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Article

Immunohistochemical detection of enteroviruses in pancreatic tissues of patients with type 1 diabetes using a polyclonal antibody against 2A protease of Coxsackievirus

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Simple Summary: The standard monoclonal antiserum against enterovirus envelop protein 1, clone 5D8/1, was proven to cross-react with other proteins, necessitating production of antiserum for immunohistochemical staining against enterovirus protein.
An antiserum against Coxsackievirus B-related protein for histological examination was raised by immunizing Coxsackievirus B-encoded 2A proteinase to rabbits. The antiserum was tested on sensitive and specific for 2A proteinase in human formalin-fixed tissues of enterovirus-induced type 1 diabetes pancreas and Coxsackievirus B-infected cultured African green monkey kidney Vero cells. The antiserum was proved to have extended performance on immunostaining in terms of sensitivity and specificity. The antiserum will contribute to exact and easy diagnosis of enterovirus infection and the serum will clarify the causative roles of enterovirus-derived 2A proteinase on beta cell destruction in type 1 diabetes.

Abstract: The need for antiserum for immunohistochemical (IHC) detection of enterovirus (EV) in formaldehyde fixed and paraffin-embedded (FFPE) specimens is increasing. The standard monoclonal antibody against EV-envelope protein (VP1), clone 5D8/1, was proven to cross-react with other proteins. Another candidate marker of EV proteins is 2A protease ($2A^{pro}$), which is coded by the EV gene and translated by host cells during EV replication. We raised polyclonal antiserum by immunizing rabbits with an 18-mer peptide of Coxsackievirus B1 (CVB1)-2A protease ($2A^{pro}$) and examined the specificity and sensitivity for EV on FFPE tissue samples. ELISA study showed a high titer of antibody for CVB1-$2A^{pro}$. IHC demonstrated that antiserum against $2A^{pro}$ reacted with CVB1-infected Vero-cells. Confocal microscopy demonstrated that $2A^{pro}$ labelled by the antibody located in the same cell with VP1 stained with 5D8/1. IHC demonstrated dense positive reactions pancreatic islets of EV-associated fulminant type 1 diabetes (FT1DM), and located in the same cell stained positive with 5D8/1. Specificity of IHC staining FT1DM pancreas was confirmed by absorption with an excessive concentration of immunized peptide. In conclusion, our study provides a new polyclonal antiserum against CVB1 $2A^{pro}$ which may be useful for detection of EV-infected human tissues stored as archive of FFPE tissue samples.

Keywords: Enterovirus, Coxsackievirus, 2A protease, polyclonal antibody, type 1 diabetes

1. Introduction

Recently, there has been a drastic increase in the clinical need for antibody against enterovirus (EV)-2A proteinase ($2A^{pro}$) as a diagnostic tool for immunohistochemical staining (IHC) of human diseases, including type 1 diabetes and chronic myocarditis [1-3]. $2A^{pro}$ is induced in EV-infected cells and cleaves EV preproteins to produce structural proteins required for viral replication [2].

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It is also implicated as an inflammatory factor for neighboring host proteins that trigger autoimmune cell destruction in CV-induced chronic myocarditis [4, 5] and type 1 diabetes [1]. However, there are no commercially available polyclonal or monoclonal antibodies against $2A^{pro}$ of EV for IHC analysis [6]. In addition, the gold standard antiserum against EV-capsid protein 1 (VP1) exhibits problematic cross reactions with other protein epitopes [7, 8]. Thus, the establishment of complicated optimized conditions that preclude cross-reactions with non-target protein epitopes is required in IHC analysis [7-9]. It is difficult to obtain positive results using the *in situ* hybridization (ISH) technique because EV RNA, especially in pancreatic tissues, is degraded by many digestive enzymes during the prolonged warm postmortem conditions that precede fixation of the samples [10]. In addition, the density of IHC by antibody (5D8/1) is relatively weak, which is a common characteristic of monoclonal antibodies.

We raised polyclonal antiserum against EV-$2A^{pro}$ and validated its specificity and reactivity against a CVB1-infected cell line and human pancreatic tissues with or without type 1 diabetes using IHC. We found that the polyclonal antibody proved to be specific to EV-$2A^{pro}$ and useful as a diagnostic tool for IHC of EV-infected formaldehyde-fixed paraffin embedded (FFPE) tissues, including type 1 diabetic pancreas.

2. Materials and Methods

2.1.Preparation of antigen and immunization of antigen

2.1.1. Preparation and immunization of CVB1-$2A^{pro}$ antigen

18-mer peptide of CVB1 $2A^{pro}$ (Fig. 1) was synthetized and conjugated with keyhole-limpet-hemocyanin (KLH). Two rabbits (weight 2.5 kg, age: 7 months, housed for12 hours light/dark cycles and free access to food and water) were immunized 5 times (1-month intervals) with CVB1 $2A^{pro}$ conjugated to KLH (500 μg), mixed with Complete-Freund's adjuvant (Sigma-Aldrich Japan, Japan). Prior to each injection, blood samples were obtained from the marginal vein of the rabbit ear, centrifuged at 2000 rpm for 10 min at 4°C and the sera was used to determine antibody titer by ELISA.

Serum was incubated with KHL at 20 μg/ml overnight at 4°C and spin down at 3000 rpm for 20 min at 4°C for evaluation of validity of antibody.

coxb1 (QEV81859.1)	909	gvyf**casrgkhyp**vifegpglievqeseyypkrfqshvllaagfsepgdcggilrcehgv	968
coxb3 (P03313.4)	912	gvyfcasknkhypisfegpglvevqeseyyprryqshvllaagfsepgdcggilrcehgv	971
coxb4 (P08292.4)	910	gvyfcaskskhypvsfegpglvevqeseyypkryqshvllatgfsepgdcggilrcehgv	969
ec13 (BAI87840.1)	915	gvyycasrnkhypvtfegpglvevqeseyypkkyqshvllaaglsepgdcggilrcehgv	974
ec6 (SPS68038.1)	918	gvyfcasknkhypvafegpglvevqeseyypkryqshvllaagfsepgdcggilrcehgv	967
he70 (ABA43708.1)	925	gvyyckhknrsypvcfegpgiqwinetdyyparyqtntllamgpcqpgdcggllvcshgv	984
svdvu (P16604.3)	912	gvyfcasrnkhypvtfegpglvevqeseyypkkhqshvllaagfaepgdcggilrcqhgv	971
bobev (AAF05693.1)	900	gvyfcksknkhypivvtppsifkieandyypermqthillgigfaepgdcggllrcehgv	959

cox, coxsackievirus; ec, echovirus; he, human enterovirus; svdvu, swine vesicular disease virus; bobev, bovine enterovirus.

Figure 1. Peptide sequences of Coxsackievirus B1 2A proteinase ($2A^{pro}$) immunized in this study. The region of the sequence chosen (bold red characters) exhibits low sequence homology from other enteroviruses.

2.2. Cell lines and viruses

Vero-cells which were grown in Dulbecco's modified Eagle medium (Gibco) at 37°C and 5% CO_2 were used for viral infection. Vero-cells were infected with CVB1 (strain number: 16-20289) at multiplicity of infection (MOI) of 0.1 and 3.0 for 24 hours and fixed with 5% formaldehyde-PBS for 24 hours and subjected to IHC examination.

2. 3. Validation of antibody

2. 3.1. Antiserum titer determination by ELISA

The peptide used for immunization was coated on 96 well plates for routine ELISA: 5 μg/mL CVB1 $2A^{pro}$-KLH solution was coated at 1 μL/well and incubated at 4 °C overnight. The wells were then washed 5 times with 0.05% Tween 20/PBS (PBS-T). Blocking

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was performed with 0.5% gelatin/PBS-T (200 µL/well) for 1 h. After 3 washes with PBS, antiserum diluted at a range from x500 – x7,812,500 (concentration range) of 100 µL/well was applied and incubated for 1 hour as the primary reaction. HRP-labeled anti-rabbit IgG (1:5000) was added 100 µL/well and incubated for 1 h at room temperature. After 3 washings, 0.5 mg/mL o-phenylenediamine dihydrochloride (Sigma-Aldrich Japan) was added at 100 µL/well and incubated for 20 min. The reaction was stopped by the addition of 50 µL/well 2 N H_2SO_4 and absorbance was measured at 490 nm.

2. 3. 2. IHC of formalin-fixed cultured cell tissues and FFPE tissues

The raised rabbit polyclonal antiserum (ET2112) was diluted at x100, x200, x400, x1600 and x3200. Vero-cells grown on 24-well glass plates were infected with CVB1 for 24 hours. The cell tissues were fixed with 5% formaldehyde-PBS. Cells were stained with $2A^{pro}$ antibodies (ET2112) (1:1600), VP1 antibodies (5D8/1, DAKO) (1: 400) (11) followed by an AMCA-conjugated donkey anti-rabbit IgG secondary antibody (1:50) (711-156-152, Jackson ImmunoResearch, PA, USA), Rhodamine (TRITC)-conjugated donkey anti-mouse IgG secondary antibody (1:200) (715-025-151, Jackson ImmunoResearch, PA, USA) and HRP-conjugated anti-mouse/rabbit secondary antibody Envision Kit (K4007, DAKO, Ready-to-use). IHC staining was visualized by confocal microscopy (FV 3000, Olympus, Tokyo, Japan). CVB1 incubated in in Dulbecco's modified Eagle medium (Gibco, Thermo Fisher Scientific Japan) and fixed by 5% formaldehyde-PBS were stained as a positive control. As disease control, autopsied pancreas from three patients who died 3-5 days after onset of fulminant type 1 diabetes (FT1DM) case due to diabetic ketoacidosis [10] was stained by IHC [10 -12]. The presence of CVB-RNA in the pancreatic islets and acinar cells of these FT1DM cases was ascertained by *in situ* hybridization (ISH) [13].

2. 3. 3. Sensitivity examination on FFPE tissues and formaldehyde-fixed cultured cells using IHC

IHC analysis on staining of 5% FFPE tissues or formaldehyde-fixed tissues and localization of CVB1 $2A^{pro}$ and EV VP1 was done as previously reported [10 -12]. Optimized condition for VP1 IHC staining was described [11]. The stained samples were observed by conventional fluorescent microscopy (DP73, Olympus, Tokyo) and confocal laser microscopy (FV3000, Olympus Tokyo) and images stored as photos were analyzed by two investigators and judged independently in a double-blind manner.

2.3.4. Specificity examination on FFPE tissues using IHC

Pancreatic sections of FFPE tissue were processed as first antibody with isotype-matched control rabbit IgG (1 : 1000) (X0903, DAKO) or pre-immune rabbit serum (1:1600), or in the absence of primary antibody to confirm the specificity of immunostaining. Non-diabetic human FFPE tissues including non-diabetic human autopsied donor's pancreas, thyroid glands, duodenum, liver, spleen, kidney, and colon were stained using antiserum against $2A^{pro}$ antigen.

2. 4. Ethics

Written, informed consent was obtained from the next of kin of the autopsied patients. The ethical committees of the Toranomon Hospital approved all procedures (No: 948, Date: 2017.5.23).

3. Results

3-1. Titers of antiserum against CVB1 $2A^{pro}$ by ELISAThe interaction between antiserum against CVB1 $2A^{pro}$ and the peptide used for immunization without carrier protein KHL was confirmed by ELISA, showing high specific binding (Fig. 2).

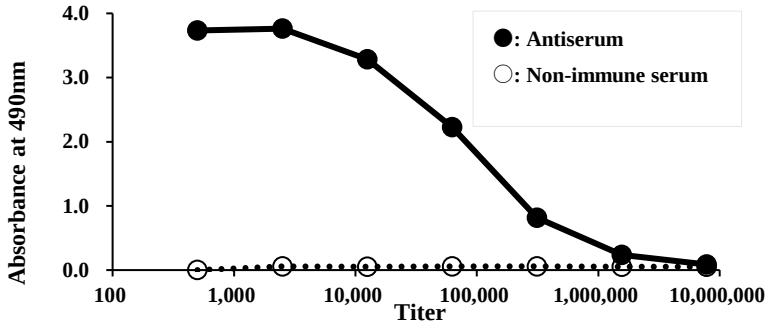

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Figure 2. ELISA analysis of interactions between antiserum against CVB1 2A^{pro} and non-immune serum and CVB1 2A^{pro} peptide immunized in the present study.

3. 2. Validation of antiserum against CVB1 2A^{pro} by IHC

3. 2. 1. 2A^{pro} in formalin-fixed tissues by IHC

Conventional and confocal fluorescence microscopy showed antiserum against CVB1 2A^{pro} stained clear immunostaining in cytosol of CVB1-infected Vero-cells for 24 hours after infection and fixed by 5% formaldehyde-PBS (Fig. 3a-i). The number of cultured cells stained for 2A^{pro} and VP1 were higher in 3.0 MOI of CVB1 infected cells than 0.1 MOI of CVB1 infected cells (Fig. 3a, b, d, e). Mock cultured cells did not show positive staining for both 2A^{pro} and VP1 (Fig. 3c, f). Confocal microscopy showed positive 2A^{pro} in cytosol of cultured cells and VP1 were stained perinuclear area in same cells in which VP1 located peri-nuclear areas and 2A^{pro} located peripheral cytosolic areas (Fig. 3g, h, i).

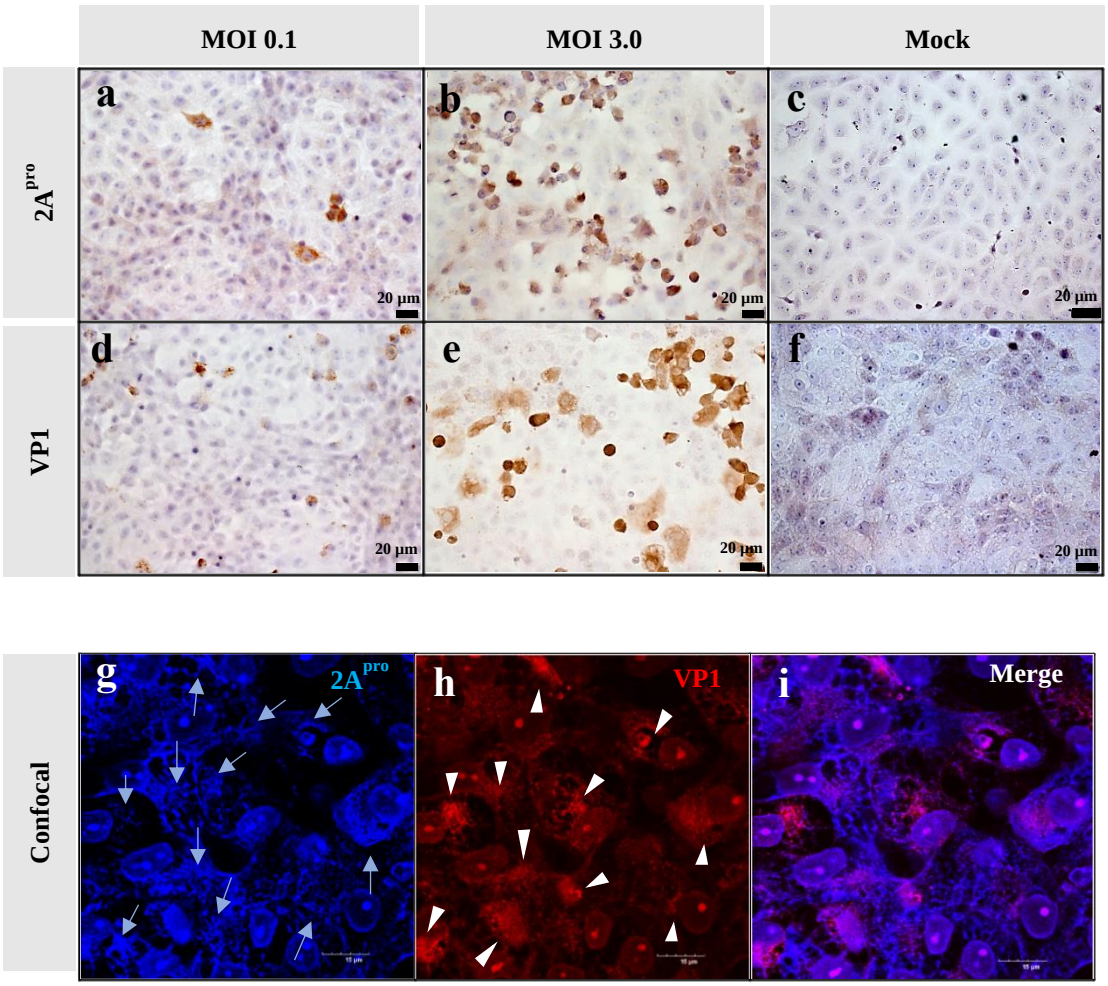

Figure 3. Polyclonal antibody ET2112 recognizes CVB1 2A^{pro} expressed in Vero-cells.

(a)-(c) Immunostaining for 2Apro antibody (ET2112) of 5% formaldehyde-PBS fixed Vero-cells incubated with CVB1. (a) 2A^{pro} is stained positive in scattered Vero-cells infected by CVB1 at MOI 0.1 and incubated for 24 hours. (b) 2A^{pro} is stained positive in some Vero-cells infected by CVB1 at 3.0 MOI and incubated for 24 hours. (c) No positive Vero-cells for 2A^{pro} in MOCK manipulated for 24 hours. Scale bar, 20 µm

(d)-(f) Immunostaining for VP1 antibody (5D8/1, DAKO) of 5% formaldehyde-PBS fixed Vero-cells incubated with CVB1. (d) VP1 was stained positive in scattered Vero-cells infected by 0.1 MOI CVB1 and incubated for 24 hours. (e) VP1 was stained positive in some Vero-cells infected by 3.0 MOI CVB1 and incubated for 24 hours. (f) No positive Vero-cells for VP1 in MOCK manipulated for 24 hour. Scale bar, 20 µm

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(g)-(i) Double immunostaining for 2Apro and VP1 in 5% formaldehyde-PBS fixed Vero-cells. (g) Cytosol of Vero-cells incubated with CVB1 at 3.0 MOI for 24 hours are stained positive for $2A^{pro}$ (allows, blue). (h) Double staining for VP1, they are characteristically stained around the nucleus (arrowheads, red). (i) Merged image of (g) and (h) shows concordant localization in the cultured Vero-cells with different cytosolic distribution. Images acquired using confocal microscopy. Scale bar, 15 µm.

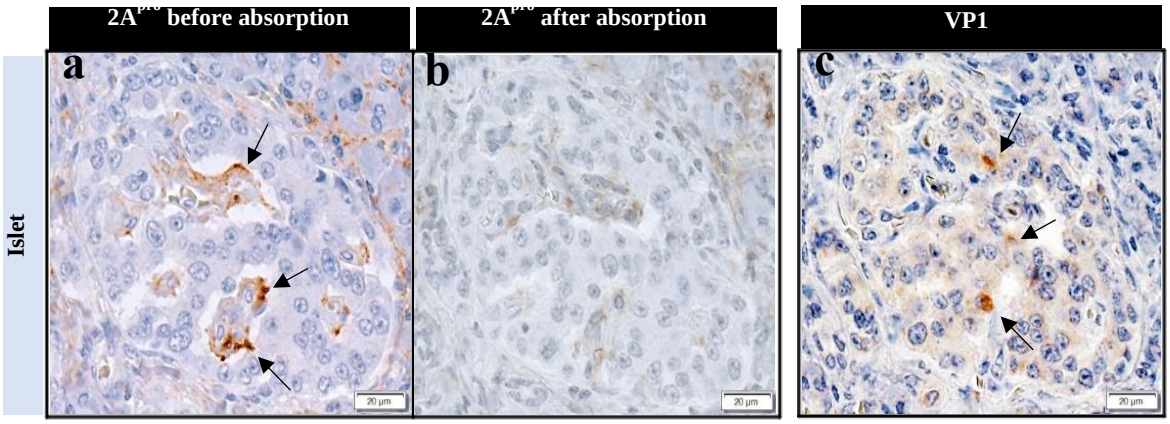

Figure 4. Absorption test of Coxsackievirus 2A proteinase ($2A^{pro}$) antibody by immunized peptide. (a) Immunohistochemical staining (IHC) for enterovirus (EV) $2A^{pro}$ in the islet of fulminant type 1 diabetes (arrows). (b) After absorption of antiserum, immunostaining of EV was completely abolished. (c) IHC staining of serial section of (a) for EV-VP1 (arrows) using 5D8D/1. Scale bar, 20 µm

3. 2. 2. IHC staining for $2A^{pro}$ and VP1 in FFPE tissues of EV-induced FT1DM

Pancreatic islets were positively stained for $2A^{pro}$ in EV-induced FT1DM (Fig.4 a). Pre-absorption with $2A^{pro}$ peptide at 10 µg/ml abolished positive immunostaining (Fig. 4b).

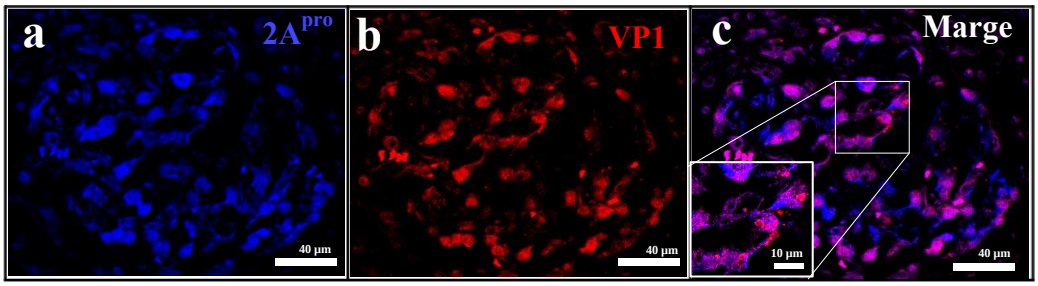

Figure 5. Double immunostaining of fulminant type 1 diabetes (FT1DM) pancreas for $2A^{pro}$ using antiserum ET2112.

a. Immunostaining of the pancreatic islet of FT1DM for $2A^{pro}$ using antiserum ET2112 (blue). Scale bar, 40 µm.

b. Immunostaining of the pancreatic islet of FT1DM for VP1 using antiserum 5D8/1 (red). Scale bar, 40 µm. c. Merged image of (a) and (b) shows that most VP1-positive islet cells are positive for $2A^{pro}$ (magenta). VP1 (red) are characteristically stained around the nucleus, while $2A^{pro}$ (blue) is stained peripheral cytosolic compartment of islet cells (inset). Scale bar, 40 µm otherwise mentioned.

Double immunostaining of FFPE-FT1DM pancreas for $2A^{pro}$ and VP1 showed concordant location of $2A^{pro}$ and VP1 in the islet cell, in which VP1 located peri-nuclear areas and $2A^{pro}$ located peripheral cytosolic areas (Fig. 5a-c), according with the findings of immune-stained CVB1 infected Vero-cells shown as Fig 3g-i.

b. CVB1 negative control tissue

Pancreases from in 14 non-diabetic controls whose islets were negative for VP1 by IHC showed negative staining for $2A^{pro}$ and VP1(data not shown). Staining of tissues from other organs than pancreases were all negative for $2A^{pro}$ and VP1 (data not shown).

Discussion

We demonstrated that a polyclonal antibody against CVB1 $2A^{pro}$ protein produced specific staining in acetone-fixed Vero-cells infected by CVB1 and the FFPE pancreatic tissues of EV-induced FT1DM. This represents an important diagnostic tool of EV IHC for FFPE tissues because there is no antiserum against CVB1 $2A^{Pro}$ for IHC staining of FFPE tissues [6]. In addition, it has been reported that the monoclonal antibody (5D8/1) (Dako) notoriously cross reacts with other protein epitopes in the pancreas [7] and requires optimized IHC conditions [8, 9]. Moreover, discordant results have been obtained between IHC and RT-PCR in acute-onset type 1 diabetic organs [14]. In general, polyclonal antibodies remain stable in various environments including alterations in pH, salt concentration, and dilution ranges associated with reduced nonspecific staining [15]. Our serum against CVB1 $2A^{pro}$ (ET2112) is polyclonal and is likely to utilize multiple epitopes that may be associated with other enterovirus subtypes, which are potentially associated with type 1 diabetes. Polyclonal antibodies are required more rigorous validation than monoclonal antibodies because they are raised against native proteins or fragment of proteins [15]. Polyclonal antibodies will be recognized multiple epitopes [15]. Some of these risks can be eliminated by absorption with immunogens used immunization of short peptide antigens. For this purpose, we synthetized short peptide of Coxsackie virus B1 $2A^{pro}$ peptide were used for antigen (Fig. 1). Furthermore, we excluded possible non-specific reaction with non-diabetic organ tissues and ascertained that antibody ET2112 dosed not react with other organ tissues.

It is generally assumed that persistent EV infection of the pancreas occurs with truncated viral RNA [16, 17] that lack active replication during the preclinical period of type 1 diabetes. EV-$2A^{pro}$ is generated during active replication of EV [2, 6]. Thus, EV-$2A^{pro}$ may be a useful marker of active EV replication in FFPE tissues, as observed in cases positive for EV-$2A^{pro}$ in the pancreas of FT1DM. An unresolved issue for EV infection in type 1 diabetes pancreas during the preclinical phase includes whether IHC with antibody raised against CVB1 $2A^{pro}$ protein is capable of discriminating between the inactive/dormant or active replicating states. In addition, further studies on pathogenetic roles of $2A^{pro}$ of EV, which potentially cleaves islet proteins and beta cell damage and subsequent autoimmunity similar to chronic myocarditis [4] await to be clarified.

Author Contributions: E. J., T. K, T. F, A. T. and S. Y. conducted to immunohistochemical staining and to the analysis of the immune-stained sample data and discussions. K.N. and S.N. prepared samples of Vero cells infected with CBV1and discussed. S.N. contributed to discussions and reviewing the manuscript. S. Y. contributed to the planning and discussions and edited the manuscript. E. J. contributed to the analysis of the data, discussions, and reviewed the manuscript. T. K. planned the study, sampled autopsied pancreases, analyzed data, and wrote and edited the manuscript. All authors approved the final version for publication. T. K. is the guarantor of this work and takes responsibility for the integrity of the data and the accuracy of the data analysis.

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Informed Consent Statement and Institutional Review Board Statement: Written, informed consent was obtained from the next of kin of the autopsied patients. The ethical committees of the Toranomon Hospital approved all procedures (No: 948, Date: 2017.5.23).

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Conflicts of Interest: The authors declare no conflict of interest.

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