Original paper

Does SOX-2 expression have a prognostic value in triple-negative breast cancer?

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Triple-negative breast cancer (TNBC) is the most aggressive subtype of breast cancer. At the cell of origin level, cancer stem cells (CSC) are the tumour initiators in breast cancer. SRY-box transcription factor 2 (SOX-2) is a CSC marker that plays a role in tumourigenesis.

The objective of this study was to evaluate the association of SOX-2 expression with histopathological parameters and clinical outcomes in TNBC patients. The study included 95 TNBC cases. An *in vitro* diagnostic SOX-2 antibody was applied to the tumoural slides in a validated automated stainer.

The expression of SOX-2 was defined as a SOX-2 H-score ≥ 1. The expression of SOX-2 was observed in 29 cases (30.5%). At a median follow-up of 76 months, SOX-2 expression was not associated with overall or disease-free survival. R-based statistical analysis determined a SOX-2 H-score cut-off of 2. Although the overall and disease-free survival rates of cases with an H-score ≥ 3 were lower than the others, the differences were not statistically significant. The percentage of SOX-2 staining is typically low, as only 1% of tumour cells exhibit CSC characteristics.

In conclusion, the prognostic significance of SOX-2 could become clear in a larger group of TNBC patients using standardized methodologies.

Key words: breast cancer, triple-negative breast cancer, cancer stem cells, SOX-2.

Introduction

Triple-negative breast cancer (TNBC) accounts for 15% of all breast cancers [1–3]. Since TNBC is not eligible for targeted therapies, treatment options include immunotherapy, chemotherapy, and radiotherapy [4]. However, due to the limited treatment protocols, only 30% of cases treated with neoadjuvant therapy result in a pathological complete response [5]. Various clinical and preclinical research centres are focusing on treatment modalities and targetable antigens in TNBC [6].

Cancer stem cells (CSC) are pluripotent cancer cells that play critical roles in recurrence and metastasis [7]. Studies based on the cell of origin identify CSC models in breast cancer [8]. Breast CSCs constitute only 0.1–1% of tumour cells. Cancer stem cells can arise from the mutation of normal stem cells or *via* epithelial-mesenchymal transition of cancer cells [9].

The SRY-box transcription factor 2 (SOX-2) is a transcription factor expressed by CSCs [10–13]. SOX-2 has been identified as a potential target molecule based on an increasing body of evidence [7, 14–16]. In breast cancer, SOX-2 has been associated with larger tumour size, lymph node metastasis (LNM), TNBC, and resistance to tamoxifen treatment [17–19]. Limited studies on TNBC have reported an association between SOX-2 and worse overall survival (OS), advanced pathological tumour stage (pT), LNM, and advanced stage [20, 21]. Metastasis, tumour proliferation, and invasion capacity of TNBC have been inhibited by enhancer of zeste homolog 2 inhibition, bufalin, and TGFB2 antisense RNA 1 (TGFB2-AS1) through the inhibition of SOX-2 and its related pathways [16, 22, 23].

The objective of this study was twofold: first, to ascertain the association of SOX-2 with the clinical outcome and the histopathological characteristics in TNBC, and, second, to compare the original research data obtained by a validated method with the existing literature.

Material and methods

In this study, the database of Izmir Katip Celebi University Ataturk Training and Research Hospital was analysed for cases of TNBC patients who underwent mastectomy between 2008 and 2020. Triple-negative breast cancer was defined as no expression of oestrogen receptor and progesterone receptor and a cerbB2 score of 0 and 1+ or 2+ but proven negative FISH HER2. Cases with neoadjuvant treatment were excluded. All the slides of the cases were retrieved from the archives and evaluated for prognostic data. A haematoxylin and eosin stained slide that demonstrated the tumour features with a high tumour content and no necrosis was chosen. The corresponding paraffin blocks were used for immunohistochemistry (IHC).

The BenchMark ULTRA IHC automated stainer was used to apply the *in vitro* diagnostic (IVD) Ultraview DAB Kit and SOX-2 (SP76) primary antibody. Two external positive controls, lung squamous cell carcinoma and IDH mutant astrocytoma, were utilized in all the slides. The immunohistochemistry-stained slides were evaluated on an Olympus BX51 light microscope by BBK and IG. The SOX-2 nuclear staining intensity was scored as follows: 3 for strong, 2 for moderate, 1 for weak, and 0 for no staining. The percentage of SOX-2 positive tumour cells in the whole slide was also recorded. The SOX-2 H-score was calculated by multiplying the intensity score and the percentage score.

Pathological data were obtained from the hospital database. Hormone receptor status and cerbB2 analyses were evaluated following the current ASCO/CAP protocol [24]. Clinical data, such as recurrence and metastasis, were provided by the Medical Oncology outpatient clinic. Survival data were collected by the death notification service. Anatomical staging was performed according to the Eighth Edition AJCC Cancer Staging Manual [25]. All tumours were histopathologically graded according to the Nottingham grading system and typed according to the 5th edition of the *WHO Classification of Tumours*, Volume 2: Breast Tumours [8, 26]. Tumour size was initially measured in the grossing room, and accurate analysis was performed by the pathologists using a light microscope. Multifocality was defined as at least two invasive breast cancers in the same quadrant, and multicentricity was defined as at least two invasive breast cancers occupying different quadrants of the same breast [27]. Perinodal extension was defined as tumour cells perforating the lymph node capsule into the peri-nodal tissue [28]. For Ki-67 proliferation index, the average score across the stained section was considered as recommended [8, 29].

The data were evaluated by a biostatistician with SPSS Statistics Standard Concurrent User V 26 and R based Jamovi version 2.3. The correlation between SOX-2 and clinicopathological data was assessed using Student's t-test and the χ^2 test. The impact of SOX-2 expression on survival data was evaluated using Kaplan-Meier and Cox regression analysis. No statistically significant cut-off for SOX-2 H-score was determined by receiver operating characteristic (ROC) and R-based cut-off determination based on the survival data. Significance was accepted if $p < 0.05$.

The study approval was obtained from the institutional review board at Izmir Kâtip Celebi University Medical Faculty (2023-GOKAE-0041). The Medical Oncology outpatient clinic obtained written, informed consent for participation and publication from all individuals in this study. This work was funded by Izmir Kâtip Çelebi University Scientific Research Projects Unit with project number 2023-TDU-TIPF-0009.

Results

Patient characteristics

The study included 95 women with TNBC. Table 1 shows the clinicopathological characteristics of the patients. Of the patients, 31.5% presented with pT1, 61.1% with pT2, 4.2% with pT3, and 3.2% with pT4. Lymph node metastasis was present in 47.4% of the patients. On analysis of pathological regional lymph nodes (pN) of the patients, 52.6% presented with pN0, 21% with pN1, 14.7% with pN2, and 11.6% with pN3. Out of the 47 pa-

tients with tumour cells in the lymph node, 19 cases (40%) showed perinodal infiltration. Twenty patients were diagnosed with stage I (21.1%), 49 patients with stage II (51.6%), and 26 patients with stage III (27.4%). Seventy-nine cases (83.2%) were histologically invasive breast cancer (IBC) no special type; 5 cases (5.3%) were mixed type, 5 cases (5.3%) were carcinoma with apocrine differentiation, 3 cases (3.1%) were metaplastic carcinoma, and 3 cases (3.1%) were lobular carcinoma. Based on the Nottingham histological grading system, 3 patients (3%) were evaluated as grade 1, 20 (21%) as grade 2, and 72 (76%) as grade 3. Lymphovascular invasion was present in 21 patients (22%) and ductal carcinoma *in situ* (DCIS) in 29 patients (31%). The median follow-up in the study was 76 months. During this period, 37 (39%) of the participants died, and 32 (34%) experienced disease progression. The overall survival rates were 96.8% at 1 year, 82.1% at 3 years, and 73.3% at 5 years. The disease-free survival (DFS) rates were 94.5% at 1 year, 68.8% at 3 years, and 62.6% at 5 years.

Table I. Clinicopathological features of the cases

Feature ($N = 95$)	Frequency N (%) or mean (min–max)
Patient age	58.4 (27–92)
Multicentricity, *n* (%)	3 (3)
Multifocality, *n* (%)	8 (8)
Tumour diameter [cm]	3 (0.7–8)
pT, *n* (%)	
1	30 (31.5)
2	58 (61.1)
3	4 (4.2)
4	3 (3.2)
Lymph node metastasis, *n* (%)	45 (47.4)
pN, *n* (%)	
0	48 (50.5)
0 (i+)	2 (2.1)
1	18 (18.9)
1mi	2 (2.1)
2	14 (14.7)
3	11 (11.6)
Perinodal infiltration, $N = 47$ (%)	19 (40)
Anatomical staging, *n* (%)	
I	20 (21.1)
II	49 (51.6)
III	26 (27.4)
Histological type, *n* (%)	
No special type	79 (83.2)
Mixed	5 (5.3)
Carcinoma with apocrine differentiation	5 (5.3)
Metaplastic carcinoma	3 (3.1)
Lobular carcinoma	3 (3.1)
Histological grade, *n* (%)	
1	3 (3)
2	20 (21)
3	72 (76)
Lymphovascular invasion, *n* (%)	21 (22)
Ductal carcinoma *in situ*, *n* (%)	29 (31)

N – the number of non-missing values, pN – pathological regional lymph nodes, pT – pathological tumour

Pathological features and survival

As the pN and anatomical stage advanced, both OS ($p = 0.006$, $p = 0.009$) and DFS worsened ($p < 0.001$, $p < 0.001$). Cases with pT3 were associated with poorer OS and DFS when compared with pT1 cases ($p = 0.009$, $p < 0.001$). Lymph node metastasis, lymphovascular invasion, and perinodal infiltration were found to worsen OS ($p = 0.037$, $p < 0.001$, $p = 0.019$) and DFS ($p = 0.001$, $p < 0.001$, $p = 0.008$). Histological type, histological grade, DCIS, and Ki-67 proliferation index were not associated with overall and/or DFS ($p > 0.05$).

Table II. SOX-2 expression features in triple-negative breast cancer

Feature ($N = 95$)	Frequency, n (%)
Expression	29 (30.5)
Nuclear expression percentages	
< 1	66 (69.5)
1–9	23 (24.3)
10–29	3 (3.1)
≥ 30	3 (3.1)
Nuclear expression intensity	
Score 0	66 (69.5)
Score 1	19 (20)
Score 2	6 (6.3)
Score 3	4 (4.3)
H-score	
Score 0	66 (69.5)
Score 1 or 2	15 (15.7)
Score 3–10	9 (9.5)
Higher than score 10	5 (5.3)

N – the number of non-missing values

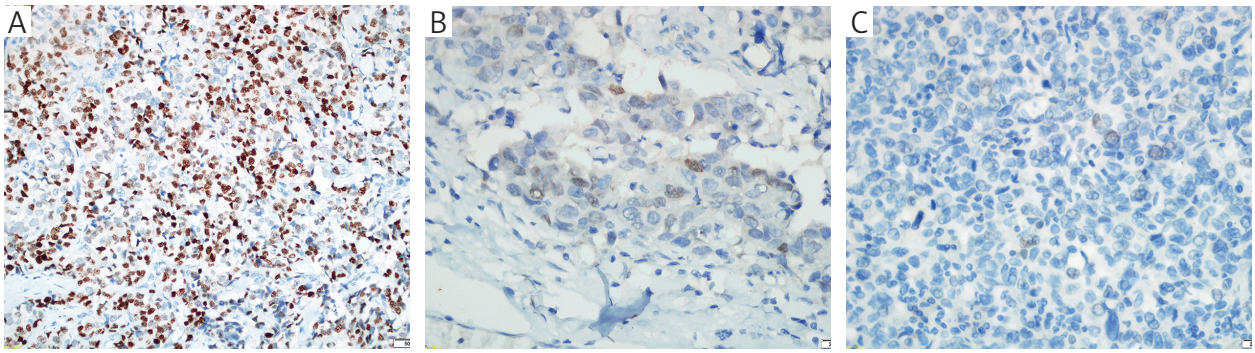

Fig. 1. A) Nuclear expression of SOX-2 with a nuclear intensity score of 3 was detected in 85% of tumour cells. B) In 25% of tumour cells, SOX-2 nuclear expression was detected with a nuclear intensity score of 2. C) SOX-2 immunohistochemistry showed staining with a nuclear intensity score of 1 in 2% of tumour cells (magnification 20×)

Table III. Association between SOX-2 nuclear expression status and clinicopathological features

Parameter	SOX-2		*p*-value
	No	Yes	
	N = 66 (%)	*N* = 29 (%)	
Age ≥ 50 years	44 (66.7)	20 (69)	0.826
Multicentricity	2 (3)	1 (3.4)	0.915
Multifocality	8 (12.1)	0 (0)	**0.013**
Tumour diameter [cm], mean	2.93	3.28	0.313
pT			0.299
1	23 (34.8)	7 (24.1)	
2	39 (59.1)	19 (65.5)	
3	2 (3)	2 (6.9)	
4	2 (3)	1 (3.4)	
Lymph node metastasis	34 (51.5)	11 (37.9)	0.222
pN			0.480
0	32 (48.5)	18 (62.1)	
1	16 (24.2)	4 (13.8)	
2	11 (16.7)	3 (10.3)	
3	7 (10.6)	4 (13.8)	
Anatomical stage			0.975
I and II	48 (72.7)	21 (72.4)	
III	18 (27.3)	8 (27.6)	
Perinodal infiltration, *n* = 47	19 (52.8)	9 (81.8)	0.074
Presence of a special histological type	14 (21.2)	2 (6.9)	0.086
Histological grade			0.595
1 and 2	17 (25.8)	6 (20.7)	
3	49 (74.2)	23 (79.3)	
Lymphovascular invasion	16 (24.2)	5 (17.2)	0.449
Ductal carcinoma *in situ*	18 (27.3)	11 (37.9)	0.299
Ki-67 proliferation index, *n* = 83, mean ±SD	50.09 ±40	52.61 ±50	0.742

N – the number of non-missing values, SD – standard deviation
Variables with statistically significant differences ($p < 0.05$) are indicated in bold letters.

SOX-2 expression and clinicopathological features

Table 2 shows the features of SOX-2 expression. SOX-2 expression was defined as nuclear expression in 1% or more of the tumoural cells in the whole slide and a nuclear intensity score of 1 or more. In 29 cases (30.5%), SOX-2 expression was present. The SOX-2 H-score was 0 in 66 cases (69.5%), 1 or 2 in 15 cases (15.7%), in the range 3–10 in 9 cases (9.5%), and higher than 10 in 5 cases (5.3%). Figure 1 shows cases with different nuclear expression percentages and densities.

Table 3 shows the clinicopathological features associated with SOX-2 nuclear expression. There was a significant association between negative SOX-2 expression and multifocal tumours ($p = 0.013$). SOX-2 expression was weakly associated with perinodal infiltration and lack of a special histological type ($p = 0.074$, $p = 0.086$). However, no association was found between SOX-2 expression and age, multicentricity, tumour diameter, pT, LNM, pN, anatomical stage, histological grade, lymphovascular invasion, DCIS, and Ki-67 proliferation index.

SOX-2 expression and survival

Figure 2 shows that there was no significant association between SOX-2 expression and OS and/or DFS ($p = 0.94$, $p = 0.62$). The SOX-2 H-score cut-off of 2 was determined using R-based analysis of the survival data. Figure 3 also shows no statistically sig-

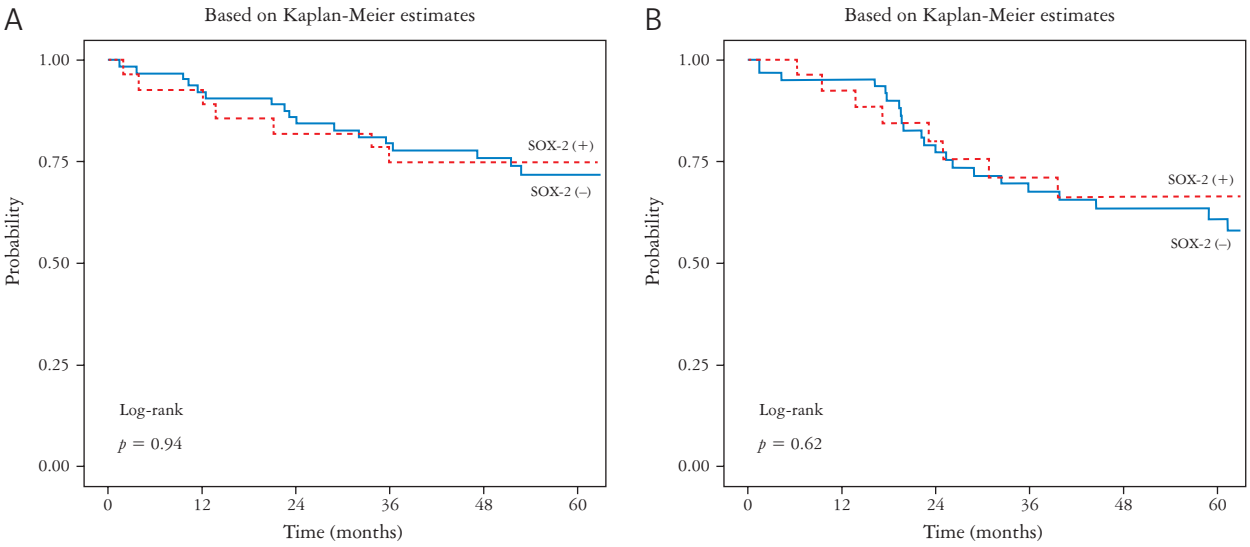

Fig. 2. Presence of SOX-2 nuclear expression was not associated with overall survival (A) or disease-free survival (B)

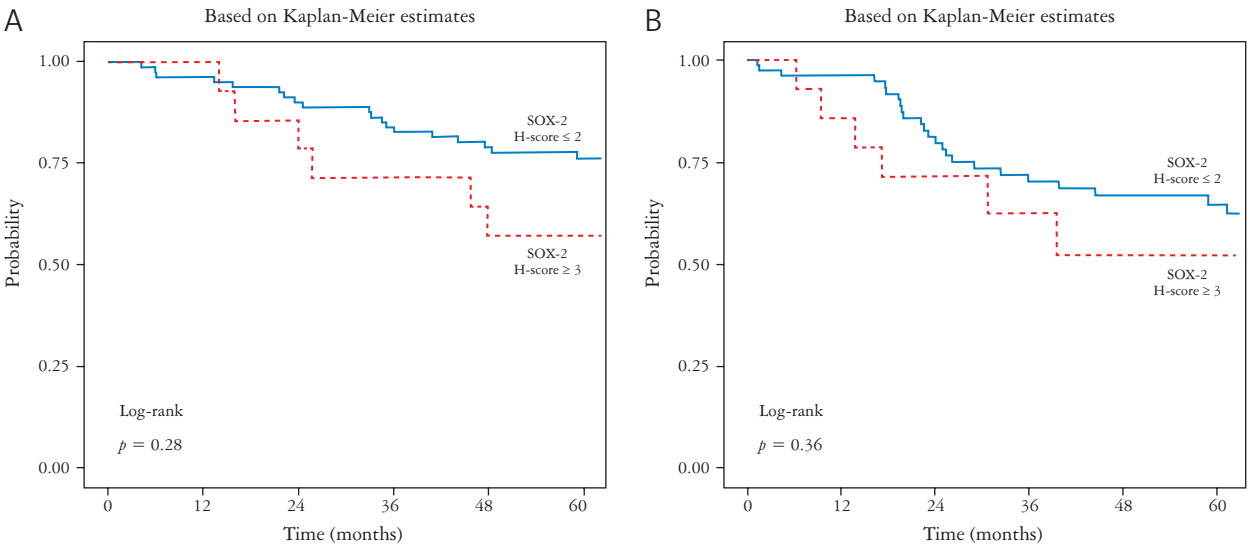

Fig. 3. A) Overall survival. B) Disease-free survival
Rates of cases with an H-score ≥ 3 were lower than the others, but without statistical significance.

nificant association between SOX-2 expression and OS and/or DFS ($p = 0.28$, $p = 0.36$). Despite the lack of significance, patients with a SOX-2 H-score higher than 2 showed lower OS rates, with 100% in 1 year, 71.4% in 3 years, and 57.1% in 5 years. For patients with a SOX-2 H-score of 2 or lower, the OS rates were 96.3% at 1 year, 84.0% at 3 years, and 76.1% at 5 years. Like OS rates, cases with SOX-2 H-scores greater than 2 had lower DFS rates, 85.7% at 1 year, 62.5% at 3 years, and 52.1% at 5 years. For cases with a SOX-2 H-score of 2 or lower, DFS rates were 96.2% at 1 year, 70.1% at 3 years, and 64.5% at 5 years.

Discussion

In CSC models of breast cancer, CSCs, which only constitute 1% of the tumour, are believed to be the tumour initiators [9]. Classical CSC markers, such as CD133, CD44, and ABCG2, are widely used but mostly nonspecific. Biomarkers such as SOX-2, Oct-4, and Nanog are expressed in stem cells and used to demonstrate the existence of CSCs [30, 31]. In breast cancer, CSCs are associated with a basal-like phenotype and TNBC [32, 33]. The SOX transcription factor family plays critical roles throughout embryonic development [34]. As CSCs represent only a small subset of cells in a tumour, they express the SOX-2 protein in a heterogeneous manner [19, 31, 35]. SOX-2 is particularly expressed in those CSCs with tumour-initiating and developing features [36, 37].

In a meta-analysis of 18 studies and 1833 cases of IBC, SOX-2 expression was found in 20.7% of cases. The expression of SOX-2 was not associated with age, menopausal status, or lymphovascular invasion. Although SOX-2 expression was not correlated with OS, it was significantly correlated with poor DFS [38]. In a meta-analysis of 1713 IBC cases, SOX-2 expression was found to be associated with larger tumour size, LNM, and higher histological grade. When grouped as TNBC and non-TNBC for research purposes, SOX-2 expression was found to be significant in the TNBC group [17].

SOX-2 is a tumour promoter of TNBC with ability to support tumour proliferation and metastasis [20]. There are only a few studies with limited data on IHC SOX-2 expression in TNBC, and the standardization of the IHC method is insufficient [20, 21, 39]. With a median follow-up of 76 months, our study aimed to show the significance of SOX-2 in TNBC cases without neoadjuvant treatment. To the best of our knowledge, this is the only research on TNBC cases that has utilized an IVD SOX-2 antibody and a validated IHC automated stainer with positive controls. Other research in the English literature used research antibodies, either with manual staining or without specifying the method and lacking positive controls or not mentioning them [20, 21, 39]. The authors define the term "standardized methodology" as an IHC study with all the slides having positive controls conducted with an IVD antibody in a validated automated stainer.

Table IV. Comparison of the methodology in the present study with three similar studies [20, 21, 39]

Parameter	Our study ($N = 95$)	Liu *et al.* ($N = 237$)	Yao *et al.* ($N = 120$)	Kamarlis *et al.* ($N = 40$)
Nuclear intensity scoring	0–3	0–4	0–3	0–4
Evaluation of the percentage	Whole slide	3 HPF	10 HPF	Whole slide
Scoring of the percentage	N/A	N/A	Score 0 = 0% 0% < Score 1 < 25% 25% < Score 2 < 50% Score 3 > 50%	Score 1 < 20% 20% < Score 2 < 50% 50% < Score 3 < 80% Score 4 > 80%
General scoring	Multiplying intensity score by percentage (H-score)	Multiplying intensity score by percentage	Aggregation of intensity score and percentage score	Multiplying intensity score by percentage score
Interpretation of SOX-2 expression	Expression: H-score 1 or higher	High expression: score 4 or higher	High expression: score 4 or higher	SOX-2 low: score 5 or lower SOX-2 moderate: score 6–10 SOX-2 high: score 11–16
SOX-2 expression rates in TNBC cases	30.5%	51.4%	In patients aged 35 years or under: 55.54% In patients aged more than 35 years: 51.23%	SOX-2 low: 47.5% SOX-2 moderate: 30% SOX-2 high: 22.5%

HPF – high-power fields, N – the number of non-missing values, N/A – not available, TNBC – triple-negative breast cancer

Our study utilized ROC and R-based cut-off determination from survival data to detect a cut-off value for the SOX-2 H-score. The SOX-2 H-score cut-off of 2 was determined, but it was not statistically significant. Although the difference was not statistically significant, cases with a SOX-2 H-score above 2 exhibited lower OS and DFS rates. This could be due to the small sample size of only 14 cases with a SOX-2 H-score higher than 2. As shown in Table 4, the evaluation of SOX-2 IHC in TNBC cases lacks standardization. Our study utilized the well-known H-score to evaluate SOX-2 expression. Therefore, we defined SOX-2 expression as nuclear expression in 1% or more of the tumoural cells in the whole slide and a nuclear intensity score of 1 or more, which corresponds to a SOX-2 H-score of 1 or more. SOX-2 expression was detected in 30.5% of TNBC cases in our study.

Different terminologies have been used to describe SOX-2 expression in different studies, such as high and/or moderate expression. Liu *et al*. reported high SOX-2 expression in 51.4% of 237 TNBC cases [20]. Yao *et al*. detected high SOX-2 expression in 55.54% of patients aged 35 years or under and in 51.23% of patients aged more than 35 years out of 120 TNBC cases [21]. In a study by Kamarlis *et al*., 40 cases of TNBC were analysed, with 47.5% showing low SOX-2 expression, 30% showing moderate expression, and 22.5% showing high expression [39]. In two studies on IBC, SOX-2 expression was defined as staining in 1% or more of the tumour [18, 40].

In comparison to IHC SOX-2 studies on TNBC cases [20, 21, 39], the SOX-2 expression rates in our study were relatively low. This may be due to the use of an IVD antibody and a specified, validated automated IHC stainer. In two studies that included slides with positive controls, SOX-2 expression was defined as nuclear staining in any tumour cell and was found in only 15% and 27% of IBC cases [41, 42].

Table 5 displays a review of the association between SOX-2 expression and clinicopathological features in TNBC. Consistent with two previous studies on SOX-2 expression in TNBC, no association was found between SOX-2 expression and age, tumour size, or histological grade [20, 21]. This study, however, is the first to demonstrate a significant association between tumour multifocality and negative SOX-2 expression. In studies by Liu *et al*. and Yao *et al*., SOX-2 expression was found to be correlated with LNM and higher anatomical stage [20, 21]. However, our study did not find such an association. Our study found, for the first time in the literature, a weak correlation between SOX-2 expression and perinodal infiltration. Additionally, we found that TNBC of a special histological type was inversely correlated with SOX-2 expression. Lengerke *et al*. observed a weak correlation between SOX-2 expression and presence of a special histological type in IBC [42].

Table V. Association of SOX-2 status with clinicopathological features in the present and in two related studies [20, 21]

Feature	Our study ($N = 95$)	Liu *et al.* ($N = 237$)	Yao *et al.* ($N = 120$)
Patient age	N/S	N/S	N/S
Multicentricity	N/S	N/A	N/A
Multifocality	**Inverse correlation**	N/A	N/A
Tumour size	N/S	N/S	N/S
Advanced pT	N/S	**Correlation**	N/A
Lymph node metastasis	N/S	**Correlation**	**Correlation**
Advanced pN	N/S	N/A	N/A
Advanced anatomical stage	N/S	**Correlation**	**Correlation**
Perinodal infiltration	Weak correlation	N/A	N/A
Histological type	N/S	N/A	N/A
Presence of special histological type	Weak, inverse correlation	N/A	N/A
Lymphovascular invasion	N/S	N/A	N/A
Ductal carcinoma *in situ*	N/S	N/A	N/A
Ki-67 proliferation index	N/S	N/A	N/A
Histological grade	N/S	N/S	N/S
Poor overall survival	N/S	**Correlation**	N/A
Poor disease-free survival	N/S	**Correlation**	N/A

N – the number of non-missing values, N/A – not available, N/S – not significant, pN – pathological regional lymph nodes, pT – pathological tumour
Variables with statistically significant differences ($p < 0.05$) are indicated in bold letters.

Liu *et al*. found that high expression of SOX-2 led to worse OS and DFS. The study included 237 cases of TNBC, but a research antibody was used, and no information on follow-up time or control methods was provided [20]. In our study, despite the long-term follow-up, there was no correlation between SOX-2 expression and survival rates.

Limitations of our study included the small number of cases, the absence of SOX-2 IHC evaluation in metastatic lymph nodes, and the inability to evaluate SOX-2 expression at the mRNA level. As neoadjuvant chemotherapy is administered even in early-stage TNBC cases, only 95 mastectomy cases with adequate tissue that did not receive neoadjuvant treatment were retrieved. Three studies that applied SOX-2 IHC to metastatic lymph nodes found that tumours expressing SOX-2 also exhibited more intense SOX-2 expression in the metastatic tumour cells [18, 42, 43].

Conclusions

The study, which had a median follow-up duration of 76 months, did not identify any association between SOX-2 and survival. Although the group with a SOX-2 H-score of 3 or more showed lower survival rates, the difference was not statistically significant. Previous research with a standardized methodology found lower SOX-2 expression levels and addressed the fact that SOX-2 expressing cells can be heterogeneous. It is worth noting that SOX-2 positivity is rare, as CSCs constitute only 1% of the tumour cells. Using a standardized methodology like in our study, future research involving larger series of TNBC cases can more conclusively demonstrate the prognostic impact of SOX-2 expression.

Disclosures

1. Study approval was obtained from the institutional review board at Izmir Kâtip Celebi University Medical Faculty (2023-GOKAE-0041). This study was conducted following the principles of the Declaration of Helsinki.
2. A preliminary version of this study was presented as an abstract at the 36th European Congress of Pathology in September 2024 in Florence, Italy. The data were subjected to evaluation by a remunerated biostatistician (MD) utilizing the software packages SPSS Statistics Standard Concurrent User V 26 and R-based Jamovi version 2.3. The authors would like to thank Prof. Dr. Gulden Diniz, MD, PhD, for her work in revising this article.
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4. Conflicts of interest: None.

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