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Original Article

Increased expression of EphA1 protein in prostate cancers correlates with high Gleason score

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Abstract: The erythropoietin-producing hepatocellular (Eph) family of receptor tyrosine kinases regulates a multitude of physiological and pathological processes. EphA1 is the first member of Eph superfamily and is involved in carcinogenesis. The aim of this study was to investigate the expression of EphA1 in prostate cancers cell lines and the tissues, then explore the correlation with the clinicopathologic parameters. The EphA1 transcript expression in prostate cancer cell lines was detected by Quantitative real-time PCR. The expression of EphA1 protein in 138 prostate cancer tissue samples and 21 benign prostate hyperplasia samples were checked by using immunohistochemical staining. EphA1 mRNA was high expressed in LNCap, moderately expressed in 22RV1 and Du145, and lost in PC3. Loss of expression of EphA1 transcript was related to hypermethylation of CpG island around the translation start site. EphA1 protein was differentially expressed in prostate cancers and hyperplasia. Increased expression of EphA1 protein was more frequently detected in prostate cancers than in hyperplasia (P = 0.02), and more often detected in prostate cancer with high Gleason score (P < 0.001). Our data indicate that EphA1 receptor may have roles in carcinogenesis and progression of prostate cancer, and can be a potentially useful target for prognostic and therapeutic application.

Keywords: Prostate cancer, EphA1, Quantitative real-time PCR, immunohistochemistry

Introduction

Prostate cancer is the most common cancer diagnosed in male and the second leading cause of cancer death among males in the United States and western countries. Approximately 192,000 men were diagnosed and 27,000 men were expected to die from prostate cancer in 2009 [1]. The incidence of prostate carcinoma has increased markedly in developing countries including China recently, which is partly due to increased population of aged males and the change in food consumption. Despite the high incidence of prostate carcinoma, only limited data are available on genetic alterations specifically involved in its initiation and progression. It is very important to develop molecular markers that can effectively detect and distinguish the progression and malignancy of prostate tumors as well as provide insights into the development and behavior of prostate cancer.

Receptor tyrosine kinases of the Eph family and Ephrin ligands play important roles in vascular development, tissue-border formation, cell migration, axon guidance and angiogenesis. Abnormal expression of Eph receptor tyrosine kinases in cancers is related to malignant transformation, tumor metastasis, tumor differentiation and outcome. EphA1, the first member of the Eph receptors tyrosine kinase family ever discovered, was isolated from erythropoietin-producing hepatocellular carcinoma cell lines and is located on chromosome 7q34 [2]. It is widely expressed in normal tissue including lung, small intestinal, kidney, bladder, thymus and colon tissue. The level of EphA1 expression in human cancers is variable with over expression of EphA1 having been observed in certain types of tumors, including ovarian carcinoma, and squamous cell carcinoma of the head and neck, and reduced expression detected in breast carcinoma cell lines, basal cell carcinomas and squamous cell carcinomas of the skin [3]. However, the role of EphA1 in the carcinogenesis of prostate carcinoma is unknown. We previously reported finding correlation between down-regulation of EphA1 in colorectal carcino-

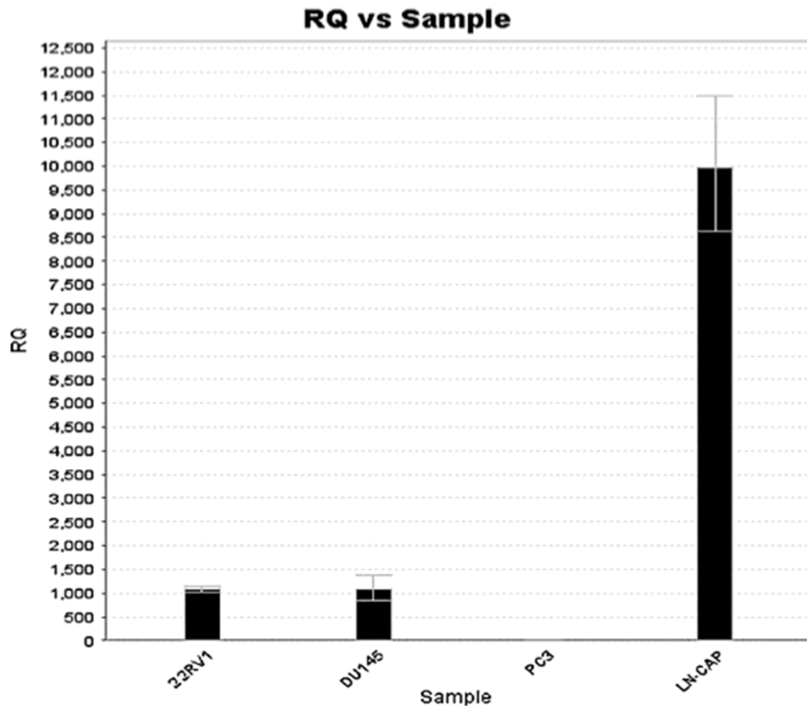

Figure 1. Expression of EphA1 transcript was detected in prostate cancer cell lines LNCap, 22 RV1, Du145 and PC3. EphA1 is highly expressed in LNCap, moderately expressed in 22RV1 and Du145 and lost in PC-3.

mas and gastric cancers and invasion and metastasis, and that reduced EphA1 expression is associated with poor overall survival [4, 5]. While another study demonstrated that epigenetic silencing of EphA1 expression in colorectal cancer is correlated with poor survival [6]. In the present study we evaluated the expression of EphA1 mRNA in prostate cancers cell lines and protein in the tissues, and then explore its association with clinicopathologic parameters.

Materials and methods

Prostate cancer cell lines and tissue samples

Prostate cell line PC-3, DU145, LNCaP, and 22RV1 were used in the present study. PC-3, DU145 and 22RV1 were cultured in RPMI 1640 medium (Invitrogen, California, USA) containing 10% fetal bovine serum (GIBCO, Invitrogen, California, USA) 100 U/ml penicillin and 100 mg/ml streptomycin in a 5% CO_2 and 95% atmosphere at 37°C, LNCaP was cultured in Ham's F12 (GIBCO, Invitrogen, California, USA).

All tissue samples were obtained between 2010 and 2012 at the Pathology Department of Jinling Hospital (Nanjing, China). 138 prostate cancer tissue samples (age ranged from 54-89 years) were obtained from patients after radical prostatectomy and 21 benign prostate hyperplasia samples (age ranged from 63-80 years) were collected from patients after transurethral resection. Formalin-fixed and paraffin-embedded tumor tissues were sectioned at 4 μm thickness and stained with hematoxylin and eosin for the pathological identification.

Quantitative real-time PCR

Quantitative real-time PCR was performed to detect the EphA1 transcript expression in prostate cancer cell lines. The protocol was used as reported before. Briefly, the sense primer and anti-sense primer for detection of EphA1 were designed according to the EphA1 mRNA sequence (GenBank accession number: NM_005232). The sense primer is 5'-ATCTTTGGGCTGCTGCTTGG-3' and the anti-sense primer is 5'-GCTTGTCCTCTCGATCCACATC-3'. The PCR products are 127 bp long. The housekeeping gene glyceraldehyde-3-phosphate dehydrogenase (GAPDH) was used as an internal control (GenBank accession number: NM_002046). The sense primer is 5'-CCAGGTGGTCTCCTCTGACTT-3' and the anti-sense primer is 5'-GTTGCTGTAGCCAAATTCGTTGT-3'. The expression level of EphA1 in prostate cancer cell lines was compared using a comparative C_T ($\Delta\Delta C_T$) as the quantitation method.

Bisulfate genomic sequencing

Two microliters of bisulfate-treated genomic DNA was PCR amplified by primer sets in a 30l reaction mixture consisting of 1x buffer (10 mM Tris-HCl, 1.5 mM $MgCl_2$, 50 mM KCl, pH 8.3), 260 of each dNTP, 400 M of primer, and 1.2 U of Taq polymerase. The PCR conditions were as

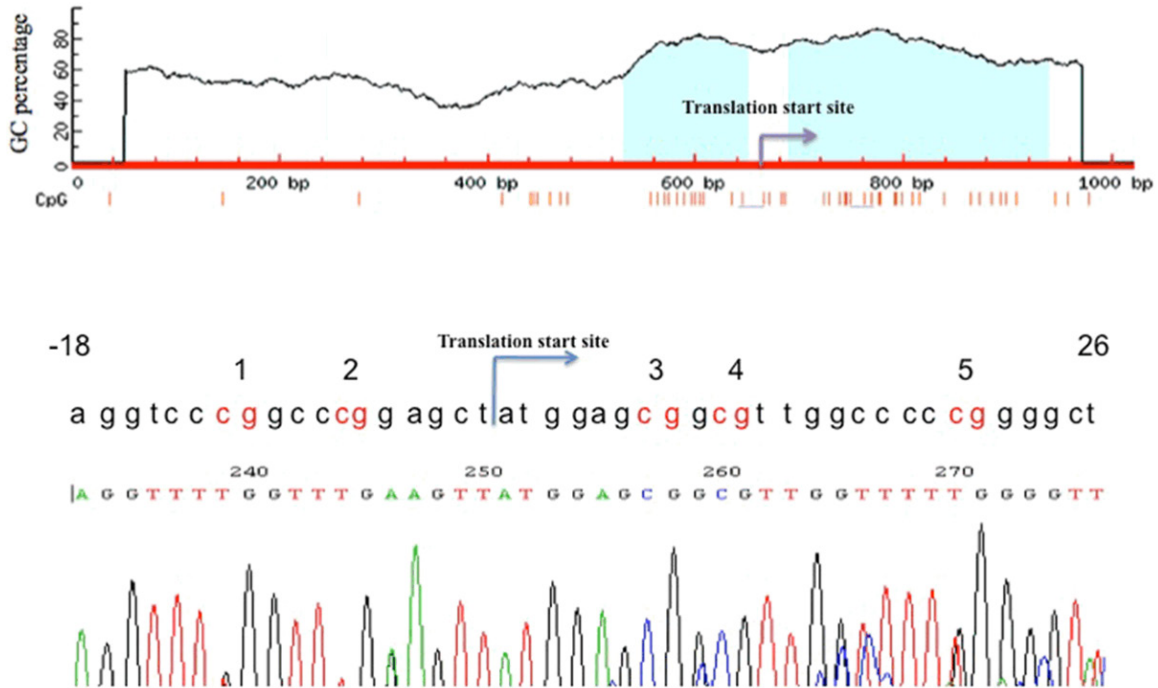

Figure 2. The methylation status of CpG island around translation start site of EphA1 in prostate cancer cell lines was checked by using direct sequencing. Up: There is a CpG island around translation start site of EphA1. Arrow shows the translation start site. Below: The sequencing shows No. 3 and 4 CG sites were methylated in PC3 cell DNA modified by sodium bisulfate.

follows: 95°C for 3 minutes, then 35 cycles of 94°C for 45 seconds, 58°C for 1 minute, 72°C for 1 minute and finally 10 minutes at 72°C. The primer sets used for bisulfate sequencing were EphA1 Mf: 5'-gttgagtttaggattagaattgg-3' (forward) and EphA1 Mr: 5'-attccctccccactc-cca-3' (reverse), PCR product = 637 bps. The direct sequencing was carried on ABI 3130 Genetic Analyser.

Immunohistochemistry

Immunostaining by the Envision method. 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 blocking non-specific sites had been blocked with 10% normal calf serum in phosphate-buffered saline for 10 min, the sections were incubated at 4°C overnight with an anti-EphA1 polyclonal antibody (ABGENT, San Diego, CA 92121, USA) at a 1:100 dilution in Antibody Diluent (ZYMED, Invitrogen, USA), then washed with PBS. Next the sections were incubated with secondary antibody (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, and any differences in the staining scores were resolved by consensus. The cytoplasm stains were considered positive. Staining intensity of EphA1 was scored using the following scale: negative, 0; weak, 1; moderate, 2; and strong, 3. Staining was semiquantitatively scored according the proportion of cells that stained by using following scale: 0, no cells stained; 1, less than 10% stained; 2, 10-50% stained and 3, more than 50% of the cells stained. The scores for expression and proportion of positive cells that stained were added. EphA1 protein expression level was assessed by comparing the score of prostate cancer cells and adjacent normal or hyperplastic prostatic gland cells.

Statistical analysis

The statistical significance of intergroup differences was evaluated by a chi-square test. All statistical analyses were performed by using SPSS 16.0 software program (SPSS, Chicago,

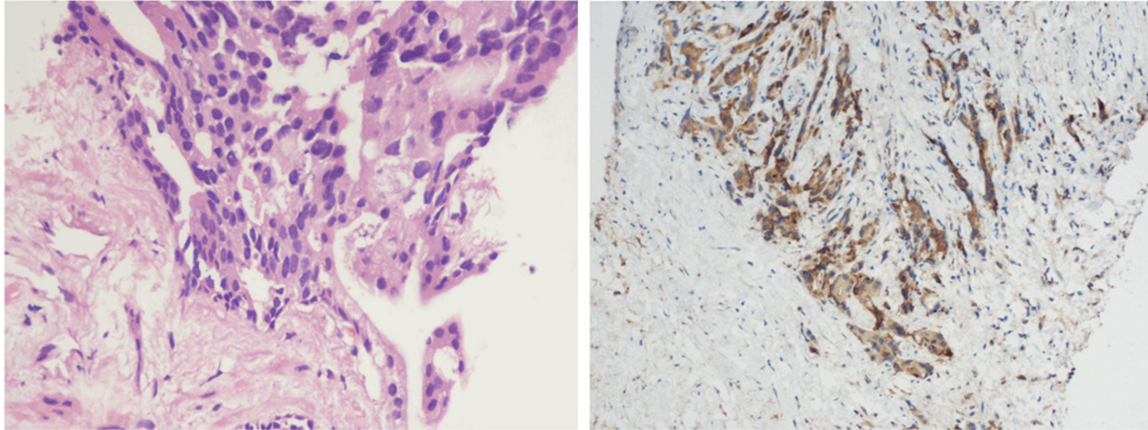

Figure 3. Immunohistochemical staining of EphA1 protein in prostate cancer with high Gleason score.

Table 1. The relation between expression of EphA1 protein and clinicopathologic parameters

EphA1 protein		Increased	No difference	Decreased	*P*
n		78 (56.5%)	42 (30.4%)	18 (13.1%)	
age	< 70 years	21	15	6	0.583
	≥ 70years	57	27	12	
TNM stage	T1-T2	48	27	12	0.903
	T3-T4	30	15	6	
Gleason score	2-6	24	6	12	< 0.001
	7-10	54	36	6	
vascular invasion	+	6	2	3	0.293
	−	72	40	15	
neural invasion	+	42	24	9	0.871
	−	36	18	9	

IL). A two-sided *P* value of less than 0.05 was considered statistically significant in all of the statistical tests.

Results

EphA1 transcript differentially expressed in prostate cancer cell lines

The expression level of EphA1 transcript was checked in four prostate carcinoma cell lines LNCap, 22RV1, Du145 and PC3. EphA1 was differentially expressed in tested prostate carcinoma cell lines (**Figure 1**). EphA1 was high expressed in LNCap, moderately expressed in 22RV1 and Du145, and lost in PC3.

Loss of EphA1 was related to hypermethylation of CpG island

There is a CpG island around the translation start site of EphA1, and the methylation status of four prostate carcinoma cell lines LNCap, 22RV1, Du145 and PC3 was checked by using direct sequencing on sodium bisulfite modified DNA. We have found that the methylated CG sites in genomic DNA of PC3 cell line (**Figure 2**). Loss of expression of EphA1 transcript was related to hypermethylation of CpG island around the translation start site.

Increased expression of EphA1 protein was more often occurred in patients with high Gleason score

The expression of EphA1 protein was detected in 138 prostate cancers and 21 benign prostate hyperplasia samples. EphA1 protein was differentially expressed inter prostate tissues samples (**Figure 3**). Increased expression of EphA1 was detected in 78 out of 138 (56.5%), decreased expression was in 18 (13.1%), and no different expression of EphA1 was in 42 out of 138 cases (30.4%). Increased expression of EphA1, decreased expression and no difference were detected in 6 (29%), 7 (33%), and 8 (38%) out of 21 prostate hyperplasia tissues correspondingly. The relation between the expression of EphA1 in prostate cancer and clinicopathologic parameters was analyzed. The increased expression of EphA1 was more

often detected in prostate cancer patients with high Gleason score ($P < 0.001$). No association of EphA1 expression with other pathological parameters was found (**Table 1**).

Discussion

The Eph family of receptor tyrosine kinases has emerged as an important player in carcinogenesis [7-16]. The expression level of EphA2 and EphB4 receptors were detected in prostate cancer cell lines and tissue samples. EphA2 is differentially expressed in prostate cancer cell lines, and over-expressed in human prostate cancers compared with benign prostate hyperplasias [17]. EphB4 is expressed in prostate cancer cell lines LNCaP, DU145 and PC3. EphB4 protein level was significantly greater in cancer as compared with matched normal epithelium, and there was a trend towards increased expression with higher grade disease [18]. EphB4 mRNA is expressed in 89% of tested prostate cancer tissues, while EphB4 protein is expressed in 66% of tumors, and 15% normal prostate tissues. EphB4 protein level is higher in PC3 cells with a metastatic potentiality [19]. The complementary expression of Eph receptors and Ephrin ligands in PC3 cells has been proved involved in migration of cells, and elevated levels of EphB3 and EphB4 could promote local invasion [7]. Yamazaki, T *et al* reported that EphA1 regulates cell morphology and motility through the ILK-RhoA-ROCK pathway [20]. EphA1 is over-expressed in hepatocellular carcinoma, elevated expression of EphA1 can promote proliferation. EphA1 receptor silencing by small interfering RNA has anti-angiogenic and antitumor efficacy in hepatocellular carcinoma [21].

In the present study, it was the first time that we examined the expression of EphA1 protein in a set of prostate cancer tissue samples and benign prostate hyperplasias, and checked the expression level of EphA1 transcript and methylation status of CpG island in four prostate cancer cell lines. Our date indicate that EphA1 is differentially expressed in prostate cancers and hyperplasias inter-samples, increased expression of EphA1 protein was found in 78 out of 138 (56.5%) patients . In benign prostate hyperplasias, increased, decreased expression and no difference were detected in 29%, 38%, and 33% correspondingly. There is a significant difference between expression pattern of EphA1 between prostate cancer and benign disease ($P = 0.02$). Increased expression of EphA1 protein was more often detected in prostate cancers compared to prostate benign diseases. The relation between expression of EphA1 protein and clinicopathologic parameters was analyzed as well. We found that increased expression of EphA1 was more often detected in patients with high Gleason score. Gleason score is considered one of the most important prognostic indictors for prostate cancer. Our data suggest a potential role of EphA1 in carcinogenesis and prognosis of prostate cancer.

Receptors tyrosine kinase of Ephs were firstly found playing a role as oncogene in carcinomas, however, data from different groups show Eph super-family may play role of tumor suppressor in certain cancers. We previously reported that EphA7 was down-regulated in colorectal cancer based on hypermethylation of CpG island [22], and differentially expressed in gastric cancer [23]. Guan *et al* reported that aberrant methylation of EphA7 in human prostate cancer [24]. More recently, we found EphA1 down-regulated in colorectal cancer and correlated with invasion and metastasis [4]. In present study, we found EphA1 may play roles as an oncogene in prostate carcinogenesis. The different roles of EphA1 protein could be interpreted as the tissue specificity. We did not found the relation between the EphA1 expression and other pathological parameters than Gleason score. This may be due to limited samples involved.

In order to explicate the mechanisms related to differential expression of EphA1 in prostate cancers, we checked the EphA1 transcript expression level and methylation status of CpG island around the translation start site in four prostate cancer cell lines. The EphA1 transcript was highly detected in LNCaP, moderately in 22RV1 and Du145, and lost in PC-3. The expression profile of EphA1 in these four prostate cancer cell lines was the same to that reported by Fox et al [12]. These four human prostate cancer cell lines were established from metastatic lesions and well characterized. We found methylation in CpG island is associated with expression of EphA1 transcript in prostate cancer cell lines. This data can in partially explain the differential expression of EphA1 protein in prostate cancers and benign

diseases. We did not investigate the methylation status of EphA1 gene in prostate cancer tissue samples, because that there were limited prostate cancer samples showed decreased expression of EphA1 (18 cases) in present study. The detailed role of methylation of EphA1 in prostate cancer need to be explored in the future.

In summery, EphA1 protein is highly expressed in prostate cancers than in benign hyperplasias, and increasingly expressed in majority of prostate cancers as compared with adjacent normal or hyperplastic prostatic gland cells. Increased expression of EphA1 protein is more often occurred in patients with higher Gleason score. Decreased expression of EphA1 in prostate cancer is associated with hypermethylation of CpG island. Our data indicate that EphA1 receptor may have roles in carcinogenesis and progression of prostate cancer, and can be a potentially useful target for prognostic and therapeutic application.

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Disclosure of conflict of interest

None.

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