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Original Article

Aberrant expression of receptor tyrosine kinase EphA7 in breast cancers

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Abstract: Receptors tyrosine kinase of Eph family play important roles in the vascular development, tissue-border formation, cell migration, axon guidance and angiogenesis. They are reported overexpressed in certain human cancers. The over-expression of Eph receptors in cancers is associated with malignant transformation, tumor metastasis, tumor progression and prognosis. Among the Eph family genes, relatively less attention has been directed toward EphA7 in human tumors. Down-regulation of EphA7 by hypermethylation was found in colorectal cancer, germinal center B-cell lymphoma, gastric cancer, and prostate cancer. The potential role of EphA7 in carcinogenesis of human breast carcinoma has not been addressed. In the present study, we tested the expression of EphA7 protein in normal breast cell line MCF-10A, breast cancer cell lines MCF-7, MDA-MB-231, SK-BR-3 and in a set of 150 invasive ductal cancer specimens by using immunohistochemical staining with an EphA7 specific polyclonal antibody. The relationship between EphA7 protein expression and clinicopathological parameters was analyzed. Loss of EphA7 expression was found in breast cancer cell lines and invasive ductal cancer specimens. The expression of EphA7 was associated with grade (P = 0.014), TNM stage (P = 0.029), lymph node metastasis (P = 0.018) and HER2 status (P = 0.005). Our data indicate that the EphA7 protein lost in most breast cancer samples. The function of EphA7 protein in carcinogenesis of breast cancer may be diverse.

Keywords: EphA7, receptor tyrosine kinase, breast cancer

Introduction

Breast cancer is one of the most common type of female cancer worldwide and represents 14% of cancer-related deaths in women [1]. Even though there has been considerable progress in the early detection, surgical therapy, hormonal and target therapy of breast cancer, there are about ~3500000 women who die from breast cancer each year. Therefore, there is an urgent need to understand the molecular mechanism and pathways that participate in the tumorigenesis, progression and prognosis of breast cancer for better and improved treatment of women diagnosed with breast cancer [2-4].

The erythropoietin producing hepatocellular carcinoma (Eph) family of receptor tyrosine kinases constitutes the largest RTK subfamily. The Eph receptors are divided into EphA and EphB based onthe sequence homology of their extra cellular domains and their affinity to bind corresponding ligands, EphrinA and EphrinB (Eph Nomenclature Committee, 1997). These receptors are located on cell surfaces and transduce signals upon binding to the ligands that typically located on the surfaces of neighboring cells. Eph receptors, like other RTKs, initiate signal transduction through autophosphorylation after ligand-receptor engagement. In general, ephrin-A and ephrin-B ligands interact with EphA and EphB receptors, but some exceptions mightexist [5, 6]. Eph receptors and their ligands ephrins are highly expressed in neural and endothelial cells. The signaling they regulated play important roles in embryonic development, axon guidance, boundary formation, hindbrain segmentation and vascular system development [5, 7]. Increasing evidence indicates that Eph receptors and ephrin ligands are involved in both physiological and pathological conditions [6, 8, 9]. The expression of

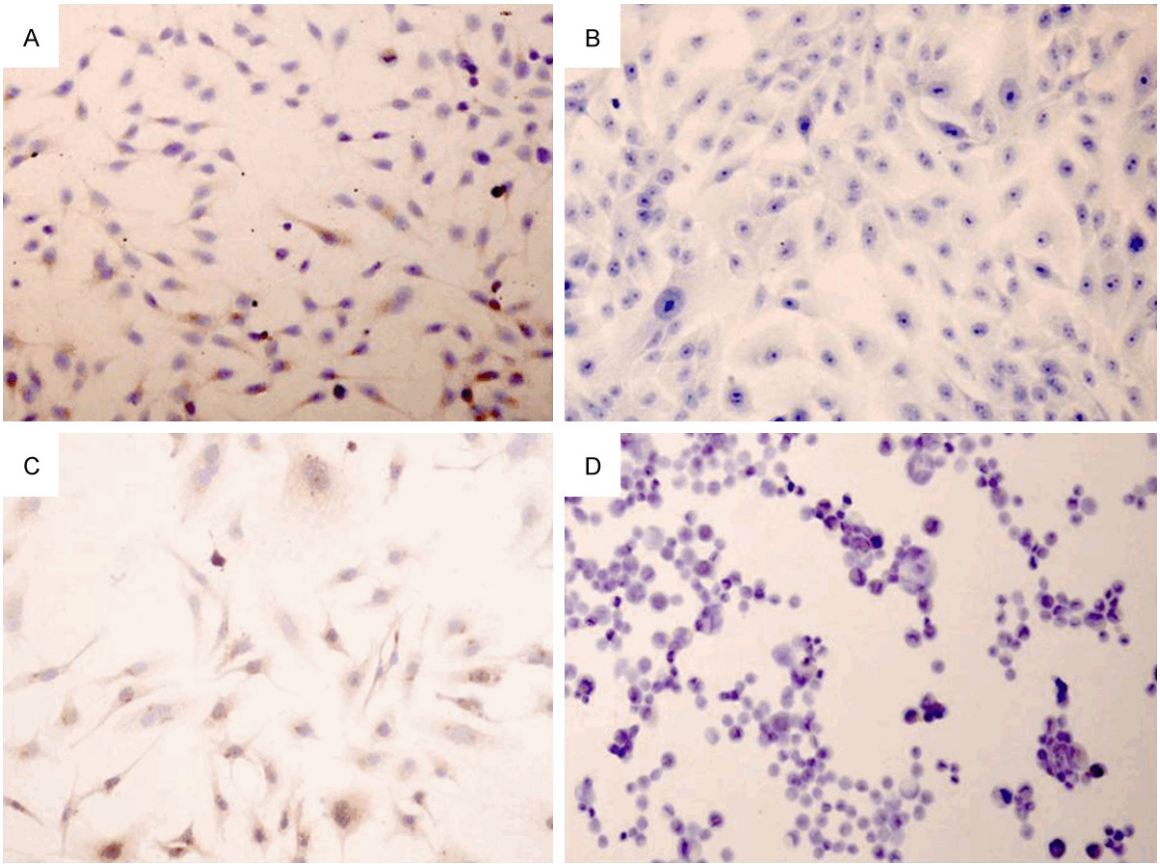

Figure 1. Expression of EphA7 in the mammary epithelial cell line MCF-10A (A: Positive staining) and in the breast cancer cell lines (B: MCF-7, negative expression; C: MDA-MB-231, moderate expression and D: SK-BR-3, negative expression).

Ephs and ephrins was explored in certain human tumors. Emerging evidence suggests their strong involvement in tumor biology, including metastasis, invasion, and angiogenesis [6, 9, 10].

The EphA7 (formerly known as Mdk1/Ebk/Ehk) was first identified in the murine nervous system, and several alternatively spliced variants existed. Human EphA7 (HEK11) was isolated from a human fetal brain liabrary and found to be distributed widely in human tissues [11]. Of the Eph family receptor, less attention has been directed to EphA7 in human tumors, and its potential role in human oncology has not been addressed. Wang *et al* previously reported that EphA7 was down-regulated in colorectal cancer and gastric carcinoma [12, 13]. In the present study, we tested the expression of EphA7 in breast cancer samples and explored the relationship with clinicopathological parameters and survival of patients.

Materials and methods

Cell lines

Mammary epithelial cell line MCF-10A, human breast cancer cell lines MCF-7, MDA-MB-231, and SK-BR-3 were purchased from the American Type Culture Collection (Manassas, VA, USA). They were maintained in the suggested medium with 10% fetal serum (FCS), 100 U/L penicillin-streptomycin and then digested with 0.25% trypsin and cryopreservation with liquid nitrogen. The cell lines were incubated at 37°C in a humidified atmosphere of 5% CO_2.

Tissue specimens

The breast cancer samples were collected from 150 patients (mean age 55.3 years, range 23-78) as part of a study approved by Municipal People's Hospital of Maanshan, China. These patients had undergone surgery without any preoperative therapy between 2009 and 2014.

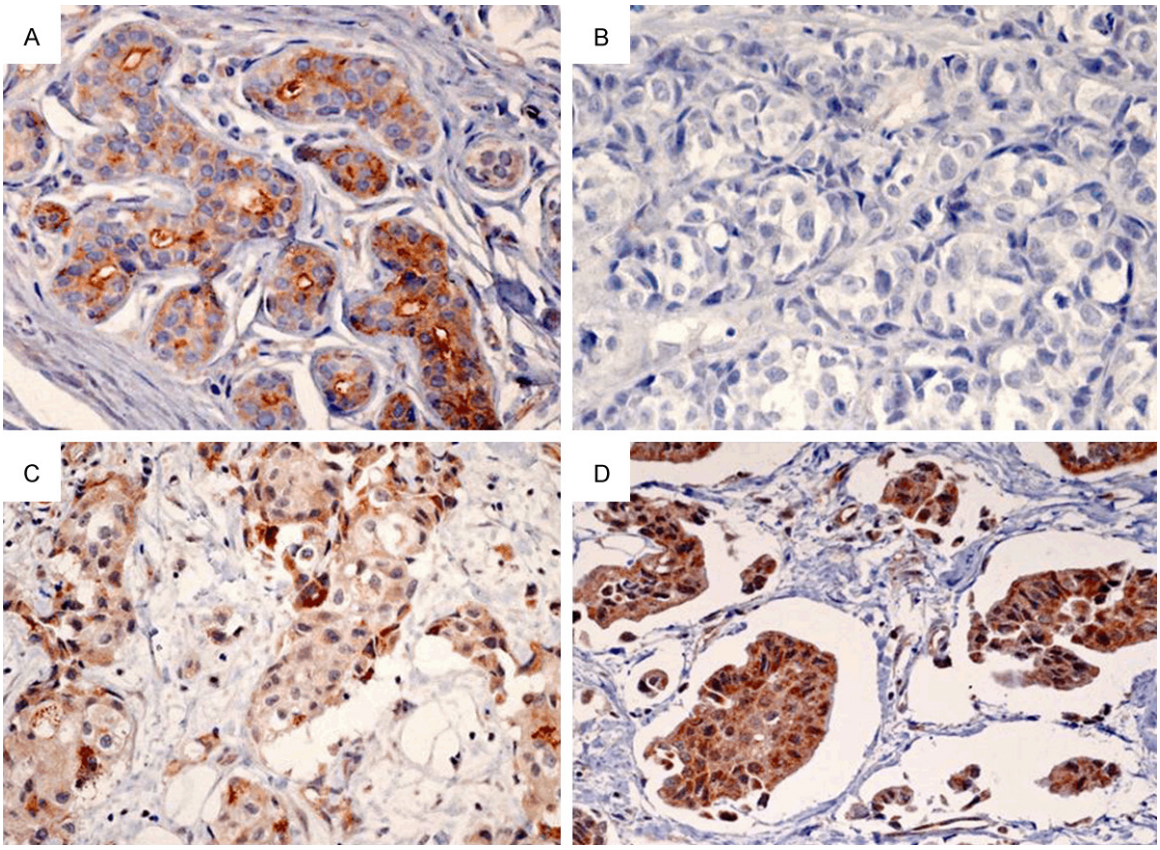

Figure 2. Detection of EphA7 protein in breast cancer tissue samples. A: Positive expression of normal breast epithelial cells. B: Negative expression in invasive ductal cancer cells. C: Moderate expression in invasive ductal cancer cells. D: Positive expression invasive ductal cancer cells.

After surgery, all patients were confirmed pathologically to express invasive ductal carcinomas (IDC). All samples were diagnosed and classified according to the World Health Organization (WHO) grading system.

Immunohistochemical staining

Sections from surgical specimens had been fixed in 10% formalin and embedded in paraffin and they were used here for immunohistochemical staining according to a standard method. Briefly, each 4-μm tissue section was deparaffinized and rehydrated. After rehydration through a graded ethanol series, the sections were autoclaved in 10 mM citrate buffer (pH 6.0) at 120°C for 2 min for antigen retrieval, then cooled to 30°C and washed with phosphate-buffered saline (PBS, pH 7.3). After endogenous peroxidase had been quenched with aqueous 3% H_2O_2 for 10 minutes and washed with PBS, the sections were incubated at 4°C overnight with a polyclonal antibody of EphA7 (Abgent, San Diego, CA, USA) at a 1:400 dilution in antibody diluent solution (Zymed, Invitrogen) and then washed with PBS. Next, the sections were incubated with secondary antibody (Dako REAL EnVision Detection System, Dako, UK) for 30 min at room temperature. Color development was performed with 3,3'-diaminobenzidine (DAB). Nuclei were lightly counterstained with hematoxylin. Two pathologists independently assessed the immunostained slides. Any difference in immunohistochemical scores was resolved by a consensus. Immunohistochemical staining of cancer cells was assessed according to the staining intensity positive cells. EphA7 expression was assessed for intensity (0 = no staining, 1 = weak, 2 = moderate, 3 = strong). The scores of 0 and 1 were defined as negative staining of EphA7, while scores of 2 and 3 were defined as positive staining of EphA7.

Statistical analysis

The statistical significance of intergroup differences was evaluated by a chi-square test. All

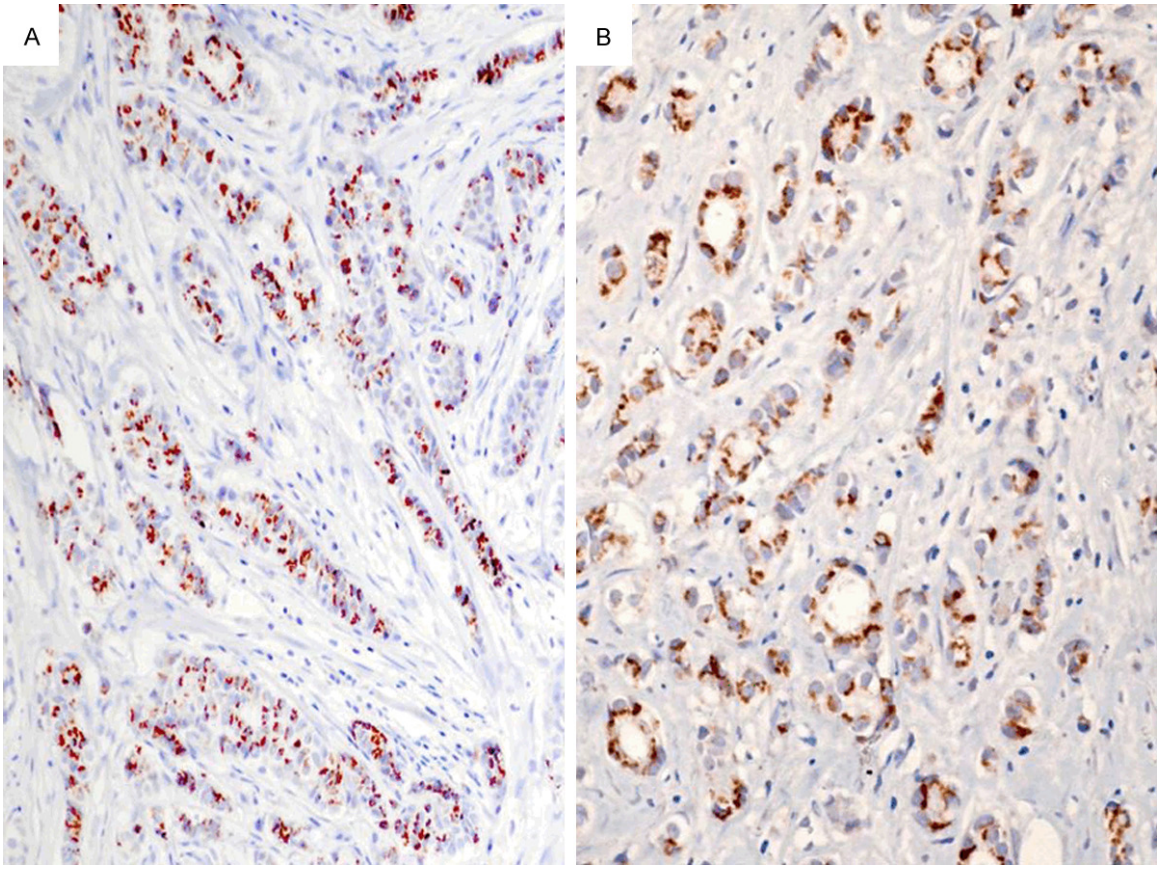

Figure 3. The EphA7 protein was positively stained in the golgiosome as brown particles.

statistical analyses were performed using SPSS software (SPSS 16.0, Chicago, IL). A two-sided *P* value of less than 0.05 was considered statistically significant.

Results

Loss of EphA7 expression in the breast cancer cell lines

The expression of EphA7 in the mammary epithelial cell line MCF-10A and in the breast cancer cell lines (MCF-7, MDA-MB-231, and SK-BR-3) was assessed using the immunohistochemical staining. The EphA7 expression was detected in MCF-10A normal cell lines, weakly expressed in MDA-MB-231, negatively expressed in MCF-7 and Sk-BR-3 (**Figure 1**).

Expression of EphA7 in breast cancer samples

Expression of EphA7 was investigated in a set of 150 breast cancer tissue samples by using a specific polyclonal antibody. Immunoreactivity for Eph A7 was observed in the cytoplasm (**Figure 2**) or in the golgiosome as brown particles (**Figure 3**). The EphA7 is a transmembrane receptor anchored on the cell membrane. However, in the present study, expression of EphA7 is dominantly detected in the cytoplasm. This phenomenon was also observed in other Eph receptors. EphA7 protein was heterogeneously expressed inter-samples both in normal luminal and tumor cells. The level of EphA7 expression was defined as reduced, non-changed, and increased expression by comparing the score of immunohistochemical staining in tumor cells with that in normal luminal cells of matched patients. The expression of EphA7 was reduced in 98 out of 150 cases (65.3%), non-changed in 36 out of 150 cases (24%), and increased expression in 16 out of 150 cases (10.7%).

The relationship between the expression of EphA7 and clinicopathological parameters

The relation between EphA7 expression and clinicopathological parameters was analyzed.

Table 1. Expression of EphA7 in 150 breast cancer tissues

Parameters	EphA7 protein expression			*P* value
	Down	Non	Up	
Age (years)				
< 50	30	16	8	0.156
≥ 50	68	20	8	
Age (years)				
1/2	62	24	16	0.014
3	36	12	0	
TNM				
1	28	4	2	0.029
2	50	16	10	
3	20	16	4	
Lymph node				
Yes	52	26	14	0.018
No	46	10	2	
Molecular subtype				
Luninal A/B	60	22	12	0.56
Her2	28	12	2	
Triple	10	2	2	
HER2 expression				
0/1	78	18	12	0.005
2	2	4	2	
3	18	14	2	
ER expression				
-	38	14	4	0.559
+	60	22	12	
PR expression				
-	48	20	6	0.482
+	50	16	10	
Ki-67 expression				
≥ 20%	38	15	4	0.502
< 20	60	21	12	

Loss of EphA7 expression was more often detected in patients with high grade (P = 0.014), early TNM stage (P = 0.029), and without lymph node metastasis (P = 0.018). No significant relationship between expression of EphA7 and age was observed (**Table 1**).

Expression of EphA7 was related to HER-2 status

Human epidermal growth factor receptor (HER-2) is a recognized prognostic and predictive marker in breast cancer. The amplification of HER-2 occurs in 18-20% of breast cancers. In our study, in 98 patients with reduced expression of EphA7, 78/98 (79.6%) cases were negative for expression of HER-2 receptor (score 0 and 1), 2/98 (2%) were moderate expression for HER-2 (score 2), and 18/98 (18.4%) cases were positive for expression of HER-2 (score 3). In 16 patients with increased expression of EphA7, 12/16 (75%) cases were negative for expression of HER-2 receptor (score 0 and 1), 2/16 (12.5%) cases were moderate expression for HER-2 (score 2), and 2/16 (12.5%) cases were positive for expression of HER-2 (score 3) (P = 0.005).

The expression of EphA7 was not associated with molecular subtypes of breast cancers

Based on immunohistochemical staining of ER, PR, HER-2 and Ki-67, St Gallen International breast cancer conference subtypes breast cancers as luminal A, B, HER-2, and Basal type. Although expression of EphA7 was related HER2 expression, no significant relationship was found between expression of EphA7 and ER, PR and Ki-67. The expression of EphA7 was not related to molecular subtypes of breast cancers.

Discussion

Data from experimental researches provide evidence that Eph receptor and their ephrin ligands play important roles in tumorigenesis and progression [14-16]. Although certain previous studies included expression analysis in patient samples, large-scale expression profiling for these receptors in relation to clinical outcome has been limited. In this study, we analyzed a set of 150 breast cancer tissue samples to identify the association between EphA7 and clinicopathological parameters. Our data show that EphA7 expression was reduced in most breast cancer samples (65.3%). Wang *et al* previously reported that EphA7 was down-regulated in colorectal cancers [13]. A significant reduction of EphA7 in human colorectal cancers was found by using semiquantitative RT-PCR. Wang *et al* examined the methylation status of the 5'CpG island around the translation start site in colon cell lines and found evidence of aberrant methylation. The expression of EphA7 in colon cancer cell lines was restored after treatment with 5-aza-2'-deoxycytidine. Guan *et al* examined a set of normal prostate tissues, benign prostate hyperplasias, and prostate carcinomas with quantitative RT-PCR,

methylation-specific PCR and immunohistochemistry [17]. Their data show down-regulation of EphA7 mRNA expression was detected in prostate carcinomas and hyperplasias. Methylation of the EphA7 was present in prostate carcinomas and hyperplasias. The present study is a preliminary one. We will examine the methylation status of CpG island of EphA7 in breast cancer tissue samples and analyze the significance of methylation associated with clinicopathological parameters.

Brantley *et al* investigated Eph and ephrin expression in breast cancer by using commercial human breast cancer tissue microarrays [18]. They found that EphA2, EphA4, EphA7, EphB4 and EphB6 protein were significantly higher expression in human invasive ductal carcinoma samples relative to normal, hyperplastic or fibroadenoma. As for expression of EphA7 in breast cancer, their results are inconsistent with that from our study. This can be partially interpreted that their data were from immunohistochemical staining on tissue microarray, which is absent matched normal luminal epithelial cells. This could affect the accurate analysis of expression level of EphA7 in breast cancer. In contrast, we used the operative tissue samples which including cancer and normal luminal cells from same patient. For the more, we stratified the EphA7 expression level as increased expression, non-changed and decreased expression by comparing expression level in tumor and normal. Brantley *et al* also found that RNA expression of EphA2, EphA4, and EphA7 negatively correlated with overall survival in human breast cancer. We should investigate the relationship between the EphA7 protein and survival in the next project.

The role of Eph and ephrin molecules in tumorigenesis and progression is complex and controversial, with evidence suggesting both tumor promoting and tumor suppressive functions [8, 10, 12]. In the present study, we analyzed the significance of EphA7 protein expression in breast cancers. Our data show that the expression of EphA7 was associated with grade, TNM stage, lymph node metastasis and HER2 status. However, it is paradoxical to predict the role of EphA7 in breast cancer as a tumor promoting or suppressive gene from our data. The detailed function of EphA7 in breast cancer should be intensively explored.

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Disclosure of conflict of interest

None.

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References

[1] Siegel R, Ma J, Zou Z and Jemal A. Cancer statistics, 2014. CA Cancer J Clin 2014; 64: 9-29.
[2] Nechuta S, Chen WY, Cai H, Poole EM, Kwan ML, Flatt SW, Patterson RE, Pierce JP, Caan BJ and Shu XO. A pooled analysis of post-diagnosis lifestyle factors in association with late estrogen-receptor positive breast cancer prognosis. Int J Cancer 2016; 138: 2088-97.
[3] Lang JE, Wecsler JS, Press MF and Tripathy D. Molecular markers for breast cancer diagnosis, prognosis and targeted therapy. J Surg Oncol 2015; 111: 81-90.
[4] Bertoli G, Cava C and Castiglioni I. MicroRNAs: New Biomarkers for Diagnosis, Prognosis, Therapy Prediction and Therapeutic Tools for Breast Cancer. Theranostics 2015; 5: 1122-1143.
[5] Egea J and Klein R. Bidirectional Eph-ephrin signaling during axon guidance. Trends Cell Biol 2007; 17: 230-238.
[6] Pasquale EB. Eph-ephrin bidirectional signaling in physiology and disease. Cell 2008; 133: 38-52.
[7] Zhang J and Hughes S. Role of the ephrin and Eph receptor tyrosine kinase families in angiogenesis and development of the cardiovascular system. J Pathol 2006; 208: 453-461.
[8] Kaenel P, Mosimann M and Andres AC. The multifaceted roles of Eph/ephrin signaling in breast cancer. Cell Adh Migr 2012; 6: 138-147.
[9] Surawska H, Ma PC and Salgia R. The role of ephrins and Eph receptors in cancer. Cytokine Growth Factor Rev 2004; 15: 419-433.
[10] Hafner C, Schmitz G, Meyer S, Bataille F, Hau P, Langmann T, Dietmaier W, Landthaler M and Vogt T. Differential gene expression of Eph receptors and ephrins in benign human tissues and cancers. Clin Chem 2004; 50: 490-499.
[11] Aasheim HC, Terstappen LW and Logtenberg T. Regulated expression of the Eph-related receptor tyrosine kinase Hek11 in early human B lymphopoiesis. Blood 1997; 90: 3613-3622.

[12] Wang J, Li G, Ma H, Bao Y, Wang X, Zhou H, Sheng Z, Sugimura H, Jin J and Zhou X. Differential expression of EphA7 receptor tyrosine kinase in gastric carcinoma. Hum Pathol 2007; 38: 1649-1656.

[13] Wang J, Kataoka H, Suzuki M, Sato N, Nakamura R, Tao H, Maruyama K, Isogaki J, Kanaoka S, Ihara M, Tanaka M, Kanamori M, Nakamura T, Shinmura K and Sugimura H. Downregulation of EphA7 by hypermethylation in colorectal cancer. Oncogene 2005; 24: 5637-5647.

[14] Guo C, Shao R, Correa AM, Behrens C, Johnson FM, Raso MG, Prudkin L, Solis LM, Nunez MI, Fang B, Roth JA, Wistuba II, Swisher SG, Lin T and Pataer A. Prognostic significance of combinations of RNA-dependent protein kinase and EphA2 biomarkers for NSCLC. J Thorac Oncol 2013; 8: 301-308.

[15] Fan M, Liu Y, Xia F, Wang Z, Huang Y, Li J, Wang Z and Li X. Increased expression of EphA2 and E-N cadherin switch in primary hepatocellular carcinoma. Tumori 2013; 99: 689-696.

[16] O'Malley Y, Lal G, Howe JR, Weigel RJ, Komorowski RA, Shilyansky J and Sugg SL. Invasion in follicular thyroid cancer cell lines is mediated by EphA2 and pAkt. Surgery 2012; 152: 1218-1224.

[17] Guan M, Xu C, Zhang F and Ye C. Aberrant methylation of EphA7 in human prostate cancer and its relation to clinicopathologic features. Int J Cancer 2009; 124: 88-94.

[18] Brantley-Sieders DM, Jiang A, Sarma K, Badu-Nkansah A, Walter DL, Shyr Y and Chen J. Eph/ephrin profiling in human breast cancer reveals significant associations between expression level and clinical outcome. PLoS One 2011; 6: e24426.