Expression of EphA2 and EphrinA-1 in vulvar carcinomas and its relation to prognosis

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Aims: To examine the expression of EphA2 and EphrinA-1 in vulvar squamous cell carcinomas and investigate their prognostic relevance.

Methods: Tumours from 224 patients with vulvar squamous cell carcinomas were investigated for expression of EphA2 and EphrinA-1 using single and double immunostaining methods.

Results: High expression (strong/moderate staining intensity) of EphA2 and EphrinA-1 was observed in 114 (51%) and 126 (56%) vulvar carcinomas, respectively. In the three cases tested using the double immunostaining method, colocalisation of EphA2 and EphrinA-1 proteins was identified in the same neoplastic cells. High EphA2 expression was significantly correlated to high expression of EphrinA-1 ($p < 0.01$) and cyclin A ($p < 0.01$), large tumour size ($p = 0.03$), deep invasion ($p < 0.01$) and higher FIGO stage ($p = 0.05$). A correlation between high EphrinA-1 expression and high levels of cyclin A ($p < 0.01$) and p21 ($p < 0.01$), deep invasion ($p < 0.01$) and higher FIGO stage ($p = 0.01$) was also seen. In univariate analysis, high expression of EphrinA-1 was associated with poor survival ($p = 0.03$). However, in the multivariate analysis neither EphrinA-1 nor EphA2 were significantly correlated to survival.

Conclusions: EphA2 and EphrinA-1 were overexpressed in 51% and 56% of the vulvar squamous cell carcinomas, respectively, and high levels of EphA2 and EphrinA-1 proteins were associated with deep tumour invasion and high FIGO stage. However, EphA2 and EphrinA-1 were not independently associated with clinical outcome in vulvar carcinomas.

Vulvar squamous cell carcinomas account for approximately 3–5% of all gynaecological cancers.¹ There are three histological subtypes: basaloid and verrucoid which are related to human papillomavirus infection, and keratinising/non-keratinising type which is not associated with human papillomavirus infection. The most important prognostic factors are tumour size and surgical node status.^{2–3} Radical surgery, which has been the standard treatment approach, is often accompanied by considerable morbidity.⁴ In an attempt to decrease the incidence of complications, a change to more individualised treatment procedures has been reported.⁵ To reduce the over-treatment as well as under-treatment of patients, new tumour markers that could predict clinical outcome would be of considerable value to design individualised treatment procedures.

Receptor tyrosine kinases have important roles in the generation and progression of human tumours.⁶ The Eph receptors are the largest subfamily of receptor tyrosine kinases and are active in regulating cell growth, survival, migration and angiogenesis.^{7–9} They are divided into two subfamilies, EphA and EphB. There are nine EphA receptors (A1–A9) and six EphB receptors (B1–B6), which preferentially bind to EphrinA and EphrinB ligands, respectively.⁹ EphA2 was initially isolated from HeLa cell cDNA library,¹⁰ whereas its main ligand EphrinA-1 was originally isolated from human umbilical vein endothelial cells as a factor induced by tumour necrosis factor α .^{11–12} EphA2 is frequently overexpressed in a wide variety of cancers, including breast,¹³ prostate,^{14–15} oesophagus,¹⁶ renal,¹⁷ lung¹⁸ and ovarian,¹⁹ whereas increased levels of both EphA2 and EphrinA-1 have been reported in cervical carcinomas,²⁰ bladder carcinomas,²¹ oesophageal carcinomas,²² gastric carcinomas²³ and ovarian carcinomas.²⁴ Furthermore, in a variety of human malignancies an association has been found between overexpression of EphA2 and EphrinA-1 and unfavourable

prognoses.^{17–20 22–24} To our knowledge, there has been no previous study of EphA2 and EphrinA-1 in vulvar carcinomas.

The aims of our study were to examine the expression of EphA2 and EphrinA-1 in a series of 224 vulvar squamous cell carcinomas and to identify predictive markers of the clinical outcome for these patients.

MATERIALS AND METHODS

Patient materials

A retrospective study of 224 patients with squamous cell carcinoma of the vulva, undergoing surgery at the Norwegian Radium Hospital in the period 1977–91, was performed. The median age at diagnosis was 70 years (range 27–96). All patients were followed until death or until five years after diagnosis. Sixty-four patients (29%) died of vulvar cancer. The Norwegian Board of Health and the Data Inspectorate approved the study. All tumours were staged according to the International Federation of Gynecology and Obstetrics (FIGO) classification.²⁵

Histological specimens were reviewed by one of the authors (JMN) who had no access to clinical information. The tumours were classified according to World Health Organization recommendations:²⁶ 205 (92%) were keratinising/non-keratinising, 14 (6%) were basaloid and 5 (2%) were verrucoid. The primary tumours of the patients have previously been investigated for the expression of p53, p16, p21, p27 and cyclins A, D1, D3 and E.^{27–29} Table 1 provides a detailed description of the tumour characteristics. As controls, samples of normal vulva were collected from 10 patients undergoing surgery for benign gynaecological diseases.

Single immunostaining method

Sections for immunohistochemistry were stained using the Dako EnVision + System, Peroxidase (DAB) (K4011, Dako

Table 1 EphrinA-1 and EphA2 immunostaining in relation to clinicopathological variables

Variables	Total n	EphA2		p Value*	EphrinA-1		p Value*
		Low	High (%)		Low	High (%)	
EphA2							
Low (negative/weak)	110				78	32 (29)	<0.01
High (moderate/strong)	114				20	94 (83)	
EphrinA-1				<0.01			
Low (negative/weak)	98	78	20 (20)				
High (moderate/strong)	126	32	94 (75)				0.09
Age (y)				0.41			
25–69	100	54	46 (46)		51	49 (49)	
70–84	103	47	56 (54)		41	62 (60)	
≥85	21	9	12 (57)		6	15 (71)	
Tumour diameter (cm)				0.03			<0.01
0.3–2.5	79	48	31 (39)		46	33 (42)	
2.6–4.0	72	31	41 (57)		32	40 (56)	
4.1–20.0	69	28	41 (59)		18	51 (74)	
Not available	4						
Depth of invasion (mm)				<0.01			<0.01
0.0–4.0	76	52	24 (32)		51	25 (33)	
4.1–8.0	73	30	43 (59)		24	49 (67)	
8.1–35.0	75	28	47 (63)		23	52 (69)	
Lymph node metastasis				0.69			0.45
None	114	58	56 (49)		53	61 (54)	
Unilateral	50	22	28 (56)		18	32 (64)	
Bilateral	24	11	13 (54)		10	14 (58)	
Not available	36						
FIGO				0.05			0.01
I	42	28	14 (33)		27	15 (36)	
II	67	31	36 (54)		26	41 (61)	
III + IV	88	39	49 (56)		33	55 (63)	
Not available	27						
Differentiation				0.32			0.27
Well	70	39	31 (44)		36	34 (49)	
Moderate	104	50	54 (52)		43	61 (59)	
Poor	50	0	0		19	31 (62)	
Infiltration of vessel				0.32			0.85
No	174	82	92 (53)		75	99 (57)	
Yes	47	26	21 (45)		21	26 (55)	
Not available	3						
p16†				0.10			0.56
Low (<5%)	154	81	73 (47)		69	85 (55)	
High (≥5%)	69	28	41 (59)		28	41 (59)	
Not available	1						
p21†				0.13			<0.01
Low (negative)	129	69	60 (47)		69	60 (47)	
High (positive)	95	41	54 (57)		29	66 (70)	
p27†				0.28			0.61
Low (<50%)	170	80	90 (53)		76	94 (55)	
High (≥50%)	54	30	24 (44)		22	32 (59)	
p53†				0.72			0.08
Low (<5%)	100	48	52 (52)		50	50 (50)	
High (≥5%)	123	62	61 (50)		47	76 (62)	
Not available	1						
Cyclin A†				<0.01			<0.01
Low (<5%)	68	45	23 (34)		42	26 (38)	
High (≥5%)	156	65	91 (58)		56	100 (64)	
Cyclin D1†				0.09			0.18
Low (negative)	166	87	79 (48)		77	89 (54)	
High (positive)	58	23	35 (60)		21	37 (64)	
Cyclin D3†				0.36			0.27
Low (<50%)	163	77	86 (53)		75	88 (54)	
High (≥50%)	61	33	28 (46)		23	38 (62)	
Cyclin E†				0.46			0.17
Low (<50%)	183	92	91 (50)		84	99 (54)	
High (≥50%)	41	18	23 (56)		14	27 (66)	

*Pearson's χ^2 .†In previous reports, p53, p16, p21, p27, cyclin A, cyclin D1, cyclin D3, and cyclin E have been studied.^{27–29}

Corporation, California, USA) and Dakoautostainer. Deparaffinised sections were microwaved in 10 mM citrate buffer pH 6.0 to unmask the epitopes, and treated with 0.03% hydrogen peroxide for 5 min to block endogenous peroxidase. The sections were incubated with polyclonal rabbit antibodies EphA2 (sc-924, 1:400, 0.5 µg IgG/ml) and EphrinA-1 (sc-911, 1:300, 0.7 µg IgG/ml) (both from Santa Cruz Biotechnology

Inc., California, USA) for 30 min at room temperature followed by incubation with peroxidase labelled polymer conjugated to goat anti-rabbit for 30 min. Tissue was stained for 10 min with 3'-diaminobenzidine tetrahydrochloride (DAB) and then counterstained with haematoxylin, dehydrated, and mounted in Diatex. All series included positive controls, which consisted of human cervical carcinomas that had been shown to express

EphA2 and EphrinA-1. Negative controls included substitution of the polyclonal antibody with normal rabbit IgG of the same concentration as the polyclonal antibody. All controls gave satisfactory results. The specificity of anti-EphA2 and anti-EphrinA-1 has been tested previously.²⁴ Cytoplasmic staining was considered positive. Four semi-quantitative classes were used to describe the intensity of staining: no staining, weak staining, moderate staining, and strong staining (fig 1). Since tumour cells stained uniformly across the samples we did not consider the fraction of tumour cells with positive staining. Protein expression was defined as high when moderate and strong staining was seen. This was based on previously published data²⁰ and staining pattern observed in normal vulvar epithelium. Sections were scored with no knowledge of clinical data.

Double immunostaining method

Double immunoenzymatic technique was performed on three cases using peroxidase as label for the first antibody and alkaline phosphatase for the second antibody. Deparaffinised sections were microwaved in 10 mM citrate buffer pH 6.0, followed by sequential incubation with different dilutions (1:200 and 1:300) of EphrinA-1 antibody (30 min), peroxidase labelled polymer conjugated to goat anti-rabbit (30 min) and DAB (10 min). The sections were microwaved in 10 mM citrate buffer pH 6.0 for 2×5 min to exclude the possibility of a cross-reaction, and then incubated sequentially with EphA2 antibody (1:400), mouse anti-rabbit IgG (Dako), rabbit anti-mouse IgG (Dako) and alkaline phosphatase mouse anti-alkaline phosphatase (Dako) for 30 min each. The alkaline phosphatase was detected using a mixture of nitroblue tetrazolium and 5-bromo-4-chloro-3-indolyl-phosphate (Promega Corp., Madison, Wisconsin, USA) for 45 min in the dark. Tissue sections were mounted in glycerine jelly.

To exclude the possibility of a cross-reaction or a non-specific colour mixing between the first and second staining sequences, the following controls were included: (a) omission of the first primary antibody; and (b) omission of the second primary antibody. These controls gave satisfactory results. Since the masking effect of the reaction product of DAB for the second primary antibody could be a problem if the same cells contained both antigens, the primary antibody applied in the first sequence was used at different dilutions. We have observed that the masking effect of DAB depends on the dilution of the first primary antibody.

Statistical analysis

Pearson's χ^2 test was used to compare the distribution of various patient characteristics by expression of EphA2 and EphrinA-1. Survival rates were calculated using the method described by Kaplan and Meier. Disease specific survival rates were based on death from vulvar cancer only. Univariate and multivariate relative risks (RRs) of dying were calculated using Cox proportional hazards regression. Patients were censored after 5 years. In the multivariate analysis, forward stepwise regression with $p=0.05$ as inclusion criteria was used. All

calculations were performed using SPSS V.12.0. A significance level of 0.05 was chosen.

RESULTS

In normal vulvar epithelium weak cytoplasmic staining for EphA2 and EphrinA-1 was present in 3/10 (30%) and 5/10 (50%) of the cases, respectively. EphA2 and EphrinA-1 stainings were seen in basal, parabasal, middle and top layers. For EphA2 and EphrinA-1 high cytoplasmic expression (strong/moderate staining intensity) was observed in 114 (51%) and 126 (56%) of the vulvar carcinomas, respectively (table 2, fig 2A,B). Using the double staining method, EphA2 and EphrinA-1 proteins were identified in the same cells (fig 2C–E). Strong EphA2 and moderate EphrinA-1 staining were observed in endothelial cells of tumour blood vessels, whereas no staining was seen in blood vessels in normal vulvar skin.

Table 1 shows the protein levels of EphA2 and EphrinA-1 in relation to clinicopathological parameters. High EphA2 expression was significantly correlated to high expression of EphrinA-1 ($p<0.01$) and cyclin A ($p<0.01$), large tumour size ($p=0.03$), deep invasion ($p<0.01$) and higher FIGO stage ($p=0.05$). There was a clear correlation between high EphrinA-1 expression and high levels of cyclin A ($p<0.01$) and p21 ($p<0.01$), deep invasion ($p<0.01$) and higher FIGO stage ($p=0.01$).

Table 3 gives the results of the univariate analysis. High expression of EphrinA-1 was associated with poor survival ($p=0.03$), whereas EphA2 gave no prognostic information. In the multivariate analyses of EphA2 and EphrinA-1, tumour diameter, depth of invasion, lymph node metastasis, p16, p53, cyclin E, age and differentiation were included as important parameters. Tumour diameter ($p<0.01$) and lymph node metastasis ($p<0.01$) retained independent prognostic significance (table 3). FIGO stage was not an independent prognostic factor when included in the multivariate analysis (data not shown).

DISCUSSION

For the first time, EphA2 and EphrinA-1 have been studied in vulvar squamous cell carcinomas. We found high levels of EphA2 and EphrinA-1 in 51% and 56% of our cases, respectively. Previously, a wide range of EphA2 overexpression (34–87%)^{15 16 18–20 22–24} and EphrinA-1 overexpression (41–61%)^{20 22 24} has been reported in many other cancer types. The differences may be due to the various tumour types studied. Others as well as our results suggest that increased expression of EphA2 and EphrinA-1 may contribute to development of human tumours, including vulvar carcinomas.

In the present work, high expression of EphA2 was significantly related to large tumour size, deep invasion and higher FIGO stage. The increase in EphA2 immunoreactivity with increasing stages is in line with the findings in some other malignancies, including ovarian,²⁴ bladder,²¹ gastric²³ and renal.¹⁷ High EphA2 expression was not associated with reduced survival in this study, which is in agreement with a small study on ovarian cancers.³⁰ High EphA2 has been correlated with poor survival in univariate analysis as well as in multivariate analysis in patients with carcinomas of the cervix,²⁰ ovaries^{19 24} and oesophagus.²² In a study of 80 oesophageal squamous cell carcinomas, Miyazaki *et al*¹⁶ found that patients with EphA2 overexpression had a poor prognosis for overall survival in univariate but not in multivariate analysis. For patients with renal cell carcinomas¹⁷ and lung carcinomas,¹⁸ overexpression of EphA2 was associated with shorter survival, but in these studies multivariate analysis of survival was not performed. Taken together, these studies and our findings suggest that the lack of prognostic significance of

Table 2 Immunostaining results for EphA2 and EphrinA-1

Expression	Number of cases	
	EphA2 (%)	EphrinA-1 (%)
Negative	43 (19)	26 (12)
Weak	67 (30)	72 (32)
Moderate	95 (42)	100 (45)
Strong	19 (9)	26 (12)

Figure 1 Immunohistochemical analysis for EphrinA-1 showing cases with no staining (A), weak staining (B), moderate staining (C) and strong staining (D).

Figure 2 Immunohistochemical analysis showing high EphA2 (A) and EphrinA-1 (B) protein expression. Double staining for EphrinA-1 (brown) and EphA2 (blue) is seen in the same tumour cells (C). Controls: omission of EphrinA-1 antibody (D) and omission of EphA2 antibody (E).

Table 3 Relative risk (RR) of dying from vulvar cancer (n = 224)

Variables	Deaths*/n	Univariate analysis		Multivariate analysis			
		RR (95% CI)†	p Value‡	EphA2		EphrinA-1	
				RR (95% CI)†	p Value‡	RR (95% CI)†	p Value‡
EphA2			0.12		0.60		
Low (negative/weak)	27/110	1.0 (-)		1.0 (-)			
High (moderate/strong)	34/114	1.5 (0.9 to 2.4)		1.2 (0.7 to 2.1)			
EphrinA-1			0.03				0.51
Low (negative/weak)	21/98	1.0 (-)				1.0 (-)	
High (moderate/strong)	44/126	1.8 (1.1 to 3.1)				1.2 (0.7 to 2.3)	
Tumour diameter (cm)			<0.01		<0.01		<0.01
0.3–2.5	10/79	1.0 (-)		1.0 (-)		1.0 (-)	
2.6–4.0	23/72	3.3 (1.6 to 6.9)		2.7 (1.1 to 6.2)		2.7 (1.2 to 6.2)	
4.1–20.0	31/69	5.8 (2.8 to 12)		4.7 (2.0 to 11)		4.5 (1.9 to 11)	
Depth of invasion (mm)			<0.01				
0.0–4.0	12/76	1.0 (-)					
4.1–8.0	26/73	2.7 (1.3 to 5.3)					
8.1–35.0	27/75	3.2 (1.6 to 6.3)					
Lymph node metastasis			<0.01		<0.01		<0.01
None	21/114	1.0 (-)		1.0 (-)		1.0 (-)	
Unilateral	20/50	3.0 (1.6 to 5.5)		2.1 (1.1 to 3.9)		2.0 (1.0 to 3.8)	
Bilateral	13/24	5.9 (3.0 to 12)		4.8 (2.3 to 9.9)		4.1 (2.0 to 8.4)	

*Number of deaths five years after diagnosis.

†95% CI of the relative risk.

‡Test for inclusion of variable.

EphA2 in vulvar carcinomas could mean that the role of EphA2 in cancer is tissue specific.

Our finding that a high level of EphrinA-1 protein expression is clearly correlated to deep invasion and higher FIGO stage is consistent with the results in bladder carcinomas.²¹ However, in ovarian carcinomas,²⁴ gastric carcinomas²³ and cervical carcinomas²⁰ EphrinA-1 expression did not correlate with disease stages. In the present study a high level of EphrinA-1 expression was associated with poorer survival in vulvar squamous cell carcinomas in univariate analysis. However, it was not significantly associated when multivariate analysis was applied. This is in agreement with other studies where a high EphrinA-1 level was correlated with poor prognosis in univariate but not in multivariate analysis in patients with ovarian carcinomas³⁰ and oesophageal carcinomas.²² In contrast, Han *et al*²⁴ did not find any association of EphrinA-1 overexpression and survival in either univariate or multivariate analysis in patients with ovarian carcinomas. Only one previous report has found that high expression of EphrinA-1 was associated with reduced survival in multivariate analysis in patients with cervical carcinomas.²⁰ Considering these different studies together, it seems controversial that overexpression of EphrinA-1 per se is of important prognostic value in tumours.

In normal cells, the EphrinA-1 ligand binds EphA2 receptor and triggers tyrosine phosphorylation of EphA2 receptors,³¹ which is followed by EphA2 degradation.³² Consistent with this, we localised low levels of EphA2 and EphrinA-1 in normal vulvar epithelium. However, high levels of EphA2 receptor and EphrinA-1 ligand were identified in the same vulvar carcinoma cells using the double immunostaining method. It is not known why high levels of both EphA2 and EphrinA-1 are seen in the same tumour cells and why EphrinA-1 does not bind to EphA2, leading to degradation of EphA2. One explanation may be that in malignant cells defective cell–cell contacts decrease the ability of EphA2 to interact with EphrinA-1, causing accumulation of non-phosphorylated EphA2.³³ High levels of non-phosphorylated EphA2 appear to promote tumour cell growth and metastasis, causing EphA2 to function as an oncoprotein in carcinogenesis.^{13–14} In melanomas EphA2 and EphrinA-1 are both overexpressed and EphrinA-1 has been shown to stimulate the growth of EphA2-expressing melanoma cell lines,

Take-home message

EphA2 and EphrinA-1 proteins have no prognostic value in vulvar carcinomas.

indicating that EphrinA-1 is an autocrine growth factor for melanomas.³⁵ Taken together, these data may imply that EphA2 and EphrinA-1 have a role in cell transformation, but the possible mechanism behind this needs further investigation.

Expression of EphA2 and EphrinA-1 has not been reported in adult blood vessels.³⁶ Our observations, identifying no expression of EphA2 and EphrinA-1 in blood vessels of normal vulvar skin, support these findings. In our series of vulvar carcinomas, however, we have found EphA2 and EphrinA-1 to be up-regulated not only in tumour cells, but also in vasculature cells. This is in agreement with previous reports detecting EphA2 and EphrinA-1 in tumour blood vessels in several human tumours.³⁶ Our results as well as those of others, indicate that EphA2 and EphrinA-1 may play a role in tumour neovascularisation, a process essential for tumour growth and malignant progression. However, more data are needed to confirm that statement.

Previously, EphA2 and EphrinA-1 have been found to be regulated by p53, p73 and p63.³⁷ We did not identify any correlation between EphA2 or EphrinA-1 and p53. However, high expression of EphA2 was correlated to high expression of cyclin A, whereas a high EphrinA-1 level was associated with cyclin A and p21. This association has not been previously reported. Therefore, further studies are needed to clarify the role of cyclin A and p21 in regulating EphA2 and EphrinA-1.

In conclusion, EphA2 and EphrinA-1 are overexpressed in 51% and 56% of vulvar squamous cell carcinomas, respectively, and high levels of EphA2 and EphrinA-1 proteins are associated with deep tumour invasion and high FIGO stage. However, EphA2 and EphrinA-1 are not independently associated with clinical outcome in vulvar carcinomas.

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