CANT1 as a novel prognostic biomarker in triple-negative breast cancer

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Abstract. Triple-negative breast cancer (TNBC) is one of the most aggressive breast cancer subtypes, with limited targeted treatment options and poor clinical outcomes. Calcium-activated nucleotidase 1 (CANT1), a calcium dependent enzyme involved in nucleotide metabolism and glycoprotein processing, has attracted limited attention in oncology and its clinical relevance in breast cancer remains unknown. The present study hypothesized that CANT1 expression may carry prognostic information in TNBC and evaluated its association with clinicopathologicalal features and survival. In the present retrospective study, 59 non-metastatic patients with TNBC who underwent curative surgery were included. CANT1 expression was assessed using immunohistochemistry and semiquantitatively scored using an H-score ranging from 1-300. Patients were categorized into three groups according to H-score (H1, 1-100; H2, 101-200; H3, 201-300). Associations between CANT1 expression and clinicopathologicalal variables were analyzed using the χ^2 or Fisher's exact test and survival outcomes were evaluated using the Kaplan-Meier method, log-rank test and Cox proportional hazards model. CANT1 expression was successfully evaluated in all tumors: 15 patients (25.4%) were classified as H1, 29 (49.2%) as H2 and 15 (25.4%) as H3. The median overall survival (OS) for the entire cohort was 40.2 months, with a median follow-up of 48.4 months. Median OS by expression group was 29.2 months in H1, 36.7 months in H2 and not reached in H3 (log-rank P=0.033). In the multivariable analysis, high CANT1 expression (H3 vs. H1) remained independently associated with improved OS (P=0.048; hazard ratio=0.149; 95% CI, 0.031-0.715). To the best of our knowledge, the present study is the first to investigate CANT1 expression in TNBC. High CANT1 expression was significantly assocaited with improved OS, suggesting that CANT1 may serve as a novel prognostic biomarker in TNBC. Further multicenter and mechanistic studies are warranted to clarify its biological role and prognostic utility.

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Abbreviations: CANT1, calcium-activated nucleotidase 1; TNBC, triple-negative breast cancer; ER, estrogen receptor; PR, progesterone receptor; IHC, immunohistochemistry; OS, overall survival

Key words: triple-negative breast cancer, calcium-activated nucleotidase 1, prognostic biomarker, immunohistochemistry, overall survival, disease-free survival, breast cancer

Introduction

Breast cancer remains the second most frequently diagnosed malignancy and a leading cause of cancer-related mortality among female patients worldwide. The average annual age-standardized incidence rate in female patients is 131.8 per 100,000. Stage at diagnosis represents the most important prognostic determinant, with 5-year relative survival rates >99% in localized disease, decreasing to approximately 87% in regional disease and 32% in metastatic disease (1). Among its molecular subtypes, triple-negative breast cancer (TNBC) accounts for 10-20% of all cases and is defined by the absence of estrogen receptor (ER), progesterone receptor (PR) and HER2 expression or amplification (2). TNBC is recognized as a biologically aggressive subtype, typically composed of high-grade, highly proliferative tumors and is associated with limited therapeutic options and a markedly worse prognosis compared with hormone receptor-positive or HER2-enriched breast cancer types, exhibiting notably lower 5-year survival rates (3).

Calcium-activated nucleotidase 1 (CANT1) is a calcium-dependent glycoprotein with nucleotidase activity that shares cDNA sequence homology with adenosine triphosphatases and diphosphatases. It is expressed in a range of human tissues, particularly in cartilage and other connective tissue, as well as epithelial organs such as the prostate, liver and kidney, and can also be secreted extracellularly (4). Functionally, CANT1 is predominantly localized

to the endoplasmic reticulum and Golgi apparatus, where it hydrolyzes nucleoside diphosphates (such as UDP and GDP), thereby regulating nucleotide homeostasis and supporting proper protein glycosylation (5). CANT1 serves a key role in cell proliferation, differentiation and cartilage matrix organization and that loss-of-function mutations in CANT1 are associated with congenital skeletal dysplasias (5,6).

Emerging evidence has suggested that elevated CANT1 expression may influence tumor biology by modulating cell proliferation, apoptosis and extracellular signaling through alterations in nucleotide metabolism and glycoprotein processing (7). However, the prognostic importance of CANT1 is context-dependent. In numerous malignancies, such as prostate cancer, higher CANT1 expression has been associated with favorable outcomes, whereas in others, including hepatocellular carcinoma, it has been associated with poor prognosis (7,8). To the best of our knowledge, CANT1 expression in breast cancer has not been characterized and no prior studies have specifically examined its role in TNBC. Given the lack of hormone receptor and HER2 signaling in TNBC, investigation of CANT1 as a potential component of alternative molecular pathways in this subtype may provide novel insights into its biological and prognostic relevance.

Therefore, the present study aimed to evaluate CANT1 expression in TNBC and investigate its association with clinicopathologicalal features and overall survival (OS). By clarifying the prognostic importance of CANT1 in this setting, the aim was to identify a potential biomarker for risk stratification in this clinically challenging breast cancer subtype. The present study specifically focused on TNBC as this subtype lacks established therapeutic targets and validated prognostic biomarkers. In hormone receptor-positive or HER2-positive breast cancer types, strong biological drivers such as ER signaling or HER2 amplification may dominate tumor behavior and potentially confound the prognostic impact of additional molecular markers, such as Ki-67, TP53 alteration or PIK3CA mutation. Furthermore, through restricting analysis to TNBC, the present study aimed to evaluate the prognostic relevance of CANT1 in a biologically homogeneous setting requiring novel biomarkers.

Materials and methods

Study design and patient cohort. Within the present retrospective, single-center study, consecutive patients with TNBC who underwent definitive breast surgery between May 2017 and December 2024 and were followed at the Department of Medical Oncology of Ankara City Hospital (Ankara, Turkey) were included. All immunohistochemistry (IHC) assessments were performed on surgical resection specimens of primary breast tumors and adjacent tissue (distance, >5 mm); preoperative core needle biopsies and metastatic tissues were not analyzed.

Eligible patients had histologically confirmed non-metastatic TNBC, available surgical tumor tissue for immunohistochemical analysis and complete clinicopathological and follow-up data. Patients with metastatic disease at diagnosis, prior malignancy, inadequate tissue samples or missing follow-up data were excluded. An initial cohort of 60 surgically treated TNBC cases was screened, with 1 case excluded due to inadequate tissue quality for reliable IHC evaluation. The final cohort comprised 59 patients, all of whom were female, with a median age of 55 years (range, 28-78 years). Clinicopathological parameters including age at diagnosis, histologic type and grade, tumor size, nodal status, lymphovascular invasion, resection margins and adjuvant treatments were retrieved from institutional electronic medical records and pathology reports.

A post hoc power calculation was performed based on the observed hazard ratio (HR) for OS between the H3 (high CANT1) and H1 (low CANT1) groups (HR=0.149; 95% CI, 0.031-0.715). Using Schoenfeld's approximation for the log-rank test with two-sided significance level α=0.05, the observed number of events between these groups (n=11) provided an estimated statistical power of 88%, demonstrating that the present study possessed adequate power to detect the observed survival difference between high and low CANT1 expression groups.

Specimen handling and sectioning. Representative formalin-fixed, paraffin-embedded blocks were selected by a breast pathologist to ensure adequate tumor cellularity and morphological preservation. Surgical specimens were fixed in 10% neutral buffered formalin at room temperature for 24-48 h. Serial 3 μm sections were cut on a Leica RM2125RT rotary microtome (Leica Biosystems), mounted on charged glass slides and baked to enhance adhesion. Scoring was performed on areas of invasive carcinoma, excluding *in situ* components, stromal elements, necrotic tissue and artifacts. When numerous tumor blocks were available, the block with the most representative viable invasive tumor was prioritized for staining; if necessary, additional blocks were evaluated and the predominant pattern was used for scoring, with heterogeneity documented qualitatively.

IHC for CANT1. IHC was performed using the BOND MAX Fully Automated IHC and ISH Staining System (Leica Biosystems) according to the manufacturer's protocol to minimize inter-assay variability. Heat-induced epitope retrieval was performed using epitope retrieval 2 solution (Tris-EDTA based buffer; alkaline pH) under controlled temperature and time settings. Endogenous peroxidase activity was quenched using the ready-to-use BOND Peroxide Block reagent (Leica Biosystems GmbH) according to the manufacturer's instructions on the BOND-MAX automated staining platform.

The primary antibody used was anti-CANT1 rabbit polyclonal (cat. no. HPA019627; Prestige Antibodies®; Merck KGaA). The antibody was applied at a 1:300 for 30 min at room temperature. Signal detection employed the Leica HRP conjugate BOND polymer refine detection system (cat. no. DS9800; Leica Biosystems). Chromogenic development with 3,3' diaminobenzidine was followed by hematoxylin counterstaining for 5 min at room temperature, graded alcohol dehydration, xylene clearing and coverslipping.

Each IHC run included negative controls prepared by omitting the primary antibody and substituting using Rabbit Negative Control Reagent (Leica Biosystems, Newcastle, United Kingdom). Internal tissue controls were derived from adjacent non-neoplastic ductal epithelium and benign breast

parenchyma , Stromal fibroblasts, lymphocytes and endothelial cells were consistently non-reactive and served as internal negative references.

Subcellular localization criteria. Based on the biochemical properties of CANT1, a lumenal enzyme primarily localized to the Golgi apparatus and endoplasmic reticulum, only cytoplasmic and perinuclear granular staining in tumor cells was considered specific. Nuclear or circumferential membranous staining, if present, was interpreted as non-specific and excluded from scoring. Cases with extensive necrosis, tissue folds, edge artifacts or inadequate antigen preservation were recut and restained; persistently suboptimal slides were excluded from analysis.

Evaluation and scoring of CANT1 immunoreactivity. When necessary, two board-certified pathologists, blinded to clinical outcomes, independently evaluated all stained slides using alight microscope (Olympus Corporation) for consensus. Staining intensity in invasive tumor cells was graded on a 4-tier scale: 0, no staining; 1+, weak cytoplasmic/perinuclear staining; 2+, moderate cytoplasmic staining; and 3+, strong coarse granular cytoplasmic/perinuclear staing (7). For each case, the percentage of invasive tumor cells demonstrating 1+, 2+ and 3+ intensity was estimated to the nearest 5%, with the sum of all intensity categories reaching ~100% of invasive tumor cells. An H-score was calculated using the following weighted formula: H-score=(1 x percentage of weakly stained cells) + (2 x percentage of moderately stained cells) + (3 x percentage of strongly stained cells).

To facilitate groupwise comparisons in subsequent analyses, H-scores were also categorized a priori as: Low (H1), 1-100; moderate (H2), 101-200; and high (H3), 201-300. These predefined H-score cut-off values were selected based on previously published IHC literature employing similar semi-quantitative H-score-based classification systems (7). Values of 0 were permitted by definition but were not observed in the present cohort.

In cases with intratumoral heterogeneity, both predominant fields and focal hotspots were examined; the final score reflected the overall distribution across the tumor area rather hotspot estimation alone. Discrepancies between observers were resolved through consensus. Percentage maps, intensity grids and representative microphotographs were archived in the digital pathology system for documentation.

Quality assurance. All IHC runs were performed in uniform batches (≤20 slides per run) using identical retrieval and detection programs to ensure technical consistency. Lot numbers, incubation times and instrument logs were recorded. Slides were examined under standardized illumination using an Olympus BX series light microscope (Olympus Corporation). To minimize preanalytical variability, blocks with prolonged fixation (>72 h), delayed fixation or gross autolysis were avoided when possible; repeat staining was performed from freshly cut sections when necessary.

Statistical analysis. Statistical analyses were performed using SPSS (version 22.0; IBM Corp.). Descriptive statistics were presented as counts and percentages for categorical variables and as medians with interquartile ranges for continuous variables. Continuous variables were analyzed using the Mann-Whitney U test, while categorical variables were assessed with Pearson's χ^2 test or Fisher's exact test, as appropriate. Survival analyses were conducted using the Kaplan-Meier method and the log-rank test with survival curves generated using the R statistical software (version 4.3.2; Posit Software, PBC). Multivariable analysis was performed using the Cox proportional hazards model. Adjusted HRs, 95% CIs and P-values were reported, with $P<0.05$ being considered to indicate a statistically significant difference.

With regard to missing data, Ki-67 values were available for 56 of 59 patients (94.9%). The correlation between CANT1 H-score and the Ki-67 proliferation index was evaluated using Pearson's correlation coefficient, as both variables were treated as continuous variables. Programmed death-ligand 1 (PD-L1) combined positive scoring (CPS) was assessable in 28 patients (47.5%), with 24 patients (40.7%) scoring as negative (CPS 0) and 4 patients (6.8%) demonstrating positive expression (CPS 1-25 or >25); 31 patients (52.5%) had unknown PD-L1 status due to unavailable tissue or incomplete testing. BRCA mutational status was available in 24 patients (40.7%), with 35 patients (59.3%) having unknown results. Missing data were handled using complete-case analysis; no imputation methods were employed given the exploratory nature of the present study and the low proportion of missingness for primary prognostic variables.

Results

A total of 59 non-metastatic patients with TNBC treated at The Department of Medical Oncology of Ankara City Hospital were included in the present study. The median age was 55 (range, 28-78 years) and comorbidities were present in 62.7% of patients. The median tumor size was 3.1 cm (range, 0.9-13.0 cm), with a median Ki-67 index of 70%. According to primary tumor stage (T), 23.7% of patients had T1, 49.2% had T2, 18.6% had T3 and 8.5% had T4 disease. Pathologically, 27.1% of patients were lymph node-negative (N0), 57.7% were N1 and 15.2% were N2. Based on pathological TNM staging8.5% were stage I, 59.3% were stage II and 32.2% were stage III (9). BRCA mutations were detected in 3.3% of patients, while the PD-L1 CPS was 1-25 in 5.1% and >25 in 1.7% of the cohort. IHC analysis of CANT1 was successfully performed in all 59 cases. Based on the predefined H-score classification, 15 patients (25.4%) demonstrated low expression (H1, 1-100), 29 patients (49.2%) showed moderate expression (H2, 101-200) and 15 patients (25.4%) exhibited high expression (H3, 201-300). No cases were completely negative for CANT1 staining. The clinical and pathological characteristics of the patients are summarized in Table I.

Among the 59 patients, 40 (67.8%) received neoadjuvant chemotherapy. The most common regimens were Adriamycin and cyclophosphamide followed by paclitaxel in 17 patients (42.5%) and Adriamycin and cyclophosphamide followed by carboplatin and paclitaxel in another 17 patients (42.5%). In addition, 4 patients (10%) received Adriamycin and cyclophosphamide alone, while 2 patients (5%) received immunotherapy-containing regimens (Adriamycin, cyclophosphamide and pembrolizumab or carboplatin, paclitaxel and pembrolizumab).

Table I. Clinical and pathological characteristics of patients in the present study.

Characteristic	No. of patients
Age, years	55 (28-78)
Sex	
Female	59 (100.0)
Comorbidity	
Yes	37 (62.7)
No	22 (37.3)
Tumor size, cm	3.1 (0.9-13.0)
Primary tumor	
T1	14 (23.7)
T2	29 (49.2)
T3	11 (18.6)
T4	5 (8.5)
Pathological lymph node	
N0	16 (27.1)
N1	34 (57.7)
N2	9 (15.2)
TNM stage	
IA	5 (8.5)
IIA	14 (23.7)
IIB	21 (35.6)
IIIA	13 (22.0)
IIIB	4 (6.8)
IIIC	2 (3.4)
Estrogen receptor	
Negative	59 (100.0)
Progesterone receptor	
Negative	59 (100.0)
HER2	
Negative	59 (100.0)
Ki-67, %	70 (5-90)
BRCA mutation status	
Wild-type	22 (37.3)
Mutant	2 (3.3)
Unknown	35 (59.3)
PD-L1 CPS	
0	24 (40.7)
1-25	3 (5.1)
>25	1 (1.7)
Unknown	31 (52.5)
CANT1 H-score category	
H-score 1 (1-100)	15 (25.4)
H-score 2 (101-200)	29 (49.2)
H-score 3 (201-300)	15 (25.4)

Age, tumor size and Ki-67 are presented as median (range). All other values are presented as n (%). T, tumor stage; CANT1, calcium-activated nucleotidase 1; PD-L1, programmed death ligand 1; CPS, combined positive scoring.

Regarding surgical procedures, 31 patients (52.5%) underwent breast-conserving surgery with axillary lymph node dissection, 16 (27.1%) underwent radical mastectomy with axillary lymph node dissection and 12 (20.3%) had breast-conserving surgery with sentinel lymph node biopsy. Among the 40 patients who received neoadjuvant chemotherapy, 12 (30%) achieved a pathological complete response, while 8 (20%) showed a partial pathological response (data not shown). The remaining 20 patients (50%) exhibited limited or minimal response to neoadjuvant treatment.

During follow-up, 26 patients (44.1%) developed distant metastases. The most common metastatic sites were multiple sites in 8 patients (30.8%), liver in 5 (19.2%), bone in 4 (15.4%), lung in 3 (11.5%), brain or leptomeningeal involvement in 3 (11.5%), skin in 2 (7.7%) and adrenal gland in 1 patient (3.8%). Among metastatic patients, the most common first-line treatments were gemcitabine alone (19.2%), gemcitabine and cisplatin (19.2%), gemcitabine and carboplatin (15.4%) as well as capecitabine alone (11.5%). Other first-line therapies included paclitaxel (7.7%), cisplatin and capecitabine (7.7%), carboplatin and paclitaxel (3.8%), radiotherapy (7.7%) and no systemic treatment (7.7%). Second-line therapy was administered to 9 patients (15.3%), most commonly vinorelbine (44.4%), followed by sacituzumab govitecan (22.2%), capecitabine (11.1%), gemcitabine (11.1%) and paclitaxel (11.1%). Third-line treatments were given to 6 patients (10.7%), including capecitabine (33.3%), sacituzumab govitecan (33.3%), gemcitabine and cisplatin (16.7%) as well as gemcitabine (16.7%). Treatment modalities and clinical courses are summarized in Table II.

CANT1 cytoplasmic staining was independently evaluated by two pathologists and graded as 0, 1+, 2+ or 3+ based on staining intensity. Fig. 1 displays representative IHC staining patterns of CANT1 in the present study cohort of 59 patients. CANT1 expression was semi-quantitatively assessed using H-scores, which combine staining intensity and the percentage of positively stained tumor cells. A total of 9 patients demonstrated strong (3+) staining in 100% of tumor cells, 15 showed moderate (2+) staining in 100% of tumor cells and 6 exhibited weak (1+) staining in 100% of tumor cells (Fig. 1). The remaining cases displayed mixed staining patterns with variable intensity distributions. Overall, 15 patients (25.4%) were classified as H-score 3, 29 patients (49.2%) as H-score 2 and 15 patients (25.4%) as H-score 1. Fig. 2 shows a Sankey diagram summarizing CANT1 staining patterns and corresponding H-score categories

The median follow-up duration was 48.4 months and the median OS (mOS) for the entire cohort was 40.2 months. According to CANT1 expression levels, mOS was 29.2 months in the H-score 1 group, 36.7 months in the H-score 2 group and not reached in the H-score 3 group. The difference in OS among CANT1 H-score groups was statistically significant (log-rank P=0.033). Kaplan-Meier survival curves according to CANT1 H-score categories are shown in Fig. 3. For patients with H-score 1, the 1-, 2- and 3-year OS rates were 93.3, 66.7 and 34.6%, respectively. For the H-score 2 group, the corresponding rates were 100, 79.3 and 58.1%, while for the H-score 3 group they were 93.3, 84.0 and 84.0%, respectively. Patients with higher CANT1 expression (H-score 3) demonstrated more favorable long-term survival compared with those with lower expression levels (log-rank P=0.033). The 1-, 2- and

Table II. Treatment modalities and clinical course of the patients.

Variable	n (%)
Neoadjuvant chemotherapy	40 (67.8)
Neoadjuvant chemotherapy regimen	
Adriamycin + cyclophosphamide/paclitaxel	17/40 (42.5)
Adriamycin + cyclophosphamide/carboplatin + paclitaxel	17/40 (42.5)
Adriamycin + cyclophosphamide	4/40 (10.0)
Adriamycin + cyclophosphamide + pembrolizumab/carboplatin + paclitaxel + pembrolizumab	2/40 (5.0)
Surgical procedure	
Breast-conserving surgery + axillary lymph node dissection	31 (52.5)
Radical mastectomy + axillary lymph node dissection	16 (27.1)
Breast-conserving surgery + sentinel lymph node biopsy	12 (20.3)
Adjuvant chemotherapy	47 (79.7)
Adjuvant chemotherapy regimen	
Capecitabine	26/47 (55.3)
Adriamycin + cyclophosphamide/paclitaxel	14/47 (29.8)
Adriamycin + cyclophosphamide/carboplatin + paclitaxel	2/47 (4.3)
Pembrolizumab	2/47 (4.3)
Adriamycin + cyclophosphamide	1/47 (2.1)
Cyclophosphamide + methotrexate + 5-fluorouracil	1/47 (2.1)
Docetaxel + cyclophosphamide	1/47 (2.1)
Adjuvant radiotherapy	51 (86.4)
Metastasis after adjuvant treatment	26 (44.1)
Site of metastasis	
Multiple sites	8/26 (30.8)
Liver	5/26 (19.2)
Bone	4/26 (15.4)
Lung	3/26 (11.5)
Brain/leptomeningeal	3/26 (11.5)
Skin	2/26 (7.7)
Adrenal gland	1/26 (3.8)
First-line treatments for metastatic disease	26 (100.0)
Gemcitabine	5/26 (19.2)
Gemcitabine + cisplatin	5/26 (19.2)
Gemcitabine + carboplatin	4/26 (15.4)
Capecitabine	3/26 (11.5)
Paclitaxel	2/26 (7.7)
Cisplatin + capecitabine	2/26 (7.7)
Carboplatin + paclitaxel	1/26 (3.8)
Radiotherapy	2/26 (7.7)
No treatment	2/26 (7.7)
Second-line treatments	9 (15.3)
Vinorelbine	4/9 (44.4)
Sacituzumab govitecan	2/9 (22.2)
Capecitabine	1/9 (11.1)
Gemcitabine	1/9 (11.1)
Paclitaxel	1/9 (11.1)
Third-line treatments	6 (10.7)
Capecitabine	2/6 (33.3)
Sacituzumab govitecan	2/6 (33.3)
Gemcitabine + cisplatin	1/6 (16.7)
Gemcitabine	1/6 (16.7)

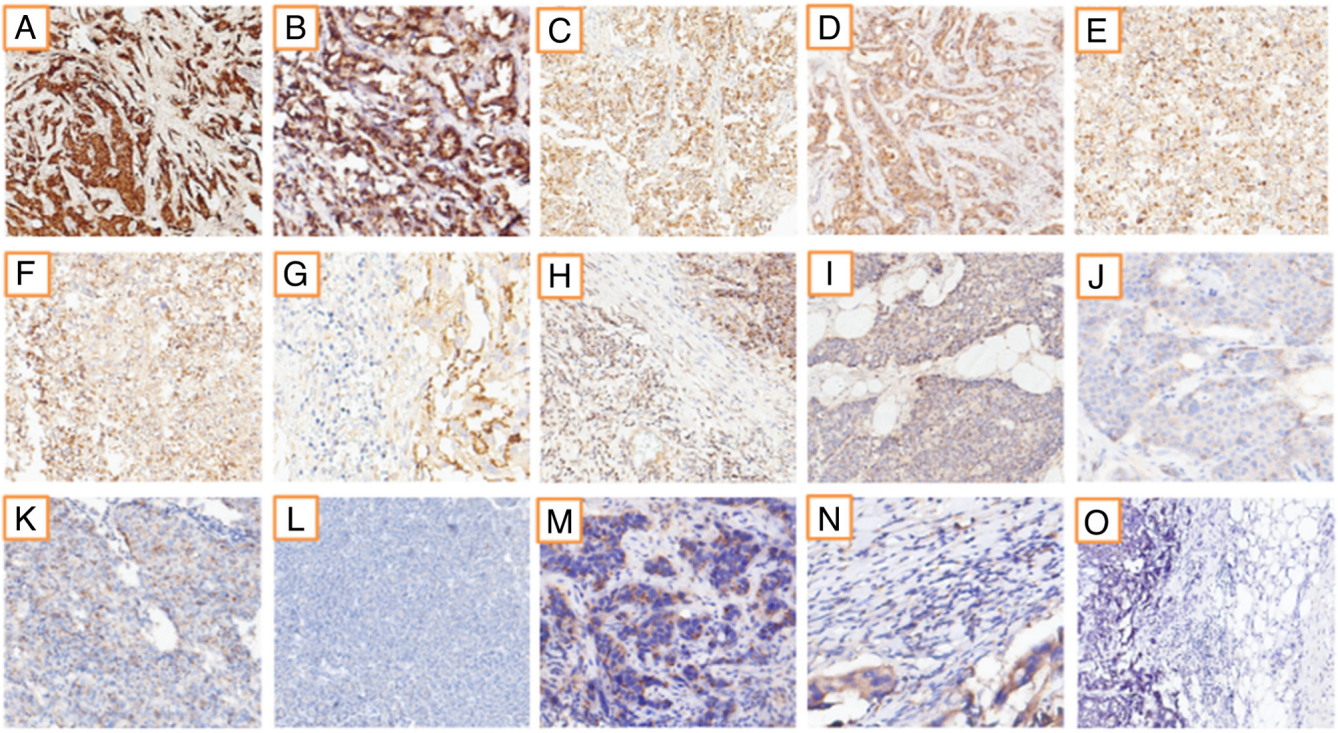

Figure 1. Representative immunohistochemical staining of CANT1 in triple-negative breast cancer showing 0 (negative), 1+, 2+ and 3+ cytoplasmic staining intensities. (A) Strong (3+) cytoplasmic and perinuclear granular positivity in 100% of invasive tumor cells. (B) Strong (3+) staining in ~80% of tumor cells. (C) Strong (3+) staining in ~70% of tumor cells. (D) Strong (3+) staining in ~70% of tumor cells. (E) Strong (3+) staining in ~60% of tumor cells. (F) Moderate (2+) cytoplasmic staining in ~60% of tumor cells. (G) CANT1 staining in peritumoral lymphocytes showing focal (~5%) positivity. (H) CANT1 staining in peritumoral lymphocytes showing diffuse (~80%) positivity. (I) Moderate (2+) cytoplasmic staining in ~40% of tumor cells. (J) Weak (1+) cytoplasmic staining in ~20% of tumor cells. (K) Weak (1+) cytoplasmic staining in ~40% of tumor cells. (L) Absence of CANT1 staining in invasive tumor cells (negative, 0). (M) Weak (1+) cytoplasmic staining in ~50% of tumor cells. (N) Peritumoral lymphocytes showing focal (~20%) positivity. (O) Peritumoral lymphocytes showing absence of staining.

3-year OS rates according to CANT1 H-score categories are summarized in Table III.

To further characterize the biological importance of CANT1 expression, its correlation with established prognostic biomarkers and clinicopathological parameters was examined. A weak negative correlation was observed between CANT1 H-score and Ki-67 proliferation index (Pearson r=-0.189; n=56). Notably, patients in the H-score 3 group (high CANT1 expression) demonstrated a mean Ki-67 index of 52.3%, which was markedly lower compared with the H-score 1 group (68.3%), suggesting an inverse relationship between CANT1 expression and tumor cell proliferative activity (Fig. 4). This finding was consistent with the improved OS observed in the high CANT1 expression group and may indicate that CANT1 contributes to suppression of proliferative signaling in TNBC. Analysis of staging parameters revealed no significant association between CANT1 H-score categories and T stage (mean T stage, H1=2.27, H2=2.28 and H3=1.87) or N stage (mean N stage, H1=1.07, H2=0.90 and H3=0.87; data not shown). These results suggest that CANT1 expression is independent of conventional anatomic staging and may represent a distinct biological pathway affecting prognosis in TNBC. With regard to PD-L1 CPS, insufficient data points precluded meaningful statistical analysis of its correlation with CANT1 expression; future studies with more complete PD-L1 assessment in larger cohorts are warranted to elucidate potential interactions between CANT1 and immune checkpoint molecule expression.

The prognostic impact of clinicopathological factors on OS was evaluated using univariate and multivariate Cox regression analyses. For multivariable analysis, only variables that demonstrated statistical significance in univariate analysis ($P<0.05$) were entered into the Cox proportional hazards model; the proportional hazards assumption was assessed using Schoenfeld residuals and was satisfied for all variables. Age was dichotomized at the median value of 55 years. In univariate analysis, patients aged ≥55 years demonstrated significantly longer OS compared with those aged <55 years (P=0.023). This association remained independently significant in multivariate analysis (P=0.024; HR=0.354; 95% CI, 0.144-0.875), indicating that a younger age (<55 years) was independently associated with worse prognosis. Other variables, including comorbidity, TNM stage, surgical approach and administration of neoadjuvant or adjuvant chemotherapy, were not significantly associated with OS in univariate analysis and thus were not included in the multivariate model. CANT1 H-score was significantly associated with OS in univariate analysis (P=0.033) and remained independently significant in the multivariate model (P=0.048). Pairwise comparisons revealed that the survival difference was primarily driven by the contrast between the H-score 3 and H-score 1 groups, whereby patients with high CANT1 expression (H3) exhibited significantly longer survival (HR=0.149; 95% CI, 0.031-0.715; P=0.017). The difference between the H-score 3 and H-score 2 groups was

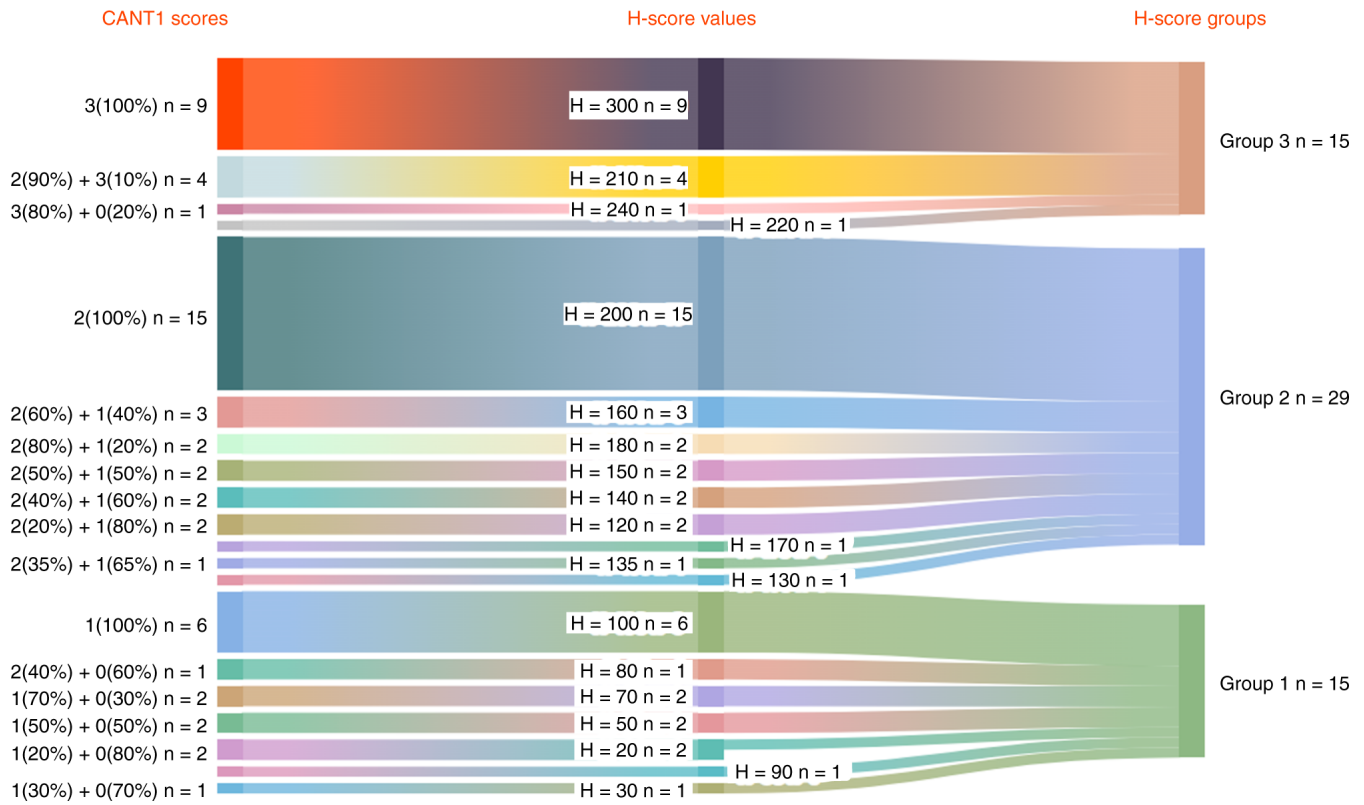

Figure 2. Sankey diagram of CANT1 expression patterns and H-score groups illustrating the relationship between semi-quantitative CANT1 staining patterns (left), calculated H-score values (middle) and predefined H-score groups (right) in 59 patients with triple-negative breast cancer. The left column represents combinations of staining intensity (1+, 2+ or 3+) and the percentage of positive tumor cells, the middle column shows the resulting H-score for each pattern (range, 20-300) and the right column shows the three H-score categories: Group 1 (H1, 1-100; n=15), group 2 (H2, 101-200; n=29) and group 3 (H3, 201-300; n=15). The width of each band is proportional to the number of patients contributing to that transition. CANT1, calcium-activated nucleotidase 1.

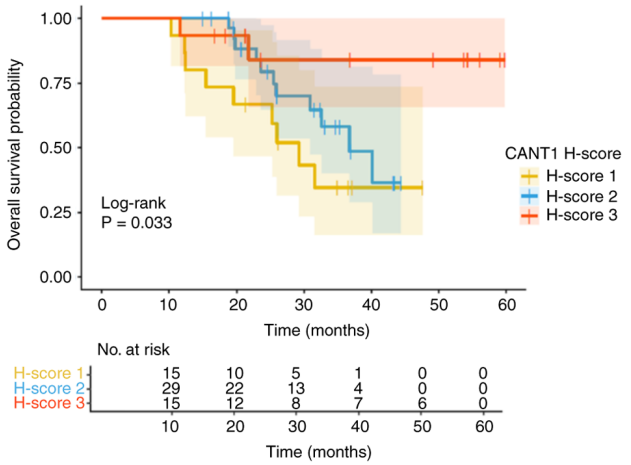

Figure 3. Kaplan-Meier overall survival curves according to CANT1 H-score categories (H1, 1-100; H2, 101-200; H3, 201-300). Vertical tick marks indicate censored observations. CANT1, calcium-activated nucleotidase 1.

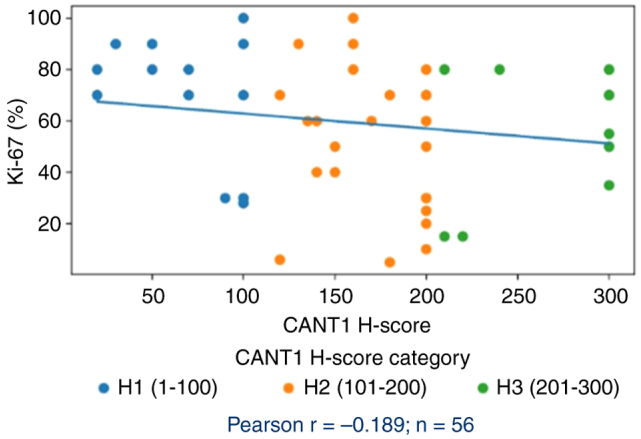

Figure 4. Correlation between CANT1 H-score and Ki-67 proliferation index. Scatter plot illustrating the relationship between CANT1 H-score and Ki-67 index in 56 patients with triple-negative breast cancer. Data points are color-coded according to CANT1 H-score categories (H1, 1-100; H2, 101-200; H3, 201-300). The solid line represents the linear regression fit, demonstrating a weak negative correlation (Pearson r=-0.189). CANT1, calcium-activated nucleotidase 1.

not statistically significant (HR=0.539; 95% CI, 0.220-1.300; P=0.172). These results are summarized in Table IV.

Discussion

TNBC is one of the most aggressive molecular subtypes of breast cancer, with limited targeted treatment options and unsatisfactory long-term outcomes despite advances in systemic therapy. In this context, reliable prognostic biomarkers that go beyond conventional anatomical staging remain an unmet clinical need. In the present cohort of 59 non-metastatic patients with TNBC, CANT1 expression, assessed by H-score,

Table III. Overall survival estimates at selected time points according to CANT1 H-score categories.

CANT1 H-score group	Time, months	No. at risk	No. of events	Survival, %	95% CI (lower-upper)
H-score 1	12	14	1	93.3	81.5-100.0
	24	9	4	66.7	46.6-95.3
	26	3	4	34.6	16.2-73.6
H-score 2	12	29	0	100.0	100.0-100.0
	24	18	5	79.3	64.7-97.3
	36	6	4	58.1	40.0-84.4
H-score 3	12	14	1	93.3	81.5-100.0
	24	8	1	84.0	65.6-100.0
	36	8	0	84.0	65.6-100.0

Survival rates are presented as percentages, with 95% CIs shown in the corresponding columns. CANT1, calcium-activated nucleotidase 1.

Table IV. Univariate and multivariate Cox regression analysis of prognostic factors for overall survival.

Variable	mOS, months	Univariate P-value	Multivariate HR (95% CI)	Multivariate P-value
Age		0.023	HR=0.354 (0.144-0.875)	0.024
<55	30.9			
≥55	NR			
Comorbidity		0.253		
Yes	31.5			
No	42.5			
Stage		0.059		
I-II	NR			
III	32.6			
Neoadjuvant chemotherapy		0.971		
Yes	NR			
No	40.1			
Adjuvant chemotherapy		0.089		
Yes	NR			
No	30.9			
Surgical procedure		0.870		
Radical mastectomy	36.8			
Breast-conserving surgery	42.4			
CANT1 H-score		0.033		0.048[a]
H-score 1	29.2		H-score 3 vs. 1, HR=0.149 (0.031-0.715); P=0.017	
H-score 2	36.7		H-score 3 vs. 2, HR=0.539 (0.220-1.300); P=0.172	
H-score 3	NR			

[a]Overall multivariate P-value for CANT1 H-score in the Cox model. CANT1, calcium-activated nucleotidase 1; mOS, median overall survival; HR, hazard ratio; NR, not reached.

25.4% of patients were classified as H-score 1, 49.2% as H-score 2 and 25.4% as H-score 3 and higher CANT1 expression (H-score 3) was associated with significantly longer OS compared with lower expression groups. In multivariable analysis, high CANT1 expression remained independently associated with improved OS, while traditional clinicopathological variables such as stage and treatment modalities were not retained as independent predictors. . Taken together, these results suggest that CANT1 expression may reflect tumor biological characteristics not indicated by conventional TNM staging and could provide additional prognostic information for risk stratification in TNBC.

At the molecular level, CANT1 encodes a calcium-dependent lumenal nucleotidase that localizes predominantly to

the endoplasmic reticulum and Golgi apparatus, whereby it regulates nucleotide metabolism and contributes to protein glycosylation and vesicular trafficking (4,5). By hydrolyzing nucleoside diphosphates such as UDP and guanosine diphosphate (GDP) into their corresponding monophosphates, CANT1 prevents the accumulation of inhibitory nucleoside diphosphates within the Golgi lumen, thereby facilitating nucleotide recycling and sustaining glycosylation reactions required for proper secretory pathway function. The clinical relevance of this pathway is illustrated by loss-of-function mutations in CANT1, which cause autosomal recessive Desbuquois dysplasia, a skeletal disorder characterized by growth restriction, limb shortening and severe abnormality of cartilage and extracellular matrix organization (10). These observations underscore the importance of CANT1 in tissue growth and matrix homeostasis, providing a plausible mechanistic basis for its involvement in tumor biology.

Beyond its intracellular role, CANT1 may also be secreted into the extracellular matrix, where its nucleotidase activity hydrolyzes extracellular nucleoside diphosphates such as UDP and GDP into monophosphates, thereby decreasing local diphosphate concentrations and potentially attenuating P2 receptor–mediated purinergic signaling (11). Through this mechanism, CANT1 may influence cell-cell communication, stromal remodeling and immune cell recruitment within the tumor microenvironment. Such context-dependent effects provide a biologically plausible explanation for why CANT1 expression might be associated with either favorable or unfavorable prognosis depending on tumor type and microenvironmental context.

Evidence from prostate cancer supports this context-dependent behavior. Gerhardt *et al* (7) systematically evaluated CANT1 expression in prostate cancer using large tissue microarrays and demonstrated marked upregulation of CANT1 in primary prostate carcinoma compared with benign tissue, with predominantly Golgi-type and cytoplasmic staining patterns. Notably, castration-resistant tumors showed decreased CANT1 expression and loss of CANT1 was associated with clinically more aggressive disease, suggesting that higher CANT1 expression may be associated with a more differentiated, less aggressive phenotype in prostate cancer (7). This pattern was concordant with the present findings in TNBC, whereby higher CANT1 expression was associated with improved OS, supporting the hypothesis that CANT1 may act as a favorable prognostic marker in at least a subset of solid tumors.

Within the present cohort, no completely CANT1-negative tumors were observed. Even in the lowest expression group, invasive carcinoma cells consistently exhibited at least weak cytoplasmic staining (1+) in ~20% of cells, resulting in minimum H-scores of 20. This near-consistent expression suggested that CANT1 may be constitutively expressed in TNBC or may represent a key feature of the TNBC phenotype. Whether this reflects a specific metabolic requirement of TNBC cells for secretory pathway homeostasis or a broader mammary tissue-specific expression pattern, could not be determined from the present data, but it underscored that prognostic information was carried not by the presence or absence of CANT1, but by the level of its expression.

By contrast, numerous studies have associated high CANT1 expression with adverse outcomes in other tumor types. Liu *et al* (8) reported that CANT1 was markedly upregulated in hepatocellular carcinoma compared with adjacent non-tumorous liver tissue and that high CANT1 levels were associated with an advanced stage, higher grade and shorter OS. Functional analyses in this study indicated that CANT1 upregulation may promote cell cycle progression, DNA replication and oncogenic signaling pathways in hepatocellular carcinoma (8). Similarly, pan-cancer bioinformatics and IHC work by Yang *et al* (12) showed that CANT1 was upregulated at both mRNA and protein levels across a number of malignancies and that elevated expression was associated with advanced T and N stages as well as worse OS and disease-specific survival in lung adenocarcinoma, kidney renal papillary cell carcinoma and lower-grade glioma, amongst others.

Yao *et al* (13) further demonstrated that high CANT1 expression was an independent predictor of poor OS in lung adenocarcinoma, both in The Cancer Genome Atlas and Gene Expression Omnibus datasets, with elevated CANT1 levels remaining notably associated with adverse prognosis after multivariable adjustment. These data stand in clear contrast with the present findings in TNBC, whereby high CANT1 expression was independently associated with improved OS. Collectively, the available evidence indicates that the prognostic impact of CANT1 is strongly tissue- and context-dependent: It may act as an oncogenic driver in HCC and lung adenocarcinoma, whereas in prostate cancer and TNBC, higher expression appears to be associated with more favorable clinical behavior.

The mechanisms underlying the protective association of high CANT1 expression in TNBC are speculative but a number of hypotheses may be considered. In the present cohort, higher CANT1 expression was accompanied by a trend toward lower proliferative activity, with the H-score 3 group exhibiting a markedly lower mean Ki-67 index compared with the H-score 1 group, consistent with the observed survival advantage. This inverse relationship suggested that CANT1 may be associated with a less proliferative, more differentiated phenotype in TNBC, potentially through its role in glycoprotein processing and maintenance of secretory pathway integrity. Enhanced CANT1-mediated glycosylation may support more orderly expression and trafficking of adhesion molecules and receptors, thereby constraining invasive behavior and metastatic potential. In addition, secreted CANT1 could modulate purinergic signaling within the tumor microenvironment, possibly reducing immunosuppressive nucleotide signaling and indirectly facilitating antitumor immune responses. Notably, the present study did not observe meaningful associations between CANT1 expression and T or N stage, suggesting that its prognostic effect is not simply a surrogate for tumor burden, but may reflect a distinct biological axis.

The present study exhibits certain limitations that should be acknowledged when interpreting the results. Its retrospective, single-center design carries the inherent risk of selection bias and incomplete capture of clinical and molecular covariates, despite systematic case identification. CANT1 expression was assessed using a semi-quantitative H-score method based on manual pathologist evaluation; although scoring was performed in a blinded fashion with consensus review, some degree of inter-observer variability cannot be excluded.

The overall sample size was modest (n=59), which limits the precision of effect estimates and the number of covariates that can be included in multivariable models. Nevertheless, a post hoc power calculation indicated 88% power to detect the observed difference between the highest and lowest H-score groups, suggesting that the main signal is unlikely to be a chance finding. Finally, the present study was designed primarily as a clinicopathological and prognostic analysis and did not include mechanistic experiments or external validation cohorts. In addition, missing data were present for certain clinical variables, including PD-L1 expression, BRCA status and Ki-67, which should be considered when interpreting the results, although the main analyses remained robust.

Furthermore, the inclusion of patients who did and did not receive neoadjuvant chemotherapy represents a source of clinical heterogeneity that may have influenced survival outcomes. While exploratory analyses did not reveal a consistent association between CANT1 expression and pathological response, this should be interpreted cautiously due to the limited sample size. Future work should validate these findings in larger, prospective, multicenter series with standardized IHC protocols, automated image analysis and comprehensive molecular profiling and to investigate the functional role of CANT1 in TNBC through both *in vitro* and *in vivo* models, as well as analyses in independent transcriptomic datasets. In addition, future studies should aim to specifically address the prognostic and biological importance of CANT1 expression in *de novo* metastatic TNBC cohorts. Given the distinct tumor biology, treatment intent and survival dynamics characterizing *de novo* metastatic disease, evaluating CANT1 in this setting may provide complementary insights and help clarify whether its prognostic impact differs between curative-intent early-stage and metastatic TNBC.

In conclusion, the present study provided primary evidence that CANT1 expression carries prognostic information in TNBC. In a cohort of 59 non-metastatic patients with TNBC treated with curative surgery, higher CANT1 expression, quantified by H-score, was independently associated with improved OS, whereas conventional clinicopathological factors did not retain significance in multivariable analysis. These findings, which contrast with reports of adverse prognostic effects of CANT1 in hepatocellular carcinoma and lung adenocarcinoma, support a context-dependent role for CANT1 in human cancer biology and suggest that it may act as a favorable prognostic marker in TNBC. Although the retrospective design and limited sample size warrant cautious interpretation, CANT1 emerges as a promising candidate biomarker for risk stratification in this high-risk breast cancer subtype. Validation in larger, independent cohorts and mechanistic studies clarifying how CANT1 modulates TNBC behavior are required before routine clinical implementation can be considered.

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Availability of data and materials

The data generated in the present study may be requested from the corresponding author.

Authors' contributions

DB and GU conceptualized the present study. DB, GU and BC were responsible for the methodology. DB, EH and SNÖC screened the hospital information system and patient medical records to identify patients with triple-negative breast cancer and extract the relevant clinical data. AC and DÖT conducted pathological examination and interpretation. SK performed tissue processing and CANT1 IHC staining. DB, ÖB and EA conducted formal analysis and statistical analysis. DB wrote the original manuscript draft. MANŞ, SY interpreted data and revised the manuscript . GU and BC provided supervision. BC contributed to the conceptualization and design of the study, and was involved in the analysis and interpretation of the data. DB and GU confirm the authenticity of all the raw data. All authors read and approved the final version of the manuscript.

Ethics approval and consent to participate

The present study protocol was reviewed and approved by The Ankara City Hospital Clinical Research Ethics Committee (approval no. TABED 1-25-1032). Due to the retrospective design and the use of archival formalin-fixed paraffin-embedded specimens, the requirement for written informed consent was waived in accordance with national regulations and institutional policy. All procedures were performed in accordance with the principles of The Declaration of Helsinki and applicable local data protection regulations.

Patient consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

Use of artificial intelligence tools

During the preparation of this work, artificial intelligence tools were used to improve the readability and language of the manuscript or to generate images, and subsequently, the authors revised and edited the content produced by the artificial intelligence tools as necessary, taking full responsibility for the ultimate content of the present manuscript.

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