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Elevated Serum Levels of SCUBE1, a Marker for Coagulation, in Patients with Breast Cancer

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Breast cancer (BC) is the most common cancer among women and a major cause of death. Signal Peptide-Cub-Epidermal growth factor domain-containing protein-1 (SCUBE1) is secreted under hypoxia and inflammatory conditions from platelet alpha granules. Its biological function is uncertain, although it may be a procoagulant substance in cancer patients. SCUBE1 is useful for identifying thrombotic diseases, including cancers and acute coronary syndromes. D-dimer reflects the relationship between coagulation activation and fibrinolysis; namely, thrombosis and D-dimer levels are closely linked. This is the first investigation of the potential diagnostic and prognostic value of SCUBE1 levels in patients with BC. Fifty patients and 33 age-matched and body mass index-matched healthy controls were enrolled. Blood samples were collected before chemotherapy regimens commenced. Serum SCUBE1 and D-dimer levels were measured before adjuvant chemotherapy and were compared to the healthy controls. SCUBE1 levels were determined using an enzyme-linked immunosorbent assay (ELISA) method. SCUBE1 and D-dimer levels were significantly higher in patients than in the controls ($p = 0.03$ and $p < 0.001$, respectively). A cut-off value of 1.55 ng/mL for SCUBE1 was associated with 62% sensitivity and 72.7% specificity and with positive predictive value of 77.5% and negative predictive value of 55.8%. Two patients with high SCUBE1 and D-dimer levels also developed pulmonary embolism. SCUBE1 may indicate hypercoagulability in patients with BC and thus help identify patients at greater risk of thrombosis and requiring anti-thrombosis treatment. SCUBE1 may also be used as an assistant test for identifying patients at risk of BC.

Keywords: breast cancer; cut-off value; D-dimer; SCUBE1; thrombosis
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Introduction

Cancer is a major health problem and cause of death worldwide. Carcinogenic chemicals, viruses, and radiation all cause mutation in DNA, resulting in uncontrolled cell proliferation and eventually in cancer. Breast cancer (BC) is the most common form of cancer in women and a cause of high mortality and morbidity worldwide (Park et al. 2014). Signal Peptide-Cub-Epidermal growth factor domain-containing protein 1 (SCUBE1) is a member of the SCUBE family. It forms part of the epidermal growth factor (EGF) super family and consists of several domain structures, such as cysteine-rich and EGF-like repeats, and CUB domain. The SCUBE family consists of three members: Scube1, Scube2, and Scube3. (Tsai et al. 2009; Zhuang et al. 2010). SCUBE1 is released in the early period of embryogenesis and is present in platelet alpha granules and endothelial cells (Tu et al. 2008). The SCUBE gene family has been shown to be expressed in the gonads, central nervous system and limb buds during mouse embryogenesis (Grimmond et al. 2000). SCUBE perform highly important role during organogenesis and branching morphogenesis (Yang et al. 2002). SCUBE1 levels have been shown to increase in inflammation and hypoxia, conditions resulting in angiogenesis in osteoblasts and bones (Yang et al. 2002). SCUBE is associated with inflammation and hypoxia-related diseases (Dai et al. 2008). Inflammation induces genetic changes and increases the risk of cancer (Mantovani et al. 2008). Several studies have shown that SCUBE1 is related to various molecules involved in

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angiogenesis, such as hedgehog (HH), transforming growth factor (TGF) beta, and platelet-derived growth factor D (PDGF-D) (Yang et al. 2002; Wu et al. 2011; Johnson et al. 2012). Mis-regulation and mutations (PTCH1 and SMO genes) in the HH signaling pathway have been reported to lead to a range of cancers (Evangelista et al. 2006).

SCUBE1 was originally separated from the complementary DNA library of human umbilical vein endothelial cells (Wu et al. 2011). It is released under hypoxia and inflammatory conditions from platelet alpha granules SCUBE1 has been identified in endothelial cells and platelets (Tu et al. 2008). Little is known about its biological function, however. Several studies have shown that SCUBE1 is a helpful biomarker in identifying acute thrombotic diseases, including acute coronary syndrome (ACS) and acute ischemic stroke (AIS) (Dai et al. 2008). SCUBE1 can be used as a biomarker in the early diagnosis of acute ischemic stroke patients (Turkmen et al. 2015). Serum SCUBE1 may therefore be of potential use in acute ischemic conditions. Individuals with cancer have an increased risk of developing thrombosis, and activation of the coagulation system accelerates cancer progression (Rickles et al. 2003). Many tumors cause coagulation dysfunction leading to production of procoagulant substances (De Cicco 2004). SCUBE1 may be a procoagulant substance in cancer.

D-dimer is a well-known parameter indicating the relationship between coagulation activation and fibrinolysis. Thrombosis and D-dimer levels are closely linked (Ferroni et al. 2012) and activation of the coagulation system causes cancer progression (Rickles et al. 2003; Ferroni et al. 2012). D-dimer is independently associated with risk of thrombosis and plays a major role in predicting the risk of venous thromboembolism (Ahlbrecht et al. 2012). BC is the most common form of cancer in women and has a high mortality rate. One etiological factor in BC-related deaths is thrombosis (Kvolik et al. 2010). Molecular and genetic studies of carcinogenesis have contributed to the development of new diagnostic and therapeutic techniques resulting in improved BC outcomes (Gunaldi et al. 2015).

The aim of this study was to evaluate serum levels of SCUBE1 and D-dimer in patients with BC and to compare them with those of healthy controls.

Materials and Methods

Fifty patients with BC admitted to the outpatient medical oncology clinic at Karadeniz Technical University, Turkey, were enrolled. Thirty-three age-matched and body mass index (BMI)-matched controls were also enrolled in this case-control study for the comparison of serum levels of SCUBE1 and D-dimer. Pregnant patients, patients with renal and hepatic function impairment, coronary insufficiency or active infections, subjects using anticoagulants or antiaggregant drugs and hospitalized patients were excluded. Patients were staged according to the Sixth Edition of the American Joint Committee on Cancer (AJCC). Blood samples were collected before chemotherapy regimens commenced. All patients were chemotherapy naive. The patients' characteristics were recorded for data analyses: age, BMI, histopathological type, menopausal status, grade, tumor invasion depth, lymph node involvement, estrogen receptor (ER), progesterone receptor (PR), human epidermal growth factor receptor 2 (HER2), lymphovascular invasion (LVI), perineural invasion (PNI), E-cadherin, and stage. Karadeniz Technical University's ethical committee approved the study and informed consent was received from all patients and controls.

Blood samples were collected in tubes containing 3.8% sodium citrate or K_3EDTA as an anticoagulant and serum separator. Samples were centrifuged at 3,000 rpm for 10 min to obtain serum supernatants. The serum samples were then stored at −80°C until biochemical analyses. A Roche Hitachi Cobas 8000 autoanalyzer (Roche, Germany) was used for routine biochemical parameters, and a Beckman Coulter autoanalyzer for CBC analysis. Prothrombin time (PT), partial thromboplastin time (PTT), International normalized ratio (INR), and D-dimer were assayed using an automatic coagulation analyzer (STA-compact, DiagnosticaStago, Asnieres, France) in a routine setting.

Measurement of SCUBE1

Levels of serum SCUBE1 were determined using an enzyme-linked immunosorbent assay kit (CUSABIO, Catalog No: CSB-E15005h), according to the manufacturer's instructions. The absorbance of samples was measured at 450 nm on a VERSA max tunable microplate reader (designed by Molecular Devices in California, USA). The results were expressed as ng/mL.

Measurement of D-dimer

Serum Levels of D-dimer were determined using an STA Compact analyzer (Houston, USA). Samples were collected into Na-citrate tubes. The results were expressed as μg/mL. The reference interval of D-dimer is 0-0.5 μg/mL.

Statistical analysis

Statistical analysis was done with NCSS (Number Cruncher statistical system) 2007&PASS (Power Analysis and Sample size), 2008 Statistical Software (Utah, USA). Descriptive statistical methods (mean, standard error, median, rate and ratio) were used. The Kolmogorov-Smirnov test was used to determine data distribution. For analysis of differences between patient and control samples, the independent samples t-test was used for data with normal distribution and the Mann Whitney U test for non-normally distributed data. Differences between groups before and after chemotherapy were determined using Wilcoxon's two related samples test. For analysis of differences between SCUBE1, D-dimer and pathological parameters (Lymph node involvement, hormone receptor status, HER2 status, LVI, E-cadherin, menopausal status, stage), ANOVA and the independent samples t-test were used for data with normal distribution and Mann Whitney U test and Kruskall Wallis test were used for data with non-normally distributed data. Pearson's correlation coefficient analysis was used to examine relationships between parameters. The area beneath the receiver operating characteristic (ROC) curves was used to show the power of a biomarker in diagnosis or exclusion of BC. Specificity, sensitivity, negative predictive value (NPV) and positive predictive value (PPV) were calculated. Results were evaluated in confidence interval. Statistical significance was accepted as $p < 0.005$ in all tests.

Table 1. SCUBE 1 and D-dimer levels of patients and control groups.

Total	Patients	Controls	p
	50	33	
Age (years) (median)	50 (32-87)	49 (34-68)	> 0.05
BMI (kg/m^2) (median)	28.04	25.10	0.37
SCUBE1 (ng/mL)	2.36 ± 2.04	0.153 ± 0.21	0.03
D-dimer (μg/mL)	1.43 ± 2.34	0.37 ± 0.15	< 0.001

SCUBE1, Signal Peptide-Cub-Epidermal growth factor domain-containing protein-1; BMI, body mass index.

Results

Fifty operated and chemotherapy naive BC patients and 33 age- and BMI-matched healthy controls were enrolled. There was no statistically significant difference in mean age between patients and controls ($p > 0.05$). Median age of patients at diagnosis was 50 years (range: 32-87 years). SCUBE1 and D-dimer levels were significantly higher in patients than in controls ($p = 0.03$ and $p < 0.001$, respectively) (Table 1, Fig.1). Incidentally, two patients with high SCUBE1 and D-dimer levels developed pulmonary embolism.

SCUBE1 and D-dimer levels were compared with the clinical parameters of patients with respect to stage, lymph node involvement, hormone receptor status, HER-2 status, lymphovascular invasion, E-cadherin status, and menopausal status. HER-2 positive patients had significantly higher SCUBE1 levels than HER-2 negative patients ($p = 0.006$). D-dimer levels were significantly higher in postmenopausal patients than in premenopausal ones ($p = 0.005$). The rest of the comparison showed no significant difference ($p \geq 0.05$) (Table 2).

Patients' platelet counts, PT, PTT and mean platelet volume (MPV) were within normal ranges. No negative or positive correlation was observed between patients' hemostatic markers (PT, PTT and INR), platelet counts, MPV and SCUBE1 or D-dimer levels. In addition, the following parameters were within normal ranges: lymphocytes, neutrophils, hemoglobin, albumin, lactate dehydrogenase (LDH), and carcinoembryonic antigen (CEA), CA15-3 (cancer antigen). No correlation was detected between each of these parameters and SCUBE or D-dimer levels ($p > 0.05$).

Area under the ROC curve (AUC), sensitivity, specificity, PPV, and NPV were calculated using ROC curve analysis. A cut-off value of 1.55 ng/mL for SCUBE1 was associated with 62% sensitivity and 72.73% specificity, and with PPV of 77.5% and NPV of 55.8% (Fig. 2).

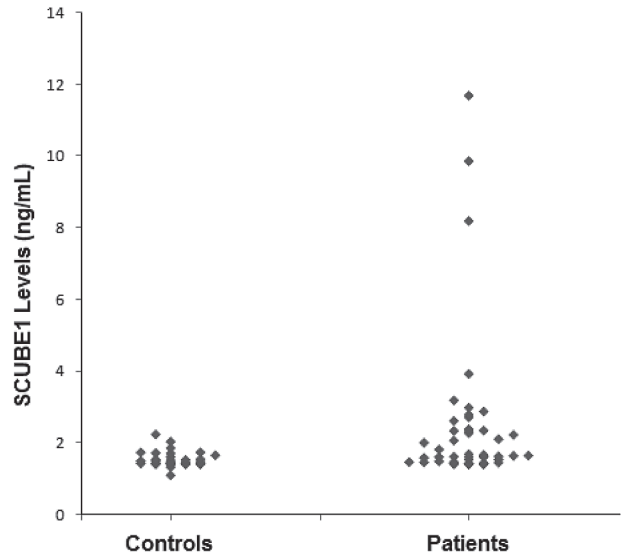

Fig. 1. Serum SCUBE1 levels in patients and healthy controls.

Discussion

BC is the most common form of cancer in women and has a very high mortality rate. Methods of screening, diagnosis and treatment all therefore need to be improved. The etiology is multifactorial, and includes obesity, history of benign breast diseases, family history of BC, early menarche, and delayed menopause (Gupta et al. 2012). These factors all compromise cellular processes, including pathways regulating gene expression, cell proliferation and apoptosis (programmed cell death) (Chandra et al. 2000).

SCUBE1 is expressed in platelets and the endothelium. It is stored in platelet α granules and is replaced on the cell surface with platelet stimulation and activation. Surface exposed platelet SCUBE1 mediates platelet-platelet agglutination under thrombotic conditions. High levels of SCUBE1 have been determined in platelets (Tu et al. 2008). Thrombotic disorders are common causes of death in BC. It is therefore essential to identify cancer patients at greater risk of thrombosis in order to initiate antithrombotic treatment early. Thrombotic disorders have high mortality and morbidity.

SCUBE1 is a novel parameter in BC. No previous published studies have investigated SCUBE1 in BC. SCUBE1 contains EGF-like repeats and a CUB domain (Grimmond et al. 2000). Very little is known about its domain and biological functions. SCUBE family members are related to HH and other extracellular signaling pathways (Kawakami et al. 2005). Mutations (PTCH1 and SMO

Table 2. Comparisons between serum SCUBE and D-dimer levels and clinical parameters in patients with breast cancer.

Variable		n	SCUBE1 (ng/mL)	D-dimer (μg/mL)
Stage	p		0.173	0.218
	1	11	2.46 ± 3.06	0.76 ± 0.58
	2	26	2.45 ± 2.01	1.26 ± 1.64
	3	13	2.08 ± 0.78	2.36 ± 3.89
Node	p		0.855	0.055
	N0	26	0.67 ± 2.73	0.70 ± 0.50
	N1	14	2.03 ± 0.83	1.92 ± 2.10
	N2	7	2.15 ± 0.64	3.26 ± 5.22
	N3	3	1.71 ± 0.32	1.21 ± 0.87
Hormone Receptors	p		0.418	0.297
	ER +	34	2.15 ± 1.29	1.11 ± 0.95
	ER +	16	3.04 ± 3.18	1.68 ± 3.72
	p		0.242	0.243
	PR +	34	2.17 ± 1.56	1.12 ± 0.95
	PR +	16	2.90 ± 2.85	1.60 ± 3.60
HER-2 Status	p		**0.006**	0.66
	positive	13	3.58 ± 3.40	0.84 ± 0.61
	negative	37	2.01 ± 1.23	1.43 ± 2.53
Lymphovascular Invasion	p		0.577	0.954
	positive	20	1.86 ± 0.54	1.14 ± 1.07
	negative	30	2.57 ± 2.44	1.51 ± 3.00
E-cadherin	p		0.591	0.321
	positive	35	2.63 ± 2.53	1.71 ± 3.60
	negative	15	2.18 ± 0.72	1.43 ± 1.02
Menopausal Status	p		0.285	**0.005**
	premenopause	25	2.13 ± 1.34	0.74 ± 0.66
	postmenopause	25	2.59 ± 2.56	2.12 ± 3.13

PR, progesterone receptor; ER, estrogen receptor; HER-2, human epidermal growth factor receptor; SCUBE1, Signal Peptide-Cub-Epidermal growth factor domain-containing protein-1.

genes) and mis-regulation of HH signaling pathways results in several types of cancer (Evangelista et al. 2006).

Several studies have reported higher levels of SCUBE1 in ACS and AIS stroke compared with controls subjects (Dai et al. 2008). SCUBE1 has been identified in the vascular endothelium, including arteries, veins, and microvessels (Yang et al. 2002). It was reported significantly higher SCUBE1 levels in patients with gastric cancer compared to healthy controls (Mentese et al. 2012).

This is the first study in the literature to investigate SCUBE1 in BC. We determined significantly higher SCUBE1 levels in patients with BC compared to healthy controls. SCUBE1 may thus be used to identify patients at high risk of thrombosis in BC. Also, SCUBE1 levels were significantly higher in HER2-positive BC patients than in HER2-negative subjects ($p = 0.006$). Elevated SCUBE1 levels may therefore be a poor prognostic factor in patients with BC. No correlations were determined between hemostatic markers and SCUBE1 levels. This may be attributed to our study including only patients with non-metastatic BC.

Thrombosis exacerbates cancer progression and reduces survival rates. Previous studies have reported higher coagulation biomarkers in BC, and several biomarkers are correlated with VTE in patients with cancer (Tas et al. 2014). D-dimer is independently related to risk of VTE and plays a major role in predicting such risk (Pabinger and Ay 2012). Also D-dimer has been identified as a marker differentiating benign disorders from malignancies in a number of trials. Studies have reported higher D-dimer

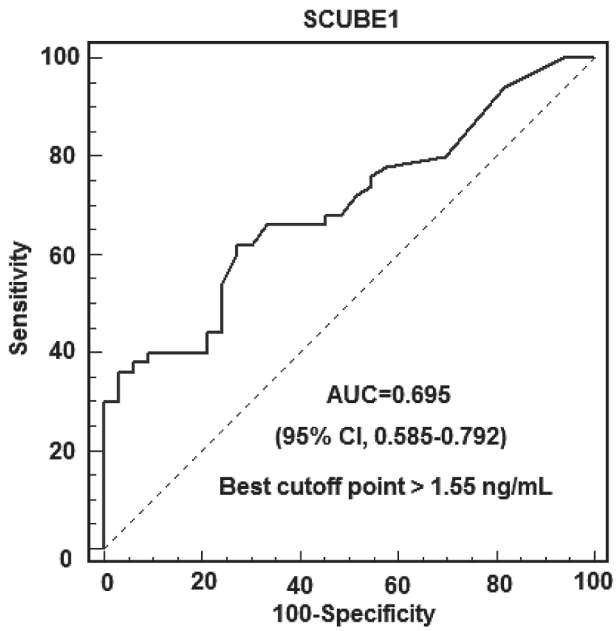

Fig. 2. Receiver operating characteristic of SCUBE1. Area under curve (AUC) for SCUBE1 is 0.695.

levels in patients with BC compared to healthy controls. D-dimer levels in this study were significantly higher in the patients than in the controls ($p < 0.001$). Our results are compatible with those studies. No correlation was observed between D-dimer and hemostatic markers and clinical characteristics of patients, except menopausal status. Interestingly, in contrast to Batschauer et al. (2010) study, we have determined that increased levels of D-dimer in postmenopausal BC patients. This finding may suggest that patients with BC (especially postmenopausal BC patients) are at greater risk of thrombosis compared to healthy controls.

Khorana (2010) first identified the association between pre-chemotherapy thrombocytosis and thrombosis, which was later confirmed by other investigators (Yang et al. 2002). We observed no correlation between platelet count and SCUBE1 or D-dimer levels. Two of our patients with high SCUBE1 and D-dimer levels also had pulmonary embolism. This supports the idea that SCUBE1 and D-dimer markers may show the risk of thromboembolism in non-metastatic BC patients.

The main limitations of this study are the low patient numbers and the exclusion of patients with metastatic diseases.

In conclusion, SCUBE1 and D-dimer levels were significantly higher in our patients than in the controls. Based on our findings and those of other studies, SCUBE1 may indicate hypercoagulability in BC patients. It may therefore be helpful for identifying patients at greater risk of thrombosis and who require anti-thrombotic treatment. Further studies are needed to achieve a better understanding of the mechanisms involved in thrombosis, to identify which markers are helpful in indicating the benefit of anti-thrombotic agents and to improve the poor prognosis associated with thrombosis in cancer.

Conflict of Interest

The authors declare no conflict of interest.

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