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Is There Any Evidence for a Viral Cause in Achalasia?

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ABSTRACT

BACKGROUND

Achalasia, as an incurable disease is defined by the lack of normal esophageal peristalsis and loss of lower esophageal sphincter relaxation due to impaired myenteric neural plexus. The exact cause of myenteric neural cells degeneration in achalasia is still unknown. One hypothesis is that certain neurotropic viruses and autoimmune factors cause the inflammatory response in myenteric network, which consequently destroy neural cells. This study was designed to find the evidence of viral causes of achalasia.

METHODS

In this case-control study, 52 patients with achalasia and 50 controls referred to Shariati Hospital, were evaluated for the genome of neurotropic viruses, HPV, and adenovirus by polymerase chain reaction (PCR) and reverse transcription (RT) PCR techniques.

RESULTS

Genome assessment of neurotropic DNA viruses turned out negative in the patients, however, the genome of HSV-1 (Herpes simplex virus) was found in tissues of six controls. No neurotropic RNA viruses were observed in the tissue samples and whole blood of both the patients and controls. Among non-neurotropic viruses, adenovirus genome was positive in tissues of two out of 52 patients and three out of 50 controls. In addition, one out of 52 patients and two out of 50 controls were positive for HPV infection in tissues.

CONCLUSION

We could not detect any significant relationship between achalasia and HPV, adenovirus, and neurotropic viruses in the cases. Nevertheless, it does not exclude the hypothesis of either an alternate viral species or resolved viral infection as the etiology of achalasia.

KEYWORDS:

Achalasia, DNA neurotropic viruses, RNA neurotropic viruses, HPV, Adenovirus

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INTRODUCTION

Achalasia, a rare esophageal motility disorder of unknown etiology, is characterized by esophageal aperistalsis and loss of lower esophageal sphincter (LES) relaxation, which results in dysphagia, impaired esophageal emptying, and poor quality of life.¹ Associated with neither race nor sex, this incurable disease can occur at any age, especially during the 3rd to 6th decades of life.^{2,3} Delayed diagnosis that usually results from non-specific symptoms in the



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Table 1: The sequences of primers for polymerase chain reaction technique

Viruses/internal control	Forward Sequence	Reverse Sequence	Size (bp*)
GAPDH (internal control)	5-GAAGGTGAAGGTCCGGAGT-3	5-GAAGATGGTGATGGGATTTC-3	225
CMV	5-GTACACGCACGCTGGTTACC-3	5-GTAGAAAGCCTCGACATCGC-3	256
VZV	5-ACGGGTCTTGCCGGAGCTGGT-3	5-AATGCCGTGACCACCAAGTATAAT-3	271
HSV-1	5-CGAAGACGTCCGGAAACAAC-3	5-CGGTGCTCCAGGATAAA-3	296
EBV	5-AACATTGGCAGCAGGTAAGC-3	5-ACTTACCAAGTGTCCATAGGAGC-3	182
JC Virus	5-AGTCTTTAGGGTCTTCTAC-3	5-GGTGCCACCTATGGAACAG-3	173
JC Virus	5-ACAGTGTGGCCAGAATTCCTACC-3	5-TAAAGCCTCCCCCAACAGAAA-3	215
HHV6	5-ATAAATTTGATGGGTTAGTGAAAAAG	5-GTC AGG ATT GGA CAT CTC TTT GT-3	205
HPV	5-TTTGTTACTGTGGTAGATACTAC-3	5-GAAAAATAAACTGTAAATCATATTC-3	141
Adenovirus	5-CAACACCTAYGASTACATGAA-3	5-CAACACCTAYGASTACATGAA-3	475
Measles	5-CGGAGCTAAGAAGGTGGATAA-3	5-CTCCCATGGCATAGCTCCA-3	444
Coxsackie Virus	5-ATT GTC ACC ATA AGC AGC CA-3	5-CCTCCGGCCCTGAATGCGGCTAA T-3	154
Coxsackie Virus	5-CAAGCACTTCTGTTTCCCCGG-3	5-ATTGTACCATAAGCAGCCA-3	435
HTLV-1&2	5-AGGGTTTGGACAGAGTCTT-3	5-AAGGACCTTGAGGGTCTTA-3	256
Brona Virus	5-GCC TTG TGT TTC TAT GTT TGC TAA TC-3	5-TTG TGG GGT TTT CCT TCT TAC TCC-3	725
Brona Virus	5-CCT CTA TCT TCA GCC ATT GTT GC-3	5-GAA ACG GGA ACA GGT CAG CAT-3	446

*bp: base pairs

initial stages of the disease, might worsen the outcomes and leads to megaesophagus, food retention, fungal esophagitis, airway stimulation, and esophageal cancer.⁴

Although the main cause of the disease remains unclear, achalasia occurs when inhibitory neurons in esophageal myenteric (Auerbach) plexus are impaired.^{2,3,5} These neurons normally produce nitric oxide (NO) and vasoactive intestinal peptide (VIP). In patients with achalasia the inflammatory response, consisting of CD3/CD8-positive cytotoxic T lymphocytes, eosinophils, and mast cells might lead to histological damage of the esophageal myenteric plexus, loss of ganglion cells, and neurofibrosis.^{5,6,7}

The internal and external factors that initiate and regulate the inflammatory processes have yet to be fully understood.^{1,8} To address these shortcomings, a few studies have hypothesized that some neurotropic viruses and autoimmune factors might cause the inflammatory response in myenteric network and eventually lead to achalasia.^{7,9-11} *Cytomegaloviruses (CMV)*, *varicella-zoster virus (VZV)*,¹² *measles virus*,¹³ *polio*,¹⁴ *herpes simplex virus 1 (HSV1)*,¹⁵ and *bornavirus*¹⁶ are among possible viral triggers.

In this case-control study, we aimed to take a step forward to explore this role by assessing the presence

of neurotropic viruses and non-neurotropic viruses in esophageal and blood samples of patients with achalasia using polymerase chain reaction (PCR).

MATERIALS AND METHODS

In a prospective study, we enrolled 52 patients with achalasia referred to Shariati Hospital, whose diseases were confirmed by endoscopy, timed barium esophagography, and manometry. The control group consisted of 50 individuals without achalasia, who were referred for endoscopy. Written informed consent was obtained from each participant. The Ethics Committees of Golestan and Tehran Universities of Medical Sciences approved the protocol of the study.

Blood samples were taken from all the case and control subjects. Four mucosal biopsy samples were taken during upper gastrointestinal (GI) endoscopy: two samples from the middle third, and two other samples from the lower third of the esophagus. Deoxyribonucleic acid (DNA) and ribonucleic acid (RNA) were extracted from the tissues and blood samples of the patients and controls. Then, the genomes of viruses were assessed by polymerase chain reaction (PCR) and reverse transcription (RT) PCR using specific primers (table 1). RNA was extracted from the samples with Trizol Reagent (Tripure isolation Re-

Table 2: Frequency of viruses in patients with achalasia and control groups

Viruses	Achalasia		control	
	Whole blood	Tissue	Whole blood	Tissue
HPV ¹	-	2	-	1
Adenovirus	-	3	-	2
HSV1 ²	-	6	-	-
JCV ³	-	-	-	-
EBV ⁴	-	-	-	-
CMV ⁵	-	-	-	-
VZV ⁶	-	-	-	-
HHV6 ⁷	-	-	-	-
HTLV1,2 ⁸	-	-	-	-
Measles	-	-	-	-
Coxsackievirus	-	-	-	-
Bornavirus	-	-	-	-

¹ Human Papilloma Virus, ² Human Simplex Virus, ³ John Cunningham virus, ⁴ Epstein Bar Virus, ⁵ Cytomegalovirus, ⁶ Varicella Zoster Virus, ⁷ Human Herpes Virus, ⁸ Human T-lymphotropic virus 1,2

agent: Roche, cat no. 11667157001). DNA was extracted from the samples with Genomic DNA purification Kit (Thermo scientific, Cat no. K0512). Then, the extracted DNA was confirmed by PCR of GAPDH (Internal control gene), which was performed using specific primers (table1). The quality of the extracted DNA was analyzed by electrophoresis in 1% agarose gel, which was stained with ethidium bromide. Consequently, it was observed with a Ultraviolet (UV) transilluminator. Finally, the quantity of the extracted DNA was measured with a spectrophotometer.

The analyses were done using SPSS software for Windows (version 17.0. Chicago: SPSS Inc.). The data were evaluated by Chi-square test and Fisher's exact test (2-sided). The results were considered statistically significant in case of p value < 0.05 .

RESULTS

The mean age of the patients with achalasia and controls was 43.5 ± 2.3 and 45.8 ± 2.3 years, respectively ($p = 0.48$). In the patients group, 21 were women and 31 were men but the controls were taken from a population with an equal number of men and women ($p = 0.329$).

The prevalence of the symptoms of achalasia was as follows: dysphagia to solid foods 98%, dysphagia to liquids 92%, regurgitation 61%, and chest pain 50%. The mean duration of achalasia symptoms in the patients was 32.3 ± 2.9 months.

Of the tissue samples of 52 patients with achalasia, one was positive for *human papilloma virus (HPV)*, and two were positive for *adenovirus*. In the tissue samples of the control group, six, three, and two subjects were positive for *Human herpes virus 1 (HSV)*, *adenovirus*, and *HPV*, respectively. Genome assessments of neurotropic DNA viruses such as *John Cunningham virus (JCV)*, *Epstein-Bar virus (EBV)*, *Cytomegalovirus (CMV)*, *Varicella-zoster virus (VZV)*, and *Human herpes virus 6 (HHV6)* were negative in both tissues and whole bloods of the patients and controls. Assessment of neurotropic RNA viruses such as *Human T-lymphotropic viruses 1 and 2 (HTLV1,2)*, *Measles*, *Coxsackie virus*, and *Bornavirus* yielded negative results in both tissue and whole blood samples of the patients and controls (table 2).

DISCUSSION

Achalasia is the most recognized motor disorder of the esophagus but its etiology is not certainly distinguished. Several studies addressed the possible role of infectious processes (e.g. viral infection), autoimmune processes, and genetic predisposing factors in the creation of this disease. In this study, we could not find any significant association between achalasia and DNA neurotropic viruses (*CMV*, *VZV*, *EBV*, *JCV*, *HSV-1*, and *HHV-6*), RNA neurotropic viruses (*bornavirus*, *coxsackievirus*, *measles*, *HTLV-1 and -2*), or non-neurotropic viruses (*adenovirus* and *HPV*).

The definite role of viral infections in development of achalasia remains unclear. While the results of some studies are in line with our research, contrary to ours, a study resulted that some viruses such as *HSV*, *Varicella zoster*, *HPV*, and *measles* were suspected to initiate the impairing events in achalasia.¹⁷ The assessment of 18 patients with achalasia and 12 control subjects by Jones and colleagues revealed that antibody titer against the measles virus had increased in the patients with achalasia.¹³ This accompaniment was also shown in the levels of *VZV* antibody titers in one third of patients with achalasia in a study by Robertson and co-workers.¹² However, this association was negative for *CMV* and *HSV-1*.¹⁴ The evaluation of T lymphocyte infiltration in the esophagus showed that lymphocytes that are reactivated by *HSV-1*, might be responsible for damaging the neurons in the myenteric plexus in the LES.¹⁷ However, the conflicting inferences might be due to the lack of identification of virus genome or antigen in related tissues. In addition, borna virus has a potential role in creating zoonotic disease and can induce severe neurobehavioral diseases.¹⁸ However, we did not find any infection with borna virus among the patient and control groups. Based on a case-control study by Sinagra and others, 80% of patients with achalasia and 66.7% of the control group were JC-PCR positive without any significant difference ($p = 0.409$).¹⁹ In our study, there was no evidence of *JCV* contamination in achalasia or control groups.

Consistent with our results, some studies have rejected the role of viruses as a cause of achalasia.^{20,21} As we have found, Niwamoto and colleagues failed to find any evidence of infection with viruses such as *CMV*, *VZV*, *EBV*, *HPV6*, or *measles* virus, which were amplified by PCR, in patients with achalasia. They found only *HSV-1* and *HSV-2* in their case and control groups.²¹ Similarly, in another study, *HPV*, *measles*, and *HSV* were negative in patients with achalasia and controls.²⁰ Birgisson and colleagues assessed herpes viruses (*HSV-1* and 2, *CMV*, *EBV*, *VZV*, and *HHV-6*), *measles*, and *HPV* sequences in a small case-control study (13 cases). Similar to our findings, they did not see any evidence of these viruses by PCR method.²⁰

The findings of our study are subject to several limitations: first, achalasia is a rare disease and our data are subject to epidemiological limitation. The second limita-

tion pertains to the cost of procedure for each sample. The conflicting results of studies addressing the role of viral infection in the pathogenesis of achalasia might result from different methods and sample sizes.²²

We used the gold standard methods, PCR and RT-PCR, to find any possible viral infection in our subjects. We plan to continue this study with a larger sample size.

CONCLUSION

In this study we aimed to evaluate the relationship between viral infection and achalasia. We could not find any significant relationship between achalasia and neurotropic DNA viruses (*CMV*, *VZV*, *EBV*, *JCV*, *HSV1*, *HHV6*), neurotropic RNA viruses (*bornavirus*, *coxsackievirus*, *measles*, *HTLV& 2*), or non-neurotropic viruses (*adenovirus* and *HPV*). Nevertheless, the possibility of either alternative viral species or resolved viral infections with disappearance of the inciting viral pathogen from the host tissue should not be ignored. Further prospective studies with larger sample sizes and more quantitative methods are needed to clarify the possible role of viruses in achalasia.

ETHICAL APPROVAL

There is nothing to be declared.

CONFLICT OF INTEREST

The authors declare no conflict of interest related to this work.

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