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Expression and prognostic relevance of endothelin-B receptor in vulvar cancer

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Abstract. Overexpression of endothelin (ET)-1 and its receptors, ET_AR and ET_BR, commonly referred to as the 'ET-axis', has been demonstrated to play a role in cancer progression for various human tumours. Based on these results we propose a similar role of the expression of the ET-axis in vulvar cancer. Expression of the ET-axis was investigated immunohistochemically using tissue microarrays with tumour samples of 68 vulvar cancer patients. Samples were obtained from patients undergoing local excision or radical vulvectomy. ET-1 expression of tumour cells correlated highly significantly with early stages of vulvar cancer (p=0.004), whereas neither ET_AR nor ET_BR expression showed any association with TNM stages. High staining levels of ET_BR in the tumour tissue were significantly related to tumour progression (p=0.01) and early metastases (p=0.09); low ET_BR staining intensity correlated with longer relapse-free survival (p=0.019). In patients with ET_BR overexpressing low-stage tumours (pT1-2) we observed a significantly reduced overall survival and disease-free survival (p=0.036 and 0.021, respectively). ET_AR expression and ET_BR expression were significantly correlative (p=0.018). Accordingly, co-expression of both receptors was related to tumour progression (p=0.022) and an increased risk for local recurrence (p=0.005). These results suggest that, in addition to established histological and clinical prognostic factors, analysis of ET-receptor and, in particular, of ET_BR expression by means of simple immunohistochemical analysis might improve prediction of the prognosis for patients with vulvar squamous cell carcinoma.

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Introduction

Malignant vulvar tumours represent 5% of all gynaecologic malignancies (1). Ninety percent of vulvar tumours are squamous cell carcinomas. In general, two groups of vulvar cancers can be distinguished depending on patients' age and the presence of human papilloma virus. Most patients (>65%) are older than 65 years (2,3). However, tumours in the other group with younger patients seem to be associated with human papilloma virus (HPV), especially HPV16. Moreover, tumours in the younger group are frequently of basaloid tumour type (4,5). Within the last years a global trend has become apparent according to which an increasing number of younger patients are developing vulvar cancer, which has been attributed to HPV (4,6). Treatment of vulvar cancer is usually surgical with radical vulvectomy and bilateral groin and pelvic lymphadenectomy, where applicable in combination with adjuvant radiotherapy (7). The five-year overall survival rate is 68%. Survival worsens with an increase in tumour stage, age and nodal stage (8,9) all of which have been established as poor prognostic factors. In addition, molecular markers are needed to enable a better discrimination of more aggressive tumour phenotypes and to potentially facilitate a targeted molecular therapy in the future.

The endothelins and their receptors, referred to as the ET-axis, play an emerging role in cancer development. Endothelin-1 (ET-1) is a vasoconstrictor that was initially isolated from endothelial cells (10). As a potent mitogen, ET-1 has direct influence on cell proliferation and a strong synergy with many growth factors that have been implicated in the progression of various tumour entities (11-14). ET-1 exerts its effects via its receptors, ET_AR and ET_BR (15). With respect to ET_BR expression in human malignancies contradictory data have been reported. Whereas ET_BR seems to function as a possible survival factor in oligodendrogliomas (16), decreased ET_BR expression was associated with poorer clinical outcome (17-19) in other cancer entities (e.g., malignant melanoma or prostate cancer).

To the best of our knowledge this is the first study that analyses expression of the ET-axis in primary vulvar cancer and correlates the results with clinicopathological and follow-up data.

Table I. Correlation of ET-1, ET_AR and ET_BR expression with clinicopathological characteristics in patients with vulvar cancer.

Parameter	Patients	(%)	ET-1 (%)	ET_A-receptor (%)	ET_B-receptor (%)
	68	100	8/68 (12)	10/68 (17)	30/68 (44)
Tumour stage			*		
pT1a	4	6	2/4 (50)	0/4 (0)	1/4 (25)
pT1b	18	26	4/18 (22)	2/18 (11)	7/18 (39)
pT2	32	47	2/32 (6)	6/32 (19)	17/32 (53)
pT3	12	18	0/12 (0)	2/12 (17)	5/12 (42)
pT4	2	3	0/2 (0)	0/2 (0)	0/2 (0)
Nodal stage					
pN0	39	57	6/39 (15)	4/39 (10)	17/39 (44)
pN1	17	25	2/17 (12)	3/17 (18)	8/17 (47)
pN2	12	18	0/12 (0)	3/12 (25)	5/12 (42)
cM stage			*		
M0	59	87	8/59 (14)	9/59 (15)	28/59 (47)
M1	9	13	0/9 (0)	1/9 (11)	2/9 (22)
Histological grading					
G1	8	12	2/8 (25)	1/8 (13)	5/8 (63)
G2	44	65	5/44 (11)	5/44 (11)	17/44 (39)
G3	16	23	1/16 (6)	4/16 (25)	8/16 (50)
Proliferation index (Ki 67) (%)					
<10	17	25	3/18 (18)	4/17 (24)	9/17 (53)
11-40	33	49	3/33 (9)	5/33 (15)	14/33 (42)
>40	18	26	2/18 (11)	1/18 (6)	7/18 (39)
Basaloid type	19	28	2/19 (11)	1/19 (5)	6/19 (32)
Keratinizing type	49	72	6/49 (12)	9/49 (18)	24/49 (49)
HPV	31	46	3/31 10)	4/31 (13)	13/31 (42)
Age					
<50	17	25	1/17 (6)	2/17 (12)	9/17 (53)
>50	51	75	7/51 (14)	8/51 (16)	21/51 (41)

*Significant correlation.

Materials and methods

Patients and tumour specimens. Sixty-eight patients who between 1988 and 2004 had been diagnosed with primary vulvar cancer at the Department of Obstetrics and Gynaecology, University of Münster, Germany, were included in the study. The patients' mean age was 62±16 years (range, 26-93 years). Treatment consisted of radical or partially radical vulvectomy. Thirty-three patients (55%) received adjuvant radiation therapy, two of them in combination with chemotherapy according to the scheme for radiochemotherapy of cervical carcinomas (20). Follow-up was performed quarterly until October 2004. Formalin-fixed and paraffin-embedded tumour-tissue blocks for all patients were obtained from the files of the Institute of Pathology (University of Münster). The presence of invasive squamous cell carcinoma was confirmed for each block selected. To ensure standardised tumour classification all slides were reviewed and reclassified according to the TNM classification [UICC, 6th edition (21)].

Tissue microarray (TMA). For construction of the tissue microarray (TMA), representative paraffin-embedded tumour tissue samples served as donor blocks. Sections were cut from each donor block and stained with haematoxylin and eosin. For each of the 68 tumour samples two morphologically characteristic areas were chosen from both the tumour invasion line and the tumour centre. These areas were circled on the H&E slides. From each of these circled regions a cylindrical

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Correlation of ET-1, ET_AR and ET_BR expression with prognostic factors in patients with vulvar cancer.

Parameter	Patients	(%)	ET-1 (%)	ET_A-receptor (%)	ET_B-receptor (%)
	68	100	8/68 (12)	10/68 (17)	30/68 (44)
Alive (recurrence-free)	20	29	3/20 (15)	2/20 (10)	6/20 (30)
Death of disease	19	28	1/19 (5)	3/19 (16)	12/19 (63)
Tumour-unrelated death	6	9	1/6 (17)	1/6 (17)	3/6 (50)
3-year survival	28/43	65	5/28 (18)	5/28 (18)	14/28 (50)
5-year survival	21/38	55	3/21 (14)	3/21 (14)	10/21 (48)
Tumour progression					*
Local recurrence	22	32	4/22 (18)	2/22 (9)	11/22 (50)
Distant metastasis	23	34	1/23 (4)	4/23 (17)	13/23 (57)

*Significant correlation.

0.6-mm core biopsy was acquired and precisely arrayed into a new recipient paraffin block using a manual tissue arrayer (Beecher Instruments, Silver Spring, MD, USA). The final tissue set consisted of one block containing 276 tumour sample cores. The presence of squamous cell carcinoma in the arrayed samples was verified on H&E sections of the TMA.

Immunohistochemistry. Four-μm sections were cut from the TMA block and mounted on poly-L-lysine-coated glass slides. After pre-treatment with a steamer (Multi-Gourmet-Steamer, Braun, type 3216) for antigen retrieval (30 min), sections were stained immunohistochemically for Ki-67 using an automated immunostainer (Dako-Autostainer). Mouse monoclonal anti-Ki-67 antibody (clone MIB-1; Dako Diagnostics, Hamburg, Germany, anti-human Ki-67 antigen, code no. M7240) was used as primary antibody in a dilution of 1:1000. The ratio of MIB-1 expressing cells (distinct nuclear staining) against the total number of cells was evaluated for each tissue cylinder (22).

Staining for ET_AR and ET_BR was also performed in a multi-step semi-automatic procedure (Dako-Autostainer; Dako Diagnostics), as previously described (14). Briefly, two sheep polyclonal antibodies for ET_AR and ET_BR were used (ET_A-receptor antiserum, product no. 210-507-C250; ET_B-receptor antiserum, product no. 210-506-C250; Affinity Bioreagents, Golden, CO, USA). For staining of ET-1, a monoclonal mouse antibody (anti-endothelin-1 MAb, clone TR.ET.48.5; Affinity Bioreagents) was used. Bladder cancer tissue known to express ET-1 and ET_AR and smooth muscle tissue with ET_BR activity were used as positive, and omission of the primary antibody was used as negative control. Semi-quantitative analysis was performed by two of the authors (E.E., M.B.), both blinded to the clinical data. The cytoplasmic immunostaining intensity of the tumour cells was categorised semi-quantitatively into 4 groups as previously described (14), namely negative (score 0), weak (score 1), moderate (score 2), and strong (score 3). The final score was designated as negative or positive in the following way: score 0-1, negative; and score 2-3, positive.

In situ hybridisation (ISH). The chromogenic ISH assays (Inform®HPV) were performed according to the manufacturer's recommendations using the BenchMark™ Automated Slide Staining System (Ventana Inform HPV Test, Tucson, AZ, USA). Patient specimens known to be positive for either oncogenic or non-oncogenic HPV were used as controls. The probe cocktails demonstrate positive hybridisation to the following 12 high-risk genotypes: 16, 18, 31, 33, 35, 39, 45, 51, 52, 56, 58 and 66, as well as 5 low-risk genotypes: 6, 11, 42, 43 and 44 (23).

Statistical analysis. Statistical analysis was performed by use of the statistical software package SPSS 11.0.1™ (SPSS Inc., USA). All clinical and pathologic parameters were correlated with IHC results by means of contingency tables and χ^2 test. For calculation of overall and disease-free survival times Kaplan-Meier estimates were generated (24). Survival curves were compared by log-rank test (25). P-values <0.05 were considered to be statistically significant.

Results

The series of 68 patients with squamous cell carcinoma of the vulva analysed in this study was well-balanced with respect to clinicopathological parameters such as age, TNM, HPV status, grading, basaloid cancer type (basaloid versus keratinising), and proliferation index (Ki-67) (Table I). Median follow-up was 26 months (range, 2-147 months). Within that time 19 patients died from vulvar carcinoma, 6 died due to non-tumour-related reasons. Fourteen patients were lost to follow-up. Overall survival was 65% at 3 years and 55% at 5 years, respectively.

Immunohistochemistry. The expression of ET-1, ET_AR, and ET_BR presented as homogenous cytoplasmic staining (Fig. 1). The staining intensity of ET-1, ET_AR and ET_BR varied between different tumours from complete absence to strong staining. Moderate or strong staining intensity (referred to as 'ET-

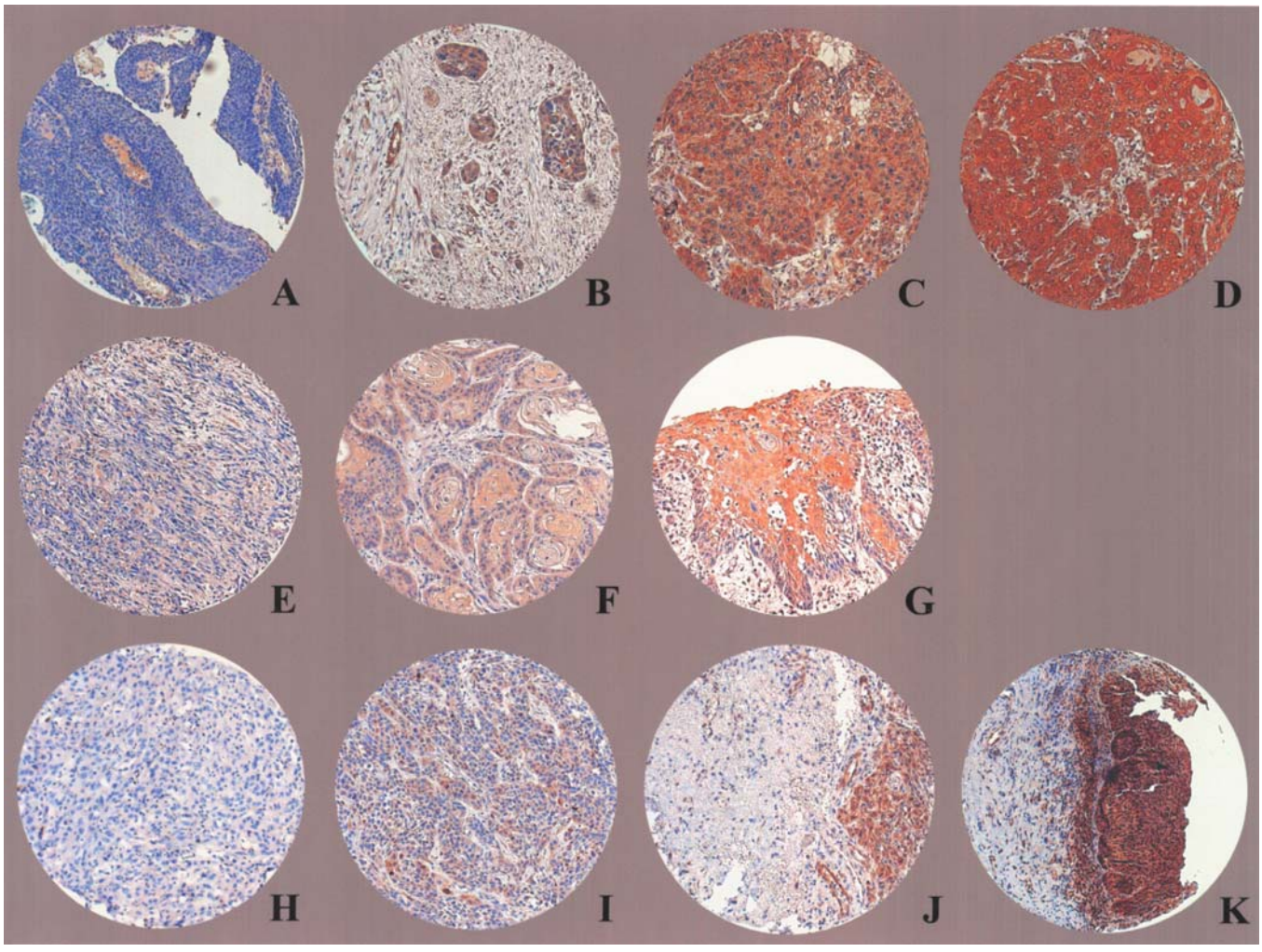

Figure 1. Immunohistochemical expression of tumours: x20 objective magnification; ET-1 (A, negative; B, weak; C, moderate; D, strong), ET_AR (E, negative; F, weak; G, moderate), ET_BR (H, negative; I, weak; J moderate; K, strong).

positive') was present for ET-1 in 8 of 68 (12%) and for ET_BR in 30 of 68 (44%) tumours. Vulvar carcinomas (10 of 68) (17%) showed a moderate staining reaction for ET_AR (Table I, Fig. 1). For ET_AR, no strong expression pattern was detected. We found a significant correlation between expression of ET_AR and ET_BR (p=0.01). Also, expression of ET-1 was associated with that of both ET receptors (ET_AR, p=0.009; ET_BR, p=0.011).

Correlation between expression of ET-axis, clinicopathological parameters and follow-up. We could not demonstrate any correlation between the expression of the ET receptors and established clinicopathological characteristics such as histological grade, tumour size, lymph node or distant metastases. In contrast ET-1 expression correlated significantly with smaller tumour size (p=0.035), whereas no ET-1 expression was present in pT3 and pT4 tumours. No correlation was found between ET-1 expression and presence of lymph node distant metastases or histological grade. Also, expression of the ET-axis did not significantly correlate with the HPV status, tumour type or proliferation index (Ki-67).

However, expression of ET_BR significantly correlated with an adverse outcome (Table II). Accordingly, ET_BR expression was more frequently detected in patients with progression of disease (p=0.01, Fig. 2), e.g. development of local recurrence or distant metastases. Consistently, patients with low ET_BR expression had a significantly longer relapse-free survival (p=0.019, Fig. 3).

Survival analysis revealed a significantly worse outcome in patients with early tumour stages (pT1+2 disease) displaying tumours overexpressing ET_BR (p=0.036, Fig. 4A) compared to those with ET_BR-negative tumours. Time to progression (TTP) was also shorter in patients with increased ET_BR expression (p=0.093). With respect to stages pT1-2 ET_BR was indicative of a significantly worse TTP (p=0.021, Fig. 4B).

Discussion

Endothelin-1 and its two receptors, ET_AR and ET_BR, have recently been demonstrated to be overexpressed in various human tumours. It has clearly been shown that the ET-axis promotes the growth and progression of carcinomas by influencing apoptosis, angiogenesis and signalling of several growth factors (26). The emerging role of the ET-axis in cancer has led to the development of selective ET-receptor antagonists

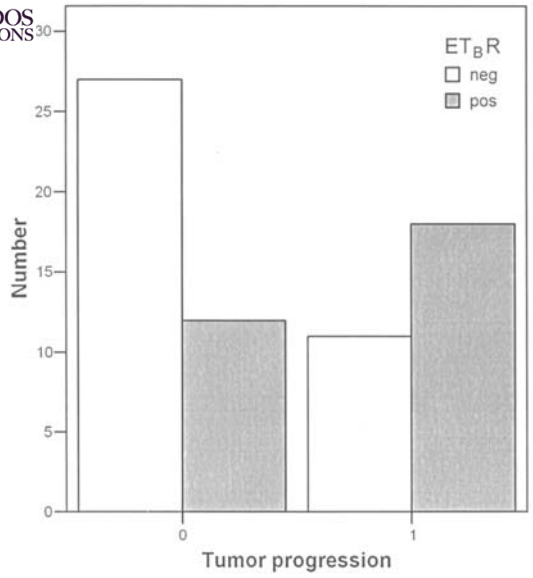

Figure 2. Significantly elevated ET_BR expression with vulvar cancer progression (p=0.01).

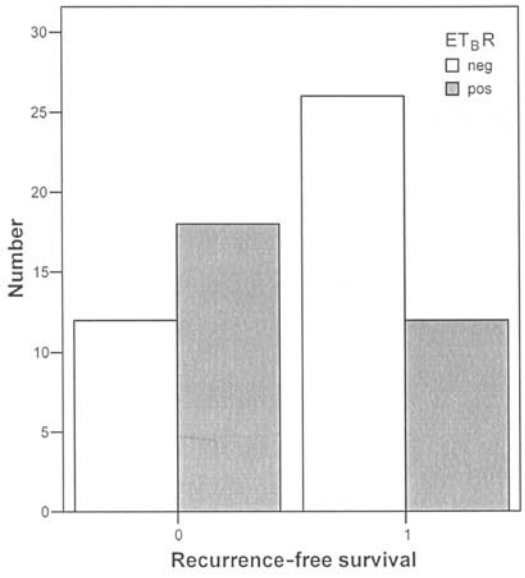

Figure 3. Significantly reduced ET_BR expression in relapse-free survival of vulvar cancer patients (p=0.019).

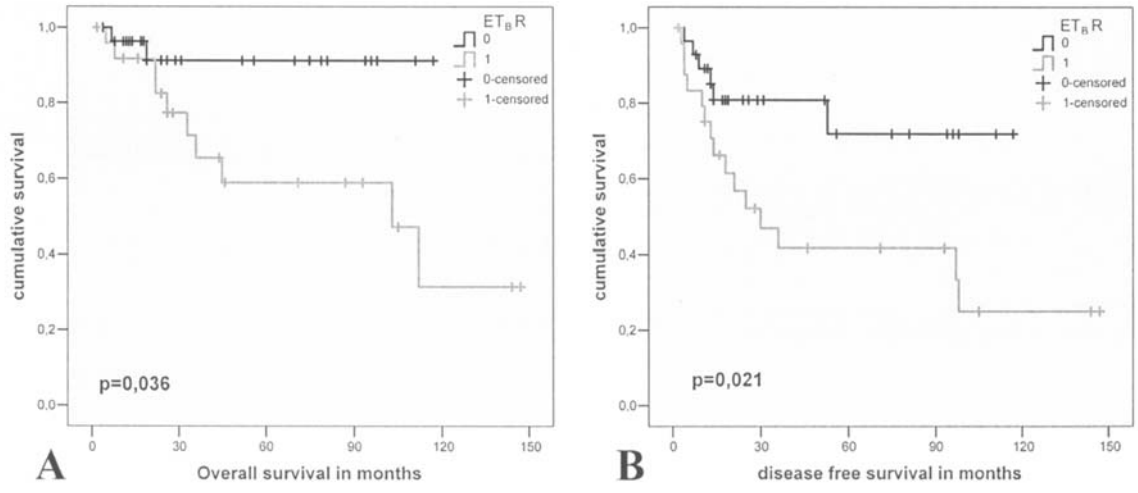

Figure 4. (A) Overall survival time of 54 patients with vulvar carcinoma pT1-2 in relation to ET_BR expression (p=0.036). (B) Disease-free survival time (DFST) of 54 patients with vulvar carcinoma pT1-2 in relation to ET_BR expression (p=0.021).

for molecular-targeted therapy. First clinical trials with a selective ET_AR antagonist showed its ability to delay disease progression in patients with hormone-refractory prostate cancer (27).

Active angiogenesis as assessed by increased microvessel density (MVD) and strong VEGF (vascular endothelial growth factor) expression has been reported to correlate with an unfavourable prognosis in vulvar cancer.

To the best of our knowledge we are the first to report on expression of ET-1, ET_AR and ET_BR in vulvar cancer. Our results indicate that the ET-axis is overexpressed in the majority of vulvar cancers. Our data are in line with recent studies on expression of the ET-axis in other tumour entities. Nelson *et al* were the first to show that the ET-axis in prostate cancer is overexpressed compared with the normal prostate gland (28). The same authors postulated a role for ET-1 as a mediator of osteoblastic response in metastatic prostate cancer. Subsequent experiments with a selective ET_AR inhibitor showed reduced bone formation in a mouse model (29). In ovarian cancer, ET-1 was demonstrated to promote tumour cell proliferation in several ovarian cancer cell lines (30), and in human ovarian tissue, expression of the ET-axis, in particular ET-1 and ET_AR expression, was significantly up-regulated in neoplastic tissue compared with normal ovarian tissue (11). Moreover, in breast cancer increased expression of ET_AR has been demonstrated to correlate with a worse prognosis and resistance to chemotherapy (31).

In our study, both ET receptors, particularly ET_BR, were overexpressed at tissue level. Our finding of frequent ET_BR overexpression in vulvar cancer is consistent with our previous data of breast cancer patients. In accordance with the breast cancer data published by Alanen *et al*, we also found ET_BR

to be expressed more frequently than ET_AR (53.4 vs. 46.5%, respectively) (14,32). In melanoma (18) and lung cancer (33) ET_BR also seems to be the predominantly expressed receptor, whereas in other tumour entities, e.g. prostate (19), ovarian (11) and cervical cancer (34), ET_AR has been reported to be overexpressed more frequently.

Prognostic relevance of ET_BR has been described in uveal melanoma where reduced ET_BR expression correlated with death from metastatic disease (17)

Our results indicate a potential role of ET_BR in cancer progression with overexpression in patients with disease progression and decreased expression with longer relapse-free survival. The significantly worse outcome in stage pT1 and pT2 patients with ET_BR overexpressing tumours suggests that analysing ET_BR expression in vulvar cancer at the time of diagnosis might help to identify patients with an unfavourable prognosis.

Our data also point to a potential therapeutic approach using specific ET_BR antagonists. To date, the selective ET_BR antagonist (BQ 788) has only been tested pre-clinically *in vitro* and in animal models. In human melanoma cells BQ 788 induced growth inhibition and cell death (35), but no effects were observed in cervical cancer cell lines (34). Also, a combined ET_AR/ET_BR antagonist (bosentan) has been demonstrated to induce apoptosis in human glioblastoma cell lines (36). Therefore an ET_BR or ET_AR/ET_BR antagonist may be helpful in vulvar carcinoma of advanced stage.

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