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Eph receptor A10 has a potential as a target for a prostate cancer therapy

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ABSTRACT

We recently identified Eph receptor A10 (EphA10) as a novel breast cancer-specific protein. Moreover, we also showed that an in-house developed anti-EphA10 monoclonal antibody (mAb) significantly inhibited proliferation of breast cancer cells, suggesting EphA10 as a promising target for breast cancer therapy. However, the only other known report for EphA10 was its expression in the testis at the mRNA level. Therefore, the potency of EphA10 as a drug target against cancers other than the breast is not known. The expression of EphA10 in a wide variety of cancer cells was studied and the potential of EphA10 as a drug target was evaluated. Screening of EphA10 mRNA expression showed that EphA10 was overexpressed in breast cancer cell lines as well as in prostate and colon cancer cell lines. Thus, we focused on prostate cancers in which EphA10 expression was equivalent to that in breast cancers. As a result, EphA10 expression was clearly shown in clinical prostate tumor tissues as well as in cell lines at the mRNA and protein levels. In order to evaluate the potential of EphA10 as a drug target, we analyzed complement-dependent cytotoxicity effects of anti-EphA10 mAb and found that significant cytotoxicity was mediated by the expression of EphA10. Therefore, the idea was conceived that the overexpression of EphA10 in prostate cancers might have a potential as a target for prostate cancer therapy, and formed the basis for the studies reported here.

1. Introduction

The development of antibody engineering has enabled a monoclonal antibody (mAb) to become a safe and effective drug for refractory diseases, such as cancer. Today, more than 30 kinds of antibody drugs are approved all over the world. Continued growth in the market is expected in the future [1]. However, the cases to which antibody drugs are applied are limited. Therefore, the development of new antibody drugs is especially needed in the cases without effective treatments, such as a triple negative breast cancer, a castration-resistant prostate cancer, as well as pancreatic cancers or malignant mesotheliomas.

Several Eph receptor family members such as EphA2 or EphB4 are highly expressed in various tumor cell types found in refractory cancers [2], and with expressions associated with tumorigenesis [3,4], proliferation [5,6], vasculogenesis [7,8] and metastasis [9,10]. Therefore, there is a current focus on the development of therapies targeted on Eph members [11]. In this context, MedImmune LLC is developing an antibody-drug conjugate against EphA2 which inhibits tumor growth *in vitro* and *in vivo* [12,13]. It has been tested in phase I to investigate the safety profile and maximum tolerated dose. However, the most recent report announced the trial was stopped halfway due to adverse events such as bleeding and liver disorders [14]. Some databases such as MOPED or PaxDb have reported that EphA2 is highly expressed in platelets and liver tissues. Therefore, the target protein needs to display specific expression in cancer tissues. However, EphA10 which we identified as a novel breast cancer-related protein is hardly expressed in normal human tissues [15] [16]. Furthermore, we also showed that an in-house developed anti-EphA10 mAb inhibited breast cancer cell proliferation at both *in vitro* and *in vivo* levels [16]. These findings

Abbreviations: EphA10, Eph receptor A10; mAb, monoclonal antibody; TMA, tissue microarray; HMEC, human mammary epithelial cell; PrEC, prostate epithelial cell; cDNA, complimentary DNA; FCS, fetal calf serum; PBS, phosphate buffered saline; IHC, immunohistochemistry; CDC, complement-dependent cytotoxicity.

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suggest that EphA10 is a promising target for breast cancer therapy. However, the only other known report was that EphA10 is expressed in the testis at the mRNA level [17]. Therefore, the potency of EphA10 as a drug target against cancers other than the breast has not been tested. Here, we report EphA10 expression in various kinds of cancer cells and the potential of EphA10 as a target in other cancer therapies.

2. Material and methods

2.1. Cell lines

The following cancer cell lines were purchased from the American Type Culture Collection (Manassas, VA): HCC70, MDA-MB-157, HCC1599, MDA-MB468, DU4475, 22Rv1, VCaP, colo201, SW620, HCT116, BxPC3, Panc1, AsPC1, H2452, H2052, H28 and Jurkat. The following cancer cell lines were purchased from the Japanese Collection of Research Bioresources Cell Bank (Osaka, Japan): RERF-LC-KJ, RERF-LC-MS, MKN1, MKN45, NEC8, NEC14, A2058, G318, Mewo and K562. PC3 and LNCaP were purchased from the Riken Bioresource Center Cell Bank (Ibaraki, Japan). Normal Human Prostate Epithelial Cells (PrEC) and Normal Human Mammary Epithelial Cells were purchased from Lonza (Basel, Switzerland). All cells were cultured at 37 °C in a humidified atmosphere of 5% CO_2 according to the provider's protocol.

2.2. Real-time PCR

Complementary DNAs (cDNAs) derived from human prostate tumors were purchased from OriGene Technologies (Rockville, MD). The PCR mixture included cDNA template, TaqMan Gene Expression Master Mix and TaqMan probe (EphA10: Hs01017018_m1 or actin-beta: Hs99999903_m1) (Life Technologies, Carlsbad, CA) and the reaction was performed according to the manufacturer's instructions. The threshold cycles were determined using the default settings. EphA10 mRNA expression levels were normalized against actin-beta.

2.3. Cell immunofluorescent staining

PrEC, PC3 and VCaP cells were seeded at 1×10^4 cells/well in Lab-Tek™ 8-well chamber slides (Thermo Fisher Scientific Inc., Waltham, MA). After 24 h, cells were washed twice with PBS, and then fixed with PBS containing 4% paraformaldehyde, pH 8.0 for 10 min. After washing with PBS, fixation was quenched with PBS containing 0.1 M glycine, pH 7.4 for 15 min. Fixed cells were blocked with PBS containing 5% FCS (blocking solution), pH 7.4 for 30 min, and then treated with the anti-EphA10 monoclonal primary antibody and isotype control antibody at 10 μg/ml in blocking solution for 1 h. Donkey anti-mouse IgG conjugated with Alexa Flour 488 (Life Technologies, Carlsbad, CA) was used as the second antibody at 2 μg/ml in the blocking solution for 1 h in the dark. Slides were mounted using a vectashield mounting medium for fluorescence with DAPI (Vector Laboratories Inc., Burlingame, CA) and analyzed with a Leica TCS SP2 confocal laser scanning microscope (Leica Microsystems GmbH, Wetzlar, Germany). Images were further processed using the Adobe Photoshop software.

2.4. Immunohistochemical (IHC) staining

TMAs with prostate tumor and normal prostate tissues (US Biomax, Rockville, MD) were deparaffinized in xylene and rehydrated in a graded series of ethanol. Heat-induced epitope retrieval was performed by maintaining the Target Retrieval Solution (Dako, Glostup, Denmark) by following the manufacturer's instructions. After treatment, endogenous peroxidase was blocked with 0.3%. The TMA slides were incubated with rabbit anti-human EphA10 polyclonal antibody (Abgent Inc., San Diego, CA) for 30 min and then with ENVISION+ Dual Link (Dako, Glostup, Denmark) for 30 min. The reaction products were rinsed three times with 0.05% Tween20/Tris buffer saline and then developed in liquid 3,3′-diaminobenzidine for 3 min. After development, sections were lightly counterstained with Mayer's hematoxylin. All procedures were performed using an AutoStainer (Dako, Glostup, Denmark). Study samples were divided into high and low expression groups based on the two criteria of distribution and quantity. In terms of distribution, the percentage of positive cells across all tumor cells

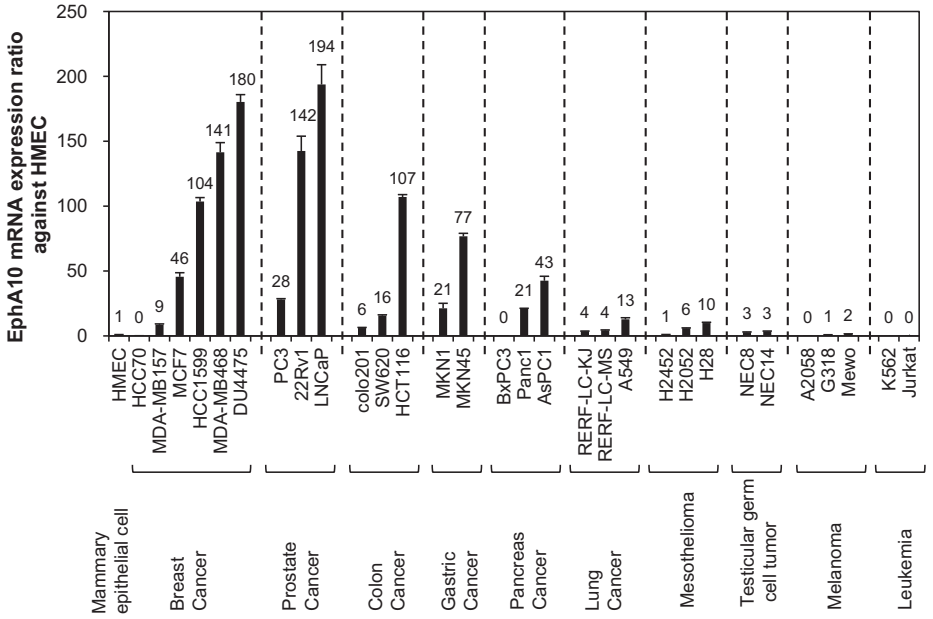

Fig. 1. Screening of EphA10 expression profile in various kinds of cancer cell lines. EphA10 expression in various kinds of cancer cells were screened by quantitative real time PCR. EphA10 expression level in each cell was normalized by actin-beta expression level and described as a ratio against EphA10 expression level in HMEC, normal human mammary epithelial primary cells. $n = 3$ in each group. Error bars represent the SD.

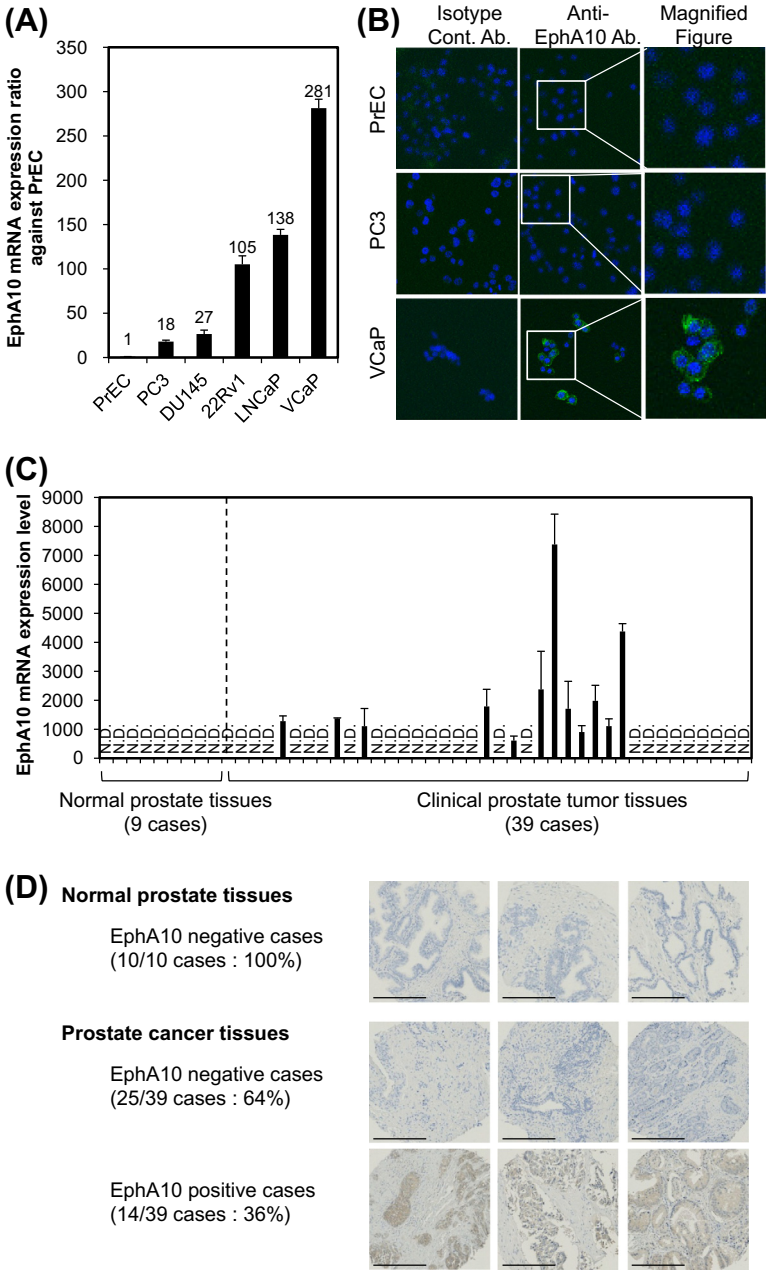

Fig. 2. EphA10 expression analysis in prostate cancer cell lines and clinical prostate cancer tissues at mRNA and protein level. (A) EphA10 mRNA expression level in prostate cancer cell lines (PC3, DU145, 22Rv1, LNCaP and VCaP) was quantified by real time PCR. It was normalized by actin-beta expression level and described as a ratio against EphA10 expression level in PrEC, normal human prostate epithelial primary cells. $n = 3$ in each group. Error bars represent the SD. (B) EphA10 protein expression in prostate cancer cell lines was analyzed by cell immunofluorescent staining. PrEC, PC3 (EphA10-mRNA low expressing cells) and VCaP (EphA10 mRNA high-expressing cells) were treated with anti-EphA10 mAb or the isotype control mAb (20 μg/ml), and then with Alexa Flour 488 conjugated anti-mouse IgG antibody. EphA10 protein expression was detected by confocal microscopy. Blue and green signals relate to DAPI and EphA10, respectively. (C) EphA10 mRNA expression levels in clinical prostate cancer tissues (39 cases) and the normal prostate tissues (9 cases) were quantified in the same method with (A). N.D. means not detectable. (D) TMAs with clinical prostate cancer tissues (39 cases) and the normal tissues (10 cases) were stained using anti-EphA10 mAb. Representative images of normal breast tissue (positive ratio: 0%), EphA10 negative cancer tissue, and EphA10 positive cancer tissues (positive ratio: 36%) are shown. Scale bar: 200 μm.

was scored as 0 (0%), 1 (1–50%), and 2 (51–100%). In terms of quantity, the signal intensity was scored as 0 (no signal), 1 (weak), 2 (moderate) or 3 (marked). Cases with a total score of more than 3 were classified into the high expression group.

2.5. Complement-dependent cytotoxicity (CDC) assay

VCaP cells were seeded at 2×10^4 cells/well in a 96 well cell culture plate (Thermo Fisher Scientific Inc., Waltham, MA) and cultured overnight. After removing the medium, antibodies (anti-EphA10 antibody or the isotype control antibody) and mouse serum as complement were added and incubated for 24 h. Cytotoxicity was evaluated using the WST-8 assay.

3. Results and discussion

3.1. EphA10 mRNA was overexpressed not only in breast cancer cell lines but also in prostate and colon cancer cell lines

In order to screen the types of cancer in which EphA10 is expressed, EphA10 mRNA expression was analyzed not only in breast cancer cells in which we had already shown EphA10

expression, but also in cell lines of colon cancer, gastric cancer, leukemia, lung cancer, melanoma, mesothelioma, pancreas cancer, prostate cancer and testicular germ cell tumors by real time PCR. EphA10 mRNA was expressed by normalizing the actin-beta expression level and represented as the ratio against normal human mammary epithelial primary cells (HMEC). Quantitative analysis demonstrated that EphA10 was expressed not only in breast cancer cells (HCC1599: 103x, MDA-MB468: 141x, DU4475: 181x), but also in prostate cancer cells (22Rv1: 142x, LNCaP: 194x) and colon cancer cells (HCT116: 107x) by more than 100 fold over human mammary epithelial primary cells (HMECs). EphA10 mRNA expression level in breast cancer cell lines was equivalent to that in prostate cancer cell lines (Fig. 1). These data suggested that EphA10 could also be associated with prostate cancers. Therefore, we next focused on prostate cancers and analyzed in more detail the expression of EphA10 at the mRNA and protein levels in cancer cell lines and clinical tissues.

3.2. EphA10 was overexpressed in prostate cancer cell lines and clinical prostate tumor tissues at mRNA and protein level

In order to examine EphA10 expression in prostate cancers, EphA10 expression at the mRNA and protein levels was evaluated in five prostate cancer cell lines (22Rv1, DU145, LNCaP, PC3 and VCaP) and normal human prostate epithelial primary cells (PrECs). Fig. 2(A) shows EphA10 was highly expressed in all cancer cell lines compared to the normal cells. Furthermore, we also analyzed EphA10 expression at the protein level in these cells. Immunofluorescent staining showed that EphA10 expression could not be detected in both PrEC and PC3 (EphA10 mRNA low-expressing cells). On the other hand, EphA10 protein expression was only observed in anti-EphA10 antibody-treated VCaP cells (EphA10 mRNA high-expressing cells), but not in the isotype control antibody-treated VCaP cells (Fig. 2(B)). These data are consistent with the pattern of EphA10 mRNA expression, further demonstrating that EphA10 was overexpressed in prostate cancer cell lines compared to the normal cells.

In order to pursue the overexpression of EphA10 in prostate cancers, we next analyzed EphA10 expression in clinical prostate cancer tissues and in normal prostate tissues. EphA10 expression at the mRNA level was first evaluated using cDNA derived from clinical prostate tumor tissues and the normal prostate tissues. A real time PCR analysis showed that EphA10 mRNA could not be amplified in all 9 normal prostate cases and 27 prostate tumor cases. In contrast, EphA10 expression was observed in 12 prostate tumor cases (approximately 31% in total cases) (Fig. 2(C)). Furthermore, we analyzed the EphA10 protein expression by IHC-staining TMA with clinical prostate cancer tissues and the normal tissues. TMA data showed that EphA10 expression was observed in 14 prostate cancer cases (approximately 36% in total cases), but not in 10 normal prostate tissues and in 25 prostate cancer cases. These data suggested that EphA10 was definitely overexpressed in prostate cancer cell lines as well as in clinical prostate tumor tissues.

We previously showed that EphA10 expression was positively associated with stage progression and lymph node metastasis in clinical breast cancers [18]. Thus, in order to evaluate the role of EphA10 overexpression in prostate cancers, we tried to analyze the relationship between EphA10 expression in clinical prostate cancer tissues and the clinical information such as the size and spread of primary tumor (pT), regional lymph node metastasis (pN), the distant metastasis (pM), and the cancer progression (pStage). Statistical analysis showed that EphA10 expression was not significantly associated with all of the above factors (Supplementary Table S1). It was reported that some Eph receptor members were overexpressed in various kinds of cancers such as breast and prostate [2], and activated by hetero-dimerizing between Eph receptors [19,20]. Therefore, in addition to focusing only on EphA10, analysis of other Eph receptors are needed in order to reveal the role of EphA10 in prostate cancers.

3.3. Anti-EphA10 mAb significantly caused complement-dependent cytotoxicity (CDC) activity dependent on EphA10 expression

In order to evaluate the potential of EphA10 as a target for prostate cancer therapy, we analyzed CDC effects of anti-EphA10 mAb on VCaP cells in which EphA10 was highly expressed. We added anti-EphA10 mAb and mouse serum as complements into VCaP cells and evaluated cytotoxicity on the next day. Fig. 3 shows that cytotoxicity in VCaP was observed only in the co-culture group of anti-EphA10 mAb and mouse serum, but not in the co-culture group of isotype control mAb and mouse serum as well as in mAb alone group. The data indicated that the cytotoxicity of anti-EphA10 mAb was dependent on EphA10 expression, and suggested that EphA10 targeted therapy might be effective in EphA10 positive prostate cancer cases.

Since molecular targeted drugs such as antibody drugs show therapeutic effects related to affinity and specificity for each antigen, it is important that the target protein display enriched expression in cancer tissues. In this respect, we previously reported that EphA10 expression was not observed in almost all normal human organs, except for testis [16]. In order to develop anti-EphA10 mAb therapy and apply it to male patients, EphA10 function in the testis should be analyzed and consider the effects of anti-EphA10 mAb on dysfunction of the testis. On the other hand, many prostate cancer patients are surgically or medically castrated with the purpose of reducing the amount of androgens which promote the growth of prostate cancer cells. However, almost all of the patients have a recurrence which is known as castration-resistant prostate cancer (CRPC). CRPCs are a bottleneck for prostate cancer therapy, because CRPCs have no effective treatment and poor prognosis. Clinical trials of antibody drugs (such as anti-CTLA4 mAb or anti-PD1 mAb) against CRPCs are currently in progress. However the therapeutic effects have been insufficient [21], emphasizing that a novel drug target is urgently needed. In this respect, EphA10 might be a promising target at least for CRPC patients, although further basic experiments are needed such as EphA10 expression analysis in CRPC cases.

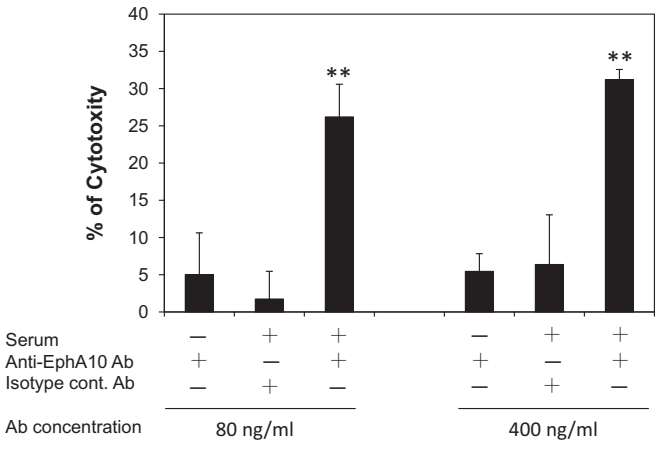

Fig. 3. Complement-dependent cytotoxicity (CDC) effects of anti-EphA10 mAb on VCaP cells. Anti-EphA10 mAb or the isotype control mAb (80 and 400 ng/ml) with/without mouse serum as complements were added to VCaP cells. After 24 h incubation, CDC effects were assessed by WST-8 assay. $^{**}p < 0.01$ vs the isotype control mAb with mouse serum. $n = 3$ in each group. Error bars represent the SD.

In conclusion, we showed that EphA10 was overexpressed in prostate cancers and suggest that EphA10 is a potential target for prostate cancer therapy.

Conflict of interest statement

The authors have no conflict of interest.

Acknowledgments

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.bbrc.2014.06.007.

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