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Validation of histological diagnostic methods for detecting endothelin B receptor expression

(Endothelin B receptor の発現を評価する適切な組織学的診断手法の確立)

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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 chemotherapy 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 over-expression 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), non-small 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-µl reaction mixture contained 1 µl of reverse transcription reaction products, 1 unit of KOD-Plus-DNA
polymerase, 5 µl PCR buffer, 5 µl of 2 mM deoxynucleotide triphosphate, 2 µl of 25 mM MgSO4 (all from Toyobo, Osaka, Japan), and 1.5 µl of each 10-µM 3' and 5' primer specific for ETBR (sense, 5'-CGGCTAGCCCTTCTGGAGCAGGTA-3' and antisense, 5'-CGCGATCTCAAGATGACGTGTA-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 50°C for 30 sec. All PCR products were electrophoresed in a 1% agarose gel and visualized by ethidium bromide staining. Bands of the same size as the positive control [PC; 293FT (pETBR)] were shown only in the 293FT (pETBR) lysate by all four antibodies, and immunostaining was found on the membrane surface with the PC by all antibodies. 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 observed 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 antisense, 5'-CGGCTAGCCCTTCTGGAGCAGGTA-3' and antisense, 5'-CGCGATCTCAAGATGACGTGTA-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 50°C for 30 sec. All PCR products were electrophoresed in a 1% agarose gel and visualized by ethidium bromide staining. Bands of the same size as the positive control [PC; 293FT (pETBR)] were shown only in the 293FT (pETBR) lysate by all four antibodies, and immunostaining was found on the membrane surface with the PC by all antibodies. 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 observed 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. 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 observed 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
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 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.
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, Ab1 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 non-specific 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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References

※本論文の掲載元は、以下のリンクを参照。

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