SOX2 autoantibodies as non-invasive serum biomarker for breast carcinoma

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Running title: SOX2 autoantibodies in breast cancer

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Conflicts of interest
None declared.

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ABSTRACT

Background: A clear association has been established between antibodies to the transcription factor sex determining region Y (SRY)-box 2 (SOX2) and small cell lung cancer. In light of the pathological role of SOX2 and its aberrant expression in breast cancer, we measured serum SOX2 autoantibodies (SOX2-Abs) in breast cancer patients.

Methods: The presence of SOX2-Abs was determined by an indirect enzyme-linked immunosorbent assay (ELISA) in sera from 282 patients with breast cancer, 78 patients with benign breast disease, and 194 healthy women.

Results: SOX2-Abs were more prevalent in patients with breast cancer (18.4%) compared with healthy women (2.6%, \( P < 0.0001 \)), and patients with benign breast disease (6.4%, \( P = 0.011 \)). The concentrations of circulating SOX2-Abs were found to discriminate between breast cancer patients and healthy controls (\( P < 0.001 \)) and between breast cancer patients and those with benign breast disease (\( P < 0.001 \)). In addition, measurement of SOX2-Abs was more effective than assays of serum tissue polypeptide-specific antigen (TPS), carcinoembryonic antigen (CEA), carbohydrate antigen (CA) 125, and CA 15-3 in distinguishing between malignant and benign breast disease. In breast cancer patients, the prevalence of SOX2-Abs was associated with a higher tumor grade (\( P = 0.021 \)) and positive nodal status (\( P = 0.021 \)).

Conclusion: The presence of SOX2-Abs in breast cancer may be of clinical value.

Impact: This study provides the first evidence for the presence of circulating SOX2-Abs in breast cancer and demonstrates their potential clinical application.
Introduction

Breast cancer remains the primary cause of cancer death among females worldwide. To date, no effective blood-based marker is available for breast cancer detection, particularly for patients with early-stage disease [1]. As part of the effort to develop additional blood-based tests for breast cancer, attention has recently focused on the host response to tumor-associated antigens (TAAs).

Evidence collected in the last few years suggests that SOX2, one of the embryonic stem cell markers, plays a role in breast carcinogenesis [2, 3]. Correspondingly, SOX2 has been shown to be aberrantly expressed in breast cancer tissues [3-5]. To date, the immunogenicity of SOX2 had mainly been investigated in individuals with small cell lung carcinoma (SCLC) [6-10]. Initially identified by serological analysis of expression cDNA libraries (SEREX) [7], the presence of SOX2 autoantibodies (SOX2-Abs) in SCLC patients has been confirmed by several subsequent studies [6-10]. The 22–33% frequency of SOX2-Abs has promising potential for clinical use. Moreover, SOX2-Abs have also been detected in meningioma [11], monoclonal gammopathy of undetermined significance (MGUS) [12], and myeloma [13]. The aforementioned findings raised the possibility that the host response to SOX2 may be spontaneously primed in breast cancer and the presence of SOX2-Abs may be of clinical significance.

Therefore, we developed an in house ELISA (a) to analyze the prevalence of serum SOX2-Abs in patients with benign and malignant breast disease and in healthy controls, (b) to determine the clinical significance of SOX2-Abs in breast cancer.
screening, and (c) to compare the performance of the SOX2-Ab assay with assays for
commonly used breast cancer markers such as serum tissue polypeptide-specific
antigen (TPS), CEA, CA 15-3, and CA 125.

Materials and Methods

Patients and samples

The majority of the total breast cancer patient cohort (n = 282) comprised patients
with early-stage breast cancer (n = 233). The control populations consisted of 194
healthy controls and 78 patients with benign breast disease (Table S1 in
Supplementary data). None of the patients with benign breast disease or the healthy
controls had a past or concomitant history of any malignancy. The clinicopathological
data for the patient population were collected. All participants were ethnic Chinese
women. Cases and controls did not differ significantly with respect to age (P = 0.074).

Sera from patients with breast cancer or benign breast disease were obtained at the
time of diagnosis at Cancer Hospital, Chinese Academy of Medical Sciences. Sera
from healthy women were obtained at Beijing Hospital. All collection procedures
followed a standardized protocol. Samples were processed within 2 h after blood
withdrawal and were stored at -80°C until use. Informed consent was obtained from
each subject, and the study protocol was approved by the Ethics Committee of the
National Center for Clinical Laboratories, and adhered to the tenets of the Declaration
of Helsinki.

ELISA for detection of SOX2-Ab
Recombinant His$_6$-tagged SOX2 protein was purified and prepared for antibody detection, and the measurement of SOX2-Abs was performed as described (Supplementary data).

All the assays were performed in duplicate. To correct for interassay variation, the results were expressed as arbitrary units (AU), as described previously [14]. To verify the ELISA results, serum samples with AU values near the assay cut-off value (see below) and a group of randomly selected serum samples were further tested for SOX2 reactivity by western blotting, as described (Supplementary data).

**CEA, CA 125, CA 15-3, and TPS assays**

Serum levels of TPS, CEA, CA 125, and CA 15-3 were measured using commercially available kits (Elecsys CEA, CA 125, and CA 15-3 Immunoassays from Roche Diagnostics, Mannheim, Germany; TPS ELISA kit from IDL Biotech AB, Bromma, Sweden), according to the manufacturer’s recommendations. The cut-off values described in the protocol for positive and negative reactivity were 110 U/L for TPS, 5 ng/mL for CEA, 35 U/mL for CA 125, and 25 U/mL for CA 15-3.

**Statistical analysis**

We used Pearson’s two-tailed $\chi^2$ test to compare proportions. The differences in the concentrations of SOX-Abs among groups were assessed by the Mann-Whitney test or Kruskal-Wallis test, as appropriate. $P$-values less than 0.05 were considered statistically significant. Statistical analyses were carried out using SPSS v. 16.0.

To assess the diagnostic performance of the SOX2-Ab ELISA in breast cancer screening, we used receiver operating characteristic (ROC) curves. The area under the
ROC curve (AUC) was estimated to evaluate the diagnostic power.

We used Venn diagrams to exam whether CA 15-3, CA 125, CEA, TPS and SOX2-Abs pinpoint overlapping group of patients.

Results

Prevalence of SOX2-Abs

Positivity was defined as 3 standard deviations (SDs) above the mean AU for normal controls. SOX2-Abs were observed to be more prevalent in patients with breast cancer (18.4%) compared with healthy women (2.6%, \( p < 0.0001 \)), and patients with benign breast disease (6.4%, \( p = 0.011 \)). The distribution of SOX2-Abs in the 3 study groups is presented in Figure 1A. The circulating levels of SOX2-Abs in patients with breast cancer were significantly higher than the levels in healthy donors (\( P < 0.001 \)) or in patients with benign disease (\( P < 0.001 \)). These results were confirmed by western blot analysis of patient sera (Figure S1). We next examined the levels of SOX2-Abs in breast cancer patients according to clinical disease stage. For this analysis, the results for patients with stage III and stage IV disease were combined because of the limited number of patients with stage IV disease (\( n = 5 \)). However, we found no significant differences in SOX2-Ab levels among the clinical stages (\( P = 0.920 \)) (Figure 1B).

Circulating SOX2-Abs and clinicopathological characteristics

Table 1 presents the associations between SOX2-Ab levels and clinicopathological variables in the 282 breast cancer patients. The presence of SOX2-Abs correlated with higher tumor grade (\( P = 0.021 \)) and nodal status (\( P = 0.021 \)). No significant association was observed between SOX2-Ab expression and patient age, T
classification, Ki-67 proliferative rate, HER2 overexpression, or hormone receptor positivity.

Comparison of serum SOX2-Abs with TPS, CEA, CA 125, and CA 15-3 assays

The serum levels of TPS, CEA, CA 125, and CA 15-3 were measured for patients with benign (n = 78) and malignant (n = 282) breast disease (Figure S2). The sensitivity and specificity were 2.1% and 100% for CA 15-3, 2.8% and 100% for CEA, 22.3% and 75.6% for TPS, and 3.6% and 92.4% for CA 125, respectively; while the sensitivity and specificity were 18.4% and 93.6% for SOX2-Abs to discriminate malignant from benign breast disease. In the Venn diagrams, we did not observe significant overlapping group of patients among these markers, except that SOX2-Abs and TPS were both positive in 10 patients (Figure S3).

Diagnostic utility of SOX2-Abs

Using ROC curves, we determined the diagnostic capability of the SOX2-Ab assay. The sensitivity of the assay was 18.4% and the specificity was 97.4% for differentiating between breast cancer patients and healthy subjects, whereas the sensitivity was 18.4% and the specificity was 93.6% for differentiating between breast cancer patients and patients with benign disease. The former had an AUC of 0.671 (95% CI: 0.623–0.719, p < 0.001) (Figure 2A), while the latter had an AUC of 0.630 (95% CI: 0.584–0.676, p < 0.001) (Figure 2B). Similar results were obtained when analyzing the ability of SOX2-Abs to discriminate between stage I–II breast cancer patients and the control groups (data not shown).
Discussion

Recently, SOX2 has drawn increasing attention for the frequency with which it elicits a spontaneous host response in cancer patients [6, 15]. The 18.4% frequency of SOX2-Abs obtained in our study on breast cancer patients is comparable to the published frequencies for SCLC and MGUS, despite the highly heterogeneous nature of breast cancer.

It is perhaps not surprising that autoimmune responses to SOX2 were detected in breast cancer patients. SOX2 has been demonstrated to promote the proliferation of tumor cells by facilitating G1/S transition [2]. Additional studies have revealed the molecular and biological basis of SOX2 involvement in early events in breast carcinogenesis by confirming its potential to induce stem cell-like cancer cells [3, 4]. In addition, SOX2 has been shown to be aberrantly expressed in breast cancer tissues, particularly in early-stage cancers [3-5], which has the potential to support a sustained memory response through ongoing antigenic exposure. Apart from the aberrant expression of SOX2, certain therapies may be responsible for the development of SOX2-Abs. It was reported that patients with multiple myeloma (MM) who undergone allogeneic stem cell transplantation (alloSCT) presented SOX2-Abs [13]; since alloSCT was demonstrated to promote immunogenicity of tumor associated antigens. It is of interest that most of the seropositive patients showed no immune response against SOX2 before treatment [13], suggesting a different underlying mechanism for the development of SOX2-Abs compared with solid tumors. In the same study, the amino acid region from 211 to 230 in SOX2 was identified as the
immunodominant epitope [13].

To date, the pathogenetic role of SOX2-Abs in tumors has not been well characterized in the literature. That is partially because the lack of correlation between SOX2 seropositivity and known clinical parameters. A large scale screening conducted in SCLC patients did not observe a significant effect of SOX2-Abs on survival [10]. The presence of SOX2-Abs was reported to be not related to disease burden in multiple myeloma [13].

Our study further revealed the clinical significance of the presence of SOX2-Abs. We found that SOX2-Abs were more prevalent in sera from patients with a higher tumor grade and positive nodal status, but there was no association between the presence of SOX2-Abs and other clinicopathological variables. Intriguingly, these results revealed a positive correlation between the presence of SOX2-Abs and previous immunohistochemical findings for breast cancer. These immunohistochemical studies demonstrated that SOX2-Abs was more prevalent in triple negative breast cancer [3, 5]. A lack of correlation between SOX2-Abs status and ER/PR receptors or HER2 overexpression was observed as well [4]. Although we did not observe SOX2-Abs to be more prevalent in triple-negative breast cancer, we did find a positive association with the less differentiated phenotypes. SOX2-Ab status was not correlated with ER/PR or HER2 overexpression, which was consistent with the results of another study on early-stage breast carcinoma [4].

To determine whether the presence of serum SOX2-Abs might be useful in breast cancer detection, we compared the sensitivity and specificity of SOX2-Ab detection
with assays for serum TPS, CEA, CA 15-3, and CA 125 in the cohort of patients with benign or malignant breast disease. Using the recommended assay cut-off values, the sensitivity of the SOX2-Ab assay (18.4%) was considerably higher than that of the CEA, CA 15-3, and CA 125 assays (all < 5%). Moreover, the SOX2-Ab ELISA showed specificity that was comparable to those of the CEA, CA 15-3, and CA 125 assays and superior to that of the TPS assay. In our study, therefore, measurement of SOX2-Abs was a more powerful diagnostic tool than these clinically “accepted” diagnostic markers. Although the diagnostic efficiency of SOX2-Abs was lower than expected, it was as effective as other well-studied biomarkers, such as autoantibodies against p53 and HER2. Lu et al. [16] reported that the detection of p53-Abs had an ROC-AUC of 0.48 ($p = 0.538$), while the combination of p53-Abs and HER2-Abs had an ROC-AUC of 0.61 ($p =0.006$), in discriminating advanced-stage breast cancer patients from non-malignant disease and healthy controls. To meet the stringent sensitivity and accuracy requirements for a diagnostic assay, we would recommend that SOX2-Abs are assayed in combination with a panel of other serum biomarkers, such as antibodies to p53, c-myc, and survivin [17-19].

In conclusion, the preliminary results presented here indicate that SOX2-Abs are not confined to SCLCs but are also present in breast cancer patients, raising the possibility that SOX2-Abs may serve as serum biomarkers for breast cancer. This study raises further questions regarding (1) whether the presence of SOX2-Abs in breast cancer and SCLC indicates that the diseases share a common pathogenic mechanism; and (2) whether SOX2-Abs could play a role as a prognostic indicator in
breast cancer. These questions should be addressed in future studies.

Acknowledgments

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References


Table 1. Association of SOX2-Abs with clinicopathological features and molecular characteristics in breast cancer patients

<table>
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<tr>
<th>Variable</th>
<th>SOX2-Ab negative</th>
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<tr>
<td></td>
<td>(n; %) n = 230</td>
<td>(n; %) n = 52</td>
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<tr>
<td>&lt;50</td>
<td>122 (53.0)</td>
<td>26 (50.0)</td>
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<td>≥50</td>
<td>108 (47.0)</td>
<td>26 (50.0)</td>
<td>0.913</td>
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<tr>
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<td>188 (81.7)</td>
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<td>III–IV</td>
<td>42 (18.3)</td>
<td>7 (13.5)</td>
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<td>135 (58.7)</td>
<td>34 (65.4)</td>
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<td>2-5 cm</td>
<td>89 (38.7)</td>
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<td>Positive</td>
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<td>36 (83.7)</td>
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Figure legends

Figure 1. (A) Distribution of SOX2-Abs in sera from patient and control groups. Scatter plots of AU values from 194 healthy controls (HC), 78 patients with benign disease (BD), and 282 patients with breast cancer (BC). The SOX2-Ab levels in patients with breast cancer were significantly higher than the levels in healthy donors ($P < 0.001$) and in patients with benign disease ($P < 0.001$). The cut-off value was set at mean + 3 SDs above the mean arbitrary unit (AU) of normal sera. (B) Distribution of SOX2-Abs in sera from patients with different stages of breast cancer. Scatter plots of absorbance values of autoantibodies from patients with stage I ($n = 118$), stage II ($n = 115$), and stage III + IV ($n = 49$) cancer. No significant differences were found among the groups. Antibody titers were standardized as AUs.

Figure 2. Diagnostic performance of SOX2-Ab measurement performed by ROC analysis. (A) The AUC of serum SOX2-Abs was 0.671 (95% CI: 0.623–0.719) for differentiation between patients with breast cancer and healthy controls. (B) The AUC of serum SOX2-Abs for differentiation between patients with breast cancer and controls (healthy controls plus patients with benign breast disease) was 0.630 (95% CI: 0.584–0.676).
Figure 1
SOX2 antoantibodies as non-invasive serum biomarker for breast carcinoma

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