

# Epipharyngeal Abrasive Therapy Down-regulates the Expression of Cav1.2: A Key Molecule in Influenza Virus Entry

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**Abstract.** *Background/Aim:* Influenza A virus (IAV) infection causes an inflammatory response to the respiratory mucosa. The viral glycoprotein hemagglutinin (HA) binds to the sialylated voltage-dependent  $Ca^{2+}$  channel (Cav1.2) in ciliated epithelium. The binding of HA and sialylated Cav1.2 is considered essential to IAV infection, entry, and IAV-induced  $Ca^{2+}$  oscillation. The epipharynx comprises the ciliated epithelium, which is the initial target for viruses that cause upper respiratory tract infections. Previously, we showed that epipharyngeal abrasive therapy (EAT), a treatment for chronic epipharyngitis in Japan, which

scratches the epipharyngeal mucosa with a cotton swab containing zinc chloride, induces squamous metaplasia. In this study, we evaluated whether squamous metaplasia by EAT affects the expression patterns of Cav1.2. *Patients and Methods:* The study subjects were seven patients who had not been treated with EAT and 11 patients who had. For the immunohistochemical assessment of the epipharyngeal mucosa, the staining intensity of Cav1.2 was described using the immunohistochemical score (IHC score). *Results:* The IHC scores for Cav1.2 in the EAT-treated group was 4.19-fold lower than those in the non-treated group ( $p=0.0034$ ). *Conclusion:* EAT down-regulates the expression of Cav1.2, a key cell surface molecule in influenza virus entry via squamous metaplasia. Thus, EAT may be a simple method for preventing influenza infection.

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**Key Words:** Epipharyngeal abrasive therapy (EAT), influenza, influenza A virus (IAV), voltage-dependent  $Ca^{2+}$  channel (Cav1.2), chronic epipharyngitis.



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Influenza A virus (IAV) is an important pathogen that causes annual seasonal epidemics around the world and often leads to unpredictable pandemics (1, 2). Hemagglutinin (HA) is a homotrimer that forms spikes on the viral lipid membrane (3). HAs from human IAVs bind epithelial cell receptors identified as glycans that are terminated by an  $\alpha$ 2,6-linked sialic acid, but no protein has been identified as a key host cell receptor for IAV so far (4, 5). Recently, it was reported that HA binds to the sialylated voltage-dependent  $Ca^{2+}$  channel Cav1.2, expressed in the airway epithelium, to

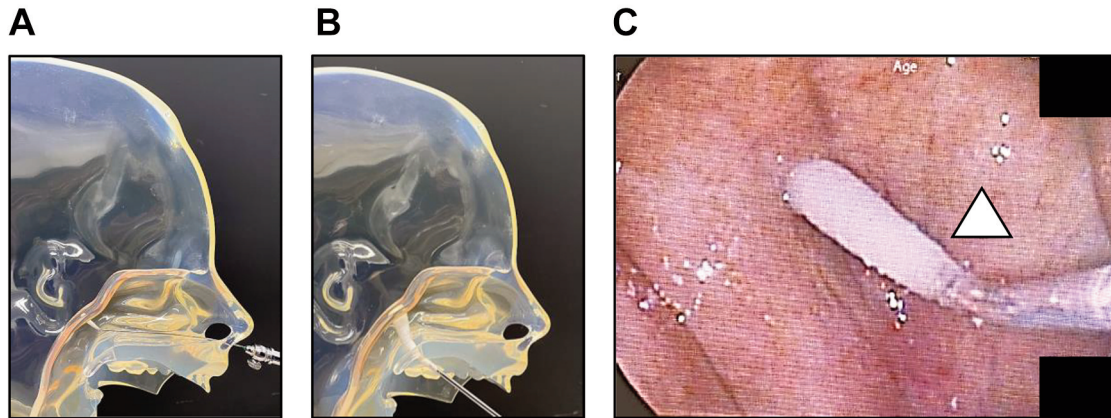


Figure 1. The method of epipharyngeal abrasive therapy (EAT). (A) Trans-nasal EAT using a sterile straight nasal cotton swab. (B) Trans-oral EAT using a pharyngeal cotton swab. (C) Endoscopic trans-nasal EAT. The entire epipharyngeal wall is scrubbed using a sterile straight nasal cotton swab soaked in 1%  $ZnCl_2$  solution. The white triangle indicates a sterile straight nasal cotton swab.

induce intracellular  $Ca^{2+}$  oscillations and subsequent IAV invasion and replication (5-7). These mechanisms suggest that the blocker of voltage-gated calcium channels (VGCC) can inhibit IAV infection (8, 9). However, since VGCC blockers have not only antiviral activity but also cardiovascular effects, further research is required for clinical application (7). The epipharynx is a main target for early virus replication of upper respiratory tract infections (URTI) (10, 11). Like other viruses that cause URTI, IAV infects humans through the ciliated epithelium in the epipharynx (12-15).

Epipharyngeal abrasive therapy (EAT) is a treatment method for chronic epipharyngitis that has been performed in Japan since the 1960s and is effective not only for suppressing local inflammation of the epipharynx but also for systemic diseases such as myalgic encephalomyelitis/chronic fatigue syndrome (ME/CFS) and Long COVID (13). EAT induces an anti-inflammatory effect on the epipharyngeal mucosa by scratching the mucous membrane with a cotton swab soaked in zinc chloride, which has a protein denaturing effect. EAT improves the severity of chronic epipharyngitis, which is determined based on endoscopic characteristics such as mucosal redness and edema and the degree of impure bleeding during EAT (13). Furthermore, continuous EAT is considered to be effective in preventing UTRI (16). In fact, we showed that continuous EAT down-regulates the expression of SARS-CoV-2 entry factors angiotensin-converting enzyme 2 (ACE2) and transmembrane protease, serine 2 (TMPRSS2), *via* squamous metaplasia (15). Since the important factors for viral invasion are present in cilia (17, 18), squamous metaplasia by EAT may reduce their expression without systemic effects induced by oral administration or infusion. In this study, to investigate whether EAT may be useful as a new method to prevent IAV infection, we confirmed the protein

expression levels of  $\alpha 2,6$ -linked sialic acid and voltage-dependent  $Ca^{2+}$  channel, a key surface molecule in influenza virus entry, in tissue samples from patients before and after EAT. EAT tended to reduce the expression of  $\alpha 2,6$  sialic acid in the epipharyngeal mucosa, but there was no significant difference in the levels before and after EAT. Importantly, we found that EAT down-regulates the expression of Cav1.2 *via* squamous metaplasia. Our results indicate that EAT inhibits IAV invasion through the epipharynx and may be effective in preventing IAV infections.

## Patients and Methods

**Patients and tissue samples.** Patient information was obtained with permission from the Ethics Committee of Fukuoka Dental College (ID: 552). The study subjects were seven patients who had not been treated with EAT and 11 patients who had, as described previously (15). All participants provided informed written consent. The research was conducted in accordance with the Declaration of Helsinki and Title 45, US Code of Federal Regulations, Part 46, Protection of Human Subjects, effective as of December 13, 2001.

**EAT.** EAT was performed as described previously (15) (Figure 1).  $ZnCl_2$  solution is commonly used as a chemical solution for EAT (13, 15, 16, 19-30). In other fields, zinc chloride is also used as a mouthwash for the treatment of oral mucositis (31, 32).

**Antibodies.** Rabbit antibody to CaV1.2 (#ACC-003) was purchased from Alomone Labs (Jerusalem, Israel). Mouse antibody to cytokeratin 13 (CK13, #NCL-CK13) was purchased from Leica Biosystems (Deer Park, IL, USA). The secondary horseradish peroxidase (HRP)-conjugated polymer anti-rabbit and anti-mouse antibodies were purchased from DAKO-Agilent Technologies Co. (Santa Clara, CA, USA).

**Immunohistochemical (IHC) staining and lectin histochemistry.** Neutral buffered formalin (10%)-fixed and paraffin-embedded tissue

blocks were cut into 4  $\mu\text{m}$  thick sections for hematoxylin-eosin and IHC staining. IHC staining was performed as previously described (15). Briefly, after antigen retrieval, each section was incubated with the primary antibody against CK13 (1:100 dilution) and Cav1.2 (1:100 dilution) at 4°C overnight. These sections were visualized using polymer detection systems. In lectin histochemistry, the sections were treated with a 0.1% hydrogen peroxide-methanol solution. Subsequently, each section was incubated with 10  $\mu\text{g}/\text{ml}$  biotin-conjugated lectins from *Sambucus sieboldiana* (SSA-biotin, J-chemical, Inc., Tokyo, Japan) for 1 h at room temperature. These sections were then incubated with HRP-conjugated streptavidin (#ab7403, Abcam, Cambridge, MA, USA) for 30 min at room temperature. The peroxidase activity was also visualized using 0.1% 3,3'-diaminobenzidine and 0.01% hydrogen peroxide in TBS. For IHC and lectin staining assessment, the staining intensity of slides was described as a staining score (scored on a scale of 0-3; 0: negative, 1: weakly positive, 2: intermediately positive, 3: strongly positive).

**Statistical analysis.** All data are expressed as the mean  $\pm$  standard error of the mean. Mann-Whitney *U*-tests were applied for comparisons between two groups. Statistical significance was set as  $p < 0.05$ .

## Results

**EAT induced squamous metaplasia without dysplasia in the epipharynx.** To confirm histological changes, focusing on the ciliary structure in the epipharyngeal mucosa due to EAT, we confirmed the changes in the expression of cytokeratin 13 (CK13) existing in mature squamous epithelial cells before and after EAT. In the EAT non-treated group, the epipharyngeal mucosa was covered with ciliated epithelium in the absence of CK13. However, the ciliated structure disappeared, and CK 13 was used to stain the cytoplasm of the keratinocytes in the areas of metaplasia in the EAT-treated group (Figure 2). No histological dysplasia has been confirmed at the squamous metaplasia site. These results show that EAT induced squamous metaplasia without dysplasia in the epipharyngeal mucosa.

**The cilia of the epipharynx highly express  $\alpha 2,6$  sialic acid, which is the binding site for IAV.** To determine the changes in the expression pattern of  $\alpha 2,6$  sialic acid, we performed lectin histochemistry using lectins from SSA, binding to sialic acid ( $\text{sia}\alpha 2\text{-}6\text{Gal}$ ), before and after EAT. The cilia of epipharynx highly expressed  $\alpha 2,6$  sialic acid in the EAT non-treated group. In the EAT-treated group, the expression of  $\alpha 2,6$  sialic acid was confirmed on the surface of the squamous metaplasia mucosa (Figure 3A). EAT tended to induce a decrease in the expression of  $\alpha 2,6$  sialic acid in the epipharyngeal mucosa, but there was no significant difference in the levels before and after EAT ( $p = 0.0630$ ; Figure 3B).

**EAT down-regulates the expression of voltage-dependent  $\text{Ca}^{2+}$  Channel Cav1.2.** To clarify whether squamous metaplasia by EAT affects Cav1.2 expression, we investigated the changes

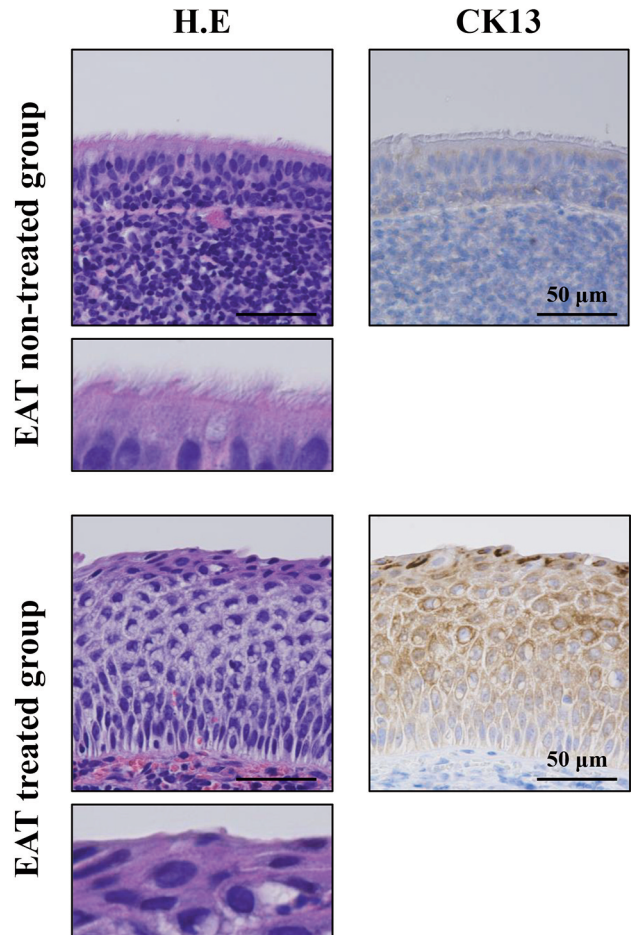


Figure 2. Protein expression patterns of Cytokeratin 13 (CK13) in patient epipharynx samples of the epipharyngeal abrasive therapy-treated and non-treated groups.

in the expression of Cav1.2 before and after EAT. The cilia highly expressed Cav1.2 at the distal end in the EAT non-treated group, whereas the expression of Cav1.2 was reduced by squamous metaplasia in the EAT-treated group (Figure 4A). The IHC scores for Cav1.2 in the EAT-treated group were 4.19-fold lower than those in the non-treated group ( $p = 0.0034$ ; Figure 4B).

## Discussion

Influenza A virus (IAV), a highly infectious respiratory pathogen causing significant morbidity and mortality across the globe, is a significant threat to global public health due to the mutation of the virus itself (33, 34). The World Health Organization estimates the potential global illness burden of influenza as up to 1 billion infections and 300,000 to 500,000 deaths annually (35, 36). The influenza

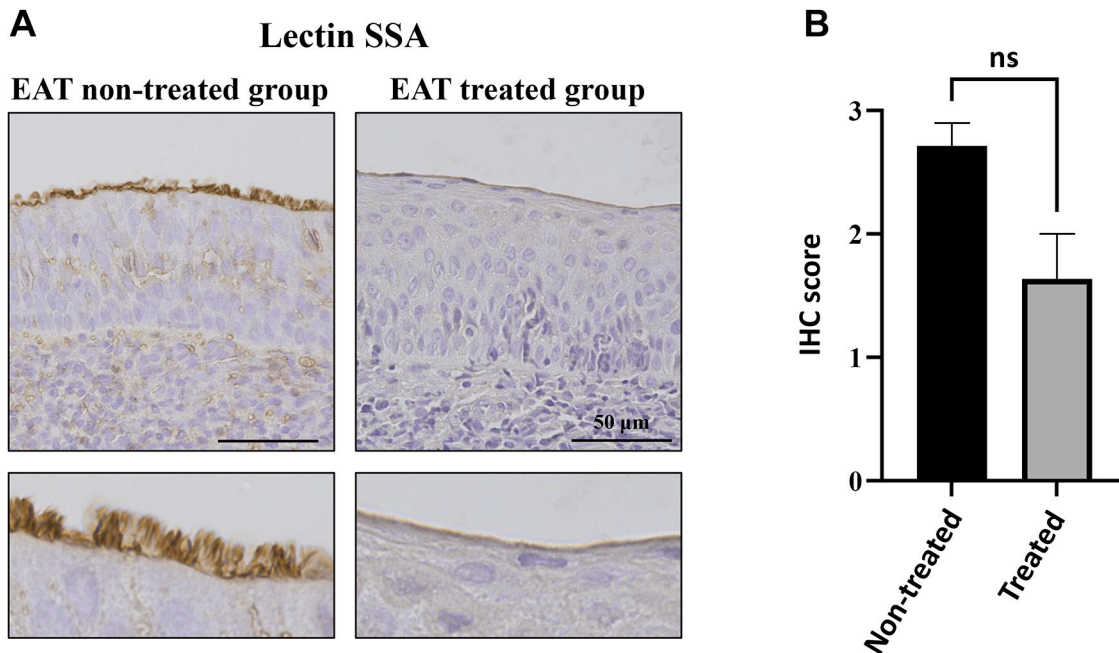


Figure 3.  $\alpha$ 2,6 Sialic acid expression in patient tissue samples without and with epipharyngeal abrasive therapy (EAT). (A) Sialic acid in  $\alpha$ 2,6 linkage was recognized with biotinylated lectin *Sambucus sieboldiana* (SSA). (B) Immunohistochemical (IHC) scores for lectin SSA expression on the epipharyngeal mucosa of the EAT-treated ( $n=11$ ) and non-treated groups ( $n=7$ ). ns: Not significant.

virus binds to the specific sialic acid on the cell surface *via* viral surface protein hemagglutinin (HA) to establish viral infection (37). The HA of influenza virus adapted to humans recognizes  $\alpha$ -2,6 sialic acid (SA) on airway ciliated epithelial cells (38-40). Like previous reports, we showed that the cilia of the epipharynx highly expressed  $\alpha$ -2,6 SA as shown by lectin histochemistry using lectins from SSA, a strong binder to  $\alpha$ -2,6 SA (Figure 3A) (41, 42). After IAV binds to the sialic acid residue of the host cell, receptor-mediated endocytosis occurs. However, it is not known which sialic acid-containing receptors are essential for IAV infection (5).

$\text{Ca}^{2+}$  is essential for virus entry, viral gene replication, and severity of the infection (43). Thus, interfering with virus-induced abnormal intracellular  $\text{Ca}^{2+}$  homeostasis may be a useful strategy in the development of antiviral drugs (7). The voltage-dependent  $\text{Ca}^{2+}$  channel's Cav1.2 proteins are the important proteins involved in electrical functions of the cell (44). The interaction of the influenza A virus hemagglutinin (HA) with host sialic acids linked to sialylated Cav1.2 on the surface of air epithelium cells induces  $\text{Ca}^{2+}$  oscillations, which trigger the initiation of virus endocytosis (5, 45). Consequently, targeting Cav1.2 required by virus infection is considered a treatment strategy with therapeutic potential, which may avoid the development of resistance (7, 46, 47).

EAT was originally developed as a treatment to reduce chronic epipharyngitis, which causes various upper

respiratory tract symptoms in Japan (13, 26). Rhinovirus, which is the primary cause of upper respiratory tract infections (URTI), is usually first detected at the epipharyngeal site, with subsequent spread of infection anteriorly to inferior turbinates, making the epipharynx an important target site for URTI (11, 48). In addition, the high prevalence of chronic epipharyngitis in patients with Long COVID after infection with SARS-CoV-2, suggests the importance of the epipharynx in the prevention of URTI (13). The mechanisms of EAT have been reported to be the anti-inflammatory effect of  $\text{ZnCl}_2$ , the blood-letting effect, and vagus nerve stimulation (25). As another mechanism of EAT, we showed that squamous metaplasia and suppression of the aggregation of inflammatory cells with submucosal fibrosis by EAT may be effective in preventing pharyngeal allergy and viral infection (15, 49). These histological changes are also confirmed in the nasal mucosa after laser treatment, aimed at suppressing antigen invasion by squamous metaplasia of the respiratory epithelium in allergic rhinitis, which is an important mechanism of EAT. In addition, EAT down-regulates the expression of SARS-CoV-2 entry factors ACE2 and TMPRSS2 *via* squamous metaplasia (15). Similarly, in this study, we found that EAT down-regulates the expression of ciliary Cav1.2, a key cell surface molecule in influenza virus entry, in the areas of squamous metaplasia, which express cytokeratin 13, highlighting the mature squamous epithelial cells (Figure 2

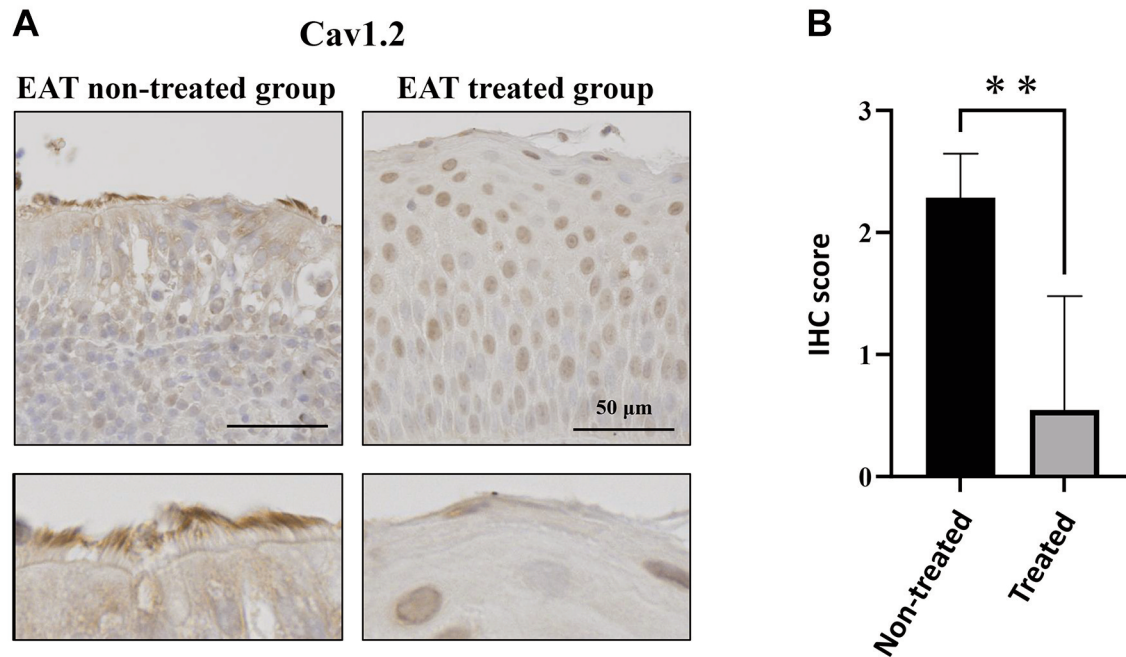


Figure 4. Protein expression patterns of Cav1.2 in patient tissue samples without and with epipharyngeal abrasive therapy (EAT). (A) Cav1.2 expression in the epipharynx of the EAT-treated and non-treated groups. (B) Immunohistochemical (IHC) scores for Cav1.2 expression on the epipharyngeal mucosa of the EAT-treated ( $n = 11$ ) and non-treated groups ( $n = 7$ ). \*\*Significantly different at  $p < 0.01$ .

and Figure 4) (50, 51). The  $\text{Ca}^{2+}$  channel blocker was reported to inhibit IAV infection in an *in vivo* study using mouse and *ex vivo* (5). On the other hand, the cardiovascular effect of voltage-gated calcium channel blockers may limit their antiviral application, and they therefore require further analysis before clinical trials (7). In these circumstances, EAT is a simple method of reducing local Cav1.2 expression. Similar to previous reports that normal squamous epithelium expresses  $\alpha 2,6$  sialic acid on the surface (52),  $\alpha 2,6$  sialic acid involved in IAV binding is present in the squamous metaplasia site (Figure 3), but it is speculated that endocytosis targeting cells is suppressed due to decreased Cav1.2 expression (5). Additionally, EAT down-regulates TMRSS2 (15), which is the major HA-activating protease of IAV in human airway cells and a potential target to treat influenza infections (53, 54). Influenza vaccination is the primary strategy for the prevention and control of influenza (55, 56). However, the seasonal influenza pandemic is caused by new influenza virus variants that emerge due to frequent antigenic drift, and annual vaccine adjustments are therefore required to prevent infection (57). On the other hand, approaches targeting the inhibition of IAV-Cav1.2 binding may also be effective against a variety of serotypes and drug-resistant strains (5). Based on the histological changes in this study, we recommend continuous EAT at least once a week for 3 months. Zinc has antibacterial activity against a variety of bacteria, and the Scientific

Committee on Consumer Safety considers that it is safe to use water-soluble zinc salts for toothpaste from the age of 6 months (58, 59). Zinc chloride used in EAT has been used as a mouthwash for the treatment of oral mucositis in cancer patients undergoing chemotherapy (31, 32). Regarding adverse events of EAT, patients may be aware of the temporary pain due to scratching during EAT, but there are no cases of significant side effects in multiple clinical research on EAT (13, 26, 27). Although the duration of the effect of EAT has not been investigated, laser treatment for allergic rhinitis that induces similar mucosal histological changes is often effective for 6 months or longer (60). Thus, it is presumed that the effect of continuous EAT will last for at least several months.

It is necessary to clarify in future research whether the mechanism of squamous metaplasia by EAT is due to the protein denaturing action of zinc chloride, physical stimulation by scratching, or both. Further clinical studies are needed on whether EAT suppresses the IAV infection rate; however, EAT may be useful as a new method to prevent IAV infection through suppressing the expression of Cav1.2.

### Conflicts of Interest

The Authors declare that they have no competing interests in relation to this study.

## Authors' Contributions

Conceptualization, K.N.; methodology, K.N., H.M., and T.Y.; validation, S.Y. (Shohei Yoshimoto); formal analysis, S.Y. (Shohei Yoshimoto); investigation, S.N., T.N. (Tatsuro Nishi), R.N. and H.T.; data curation, K.N.; writing—original draft preparation, K.N.; writing—review and editing, T.T.; supervision, O.H., S.Y. (Susumu Yasumasu), K.H., S.S., and T.N. (Takashi Nakagawa); project administration, K.N. All Authors have read and agreed to the published version of the manuscript.

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