spindle-associated protein; PR, progesterone receptor.
P<sup>a</sup> value was calculated using Pearson's χ.  
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Fig 2. Low expression of NuSAP1 favored DFS, particularly for TNBC patients. Cumulative DFS curves for breast cancer patients classified as the total group (a) training set: NuSAP1+ (n = 108) and NuSAP1- (n = 140); validation set: NuSAP1+ (n = 88) and NuSAP1- (n = 112); overall: NuSAP1+ (n = 196) and NuSAP1- (n = 252); and (b) overall population luminal A, NuSAP1+ (n = 41) and NuSAP1- (n = 58); luminal B, NuSAP1+ (n = 53) and NuSAP1- (n = 55); HER-2 overexpression, NuSAP1+ (n = 33) and NuSAP1- (n = 35); triple-negative NuSAP1+ (n = 69) and NuSAP1- (n = 79) subgroups.

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contrast, positive BRCA1 expression was related to improved DFS \( (P = 0.031) \). Furthermore, the multivariate analysis found lymph nodes status, NuSAP1, and BRCA1 expression to be related to DFS in TNBC (Table 5).

**Prognostic value of the combined expression of NuSAP1 and BRCA1 for DFS in TNBC**

Subsequently, we evaluated the combined predictive value of NuSAP1 and BRCA1 for DFS. All TNBC patients were classified into the following four subgroups: NuSAP1+/BRCA1+ \( (n = 22) \), NuSAP1+/BRCA1- \( (n = 48) \), NuSAP1-/BRCA1+ \( (n = 21) \), and NuSAP1-/BRCA1- \( (n = 59) \). As shown in Fig 3b, the NuSAP1+/BRCA1- patients exhibited worse DFS than the NuSAP1-/BRCA1+ \( (P = 0.008) \) subgroup.

**Predictive risk model of the combined expressions of NuSAP1 and BRCA1 for DFS in TNBC**

Next, we sought to evaluate the capability of the combination of NuSAP1 and BRCA1 to identify the patients with TNBC who were more likely to experience DFS events. In the absence of NuSAP1 and BRCA1, the traditional model exhibited modest prognostic accuracy with a bootstrap-corrected AUC value of 0.612 (Fig 4a, 95% CI: 0.488–0.699). The addition of NuSAP1 and BRCA1 expression to the traditional model significantly improved the bootstrap-corrected AUC value to 0.778 (Fig 4a, 95% CI: 0.665–0.838). The traditional model (\( M_{\text{traditional}} \)) was

\[
M_{\text{traditional}} = -0.025 \cdot \text{Age} + 0.601 \cdot \text{Menopausal Status} + 0.767 \cdot \text{Lymph Node Status}.
\]

The combined model (\( M_{\text{combined}} \)) was

\[
M_{\text{combined}} = -0.006 \cdot \text{Age} + 0.5290 \cdot \text{Menopausal Status} + 0.775 \cdot \text{Lymph Node Status} + 1.479 \cdot \text{NuSAP1} - 0.942 \cdot \text{BRCA1}.
\]

The optimal cutoff
value of the ROC curve was 0.821. The TNBC patient cohort was subsequently reclassified as high risk (risk score > 0.821, n = 80) or low risk (risk score ≤ 0.821, n = 70). The survival curves revealed a significant difference in survival between the two groups (Fig 4b, P < 0.01).

**Discussion**

As a cell cycle-related protein, NuSAP1 plays a vital role in mitosis, and aberrant NuSAP1 expression results in abnormal mitotic spindles. NuSAP1 is upregulated and related to poor

| Table 4. Univariate analysis for disease-free survival in TNBC. |
|---------------------------------------------------------------|
| **Age**                                                      |
| <50 years                                                   | 1          |
| >50 years                                                   | 0.814(0.429–1.543) | 0.528 |
| **Menopausal status**                                       |
| Premenopause                                                | 1          |
| Postmenopause                                               | 1.771(0.923–3.395) | 0.085 |
| **Tumor size**                                              |
| ≤2cm                                                        | 1          |
| >2, ≤5cm                                                    | 1.577(0.796–3.123) | 0.192 |
| >5cm                                                        | 3.272(1.074–9.966) | 0.037 |
| **Lymph node status**                                       |
| Negative                                                    | 1          |
| Positive                                                    | 2.137(1.129–4.042) | 0.020 |
| **Grade**                                                   |
| 1 or 2                                                       | 1          |
| 3                                                           | 1.035(0.535–2.001) | 0.920 |
| **BRCA1**                                                   |
| Negative                                                    | 1          |
| Positive                                                    | 0.383(0.160–0.915) | 0.031 |
| **NuSAP1**                                                  |
| Negative                                                    | 1          |
| Positive                                                    | 4.136(1.956–8.747) | 0.000 |

**Abbreviations**: BRCA1, breast cancer type 1 susceptibility protein; NuSAP1, Nucleolar spindle-associated protein.

$P_a$ value was calculated using Pearson’s $\chi^2$.

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| Table 5. Multivariate analysis for disease-free survival in TNBC. |
|---------------------------------------------------------------|
| **Age**                                                      |
| <50 years                                                   | 0.994(0.424–2.330) | 0.989 |
| **Menopausal status**                                       |
| Premenopause                                                | 1.336(0.560–3.189) | 0.514 |
| Postmenopause                                               | 2.171(1.126–4.186) | 0.021 |
| **Lymph node status**                                       |
| Negative                                                    | 0.390(0.162–0.940) | 0.036 |
| **BRCA1**                                                   |
| Negative                                                    | 4.388(2.048–9.400) | 0.000 |
| **NuSAP1**                                                  |
| Negative                                                    | 0.994(0.424–2.330) | 0.989 |

**Abbreviations**: BRCA1, breast cancer type 1 susceptibility protein; NuSAP1, Nucleolar spindle-associated protein.

$P_a$ value was calculated using Pearson’s $\chi^2$.

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Dilek Colak et al. indicated that the NuSAP1 gene might be involved in the carcinogenesis and progression of breast cancer. To further investigate the correlation between NuSAP1 expression and breast cancer prognosis, particularly for different subtypes, we constructed two sets of TMAs that contained 450 stage I to III primary breast cancer tissues and determined the NuSAP1 expressions via immunostaining. A KM plot was constructed to evaluate the prognostic value of NuSAP1, and high levels of NuSAP1 expression were found to be related with poor DFS for all patients. Specifically, this association was more significant in the triple-negative subgroup. No significant difference was observed between the Luminal A, Luminal B and HER-2 overexpression subgroups. Therefore, the association between NuSAP1 and DFS might have primarily derived from the triple-negative subgroup.

TNBC is a specific subtype of breast cancer that is negative for ER, PR and HER-2 expression. Due to the high level of heterogeneity and the lack of well-defined molecular targets, the treatment of TNBC has long been a challenge. Recently, Brian D. Lehmann et al. examined gene expression profiles and further divided TNBC into six subtypes that included two basal-like (BL) subtypes, an immunomodulatory subtype, a mesenchymal subtype, a mesenchymal stem-like subtype and a luminal androgen receptor subtype. The basal-like subtypes included BL1 and BL2, both of which exhibited increased expressions of cell cycle and DNA damage response genes. Lehmann et al. also primarily observed BRCA1 enrichment in the BL1 subtype. In our study, high NuSAP1 expression levels indicated poor prognosis, which is consistent with the emerging role of NuSAP1 as a modulator of the relationship between the bundling of spindle microtubules and cancer. In contrast, decreased BRCA1 expression indicated a poor prognosis in the TNBC group, as previously described. In the current study, the DFS of TNBC patients could be stratified by the NuSAP1 and BRCA1 expression.
status. These findings indicated that the combination of these two molecular markers provided additional prognostic information. Thus, NuSAP1 might be a biomarker for TNBC.

The univariate and multivariate analysis demonstrated that NuSAP1 and BRCA1 were both independent prognostic factors of DFS in TNBC. Furthermore, a risk model that incorporated these two proteins could classify the TNBC patients into two recurrence risk categories. To the best of our knowledge, this study is the first to verify the prognostic value of the combination of NuSAP1 and BRCA1 in TNBC.

Our results are limited by the restricted sample size, particularly regarding TNBC. Therefore, our results should be validated in larger and consistent cohorts of breast cancer patients. TNBC were divided into six subgroups and subsequent investigations were needed to verify the specific type of NuSAP1 function.

In conclusion, our study confirmed the prognostic value of NuSAP1 in breast cancer. The combination of NuSAP1 and BRCA1 improved the DFS prediction accuracy in TNBC. Our findings may be used to advance the classification and treatment of specific breast cancer patients.

Supporting Information

S1 Fig. Kaplan-Meier estimates of the OS according to NuSAP1 expression. Cumulative OS curves for breast cancer patients (a) training set: NuSAP1+ (n = 108) and NuSAP1- (n = 140) patients; Validation set: NuSAP1+ (n = 88) and NuSAP1- (n = 112) patients; all patients: NuSAP1+ (n = 196) and NuSAP1- (n = 252) patients and (b) luminal A: NuSAP1+ (n = 41) and NuSAP1- (n = 58); luminal B: NuSAP1+ (n = 53) and NuSAP1- (n = 55); HER2-overexpression: NuSAP1+ (n = 33) and NuSAP1- (n = 60); triple-negative: NuSAP1+ (n = 69) and NuSAP1- (n = 79) patients.

S2 Fig. Kaplan-Meier estimates of the OS according to BRCA1 expression in the TNBC subgroup. Cumulative DFS curves for TNBC patients classified as BRCA1+ (n = 43) and BRCA1- (n = 107).

S1 Table. Correlations between NuSAP1 and BRCA1 expression in the TNBC subgroup.

S2 Table. Correlations of the clinicopathologic variables with the expressions of BRCA1 in the TNBC subgroup.

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Author Contributions

Conceived and designed the experiments: LC L Yang XH ZMS. Performed the experiments: LC L Yang FQ SL L Yao XLY. Analyzed the data: LC L Yang XLY. Contributed reagents/materials/analysis tools: XH L Yao ZMS. Wrote the paper: LC L Yang. Collected data: LC L Yang FQ SL L Yao. Evaluated the data: FQ SL.
References

1. Hassan MS, Ansari J, Spooner D, Hussain SA. Chemotherapy for breast cancer (Review). Oncology reports. 2010; 24(5):1121–31. PMID: 20878101.

2. Fan L, Strasser-Weippl K, Li J, J J, St Louis J, Finkelstein DM, Yu KD, et al. Breast cancer in China. The Lancet Oncology. 2014; 15(7):e279–90. doi: 10.1016/S1470-2045(14)70567-9 PMID: 24872111.

3. Carey LA, Dees EC, Sawyer L, Gatti L, Moore DT, Collichio F, et al. The triple negative paradox: primary tumor chemo sensitivity of breast cancer subtypes. Clinical cancer research: an official journal of the American Association for Cancer Research. 2007; 13(8):2329–34. doi: 10.1158/1078-0432.CCR-06-1109 PMID: 17438091.

4. Voduc KD, Cheang MC, Tyldesley S, Gelmon K, Nielsen TO, Kennecke H. Breast cancer subtypes and the risk of local and regional relapse. Journal of clinical oncology: official journal of the American Society of Clinical Oncology. 2010; 28(10):1684–91. doi: 10.1200/JCO.2009.24.9284 PMID: 20194857.

5. Severson TM, Peeters J, Majewski I, Michaut M, Bosma A, Schouten PC, et al. BRCA1-like signature in triple negative breast cancer: Molecular and clinical characterization reveals subgroups with therapeutic potential. Molecular oncology. 2015. doi: 10.1016/molonc.2015.04.011 PMID: 26004083.

6. Timms KM, Abkevich V, Hughes E, Neff C, Reid J, Morris B, et al. Association of BRCA1/2 defects with genomic scores predictive of DNA damage repair deficiency among breast cancer subtypes. Breast cancer research: BCR. 2014; 16(6):475. doi: 10.1186/s13058-014-0475-x PMID: 25475740; PubMed Central PMCID: PMC4308910.

7. Hill SJ, Clark AP, Silver DP, Livingston DM. BRCA1 pathway function in basal-like breast cancer cells. Molecular and cellular biology. 2014; 34(20):3828–42. doi: 10.1128/MCB.01646-13 PMID: 25092866.

8. Ricciardi GR, Adamo B, Ieni A, Licata L, Cardia R, Ferraro G, et al. Androgen Receptor (AR), E-Cadherin, and Ki-67 as Emerging Targets and Novel Prognostic Markers in Triple-Negative Breast Cancer (TNBC) Patients. PloS one. 2015; 10(6):e0128368. doi: 10.1371/journal.pone.0128368 PMID: 26039245.

9. Zhang L, Fang C, Xu X, Li A, Cai O, Long X. Androgen receptor, EGFR, and BRCA1 as biomarkers in triple-negative breast cancer: a meta-analysis. BioMed research international. 2015; 2015:357485. doi: 10.1155/2015/357485 PMID: 25695063; PubMed Central PMCID: PMC4324735.

10. Burstein MD, Tsimelzon A, Poage GM, Covington KR, Contreras A, Fuqua SA, et al. Comprehensive genomic analysis identifies novel subtypes and targets of triple-negative breast cancer. Clinical cancer research: an official journal of the American Association for Cancer Research. 2015; 21(7):1688–98. doi: 10.1158/1078-0432.CCR-14-0432 PMID: 25208879; PubMed Central PMCID: PMC4362882.

11. Ribbeck K, Groen AC, Santarella R, Bochnak MT, Raemaekers T, Kocher T, et al. NuSAP, a mitotic RanGTP target that stabilizes and cross-links microtubules. Molecular biology of the cell. 2006; 17(6):2646–60. doi: 10.1091/mbc.E05-12-1178 PMID: 16571672; PubMed Central PMCID: PMC1474800.

12. Raemaekers T, Ribbeck K, Beaudouin J, Annaert W, Van Camp M, Stockmans I, et al. NuSAP, a novel microtubule-associated protein involved in mitotic spindle organization. The Journal of cell biology. 2003; 162(6):1017–29. doi: 10.1083/jcb.200302129 PMID: 12963707; PubMed Central PMCID: PMC2172854.

13. Verbakel W, Carmeliet G, Engelborghs Y. SAP-like domain in nucleolar spindle associated protein mediates mitotic chromosome loading as well as interphase chromatin interaction. Biochemical and biophysical research communications. 2011; 411(4):732–7. doi: 10.1016/j.bbrc.2011.07.015 PMID: 21782797.

14. Iyer J, Moghe S, Furukawa M, Tsai MY. What's Nu(SAP) in mitosis and cancer? Cellular signalling. 2011; 23(6):991–8. doi: 10.1016/j.cellsig.2010.11.006 PMID: 21118112.

15. Satow R, Shitashige M, Kanai Y, Takeshita F, Ojima H, Jigami T, et al. Combined functional genome survey of therapeutic targets for hepatocellular carcinoma. Clinical cancer research: an official journal of the American Association for Cancer Research. 2010; 16(9):2518–28. doi: 10.1158/1078-0432.CCR-09-2214 PMID: 20388486.

16. Bidkhor G, Narimani Z, Hosseini Ashitian S, Moeini A, Nowzari-Dalini A, Masoudi-Nejad A. Reconstruction of an integrated genome-scale co-expression network reveals key modules involved in lung adenocarcinoma. PloS one. 2013; 8(7):e67552. doi: 10.1371/journal.pone.0067552 PMID: 23874426; PubMed Central PMCID: PMC3708931.

17. Espinosa AM, Alfaro A, Roman-Basaure E, Guardado-Estrada M, Palma I, Serralde C, et al. Mitosis is a source of potential markers for screening and survival and therapeutic targets in cervical cancer. PloS one. 2013; 8(2):e55975. doi: 10.1371/journal.pone.0055975 PMID: 23405241; PubMed Central PMCID: PMC3566100.
18. Ryu B, Kim DS, Deluca AM, Alani RM. Comprehensive expression profiling of tumor cell lines identifies molecular signatures of melanoma progression. PloS one. 2007; 2(7):e594. doi: 10.1371/journal.pone.0000594 PMID: 17611626; PubMed Central PMCID: PMC1895889.

19. Stuart JE, Lusis EA, Scheck AC, Coons SW, Lai A, Perry A, et al. Identification of gene markers associated with aggressive meningioma by filtering across multiple sets of gene expression arrays. Journal of neuropathology and experimental neurology. 2011; 70(1):1–12. doi: 10.1097/NEU.0b013e3182018f1c PMID: 21157382; PubMed Central PMCID: PMC3839953.

20. Gulzar ZG, McKenney JK, Brooks JD. Increased expression of NuSAP in recurrent prostate cancer is mediated by E2F1. Oncogene. 2013; 32(1):70–7. doi: 10.1038/onc.2012.27 PMID: 22349817; PubMed Central PMCID: PMC3360134.

21. Colak D, Nofal A, Albakheet A, Nirmal M, Jeprel H, Eldali A, et al. Age-specific gene expression signatures for breast tumors and cross-species conserved potential cancer progression markers in young women. PloS one. 2013; 8(5):e63204. doi: 10.1371/journal.pone.0063204 PMID: 23704896; PubMed Central PMCID: PMC3660335.

22. Goldhirsh A, Wood WC, Coates AS, Gelber RD, Thurlimann B, Senn HJ, et al. Strategies for subtypes—dealing with the diversity of breast cancer: highlights of the St. Gallen International Expert Consensus on the Primary Therapy of Early Breast Cancer 2011. Annals of oncology: official journal of the European Society for Medical Oncology / ESMO. 2011; 22(8):1736–47. doi: 10.1093/annonc/mdr304 PMID: 21709140; PubMed Central PMCID: PMC3144634.

23. Thompson ME, Robinson-Benion CL, Holt JT. An amino-terminal motif functions as a second nuclear export sequence in BRCA1. The Journal of biological chemistry. 2005; 280(23):21854–7. doi: 10.1074/jbc.M502676200 PMID: 15811849.

24. Rebbeck TR, Mitra N, Wan F, Sinilnikova OM, Healey S, McGuffog L, et al. Association of type and location of BRCA1 and BRCA2 mutations with risk of breast and ovarian cancer. Jama. 2015; 313(13):1347–61. doi: 10.1001/jama.2014.5985 PMID: 25849179.

25. Lehmann BD, Bauer JA, Chen X, Sanders ME, Chakravarthy AB, Shyr Y, et al. Identification of human triple-negative breast cancer subtypes and preclinical models for selection of targeted therapies. The Journal of clinical investigation. 2011; 121(7):2750–67. doi: 10.1172/JCI45014 PMID: 21633166; PubMed Central PMCID: PMC3127435.

26. Khoshnaw SM, Rakha EA, Abdel-Fatah TM, Nolan CC, Hodi Z, Macmillan DR, et al. Loss of Dicer expression is associated with breast cancer progression and recurrence. Breast cancer research and treatment. 2012; 135(2):403–13. doi: 10.1007/s10549-012-2169-3 PMID: 22821364.

27. Guler G, Himmetoglu C, Jimenez RE, Geyer SM, Wang WP, Costinean S, et al. Aberrant expression of DNA damage response proteins is associated with breast cancer subtype and clinical features. Breast cancer research and treatment. 2011; 129(2):421–32. doi: 10.1007/s10549-010-1248-6 PMID: 21069451; PubMed Central PMCID: PMC3677189.