Expression of matrix metalloproteinase-2 in serous borderline ovarian tumors is associated with noninvasive implant formation

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Summary

Objective: To investigate matrix metalloproteinase (MMP) proteolytic and vascular endothelial growth factor (VEGF) and receptor (VEGFR-1, VEGFR-2) angiogenetic capacity in serous borderline ovarian tumors (S-BOTs) for women with and without noninvasive implants.

Methods: The population was made up of 99 patients with S-BOTs as the primary diagnosis between 1985 and 1995, 44 of whom had noninvasive implants and 55 without implants. MMP-2, MMP-14, the type-2 tissue inhibitor of MMPs (TIMP-2), and VEGF and receptors (VEGFR-1, VEGFR-2) were examined by immunhistochemistry.

Results: Strong positive (+++) MMP-2 staining was found more frequently in women with primary S-BOTs and noninvasive implants (76%) than in those without implants (53%; $p < 0.05$). In contrast, staining for MMP-14 and TIMP-2 was not significantly different in the two groups. Furthermore, expression of MMP-2, MMP-14, and TIMP-2 was similar in primary tumors and in their noninvasive implants. Most tumors in both groups had no VEGF expression (84% in the noninvasive implant group and 82% in the group without implants), while moderate (++) to strong (+++) expression of VEGFR-1 and VEGFR-2 was detected in 79% and 94% of the two tumor groups, with no significant difference between the groups.

Conclusions: Enhanced MMP-2 was seen in primary S-BOT with noninvasive implants. The presence of noninvasive implants was prognostic for disease-free survival.

Key words: MMP-2, MMP-14, TIMP-2, VEFG, VEGFR-1, VEGFR-2; Ovarian neoplasm; Borderline tumor; Population-based; Epithelial; Noninvasive implants.

Introduction

Serous borderline ovarian tumors (S-BOTs) [1] are less common than serous epithelial ovarian cancer, with rates of 4.6 and 12.8 cases per 100,000 women/years, respectively, for all histological subtypes in Norway during 2002 [2]. The serous subtype comprise 56% of all BOT [3]. Sixty-three percent of invasive epithelial ovarian cancers are of the serous type [3]. The 5- and 10-year survival rates for women with S-BOTs are better than those for women with any stage of serous epithelial ovarian cancer. S-BOTs with invasive implants have inferior survival compared to those with noninvasive implants or no implants [4, 5].

A series of cellular events take place before tumor spreading. The cellular steps involve proteolytic degradation of extracellular matrix components [6-8]. Many enzymes catalyze this process, including factors of the matrix metalloproteinase pathway (MMP). Initially, it was believed that MMPs were involved in local tumor invasion and migration at sites of tumor dissemination, however, there is growing evidence that they have a role as signaling molecules, and they are important for the creation and maintenance of a microenvironment that facilitates cellular proliferation and angiogenesis in primarily tumors and at metastatic sites [9-11].

One of the major structural components of the basement membrane, type IV collagen, is a substrate for the gelatinase MMP-2 [12] which is heavily degraded during tumor cell invasion. Like most MMPs, MMP-2 is secreted as a proenzyme, which is processed extracellularly to generate an active gelatinase [13, 14]. This activation process is complex [15], involving a membrane-bound MMP called membrane type-1 MMP or MMP-14 [13, 14]. A specific MMP-2 inhibitor, the type-2 tissue inhibitor of MMPs (TIMP-2) [16], probably exercises its inhibitory function near the MMP-14 binding sites, and MMP-2 activation also involves TIMP-2 bound to MMP-14 [17].

The functional role of angiogenesis in ovarian tumor progression is debated [18]. Vascular endothelial growth factor (VEGF) has diverse regulatory functions in epithelial ovarian cancer, one of which, tumor proliferation, has been thoroughly studied [19, 20]. VEGF expression has been shown to be greater in advanced stage primary epithelial ovarian cancer and in omentum metastases than

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in benign ovarian tumors or normal ovarian tissue [21]. Receptors for VEGFs may therefore be implicated in ovarian tumor progression.

The development of inhibitors of MMPs with low relative molecular mass offers the potential of preventing tumor invasion and angiogenesis, thus inhibiting tumor progression [22]. Furthermore, therapeutic targeting of VEGF receptors by receptor tyrosine kinase inhibitors has been shown to inhibit malignant progression in experimental ovarian cancer models [21].

S-BOT is characterized by atypical epithelial proliferation without destructive stromal invasion at the primary site, which distinguishes this entity from invasive carcinoma [23, 24]. Some S-BOT tumors contain noninvasive peritoneal implants, comprising serous epithelial proliferation involving the peritoneal surface [24]. The development of noninvasive implants in S-BOT is not well understood. Recently a new theory has been proposed, describing two different pathways for the development of epithelial ovarian cancer. One comprises serous cystadenomas that evolve slowly via S-BOTs to low-grade epithelial ovarian cancer. This "low-grade" development involves mutations in K-ras. The other suggests that ovarian cancer develops rapidly from inclusion cysts into highly invasive and widespread disease via a "high-grade" pathway [25-28]. A precursor has so far not been identified in the latter. It frequently contains P53 mutations and very seldom KRAS mutations.

Gotlieb *et al.* found that women with S-BOTs were younger than women with early-stage invasive ovarian cancer [29]. S-BOT tumors were more frequently of the serous type and less frequently had mutations in BRCA1 and BRCA2, suggesting that S-BOT is an unique biological entities, different from highly invasive ovarian cancer, developed via the "high-grade" pathway [28].

We investigated the possible regulatory effects of the MMP-2/MMP-14/TIMP-2 complex and VEGF and its receptors (VEGFR-1, VEGFR-2) by comparing tumor expression in S-BOTs with and without noninvasive implants. We also investigated the prognostic effect from the presence of noninvasive implants on disease-free survival.

Materials and Methods

Patients

The study population consisted of 99 patients with S-BOTs of the typical type [23], 44 with noninvasive implants and 55 without noninvasive implants. The patients had the primary diagnosis between 1985 and 1995 and were selected randomly from the population-based Norwegian Cancer Registry. In this period totally 632 patients were diagnosed with S-BOTs in Norway, 550 without implants and 82 with implants. The age distribution was similar in study and register groups. Tumors with implants had a separate code in the main database of the Norwegian Cancer Registry, making them easy to identify. The distinction between invasive and noninvasive tumors was done by two pathologists at The Norwegian Radium Hospital prior to the analyses.

The presence of prognostic factors assessed prior to or at the time of surgery was extracted from patient records and included age, parity, hereditary cancer (any first or second degree family member with cancer), other cancers before diagnosis (yes, no), symptoms before diagnosis (present, not present), atypia (mild, moderate, high), ploidy (diploid, aneuploid, other), largest tumor diameter (in mm), ascites (< 200 ml, ≥ 200 ml), serum CA125 (< 35 Uk/l, ≥ 35 Uk/l), and adjuvant chemotherapy given postoperativly (yes, no) [30]. The omentum had not been removed during primary surgery in 23 patients in the nonimplant group.

Tumor specimens

Formalin-fixed, paraffin-embedded tissue blocks were collected from 19 pathological departments in Norway. The mean and median storage periods were 15 years and two months (range, 9 years and 7 months to 20 years). At primary surgery pathologists at The Norwegian Radium Hospital assessed the histological diagnoses for 94 of the patients in the cohort. A senior pathologist diagnosed 74 specimens. The diagnosis of noninvasive implants was defined as lacking all of the following three histological features: invasion of underlying normal tissue, micropapillary architecture, and solid epithelial nests surrounded by clefts [31]. Prior to the present investigation, one pathologist (JMN) re-evaluated and confirmed the diagnosis in each case.

Immunhistochemistry

MMP-2, MMP-14, TIMP-2, VEGF, VEGFR-1, and VEGFR-2 were identified in primary S-BOTs and corresponding noninvasive peritoneal implants by immunohistochemistry. The antibodies and immunostaining conditions are listed in Table 1. The tissues were sectioned (4 μm) with a Microm HM355 microtome, mounted on Superfrost plus slides (Menzel, Germany), and air-dried for 24 h at 37°C. Sections were deparaffinized in xylene and rehydrated in graded series of alcohol to distilled water. Subsequently, antigens were retrieved by heating in 10 mmol/l Tris/EDTA buffer (pH 9.0) in a microwave oven at the highest power (850 W). Polymer EnVision System (Dako, Glostrup, Denmark) was used for detection. The antigen-antibody-enzyme complexes were visualized with diaminobenzidin. Sections were counterstained with hematoxylin. Slides with no microvessel staining for antibodies were treated as missing in the analyses (93 out of 858 slides). Appropriate negative (by omitting the primary antibody) and positive (placenta) controls were used in each staining run. We observed expression of MMP-2, MMP-14, TIMP-

Table 1. — *Antibodies used in immunochemistry in primary S-BOTs and associated noninvasive implants.*

Antibody (clone)	Source	Dilution	HIER
MMP-2 (po)	LabVision Corporation, Fremont, CA, USA	1:400	A
MMP-14 (po)	LabVision Corporation, Fremont, CA, USA	1:300	A
TIMP-2 (po)	LabVision Corporation, Fremont, CA, USA	1:100	A
VEGF (C-1)	Santa Cruz Biotechnologies, Santa Cruz, CA, USA	1:50	A
VEGFR-1 (po)	Santa Cruz Biotechnologies, Santa Cruz, CA, USA	1:400	A
VEGFR-2 (CH-11)	Chemicon, Temecula, CA, USA	1:200	A

HIER, heat-induced epitope retrieval; A, 4 x 5 min in Tris/EDTA buffer.

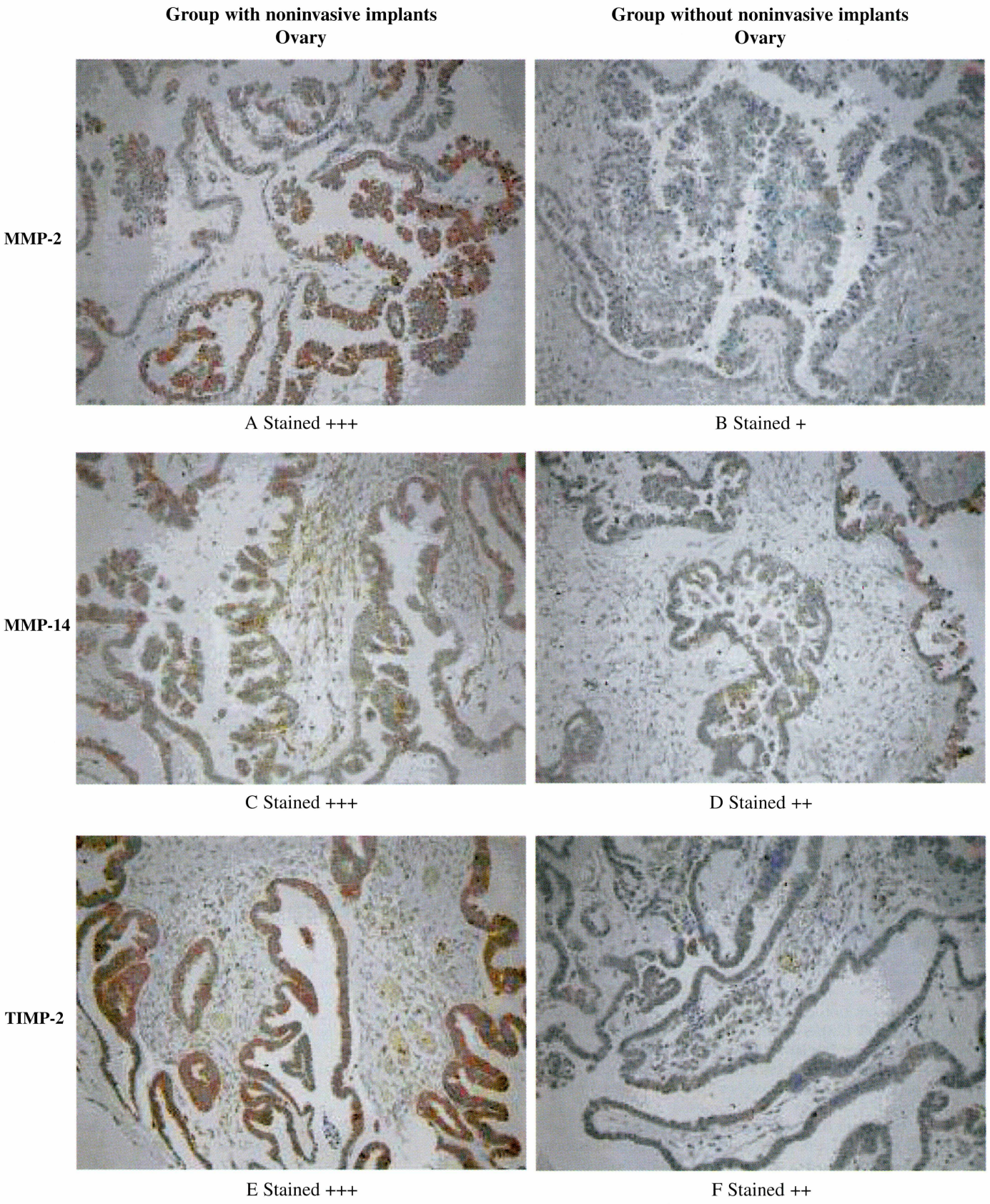
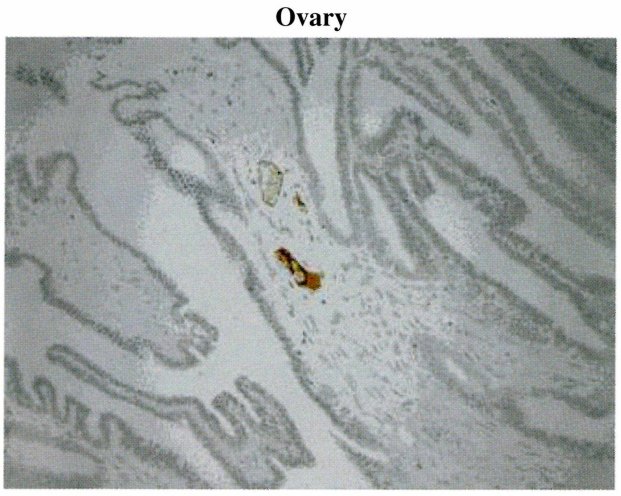


Figure 1. — The panels show primary S-BOTs with and without noninvasive implants stained with antibodies to MMP-2, MMP-14, and TIMP-2.



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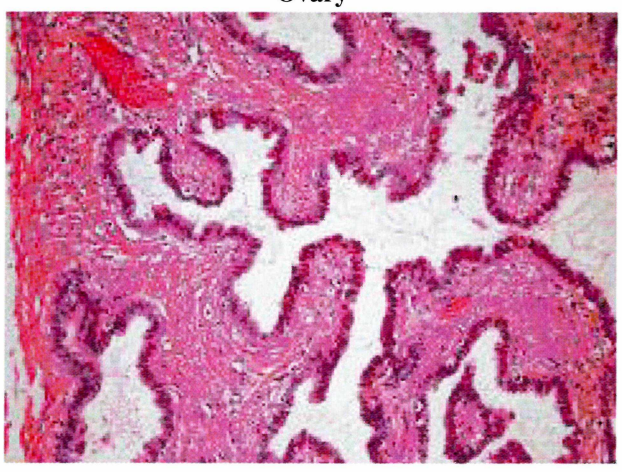

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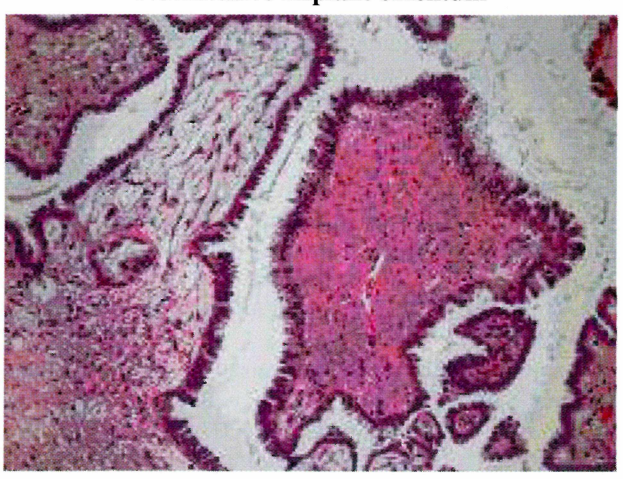

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Figure 2. — Panels A shows VEGF staining in primary S-BOTs. Panel B shows hematoxylin staining of primary S-BOTs and panel C shows hematoxylin staining of a noninvasive implant in the omentum.

Table 2. — *Characteristics of 99 women with primary S-BOTs with and without noninvasive implants.*

Characteristic	Implant group n = 44 (%)	Nonimplant group n = 55 (%)	p value*
Age (years)			
Median	46	49	
< 40	15 (34)	15 (27)	0.4
40-49	14 (32)	13 (24)	
50-59	5 (11)	12 (22)	
> 60	10 (23)	15 (27)	
FIGO stage			
IA	0	37 (67)	< 0.01
IB	0	10 (18)	
IC	0	8 (15)	
IIA	8 (18)	0	
IIB	6 (14)	0	
IIIA	22 (50)	0	
IIIB	6 (14)	0	
IIIC	2 (5)	0	
Parity (median = 2)			
P0	12 (32)	5 (13)	0.1
P1	9 (24)	8 (20)	
P2	8 (21)	17 (43)	
P ≥ 3	9 (24)	10 (25)	
Unknown	6	15	
Family history of cancer			
Yes	19 (83)	6 (50)	< 0.05
No	4 (17)	6 (50)	
Unknown	21	43	
Other cancer			
Yes	1 (2)	3 (6)	0.4
No	43 (98)	52 (95)	
Symptoms			
Yes	32 (91)	38 (79)	0.1
No	3 (9)	10 (21)	
Unknown	9	7	
Atypia			
Mild	13 (31)	17 (32)	0.9
Moderate	25 (60)	30 (57)	
High	4 (10)	6 (11)	
Unknown	8	5	
Ploidy			
Diploid	24 (92)	18 (75)	0.1
Aneuploid	1 (4)	6 (25)	
Other	1 (4)	0	
Unknown	18	31	
Largest primary tumor diameter in mm (median = 70)			
<50	11 (25)	9 (16)	0.1
50-100	21 (48)	37 (67)	
> 100	12 (27)	9 (16)	
Ascites			
< 200 ml	37 (90)	53 (100)	< 0.05
≥ 200 ml	4 (10)	0	
Unknown	3	2	
CA-125			
< 35	16 (50)	11 (73)	0.1
≥ 35	16 (50)	4 (27)	
Unknown	12	40	
Chemotherapy primarily			
Yes	31 (71)	9 (20)	< 0.01
No	13 (30)	36 (80)	
Unknown	0	10	
Recurrence			
Yes	5 (11)	2 (4)	0.1
No	39 (89)	53 (96)	
Death due to recurrence			
Yes	2 (5)	0	0.1
No	42 (96)	55 (100)	
Death due to other cause			
Yes	3 (7)	10 (18)	0.1
No	41 (93)	45 (82)	

*Pearson chi-square test; not known omitted from analyses.

2, VEGFR-1, and VEGFR-2 also in the stromal cells, which was considered as internal positive controls. The VEGF staining of the small vessels in the slides was used as an internal positive control.

The immunohistochemistry reaction was evaluated semiquantitatively, < 1% positive tumor cells being recorded as negative (0), intense staining in 2-10% of tumor cells as weakly positive (+), intense staining in 11-50% of tumor cells as moderately positive (++), and a positive reaction in > 51% of tumor cells as strongly positive (+++) (Figures 1, 2; Tables 5 and 6).

Cytoplasmic, nuclear, and membrane immunoreactivity of epithelial cells was scored by one of the authors for all stained slides (n = 858). A second author gave blinded scores, with an inter-observer agreement of complete match in 21 specimens out of 28 (kappa, 0.65) [32]. All slides were coded and evaluated by the two pathologists without knowledge of the patients' clinical status.

Statistical analyses

All analyses were performed with SPSS for Windows version 12.0.1 (SPSS Inc, Chicago, Illinois, USA). The Pearson chi-square test was used to compare age distribution, frequencies of prognostic factors, chemotherapy, recurrence status and cause of death according to implant status (Table 2).

The Mann-Whitney test was used to test for differences in immunohistochemistry staining between the noninvasive implant group and the group without implants (Table 3). The Wilcoxon signed rank test was used to assay any difference in immunohistochemistry staining between primary tumors and their associated noninvasive implants. The patterns of disease-free survival of patients with and without noninvasive implants were displayed in a Kaplan-Meier plot, and a log-rank test was used to test differences in disease-free survival between the groups as shown in Figure 3 [32].

Ethics

The Regional Ethical Committee for Medical Research in Norway, the Norwegian Data Inspectorate, and the Norwegian Directorate of Health approved the design of the study.

Table 3. — *Expression of MMP-2, MMP-14, and TIMP-2 in primary S-BOTs with and without noninvasive implants.*

Expression	Implant group n = 44 (%)	Non implant group n = 55 (%)	p value*
MMP-2			
Negative 0	1 (3)	2 (4)	< 0.05
Positive +	2 (5)	5 (9)	
Positive ++	6 (16)	18 (34)	
Positive +++	28 (76)	28 (53)	
Not evaluable	7	2	
MMP-14			
Negative 0	0	0	0.1
Positive +	0	4 (7)	
Positive ++	4 (11)	8 (15)	
Positive +++	33 (89)	42 (78)	
Not evaluable	7	1	
TIMP-2			
Negative 0	0	0	0.1
Positive +	1 (3)	3 (6)	
Positive ++	1 (3)	6 (11)	
Positive +++	35 (95)	45 (83)	
Not evaluable	7	1	

*Mann-Whitney test.

Table 4. — *Location of peritoneal noninvasive implants in 44 patients with primary S-BOTs.*

Location of implants*	No. of implants* (n = 97) (%)
Mean	2.2
Median	2
Tube	15 (15)
Uterus serosa	5 (5)
Bladder serosa	2 (2)
Pelvic peritoneum/ Fossa Douglasi	10 (10)
Ileum serosa	1 (1)
Colon serosa	1 (1)
Rectum serosa	2 (2)
Omentum	61 (63)

* Described in the pathological reports

Results

Patient characteristics related to presence or absence of noninvasive implants at the time of primary surgery are shown in Table 2. S-BOTs without implants were found in a non-significantly larger fraction of patients younger than 50 years of age (66%) than patients older than 50 years of age (51%). A family history of cancer was found more often in the noninvasive implant group than in the group without implants (83% vs 50%, $p < 0.05$).

All patients with more than 200 ml of ascites were in the noninvasive implant group ($p < 0.05$). Most of the patients in the noninvasive implant group had received chemotherapy (71% vs 20%, $p < 0.01$), with platinum (n = 35) or thifosyl (n = 3).

Table 3 shows strong positive (+++) MMP-2 staining in a higher percentage of cases of S-BOTs with noninvasive implants (76%) than in those without implants (53%) ($p < 0.05$). In contrast, staining for MMP-14 and TIMP-2 was not significantly different in primary S-BOTs with and without implants. Furthermore, expression of MMP-2, MMP-14, and TIMP-2 in primary tumors was similar to that of corresponding noninvasive implants (data not shown). No differences in the expression of VEGF, VEGFR-1, or VEGFR-2 were found between primary S-BOTs with and without noninvasive implants (data not shown). Most of the tumors in both groups were negative for VEGF expression (84% in the noninvasive implant group and 82% in the group without implants), while moderate to strong expression of VEGFR-1 and VEGFR-2 was detected in 79% and 94% of tumors from both patient groups, with no significant difference between them (data not shown).

The noninvasive implant lesions were located at nine different sites but were most frequent in the omentum (63%) (Table 4). Twenty-three patients had only one implant, while 21 patients had two to ten implants (mean 2.2) described in the pathology report.

Figure 1 shows S-BOTs stained with antibodies to MMP-2, MMP-14, and TIMP-2. Figure 2 shows primary S-BOTs and noninvasive implants stained with antibodies to VEGF, as well as two hematoxylin-stained slides from S-BOTs.

Table 5. — *Clinical characterestics of seven patients with recurrent disease from primary S-BOTs.*

Patient	Time after primary diagnosis (months)	Age at primary diagnosis (years)	Death due to recurrence	Invasivness of recurrence	Ascites (ml)	Atypia	Ploidy	CA-125	Stage	No. of implants
1	74	30	No	Invasive G1*	No	Moderate	Aneuploid	11	IA	0
2	56	45	No	S-BOT	No	**	Diploid	21	IIA	1
3	119	22	No	S-BOT	No	Moderate	Diplod	12	IC	0
4	45	35	Yes	Invasive G2*	3000	**	**	3016	IIIA	1
5	37	18	No	S-BOT	No	**	Diploid	1068	IIIA	2
6	21	33	No	S-BOT	No	Moderate	**	**	IIB	2
7	89	44	Yes	Invasive G2*	No	Mild	Aneuploid	225	IIIA	3

* G: Grade; ** Missing data.

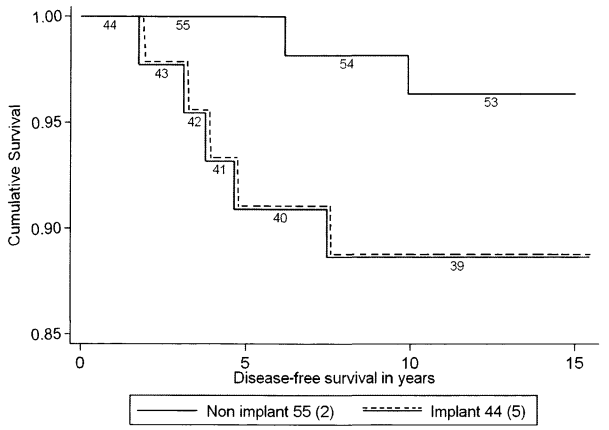

Figure 3. — Disease-free survival of patients with S-BOTs; 44 with noninvasive implants and 55 without. Five patients with implants and two without relapsed.
Log-rank test p = 0.1.

The 10-year disease-free survival rate was nonsignificantly poorer for patients with noninvasive implants (89%) compared to those without implants (96%; p = 0.1) (Figure 3). Seven patients (7%) developed recurrent disease; the characteristics are shown in Tables 5 and 6. All patients with recurrence were premenopausal (18-45 years) (Table 5). Three patients had recurrence as invasive serous papillary cancer 45, 74 and 89 months after primary surgery, one as well differentiated and the two others as moderately differentiated cancer. Two of these women died of the disease 118 and 129 months after primary surgery. Both patients showed strong staining for MMP-2 in the noninvasive implants, and one had moderate staining (++) in the ovaries whereas the other had strong staining (+++) (Table 6). Both patients had spread to the omentum and increased serum CA-125 (225 and 3016 kU/l) at primary surgery. Both were treated with platinum after primary surgery.

Another four patients relapsed with S-BOTs 21-119 months after primary surgery. They were all alive on 1 January 2006. Two patients without implants relapsed, both incompletely staged at primary surgery. One patient had an aneuploid tumor, FIGO Stage IA, whereas the other had a diploid tumor, Stage IC, at primary surgery.

Table 6. — *Immunhistochemical characteristics of primary S-BOTs from seven patients with recurrence.*

Patient	Ovary MMP-2	Ovary MMP-14	Ovary TIMP-2	Implant MMP-2	Implant MMP-14	Implant TIMP-2
1	0	+++	+	0	+++	+
2	+++	+++	+++	*	*	*
3	+++	+++	+++	+++	+++	+++
4	++	+++	+++	+++	+++	+++
5	*	*	*	+++	+++	+++
6	+++	+++	+++	+++	+++	+++
7	+++	+++	+++	+++	+++	+++

* Not evaluable.

Discussion

In this study, strong MMP-2 staining was found by immunohistochemistry more frequently in primary S-BOTs with noninvasive peritoneal implants than in S-BOTs without implants. No apparent differences in staining for MMP-14 or TIMP-2 were observed between the two groups. The expression profiles for each factor of the MMP-2/MMP-14/TIMP-2 complex were similar in the primary tumors and in their corresponding noninvasive implants. From these results we suggest that the overall signaling of the MMP-2/MMP-14/TIMP-2 complex indicates enhanced MMP-2 proteolytic activity in the interaction between ovarian tumor cells and the peritoneum, resulting in formation of S-BOTs with noninvasive implants.

No differences in the expression of VEGF or its receptors were found between primary S-BOT with and without noninvasive implants. Previously it was suggested that the VEGF signaling pathway is involved in implant development [21]. It has also been suggested that MMPs have a role in facilitating tumor angiogenesis in both primary invasive tumors and metastasis [9-11, 33, 34], which might be in accordance with our data.

Many specimens with noninvasive implants did not stain, possibly because they contained too few tumor cells spread across the slide. The quality of staining for specimens with enough tumor cells was good. The lack of staining might have been due to heterogeneous staining of the antigens from the paraffin-embedded tissues. This is in accordance with what Ogawa and co-workers found [35].

Limited investigations on MMP and VEGF expression have been performed on S-BOTs, however, the research on invasive ovarian and other cancers is more substantial. Conflicting results have been obtained with regard to the

expression of MMP-2 and MMP-9 in normal ovaries and S-BOTs [36, 37]. Davidson et al. concluded that MMP-2, MMP-9, MMP-14, and TIMP-2 were valid markers of poor survival from advanced stage ovarian cancer [38, 39]. Lengyel and co-workers showed that MMP-9 predicted better survival of advanced ovarian cancer patients without residual tumor [40]. In contrast, Wu *et al.* found no significant relationship between activated MMP-2 and invasiveness, metastasis, and disease-free progression between different patient groups [41].

The intensity of MMP-2 staining on primary gastric tumors cells correlated significantly with the number of lymph node metastases [42]. In breast cancer, stronger expression of MMP-2, MMP-3, and MMP-9 was found in brain metastases compared to normal brain tissue [43]. Stronger expression of MMP-2 was found in the primary tumors than in the pleural effusions from breast cancer within the same patients [44]. In many studies, enhanced immunohistochemical staining of MMPs in the invasive primarily tumors and high serum levels of MMPs were associated with metastases and poor outcome [40, 41, 45, 46].

Our findings give support to the theory that S-BOTs might evolve to a low-grade invasive ovarian cancer [28, 47]. Three patients in our study had recurrent disease as well or moderately differentiated serous papillary cancer. We hypothesize that enhanced expression of MMP-2 plays a role in noninvasive implant formation and in malignant transformation to highly differentiated cancer along the "low-grade" pathway described by Singer *et al.* [47]. We found that both patients that had died of the disease had strong tumor staining of MMP-2, MMP-14, and TIMP2. The patient still alive with recurrence of highly differentiated cancer did not show tumor staining for MMP-2. Strong staining of MMP-2 might be a prognostic factor.

The patients with implants in our cohort were younger than those without implants, and more patients in the noninvasive implant group had a family history of cancer. Only two patients died of invasive disease. Both had noninvasive implants primarily. The presence of noninvasive implants might be a prognostic factor in S-BOT. Gilks *et al.* found that only patients with implants relapsed [48]. According to Zanetta, 2% of S-BOTs will recur as invasive cancer, which is in accordance with the present finding [49]. Although many will consider recurrence after five years as new cancer, we consider relapse after five years as further development of the primary tumor.

The patients in the present study were selected from a well-defined population-based national cancer registry. A high proportion of all patients in the study period were selected for the study, so that the study population was representative for S-BOT patients with and without noninvasive implants. Two pathologists with a special interest in S-BOTs reevaluated the histological diagnoses, one shortly after the primary operation and the other before the present study, ensuring the validity of the S-BOT diagnosis. The number of implants found per patient in our study is in accordance with the number of implants found by Bell *et al.* [31].

Further studies should be carried out in order to describe the biological mechanisms of MMPs in S-BOTs [50]. A comparison of MMP staining should be done between ovarian cystadenomas without atypia, S-BOTs without implants, S-BOTs with noninvasive implants, S-BOTs with invasive implants, and grade 1 serous papillary ovarian cancer.

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