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Serum Direct Bilirubin as a Biomarker for Breast Cancer

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Background: The role of serum total bilirubin (TB) in cancer has been a subject of controversy, as has the role of its subtypes, particularly serum direct bilirubin (DB). The aim of the present study was to investigate the association between serum DB levels and breast cancer, as well as to assess the diagnostic utility of serum DB in breast cancer.

Methods: A total of 5299 patients diagnosed with breast cancer for the first time at Taizhou Hospital of Zhejiang Province were included in the study, and 10028 healthy physical examination subjects were included as healthy controls. Logistics regression was used to investigate the relationship between serum DB and breast cancer, and the value of serum DB in the diagnosis of breast cancer was assessed by means of receiver operator characteristic (ROC) curve analysis.

Results: The serum DB concentration in the breast cancer group was significantly higher than the healthy controls ($P < 0.001$). Multivariate logistic regression results show that serum DB was an independent risk factor for breast cancer (odds ratio [OR]=4.504, 95% confidence interval [CI]: 4.200–4.831). Subjects with a serum DB concentration in the fourth quartile had a higher risk of breast cancer occurrence compared to those in the first quartile after adjusting for age (OR = 7.155, 95%CI: 6.474–7.907). The optimal cut-off value of serum DB for diagnosing breast cancer was determined to be 2.75 μmol/L, with an area under the curve (AUC) of 0.712 (95% CI: 0.703–0.722). This value exhibited good specificity (77.0%) and negative predictive value (77.8%).

Conclusion: Serum DB was identified as a risk factor for breast cancer, demonstrating good diagnostic potential for the disease. These findings suggest that serum DB could serve as a promising serum molecular marker for breast cancer.

Keywords: direct bilirubin, breast cancer, biomarker, risk, diagnostic

Introduction

Breast cancer is the most commonly diagnosed and deadliest cancer among women both in China and globally.[1] As of 2020, breast cancer constituted 11.7% of all new cancer cases in both men and women, and accounted for 6.9% of all cancer-related deaths.[2,3] At present, commonly used diagnostic methods for breast cancer include mammography, positron-emission tomography (PET), single-photon emission computed tomography (SPECT), computed tomography (CT), and magnetic resonance imaging (MRI).[4,5] These imaging techniques often exhibit moderate diagnostic specificity or positive predictive values, yet the sensitivity of these methods can be diminished by high breast tissue density.[6] Several biomarkers for diagnosis and prognosis of breast cancer have been developed, such as DNA methylation, MicroRNAs, oestrogen receivers, progesterone receivers, HER2, Ki67, Nottingham Prognostics Index (NPI) and machine learning system.[7–11] Diagnostic biomarkers are particularly useful for women with uncertain biopsy results.[12] Yet, there are no biomarkers recommended for routine diagnosis of breast cancer. Therefore, it is crucial to investigate effective molecular markers that can guide treatment decisions.

Bilirubin, composed of an open chain structure consisting of four pyrrole-like rings (tetrapyrrole), is derived from the breakdown of heme, primarily from hemoglobin.[13] The liver is involved in the physiological metabolism of bilirubin,

Received: 14 August 2024
Accepted: 31 October 2024
Published: 7 November 2024

with a portion of bilirubin's decomposition products participating in enterohepatic urobiliary circulation and a portion being excreted in urine by the kidneys.[14] Disorders in the synthesis or breakdown of bilirubin, or obstruction in the biliary system, can elevate blood bilirubin levels, resulting in conditions such as acute hepatitis and cirrhosis. Elevated bilirubin levels can also lead to bilirubin entering the central nervous system, causing encephalopathy.[15] Bilirubin is also considered one of the most effective endogenous antioxidants and a powerful immunosuppressive agent.[16] Recent studies have shown that bilirubin has a protective effect on cardiovascular disease. The lower the serum bilirubin concentration, the higher the risk of coronary heart disease.[17,18] Slightly elevated serum bilirubin levels may reduce cancer risk,[19] such as colorectal cancer.[20] Serum total bilirubin (TB) can be subdivided into indirect bilirubin (IB) and direct bilirubin (DB) based on testing methods. Certain studies suggest that while TB may have the potential to lower cardiovascular risk, DB has been identified as a risk factor for cardiovascular disease.[21] These findings highlight the controversial role of TB in human health, particularly in the analysis of its subclasses. While studies have examined the prognosis of breast cancer patients in relation to TB levels,[22] the relationship between bilirubin and its subtypes and the incidence of breast cancer remains unexplored. The aim of the present study was to investigate the association between DB, a subtype of bilirubin, and breast cancer within the Chinese population.

Patients and Methods

Patients

The patient group in the present study was first diagnosed with breast cancer at Taizhou Hospital of Zhejiang Province from December 30, 2011 to April 26, 2024. The inclusion criteria for the patient group were women aged 20 years or older who had undergone surgery for breast cancer and received a diagnosis confirmed by pathology. Exclusion criteria included severe deficiencies in clinical or laboratory information, significant liver or blood diseases, and male gender. Initially, 6045 patients with breast cancer were identified. Subsequently, 320 patients were excluded due to missing clinical information, 32 patients due to other cancers, 29 patients due to severe liver disease, 26 patients due to hemolytic disease, 307 patients due to missing laboratory data, and 32 male patients were also excluded. Finally, a total of 5299 patients were included in the study, with ages ranging from 20 to 92 years old. From January 1, 2022 to December 31, 2022, female participants undergoing health examinations throughout the year were selected as healthy controls. Individuals with cancer, severe liver disease, or hemolytic disease were excluded from this population. The healthy control group consisted of 10,028 individuals, with ages ranging from 20 to 94 years old. The study methodology was approved by the Medical Ethics Committee of Taizhou Hospital in Zhejiang Province, affiliated with Wenzhou Medical University (#K20221129). Due to the retrospective nature of the study, informed consent from patients was exempted by the Medical Ethics Committee of Taizhou Hospital in Zhejiang Province, as the research did not impact patient health or privacy.

Methods

All samples were collected in the morning from patients or healthy examiners who fasted. Serum samples were centrifuged at 3500 rpm for 5 min. A Beckman Coulter AU5800 autoanalyzer was used to detect the serum concentrations of γ-glutamyl transpeptidase (GGT), alanine aminotransferase (ALT), alkaline phosphatase (ALP), aspartate aminotransferase (AST), TB, DB, total protein (TP), albumin (ALB), triglycerides (TG), total cholesterol (TC), blood urea nitrogen (BUN), serum creatinine (Scr), uric acid (UA), and glucose (GLU). A Beckman DxI 800 automated chemiluminescence analyzer was used to analyze the concentration of carcinoembryonic antigen (CEA), cancer antigen 153 (CA153), cancer antigen 125 (CA125), cancer antigen 199 (CA199), alpha-fetoprotein (AFP) in serum.

Statistical Analysis

To compare laboratory parameters between breast cancer patients and the healthy control group, the independent sample *t*-test was employed for normally distributed data, expressed as mean ± standard deviation. For non-normally distributed

data, the Mann–Whitney *U*-test was used and results were reported as p50% (p25%, p75%). Logistics regression analysis was used to evaluate the risk factors for breast cancer, Laboratory parameters were treated as categorical variables based on quartiles in the logistic regression analysis. Variables with a significance level of $P < 0.1$ in the univariate logistic regression analysis were included in the multivariate logistic regression model. All subjects were divided into four groups according to the quartiles of serum DB concentration: Q1: DB ≤ 1.80 μmol/L; Q2: 1.80 < DB ≤ 2.30 μmol/L; Q3: 2.30 < DB ≤ 3.10 μmol/L; and Q4: DB > 3.10 μmol/L. Diagnostic accuracy was evaluated using receiver operating characteristic (ROC) curves. A *P* value < 0.05 was considered statistically significant. All statistical analyses were performed using SPSS software (v. 24.0).

Results

Baseline Characteristics of Subjects

The analysis included 5299 breast cancer patients (53 ± 11 years) and 10028 healthy control subjects (51 ± 14 years). As shown in Table 1, the age of breast cancer patients was significantly higher than that of healthy people (*P* value <0.001). Table 1 shows that serum GGT, ALP, AST, DB, TG, GLU, and CEA levels in patients with breast cancer were significantly higher than those in the healthy control group (all *P* value <0.05), while the serum concentrations of ALT, TB, TP, TC, UA, and CA125 were significantly lower than those in the healthy control group (all *P* value <0.01).

The Risk Factors for Breast Cancer by Logistic Regression Analysis

The univariate logistic regression results in Table 2 show that age (odds ratio [OR] = 1.105, 95% confidence interval [CI]: 1.072–1.139), GGT (OR = 1.044, 95% CI: 1.013–1.076), ALP (OR = 1.073, 95% CI: 1.042–1.106), AST (OR = 1.051, 95% CI: 1.021–1.082), DB (OR = 1.958, 95% CI: 1.894–2.023), ALB (OR = 1.098, 95% CI: 1.064–1.132), TG

Table 1 Baseline Characteristics of the Study Participants

Variables	Health (N = 10,028)	Breast Cancer (N = 5299)	*P* value
Age, Year	51 ± 14	53 ± 11	<0.001
GGT, U/L	17 (13, 25)	18 (13, 26)	0.012
ALT, U/L	16 (12, 22)	15 (11, 21)	<0.001
ALP, U/L	73 (59, 89)	75 (60, 92)	<0.001
AST, U/L	21(18, 25)	21 (18, 26)	0.004
TB, μmol/L	12.3 (10.1, 15.2)	11.6 (9.0, 15.0)	<0.001
DB, μmol/L	2.1 (1.7, 2.7)	3.0 (2.2, 4.1)	<0.001
TP, g/L	74 ± 4	73 ± 6	<0.001
ALB, g/L	44 ± 3	44 ± 4	0.283
TC, mmol/L	5.5 ± 1.1	5.0 ± 1.0	<0.001
TG, mmol/L	1.15 (0.83, 1.66)	1.28 (0.91, 1.88)	<0.001
Scr, μmol/L	57 (52, 63)	58 (51, 65)	0.089
BUN, mmol/L	4.8 ± 1.2	4.8 ± 1.5	0.258
UA, μmol/L	292 ± 64	273 ± 73	<0.001
GLU, mmol/L	4.89 (4.57, 5.33)	5.38 (4.98, 5.91)	<0.001
AFP, ng/mL	2.55 (1.88, 3.56)	2.59 (1.90, 3.62)	0.193
CA125, U/mL	12.9 (9.5, 18.2)	12.6 (9.4, 17.8)	0.007
CA153, U/mL	8.6 (6.4, 12.6)	8.5 (6.5, 12.2)	0.379
CA199, U/mL	6.1 (3.1, 12.1)	6.1 (3.1, 12.2)	0.935
CEA, ng/mL	1.46 (1.00, 2.20)	1.55 (1.04, 2.34)	<0.001

Abbreviations: GGT, γ-glutamyl transpeptidase; ALT, alanine aminotransferase; ALP, alkaline phosphatase; AST, aspartate aminotransferase; TB, total bilirubin; DB, direct bilirubin; TP, total protein; ALB, albumin; TC, total cholesterol; TG, triglycerides; Scr, serum creatinine; BUN, blood urea nitrogen; UA, uric acid; GLU, glucose; AFP, alpha-fetoprotein; CA125, cancer antigen 125; CA153, cancer antigen 125; CA199, cancer antigen 199; CEA, carcinoembryonic antigen.

Table 2 Univariate and Multivariate Logistic Regression Analyses of Risk Factors for Breast Cancer

Variables	Univariate Analysis		Multivariate Analysis	
	OR (95% CI)	*P* value	OR (95% CI)	*P* value
Age, Year	1.105 (1.072, 1.139)	<0.001	1.054 (1.005, 1.105)	0.030
GGT, U/L	1.044 (1.013, 1.076)	0.005	1.047 (0.994, 1.103)	0.083
ALT, U/L	0.918 (0.892, 0.946)	<0.001	0.834 (0.795, 0.876)	<0.001
ALP, U/L	1.073 (1.042, 1.106)	<0.001	0.994 (0.951, 1.038)	0.777
AST, U/L	1.051 (1.021, 1.082)	0.001	1.021 (0.979, 1.065)	0.330
TB, μmol/L	0.865 (0.841, 0.891)	<0.001	0.322 (0.302, 0.344)	<0.001
DB, μmol/L	1.958 (1.894, 2.023)	<0.001	4.504 (4.200, 4.831)	<0.001
TP, g/L	0.829 (0.804, 0.855)	<0.001	0.688 (0.655, 0.722)	<0.001
ALB, g/L	1.098 (1.064, 1.132)	<0.001	1.248 (1.188, 1.314)	<0.001
TC, mmol/L	0.663 (0.643, 0.684)	<0.001	0.836 (0.799, 0.874)	<0.001
TG, mmol/L	1.175 (1.140, 1.211)	<0.001	1.443 (1.374, 1.516)	<0.001
Scr, μmol/L	1.022 (0.992, 1.052)	0.156		
BUN, mmol/L	0.993 (0.964, 1.023)	0.657		
UA, μmol/L	0.767 (0.744, 0.791)	<0.001	0.798 (0.763, 0.834)	<0.001
GLU, mmol/L	1.952 (1.886, 2.020)	<0.001	2.067 (1.966, 2.173)	<0.001
AFP, ng/mL	1.027 (0.994, 1.060)	0.110		
CA125, U/mL	0.964 (0.934, 0.996)	0.026	0.965 (0.925, 1.007)	0.089
CA153, U/mL	0.980 (0.949, 1.012)	0.220		
CA199, U/mL	0.997 (0.965, 1.030)	0.856		
CEA, ng/mL	1.092 (1.058, 1.127)	<0.001	1.080 (1.035, 1.127)	<0.001

Abbreviations: GGT, γ-glutamyl transpeptidase; ALT, alanine aminotransferase; ALP, alkaline phosphatase; AST, aspartate aminotransferase; TBil, total bilirubin; DB, direct bilirubin; TP, total protein; ALB, albumin; TC, total cholesterol; TG, triglycerides; Scr, serum creatinine; BUN, blood urea nitrogen; UA, uric acid; GLU, glucose; AFP, alpha-fetoprotein; CA125, cancer antigen 125; CA153, cancer antigen 125; CA199, cancer antigen 199; CEA, carcinoembryonic antigen; OR, odds ratio; CI, confidence interval.

(OR = 1.175, 95% CI: 1.140–1.211), GLU (OR = 1.952, 95% CI: 1.886–2.020), CEA (OR = 1.092, 95% CI: 1.058–1.127) were risk factors for breast cancer. Conversely, the ALT (OR = 0.918, 95% CI: 0.892–0.946), TB (OR = 0.865, 95% CI: 0.841–0.891), TP (OR = 0.829, 95% CI: 0.804–0.855), TC (OR = 0.663, 95% CI: 0.643–0.684), UA (OR = 0.767, 95% CI: 0.744–0.791), and CA125 (OR = 0.964, 95% CI: 0.934–0.996) were protective factors for breast cancer. The multivariate regression analysis results in Table 2 show that the following were independent risk factors for breast cancer: age (OR = 1.054, 95% CI: 1.005–1.105), DB (OR = 4.504, 95% CI: 4.200–4.831), ALB (OR = 1.248, 95% CI: 1.188–1.314), TG (OR = 1.443, 95% CI: 1.374–1.516), GLU (OR = 2.067, 95% CI: 1.966–2.173), and CEA (OR = 1.080, 95% CI: 1.035–1.127). Meanwhile, the following were independent protective factors for breast cancer: ALT (OR = 0.834, 95% CI: 0.795–0.876), TB (OR = 0.322, 95% CI: 0.302–0.344), TP (OR = 0.688, 95% CI: 0.655–0.722), TC (OR = 0.836, 95% CI: 0.799–0.874), and UA (OR = 0.798, 95% CI: 0.763–0.834). All subjects were divided into four groups according to the quartiles of serum DB concentration: Q1: DB ≤ 1.80 μmol/L; Q2: 1.80 < DB ≤ 2.30 μmol/L; Q3: 2.30 < DB ≤ 3.10 μmol/L; and Q4: DB > 3.10 μmol/L (Table 3). In the unadjusted model, the relative risk of developing breast cancer in the Q2 group, compared to the Q1 group, was 0.925 (95% CI: 0.829–1.033) times, lacking statistical

Table 3 The Relationship Between Serum DB and Breast Cancer

Model	Q1: DB ≤ 1.80 μmol/L	Q2: 1.80 < DB ≤ 2.30 μmol/L	Q3: 2.30 < DB ≤ 3.10 μmol/L	Q4: DB > 3.10 μmol/L
Unadjusted	1	0.925 (0.829, 1.033)	1.644 (1.486, 1.819)	6.993 (6.331, 7.725)
Model 1	1	0.925 (0.828, 1.032)	1.667 (1.506, 1.845)	7.155 (6.474, 7.907)

Note: Results were presented using OR (95% CI). Model 1 was adjusted by age.
Abbreviation: DB, direct bilirubin; OR, odds ratio; CI, confidence interval.

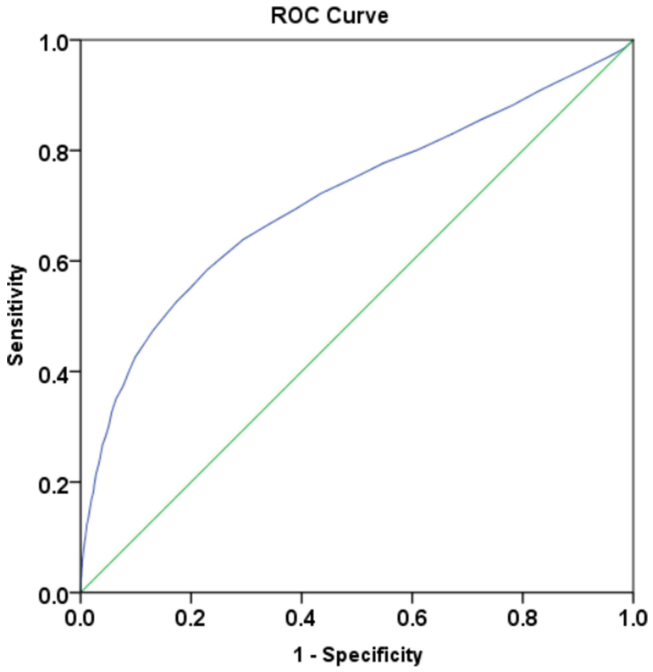

Figure 1 Receiver operating characteristic (ROC) curve of serum DB diagnostic breast cancer.

significance. However, the risk of breast cancer in the Q3 and Q4 groups was notably higher, with relative risks of 1.644 (95% CI: 1.486–1.819) and 6.993 (95% CI: 6.331–7.725) times that of the Q1 group, respectively, both statistically significant. After age adjusted, the relative risk of breast cancer in the Q2 group, compared to the Q1 group, was 0.925 (95% CI: 0.828–1.032) times, without statistical significance. However, the risks of breast cancer in the Q3 and Q4 groups were found to be 1.667 (95% CI: 1.506–1.845) times and 7.155 (95% CI: 6.474–7.907) times of that in the Q1 group, respectively, both statistically significant.

The Diagnostic Value of serum DB for Breast Cancer

The optimal cut-off value of serum DB in the case of diagnosed breast cancer was 2.75 μmol/L. The ROC curve is shown in Figure 1. The area under the curve (AUC) of DB was 0.712 (95% CI: 0.703–0.722), and the sensitivity, specificity, positive predictive value and negative predictive value were 58.5%, 77.0%, 57.4%, and 77.8%, respectively.

Discussion

In the present study, it was observed that breast cancer patients had significantly lower TB levels and significantly higher DB levels compared to healthy controls. Traditionally, elevated serum bilirubin levels have been associated with liver or biliary dysfunction. Elevated DB levels indicate impaired excretion of bilirubin from the biliary tract. In bilirubin metabolism, unconjugated bilirubin (indirect bilirubin, IB) undergoes transformation in hepatocytes, conjugating with glucuronic acid to form conjugated bilirubin (direct bilirubin, DB). DB is water-soluble and easily excreted from the body. Recent studies increasingly highlight the beneficial aspects of bilirubin for physical health. Slightly elevated serum TB levels have been linked to increased lifespan,[23] and may also reduce the risk of cardiovascular disease and cancer.[19] However, several studies have shown that serum DB is a risk prognostic factor for colorectal cancer.[24,25] Previous research has indicated that TB and DB may have contrasting monitoring or prognostic roles in diseases. Our results reveal that serum TB was independently associated with a reduced risk of breast cancer, whereas DB was independently associated with an increased risk of breast cancer. This suggests that TB and DB levels play distinct roles in the monitoring and prognostic evaluation of breast cancer. Studies have also demonstrated a decreased risk of mortality among patients with non-metastatic breast cancer who have higher levels

of TB.[22] The protective function of bilirubin can be explained by its immunomodulatory, antioxidant and anti-proliferative effects.[26] At present, the internal correlation mechanism between DB and breast cancer remains relatively unclear. One study parallels the present findings in breast cancer, indicating that TB within normal ranges may exert a protective effect, largely due to mild to moderate elevations in IB levels. Conversely, DB was linked to an increased risk of coronary heart disease incidence in a dose-response manner.[21] Moreover, another study highlighted that baseline DB serves as an independent risk factor for mortality in idiopathic pulmonary arterial hypertension.[27] These findings collectively underscore the potential risks associated with elevated DB levels in various diseases.

X-ray breast imaging and ultrasound imaging are commonly used technique for the diagnosis and screening of breast cancer.[28,29] Research has demonstrated that mammography exhibits significantly higher sensitivity (93.0%) compared to (86.0%).[30] However, while both modalities demonstrate good sensitivity in breast cancer diagnosis, they lack specificity and sensitivity in dense breast tissue. Consequently, the combination of mammography and ultrasound represents an effective approach in clinical practice.[31] At present, some serum markers for breast cancer have been developed. Conventional serum tumor markers such as CA153 and CEA are the most commonly used in clinical diagnosis and disease monitoring of breast cancer, yet they face the problem of limited sensitivity and specificity.[32,33] A study with healthy individuals as the control group showed that the AUC of CA153 and CEA in the diagnosis of breast cancer was 0.57 and 0.62,[33] respectively. In this study, the AUC of CA153 and CEA in the diagnosis of breast cancer was determined to be 0.50 and 0.53, respectively, consequently supporting prior research indicating their restricted utility in breast cancer diagnosis[34] The present study further confirms the limited diagnostic efficacy of these markers in breast cancer. Therefore, it is imperative to identify and validate new effective blood markers for breast cancer. Subsequently, the study revealed that serum DB presents superior diagnostic value in breast cancer, as indicated by a high AUC (0.712), specificity (77.0%) and negative predictive value (77.8%). Although the diagnostic efficacy of DB is inferior to that of ultrasound and X-ray, its identification represents a significant milestone in the exploration of blood markers for breast cancer. In addition, DB is a degradation product of hemoglobin, cytochrome enzymes, and other iron porphyrin compounds, and is excreted through the biliary tract after liver treatment. As an endogenous molecular marker, changes in serum DB in cancer patients suggest a potential connection between the pathogenesis of cancer and the metabolism of iron porphyrin or changes in liver and biliary function.

It was also found that age, ALB, TG, GLU and CEA were independent risk factors for breast cancer, while ALT, TP, TC and UA were independent protective factors. Winters et al showed that the incidence and mortality rate of breast cancer increases proportionally with age, with the disease peaking around age 60.[35] ALB, TP and ALT are all markers related to liver function, and since the liver is involved in bilirubin metabolism, their levels may reflect liver health, which could be associated with breast cancer risk. Regarding serum TG and TC, there has been some controversy regarding their relationship with breast cancer risk. Some studies suggested that elevated levels of triglycerides and TC are risk factors for predicting breast cancer,[36,37] while another study showed that the TC level was inversely proportional to the risk of breast cancer.[38] Studies have shown that elevated GLU levels are a risk factor for breast cancer in pre - and postmenopausal women,[39] and women with T2DM were more likely to be diagnosed with breast cancer.[40] CEA is a tumor marker that is broadly used across various types of cancers. In the context of breast cancer, CEA levels in breast ductal secretions have been found to possess significant diagnostic value.[41] Preoperative serum CEA levels are independently associated with the molecular subtypes of breast cancer. In the present study, UA was identified as a protective factor for breast cancer. This may be because UA is also a powerful antioxidant,[42] which plays a protective role in cancer by inhibiting the production of oxygen free radicals, lipid peroxidation and other mechanisms.[43]

The present study possesses several strengths and limitations. It benefits from a substantial dataset, encompassing 5299 patients in the experimental group and 10,028 individuals in the healthy control group. However, there was limited analysis on the diagnostic utility of DB in relation to breast cancer. Nonetheless, the research has expanded the repertoire of blood tumor markers for breast cancer, enhancing understanding in this area. One notable limitation is that the present study was single-center, which may impact the generalizability of the findings. Additionally, the focus was on non-metastatic patients undergoing surgery for breast cancer, representing a cohort that predominantly consists of early-stage

patients. In addition, it is important to note that research involving healthy individuals as the control cohort is primarily suitable for disease screening, rather than for differential diagnosis.

In conclusion, our study found that TB is an independent protective factor for breast cancer, while DB is a risk factor. Although the diagnostic efficacy of serum DB in breast cancer may not match that of X-ray breast imaging and ultrasound imaging, it holds greater diagnostic value when compared to conventional serum tumor markers. Meanwhile, changes in the level of DB in the serum of breast cancer patients are indicative of alterations in metabolism of porphyrin-containing compounds or changes in liver and biliary tract function. Further research is necessary to elucidate and confirm the associated mechanisms.

Data Sharing Statement

Dataset used during the current study is available from the corresponding author on request.

Ethical Approval and Informed Consent

This research did not affect the patients' health and privacy. All procedures performed in the studies involving human participants accorded with the ethical standards of the Medical Ethics Committee of Taizhou Hospital of Zhejiang Province (#K20221129), and with the 1964 helsinki Declaration and its later amendments, or other comparable ethical standards.

Author Contributions

All authors made a significant contribution to the work reported, whether that is in the conception, study design, execution, acquisition of data, analysis, and interpretation, or in all these areas; took part in drafting, revising, or critically reviewing the article; gave final approval of the version to be published; have agreed on the journal to which the article has been submitted; and agree to be accountable for all aspects of the work.

Funding

The Taizhou Social Development Science and Technology Project (#23ywa06), the Medical Health Science and Technology Project of Zhejiang province (#2024KY529) and the Taizhou Enze Medical Center (group) Technology Plan Major Project (#19EZZDC9) supported this work.

Disclosure

The authors declare that they have no competing interests.

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