

# Activation of the receptor protein tyrosine kinase EphB4 in endometrial hyperplasia and endometrial carcinoma

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Received 29 May 2002; revised 1 October 2002; accepted 22 October 2002

**Background:** Members of the Eph family of tyrosine kinases have been implicated in embryonic pattern formation and vascular development; however, little is known about their role in the adult organism. We have observed estrogen-dependent EphB4 expression in the normal breast suggesting its implication in the hormone-controlled homeostasis of this organ. Since the endometrium is a similarly hormone dependent organ and endometrial carcinoma is thought to result from estrogenic stimulation, we have investigated EphB4 expression in normal human endometrium and during its carcinogenesis.

**Patients and methods:** EphB4 expression was analyzed immunohistochemically in 26 normal endometrium specimens, 15 hyperplasias and 102 endometrioid adenocarcinomas and correlated with clinical and prognostic tumor characteristics.

**Results:** In normal endometrial tissue no EphB4 protein was detected. Strikingly, we observed a drastic increase ( $P < 0.0001$ ) in the number of EphB4 protein-expressing glandular epithelial cells in the majority of hyperplasias and carcinomas. Moreover, we found a statistically highly significant positive correlation between EphB4 expression and post-menopausal stage of the patient ( $P = 0.007$ ).

**Conclusions:** These findings indicate that in the endometrium, EphB4 is an early indicator of malignant development and, thus, EphB4 may represent a potent tool for diagnosis and therapeutic intervention.

**Key words:** endometrial cancer, immunohistochemistry, tyrosine kinase

## Introduction

Carcinoma of the uterine corpus is one of the most common gynecological malignancies in the Western world [1]. Excessive estrogen exposure has been linked to the development of endometrial hyperplasias, which has a considerable risk of malignant progression when associated with atypical cytological features [2]. Patients treated with tamoxifen for breast cancer, exhibit a two-fold higher risk of endometrial cancer because of its estrogen-like effects [3]. The uterus is unique compared with most other organs, since processes such as growth, tissue organization and cell death observed in other organs during embryonic development occur in the endometrium throughout adult life. A better understanding of the mechanisms controlling its growth is clearly needed to allow the development of new prognostic and therapeutic tools and to improve clinical management of endometrium-related diseases.

Receptor tyrosine kinases (RPTKs) comprise 14 distinct members, the largest of which is the Eph family group [4]. The

Eph receptors are grouped into two major subclasses, EphA and EphB, based on sequence homologies and ligand binding specificities [5]. Their ligands, the ephrins, are membrane anchored by either a GPI (glycosylphosphatidylinositol) linkage (ephrin-A) or a transmembrane region (ephrin-B). Ephrin-A ligands interact preferentially with receptors of the EphA subclass and ephrin-B ligands with receptors of the EphB subclass [6]. Given the membrane bound localization of both the ligands and the receptors, signaling must function in the cell-to-cell range rather than in long-range communication [7]. Furthermore, interaction between EphB receptors and their ligands can provoke bi-directional signaling and mutual cell–cell communication, since ephrin-B ligands have been shown to undergo phosphorylation on conserved intracellular tyrosines upon receptor interaction [8, 9].

Eph receptors and ephrin ligands play a pivotal role in pattern formation, cell aggregation and migration, as well as segmentation during embryonic development [10]. In particular, Eph family members, including EphB4, are instrumental in the development of the embryonic capillary network [10]. Little is known about Eph functions in adult organisms; however, it is conceivable that this receptor–ligand family is involved in morphogenic processes persisting in adult organs, such as those occurring in the endometrium.

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We have previously described hormone-dependent and cell type-specific expression of the EphB4 receptor during normal mouse mammary gland development [11]. In the human breast, EphB4 was expressed in the luminal epithelial cells of normal breast cells [12] and a drastic reduction of EphB4 expression was observed in almost all invasive carcinomas analyzed. Furthermore, we found a highly significant correlation between EphB4 positivity and low histological grade of the tumors [13]. Like in the breast, hormonal changes throughout the menstrual cycle induce the endometrium epithelium into proliferation, differentiation and cell death by apoptosis [14]. Any escape from this tightly regulated equilibrium of growth and regression may eventually lead to malignant transformation.

In search of putative regulatory mechanisms affecting the pathogenesis of endometrial cancer, we investigated by immunohistochemistry the expression of EphB4 in normal endometrium, in endometrial hyperplasia and in a series of endometrioid adenocarcinomas. Furthermore, the level of EphB4 expression in cancer as defined by semiquantitative analyses was correlated to patient survival.

## Patients and methods

### Patient material

The study included 117 patients treated for endometrial carcinoma from January 1988 to December 1996 at the Department of Obstetrics and Gynecology of the University of Bern [15]. Patients with prior malignant disease and metastasis at presentation were ineligible. Papillary serous or clear-cell carcinomas were excluded from the studies because they represent distinct entities at the morphological, molecular and clinical level. Patients were surgically staged according to the 1988 International Federation of Gynecology and Obstetrics (FIGO) classification [16]. Surgical procedures required peritoneal cytology, abdominal exploration, extrafascial hysterectomy, bilateral salpingo-oophorectomy and selective pelvic and aortic node dissection. The decision to perform lymphadenectomy and the extent of node dissection were influenced only by patient age and their general state of health. Fifteen patients were excluded because of insufficient material. One hundred and two patients (mean age at diagnosis  $66.1 \pm 12.4$  years, range 37–91) with endometrial carcinoma were included in this study. Fifty-seven patients (55.9%) had a lymphadenectomy and 90 patients (88.2%) were post-menopausal. The distribution of surgical FIGO stages and grade of tumor are summarized in Table 1. High-risk patients were defined by deep myometrial invasion of >50%, invasion of the cervix or an undifferentiated tumor (G3). Patients with negative pelvic and periaortic lymph nodes did not receive external beam radiation despite the presence of risk factors. High-risk patients who did not undergo lymphadenectomy received external beam radiation with 4600–5000 cGy in a four-field box technique to the whole pelvis. Brachytherapy of the vaginal vault was given except for patients with G1 tumors invading less than half of the myometrium (IA G1 and IB G1). Routine follow-up consisted of clinical review at 3-month intervals during the first 2 years and 6-monthly visits thereafter. The median follow-up of the patients was 62.7 months (range 1–140 months). Additionally, we analyzed tissue samples showing endometrial hyperplasia taken from resection specimens of 15 patients (mean age  $61.7 \pm 12.1$  years, range 45–83) who underwent surgery for causes other than cancer. Hyperplasias were classified as follows: six simple, six complex and three complex atypical. Eleven of the 15 patients (73.3%) were post-menopausal. Finally, we included samples from 26 patients (mean age  $53.3 \pm 19.0$  years, range 26–97) with histo-

**Table 1.** Clinical features of carcinomas ( $n = 102$ )

Stage	
IA	11
IB	53
IC	24
IIA	2
IIB	4
IIIA	2
IIIB	–
IIIC	6
Grade	
G1	18
G2	75
G3	9

logically normal endometrium, either in the proliferative phase or atrophic. Eleven (42.3%) of 26 patients were post-menopausal.

### Immunohistochemical studies

Tissue specimens were fixed in phosphate-buffered 4% formaldehyde, paraffin embedded and processed for routine diagnostic. Sections (4  $\mu$ m) were de-waxed, rehydrated and washed three times for 5 min at room temperature in TBS (25 mM Tris–HCl, pH 7.5, 140 mM NaCl). Antigen retrieval was performed by boiling in a microwave oven (Miostar, Migros, Switzerland) in 10 mM citrate buffer for 8 min at 850 W and twice at 410 W for 5 min. After washing in TBS (three times for 5 min), endogenous peroxidase was blocked by incubation in 0.3% H<sub>2</sub>O<sub>2</sub> in TBS for 10 min at room temperature. Incubation with the primary antibodies was done overnight at 4°C. Antibodies were diluted in either 5% TNA (50 mM Tris–HCl, 140 mM NaCl, 0.5% sodium caseinate, 15 mM NaN<sub>3</sub>) (anti-EphB4, 1:100) or TBS (anti-estrogen receptor, 1:25). All antibodies were obtained commercially: anti-EphB4 (clone C-16; Santa Cruz Biotechnology, Santa Cruz, CA, USA); anti-estrogen receptor (clone 6F11; Novocastra, Newcastle upon Tyne, UK). After three washes in TBS, sections were incubated with biotinylated swine anti-rabbit or rabbit anti-mouse antibodies (DAKO, Copenhagen, Denmark) for 1 h at room temperature. After three washes in TBS, sections were incubated with avidin and biotin–horseradish peroxidase complex for the detection of estrogen receptor or with avidin and biotin–alkaline phosphatase complex for the detection of the EphB4 protein. Peroxidase activity was localized by incubation in substrate solution [3,3'-diaminobenzidine tablets (Sigma, Buchs, Switzerland) dissolved in 100 mM imidazole, 100 mM NaCl, 20 mM citric acid (pH 7.0) containing 0.005% H<sub>2</sub>O<sub>2</sub>] for 8–10 min at room temperature. Phosphatase activity was localized by incubation in naphtol and fuchsin substrate solution for 20 min at room temperature. Sections were counterstained with haemalaun for 30 s and mounted in Aquatex (Merck, Darmstadt, Germany). The primary antibody was omitted in control sections.

### Evaluation procedures

The number of immunoreactive cells was semiquantitatively estimated by two independent investigators (E.K. and G.B.) who were blinded with regard to the results of tumor stage, clinical variables and follow-up data. First, areas with intense staining were selected at low magnification with a 4 $\times$  objective. Then semiquantitative analyses were performed with the 40 $\times$  objective in these preselected areas: '+' corresponds to <10% positive cells, '++' to

10–50% positive cells and ‘+++’ to >50% positive cells. For every sample, at least 100, and usually >1000 cells were analyzed.

### Statistical analysis

SPSS statistical software (SPSS, Chicago, IL, USA) was used for statistical analysis. The significance of differences between individual groups was analyzed using Fisher’s exact test or the Pearson chi-square test. The relationship between the dependent variable EphB4 and the other independent variables was examined using the multiple logistic regression model for polytomous data with ordinal scale [17]. The full model was reduced by a backward elimination procedure to get to the final model. Analyses of survival were performed using the Kaplan–Meier method [18]. Survival distributions were compared with the log-rank test. Values of  $P < 0.05$  were considered statistically significant.

## Results

### EphB4 expression in normal endometrium and in endometrial hyperplasia

EphB4 expression in normal endometrium was absent in all but one of the 26 samples examined (Figure 1A). In one case, EphB4 expressing cells were restricted to one single gland. Conversely, EphB4 expression was detected in the majority of specimens with endometrial hyperplasia analyzed ( $P < 0.0001$ ). EphB4 protein was confined to the glandular epithelial cells, with membrane-associated staining indicative for the trans-membranal localization of the EphB4 receptor (Figure 1B). Table 2 shows the expression of EphB4 according to the hyperplasia WHO classification. More cells expressing EphB4 could be observed in atypical hyperplasia than in simple hyperplasia. However, the low number of cases did not allow definitive conclusions to be drawn, as corroborated by the statistical analysis ( $P = 0.505$ ).

### EphB4 expression in endometrial carcinoma

In order to investigate the involvement of EphB4 in human endometrial carcinogenesis, we analyzed its expression in 102 endometrial carcinomas. Immunohistochemical staining revealed that the majority of endometrial carcinomas, in contrast to normal epithelium, exhibited positive EphB4 expression ( $P < 0.0001$ ). These neoplastic cells exhibited a membrane-associated localization of EphB4 protein, as observed in the hyperplastic epithelium (Figure 2A). EphB4 protein expression was detected in 67.7% of carcinomas, irrespective of the tumor stage (Table 3). In most cases, EphB4 was not equally expressed in all tumor cells but was stronger at the interface between neoplastic cells and tumor stroma (Figure 2B). We have used multiple logistic regression to

**Table 2.** Percentage of epithelial cells expressing EphB4 in endometrial hyperplasia

	EphB4 expression (%)				<i>n</i>
	0	1–10	10–50	>50	
Simple	3	2	1	–	6
Complex	2	3	1	–	6
Complex atypical	–	2	1	–	3

**Table 3.** Epithelial cell expression of EphB4 in normal endometrium, endometrial hyperplasia and endometrial cancer

	EphB4 expression (%)				<i>n</i>
	0	1–10	10–50	>50	
Normal	25	1	–	–	26
Hyperplasia	5	7	3	–	15
Cancer	33	27	40	2	102

**Table 4.** Relationship between membrane accumulation of EphB4, patient characteristics and prognostic factors in endometrial cancer ( $n = 102$ )

Variable	Chi-square	<i>P</i> value <sup>c</sup>
Grade of tumor	0.001	0.971
Estrogen receptor	0.003	0.959
Body mass index <sup>a</sup>	0.130	0.719
Depth of invasion <sup>b</sup>	1.121	0.290
Menopausal state	7.317	<b>0.007</b>

<sup>a</sup>Body mass index = weight in kg/(height in m)<sup>2</sup>.

<sup>b</sup>Endometrium /  $\leq$  ½ myometrium /  $>$  ½ myometrium.

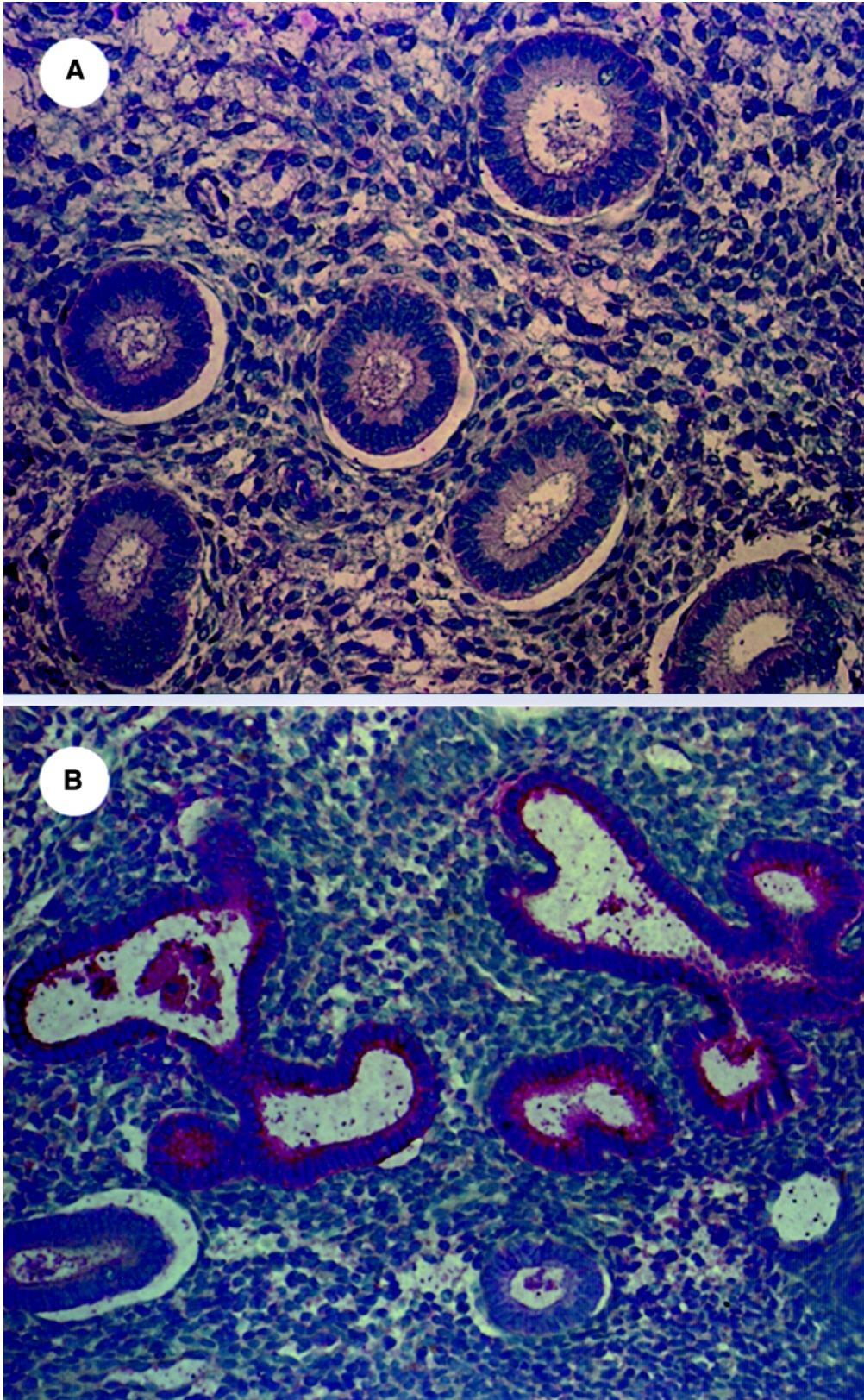
<sup>c</sup> $P < 0.05$  was required for significance and is presented in bold.

correlate EphB4 expression with a variety of clinical and prognostic tumor characteristics (Table 4). We found a significant relationship between positive EphB4 expression and post-menopausal stage ( $P = 0.007$ ). There was no significant relationship with patient characteristics such as body mass index ( $P = 0.719$ ) or with other prognostic factors, such as tumor stage ( $P = 0.150$ ), tumor grade ( $P = 0.971$ ) and estrogen receptor (ER) expression ( $P = 0.959$ ). Similarly, no relationship could be found with the clinical outcome, such as shorter recurrence-free survival ( $P = 0.402$ ) (Figure 3).

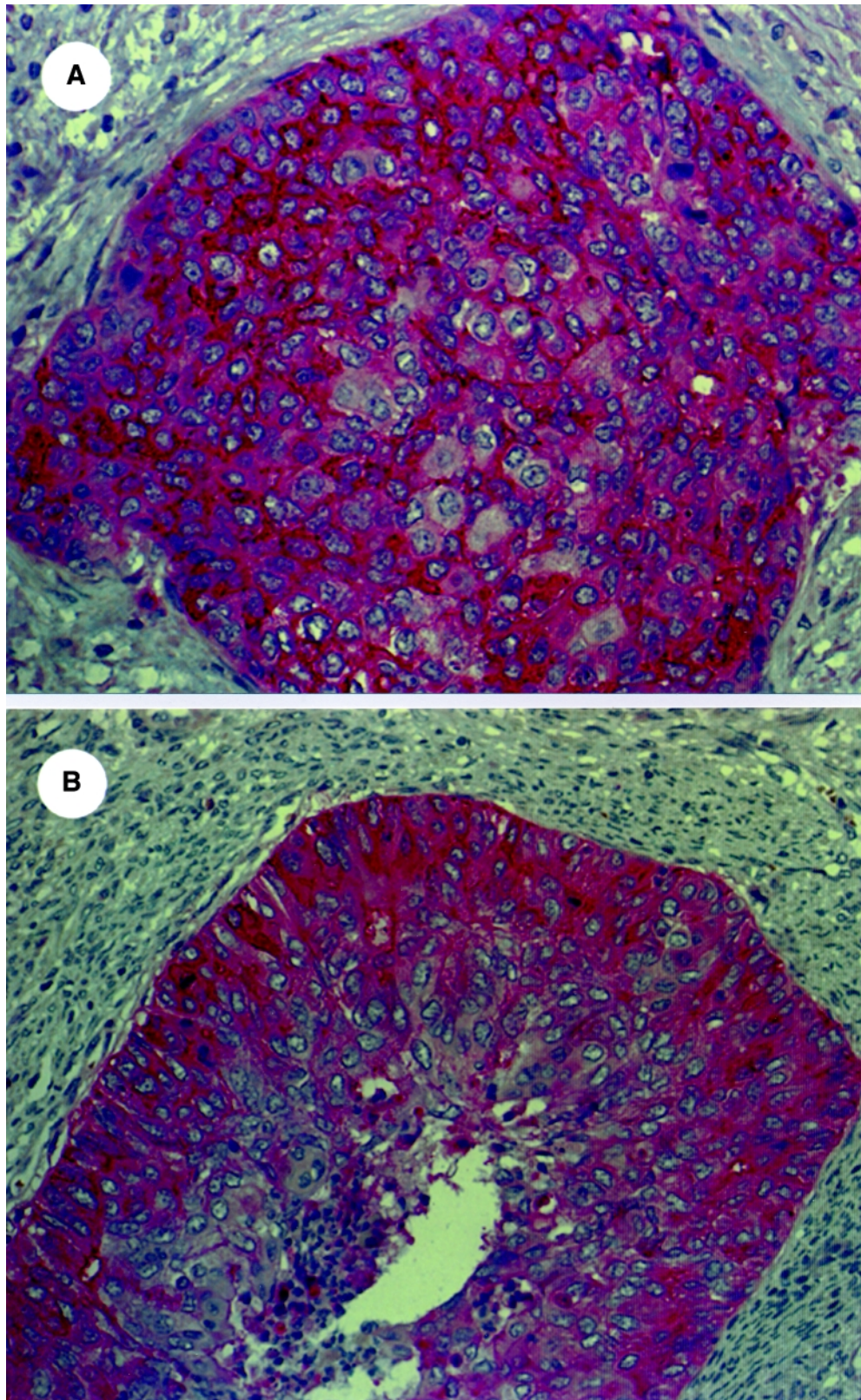
## Discussion

The present immunohistochemical studies show that EphB4 protein is absent in normal endometrium whereas it is found in epithelial cells of endometrial hyperplasia and endometrial carcinoma as indicated by membrane staining. Endometrial hyperplasia results from persistent prolonged estrogenic stimulation. Some of these lesions progress to atypical hyperplasia and eventually to endometrial adenocarcinoma [19]. In our study, we observed a higher expression of EphB4 in patients with complex atypical hyperplasia compared with simple hyperplasia, although this difference was not statistically significant, most probably due to the small number of patients. To our knowledge, this is the first study reporting an increased expression of EphB4 in endometrial hyperplasia. Despite the low number of patients investigated, our results suggest that expression of EphB4 may occur early in the progression from hyperplastic to neoplastic endometrium.

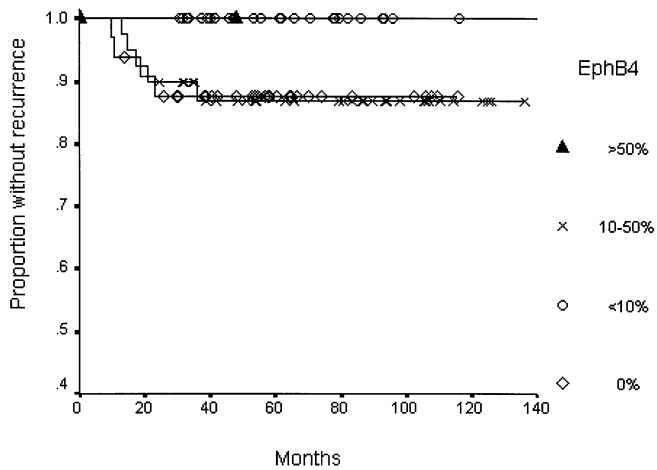
Overexpression of EphB4 has been described in colon cancer [20] and in a variety of human lung and breast cancer cell lines [12, 21], but only a few studies have addressed the expression of



**Figure 1.** (A) Normal endometrial tissue does not express EphB4. (B) Endometrial hyperplasia shows membrane expression of EphB4 protein. Absence of EphB4 expression in normal appearing endometrial glands (bottom).



**Figure 2.** (A) Endometrial carcinoma with strong epithelial expression of EphB4. (B) Endometrial carcinoma with stronger expression at the interface between cancer cells and stroma suggesting interaction between the tumor cells and the surrounding extracellular matrix.



**Figure 3.** Recurrence-free survival rates according to EphB4 expression (percentage of positive cells).

EphB4 during carcinogenesis. Nevertheless, in experimentally-induced invasive mouse mammary tumors, overexpression of EphB4 suggested its involvement in the carcinogenic process [22]. In endometrial carcinoma we demonstrated a significant increase of EphB4 expression compared with normal tissue. Takai et al. [23] have recently analyzed 20 endometrial carcinomas, and in analogy to our results, induction of EphB4 expression was found in all samples. In these studies, EphB4 expression correlated with poor prognostic factors, such as grade and myometrial invasion. Although our study included a higher number of patients, this association could not be confirmed.

It has been suggested that the roles of Eph receptors and their ephrin ligands include the promotion of angiogenesis and the modulation of cell–matrix attachment [24]. Interactions between Eph receptors and ephrin ligands may result in destabilization, which can also affect cell–matrix attachment, and thereby promote invasion and metastasis [25]. Indeed, in endometrial carcinoma, ephrin-B2, the EphB4 ligand, is almost always expressed in cancer cells neighboring EphB4-positive cells, which suggests autocrine and/or paracrine activation [23].

The implication of EphB4 in embryonic vascular network formation is demonstrated by the fact that in the developing vasculature EphB4 is exclusively expressed in embryonic veins, whereas expression of one of its ligands, ephrin-B2, is limited to arteries [26]. Moreover, in tumor angiogenesis expression of EphA2 and its ligand ephrin-A1 was found to be up-regulated not only in tumor blood vessels but also in tumor cells, suggesting interaction between the endothelium with the surrounding tumor cells [25]. In accordance with this hypothesis, we have observed that EphB4 expression in endometrial carcinoma was stronger at the interface between cancer cells and stroma where possibly angiogenesis is most critical for progression and survival of endometrial carcinoma. It remains to be further clarified whether EphB4 expression results in the promotion of angiogenesis during endometrial carcinogenesis.

In the mouse mammary gland, the expression of EphB4 and its ligand ephrin-B2 is induced by estrogens, whereas no receptor or ligand proteins are detected in ovariectomized mice [27]. In the

normal human breast epithelium, the highest portion of EphB4-positive epithelial cells is found during the estrogen dominated follicular phase of the cycle and EphB4 expression is drastically reduced in breast cancer [13]. The endometrium and the breast epithelium both undergo cyclic proliferation and differentiation, and exposure to estrogen has been identified as one of the major risk factors for the development of both cancers. Surprisingly, the opposite was true in the endometrium, where no EphB4 expression could be detected in normal tissue. Furthermore, in endometrial carcinoma the highest portion of cells expressing EphB4 is found in post-menopausal patients with low estrogen levels. These results suggest that estrogen down-regulates EphB4 protein in normal and malignant endometrium. Tamoxifen is a well-known estrogen agonist on the endometrium and an antagonist on breast tissue [3]. These opposite effects are mainly explained by its action on the two estrogen receptor subtypes, ER $\alpha$  and ER $\beta$ , which are differentially expressed in breast and endometrial epithelial cells [28]. It has recently been demonstrated that ER $\alpha$  is able to bind to insulin-like growth factor 1 receptor (IGF-1R) leading to its autophosphorylation and thus activation [29]. Conceivably, regulation of EphB4 expression is affected by a similar ER subtype-specific mechanism.

In summary, we have shown that EphB4 is expressed early during endometrial carcinogenesis and therefore could represent an indicator for malignant transformation. EphB4 expression, however, does not appear to have prognostic significance in endometrioid carcinomas. Additional studies are clearly needed to clarify this issue.

## Acknowledgements

This work was supported by the Bernese Cancer League and the Swiss National Science Foundation (grant No. 31-65322.01).

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