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ORIGINAL ARTICLE

# TFF3 Expression as Stratification Marker in Borderline Epithelial Tumors of the Ovary

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**Abstract** Borderline tumors (BOT) of the ovary account for 10% to 20% of ovarian neoplasms. Like ovarian cancer, BOT encompass several different histological subtypes (serous, mucinous, endometrioid, clear cell, transitional cell and mixed) with serous (SBOT) and mucinous (MBOT) the most common. Current hypotheses suggest low-grade serous carcinoma may develop in a stepwise fashion from SBOT whereas the majority of high grade serous carcinomas develop rapidly presumably from inclusion cysts or ovarian surface epithelium. The pathogenesis of mucinous ovarian tumors is still puzzling. Molecular markers could help to better define relationships between such entities. Trefoil factor-3 (TFF3) is an estrogen-regulated gene associated with prognosis in different types of cancer. It has also been included in a recent marker panel predicting subtypes of ovarian carcinoma. We analyzed the expression of TFF3 by immunohistochemistry in a cohort of 137 BOT and its association with histopathological features. Overall expression rate of TFF3 was 21.9%. None of the BOT with serous and endometrioid histology displayed strong TFF3 expression. On the other hand, TFF3 was highly expressed in 61.4% of MBOT cases and 33.3% of BOT with mixed histology ($P < 0.001$) suggesting a potential function of the protein in that subtypes. Associations of TFF3 expression with FIGO stage and micropapillary pattern were significant in the overall cohort but confounded by their correlation with histological subtypes. The highly specific expression of TFF3 in MBOT may help to further clarify potential relationships of tumors with mucinous histology and warrants further studies.

**Keywords** Ovarian cancer · Borderline tumors · Histological subtypes · Prognosis

## Introduction

Borderline ovarian tumors (BOT) differ from ovarian cancer by absence of stromal invasion. They do not clearly fall into benign or malignant categories, their pathogenesis is still not well understood [1]. BOT account for approximately 10% to 20% of all ovarian neoplasms [2, 3]. Like epithelial ovarian cancer, BOT encompass several different histological subtypes (serous, mucinous, endometrioid, clear cell, transitional cell and mixed epithelial cell) [4]. However, serous (53%–75%) and mucinous (25%–43%) BOT are by far the most common [5, 6]. In general, BOT have an excellent prognosis with an overall recurrence rate between 3% and 10% [7–10]. BOT differ markedly from invasive ovarian cancers and the distinct phenotypes associated with BOT and high-grade serous ovarian carcinomas suggest that these lesions may have different origins. Several aspects support this hypothesis including the high frequency of KRAS or BRAF mutations in BOT and low-grade carcinomas that are less common in high-grade serous carcinomas [11, 12] as well as the wild-type status of p53 in BOT and low-grade cancers, which is often mutated in high-grade tumors [13]. Nevertheless, recurrence of BOT can be also malignant mostly representing low-grade carcinomas [14]. A recent study showed that 20% of recurrences from primarily diagnosed BOT are invasive

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carcinomas [5]. Whether these recurrences are considered to be de novo carcinomas or direct transformation from BOT is still a matter of debate [5, 15, 16]. Shih et al. hypothesized that low-grade serous carcinoma develops in a slow stepwise fashion from serous BOT (SBOT) and intra- epithelial carcinoma, whereas the majority of high-grade serous carcinomas develop rapidly, presumably from precursor lesions that originates as intraepithelial carcinomas in the fallopian tubes, reviewed in [1, 17]. The pathogenesis of mucinous ovarian tumours is still mysterious [18]. Mucinous carcinomas are typically heterogeneous, containing foci of mucinous cystadenoma admixed with atypical proliferative tumour and obvious carcinoma. A study using Laser capture microdissection have shown the identical KRAS mutation in all three components (adenoma, atypical proliferative, and carcinoma), supporting their clonal relation [19]. This provides in term evidence that mucinous cystadenomas are possible precursor lesions for mucinous carcinomas. How mucinous BOT (MBOT) develop from mucinous cystadenomas and what may be the causes and mechanism of this malignant transformation is still not well defined [20]. Further studies of molecular markers may help to better define the relationships of the different subtypes of premalignant and malignant forms of ovarian tumours.

Trefoil factor-3 (TFF3) is an estrogen-regulated oncogene. Its expression has been demonstrated to be associated with prognostic factors in a multitude of different types of cancer, e.g. estrogen receptor positive breast cancer [21]. Recent studies has also shown that TFF3 expression is increased in carcinoma and is involved in tumor cell growth, scattering, invasion and metastasis [22–26]. The number of studies on TFF3 in ovarian cancer is limited, but its expression may have protective effects on epithelial cells and was associated with a variable but statistically significant risk of cancer recurrence [27]. Furthermore, TFF3 has been included in a marker panel predicting subtype of ovarian carcinoma [28]. TFF3 expression in BOT has not yet been thoroughly studied. Therefore, we have analyzed TFF3 by immunohistochemistry in a cohort of 137 borderline tumors of the ovary and its association with various histopathological features.

## Materials and Methods

### Patients and Samples

All analyses were performed according to the "REporting recommendations for tumourMARKer prognostic studies" (REMARK). [29] A corresponding REMARK diagram is given in Supplementary Fig. S1.The Local Research Ethics Committees approved studies of human tissue and samples were processed anonymously.

Formalin-fixed, paraffin-embedded (FFPE) tissue samples of 156 ovarian borderline tumors patients were retrieved from the Senckenberg's Institute of Pathology, University Frankfurt and were reviewed by an experienced second gynecologic pathologist (RA). Diagnosis and tumor grading was performed according to the current criteria of the World Health Organization (WHO) [30].

**Table 1** Sample characteristics

| Characteristic according second pathology | | n= | % |
|---|---|---|---|
| Subtype | serous | 82 | 59,9% |
| | mucinous | 44 | 32,1% |
| | endometroid | 2 | 1,5% |
| | mixed | 9 | 6,6% |
| FIGO stage | IA | 42 | 30.7% |
| | IB | 8 | 5.8% |
| | IC | 53 | 38.7% |
| | II | 7 | 5.1% |
| | III | 6 | 4.4% |
| | IV | 1 | 0.7% |
| | n.a. | 20 | 14.6% |
| Implants | no | 125 | 91,2% |
| | yes | 12 | 8,8% |
| Micropapillary pattern | no | 106 | 77,4% |
| | partially | 24 | 17,5% |
| | yes | 7 | 5,1% |
| Presence of in situ carcinoma* | no | 124 | 90,5% |
| | yes | 13 | 9,5% |
| Macroinvasion† | no | 123 | 92.5% |
| | yes | 10 | 7,5% |
| Microinvasion | no | 134 | 97,8% |
| | yes | 3 | 2,2% |

*according to WHO criteria: Cribriform glands measuring 5 mm in one dimension and nuclear atypia greater than that allowed in SBOT [30]

† macroinvasion refers to invasive carcinoma with underlying borderline tumor in the histology

### Histopathological Evaluation and Immunohistochemistry

Paraffin sections (2 μm) were mounted on Superfrost Plus slides, dewaxed in xylene and rehydrated through graduated ethanol to water. Antigens were retrieved by microwaving sections in 10 mM citrate buffer (pH 6.0) for 20 min at 800 W. Blocking was performed using antibody dilution buffer (DCS-Diagnostics, Hamburg, Germany) at room temperature for 15 min. Subsequently, the TFF-3 antibody was diluted 1:100 in this buffer. Sections were incubated with the TFF-3 antibody for 1 h at room temperature. For negative control, the primary antibody was replaced with phosphate-buffered saline. For secondary antibody incubation and detection, the Dako REAL Detection System Alkaline Phosphatase/RED Rabbit/Mouse (Dako, Glostrup, Denmark) was used following the protocol of the supplier and sections were

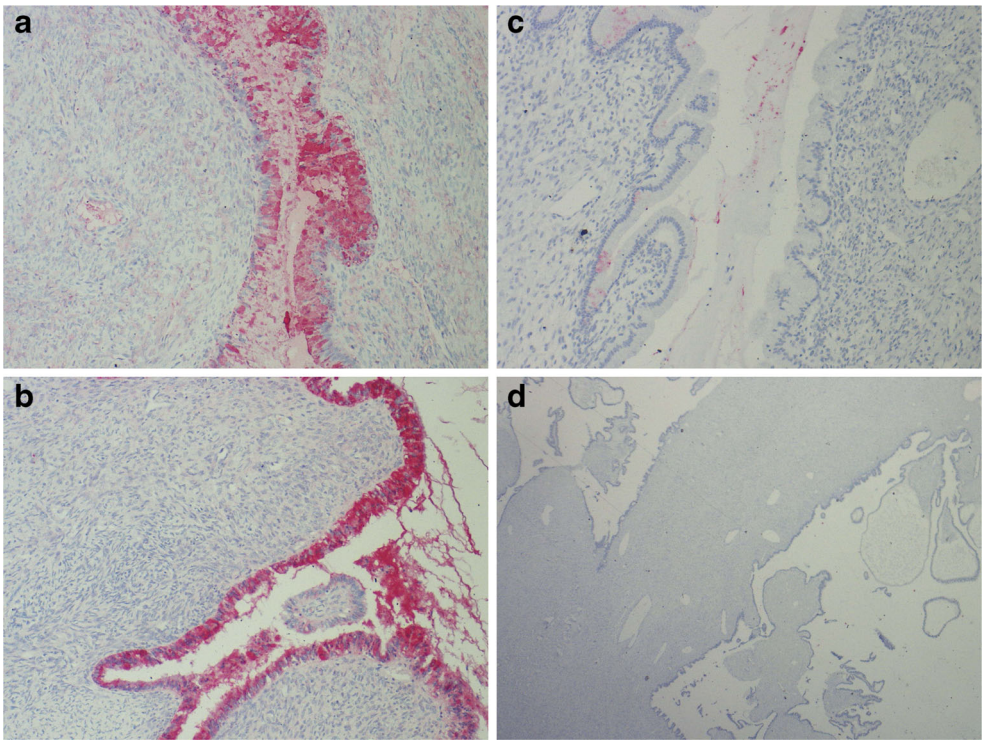


**Fig. 1** Immunohistochemichal detection of TFF3 expression in borderline tumors of the ovary. Mucinous borderline (**a**) and serous tumor (**b**) of the ovary showing strong cytoplasmic positivity for TTF3 Original magnification 10X10. (**c**), (**d**): Mucinous borderline and serous tumor of the ovary showing no positivity for TTF3. Original magnification 10X10

counterstained with Hematoxylin Solution, Gill No. 3 (Sigma-Aldrich GHS332). The mouse monoclonal antibody directed against TFF-3 (ab57752, lot GR71649–1) was obtained from Abcam (Cambridge UK).

TFF3 were scored semiquantitatively based on the staining intensity (SI). SI was assigned as 0, negative; 1, weak; 2, moderate; or 3, intense. A combined intensity score (CIS) was calculated as: CIS = SI × PP/100 (with PP as percentage of stained cells). All assessments were made blinded with respect to clinical patient data.

### Statistical Analysis

Chi-Square and Fisher's Exact Test were used to determine significance of categorical variables, Mann-Whitney U-Test for the analysis of continuous variables. All *p*-values are two-sided and 0.05 was applied as significance level. Subjects with missing values were excluded from the analyses. All analyses were performed using SPSS Statistics Version 22 (IBM Corp.).

## Results

### Cohort

We retrospectively identified 156 cases of borderline tumor of the ovary (BOT) from pathology records. For 139 samples sufficient archival material was present for standard hematoxylin-eosin staining and immuno-histochemistry using a monoclonal anti-TFF3 antibody. However, on reevaluation one of the 139 samples was re-characterized as adenoma of the ovary and for one BOT only material from implants was available, leaving a total of 137 BOT samples for analysis.

### Sample Characteristics of the Cohort

We finally studied a cohort of 137 BOT with validated diagnosis by a second pathologist and sufficient archival material for immuno-histochemical analysis. Median age of patients was 49.0 years (IQR 34.5–63.5). Additional sample

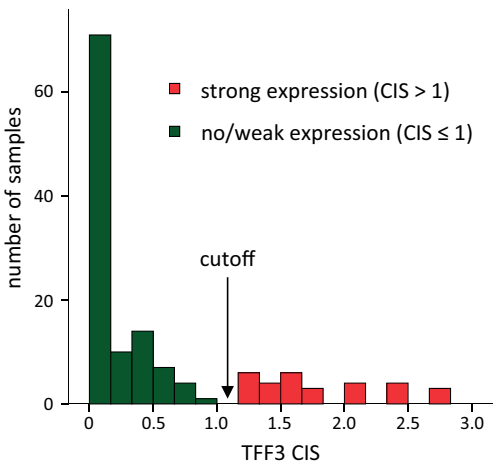


**Fig. 2** Distribution of TFF3 CIS score among 137 BOT

characteristics are given in Table 1. The majority of the samples were either of serous (59.9%, SBOT) or mucinous subtype (32.1%, MBOT). The high frequency of mucinous histology in BOT as compared to EOC has also been described by others [5]. FIGO stage for most of the patients were either IA (30.7%) or IC (38.7%). Micropapillary pattern was observed for 22.6% (17.5% partially) and implants were detected among 8.8% of the patients (Table 1).

### TFF3 Expression in Borderline Tumors of the Ovary

We next studied TFF3 expression by immuno-histochemical analysis of tissue samples from all 137 borderline tumors from Table 1. Representative examples of TFF3 staining results are shown in Fig. 1. TFF3 localized to cell cytoplasm in all tumors with positive staining results. Fibroblasts, endothelial cells and dendritic cells of surrounding lymphoid tissue stained negative for TFF3. Intensity of staining and percentage of stained cells were scored separately and combined as a immuno-histochemical score (CIS, see Methods section). Based on the distribution of CIS shown in Fig. 2 we selected CIS > 1 as a cutoff for TFF3 expression. This cutoff resulted in 30 cases (21.9%) with strong positive TFF3 expression and 107 cases (78.1%) with weak or no expression. We then compared this classification with sample characteristics as presented in Table 2. In the studied cohort the overall TFF3 expression rate was 21.9%. None of patients with serous and endometrioid BOT (82 patients and 2 patients, respectively) showed strong expression of TFF3. On the other hand, TFF3 was expressed in 61.4% (27 Patients) of MBOT cases and 33.3% of BOT with mixed histology ($p < 0.001$; Table 2). Another significant finding was the correlation of TFF3 expression to FIGO stage. In our analyzed samples all patients with disease exceeding the ovaries (FIGO II-IV) were TFF3 negative compared to patients with disease confined to the ovaries having 24.3% with positive TFF3 expression ($P = 0.038$). However, this difference was no more significant when controlling for the confounding effect of TFF3 expression between histological subtypes.

We found no significant differences regarding patients' age, presence of in situ carcinoma, macro- or microinvasion, and the presence of implants between samples positive and negative for TFF3, respectively (Table 2). In contrast, TFF3 was highly correlated to mucinous subtype of BOT with no sample of serous histology showing strong TFF3 expression ($P < 0.001$; Table 2). Accordingly, we also detected no strong TFF3 expression in BOTs displaying a micropapillary pattern which was only observed in SBOT.

## Discussion

The Trefoilfactors (TFFs) are soluble proteins, encompassing small (12–22 kD) peptides, which have a common three looped structure formed by inter-chain disulphide bond. TFF2 was the first discovered member of this family [31].

**Table 2** TFF3 expression in borderline tumors of the ovary

| Sample characteristic | | TFF 3 no/weak (CIS ≤ 1) | | TFF 3 Strong (CIS > 1) | | *P*-Value |
|---|---|---|---|---|---|---|
| Frequency | | 107 | 78.1% | 30 | 21.9% | |
| Median age | (95% CI) | 49.0 | | 58.5 | | $P = 0.41$ |
| FIGO stage | I | 78 | 75.7% | 25 | 24.3% | **$P = 0.038$** |
| | II-IV | 14 | 100% | 0 | 0% | |
| Subtype | serous | 82 | 100% | 0 | 0% | **$P < 0.001$** |
| | mucinous | 17 | 38,6% | 27 | 61.4% | |
| | endometroid | 2 | 100% | 0 | 0% | |
| | mixed | 6 | 66,7% | 3 | 33,3% | |
| | Total | 107 | 78,1% | 30 | 21,9% | |
| Presence of in situ carcinoma | no | 97 | 78,2% | 27 | 21,8% | $P = 1.0$ |
| | yes | 10 | 76,9% | 3 | 23,1% | |
| Micropapillary pattern | no | 76 | 71,7% | 30 | 28,3% | **$P = 0.004$** |
| | partially | 24 | 100% | 0 | 0% | |
| | yes | 7 | 100% | 0 | 0% | |
| Macroinvasion | no | 95 | 77,2% | 28 | 22,8% | $P = 1.0$ |
| | yes | 8 | 80% | 2 | 20% | |
| Microinvasion | no | 105 | 78,4% | 29 | 21,6% | $P = 0.53$ |
| | yes | 2 | 66,7% | 1 | 33,3% | |
| Implants | no | 95 | 76,0% | 30 | 24% | $P = 0.68$ |
| | yes | 12 | 100% | 0 | 0% | |

Significant *P*-Values are given in bold.

Although TFFs are mostly found in the gastrointestinal tract, their expression and specially that of TFF3 has been also detected on all mucin-secreting tissues. Data suggest that the TFFs may play a role in different functions as proliferation, migration and angiogenesis. These are crucial processes for wound healing and tumorigenesis [25]. TFF3 is the last described mammalian member of the trefoil factor family. The peptide was cloned from rat intestinal epithelial cells during a search for proteins that contributed to the regulation of proliferation and differentiation among intestinal epithelial populations, and was consequently named intestinal trefoil factor [31]. TFF3 contains one trefoil domain consist of 59 amino acids and has a molecular weight of approximately 6.6 kD (monomer) or 13 kD (dimer) [32]. The physiological function of TFF3 in the Ovaries is still not fully understood [33], but it may play a role in tumour development and progression in different tumour entities [26, 34, 35].

In the present study, we analyzed the expression of TFF3 in a cohort of 137 borderline tumors of the ovary (BOT). A strength of our study is the large sample size, the use of a central pathology as well as the blinded re-evaluation by a second pathologist. Limitations however include the retrospective design of the analysis and the missing follow up of the patients.

In our analysis we found a highly significant association of strong TFF3 positivity with mucinous histology (MBOT) ($P < 0.001$). Interestingly, this finding was only evident in 61.4% of patients with mucinous histopathology, whereas 38.6% of MBOT did not or only weakly express the gene. According to the dualistic model for ovarian carcinogenesis the origin of mucinous tumours is puzzling, unlike serous, endometrioid, and clear cell tumours, they do not display a müllerian phenotype. Although it has been argued that these mucinous tumours bear some relationship with the endocervix, the mucinous epithelium that characterizes these neoplasms more closely resembles gastrointestinal mucosa [36]. Seidmann et al. speculated that Brenner and mucinous tumours originate from microscopic transitional cell nests at the tubal-mesothelial junction [37]. These tumours grow and their mucinous component becomes dominant. They compress and eventually obliterate the adjacent ovary giving the appearance that they arose in the ovary [37]. Whether the variation of TFF3 expression in MBOTs reflects their different origin or not, requires further investigations.

Several factors have been associated with prognosis of BOT. Surgical pathological stage and classification of extra-ovarian disease into invasive and non-invasive implants are the most important prognostic indicators for SBOTS [12]. Data concerning predictive and prognostic factors for MBOT are scarce [38]. A correlation of higher TFF3 expression and poor prognosis was found in gastrointestinal tumors [39]. On the other hand TFF3 expression was found to be associated with a good prognosis in endometrioid adenocarcinoma of the uterus with longer recurrence free survival and longer overall survival in comparison to those with negative TFF3 expression [24]. In our study we detected an inverse association of TFF3 expression with FIGO stage and micropapillary pattern, both markers of poor prognosis. However, these associations seem to result from confounding of the restricted expression in MBOT cases. When we controlled for histological subtypes no significant differences were observed anymore.

Taken together, our analysis strongly links TFF3 expression to MBOT and thus suggests a potential function of the protein in that histological subtype. Since the carcinogenesis of mucinous tumors is still enigmatic, molecular markers may help to unveil their pathogenesis and further studies on TFF3 in that entity are warranted.

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**Compliance with Ethical Standards**

**Conflict of Interest** The authors have declared no conflicts of interest.

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