Autophagy-related 16-like 1 is influenced by human herpes virus 1-encoded microRNAs in biopsy samples from the lower esophageal sphincter muscle during per-oral endoscopic myotomy for esophageal achalasia

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Abstract. Esophageal achalasia is characterized by abnormal peristalsis of the esophageal body and impaired relaxation of the lower esophageal sphincter (LES); however, its etiology remains unknown. One of the potential causes of esophageal achalasia is herpes simplex virus type 1 (HSV-1). Following infection with HSV-1, a complex interaction between the autoimmune and inflammatory responses is initiated. Viral microRNAs (miRNAs/miRs) serve a crucial role in this interaction. In the present study, the expression of E3 ubiquitin-protein ligase component n-recognition 1 (*UBR1*) and autophagy-related 16-like 1 (*ATG16L1*) was assessed in patients with sporadic and classic achalasia as potential targets of the viral miRNAs. We assessed the mRNA levels of target transcripts using reverse transcription-quantitative PCR. *UBR1* expression was slightly decreased, although the difference was not significant. However, *ATG16L1* expression was significantly decreased in the LES. In conclusion, *ATG16L1* expression was reduced in the LES of achalasia patients; therefore, *ATG16L1* might be a target of HSV1-miR-H1, and its reduction could be related to the disease mechanism.

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Introduction

Esophageal achalasia is an archetypal esophageal motility disorder, which is characterized by impaired relaxation of the lower esophageal sphincter (LES) and abnormal peristalsis of the esophageal body. Esophageal achalasia can result in an impaired ability to digest food and can thus reduce a patient's quality of life (1-3).

Although this disease was first reported ~300 years ago, its etiology remains unknown (4). Current treatment strategies aim to reduce LES pressure. Methods of treatment include endoscopic balloon dilation, botulinum toxin injection, laparoscopic Heller's myotomy, and in serious advanced cases, surgical resection of the affected esophagus (5). However, in recent years, per-oral endoscopic myotomy (POEM) has been established as an alternative minimally invasive method of treating esophageal achalasia (6). This treatment is effective and safe, even in elderly patients, allowing for short and long-term prognoses (6,7). Sato *et al* (8) performed per-oral endoscopic biopsies from the muscle layer during POEM, called POEM-b. According to their study, histopathological and immunohistochemical analysis of POEM-b samples showed neurodegenerative signatures rather than inflammatory infiltrates in the muscular layer (8). There was a tendency for preservation of interstitial cells of Cajal in patients with type III achalasia, whereas more severe fibrosis was observed in patients with type I achalasia, based on the Chicago classification criteria high-resolution manometry (HRM) (8,9).

Currently, the proposed causal factors are varied and multifactorial, and are hypothesized to involve complex interactions between the autoimmune and inflammatory response, and this may be initiated by viral infections in patients who are genetically susceptible (2). Causal viral agents include (but not limited to) herpes simplex virus, which is a neurotropic virus that exhibits a predilection for squamous epithelium, as well as varicella-zoster, measles and human papillomavirus (4,5,7,10). Moreover, it has been reported that HSV type 1 (HSV-1) DNA and RNA is detectable in all tissues from patients

with achalasia, but not in the control tissues (11). Therefore, HSV-1 infection may be considered particularly relevant in the development and/or progression of achalasia.

microRNAs (miRNAs/miRs) are single-stranded RNAs that regulate gene expression and serve crucial roles in numerous physiological and pathological processes, including viral infections and antiviral response (12-15). Certain viruses, particularly herpes viruses (including HSV-1), express miRNAs, although their pathological roles are not completely understood (12,15). In our previous study, it was shown that expression of HSV1-miR-H1-3p in the esophageal mucosa of patients with achalasia was increased (1). However, the analysis and understanding of the miRNA expression profiles in the muscular layer of the LES are still being elucidated. HSV1-miR-H1 can directly target E3 ubiquitin-protein ligase component n-recognition 1 *in vitro* (*UBR1*) (14). This miR-mediated downregulation of the ubiquitin-proteasome system results in the accumulation of neurodegenerative-associated protein fragment β amyloid (15). Subsequently, the autophagy pathway is influenced, which is essential for host defense against viral infection; in particular, autophagy-independent antiviral functions of autophagy-related genes (ATGs) have been reported to be activated (16-18). For example, ATG 16-like 1 (ATG16L1), which is part of the ATG5-ATG12-ATG16L1 complex, is activated following interferon-γ treatment (18). Finally, autophagy also increases interleukin-1β (IL-1β) secretion (19).

The aim of the present study was to analyze the mRNA expression levels of *UBR1*, *ATG16L1* and *IL-1B* as potential targets for viral miRNAs, and to investigate the mechanisms underlying onset of achalasia.

Materials and methods

Ethical considerations. Written informed consent was obtained from all patients. The study protocol followed the ethical guidelines of the Declaration of Helsinki and was approved by the Nagasaki University Ethics Committee (approval no. 110328329).

Per-oral endoscopic muscular biopsy sampling during POEM. The standard POEM procedure was performed as previously described (6). Briefly, the following steps were followed: Submucosal injection and mucosal incision, submucosal tunneling, selective myotomy for the inner circular muscle and then closing of the mucosal entry. All patients who underwent POEM were under general anesthesia and endotracheal intubation with positive pressure ventilation, and included patients who underwent surgery between October 2011 and June 2012 at the Showa University Koto-Toyusu Hospital. Patients with any severe underlying illnesses, such as cancer, or those who could not tolerate general anesthesia due to other diseases were excluded. Each patient was diagnosed with sporadic and classic achalasia by routine analysis, including barium follow through, upper gastrointestinal endoscopy and manometry. An incision was subsequently made in the circular muscle bundle from the entrance to the LES, where the two muscular biopsies were performed using both ends of the biopsy forceps. As controls, biopsy samples were collected from the LES of patients whose excised esophagogastric junction (EGJ) was used. The control group consisted of patients with esophageal cancer requiring surgical resection whose cancer lesions did not reach the LES. Patients were successfully treated with esophagectomy in all control patients, and immediately after removal of the esophagus, including the LES, they deployed the resected specimens in a longitudinal direction. Positional identification of the EGJ in the macroscopic findings was uncomplicated. Following confirmation from a physician endoscopist with extensive experience in the POEM procedure, ~2 mm of tissue was collected from the inner circular muscle at the position where the LES appeared to be directly above the EGJ from the mucosal side using a pointed blade. Patients in the control group did not undergo evaluation of esophageal peristalsis by HRM, but medical examinations, including barium follow-through, did not show any symptoms or signs suggestive of abnormal esophageal motility. All samples were immediately placed in 1 ml RNAlater® reagent (Ambion; Thermo Fisher Scientific, Inc.) and stored at -80˚C until subsequent RNA isolation. Reverse transcription-quantitative (RT-q)PCR was performed on samples from 6 control (5 males and 1 female; age range 35-69 years; median age, 66) and 11 achalasia cases (7 males and 4 females; age range 27-78 years old; median age, 40, which included 6 smokers). Based on the Descriptive Rules for Achalasia of the Esophagus (20), there were 8 straight-types and 3 sigmoid-types; and one patient with grade I achalasia and 10 patients with grade II achalasia.

RT-qPCR. cDNAs were prepared from total RNA using a High-Capacity cDNA Reverse Transcription kit (cat. no. 4374966, Thermo Fisher Scientific Inc.). Reverse transcription reactions were performed in reactions containing 5 μl total RNA, 1x RT buffer, 4 mM dNTP mix, 1x RT random primers, 50 units MultiScribe™ reverse transcriptase, 20 units RNase inhibitor and nuclease-free water added up to 20 μl. Reactions were performed at 25˚C for 10 min, followed by 37˚C for 120 min and 85˚C for 5 min. Primer sequences for quantitative PCR were as follows: *UBR1* forward, 5'-CTTCGCTGTGCT GCATTGTT-3' and reverse, 5'-TCTAGGGTACCTGACCAC GG-3'; *ATG16L1* forward, 5'-CAGGCACGAGATAAGTCC CG-3' and reverse, 5'-AACTCCCCACGTTTCTTGTGT-3'; *IL-1β* forward, 5'-CAGCTACGAATCTCCGACCAC-3' and reverse, 5'-GGCAGGGAACCAGCATCTTC-3'; and *β-actin* forward, 5'-GCATCCTCACCCTGAAGTA-3' and reverse, 5'-TGTGGTGCCAGATTTTCTCC-3'. qPCR reactions were performed in 20 μl aliquots containing 1 μl RT product with 4 μl LightCycler® FastStart DNA MasterPLUS SYBR Green I (cat. no. 03515869001, Roche Diagnostics,), 0.5 μM of each primer and 14.6 μl nuclease-free water. The reactions were carried out in a Real Time PCR LightCycler 1.5 Complete system (Roche Diagnostics). The thermocycling conditions were: Denaturation at 95˚C for 10 min; followed by 45 cycles of 95˚C for 10 sec, 60˚C for 10 sec and 72˚C for 10 sec. The quantification cycle (Cq) was recorded for mRNA amplification using LightCycler Software version 3.5.28 (Roche Diagnostics), and *β-actin* was used as an endogenous control for data normalization. Relative expression was calculated using the following formula $2^{-\Delta\Delta Cq}=2^{-(\Delta Cq,\ \text{reagent treatment}-\Delta Cq,\ \text{control})}$.

Statistical analysis. Differences between two groups were compared using an unpaired one-tailed Student's t-test. Data

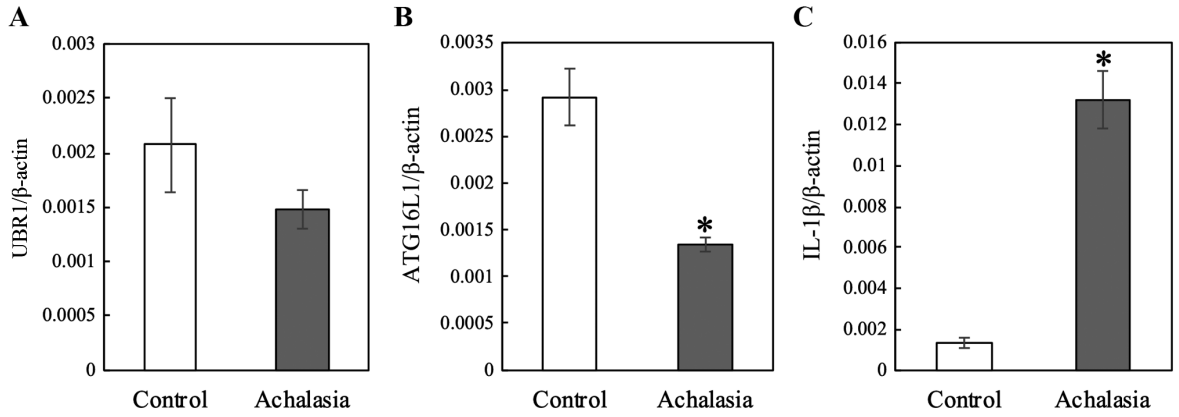

Figure 1. Relative mRNA expression levels. mRNA expression levels of (A) *UBR1*, (B) *ATG16L1* and (C) *IL-1β* using *β-actin* as the endogenous control. *P<0.01. *UBR1*, E3 ubiquitin-protein ligase component n-recognition 1; *ATG16L1*, autophagy-related 16-like 1; *IL-1β*, interleukin-1β.

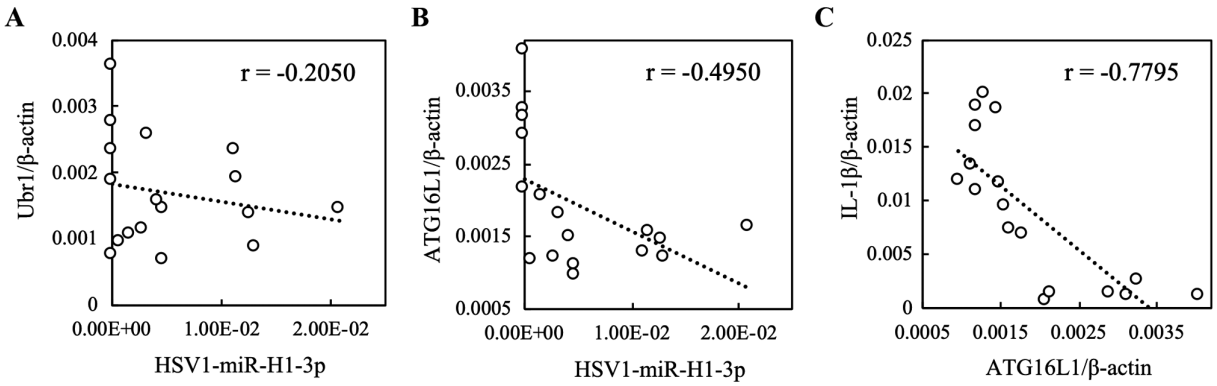

Figure 2. Correlation coefficient analysis. Correlation between (A) HSV1-miR-H1 and *UBR1*, (B) HSV1-miR-H1 and *ATG16L1*, and (C) *ATG16L1* and *IL-1β*. *UBR1*. E3 ubiquitin-protein ligase component n-recognition 1; *ATG16L1*, autophagy-related 16-like 1; *IL-1β*, interleukin-1β.

are presented as the mean ± standard error of the mean. Correlations were calculated using Pearson's correlation coefficient analysis. Statistical analysis was performed using StatFlex version 7 (Artec Co., Ltd.). P<0.05 was considered to indicate a statistically significant difference.

Results

RT-qPCR was performed to assess the mRNA expression levels of UBR1, ATG16L1 and IL-1β in samples from 6 controls and 11 achalasia patients. The duration of disease in the achalasia patients was 3-240 months; mean, 100 months and 3 patients complained of chest pains. Based on the Descriptive Rules for Achalasia of the Esophagus (20), there were 8 straight-types and 3 sigmoid-types; and one patient with grade I achalasia and 10 patients with grade II achalasia. *UBR1* mRNA levels were decreased, although not significant, (P=0.1561), and the expression levels of *ATG16L1* were significantly decreased (P=0.0028) in the LES of patients compared with the control (Fig. 1). In contrast, *IL-1β* expression was significantly increased (P<0.0001) in the LES of patients compared with the control (Fig. 1). In our previous study, it was shown that relative HSV1-miR-H1-3p expression levels were significantly higher in the LES samples of patients with achalasia compared with the controls (1). The cohort used in the present study was the same as that used in our previous study (1). As shown in Fig. 2, the correlation between hsv-miR-H1-3p and *UBR1* was not observed (r=-0.2050; P=0.4300). However, a weak correlation was observed between HSV1-miR-H1-3p and *ATG16L1* (r=-0.4950; P=0.0434). Furthermore, a strong correlation was observed between *ATG16L1* and *IL-1β* (r=-0.7795; P=0.0002; Fig. 2).

The relationship between the mRNA expression levels and the patients' clinical parameters were assessed. A positive correlation was found between patient age and *IL-1β* expression (r=0.6031; P=0.0495; Fig. 3). However, *UBR1* and *ATG16L1* levels were not significantly associated with age; there were no significant associations between the expression levels of the three mRNAs assessed with sex, smoking status, type of achalasia or duration of the disease (Fig. S1).

Discussion

Esophageal primary achalasia is characterized by aganglionosis or loss of myenteric neurons (2). These features are predominantly caused by the degeneration of inhibitory neurons in the Auerbach's plexus (20). At present, causal factors of idiopathic achalasia are diverse and multifactorial, and they may be involved in complex interactions of autoimmune responses, degenerative neuronal processes, viral infections and the genetic susceptibility of individuals (1,4).

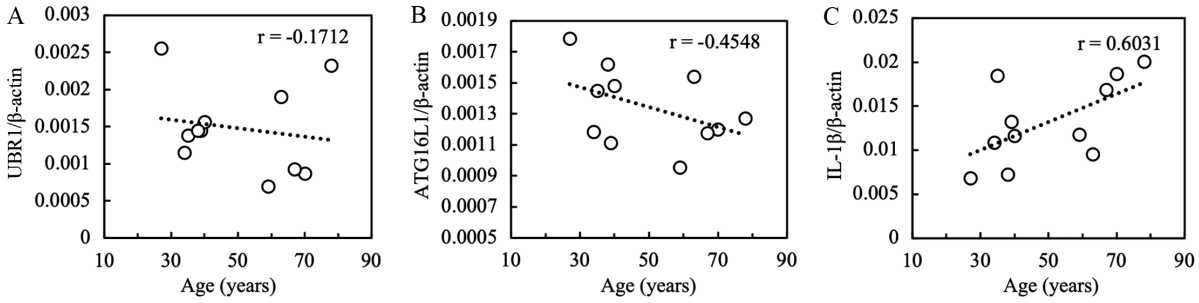

Figure 3. Relationship between mRNA expression levels and patient age. mRNA expression levels of (A) *UBR1*, (B) *ATG16L1* and (C) *IL-1β* are shown. The correlation coefficients were calculated using a Pearson's correlation analysis. *URB1*, E3 ubiquitin-protein ligase component n-recognition 1; *ATG16L1*, autophagy-related 16-like 1; *IL-1β*, interleukin-1β.

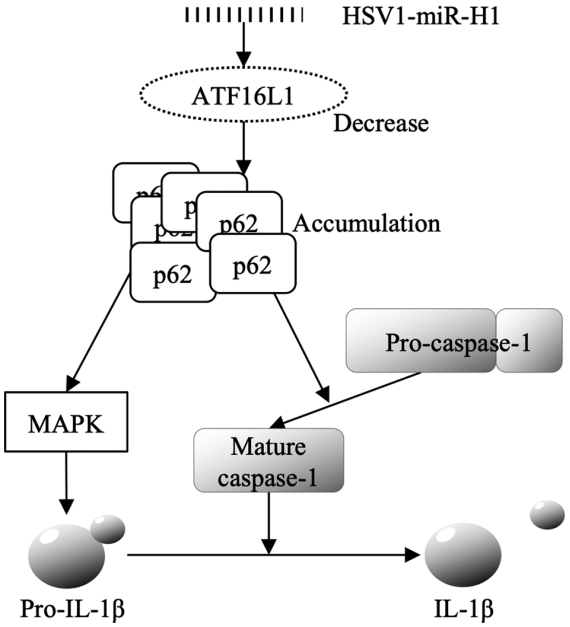

Figure 4. Hypothesized mechanism underlying upregulation of IL-1β expression through ATG16L1 via HSV1-miR-H1. HSV1-miR-H1 decreases ATG16L1, resulting in accumulation of p62. The abundance of p62 induces upregulation of pro-IL-1β via MAPK activation and activates Caspase-1. Maturation of pro-IL-1β to IL-1β is then induced by MAPK and Caspase-1. *ATG16L1*, autophagy-related 16-like 1; *IL-1β*, interleukin-1β.

HSV-1 is a member of the *Herpesviridae* family, and has been proposed as the most likely candidate as a primary target for treatment or management of esophageal primary achalasia, taking into account its neural cell tropism and predilection for squamous epithelium (5,12,15). HSV-1 has a life cycle with two distinct programs consisting of productive and latent phases (13). Certain viral miRNAs can downregulate specific targets or promote viral genome stability, translation and RNA accumulation (13). In our previous study, it was shown that expression of HSV1-miR-H1, an HSV-1 miRNA, was increased in the LES of achalasia patients (1). Hence, the muscular layer of LES may act as a reservoir for HSV-1 and serve as a region for expression of viral miRNAs in patients with achalasia. Additionally, HSV1-miR-H1 is a latency-associated transcript (LAT) that is a non-coding viral miRNA. LAT-derived miRNAs interfere with viral metabolites and regulate the host immune response (13,15). In the present study, it was hypothesized that *UBR1* and *ATG16L1* were the direct targets of HSV1-miR-H1, as the downregulation of the ubiquitin-proteasome system results in the accumulation of neurodegenerative-associated protein fragment β-amyloid (15), and the downregulation of autophagy-mediated viral clearance is advantageous for the survival of viruses (16-18). In the present study, *UBR1* showed decreased expression (although not significant), whereas *ATG16L1* was significantly downregulated at the site of LES. Furthermore, there was a weak correlation between hsv-miR-H1-3p and *ATG16L1* levels, but not *UBR1*. These data suggest that *ATG16L1* is the target of HSV1-miR-H1. In contrast, IL-1β expression was upregulated in the LES, and the inflammatory pathway may have been influenced by viral miRNAs. A correlation between *IL-1B* mRNA expression levels and patient age was also observed. However, additional studies with larger cohorts are required to determine if these observations are generalizable. Additionally, the studied mRNAs are only part of a complex of virus-host interactions, and further studies on the involvement of other transcripts are required. The construction of a achalasia mouse model is also important in obtaining a deeper understanding of the pathogenesis *in vivo*.

A strong correlation was verified between *ATG16L1* and *IL-1β* in the present study. The relationship between HSV1 and autophagy (21-24) and between autophagy and IL-1β production (25-28) have been reported previously. Notably, p62, a selective autophagy receptor, accumulates in ATG16L1 deficient cells, and p62 activates MAPK and Caspase-1, in-turn increasing IL-1β production (29). Based on these previous studies, the mechanism underlying upregulation of IL-1β expression through ATG16L1 via HSV1-miR-H1 hypothesized in the present study is presented in Fig. 4. HSV1-miR-H1 decreases ATG16L1, and the accumulation of p62 is induced by the decrease in ATG16L1. The abundance p62 induces pro-IL-1β via MAPK activation and activates Caspase-1 simultaneously. IL-1β secretion in induced by MAPK and Caspase-1 (Fig. 4).

There are several limitations to this pilot study. As the controls, tissues from non-motility patients with upper gastrointestinal carcinomas not affecting the EGJ, including LES were used. However, the ideal samples would be tissues from healthy individuals, although there are ethical issues related to obtaining such muscular samples. The number of samples was small and each sample in this study was from a patient diagnosed by classical criteria, primarily based on typical findings using barium. Additionally, all samples were used for RNA extraction due to the small sample size; thus protein expression

analysis could not be performed. Finally, whether the influence of ATG16L1 was direct or indirect was not determined. To overcome these problems, a prospective study with a larger sample size and detailed pathological analysis is required.

In conclusion, the levels of *ATG16L1*, a target of HSV1-miR-H1, are reduced in the LES of achalasia patients, and this reduction could be the cause of the esophageal motility disorder.

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Availability of data and materials

All data generated and/or analyzed in the present study are included in the published article.

Authors' contributions

TK and AY made substantial contributions to acquisition of data, analysis and interpretation of data, and to drafting of the manuscript. YI made substantial contributions to analysis and interpretation of the data, and to drafting of the manuscript. HIk, TS, SU and HM contributed to acquisition of the data. KN and HIn contributed to conception and design of the study. HIs made substantial contributions to conception and design of the study, and to drafting of the manuscript. All authors read and approved the final manuscript.

Ethics approval and consent to participate

Written informed consent was obtained from all patients. The study protocol followed the ethical guidelines of the Declaration of Helsinki and was approved by the Nagasaki University Ethics Committee (approval no. 110328329).

Patient consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

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