Absence of Measles Virus Detection from Stapes of Patients with Otosclerosis

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Sponsorships or competing interests that may be relevant to content are disclosed at the end of this article.

Abstract
Objective. To determine molecularly the presence of measles virus genetic material in the stapes of patients with otosclerosis.

Study Design. A cross-sectional study.

Setting. A tertiary referral hospital.

Subjects and Methods. Genetic material was extracted from the stapes of patients with otosclerosis (n = 93) during the period from March 2011 to April 2012. The presence of viral measles sequences was evaluated by the real-time reverse transcriptase polymerase chain reaction (RT-PCR). The expression of the CD46 gene was determined.

Results. Ninety-three patients were included in the study. No sample was positive for any of 3 measles virus genes (H, N, and F). Measles virus RNA was not detected in any sample by real-time RT-PCR. CD46 levels were positive in 3.3% (n = 3) and negative in 96.7% (n = 90).

Conclusion. This study does not support the theory of measles virus as the cause of otosclerosis. It is necessary to do more research about other causal theories to clarify its etiology and prevention.

Keywords
otosclerosis, stapes, measles virus, reverse transcriptase polymerase chain reaction

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Otosclerosis is a disorder of bone remodeling that affects the homeostasis of the otic capsule, whose prevalence is 0.3% to 0.4% in the general population, causing progressive conductive and sensorineural hearing loss. It is considered a multifactorial etiology disease of which several theories have been postulated as follows: genetics, hormonal, autoimmune, bone remodeling theory by bone morphogenetic proteins (BMPs) or transforming growth factor–β (TGF-β), and the viral theory. The latter is linked to the measles virus, described by McKenna et al since 1986; these authors detected viral sequences in footplate fragments by molecular biology techniques, which paved the way for similar studies that support this theory.

The measles virus is a single-stranded RNA virus of the paramyxovirus family that has hemagglutinin on the viral surface and a glycoprotein with a lateral cleft that functions as a sialic acid receptor. The inside part of the protein is involved in binding to CD46 lymphocyte receptors, which have been implicated in viral infection. Transmembrane agent CD46 is known as one of the major cellular receptors of the measles virus and possesses cofactor activity capable of inactivating the components of complements C3b and C4b by serum factor I; the latter protects the host cells from damage by the complement, thereby creating a persistent infection. The CD46 receptor is strongly expressed in epithelial cells, fibroblasts, and chondrocytes; moderately in lymphocytes and endothelium; and slightly in osteoclasts and osteocytes.

Karosi et al found that in active otosclerosis, there is an increase in CD46 expression in osteoclasts, while in negative samples for measles with stapes fixation due to another cause or in normal stapes, there is weak CD46 immunoreaction in osteocytes and fibroblasts. Liktor et al found 4 new CD46 isoforms in otosclerosis and postulated that...
otosclerosis has a CD46-associated expression pattern. Based on all of the studies conducted, it is proposed that otosclerosis should be considered a specific organic disease induced by the measles virus.8-12 However, the results of viral detection in the stapes of patients with otosclerosis remain unclear because some studies do not validate the presence of the virus, as demonstrated by Komune et al13 and Grayeli et al14 in their works, in which no evidence of the virus was detected in stapes samples of patients with otosclerosis by molecular methods or cell cultures. Thus, this theory continues to remain uncertain.

This work aimed to determine the presence of genetic material of the measles virus in the stapes of patients with a diagnosis of otosclerosis.

Methods

Ninety-three patients were included with a diagnosis of otosclerosis at the National Institute of Rehabilitation from March 2011 to April 2012. Eighty-three patients had bilateral disease and 10 had unilateral disease, among whom 57 were women and 36 men, with a median age of 42 years (range, 24-63 years). Demographic and clinical variables were obtained. The diagnosis of otosclerosis was performed preoperatively and clinically, with the disease course manifested by slowly progressive hearing loss, normal otoscopy, tonal audiometry with mixed or conductive hearing loss with an air-bone gap >20 db, and the absence of stapedial reflexes. The diagnosis was confirmed during the surgical procedure when verifying the stapes fixation and immobility.

Genetic Material Extraction

We extracted the stapes from all patients and placed them in saline solution at 4°C to 8°C, and the samples were immediately transported to the laboratory for treatment in cold saline. The genetic material was obtained as previously described15; the tissue was promptly macerated and subsequently lysed with a nucleic acid stabilizer, and the RNA was extracted into the NucliSENS easyMAG semi-automated kit (bioMérieux, Marcy l’étoile, France), eluting in a volume of 50 μL. The extraction was preserved at –70°C. RNA viability was corroborated in agarose gel.

Real-Time Reverse Transcriptase Polymerase Chain Reaction Assay for Viral RNA Detection

This was conducted using the Superscript One-Step reverse transcriptase polymerase chain reaction (RT-PCR) kit with Taq Platinum polymerase (Invitrogen, Carlsbad, California), using 10 pmol of each oligonucleotide and 10 ng of the extracted RNA, in a final volume of 25 μL. The Ct value was employed as the cutoff point to discard negative samples. The conditions used in the thermal cycler were the following: retrotranscription at 48°C for 30 minutes, followed by 1 cycle at 95°C for 10 minutes, 40 cycles of 95°C for 15 seconds, and at 60°C for 60 seconds. Three hemagglutinin (H), nucleoprotein (N), and fusion protein (F) genes were identified, and the human RNAsaP gene was identified as control for sample viability. The oligonucleotide sequences and probes used in this study are described in Table 1. For CD46 gene expression, the mixing reaction contained 10 pmol of each oligonucleotide, 5 pmol of each probe, 0.5 μL of SSIII RT/PlatinumTaq Mix, 12.5 μL of 2× PCR Master Mix, and 10 ng of RNA; the final volume was 25 μL. The conditions used were 94°C for 2 minutes, 45 cycles of 94°C for 15 seconds, and at 60°C for 30 seconds, as well as 1 step at 72°C for 5 minutes. A 7500 Real-Time PCR Thermocycler (Applied Biosystems, Foster City, California) was used. The glyceraldehyde 3-phosphate dehydrogenase gene (GAPDH) was used as the constituent gene.

Ethics

The protocol was reviewed and authorized by the Ethics Committee of the National Institute of Rehabilitation with registration number 9/12, and all patients signed informed consent for their participation in the study.

Statistics

Descriptive statistical analysis was performed using the Prism version 6 statistical software package (GraphPad Software, La Jolla, California).

Results

Ninety-three patients were included in the study; only 1 specimen was obtained per patient. Demographic variables are shown in Table 2. Otosclerosis was sporadic in 50 cases, and 43 had a family history of otosclerosis or hearing loss. Twenty-five patients (26.8%) reported a history of measles disease, 27 (29%) did not know whether they had had measles, and 41 (44%) denied having measles. Only 2 (2.1%) of the women had increased hearing loss during pregnancy. Fifty-five patients (59%) underwent stapes surgery with a stapedotomy technique, obtaining only the suprastructure of the stapes; 21 (22.5%) had hemiplatinectomy where the suprastructure was obtained with half of the footplate; and in 17 patients (18.2%), the entire stapes was obtained by total footplate resection.

No sample was positive for any of the 3 measles virus genes (H, N, and F). The human RNAsaP gene was positive in all samples. CD46 receptor messenger RNA (mRNA) expression was positive in 3 of 93 patients (3.3%), of whom 2 were men aged 24 and 30 years with bilateral disease with sample from stapes’ suprastructure, and the other was one-half footplate of a 38-year-old woman with bilateral disease.

Discussion

The measles virus is a single-stranded RNA virus, whose genome has 6 genes encoding different proteins: nucleocapsid (N), phosphoprotein (P), matrix protein (M), fusion protein (F), hemagglutinin (H), and large proteins (L). The virus is serologically monotypic but, based on the sequences of the N and H genes, 8 wild viruses, designated A, B, C, D, E, F, G, and H, have been classified. Observation of filamentous structures that were morphologically similar to the nucleocapsid virus in the endoplasmic reticulum of osteoblast-like cells in the osteoclastic lesions of stapes from patients with otosclerosis led McKenna et al2 to
propose the viral etiology of this disease. Since that time, a large number of studies have reported the detection of measles virus in stapes samples of patients with otosclerosis by means of RT-PCR and immunohistochemical analysis. In addition, Arnold et al analyzed patients with otosclerosis hospitalized in Germany from 1993 to 2004 and concluded that there is a statistically significant lower incidence of otosclerosis in patients who had been vaccinated against measles than in those who had not been. However, the complete mRNA sequence of the virus has, to our knowledge, never been reported, nor has it been possible to isolate it from a patient with this pathology.

Grayeli et al reported that they did not detect RNA from the measles virus in any sample of stapes from patients with otosclerosis (n = 35). Similarly, Komune et al did not detect any evidence of virus in stapes samples of patients with otosclerosis by RT-PCR or cell cultures. In agreement with the previous works, we did not detect the viral RNA in the stapes samples by means of the RT-PCR method, which even possesses higher sensitivity than the conventional method that was employed in other studies in which the authors detected it. 

### Table 1. Oligonucleotide and Probe Sequences.

<table>
<thead>
<tr>
<th>Gene</th>
<th>Oligonucleotides and Probe Sequences</th>
<th>Length, pb</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>Hemagglutinin</td>
<td>5'-TTC ATC GGG CAG CCA TCT AC-3' 5'-CTC TGA GGT GTC CTC AGG CC-3'</td>
<td>150</td>
<td>Bühlmann et al</td>
</tr>
<tr>
<td></td>
<td>FAM-CGG CAG AGA TCC ATA AAA GCC TCA GCA C-BHQ</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Nucleoprotein</td>
<td>5'-TCA GTA GAG CGG TTG GAC CC-3' 5'-GCG CCG GTT TCT CTG TAG CT-3'</td>
<td>151</td>
<td>Bühlmann et al</td>
</tr>
<tr>
<td></td>
<td>FAM-CAA ACA GAG TCG AGG AGA AGC CAG GGA-BHQ</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Fusion protein</td>
<td>5'-TGA CTC GTT CCA GCC ATC AA-3' 5'-TGG GTG ATC ATT GCA TTA AGT GCA-3'</td>
<td>150</td>
<td>Bühlmann et al</td>
</tr>
<tr>
<td></td>
<td>FAM-CTG CAC GAG GTG AGA GAT CGC AGA ATA CAG-BHQ</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Human ARNase P</td>
<td>5'-AGATTGAGACCTGCGAGCG-3' 5'-GAGCCGCTGTCCTCACAAGT-3'</td>
<td>160</td>
<td>Hui Donga et al</td>
</tr>
<tr>
<td></td>
<td>FAM-TTCTGACCTAGGGCTCTGCG-BHQ</td>
<td></td>
<td></td>
</tr>
<tr>
<td>CD46</td>
<td>GAG TGT AAGTTGCTAAAA TTGCG GGAGTG GGT GAT TTAGTC TTGGAA FAM-GGTCAATTGCTTGAGTGG-BHQ</td>
<td>200</td>
<td>Nolan et al</td>
</tr>
<tr>
<td>GAPDH</td>
<td>CATGAGTCCTCTCAGGATACC GTGAAACATGAGAAGGATGTGACACAG CY5-CCTCAAGATCATCAGCAATGCTCTCG-BHQ</td>
<td>146</td>
<td></td>
</tr>
</tbody>
</table>

Abbreviations: BHQ, nonfluorescent quencher (Black Hole Quencher); FAM, 6-carboxyl fluorescein; GAPDH, glyceraldehyde 3-phosphate dehydrogenase.

### Table 2. Demographic Values of Patients with Otosclerosis by Gender.

<table>
<thead>
<tr>
<th>Variable</th>
<th>Women (n = 57)</th>
<th>Men (n = 36)</th>
</tr>
</thead>
<tbody>
<tr>
<td>History of measles</td>
<td>16 positive</td>
<td>9 positive</td>
</tr>
<tr>
<td></td>
<td>24 negative</td>
<td>17 negative</td>
</tr>
<tr>
<td></td>
<td>17 Unknown</td>
<td>10 Unknown</td>
</tr>
<tr>
<td>Stapedial reflex</td>
<td>57 absent</td>
<td>36 absent</td>
</tr>
<tr>
<td>Type of sample</td>
<td></td>
<td></td>
</tr>
<tr>
<td>12 suprastructure with full footplate</td>
<td>8 suprastructure with half-footplate</td>
<td></td>
</tr>
<tr>
<td>13 suprastructure with half-footplate</td>
<td>8 suprastructure with half-footplate</td>
<td></td>
</tr>
<tr>
<td>32 suprastructure</td>
<td>23 suprastructure</td>
<td></td>
</tr>
<tr>
<td>Laterality</td>
<td>50 bilateral</td>
<td>33 bilateral</td>
</tr>
<tr>
<td></td>
<td>7 unilateral</td>
<td>3 unilateral</td>
</tr>
<tr>
<td>Median age (minimum-maximum), y</td>
<td>43 (26-63)</td>
<td>40 (24-63)</td>
</tr>
</tbody>
</table>
in 2 cycles with complete reproducibility. Investigation groups that have found genomic sequences have amplified the nucleoprotein component using a large number of amplification cycles (35-40 cycles); in these cases, the amplification tests have been repeated 2 to 3 times for each sample with a low reproducibility index.\textsuperscript{3,20,23,30} It can be argued that copies of the measles virus may be very low in the otosclerosis tissue for detection; however, that would lead us to wonder whether such a low titer, not detectable by PCR, may produce chronic disease.

On the other hand, the concept that measles infection is a cause of otosclerosis is not supported by the observation that otosclerosis is rare in Africans, in whom the incidence of measles is high.\textsuperscript{31}

In addition, viral particle localization reported by McKenna et al\textsuperscript{2} in otosclerotic tissue cells is very different from localization in the infected cells of other tissues found in the cytoplasm and not in the endoplasmic reticulum. The cellular infection of measles virus is carried out through receptors, with that of SLAM (signaling lymphocyte activating protein, CD150) participating mainly in the viral infection; however, many immunohistochemical studies have shown that infection in the cells of the otic capsule is very weak for the SLAM receptor and very high for the CD46 receptor.\textsuperscript{3} This assumes that cellular penetration by the measles virus occurs through the CD46 receptor in the otic capsule. The CD46 receptor is a type 1 transmembrane glycoprotein that has different known isoforms that are expressed by all nucleated cells to a different extent.\textsuperscript{5} In support of the negative finding of measles virus particles in this study, CD46 receptor expression was very weak in most of the samples (96.7%), thus reinforcing the absence of the virus and of viral transcription factors that could increase the expression of these receptors. The detection of CD46 in our study was performed through specific primers without the analysis of isoforms, which reduces specificity to positive results, even though it does not diminish the sensitivity of the negative results that strengthen the nonexistence of the virus in patients with otosclerosis analyzed in this study.

Other theories that could support the presence of the viral RNA detected in other studies would be genetics and autoimmunity but, in this case, the virus would not act as an infecting agent but as an autoimmune trigger due to molecular mimicry by genetic predisposition. However, this would be very difficult to demonstrate because there is a lack of animal models for otosclerosis.\textsuperscript{32} Ayala-Peña et al\textsuperscript{29} demonstrated, in an animal model on infection of bone cells by the measles virus, that it may infect osteoblasts in vitro, causing osteogenic differentiation as measured by an increase in differentiation biomarkers in the infected cells, such as morphogenetic proteins, bone sialoprotein (BSP), and alkaline phosphatase in the cells infected by the virus.

One study limitation comprised the surgery technique for sample obtention because 55 (59.1%) samples derived from the stapes suprastructure, 21 (22.5%) were from the suprastructure with half of the footplate, and 17 (18.2%) of stapes corresponded to the full footplate. All of this makes us consider the possibility that samples that included only the stapes suprastructure were negative for the genes of the measles virus because there was no footplate involved in the analysis. However, 38 (40.8%) samples had a footplate, either half or complete, and these were also negative. Another limitation is that we did not perform electron microscopy to search for the filamentous structures of the measles virus; however, we consider that RT-PCR is highly sensitive for the detection of viral RNA and would permit us to do without electron microscopy. In addition, we were unable to obtain perilymph to measure IgG concentrations. With respect to the remaining theories on the etiology of otosclerosis, it would be of great interest to investigate the genetic theory, due to the large number of patients who had a history of hearing loss, so that we could search for OTSC1-OTSC8 genes in the same samples of our patients and investigate more on the characterization of these to understand what improves the type of inheritance and the pathogenesis. Another genetic link has comprised osteogenesis imperfecta, where otosclerosis is characterized as a local expression of the disease; thus, it is also possible to detect abnormalities in the COL1A1 gene in patients with otosclerosis and to compare it with patients with the abnormalities found in osteogenesis imperfecta.

**Conclusion**

This study supports that otosclerosis is not related to chronic infection with the measles virus. Further studies are needed to detect the differences and to further investigate other theories of causality of otosclerosis to clarify its etiology and to act in the prevention of the disease.

**Acknowledgments**

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**Author Contributions**

Maria de Lourdes Flores-García, conception and design of the work, acquisition, analysis and interpretation of the data, drafting and final approval; Claudia Adriana Colín-Castro, data analysis, drafting, final approval and accountability for the work; Mario Sabas Hernández-Palestina, conception and design of the work, drafting, final approval, accountability for the work; Roberto Sánchez-Larios, conception and design of the work, drafting, final approval, accountability for the work; Rafael Franco-Cendejas, data analysis, drafting, final approval, accountability for the work.

**Disclosures**

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**References**


