Research Article

Can serum SCUBE1 levels be useful in the diagnosis of bladder cancer?

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Abstract

Bladder cancer (BC) is the most common malignancy of the urinary system and the sixth most prevalent cancer in both men and women. There is currently no biomarker identified to facilitate the diagnosis of BC and which can be considered as the gold standard. The aim of this study was to determine the diagnostic significance of serum signal peptide-CUB-EGF domain-containing protein 1 (SCUBE1) levels in patients newly diagnosed with BC and to compare the sensitivity and specificity of SCUBE1 with those of carbonic anhydrases IX (CAIX), which has previously been shown to be positive in BC. SCUBE1 and CAIX levels were investigated using enzyme-linked immunosorbent assay (ELISA) in serum samples from 19 patients with bladder cancer and 25 healthy peers. Levels of both were significantly higher in the BC group compared with the control group (p=0.0001). Based on ROC analysis, SCUBE1 emerged as a sensitive test, similarly to CAIX, for identifying BC. These findings suggest that increased SCUBE1 levels may be a useful addition to clinical findings of disease in the diagnosis of BC patients.

Keywords: bladder cancer, carbonic anhydrase, SCUBE1, CAIX

1. Introduction

Cancer is a major public health problem worldwide and the second leading cause of death in the USA. Some 1.8 million new cancer cases and 606,500 cancer-related deaths are projected to occur in the USA in 2020 (1). Bladder cancer (BC) is the most common malignancy of the urinary system and the sixth most prevalent cancer in both men and women. More than half a million people were diagnosed with BC worldwide in 2018, and 200,000 died from the disease (2). It is estimated that approximately 81,400 new cases of BC will be diagnosed in adults in the USA in 2020, of which 17,980 will be fatal (1). BC generally arises from epithelial cells, and more rarely from mesenchymal cells. The best-established and most important risk factor for BC development is smoking. Chemicals in tobacco chemicals increase the expression of proteins involved in inflammation, and activate genetic and epigenetic pathways, thereby adversely affecting the cell cycle by inducing uncontrolled cell proliferation. BC is a complex disease involving various molecular and pathological pathways, and therefore exhibits different behaviors depending on the clinical staging of the tumor and the molecular type. The diagnosis and monitoring of BC largely involve invasive tests involving periodic cystoscopy. Although this is a reliable method, the procedure is highly uncomfortable for the patient and causes comorbidity. Due to this disadvantage of the method, studies on developing new biomarkers have been accelerated by taking into account the metabolic events that play a role in the pathogenesis of BC. Biomarkers associated with hypoxia, inflammation and oxidative stress have been shown to be capable of use in the diagnosis of BC (2-4). However, there is currently no biomarker identified to facilitate the diagnosis of BC and which can be considered as the gold standard (2).

Carbonic anhydrases (CAs) are mostly zinc-containing metalloenzymes which catalyze the reversible hydration of carbon dioxide. They also play a role in the development stages of various cancers. Carbonic anhydrase IX (CAIX), which can be induced by hypoxia and is one of the best cellular biomarkers of hypoxia, plays an important role in pH regulation in cancer cells. By regulating intracellular and extracellular pH, it helps these cells adapt to adverse conditions in the tumor microenvironment (5). Previous studies have shown that CAIX is a biomarker capable of use in the diagnosis of various cancers, including BC (6-10).

Signal peptide-CUB-EGF domain-containing protein 1 (SCUBE1) was discovered in the early 2000s. The production of this cell surface glycoprotein begins in early embryogenesis, and it is present in platelets and endothelial cells (11). SCUBE1 consists of nine consecutively edited EGF-like repeats following an N-terminal signal peptide sequence, an intermediate region, three cysteine-rich repeat motifs, and a

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CUB region at the C terminal (12). These secreted proteins exhibit confirmed interactions with the angiogenesis-related signal system. Like other proteins containing EGF and CUB domains, they are involved in stages, such as organogenesis and morphogenesis. As with cell surface and other secreted glycoproteins, they are expressed in tissues with rich blood supplies and primary osteoblasts and bones. SCUBE genes have been shown to be expressed from developing tissues, such as gonads, the central nervous system, dermomyotome, digital mesenchyme, and limb buds during mouse embryogenesis. In addition to embryonic expression, SCUBE1 has been found to be expressed in endothelium and platelets (13). SCUBE1 has recently been demonstrated in various carcinomas, such as gastric and renal cell carcinoma (9,12). However, there is no study examining SCUBE1 levels in BC patients. The aim of this study was to determine the diagnostic significance of serum SCUBE1 levels in patients newly diagnosed with BC and to compare the sensitivity and specificity of SCUBE1 with those of CAIX, previously shown to be positive in BC.

2. Materials and methods

2.1. Study group

Informed consent was obtained from all patients and controls. Approval for the study was granted by the local ethics committee. Nineteen patients newly diagnosed with BC with stage T2 were included as the study group and 25 healthy peers as the control group. Coronary or liver failure, chronic inflammatory diseases or anemia, receiving chemotherapy, or using oral contraceptives and anticoagulants were excluded from the study in the selection of control and patient groups. Control groups were randomly selected from among patients with no history of urothelial and other malignancy, after the detailed medical history, laboratory and radiology results were evaluated. Patients were selected from individuals presenting to the urology clinic. The urothelial cancer diagnose in patients was based on laboratory examinations, radiological imaging in line with their complaints and transurethral resection of bladder cancer. Tumor grade was determined using the 2016 World Health Organization classification of Tumours of the Urinary System and Male Genital Organs system (14) and the pathologic stage using the 2017 American Joint Committee on Cancer eighth edition cancer staging manual system (15). The tumour, node, metastasis classification (TNM) of the patients was Ta: Non-invasive papillary carcinoma, Tis: Carcinoma in situ: 'flat tumour' and T1: Tumour invades subepithelial connective tissue. All the patients present with papillary urothelial carcinoma. The urothelial tumors were noninvasive in all patients. The noninvasive urothelial tumors included low-grade papillary urothelial carcinomas in this study. In addition, by examining the patients' records, data on demographic information, smoking history, medical history, tumor recurrence, metastasis, treatment, and clinical outcome were collected. Five-milliliter blood samples from each individual were placed into vacutainer tubes without anticoagulant. These were then centrifuged at 1800xg for 10 minutes. Serum

samples were stored at -80ºC until being used for measurements.

2.2. Determination of serum CAIX levels

Serum CAIX levels were determined using enzyme-linked immunosorbent assay (ELISA) kits (R&D Systems, Catalog No: DCA900, Minneapolis, USA) according to the manufacturer's recommendations. The absorbance of the samples was measured at 450 nm wavelength on a microplate reader (Molecular Devices Versamax, California, USA). The results are given in pg/mL. The intra-assay CV reliability of this ELISA method was found to be <3% and the confidence of the inter-assay CV was <6%.

2.3. Determination of serum SCUBE1 levels

Serum SCUBE1 levels were determined using ELISA kits (Cusabio, Catalog No: CSB-E15005h, Wuhan, China) according to the manufacturer's recommendations. The absorbance of the samples was measured at 450 nm wavelength on a microplate reader (Molecular Devices Versamax, California, USA). The results are given in ng/mL. The confidence of the intra-assay CV of this ELISA method was <8% and the confidence of the inter-assay distribution was <10%.

2.4. Statistical analysis

The results were expressed as mean±standard deviation for normally distributed variables and as median (interquartile range [IQR]) values for non-normally distributed variables. Statistical analysis was performed on Statistical Package for the Social Sciences (Version 23.0, NY, USA) statistical software. Compatibility with normal distribution was determined using the Kolmogorov-Smirnov test. Differences between the two groups were analyzed using Student's t-test for normally distributed data and the Mann-Whitney U test for non-normally distributed data. Chi square test was applied to evaluate the smokers' data among the groups. Receiver operating characteristic (ROC) curves were used to detect the discriminatory dominance of CAIX and SCUBE1 for the identification of BC. Sensitivity and specificity were determined from ROC graphs for CAIX and SCUBE1. $p < 0.05$ was regarded as significant.

3. Results

Forty-four individuals were enrolled in the study. SCUBE1 and CAIX levels were determined in serum samples from 19 BC patients [9 male, 10 female; median age 56 (51.0-62.0) years] and 25 controls [12 male, 13 female; median age 53 (50.0-62.0) years]. No significant difference was observed between patients and controls in terms of age (p>0.05). The clinical and biochemical parameters of BC and control groups were given in Table 1 and Fig. 1. Mean SCUBE1 levels were 18.6±6.20 ng/mL in the BC patients and 8.61±5.22 ng/mL in the control group and were statistically significantly higher in the BC patients (p=0.0001). Median (IQR) CAIX values were 54.1 (40.5-114.6) pg/mL in the BC patients and 22.3 (10.6-36.1) pg/mL in the control group. CAIX levels were statistically

significantly higher compared to the control group (p=0.0001). ROC curve analysis was also used to quantify serum SCUBE1 and CAIX levels. Values for cut-off points, AUC, sensitivity and specificity for individual parameters are shown in Fig. 2. The significant power of SCUBE1 was similar to that observed in CAIX (p= 0.0001).

Table 1. Comparison of biochemical parameters in BC and control groups

Parameters	BC (n:19)	Control (n:25)	p
BMI (kg/m^2)	26.4±2.81	25.7±3.50	0.240
Smoker, n (%)	13 (68.4)	10 (40)	0.136*
SCUBE1 (ng/mL)	18.6±6.20	8.61±5.22	0.0001**
CAIX (pg/mL)	82.8±65.4 54.1 (40.5-114.6)	27.1±19.3 22.3 (10.6-36.1)	0.0001***

Data were expressed as: mean±SD, median (inter quartile range for 25-75%). *p shows differences between control and cancer according to Chi square test, **p shows differences between control and cancer according to student t test, ***p shows differences between control and cancer according to Mann-Whitney U test.
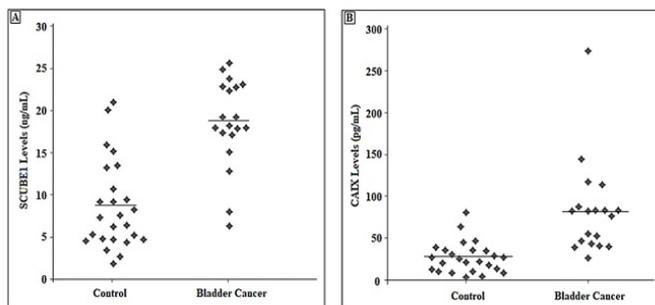

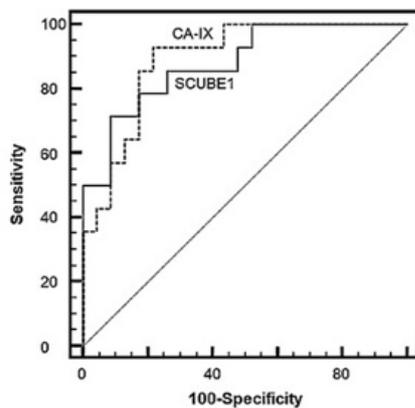**Fig. 1.** SCUBE1 (A) and CAIX (B) levels in the bladder cancer and control groups

Fig. 2. ROC curve analysis of CA-IX and SCUBE1 levels in patients with bladder cancer and their AUC, p values, sensitivity and specificity

4. Discussion

Bladder cancer entails the highest costs per patient of all cancers, due to the diagnostic protocols currently in use and the fact that many patients live long after diagnosis. The gold standard for the diagnosis of BC remains direct visualization of the urothelium using conventional white light cystoscopy. Urine cytology continues to play an important role in the diagnosis and follow-up of BC. In addition, the US Food and Drug Administration (FDA)-approved UroVysion and nuclear matrix protein 22 (NMP22) are also used in the diagnosis and follow-up of BC in clinical applications (16). Despite the existing diagnostic markers used in BC diagnosis, there is still a need for non-invasive, simple, and inexpensive tests capable of use in the clinical setting.

Hypoxia is a consequence of the rapid growth of many tumors, including BC, and is an important regulator of gene expression. CAIX, a hypoxia-dependent member of the carbonic anhydrase family, plays a role in intracellular pH, cell proliferation, and cell content, and regulates tumor progression (17). Hypoxia leads to overexpression of some genes mediated by hypoxia-inducible factor 1. This overexpression also triggers CAIX expression. Although weak CAIX expression is observed in the normal gastric mucosa, small intestine, biliary tract, and seminal canals, its expression has not been observed in other organ systems, including the urinary tract. Surprisingly, CAIX is abundantly expressed as a direct result of hypoxia in a large number of cancers, but it is also affected by other means and by genetic disorders (18). CAIX levels in the circulation have been investigated in various cancers such as renal cell, gastric and breast cancer, and high levels have been determined (6-9). However, Hyrsl *et al*. determined that serum CAIX did not exceed normal levels in transitional cell carcinoma patients (10). In the present study, serum CAIX levels were higher in patients with BC than in the healthy controls. This finding supports the possibility of CAIX being used in the diagnosis of BC, in agreement with the previous literature.

SCUBE1, a cell surface glycoprotein belonging to the epidermal growth factor (EGF) superfamily, functions as a newly defined platelet-endothelial adhesion molecule (19). An immunohistochemically soluble adhesion molecule, SCUBE1 has been detected in the subendothelial matrix of advanced atherosclerotic lesions in humans. This protein is regarded as a novel biomarker of platelet activation in acute thrombotic diseases (20). For the first time in the literature, serum SCUBE1 values in BC patients were found to be statistically higher than in the healthy control group in this study. An AUC value of 0.879 for SCUBE1 was associated with 71% sensitivity and 92% specificity, while an AUC value of 0.891 for CAIX was associated with 93% sensitivity and 78% specificity. In previous studies evaluating potential biomarkers in bladder cancer, Sakinewicz *et al.* reported that podoplanin exhibits 72% sensitivity and 69% selectivity (21), while Tokarzewicz et al demonstrated that cystatin C shows 87% sensitivity and 92% selectivity in patients with BC (22). Wang *et al*. also reported that bladder cancer-specific antigen-1 exhibits 74% sensitivity and 69% selectivity (23). Interestingly, in a recently published study, Guszcz *et al.* showed that the plasma aromatase biomarker exhibited 100% sensitivity and 100% selectivity in their study of 78 patients with BC and 18 healthy controls (24). From this point of view, it is seen that the results obtained in the study are compatible

with the values obtained for other biomarkers in BC patients.

Based on these results, the sensitivity and specificity of SCUBE1 appear to be quite similar to those of CAIX. In addition to its embryonic expression, SCUBE1 has been found to be expressed in the endothelium and platelets (13). SCUBE1 is stored in inactive platelet α-granules. Thrombin is transported to the cell surface as a result of platelet stimulation and activation through various stimulants, such as inflammation and hypoxia. SCUBE1 is transported to the platelet surface under thrombotic conditions. It is then separated from the platelet surface and released into the circulation in the form of small, soluble particles and is included in the thrombus (25). There is a known association between thrombosis and cancer and the presence of tumor cells in the thrombus (26). Coagulation dysfunction can be considered primary evidence of malignancy (27). Many tumors stimulate the clotting cascade and trigger the production of procoagulants, resulting in an inflammatory response. This inflammatory response increases the release of more procoagulants from tumor cells (28). Circulating SCUBE1 levels have been found to increase in various diseases involving platelet activation, such as acute coronary syndrome, and acute ischemic stroke (19). An increase in plasma SCUBE1 levels has also been observed in patients with such pathological conditions as acute mesenteric ischemia (29), end stage renal failure (30), Crimean-Congo hemorrhagic fever (31), renal cell cancer (9), breast cancer (32) and stomach cancer (12).

The main limitation of this study is the low number of patients. Further studies are needed to examine SCUBE1 levels and relationship by increasing the number of patients and adding cases of low and high grade papillary urothelial carcinomas. The precise presentation of the findings can be better understood in the longitudinal follow-up of a larger group of patients.

The principle conclusions from the new findings from this study are that SCUBE1 is as sensitive and specific as CAIX in the circulation in the diagnosis of BC, and that the use of SCUBE1 together with CAIX can also help support clinical findings in the diagnosis of BC.

Conflict of interest
None to declare.

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