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Expression of Ephb2 And Ephb4 in Breast Carcinoma

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Eph receptor tyrosine kinases and their cell-surface-bound ligands, the ephrins, play key roles in diverse biological processes. Eph receptors comprise the largest family of receptor tyrosine kinases consisting of eight EphA receptors (with five corresponding ephrinA ligands) and six EphB receptors (with three corresponding transmembrane ephrinB ligands). Originally identified as neuronal pathfinding molecules, EphB receptors and ephrinB ligands are later proved to be crucial regulators of vasculogenesis and embryogenesis. More studies indicate that Eph receptors are involved in angiogenesis and tumorigenesis. This study aimed to investigate the expression of EphB2 and EphB4 in breast carcinomas. Semi-quantitative RT-PCR and immunohistochemistry were used to examine the expression patterns of EphB2 and EphB4. Clinicopathological and survival correlations were statistically analyzed in a series of 94 breast carcinomas, 9 normal specimens and 4

breast carcinoma cell lines. 1(1%), 16(17%), 29(31%), 48(51%) of the 94 tumors were negative, weak, moderate and strong EphB2 protein expression, respectively. 6(6%), 27(29%), 28(30%), 33(35%) of the tumors were negative, weak, moderate and strong EphB4 expression, respectively. Both EphB2 and EphB4 RT-PCR products could be detected in all specimens. Increased EphB2 protein expression was negatively associated with overall survival, and there was a trend that increased EphB2 protein expression was correlated with shorter disease free survival, while EphB4 protein expression was associated with histological grade and stage. EphB4 membrane staining was increased with S phase fraction and associated with DNA aneuploidy. These findings indicate that both EphB2 and EphB4 are involved in the development of breast cancer and that both molecules could be potential predictive markers. (Pathology Oncology Research Vol 10, No 1, 26–33)

Keywords: EphB2, EphB4, breast carcinoma, RT-PCR, immunohistochemistry

Introduction

Several important extracellular signal proteins act through receptor tyrosine kinases (RTKs), being involved in cell growth, differentiation, and migration.¹ The Eph receptor family is one of the RTK families. Based on ligand binding differences, the Eph receptors are divided into two subfamilies: EphA (A1-A8) and EphB (B1-B6) receptors. EphA is bound by ephrinA (A1-A5), which is anchored to membrane by GPI, glycosyl-phosphatidylinositol. EphB is bound by ephrinB (B1-B3), which is anchored to the membrane by the transmembrane domain.

Only EphA4 is an exception. It can bind both ephrinA and most of the ephrinB ligands.² There are two ways for Eph family members to mediate signal transduction: forward and reverse signalling. EphrinB ligands have tyrosine residues in the cytoplasmic domain. When interacting with receptors, their tyrosine sites are also phosphorylated, then a reverse signalling can be initiated. EphrinB ligands can also transduce signals by a phosphorylation-independent way. Ephrin A, lacking this structure, can transduce signals by other mechanisms, through integrin and the Src family member, Fyn.³⁻⁵

Eph receptors and their ligands have been found to play important roles in fundamental processes of the nervous system, such as axon guidance, axon fasciculation and neural crest cell migration.⁶⁻⁹ Recently, investigations have shown that Eph receptors and their ligands, like many other transmembrane receptor tyrosine kinases and their ligands, such as vascular endothelial growth fac-

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Table 1. Clinicopathological features for breast carcinoma patients (n=94)

<i>Histological grades</i>	<i>N(94)</i>
Grade 1	7
Grade 2	45
Grade 3	42
<i>Stage</i>	<i>N(91)*</i>
I	34
II	51
III	4
IV	2
<i>DNA ploidy</i>	<i>N(91)*</i>
Diploidy	33
Aneuploidy	58

*Information was missing in three cases.

tors(VEGFs), platelet-derived growth factor(PDGF-B) and angiopoietins, are involved in angiogenesis.^{10,11} Tumor vascular density is associated with malignant progression and a poor prognosis for breast and pancreatic cancer.¹²⁻¹⁴ EphA receptors are reported to be involved in development of breast, esophageal squamous cell, and prostate carcinomas.¹⁵⁻¹⁷ Furthermore, EphB2 and EphB4 have been indicated to be associated with tumorigenesis.¹⁸⁻²⁰

EphB2 is located at 1p35-1p36.1.¹⁸ High mRNA expression level is found in many carcinoma cell lines such as gastric, esophageal, colon, renal, teratocarcinoma and choriocarcinoma.²¹ EphB2 protein overexpression has been observed in carcinomas of gastric, colon, lung cancer, and neuroblastomas.²²⁻²⁶ EphB4, located at chromosome 7,²⁷ and initially isolated from a human hepatocellular carcinoma cell line, Hep3Ba, is reported to be highly expressed in many tumor tissues, such as in endometrial and colon cancers.^{28,29} However, was also found a drastic reduction of EphB4 protein expression in invasive breast carcinoma.³⁰

Table 2. Primers of EphB2, EphB4 and GAPD.

<i>Gene</i>	<i>Access no of gene bank</i>	<i>Primer pairs</i>	<i>Exon</i>	<i>Flanking sequence</i>	<i>Length (bp)</i>	<i>Reference</i>
EphB2	NM_004442	F:5'-AAA ATT GAG CAG GTG ATC GG-3' R:5'-TCA CAG GTG TGC TCT TGG TC-3'	10~11	1885-2107	223	22
EphB4	NM_004444	F:5'-GTC TGA CTT TGG CCT TTC CC-3' R:5'-TGA CAT CAC CTC CCA CAT CA-3'	13~14	2353-2530	178	22
GAPD	NM_002046	F1:5'-CCT CAA GAT CAT CAG CAA TGC-3' R1:5'-TGG TCA TGA GTC CTT CCA CG-3'			101	

We have examined the mRNA and protein expression status of EphB2 and EphB4 in breast carcinomas and breast carcinoma cell lines, and analyzed associations with patients' clinical data.

Materials and Methods

Breast carcinoma cell lines

Four commercially available breast carcinoma cell lines (MCF-7, MDA-MB-231, SK-BR-3 and T47-D) were applied in this study. Immunohistochemistry and RT-PCR were also used to characterize the status of mRNA and protein expression level of EphB2 and EphB4.

Clinical materials

Tumor tissues from 94 patients with breast carcinoma operated at Örebro Medical Center Hospital during the period of 1987-1992 were enrolled in this study. All specimens were divided into two parts. One was embedded in paraffin and used for immunohistochemistry. Another was frozen and used for RNA isolation and PCR. Detail clinical information and follow-up data were obtained from the medical records (*Table 1*). All histological slides were re-examined, classified and graded according to the criteria published by Elston and Ellis.³¹ In addition, 9 normal breast tissue samples obtained from the surgically removed breast cancer samples at the Norwegian Radium Hospital, Norway were included in this study.

Microselection

The microselection method³² was used in this study, aiming to avoid normal elements and areas with prominent infiltration of lymphoid cells.

Four μ frozen sections were made and stained with hematoxylin and eosin. An area of tumor cells on the 4 μ m frozen sections were selected under light microscopical examination. Corresponding areas on the frozen block were then marked and orientated. Thereafter, the blocks were trimmed to get the tumor cell areas. The trimmed

frozen blocks were re-embedded with OCT compound (Tissue-Tek, Torrance, California, USA) and new 4 μ m frozen sections were made from the re-embedded block to ensure that no normal epithelial cells were inside the selected areas. Then 20 μ m frozen sections were cut and collected into cooled Ependorf tubes. After section collection, an additional 4m frozen-section was made to control morphology.

RNA isolation

Total RNA was isolated from the microselected material and cultured breast carcinoma cell lines using RNeasy total RNA isolation kit (Qiagen, West Sussex, UK) following the suppliers protocol.

RT-PCR

Two primer pairs for EphB2 and EphB4 mRNA, previously reported by Tang²² and one primer pair for GAPD as internal control were applied in the present study (Table 2). The primers of EphB2 and EphB4 were designed for detection of the sequence of cytoplasmic tyrosine kinase in the catalytic domain. The Qiagen OneStep RT-PCR (Qiagen Inc.Valencia, USA) was used. The exponential phase of PCR amplification was obtained by optimizing the amount of RNA templates and cycle conditions.

The RT-PCR was performed in a 25 μ l volume, with 9.4 μ l RNase-free water, 5 μ l 5xQiagen OneStep RT-PCR Buffer, 1 μ l dNTP mix, 5 μ l 5xQ-Solution, 0.6 μ M forward and reverse primers of detected gene, 0.2 μ M GAPD primers, 1 μ l Qiagen OneStep RT-PCR enzyme mix and 1 μ l template RNA. The thermal cycle conditions were: 50°C for 30 minutes incubation for reverse transcription, following by RT-PCR enzyme activation 95°C for 15 minutes, 35 cycles at 95°C for 1minute, 57°C for 1 minute and 72°C for 1 minute, and finally 10 minutes extension at 72°C. Water instead of template RNA was used as negative control and positive cell line RNA as positive control. Both positive and negative controls were applied in each running.

Photographs of the PCR results were analyzed by reading the intensities of the amplified EphB2 and EphB4 bands. The housekeeping gene GAPD was used to control an equal loading of RNA for each sample. The ratio of the intensities between EphB2/EphB4 and GAPD PCR band was recorded and divided into 3 grades: low transcript, +; moderate transcript, ++ and high transcript, +++.

Immunohistochemistry

Automatic immunostaining with Optimax Automated Cell Staining System Plus (BioGenex, San Raman, CA, USA) was performed after optimizing antibody dilutions.

The 5 μ m sections made from the paraffin blocks were dried in a 56°C incubator for 1 hour. Then the sections were deparaffinized, rehydrated and incubated with 0.3% hydrogen peroxide in methanol for 30 minutes to block the endogenous peroxidase activity. The antigen retrieval of the sections was done in a microwave in a citrate-buffer solution for 4x5 minutes. The sections were incubated with the primary antibodies EphB2(1:150) and

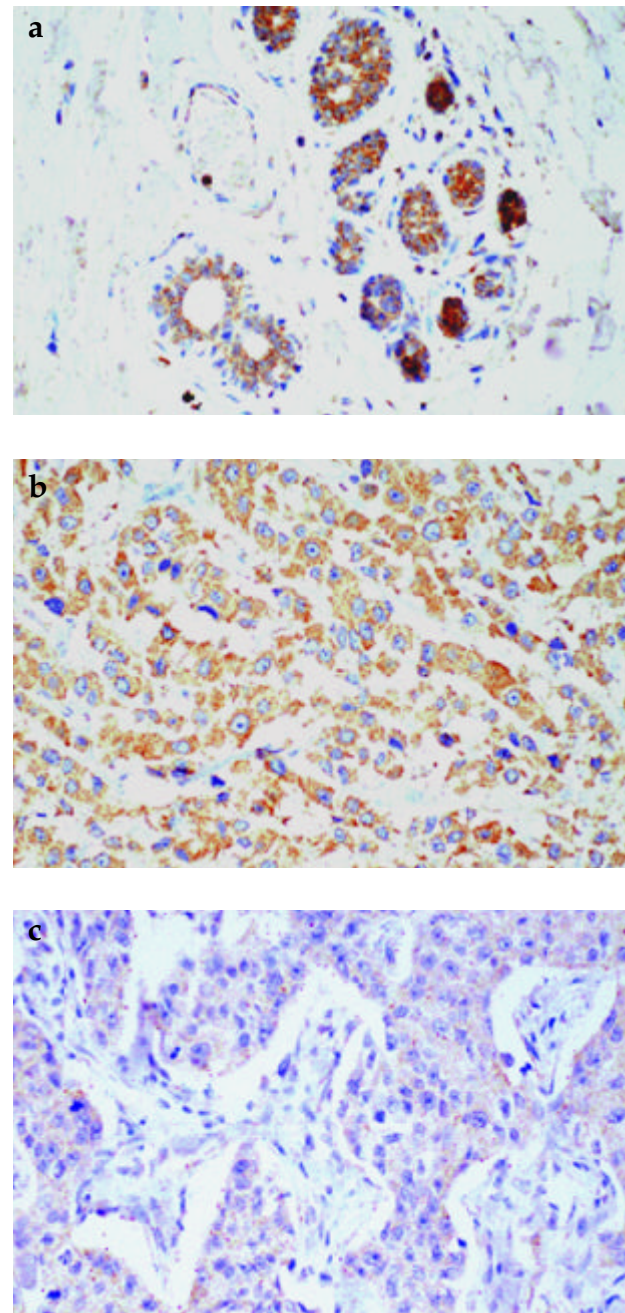


Figure 1. Strong expression of EphB2 in normal breast glandular structure (a, x20). Strong (b, x20) and moderate (c, x20) staining of EphB2 protein in breast carcinoma.

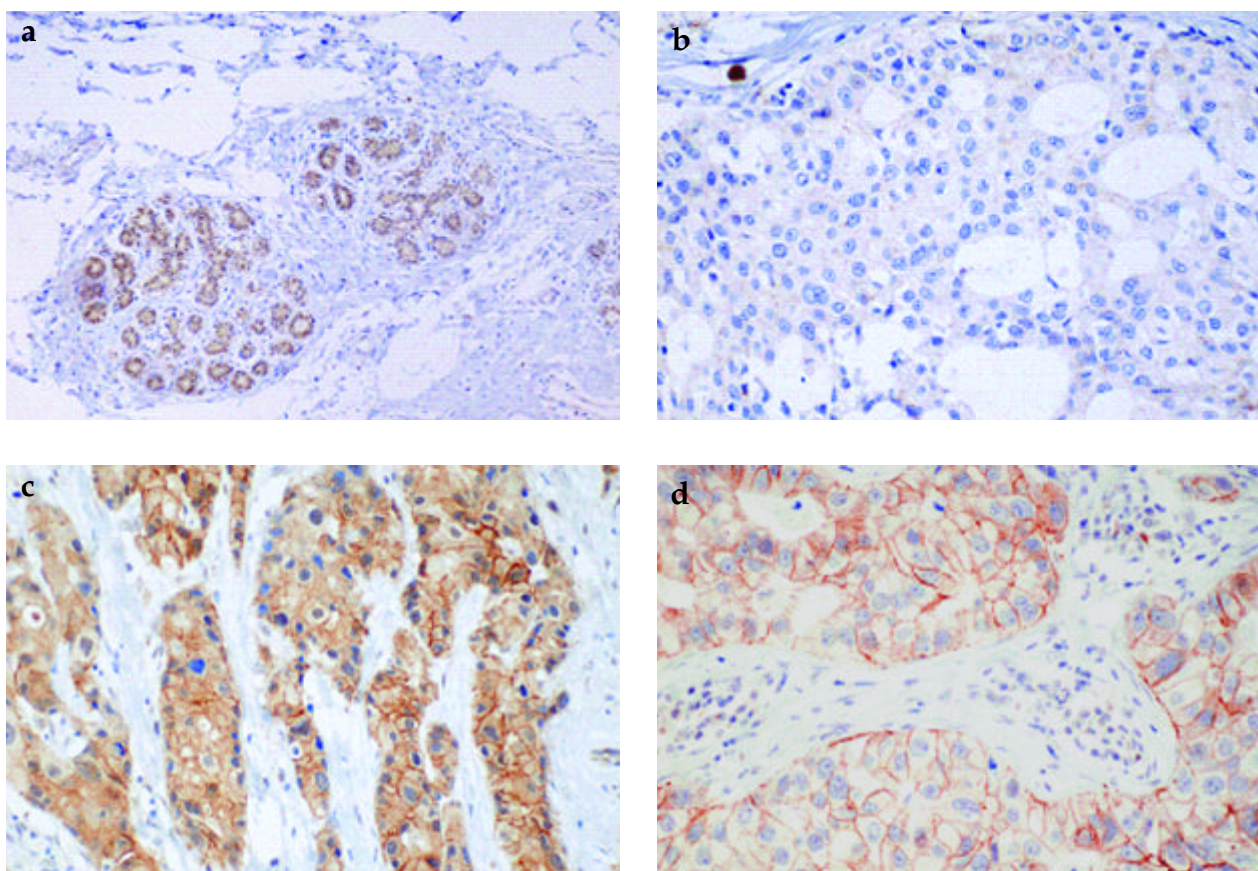


Figure 2. Strong expression of EphB4 in normal breast glandular structure (a, x10), weak expression of EphB4 in breast carcinoma (b, x20), strong expression in both membrane and cytoplasm (c, x20) and strong membrane expression (d, x20).

EphB4(1:200), respectively(both from Santa Cruz Biotechnology, Inc). Then the slides were incubated with biotinylated secondary antibody for 30 minutes. The sections were treated with streptavidin peroxidase for 20 minutes and stained for 5 minutes with 0.05% DAB freshly prepared in 0.05M Tris-HCL buffer at pH7.6 containing 0.01% hydrogen peroxide. Each staining batch had positive and negative controls. The anti-EphB2(sc-1763) is a goat polyclonal antibody against a peptide mapping at the carboxy terminus, and the anti-EphB4(sc-5536) is a rabbit polyclonal antibody against an extracellular domain of EphB4.

The immunohistochemistry was evaluated by staining intensity on a four-level scales: negative as 0, weak as 1, moderate as 2 and strong as 3.

Statistic analysis

The statistical significance of intergroup difference was evaluated by a chi-square test, and survival analysis was carried out using the Kaplan-Meier method. P-value<0.05 was considered statistically significant.

Results

Immunohistochemistry

All of the four breast carcinoma cell lines revealed a strong immunostaining in cytoplasm with both EphB2 and EphB4 antibodies. For the 9 normal breast tissues, all were strongly stained for EphB2 in the cytoplasm (*Figure 1a*), occasionally in the nucleus, and 3 were weakly, moderately, strongly stained for EphB4 (*Figure 2a*).

In breast carcinomas, the EphB2 protein expression was mainly cytoplasmic, not only in cancer tissues but also in the adjacent normal looking ductal epithelial cells. 1 (1%), 16 (17%), 29 (31%) and 48 (51%) of the 94 tumors were negative, weak, moderate and strong expression of EphB2, respectively (*Figure 1b and c*). The EphB4 protein was also mainly cytoplasmic (*Figure 2b*). 6 (6%), 27 (29%), 28 (30%) and 33 (35%) of the 94 tumors were negative, weak, moderate and strong expression of EphB4, respectively. 22 of the 94 tumors showed cell membrane staining of EphB4 (*Figure 2c and d*). In some cases, the cytoplasmic staining of tumor cells was stronger in the invasive front than that in the center of tumor areas.

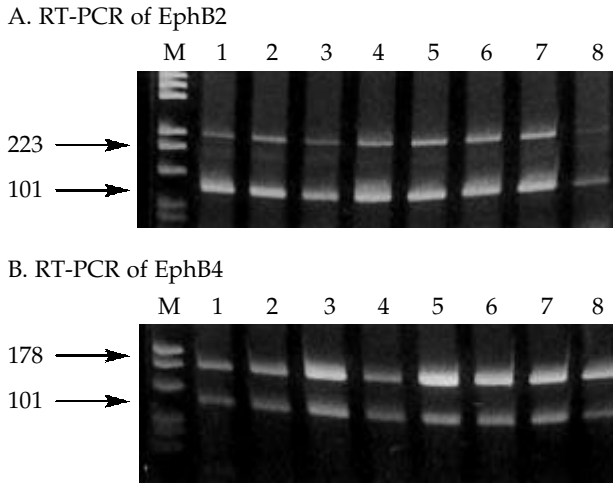


Figure 3. Photographs of EphB2 and EphB4 RT-PCR products in breast carcinomas. For EphB2 (top): lane 1, +; lane 2, +++; lane 3, ++; lane 4, +; lanes 5-7, ++ and lane 8, +. For EphB4 (bottom): lane 1, +; lane 2, +; lane 3, +++; lane 4, +; lane 5, +++ and lanes 6-8, ++. M: pGEM[®] DNA Markers (G174A, Promega)

In addition, a strong cytoplasmic staining for both EphB2 and EphB4 was seen in endothelial cells of tumor areas.

RT-PCR

RT-PCR was performed in 37 carcinomas and 9 normal tissue specimens. Both EphB2 and EphB4 PCR products were present in all of these tissues (Figure 3).

Table 3. Correlation between EphB2 RNA transcript and protein expressions

Cytoplasmic immunoreactivity	EphB2 PCR			Total
	+	++	+++	
Negative\weak expression	9	1	0	10
Moderate expression	0	6	5	11
Strong expression	0	2	14	16
Total	9	9	19	37

Table 4. Correlation between EphB2 cytoplasmic staining and clinical stage

Cytoplasmic immunoreactivity	Stage				Total
	1	2	3	4	
Negative	0	1	0	0	1
Weak	9	6	0	0	15
Moderate	11	15	2	1	29
Strong	14	29	2	1	46
Total	34	51	4	2	91

For EphB2, RNA expression was associated with its protein level ($p < 0.001$) (Table 3), while association between protein and RNA expression of EphB4 was not significant ($p = 0.711$).

The four breast carcinoma cell lines were also positive for the two genes detected with RT-PCR, and no significant difference in mRNA expression levels of EphB2 or EphB4 among the cell lines was found.

Correlation between EphB2/EphB4 expression and clinicopathological features

Increased EphB2 protein expression was negatively associated with overall survival ($p = 0.044$) (Figure 4a) and there was a trend that increased EphB2 protein expression was also correlated with disease free survival ($p = 0.081$) (Figure 4a), but not with clinical stage and histological grade (Table 4,5).

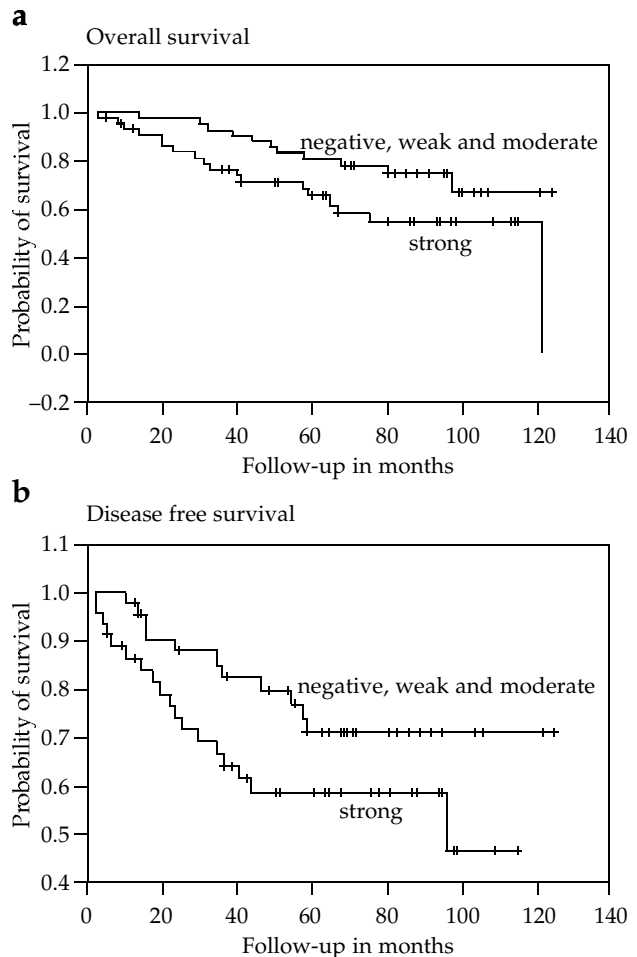


Figure 4. Kaplan-Meier survival plots. (a): a significant association between strong EphB2 protein expression and poorer overall survival; (b): a statistical trend showing an association between increased expression of EphB2 and poorer disease free survival.

The expression of EphB4 protein was increased with the clinical stage ($p=0.007$) and histological grade ($p=0.036$) (Table 6,7). There was no association however in terms of patients' survival. EphB4 membrane staining was positively associated with DNA aneuploidy ($p=0.029$) and increased S phase fraction ($p=0.016$).

No significant difference between RNA /protein expression of EphB2/EphB4 and age, tumor size, metastases and ER status was observed.

Discussion

Eph receptors and their ligands are important in regulating cell-cell interactions by initiating unique bidirectional signal transductions. Eph receptor-ephrin complex can form special crystal structure. For example, each ephrinB2

ligand interacts with two EphB2 receptors and each EphB2 receptor can react with two ephrinB2 ligands as well. This complex forms a ring-like structure, which is fundamental for initiating Eph-ephrin signaling.³³

When Eph receptors are activated by their ligands, multiple tyrosine sites are phosphorylated and the kinase domains are activated.³⁴ The phosphorylated tyrosines serve as docking sites for down-stream SH2 domain-containing or PDZ domain containing proteins or adaptors, including p59fyn, PI3-kinase, Grb2, Grb10, Nck, Src-like protein and SHEP1.³⁵⁻⁴¹ Activated EphB2 can activate the Ras binding protein, AF6, the low-molecular weight phosphotyrosine phosphatase (LMW-PTP) and the docking protein p62.^{36,42,43} Through protein interaction cascades, Eph family influences cell behavior and cellular biological activities,⁴³ but at the same time, EphB receptors are of the autoinhibitory capability through which receptor kinases are controlled.⁴⁴

Chromosome 1p35-36, on which EphB2 is located, is a frequent site of allelic loss in colorectal tumors,^{45,46} indicating that EphB2 could be a tumor suppressor gene. However, no mutation has been found in any of its exons.⁴⁷ And our present results support the notion that EphB2 plays important role in breast carcinoma progress. In our study, EphB2 protein was expressed in all normal tissue specimens, but highly expressed in 51% of the breast carcinomas. High expression of EphB2 was associated with patients' poor overall survival and there was a clear trend that high levels of EphB2 protein were negatively associated with disease-free survival by Kaplan-Meier test, although no significant association with clinical stage and histological differentiation was found.

Membrane staining of EphB4 was found in 22 of the 94 breast carcinomas. Among the 22 tumors, adjacent normal breast glandular structures in 5 of 22 tumors demonstrated membrane immunostaining as well. A similar finding was also reported in normal endometrium, endometrial hyperplasia and endometrial carcinoma.²⁸ We observed a significant association between EphB4 positive expression and increased S phase fraction and DNA aneuploid; but no associations with tumor differentiation, stage and ER were found. EphB4 protein expression was positively associated with increased histological grade of the breast carcinomas, contrary to the results previously reported by Berclaz.³⁰

In our study EphB4 RNA transcripts were detected in both normal and breast carcinomas. In some tumors, RNA transcripts were detected, but protein immunoreactivity was negative. There was no association between EphB4 protein expression and RNA level in our study. It may be due to the fact that expression of EphB4 protein could be regulated or influenced by post-transcription factors, such as estrogen. Berclaz et al²⁸ have reported that EphB4 protein may be down-regulated in normal and malignant

Table 5. Correlation between EphB2 cytoplasmic staining and histological grade

Cytoplasmic immunoreactivity	Histological grade			Total
	1	2	3	
Negative	0	0	1	1
Weak	1	9	6	16
Moderate	3	15	11	29
Strong	3	21	24	48
Total	7	45	42	94

Table 6. Correlation between EphB4 cytoplasmic staining and clinical stage

Cytoplasmic immunoreactivity	Stage				Total
	1	2	3	4	
Negative	3	1	2	0	6
Weak	8	18	1	0	27
Moderate	13	12	0	2	27
Strong	10	20	1	0	31
Total	34	51	4	2	91

Table 7. Correlation between EphB4 cytoplasmic staining and histological grade

Cytoplasmic immunoreactivity	Histological grade			Total
	1	2	3	
Negative	1	2	3	6
Weak	2	12	13	27
Moderate	1	21	6	28
Strong	3	10	20	33
Total	7	45	42	94

endometrium, but highly expressed in the endometrial carcinoma cells in the post-menopausal patients with low estrogen level. The experiments in ovariectomy mice indicate that the expression of EphB4 is dependent on estrogen in mammary development and its expression is decreased during estrus cycle, completely absent in the anestrus phase, although its RNA expression was not significantly affected by ovariectomy.¹⁹

EphB2 and EphB4 were expressed in most of the vascular endothelial cells in both normal breast and carcinoma tissues in our present study. It is known that Eph receptors, such as EphB2, B4 and their ligands are critical for vascular development during embryogenesis, and ephrinB2 and EphB4 are markers for the endothelium of primordial arterial and venous vessels, respectively.^{48,49} Mutant mice lacking ephrinB2 or EphB4 die before day 11 of embryonic development with vascular defects. Our results support the notion that EphB2 and EphB4 are involved in angiogenesis in breast carcinoma.

In summary, we have shown that EphB2 protein expression is negatively associated with both overall survival and disease free survival, while EphB4 protein expression is positively associated with increased histological grade and stage. Furthermore, membrane staining of EphB4 is associated with higher S phase fraction and aneuploidy. These findings indicate that EphB2 and EphB4 are involved in the progression of breast cancer and that EphB2 is correlated with patients' prognosis.

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