Virchows Arch (2005) 446: 505–510
DOI 10.1007/s00428-005-1240-4

ORIGINAL ARTICLE

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Analysis of trefoil factor family protein 1 (TFF1, pS2) expression in chronic cholecystitis and gallbladder carcinoma

Received: 5 December 2004 / Accepted: 22 February 2005 / Published online: 9 April 2005

Abstract Trefoil factor family protein 1 (TFF1, pS2) interacts with mucins to protect gastrointestinal epithelium against injury and contributes to mucosal repair by promoting epithelial cell migration and restitution. Moreover, TFF1 has antiproliferative and anti-apoptotic effects and promotes cell scattering and invasion. We investigated TFF1 expression in healthy and inflamed non-neoplastic gallbladder mucosa as well as in gallbladder carcinomas (n=57) and corresponding metastases (n=18), using a tissue microarray technique. TFF1 immunoreactivity was absent in healthy mucosa, focally observed in epithelium with inflammatory changes and present in 35% of primary and 24% of metastatic cancer tissues. Immunoreactivity significantly decreased with increasing tumour stage (P=0.009) and increasing tumour grade (P=0.001). Patients with TFF1 positive tumours showed a more favourable outcome compared to patients with TFF1 negative tumours in univariate analysis (P=0.006). However, multivariate analysis proved resection status and tumour grade as the only independent prognostic factors. In conclusion, TFF1 is expressed in inflamed non-neoplastic gallbladder epithelium and in low stage and low grade gallbladder carcinomas. Thus, TFF1 may be the missing link between gallstones, chronic cholecystitis and gallbladder cancer. Further studies are needed to evaluate whether TFF1 immunostaining can be used as a diagnostic tool to identify patients with a more favourable outcome.

Keywords TFF1 · pS2 · Gallbladder carcinoma · Pathogenesis · Prognosis

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Introduction

Carcinoma of the gallbladder represents the most common malignant tumour of the biliary tract and the sixth most common cancer of the gastrointestinal tract [4]. Overall, this tumour entity shows a strong female predominance, with incidence rates up to 3 times higher among women than men. Prognosis is generally poor with a 32% 5-year survival rate for lesions confined to the gallbladder mucosa and a 10% 1-year survival rate for more advanced stages [13]. The main prognostic factors for gallbladder cancer are resection status and TNM stage [8, 23], and there is a correlation between the level of tumour invasion and the presence of lymph node metastases [13]. However, since gallbladder cancer is difficult to cure by surgery alone, identification of new prognostic biomarkers may help to identify patients who might benefit from additional therapy.

The trefoil factor family protein 1 (TFF1), originally called pS2, was first identified in the estrogen receptor positive MCF-7 human breast cancer cell line [20]. It belongs to a family of small protease-resistant proteins known as trefoil ("three leaves") peptides because of their distinctive three-loop structure formed by intrachain disulfide bonds. Three members of this family have been identified in humans: TFF1 (pS2), TFF2 or human spasmolytic polypeptide (SP) and TFF3 or intestinal trefoil factor (ITF) [35]. Depending on the methylation status of their proximal promoter regions, the TFF peptides are expressed in a site-specific pattern along the gastrointestinal tract [25]. Thus, TFF1 is expressed primarily in the stomach (superficial and foveolar epithelium) and TFF2 in the stomach (mucous neck

cells) and in the duodenum (Brunner's glands), whereas TFF3 predominates in the small and large intestine (goblet cells) [35].

Physiologically, TFFs are abundantly secreted peptides that interact directly with mucins to stabilize the mucous layer lining the gastrointestinal tract, thus protecting the epithelium against injury and maintaining the integrity of the mucosal barrier [37]. Moreover, TFFs contribute to mucosal repair promoting epithelial cell migration and mucosal restitution, and increased TFF expression has been demonstrated in response to epithelial damage and inflammation [35]. According to recent in vitro data, TFF1 is able to inhibit both proliferation (by delaying G1-S phase transition) and apoptosis (via the decrease of caspase-3, -6, -8, and -9 activities) in gastrointestinal epithelial cells [3]. Thus, TFF1 has an important role in regulating the balance between gastrointestinal cell proliferation, death and differentiation.

However, TFFs may also play a role in tumour biology. Support for this function has come mainly from observations that TFF levels in malignant tissues differ from that of corresponding normal tissues. Thus, TFFs are variably expressed by many types of human cancer, including breast, prostatic, gastric, colorectal and pulmonary adenocarcinomas [18].

In the biliary tract, TFF1 expression is increased in biliary diseases in response to injury, as is seen in epithelial damage elsewhere in the gastrointestinal tract [30, 33]. With respect to gallbladder cancer, only two studies investigating TFF1 expression exist which have yielded conflicting results and have not correlated pathologic data with clinical follow-up [26, 31]. Therefore, the current study was designed to investigate the expression of TFF1 in a large series of gallbladder cancers with respect to associations with tumour stage, tumour grade and histological subtype as well as impact on carcinogenesis and patient outcome.

Materials and methods

Case selection

Formalin-fixed and paraffin-embedded specimens of 57 primary gallbladder carcinomas from consecutive patients (40 women and 17 men; male/female ratio 2.4:1) operated between 4/1984 and 12/2002 were chosen for analysis. The mean and median age of patients at surgery were 71 and 72 years (range 35–91 years), respectively. Resection categories of primary tumours were R0 in 28 (49%), R1 in 16 (28%) and R2 in 13 (23%) cases, respectively. Corresponding syn-/metachronous lymph node (n=11) and hepatic (n=7) metastases were included in the study. PT categories were adjusted according to the UICC 2002 issue of the TNM-system [32]: stage pT1a was present in one (2%), pT1b in four (7%), pT2 in 23 (40%), pT3 in 27 (47%) and pT4 in two (4%) cases, respectively. For the conventional histopathologic examination of tumour specimens H&E-stained sections were used. Tumour grades and histological subtypes (variants) were evaluated according to WHO guidelines [1]. Ten cases of non-neoplastic, healthy and inflamed gallbladder tissue were analyzed for comparison. Ethical committee approval and informed consent are not required for retrospective studies dealing with archival material at our institution.

Immunohistochemistry

For immunohistochemical analysis, a tissue microarray technique was used which allows staining of a large number of specimens on one slide. One might argue that the analysis of only small tumour samples could yield unreliable data. However, this objection has been disproved by former studies [22]. Tissue microarrays (TMAs) were prepared using a manual tissue arraying instrument (Beecher, Silver Spring, Md., USA). The details of this technique have been described previously [11]. With respect to the well known heterogeneity of cancer tissues, between three and five cylindrical core biopsies, 0.6 mm in diameter, were taken from different sites of each tumour which had been selected on the original tumour slides to include all patterns of differentiation. Sections of 4 μm were mounted on Superfrost slides for immunohistochemical analysis using an automated immunostainer (DAKO-Autostainer, Universal Staining System; DAKO, Glostrup, Denmark). TMA sections were deparaffinized, rehydrated in graded alcohols and treated for 5 min with 1% H_2O_2. Sections were submitted to microwave antigen retrieval (30 min 160 W in 0.01 M sodium citrate buffer pH 6.0) and subsequently incubated for 30 min with a monoclonal mouse antibody anti-human TFF1 (pS2) antibody (Clone BC04, 1:50, DAKO). Binding of the primary antibody was assessed by the DAKO LSAB2 System HRP (AEC) Detection kit.

Immunohistochemical evaluation and controls

Immunohistochemical staining was evaluated in a semiquantitative fashion independently by two investigators (P. K. and C.L.) who were blinded regarding the clinicopathologic data, especially pT-stage and patients' outcome. Discrepancies were resolved by simultaneous reexamination of the slides by both investigators using a double-headed microscope. A distinct cytoplasmic staining was considered positive, and immunoreactivity was categorized as "focal" (+; <10% of tumour cells positive), "moderate" (++; 10–50%), or "extensive" (+++; >50%). Sections of breast cancer tissue served as positive controls. Negative controls included omission of the primary antibody and incubation with DAKO ChemMate Antibody Diluent.

Statistical analysis

Subgroups according to pT-stage, tumour grade and histological subtype were compared with respect to possible differences in immunoreactivity using the Chi-square test or Fisher's exact test, respectively. Patient outcome was investigated using the Kaplan–Meier method and compared by

the log-rank test. For multivariate testing a Cox's proportional hazards regression model for pT-stage, tumour grade, R-status of surgery and TFF1 expression was performed.

Results

Histopathology

Conventional histopathological evaluation showed tubular adenocarcinoma in 43/57 (75%) cases, whereas the remaining 14 tumours displayed either a papillary (n=5; 9%), mucinous (n=2; 4%), clear cell (*n*=2; 4%), signet-ring cell (*n*=1; 2%) or adenosquamous (*n*=4; 7%) morphology. WHO grading revealed 16 (28%) well differentiated (G1), 19 (33%) moderately differentiated (G2), 21 (37%) poorly differentiated (G3) carcinomas and one (2%) undifferentiated (G4) carcinoma. Lymphatic and venous invasion were detected in 41/57 (72%) and 16/57 (28%) primary tumours, respectively. In general, the histology of metastases was identical to that of corresponding primary tumours.

Immunohistochemistry

Cancer tissue allowing a reliable evaluation of TFF1 immunostaining was present in 52/57 (91%) primary tumours and 17/18 (94%) metastases, respectively. Overall, TFF1 immunoreactivity was seen in 18/52 (35%) primary cancer tissues (Fig. 1a) with only four tumours showing extensive immunostaining (Table 1). TFF1 expression was independent of gender, since immunoreactivity was seen in 14/38 (37%) female and 4/14 (29%) male patients. Regarding metastases, TFF1 immunoreactivity was seen in 4/17 (24%) cases, with three metastases showing only focal and one moderate immunostaining. Comparison of primary and syn-/metachronous metastatic cancer tissues yielded identical staining results in ten cases. In four cases, the primary tumours showed focal TFF1 immunoreactivity, whereas the corresponding metastases lacked immunostaining. In the remaining three cases focal TFF1 immunoreactivity of metastatic tissues was seen, whereas the corresponding primary tumours lacked immunostaining. Non-neoplastic gallbladder mucosa showed focal epithelial TFF1 expression in areas of inflammation (Fig. 1b), whereas healthy mucosa lacked TFF1 immunoreactivity.

TFF1 immunoreactivity of primary tumours decreased with increasing stage (13/24 (54%) pT1/pT2 versus 5/28 (18%) pT3/pT4; *P*=0.009) and increasing grade (16/30 (53%) G1/G2 versus 2/22 (9%) G3/G4; *P*=0.001), but was not found to be significantly associated with angioinvasion (7/14 (50%) L0/V0 versus 11/38 (29%) L1 and/or V1; *P*=0.2). Due to sample size, no significant association between TFF1 immunoreactivity and histological subtypes were found. However, it appears worth mentioning that 4/5 (80%) papillary adenocarcinomas showed at least moderate immunoreactivity.

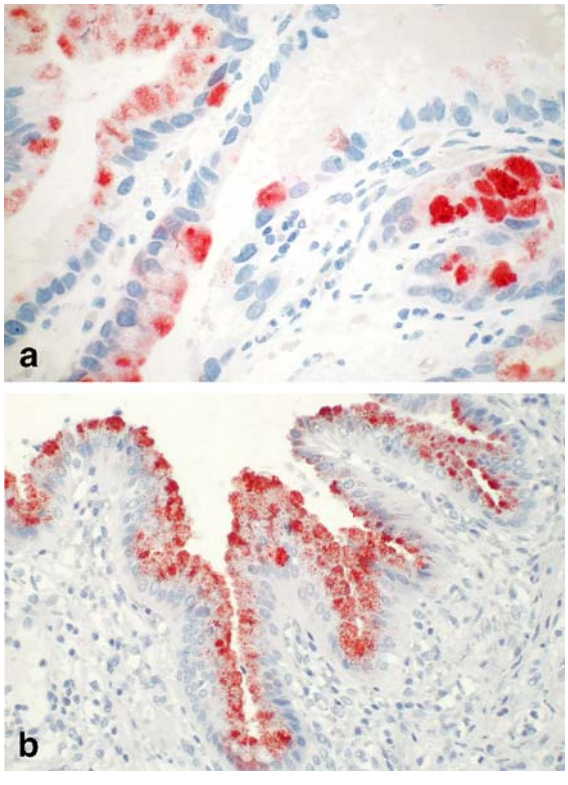

Fig. 1 Distinct cytoplasmic TFF1 immunoreactivity in a moderately differentiated tubular adenocarcinoma of the gallbladder (A, original ×400) and in non-neoplastic gallbladder epithelium with inflammatory alterations (B, original ×150)

Survival analysis

Follow-up data were available for all patients. The overall median survival time of all patients was 45 weeks (range 0.4–604) with a 1-year survival rate of 34% and 5-year sur-

Table 1 TFF1 immunoreactivity (+,<10% of cells positive; ++, 10–50% of cells positive; +++, >50% of cells positive) of primary gallbladder carcinomas related to tumour stage and tumour grade

Stage/grade	Gallbladder carcinomas (*n*=52)	
	N	%
Negative	34/52	65
+	5/52	10
++	9/52	17
+++	4/52	8
Overall positive	18/52	35
pT1a	1/1	100
pT1b	2/4	50
pT2	10/19	53
pT3	4/26	15
pT4	1/2	50
G1	8/13	62
G2	8/17	47
G3	2/21	10
G4	0/1	0

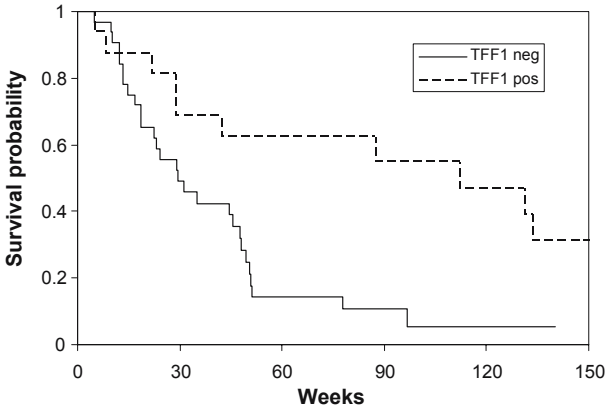

Fig. 2 Overall survival (weeks) of patients with gallbladder carcinoma related to TFF1 immunoreactivity (P=0.006; log-rank test)

vival rate of 6%, respectively. There were four (7%) procedure related deaths (within 28 days): two patients with pulmonary embolism, one patient with pneumonia and one patient with cholangitis and consecutive sepsis. Forty-four (77%) patients died (range 10–175 weeks, median=31 weeks) because of tumour progression, eight (14%) patients died (range 12–604 weeks, median=44 weeks) from causes unrelated to gallbladder cancer without evidence of residual tumour, and one (2%) patient currently is alive, but suffering from metastatic disease (90 weeks after surgery). Patients who underwent complete resection (R0) had a more favourable overall median survival (78 weeks, range 14–604) compared with patients with R1 resection (21 weeks, rage 5–90) and R2 resection (18 weeks, range 0.4–42), respectively (P<0.001, log-rank test). The 1-year survival rate was 63% for patients with R0 resection, 7% for R1 resection and 0% for R2 resection status, respectively. The 5-year survival rate of patients with R0 status was 11% (n= 6), whereas no patients with R1- or R2-resection were alive 5 years after surgery.

Regarding TFF1 expression, patients with TFF1 positive tumours showed a significantly longer median survival time (113 weeks, range 2–604) compared with patients with TFF1 negative tumours (30 weeks, range 0.4–140). Moreover, 57% of patients who died from causes unrelated to cancer had TFF1-positive tumours compared with 30% of patients who died due to tumour progression (P=0.006; log-rank test; Fig. 2). However, multivariate analysis proved only resection category >0 (RR 12.2, CI=4.5–33.6; P<0.001) and tumour grade >2 (RR 3.1, CI=1.4–7.0; P=0.005) as independent prognostic factors regarding survival, whereas both tumour stage and TFF1 lacked independent influence on patient outcome.

Discussion

TFF1 is variably expressed by many types of human cancer, including breast, prostatic, gastric, colorectal and pulmonary adenocarcinomas [18]. Two general patterns have emerged: TFFs either are overexpressed in tumours, whereas normal counterpart tissues express no or low levels of TFFs (e.g. breast, colon, prostate), or they are absent or reduced in tumours in contrast to high levels in the respective non-neoplastic tissue (e.g. stomach). It has been proposed by Lefebvre et al. [14] that TFF1 is a tumour suppressor gene. In support of this idea, TFF1 "knock-out" mice (*TFF1*−/−) constantly develop gastric adenomas and 30% develop invasive adenocarcinomas [14]. Accordingly, TFF1 gene inactivation by deletion, missense mutation and promoter methylation has been documented in human gastric cancer in several studies [9]. Interestingly, TFF1 protein expression is frequently down-regulated in intestinal-type gastric cancer, whereas the TFF1 protein is commonly expressed in diffuse-type gastric cancer cells [10, 15, 19]. This finding is supported by some recent in vitro data showing TFF1-mediated cell scattering and activation of invasion by kidney and colonic cancer cells as well as TFF1-induced angiogenesis implicating cyclooxygenase-2 and EGF receptor signalling, thus linking auto-/paracrine TFF1 expression to cancer progression [5, 27, 28].

Only two studies investigating TFF1 expression in gallbladder cancer exist [26, 31]. Seitz et al. [31] reported TFF1 immunostaining in up to 86% of cancer cases and in non-neoplastic mucosa with inflammatory alterations, whereas healthy gallbladder epithelium constantly lacked TFF1 expression. Roa et al. [26], however, found TFF1 expression in only 32% of cancer cases and in 60% of non-tumoural gallbladders. No associations with either stage or grade were reported and no correlation with clinical follow-up was performed.

According to our data, TFF1 immunostaining is absent in healthy gallbladder mucosa, but present in non-neoplastic mucosa with inflammatory changes and expressed in about one-third of primary gallbladder cancers as well as in about a quarter of cancer metastases. The similar expression in primary and metastatic tumour tissues has already been noted in colorectal carcinomas and corresponding metastases [6], indicating that pS2 may not contribute to the metastatic process. In our study, TFF1 expression by primary tumors decreased with increasing tumour stage and grade. Moreover, patients with TFF1-positive tumours showed a more favourable outcome compared to patients with TFF1-negative tumours in univariate analysis. Further studies including more patients are needed to decide whether TFF1 expression in gallbladder cancer might independently influence patient outcome.

However, our findings can be related to a recent study by Sasaki et al. [30], who examined the participation of TFF1 in the development and progression of intrahepatic cholangiocarcinoma associated with hepatolithiasis. According to their data, TFF1 expression was found to be increased in hepatolithiasis compared with control livers. In biliary epithelial dysplasia and in non-invasive cholangiocarcinoma, TFF1 was extensively expressed, whereas its expression was significantly decreased in invasive cancers, possibly related to methylation of the TFF1 promoter region [30]. A similar correlation between loss of TFF1 expression and tumour dedifferentiation and/or progression has been reported for breast [2], pancreatic [29] and endo-

metrial [12] adenocarcinomas, whereas positive TFF1 immunostaining has been correlated with poor prognosis in pulmonary adenocarcinomas [7] and transitional cell bladder tumours [17]. With respect to gastric cancer, Suarez et al. [34] reported a significant association between high intratumour TFF1 levels and unfavourable outcome, whereas two other groups failed to detect any impact on survival [19, 21].

The pathogenesis of gallbladder cancer has been related to cholelithiasis and chronic infection. Gallstones are found in almost all cases of gallbladder cancer [13] and chronic inflammation may contribute to the progression from epithelial dysplasia (intraepithelial neoplasia) to invasive carcinoma [36]. On the molecular level, loss of p16 and reduced p21(WAF1/CIP1) expression, as well as p53 overexpression are frequent events in gallbladder carcinogenesis [16, 24]. However, the pathogenetic mechanisms concerning the relationship between gallstones (and chronic inflammation) and gallbladder cancer are poorly understood.

Our results and recent data in the literature strongly suggest that TFF1 expression may play a role in the relationship between gallstones, chronic cholecystitis and gallbladder cancer. Gallstones and other agents causing chronic cholecystitis lead to alterations of gallbladder epithelium which are associated with TFF1 overexpression. TFF1 has a motogenic potential and can be regarded as an auto-/paracrine scatter factor promoting invasion (and angiogenesis). Moreover, inhibition of apoptosis by TFF1 may interrupt the death signal that is usually encountered by motile cells when they detach from the matrix. Once the first steps of cancer development are established, the antiproliferative effect of TFF1 may impede tumour growth. Thus, decreased TFF1 expression, possibly due to epigenetic effects, such as methylation of the TFF1 promoter region, may lead to increased tumour cell proliferation, promoting cancer progression.

In conclusion, TFF1 protein is absent in healthy gallbladder mucosa, but present and up-regulated in inflamed gallbladder epithelium and expressed in increased amounts in both primary and metastatic gallbladder cancers. TFF1 expression of primary tumours decreases with increasing tumor stage and grade. Moreover, patients with TFF1-positive tumours show a more favourable outcome compared to those with TFF1-negative tumours in univariate analysis. Finally, TFF1 may play a role in the relationship between gallstones, chronic cholecystitis and gallbladder cancer.

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