Validation of histological diagnostic methods for detecting endothelin B receptor expression

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Abstract. Pancreatic ductal adenocarcinoma (PDAC) has an extremely poor prognosis. Recently, it was reported that the endothelin B receptor (ETBR) of tumor endothelial cells prevents antitumor immunity. However, the immunohistochemistry (IHC) conditions required to detect ETBR expression remain unclear. The aim of the present study was to confirm the appropriate conditions for IHC for ETBR using ETBR cDNA and transfectant cells and to assess ETBR expression in PDAC patients. An ETBR-expressing cell was established as an objective positive control and the detectability of ETBR expression was evaluated using several types of anti-ETBR antibodies. ETBR mRNA expression was then studied. Finally, ETBR expression was examined in human PDAC tissue using IHC. As a result, four different anti-ETBR antibodies recognized the cell surface ETBR appropriately. A non-specific reaction was shown in the detection of ETBR in normal human tissues. ETBR mRNA expression was weakly detected only in the adrenal gland. No biologically significant correlation was observed in the ETBR-IHC of human PDAC sections. In conclusion, it is necessary to perform IHC using an appropriate control to assess the tissue expression of ETBR.

Introduction

Pancreatic ductal adenocarcinoma (PDAC) is one of the most difficult-to-treat types of cancer and has an extremely poor prognosis (1,2). The only effective treatment for PDAC is surgical resection. Research and development of chemo-therapy and immunotherapy are progressing, but their clinical utility remains insufficient (1,2). To classify the poor prognosis group of PDAC, an immunohistological analysis using paraffin sections from surgical specimens was developed

as a new molecular biological method (3,4). Endothelin B receptor (ETBR) is a glycoprotein that consists of 442 amino acid residues and penetrates through the cell membrane seven times (5). It has been reported that ETBR of tumor endothelial cells prevents antitumor immunity. Specifically, ETBR overexpression prevents the endothelial barrier to T cell homing to tumors in human ovarian cancer (6). It was previously reported in our facilities that the existence of tumor-infiltrating T lymphocytes in PDAC tissue served as a significant factor related to good prognosis (3). The role of the endothelin axis in a cancer domain other than ETAR of prostate cancer has rarely been studied (7,8). Several studies have examined breast cancer (9), melanoma (10,11), glioblastoma (12), nonsmall cell lung (13), colon (14-16), gastric (17) and squamous cell carcinoma (18). Therefore, we sought to examine ETBR expression in PDAC tissue. However, no studies have reported the conditions needed for immunohistochemistry (IHC) to detect ETBR expression. Therefore, the aim of the present study was to confirm the appropriate conditions required for IHC of ETBR using ETBR cDNA and transfectant cells, as well as to assess ETBR expression in PDAC patients.

Materials and methods

Human tissue samples, culture cells and xenografts. Frozen normal human tissues were received from the Department of Gastroenterological Surgery II, Hokkaido University Graduate School of Medicine, Sapporo, Japan. PBMCs were obtained from healthy Japanese adult volunteers. HEK293 and 293FT cells were purchased from Invitrogen Corporation (Carlsbad, CA, USA). The human pancreatic cancer cell line PANC-1 was provided by RIKEN (Tsukuba, Japan), PK-9 and PK-45P were from Tohoku University (Sendai, Japan), and SUIT-2 was from Health Science Research Resources Bank (Osaka, Japan). Human pancreatic cancer xenografts were established by subcutaneous injection of these cell lines into female BALB/c-SCID mice.

ETBR subcloning. Total RNA from normal human adrenal gland was extracted by the RNeasy Mini kit (Qiagen, Tokyo, Japan). The cDNA synthesis reaction was performed as previously described (19). ETBR-cDNA was amplified by PCR. Briefly, each 50- μ l reaction mixture contained 1 μ l of reverse transcription reaction products, 1 unit of KOD-Plus-DNA

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polymerase, 5 μ l PCR buffer, 5 μ l of 2 mM deoxynucleotide triphosphate, 2 μ l of 25-mM MgSO₄ (all from Toyobo, Osaka, Japan), and 1.5 μ l of each 10- μ M 3' and 5' primer specific for ETBR (sense, 5'-CGGCTAGCCCTTCTGGAGCAGGTA-3' and antisense, 5'-CGCGGATCCTCAAGATGAGCTGTA-3'). ETBR cDNA was amplified for 30 cycles. Conditions for ETBR PCR were: 94°C for 15 sec, 40°C for 30 sec and then 68°C for 3 min. All PCR products were electrophoresed in a 2.0% agarose gel and visualized by ethidium bromide staining. Plasmids expressing ETBR were generated by the PCR amplification of ETBR cDNA and cloning into the *Nhe*I and *Bam*HI sites of pcDNA3.1(+) (Invitrogen).

Transfection. Subsequently, pcDNA-3.1(+)-ETBR-IRES-GFP (pETBR) was transfected into 293FT or HEK293 cells using Lipofectamine[®] 2000 (Invitrogen). Transfected cells were incubated at 37°C in a 5% CO₂ incubator for 20 h prior to harvest.

Semi-quantitative reverse transcription-polymerase chain reaction (RT-PCR). Total RNA extraction and cDNA synthesis of normal human tissues were performed as described above. Multiplex PCR was performed using primers specific for ETBR or glyceraldehyde-3-phosphate dehydrogenase (GAPDH). Briefly, each 20- μ l reaction mixture contained 1 μ l reverse transcription reaction products, 0.2 µl Taq DNA polymerase, 4 µl reaction buffer (both from Promega, Madison, WI, USA), 0.5 µl of 10-mM deoxynucleotide triphosphate, and 0.5 μ l of each $10-\mu M 3'$ and 5' primer specific for ETBR (as described above) and GAPDH (sense, 5'-ACCCCTTCATTGACCTCAACT-3' and antisense, 5'-TGAGTCCTTCCACGATACCAA-3'). ETBR and GAPDH cDNA was amplified for 25, 30, 35 and 40 cycles. Conditions for ETBR and GAPDH PCR were: 95°C for 30 sec, 50°C for 80 sec, and then 72°C for 5 min. All PCR products were electrophoresed in a 2.0% agarose gel and visualized by ethidium bromide staining.

Western blot analysis. Western blot analysis was performed to analyze ETBR and GFP protein expression in transfected 293FT and HEK293 cells, normal human tissues, human pancreatic cancer cell lines and xenografts. Briefly, lysates from cells, tissues and xenografts were resolved using 15% sodium dodecyl sulfate-polyacrylamide gel electrophoresis and then transferred to polyvinylidene fluoride microporous membranes (Millipore, Billerica, MA). Anti-ETBR rabbit polyclonal antibodies (Ab1, 3, 4), mouse monoclonal antibody (Ab2), and anti-GFP and anti- β -actin mouse monoclonal antibodies were used as the primary antibodies. Peroxidaseconjugated goat anti-rabbit or mouse IgG was used as the secondary antibody (1:10,000 dilution). The detection of bound antibodies was performed using the electrogenerated chemiluminescence (ECL) system (Amersham, Aylesbury, UK).

Flow cytometry. Flow cytometry was performed by FACSCalibur (BD Biosciences, Franklin Lakes, NJ, USA). Briefly, pETBR-transfected 293FT cells [293FT (pETBR)] were harvested by 0.25% trypsin, and then anti-ETBR antibodies (Ab1-4) were used as the primary antibody. PE-conjugated goat anti-rabbit or mouse IgG (Beckman Coulter, Fullerton, CA, USA) was used as the secondary antibody.

Patients and tissue specimens. Tumor specimens were obtained from the same 80 patients reported previously (3). This examination was carried out with the approval of the Hokkaido University Ethics Committee.

IHC. Immunohistochemical reactions were carried out using the universal immunoenzyme polymer method. Sections were deparaffinized with xylene, rehydrated through a graded series of ethanol/water and treated in a pressure cooker for 20 min. Endogenous peroxidase activity was blocked by 30-min incubation with 0.3% hydrogen peroxide in methanol. After washing in TBS-T, specimens were saturated with 10% normal goat serum [Histofine MAX-PO (Multi) kit; Nichirei Corp., Tokyo, Japan] for 30 min. Sections were then incubated overnight with anti-ETBR antibodies (Ab1-4) at 4°C. After three additional washes, the sections were incubated with Histofine Simple Stain MAX-PO (Multi) (Nichirei Corp.) for 20 min at room temperature. Reaction products were observed by incubation for ~5 min with 3,30-diaminobenzidine tetrahydrochloride (Nichirei Corp.). Sections were counterstained in hematoxylin for 1 min and then mounted in Marinol (micro slides; Muto-Glass, Tokyo, Japan).

Results

Establishment of ETBR-expressing cells. Three vectors were constructed as follows (Fig. 1A): pcDNA3.1(+) (pEmpty Vector), pcDNA3.1(+)-IRES-GFP (pGFP), and pcDNA3.1(+) -ETBR-IRES-GFP (pETBR). Four sense and antisense primers were synthesized (Fig. 1B), and the sequence after subcloning was confirmed (data not shown). The amino acid sequence corresponding to the ETBR base sequence was equal to the ETBR sequence described in The National Center for Biotechnology Information (NCBI) (Fig. 1C). Transfection was then performed, and the detectability of ETBR expression by western blot analysis (WB), flow cytometry and IHC was evaluated. On WB, the expression of GFP protein was confirmed in both 293FT (pGFP) and 293FT (pETBR) lysates. For the ETBR protein, bands (100, 37, 25 kDa) were shown only in the 293FT (pETBR) lysate by all four ETBR antibodies. The density of the 25-kDa band appeared different with each antibody (Fig. 2). HEK293 cell lysates showed lower density bands of the same size as compared with 293FT. In the normal human tissue sample, specific bands of the same size as the positive control [PC; 293FT (pETBR)] were not observed with all tissue samples. On the other hand, different size bands from PC were found. This tendency was different with each antibody (Fig. 2). On flow cytometry, ETBR expression of 293FT (pETBR) was observed with all antibodies. However, the ratio of ETBR positivity varied by antibody (Fig. 3). On IHC, no immunostaining was observed with the negative control [NC; 293FT (pGFP)] by all antibodies, and immunostaining was found on the membrane surface with the PC by all antibodies. No immunostaining was seen with isotype IgG (Fig. 4). This result remained the same under different pH conditions (data not shown). In normal human tissues, excluding the adrenal gland, obvious immunostaining such as 293FT (pETBR) was not shown. In the adrenal gland, the staining region (cortex or medulla) and intensity were different with each antibody (Fig. 4). These



I MQPPPSLCGR ALVALLLACG LLGVWGEERG FPPDKATPLL QTAEIMTPPT KTLWPKGSNA
61 SLARSLAPAE VPKGDRTAGS PPRTISPPPC QGPIEIKETF KYINTVVSCL VFVLGIIGNS
121 TLLRIIYKNK CMRNGPNILV ASLALGDLLH IVIDIPINVY KLLAEDWPFG AEMCKLVPFI
181 QKASVGITVL SLCALSIDRY RAVASWSRIK GIGVPKWTAV EIVLIWVVSV VLAVPEAIGF
241 DIITMDYKGS YLRICLLHPV QKTAFMQFYK TAKDWWLFSF YFCLPLAITA FFYTLMTCEM
301 LRKKSGMQIA LNDHLKQRRE VAKTVFCLVL VFALCWLPLH LSRILKLTLY NQNDPNRCEL
361 LSFLLVLDYI GINMASLNSC INPIALYLVS KRFKNCFKSC LCCWCQSFEE KQSLEEKQSC
421 LKFKANDHGY DNFRSSNKYS SS

Figure 1. Subcloning and transfection of ETBR. (A) ETBR-cDNA was synthesized, and pcDNA3.1(+) (pEmpty Vector, upper), pcDNA3.1(+)-IRES-GFP (pGFP, middle) and pcDNA3.1(+)-ETBR-IRES-GFP (pETBR, lower) were constructed. (B) Sequence analyses were performed using 4 sense (S1-4) and 4 antisense (AS1-4) primers. (C) Amino acid sequence determined from the cloning.

staining results changed under different pH conditions (data not shown).

Analysis of ETBR mRNA expression. The mRNA of normal human tissues was extracted, and semi-quantitative RT-PCR was performed with the ETBR primer. ETBR mRNA expression was weak only in the adrenal gland sample (Fig. 5).

Evaluation of ETBR expression in human PDAC cell lines and tumor specimens. On WB, no specific band of ETBR was shown by two antibodies (Fig. 6A). On IHC, immunostaining was shown only in the adrenal medulla section by Ab1, maintaining consistency between cell cultures and xenografts (Fig. 6B). Thus, it was considered that Ab1 was more suitable for IHC than Ab2 as almost all immunostaining by Ab2 was shown to be non-specific. Ab1 was then used for IHC of PDAC tumor specimens. No biologically significant staining was shown in the vascular endothelial cells and the pancreatic cancer cells. Some staining was shown in the pancreatic acinar cells and pancreatic islets (Fig. 7).

Discussion

An appropriate ETBR gene expression system was created, with 293FT (pETBR) as the PC and 293FT (pGFP) as the NC. Although the theoretical molecular weight of ETBR protein is 49.64 kDa, the results of WB revealed several bands of different sizes. Although all the bands (25, 37, 100, 200 kDa) of PC differed from the theoretical molecular weight of ETBR, it is appropriate to consider all these bands are ETBR. The PC



Figure 2. Western blot analysis of HEK293FT, HEK293 transfectants and normal human tissues by 4 ETBR antibodies (Ab1-4). G, 293FT (pGFP); E, 293FT (pETBR); Ad, adrenal gland; Li, liver; Pa, pancreas; Lu, lung; PB.PBMCs.



Figure 3. Flow cytometry of HEK293FT transfectants by 4 antibodies. Numbers represent the percentage of GFP and ETBR double-positive cells.



Figure 4. Immunohistochemistry of HEK293FT transfectants or normal human tissues by 4 antibodies. pH 7.0. G, 293FT (pGFP); E, 293FT (pETBR); Ad, adrenal gland; Li, liver; Pa, pancreas; Lu, lung; PB, PBMCs.



Figure 5. Semi-quantitative reverse transcription-polymerase chain reaction (RT-PCR) of HEK293FT transfectants or normal human tissues by ETBR primers for 35 cycles. Positive control (PC), pcDNA3.1(+)-IRES-GFP-ETBR; negative control (NC), DDW; O.L., one step ladder (marker); G, 293FT (pGFP); E, 293FT (pETBR); Ad, adrenal gland; Li, liver; Pa, pancreas; Lu, lung; PB, PBMCs.



Figure 6. WB and IHC of PDAC cell lines. (A) WB of PDAC cell lines or normal human tissues by two ETBR antibodies. (B) IHC of PDAC cell line xenografts or normal human adrenal gland tissue. C, culture cells; X, xenograft; M, marker; Lu, lung; Ad; adrenal gland; WB, western blot analysis; IHC, immunohistochemistry; PDAC, pancreatic ductal adenocarcinoma; ETBR, endothelin B receptor.



Figure 7. Statistical analysis of IHC of PDAC tumor specimens. Upper panel, positive ETBR expression; lower panel, negative ETBR expression. IHC, immunohistochemistry; PDAC, pancreatic ductal adenocarcinoma; ETBR, endothelin B receptor.

was the NC with the addition of the ETBR gene. Therefore, all bands seen in the PC that were not seen in the NC must be ETBR. Therefore, it is logical that these bands did not vary with the different antibodies.

It has been reported that ETBR can form a homodimer (20,21). In this case, the homodimer shows a size of 100 kDa, and polymerization of the homodimer or a combination of the homodimer and other membrane proteins can show a size exceeding 200 kDa. If one assumes that although ETBR homodimer is comparatively stable biochemically, monomer is unstable and is cleaved by a specific site during protein extraction, the band of ETBR may also appear with sizes of 25 and 37 kDa. Splicing variants (22,23) or glycosylation modification may explain the band size variation from the theoretical molecular weight of ETBR. Since the band of the PC differed from the theoretical molecular weight, examination of the type and concentration of reagent in the case of protein extraction was also performed. It was found that the band of the PC was similarly different from the theoretical figure.

The adrenal gland can serve as a PC of IHC under appropriate conditions due to ETBR-mRNA expression. Of the four ETBR antibodies, Abl was considered to be the most suitable antibody for IHC, as adrenal gland was stained and other tissues were not stained as much (Fig. 4). The examination results for PDAC cell lines suggest that both cultured cells and xenograft tumors may have no biologically significant expression of ETBR. The assessment data on the PDAC tumor specimens demonstrate that both pancreatic cancer cells and vascular endothelial cells have no overexpression of ETBR. Furthermore, it is possible that some intracellular enzymes may contribute to the non-specific staining observed in gland tissue such as pancreatic islets.

Based on the present results, ETBR appears to have no biological significance in PDAC tissue, as non-staining of IHC was regarded as low expression of ETBR.

In IHC, if positive and negative controls with high objectivity are not used, there can be a risk of misleading nonspecific staining for the expression of target molecules.

In conclusion, ETBR expression in human PDAC tissues may not be detected by IHC based on the strict objective controls we have established and, therefore, it was not considered to reflect the grade of malignancy of PDAC.

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