# Overexpression of *EphA4* gene and reduced expression of *EphB2* gene correlates with liver metastasis in colorectal cancer

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**Abstract.** The Eph receptors, members of a large family of transmembrane receptor tyrosine kinases, play important roles in a variety of biological functions. Recent studies have suggested that EphA4 and EphB2 participate in the growth and development of various carcinomas. This study examined the relationship of *EphA4* and *EphB2* gene expression to clinicopathological factors, especially metastasis, in patients with colorectal cancer. We studied surgical specimens of cancer tissue and adjacent normal mucosa obtained from 205 patients with untreated colorectal cancer. The relative expression levels of *EphA4* and *EphB2* mRNA in the specimens were measured by quantitative real-time, reverse-transcription polymerase chain reaction. The relative expression level of *EphA4* mRNA was higher in the presence than in the absence of liver metastasis, whereas the relative expression levels of *EphB2* mRNA were similar. Analysis of the relationship between clinicopathological features and gene expression showed that high expression of the *EphA4* gene and low expression of the *EphB2* gene correlated with liver metastasis. There was no correlation between *EphA4* and *EphB2* gene expression. Our results suggest that overexpression of the *EphA4* gene and reduced expression of the *EphB2* gene might promote liver metastasis in colorectal cancer. Overexpression of the *EphA4* gene and reduced expression of the *EphB2* gene may thus be a useful predictor of liver metastasis in patients with colorectal cancer.

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## Introduction

The Eph receptor family constitutes one of the largest groups of transmembrane receptor tyrosine kinases (1). They are activated by a second family of cell surface-anchored ligands, the ephrins, which are attached to the plasma membrane via either a glycosylphosphatidylinositol (GPI) linkage (type A) or a transmembrane sequence (type B). The Eph receptors are also divided into type A or type B according to their ligand-binding specificities. In general, type A receptors bind type A ephrin ligands, and type B ephrin ligands stimulate type B receptors. One molecule that shows an exception to this rule is EphA4, which can bind and respond to type B as well as type A ephrin ligands (2). These Eph receptors and their ligands have been implicated in a variety of biological functions, including axon guidance and migration of neural crest cells in the nervous system, establishment of segmental boundaries, and formation of angiogenic capillary plexi (3-7). Among Eph receptor family members, EphA4 and EphB2 are frequently overexpressed or functionally altered in many types of cancers, suggesting a role in tumor progression or angiogenesis (8-14).

In this study, we measured expression levels of the *EphA4* and *EphB2* genes in 205 pairs of cancer tissue and adjacent normal mucosa obtained from patients with colorectal cancer. To evaluate the clinical significance of EphA4 and EphB2, we examined correlations between the relative expression of these genes and clinicopathological features.

## Materials and methods

*Patients and samples.* We studied surgical specimens of cancer tissue and adjacent normal mucosa obtained from 205 patients with untreated colorectal cancer. The patients underwent surgery at Yokohama City Medical Center, Gastroenterological Center and at Kanagawa Cancer Center between 2002 and 2006. Informed consent was obtained from each patient and the ethics committees of Yokohama City Medical Center and Kanagawa Cancer Center approved the protocol before

Table I. PCR primers and conditions.

| Gene | Primer | Temperature (˚C) | Product size (bp) |
|---|---|---|---|
| EphA4 | 5'-AGTCCTTCTGGTCTCTGTCTC-3'<br>5'-CTTCATCCGCTTCTTGTTTGG-3' | 60 | 116 |
| EphB2 | 5'-GCTTTCTGCTTACTGACTTAGG-3'<br>5'-GGTGGGAGGAGGGAAGAG-'3 | 60 | 105 |
| ß-actin | 5'-AGTTGCGTTACACCCTTTCTTGAC-3'<br>5'-GCTCGCTCCAACCGACTGC-3' | 60 | 171 |

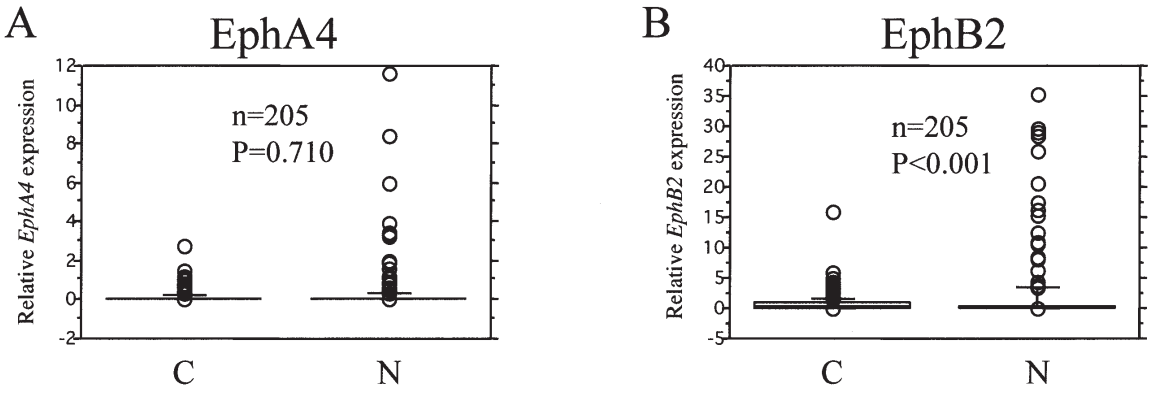


Figure 1. Comparison of *EphA4* and *EphB2* mRNA expression levels between colorectal cancer tissue and adjacent normal mucosa. P-values were calculated by the Wilcoxon test. *EphB2* gene expression levels were higher in adjacent normal mucosa than in cancer (P<0.001). *EphA4* gene expression levels did not differ significantly between cancer and adjacent normal mucosa.

initiation of the study. All tissue samples were embedded in O.C.T. compound (Sakura Finetechnical Co., Ltd., Tokyo) and immediately stored at -80˚C until use. No patient had any other malignancies. The histopathological features of specimens stained with hematoxylin and eosin were examined and sections that consisted of >80% cancer cells were used to prepare total RNA.

*Quantitative real-time, reverse-transcription polymerase chain reaction (PCR).* Total RNA isolated from colorectal cancer and adjacent normal mucosa was prepared with the use of Trizol (Gibco, Life Tech, Gaithersburg, MD). Complemetary DNA (cDNA) was synthesized from 2 $\mu$g of total RNA with an iScript cDNA synthesis kit (Bio-Rad Laboratories, Hercules, CA). After synthesis, the cDNA was diluted at 1:4 with water and stored at -20˚C until use. Quantitative real-time PCR was performed with an iQ SYBR-Green Supermix (Bio-Rad Laboratories). PCR reactions were carried out in a total volume of 15 $\mu$l containing cDNA derived from 75 ng of mRNA, 0.27 $\mu$M of each primer, 7.5 $\mu$l of iQ SYBR-Green Supermix containing dATP, dCTP, dGTP, and dTTP at concentrations of 400 $\mu$M each and 50 U/ml of iTag DNA polymerase. The PCR consisted of 10 min at 94˚C, followed by 50 cycles of denaturation of the cDNA for 30 sec at 94˚C, annealing for 30 sec at an appropriate temperature (Table I) and a primer extension for 1 min at 72˚C followed by 10 min at 72˚C. The PCR primer sequences of *EphA4*, *EphB2* and ß-actin, used as internal controls, are shown in Table I.

*Statistical analysis.* Gene expression levels of colorectal cancer were compared with those of adjacent normal mucosa by the Wilcoxon test. The relationship between gene expression and potential explanatory variables, including age, gender, tumor size, histological type, depth of invasion, lymph node metastasis, location, lymphatic invasion, venous invasion and liver metastasis were evaluated with the $\chi^2$ test. Associations between variables were assessed using the Mann-Whitney U test. Correlation coefficients between different variables were calculated by simple regression analysis. All statistical analyses were performed using Statview J 5.0 software (Abacus, CA). Two-sided P-values were calculated and a difference was considered significant if the P-value was <0.05.

## Results

*Comparison of EphA4 and EphB2 mRNA expression between colorectal cancer tissue and adjacent normal mucosa. EphB2* gene expression levels were higher in adjacent normal mucosa than in cancer (P<0.001) (Fig. 1B). *EphA4* gene expression levels were similar in cancer and adjacent normal mucosa (P=0.710) (Fig. 1A).

*Relationship of EphA4 and EphB2 gene expression levels to clinicopathological features.* Expression levels of the *EphA4* and *EphB2* genes were categorized as low or high according to their median values. The relationship between the expression of these genes and clinicopathological features were then examined. Expression levels of the *EphA4* and *EphB2* genes

Table II. Relationship between expression of the *EphA4*, *EphB2* genes and clinicopathological features.

| Variables/categories | *EphA4* expression | | P-value | *EphB2* expression | | P-value |
|---|---|---|---|---|---|---|
| | low (n=103) | high (n=102) | | low (n=103) | high (n=102) | |
| Age | 65.4±10.5 | 66.3±11.1 | 0.534 | 66.0±10.9 | 65.6±10.7 | 0.912 |
| Gender | | | | | | |
| Male | 60 | 52 | 0.296 | 55 | 57 | 0.721 |
| Female | 43 | 50 | | 48 | 45 | |
| Size | | | | | | |
| ≤5 cm | 52 | 63 | 0.104 | 62 | 53 | 0.235 |
| >5 cm | 51 | 39 | | 41 | 49 | |
| Histological type | | | | | | |
| Well differentiated | 26 | 35 | 0.258 | 30 | 31 | 0.864 |
| Moderately differentiated | 64 | 52 | | 60 | 56 | |
| Poorly differentiated | 13 | 15 | | 13 | 15 | |
| Depth of invasion | | | | | | |
| T1 | 10 | 9 | 0.071 | 6 | 13 | 0.059 |
| T2 | 38 | 56 | | 52 | 42 | |
| T3 | 48 | 32 | | 36 | 44 | |
| T4 | 7 | 5 | | 9 | 3 | |
| Lymph node metastasis | | | | | | |
| Absent | 49 | 46 | 0.722 | 47 | 48 | 0.838 |
| Present | 54 | 56 | | 56 | 54 | |
| Location | | | | | | |
| Colon | 54 | 58 | 0.524 | 63 | 49 | 0.059 |
| Rectum | 49 | 44 | | 40 | 53 | |
| Lymphatic invasion | | | | | | |
| Absent | 71 | 63 | 0.281 | 70 | 64 | 0.376 |
| Present | 32 | 39 | | 32 | 38 | |
| Venous invasion | | | | | | |
| Absent | 34 | 43 | 0.176 | 37 | 40 | 0.626 |
| Present | 69 | 59 | | 66 | 62 | |
| Liver metastasis | | | | | | |
| Absent | 78 | 63 | 0.031 | 64 | 77 | 0.039 |
| Present | 25 | 39 | | 39 | 25 | |

were unrelated to age, gender, tumor size, lymph node metastasis, lymphatic invasion and venous invasion. High expression of the *EphA4* gene and low expression of the *EphB2* gene correlated with liver metastasis (P=0.031, 0.039) (Table II).

*Relationship of EphA4 and EphB2 gene expression levels to liver metastasis*. The highest rate of liver metastasis was associated with high expression of the *EphA4* gene and low expression of the *EphB2* gene (Fig. 2).

*Associations of EphA4 and EphB2 gene expression with lymph node metastasis in patients with colorectal cancer*. There was no significant association between the expression level of either gene and the presence or absence of lymph node metastasis (Fig. 3).

*Associations of EphA4 and EphB2 gene expression with liver metastasis in patients with colorectal cancer. EphA4*

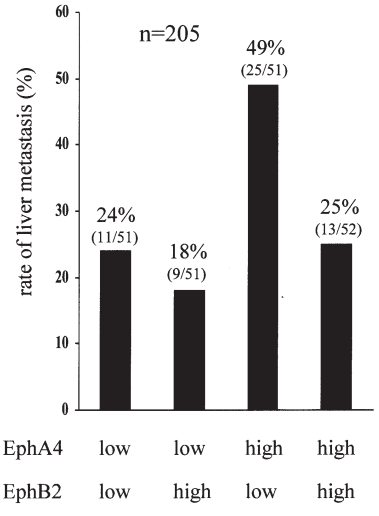


Figure 2. Relationship of *EphA4* and *EphB2* gene expression levels to liver metastasis. The highest rate of liver metastasis was associated with high expression of the *EphA4* gene and low expression of the *EphB2* gene.

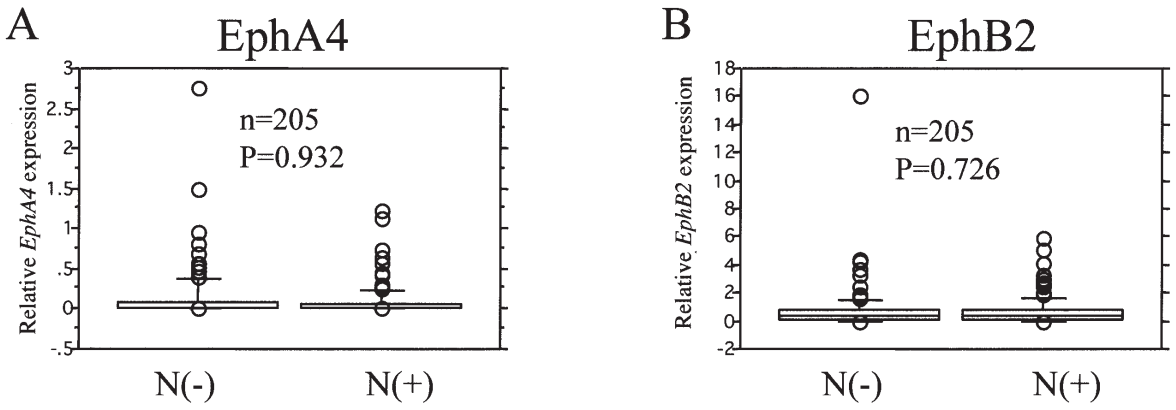


Figure 3. Associations of *EphA4* and *EphB2* gene expression with lymph node metastasis in 205 patients with colorectal cancer. Box boundaries, the 25th and 75th percentiles of the observed values; capped bars, the 10th and 90th percentiles; solid line, median. P-values were calculated by the Mann-Whitney U test. There was no correlation between the expression level of either gene and the presence or absence of lymph node metastasis.

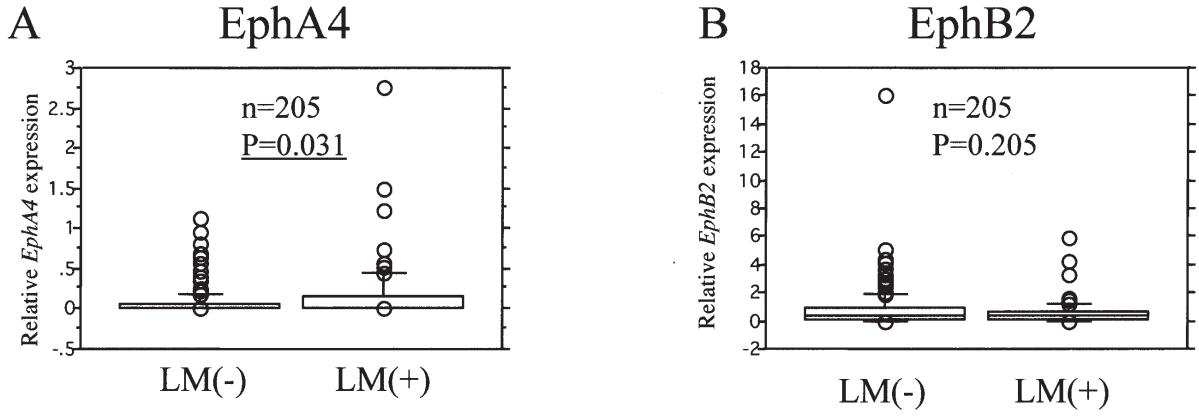


Figure 4. Associations of *EphA4* and *EphB2* gene expression levels with liver metastasis in 205 patients with colorectal cancer. Box boundaries, the 25th and 75 th percentiles of the observed values; capped bars, the 10th and 90th percentiles; solid line, median. P-values were calculated by the Mann-Whitney U test. *EphA4* gene expression levels were higher in the presence than in the absence of liver metastasis (P=0.031).

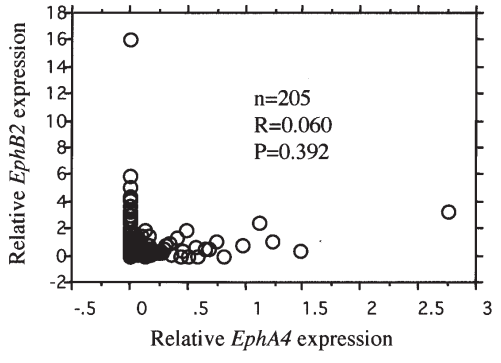


Figure 5. Correlation between *EphA4* and *EphB2* gene expression levels in colorectal cancers. Each gene expression level is relative to that of the ß-actin gene. There was no correlation between *EphA4* and *EphB2* expression levels.

gene expression levels were higher in the absence than in the presence of liver metastasis (P=0.031) (Fig. 4A).

*Correlation between EphA4 and EphB2 gene expression.* The correlation between *EphA4* and *EphB2* gene expression levels is shown in Fig. 5. There was no correlation between *EphA4* expression and *EphB2* expression.

## Discussion

Receptor tyrosine kinases and their ligands play critical roles in the regulation of a variety of cell activities, including cellular survival, proliferation, differentiation and tissue organization (15). Eph receptors and their ligands, ephrins, are indeed involved in several cell processes during embryonic development, such as pattern formation, cell aggregation and migration, segmentation, neural development, angiogenesis, and vascular hierarchical remodeling (3-7). The overexpression of some Eph receptor family members has an important role in the development and progression of various cancers. In particular, EphA4 and EphB2 overexpression is frequently associated with human invasive cancers (10-12,16,17).

In this study, we examined expression levels of the *EphA4* and *EphB2* genes in colorectal cancer and in adjacent normal mucosa. We also studied the relationship of these gene expression levels to clinicopathological features, as well as correlations among the expression of these genes.

Several previous studies have compared *EphA4* and *EphB2* mRNA expression levels between colorectal cancer tissue and adjacent normal mucosa. Ashida *et al* (17) found that the expression of *EphA4* is significantly higher in human prostatic cancer than in adjacent normal prostatic epithelium. Liu *et al*

(18) reported that the expression of EphB2 mRNA is lower in nonmalignant cell lines than in the colon cancer cell lines *in vitro*. Mao *et al* (19) showed that the expression of *EphB2* is higher in colorectal cancer tissue than in normal colorectal tissue (n=11). However, Guo *et al* (20) found that the expression of EphB2 protein is significantly higher in normal colorectal mucosa than in colorectal cancer. Our study (n=205) demonstrated that *EphB2* gene expression levels were higher in adjacent normal mucosa than in colorectal cancer tissue. This finding is consistent with the results of a previous study showing that EphB2 suppresses carcinogenesis, including the transition from colorectal adenoma to carcinoma (20). In contrast, *EphA4* gene expression levels did not differ significantly between cancer and adjacent normal mucosa.

A previous study examining the relationship between clinicopathological features and gene expression levels, found no significant correlation between EphA4 expression and the histological type of pancreatic ductal adenocarcinoma (16). This result was unexpected because the expression of exogenous EphA4 promotes the growth of pancreatic ductal adenocarcinoma cells (16). In our study, there was no significant correlation of EphA4 expression with tumor size, histological type, invasion, or lymph node metastasis in colorectal cancer. However, high *EphA4* gene expression correlated with liver metastasis.

As for EphB2, Wu *et al* (21) reported that *EphB2* gene expression does not correlate with clinical stage or histological grade in breast cancers. Guo *et al* (20) found that low expression of EphB2 correlates with invasion and metastasis in colorectal cancers. In our study, reduced *EphB2* gene expression correlated with liver metastasis in colorectal cancer. This result is considered reasonable, because EphB2 receptor activity suppresses colorectal cancer progression and metastasis (22,23). Thus, overexpression of the *EphA4* gene and reduced expression of the *EphB2* gene is associated with liver metastasis in colorectal cancer.

When expression levels of the *EphA4* and *EphB2* genes were contrasted with the presence or absence of lymph node metastasis, no correlation was noted for either gene. We also examined potential correlations of gene expression levels with the presence or absence of liver metastasis. Iiizumi *et al* (16) reported that EphA4 contributes to properties such as invasiveness or metastasis in a wide range of malignancies. Thorstensen *et al* (22) found that loss of heterozygosity at the *EphB2* locus was frequently associated with liver metastasis. In our study, *EphA4* gene expression levels were higher in the presence than in the absence of liver metastasis. This finding suggested that overexpression of *EphA4* mRNA might contribute to liver metastasis in colorectal cancer.

We then examined correlations between EphA4 and EphB2 gene expression in colorectal cancers. There was no significant correlation between the expression levels of these genes.

In conclusion, our results show that overexpression of the *EphA4* gene and reduced expression of the *EphB2* gene correlates with liver metastasis in colorectal cancer. Overexpression of the *EphA4* gene and reduced expression of the *EphB2* gene may thus be a novel marker or predictor of liver metastasis.

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