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Original Article

Clinicopathological significance of stanniocalcin-2 expression in adenocarcinomas of biliary tract

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Abstract: Background: Despite all efforts, patients with biliary tract cancer (BTC) still share a dismal outcome. Based on its lack of specific symptoms, BTCs are often diagnosed in an advanced stage. Molecular biomarkers augur to overcome this misery, but none has entered clinical routine yet. We sought to investigate the prognostic relevance of Stanniocalcin-2 (STC2), which has shown encouraging results in other tumors, for its potential as biomarker in BTC. Material and methods: We first evaluated STC2 expression and its prognostic properties on mRNA level in silico using the TCGA (The Cancer Genome Atlas) database. In a second step, we validated the results using STC2 immunohistochemistry in a tissue microarray consisting of an independent cohort of BTCs who underwent resection at our center from 2000-2012. Results: TCGA data of 36 cholangiocellular carcinomas (CCC) revealed a significantly higher expression level of STC2 compared to normal tissue ($P < 0.001$) and a significant correlation to lymphatic infiltration ($P = 0.020$), but no impact on overall survival. In our cohort consisting of 53 patients with CCC and 11 patients with gallbladder cancer (GBC), STC2 also showed an over expression in CCC and GBC tissue and a correlation to lymphatic metastasis ($P = 0.038$). Patients with STC2 positive tumors had a significant worse overall survival ($P = 0.007$) and STC2 was an independent prognostic marker ($P = 0.022$). Conclusion: Valid biomarkers for BTC are desperately needed. STC2 has proven its potential as a prognostic marker in other tumors and showed promising observation in our study. Nonetheless, these findings have to be validated in a prospective cohort.

Keywords: Stanniocalcin-2, cholangiocellular carcinoma, gallbladder cancer, biliary tract cancer, prognosis

Intoduction

Biliary tract cancers (BTC) are aggressive malignant tumors, represented by cholangiocellular carcinomas (CCC) and gallbladder cancer (GBC), which arise from the epithelium of the biliary ductal system. In spite of different locations of these lesions, all BTCs originate from cells of a common embryonic background and share distinct morphologic and molecular changes. This is why intra- and extrahepatic CCC and GBC show a marked commonality regarding their enormously aggressive behavior [1]. BTCs account for ~3% of all gastrointestinal malignancies and show a rising incidence in Western countries [2]. CCCs are subdivided-based on its anatomic location-into intrahepatic, perihilar and distal CCCs. The only curative option for this cancer entity is a complete surgical resection, if the tumor is diagnosed early enough. Additionally, liver transplantation may rarely be an option for CCC patients with localized, perihilar disease [1]. In advanced disease, chemotherapy or locoregional therapies are applied in a palliative setting. Despite all scientific efforts and new treatment options like targeted therapies, BTCs still have a dismal 5-year prognosis [3]. Although most BTCs arise *de novo*, several risk factors have been identified. The main risk factor comprisesa chronic bile duct inflammation [4], primary sclerosing cholangitis [5], chronic hepatitis B and C infections [4], parasitic bile duct infection [6], biliary-hepatic drainage or hepatolithiasis [7]. Furthermore, several genetic polymorphisms and genetic driver mutations raise the risk of BTCs development [8]. In the battle for better survival promising biomarkers and therapeutic

targets have been identified [1], but none has been established in the clinical setting so far.

Originally, the glycoprotein mammalian Stanniocalcin-1 and -2 (STC1 and STC2), have been described in bony fish for its role in calcium homeostasis [9]. The hormone STC was secreted by the "Corpuscles of Stannius", an endocrine gland located close to the kidneys. In humans, both subtypes (STC1 and STC2) show an almost ubiquitous expression with high concentrations in the kidney, skeletal muscles, pancreatic and ovarian tissue [10].

In humans, STC1 and STC2 are glycoproteins found to be involved in a variety of cell functions. They may influence cellular calcium homeostasis in a putative autocrine and/or paracrine manner, mediate pro-and anti-apoptotic effects, conduct cell responses to unfolded proteins and oxidative stress, and have impact on sub-cellular functions. Recently, STCs have also been associated with inflammation, carcinogenesis, invasiveness and epithelial-mesenchymal transition (EMT) [11-14]. Some authors even proposed STC's role as a possible tumor suppressor gene [14].

Based on reports about STC2s' prognostic potential in other cancer types [15-19] we sought to evaluate the expression level of STC2 in BTCs tissue and its correlation with prognosis.

Material and methods

CCC's data in The Cancer Genome Atlas (TCGA)

In a first step, we tested our hypothesis in data from a TCGA cohort of CCC patients (training cohort). Despite our own data, the results shown here are in part based upon data generated by the TCGA Research Network (http://cancergenome.nih.gov/). This data base contains enormous data about DNA aberrations and is publicly accessible.

BTC samples and patients

We then performed a wet bench validation of the data retrieved from TCGA computations in an independent cohort (testing cohort). We included archival, formalin-fixed and paraffin-embedded (FFPE) tissue specimens from 64 patients with CCC and 19 patients with GBC. All samples were stored within routine pathology procedure after surgery performed for these lesions from January 2000 till May 2012 at the University Hospital of Bonn. Dead of disease was set as the clinical end-point. Clinical data and overall survival were obtained according to medical reports from our prospectively maintained hepatopancreatobiliary surgery database. Clinical follow-up was conducted on an established protocol with presentation all 3 months for the first year after operation, every 6 months in the second year and third year and every 12 months, thereafter. Median follow-up time was 21 months (range 0-104 months). Overall survival was defined as the time from initial surgery to patients' death or last follow-up. BTCs were classified according to the latest guidelines [20, 21]. The study has been approved by the Institutional Review Board (IRB) at the University Hospital of Bonn (vote No. 379/13).

Tissue microarrays (TMA)

After definition of the studies cohort one to six tissue cores (containing tumorous and non-tumorous tissue) with a core diameter of 1 mm were prepared for each tumor. Matched normal liver tissue was sampled distant from the tumor in order to harvest normal epithelium without any tumor associated morphologic or molecular alterations. 11 specimens of the 64 CCC samples and 9 of the 19 GBC samples showed no tumor or only minor portions of tumorous tissue in TMA cores leaving 53 CCC patients and 11 GBC patients for further analysis.

Immunhistochemistry (IHC)

Immunohistochemical staining of STC2 was performed with a Ventana Benchmark automated staining system (Ventana Medical Systems, Tucson, AZ, USA), following the manufacture's protocol, using a Stanniocalcin 2 Polyclonal Antibody (Proteintech, 10314-1-AP, 1:100). Slides were counterstained with haematoxylin, dehydrated, and mounted.

We used a distinct scoring system for the quantification of STC2 staining as previously described [22-24]. Briefly, each slide was scored according to intensity of staining (0 = no staining, 1 = weak, 2 = moderate, 3 = strong) and the amount of positively stained epithelial tumor cells or matching non-tumorous bile duct

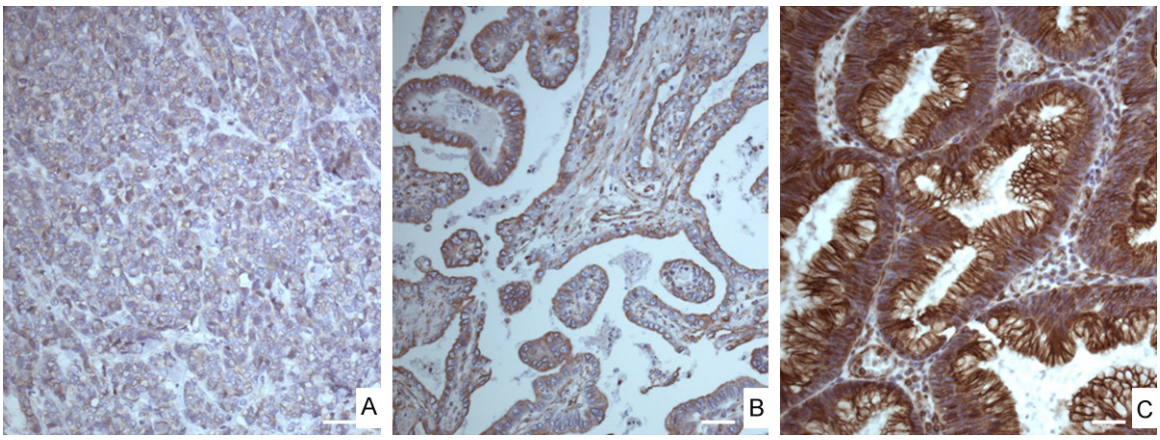

Figure 1. Criteria for STC2 expression intensity scoring. Representative areas of immunohistochemical stained CCC for weak (A), moderate (B) and strong (C) staining. Scale bar: 100 µm.

Table 1. Correlations of STC2 expression with clinicopathologic features of cholangiocarcinoma

Factor	Category	STC2-Score		χ^2	*P*-value
		Positive *n=34*	Negative n=19		
Age (women vs. man)		62 ± 9.6	66 ± 9.1		0.11#
Gender	Male	19	13	0.80	0.37
	Female	15	6		
CCC Classification	Intrahepatic	20	7	2.84	0.24†
	Perihilar	11	8		
	Distal	3	4		
Histologic grade	Well, moderate	17	9	0.34	0.85
	Poor	17	10		
T-classification	0	11	8	0.50	0.48
	1	23	11		
N-classification[1]	0	14	14	4.31	0.038*
	1	18	5		
M-classification	0	28	17	0.49	0.49†
	1	6	2		
Venous invasion[1]	0	19	13	0.37	0.55†
	1	8	3		
Lymphatic invasion[1]	0	17	11	0.10	0.75†
	1	6	3		
Perineural invasion[1]	0	9	4	0.99	0.32†
	1	12	11		

#Fisher's t-Test, †Likelihood-Ratio, *P < 0.05, N-classication had 2 missing data sets, venous invasion had 10 data sets missing, lymphatic invasion had 16 missing data sets, perineural invasion had 17 missing data sets.

epithelial cells (1 = 0-10%, 2 = 10-50%, 3 = 50-75%, 4 ≥ 75%), see **Figure 1**. The product of intensity score and percentage grade represented the overall STC2 score (0-12). STC2 score values ≥ 4 were considered positive and values ≤ 3 were considered negative and was used for further analysis. IHC stainings for STC2 were independently scored by 2 investigators blinded to the clinical parameters. In case of divergent results, respective cases were discussed among pathologists and consent was achieved.

Statistical analysis

Continuous variables were expressed as median (range) and compared using the Mann-Whitney U test. Categorical variables were compared using the χ^2 (Chi square) test or Fisher's exact test, where appropriate. Values are expressed as mean or median and range, unless otherwise stated. Patient survival and the differences in patient survival were determined using the Kaplan-Meier method and the log-rank test. A Cox regression analysis (proportional hazard model) was performed for the multivariate analyses of prognostic factors. Differences of P < 0.05 were considered statistically significant. Analyses were performed

Table 2. Univariate and multivariate cox proportional hazards analyses of overall survival in 53 cholangiocarcinoma patients

	Univariate Cox		Multivariate Cox	
Clinico-pathologic parameter/biomarker	Hazard ratio [95% CI]	*p*-value	Hazard ratio [95% CI]	*p*-value
Age	1.01 [0.97-1.05]	0.66	-	-
Gender (women vs. man)	0.73 [0.37-1.43]	0.36	-	-
Histologic grade (G3/4 vs. G1/2)	1.16 [0.68-1.99]	0.59	-	-
T-Classification (pT3/4 vs. pT1/2)	1.73 [1.09-2.72]	0.018*	0.70 [0.34-1.44]	0.059
N-Classification (pN1 vs. pN0)	3.41 [1.67-6.97]	0.001*	4.21 [1.39-12.56]	0.011*
M-Classification (pM1 vs. pM0)	1.70 [0.70-3.92]	0.21	-	-
Venous infiltration (pV1 vs. pV0)	1.33 [0.62-2.84]	0.47	-	-
Lympahatic infiltration (pL1 vs. pL0)	2.50 [0.44-2.32]	0.018*	3.18 [1.11-9.10]	0.031*
Perineural infiltration (pPn1 vs. pPn0)	1.01 [1.02-3.55]	0.98	-	-
STC2 Score (positive vs. negative)	2.50 [1.31-10.97]	0.014*	5.05 [1.27-20.08]	0.022*

*P < 0.05.

using SPSS computer software (SPSS version 23, IBM Corp, NY, USA).

Results

CCC's STC2 mRNA expression level retrieved from TCGA

Data plot of *STC2* expression in CCC patients (n = 36) with matching normal bile duct epithelial tissue (n = 9) from TCGA revealed a significant higher *STC2* mRNA expression level in CCC in comparison to matching normal bile duct epithelium (312.78 vs 24.24, P < 0.001). *STC2* expression significantly correlated with lymphatic metastasis (r = 0.412, P = 0.021), which is a well established prognostic parameter in CCC [21, 25]. In the 36 CCC patients of the TCGA data system, total *STC2* mRNA expression level did not show a prognostic value (34.0 vs 36.4 months of survival, log rank P > 0.05, HR 0.99, 95% CI 0.99-1.00, P > 0.05).

Validation of STC2 expression in an independent cohort

Analysis of immunohistochemical STC2 expression in an independent cohort of CCC patients from the University Hospital Bonn showed a clear demarcation of malignant epithelial bile duct cells in TMA cores. In total, 34 of 53 CCCs (64.4%) showed a positive STC2-Score, while only 2 of 53 (3.8%) matched normal biliary epithelium showed a positive SCT2 staining. These results go along with previously reported rates of STC2 staining rates in hepatocellular carcinoma (60.83%) and nasopharyngeal carcinomas (69.1%) [22, 23]. Accordingly, 8 of 11 GBC specimens (72%) had well demarked STC2 positive tumor cells while only 1 of 11 NAT tissue samples (9%) presented STC2 positive.

STC2 correlates with clinicopathological features of CCC

Clinicopathological features were analyzed in relation to STC2 expression level determined by immunohistochemical staining. There was no significant correlation between STC2 expression and age, gender, CCC classification, histologic grade, pathological T-classification or M-classification, venous invasion, lymphatic and perineural invasion (all P > 0.05), see **Table 1**. In contrast, STC2 expression was higher in patients with proven lymph node infiltration (18/32, 56.3% vs 5/19, 26.3%, P = 0.038) and showed a positive correlation (r = 0.291, P = 0.038). These results mirror the significance of lymphatic infiltration for survival as reported for extrahepatic CCC by Murakami *et al.* [21, 25]. Corresponding, univariate and multivariate analysis revealed a high impact of lymphatic infiltration and N-classification on overall survival (HR 4.21, 95% CI 1.39-12-56, P = 0.011 and HR 3.17, 95% CI 1.11-9.10, P = 0.031), see **Table 2**.

STC2 as prognostic factor in CCC

Univariate Cox proportional hazard analysis identified overall STC2 expression, T-classification, N-classification, and lymphatic invasion as adverse prognostic factors for overall sur-

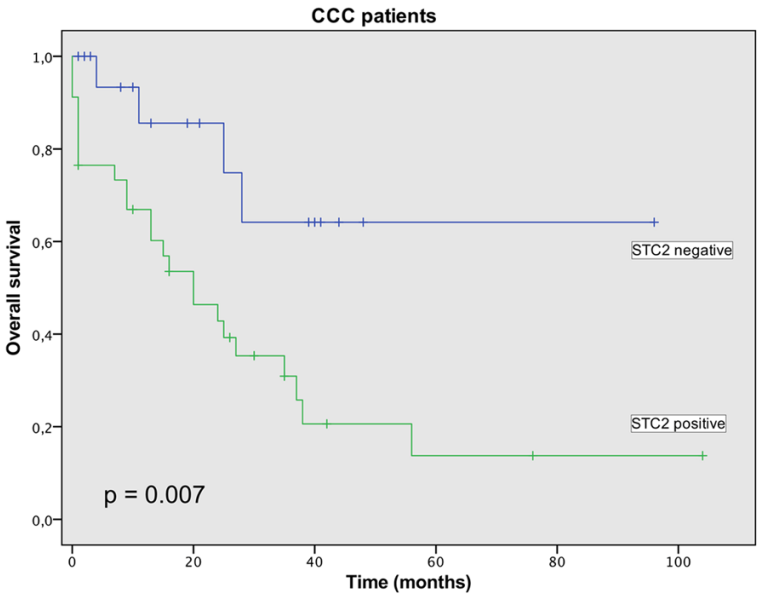

Figure 2. STC2 positive tumors have a worse overall survival. Kaplan Meier-survival analysis of CCC patients of our cohort stratified by STC2 expression. Patients were classified into STC2 negative or positive according to STC2 immunohistochemistry.

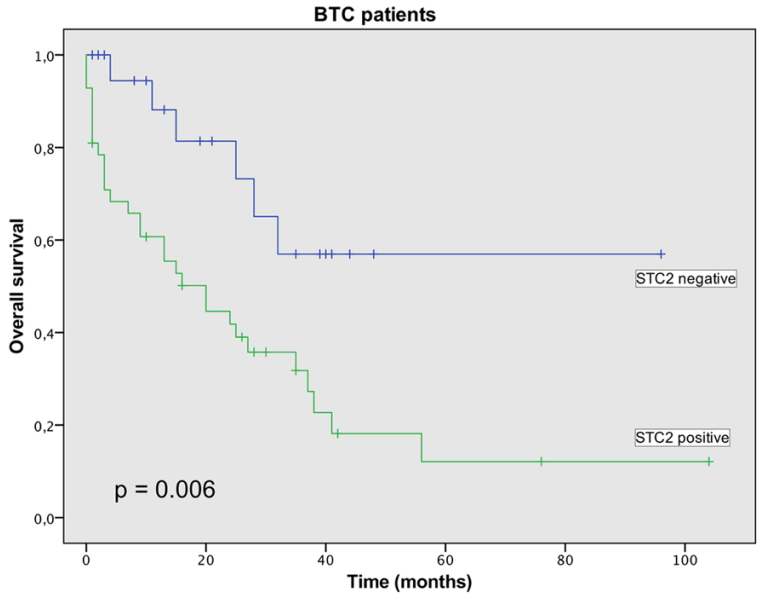

Figure 3. STC2 positive tumors have a worse overall survival. Kaplan-Meier survival analysis of BTC patients (CCC and GBC) of our cohort stratified by STC2 expression. Patients were classified into STC2 negative or positive according to STC2 immunohistochemistry.

vival in CCC patients after surgery. Besides lymphatic infiltration and N-classification, STC2 expression also displayed an affection on overall survival on multivariate analysis (HR = 5.046, 95% CI = 1.27-20.08, P = 0.022), see **Table 2**. Concordantly, Kaplan-Meier analysis showed better survival for patients with STC2 negative tumors, see **Figure 2**. The combinded analysis of patients with GBC and CCC tumors, that are STC2 positive, revealed a worse prognosis for STC2 positive BTCs in survival analysis (63.5 vs 28 months, P = 0.006, HR = 3.19, CI = 1.33-7.69, P = 0.01), see **Figure 3**.

Investigating our sub-cohort of 11 GBT patients, STC2 expression in IHC was also elevated in tumor tissue in comparison to matching non tumorous epithelial tissue (P = 0.041). However, STC2 showed no influence on overall survival (17 vs 27 months, logrank P > 0.05; HR = 1.702, 95% CI 0.31-9.35, P > 0.05), although sample numbers were too small to interpret these data. As recommended by Bridgewater et al. and Wistuba *et al.*, we therefore decided to separate patients with gallbladder cancer from patients with distal, perhilar and intrahepatic CCC [20, 26].

Discussion

BTC continues to pose a critical challenge to clinicians and patients since no effective therapies have evolved thus far. While the molecular basis for biliary carcinogenesis is more and more elucidated [27, 28], there is a lack of translational successes that truly help alleviate the suffering from this highly lethal group of tumors. Despite all research efforts, BTC's 5-year mortality rate still remains under 10% although targeted therapies promise new hope in this deadly disease [29].

Encouraged by previous studies evidencing a biomarker role of STC2 in other malignancies [15-19] we investigated its impact on BTC. In this study, STC2 was significantly overexpressed in CCC on transcriptional level. These findings were validated using immunolabeling in a single center series of BTC.

Tumors with high expression of STC2 were associated with higher rates of lymphatic infiltration and lymph node metastasis in our cohort. This observation has to be stressed because no prognostic value of age, gender, tumor histology and histologic grading have been proven so far in CCC [21, 30]. In contrast, several studies emphazized that the number of infiltrated lymph nodes might be an independent prognostic factor [25], although CCC subtypes show different patterns of metastatic spread [30]. Suggesting a potential role of STC2 in metastasis, Volland *et al.* have shown that STC2-transfected neuroblastoma cells have an increased invasive potential and display higher activity of collagen-degrading matrix metalloproteinase 2 (MMP2) [31]. Correspondingly, Law *et al*. were able to demonstrate that STC2 transfected human ovarian cells showed higher levels of proteases expression, resulting in a more invasive behavior and an initiation of epithelial-mesenchymal transition (EMT) via activation of hypoxia-inducible factor 1-alpha (HIF-1α) [32]. These results may explain the higher lymphatic invasion in STC2 positive CCC cells and emphasizes the possible prognostic value of STC2. Patients with STC2 positive tumors might benefit from adjuvant chemotherapy in combination with radiotherapy or local brachytherapy as very recently proposed for patients with extrahepatic CCC in a phase II trial [33].

In contempt of their potential functional role in metastasis, STC2 have been proposed to be involved in carcinogenesis, apoptosis and cell proliferation. Hence, STC2 inhibition of apoptosis has been consistently found in the human ovarian cancer cell line (SKOV3) [32, 34] and may reflect STC2s' inhibition of plasma membrane store-operated calcium entry (SOCE) [35]. One hallmark of cancer is the ability of solid tumors to survive in an environment of nutrient and oxygen deprivation, resulting in their unhindered growth [36]. In response to exogenous and endogenous pressure, tumor cells change their metabolism and influence their microenvironment to survive [37]. In this context, STC2 has been identified among the most up-regulated proteins in response to glutamine and glucose deprivation [38]. In ovarian cancer cells, hypoxia induced STC2 up-regulation seems to be HIF-1α dependent [32], while STC2 regulates the expression of cyclin D1 and extracellular signal-regulated kinase 1/2 (ERK1/2) in HCC cells [39].

Previously, STC2 expression levels have been shown to influence the outcome in a variety of different cancer entities. High STC2 expression levels have been reported to have a worse prognosis in gastric cancer [15], colorectal cancer [16], renal cell carcinoma [17], esophageal squamous-cell cancer [18] and laryngeal squamous cell carcinoma [19], which is in line with our results. In contrast, high STC2 expression has been shown to improve survival in a subgroup of estrogen receptor positive breast cancer patients [40-42]. These discrepancies may be explained by a tissue or tumor specific role of STC2 and by the fact that hormone-dependent tumors show different growth pattern. The precise function of STC2 in distinct tumor entities is not yet completely understood and issue of ongoing research [12, 43].

We are aware of certain limitations of our study. First of all, the total number of patients and the retrospective fashion of our study hamper conclusions with sufficient evidence. Thus, our findings have to be interpreted with caution until validation in alarger cohort of BTC patients. According to the recently updated guidelines, CCC management critically relies on its anatomic location. Because of our small sample size, we were not able to statistically distinguish between the three subtypes of CCC (intrahepatic, perihilar and distal CCC) [20, 21].

While STC2 is apparently not cancer-specific it may still serve as a biomarker in BTC: One of the major diagnostic techniques in the diagnostic workup of an unclear bile duct stenosis isendoscopic retrograde cholangio-pancreatography (ERCP). It helps to identify the bile duct stenosis and enables cytological sampling by material for brush and direct biopsies for histological workup. Therefore, it may be of interest to stain brush cytological sample obtained during ERCP in BTC patients, because STC2 even if not cancer specific, may help to identify BTC cells. Furthermore, STC2 may help identify patients with particular poor outcome for individualizing the therapy of BTC.

In conclusion, the current study identifies STC2 as a potential new marker for postoperative outcome in patients with bilary tract cancer. STC2 positive tumors show a higher degree of lymphatic infiltration and have a worse overall survival. Thus, STC2 together with established clinicopathological features may be used as an ancillarybiomarker to characterize patients more amenable for adjuvant therapy.

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Disclosure of conflict of interest

None.

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