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Detection of herpesviruses 1-6 and community-acquired respiratory viruses in patients with chronic rhinosinusitis with nasal polyposis.

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Running title: herpesviruses and CARV in nasal polyposis.

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ABSTRACT

Objective. To evaluate the prevalence of human herpesviruses 1-6 and community-acquired respiratory viruses (CARVs) in specimens from patients with nasal polyposis undergoing functional endoscopic sinus surgery (FESS) and investigate the potential clinical role.

Methods. Viral occurrence was evaluated by molecular methods in polyps, turbinate mucosa, pre- and post-operative scraping specimens from 35 consecutive patients at different time points in relation to FESS.

Results. Overall, 21 patients (60%) were positive to at least one virus in at least one specimen; in particular, 12.1% of all specimens for HHV-6 (3/35 polyps, 11/31 turbinates, 1 pre-surgical scraping) and 10.5% for EBV (8/35 polyps, 3/31 turbinates, 1/29 pre- and 1/29 post-surgical scraping), followed by CMV and HSV-1 (both 1.6%; 1/35 polyps, 1/29 post-surgical scraping and 12/35 polyps, respectively). EBV-positivity tended to be higher in polyps, as well as HHV-6 in adjacent healthy turbinate mucosa, although no significant association was found. Only one pre-operative cytological specimen was positive to parainfluenza virus-1.

Conclusion. No association between the development of nasal polyps, herpesviruses and CARVs seem to exist. However, the higher EBV frequency in polyps could suggest a causative role or persistence in the inflammatory lymphoid tissue.
INTRODUCTION

Nasal polyps are a common chronic disease of nasal and paranasal sinus mucosa, which affects approximately 4% of general population. These benign lesions are characterized by inflammation-induced mucosal swelling, inflammatory cell infiltration, and subepithelial edema. Nasal polyps are usually associated with chronic rhinosinusitis (CRSwNP) and most common symptoms are obstruction, rhinorrhea, anosmia, facial pain and headache [1]. Medical therapy consists of intra-nasal steroids [2] and antibiotics [3]; nevertheless functional endoscopic sinus surgery (FESS) is often necessary, despite of 70% chance of recurrence [4]. Pathogenesis and molecular mechanisms underlying CRSwNP are poorly known; several factors have been investigated, including Kirsten rat sarcoma (K-RAS) codon 12 mutations/increased expression [5], elevated expression of Vascular endothelial growth factor (VEGFA) and Transforming growth factor-B1 (TGFβ1) [6,7], as well as clinical features, including allergy, asthma, immunodeficiency and chronic sinus infections [8,9]. Viral infections have been hypothesized to play a role in the pathogenesis, progression and recurrence of CRSwNP [10]. While human papillomavirus has been associated rather to neoplastic lesions [11-13], very few studies have investigated the role of herpesviruses and community-acquired respiratory viruses (CARVs) with no definitive conclusions [14]. The Herpesviridae family encompasses several DNA viruses, that are able to establish lifelong latent infections and reactivate in immunocompromised conditions; in particular, human herpesviruses 1-6 (including HSV-1 and 2, VZV, EBV, CMV) are highly seroprevalent and have been associated to upper airway infections.

The aim of this study was to evaluate human herpesviruses 1-6 and CARVs prevalence by molecular methods in nasal polyps, adjacent inferior/middle turbinates, pre- and post-operative nasal scraping from patients undergoing FESS.
Materials and Methods

Study population consisted of 35 consecutive patients (M/F, 25/10; mean age±standard deviation, 6250.3±15.4 years; range, 23-77) with CRSwNP undergoing FESS between September 2011 and April 2012 (Table 1). All the patients gave their informed consent and the study was approved by the institutional review board. Diagnosis of CRSwNP was made on the basis of European Position Paper on Rhinosinusitis and Nasal Polyps (EPOS) 2012 criteria [1]. In details, diagnostic criteria included inflammation of the nose and the paranasal sinuses characterized by two or more symptoms, one of which being either nasal blockage/obstruction/congestion or nasal discharge (anterior/posterior nasal drip); facial pain/pressure; reduction/loss of smell for ≥12 weeks; supported by endoscopic signs of nasal polyps, mucopurulent discharge, oedema/mucosal obstruction and/or computed tomography changes. All patients were affected by CRS with multiple polyps arising from the middle turbinate, middle meatus, or ethmoidal sinuses and were classified as grade II-III according to the Mackay and Lund system [15]. Pediatric subjects, HIV-seropositive individuals, patients with cystic fibrosis, immotile cilia syndrome, allergic fungal rhinosinusitis and inverted papilloma were excluded. The following time points were considered: T0 (one month pre-surgery) clinical history for allergy and asthma (previous investigation by prick test) and pre-operative cytological specimen; T1 (surgery) collection of two bioptic samples of polyps and the adjacent inferior/middle turbinates without polyposis; T2 (one month post-surgery) post-operative cytological specimen (Figure 1). Polyp specimens were collected from maxillary or ethmoid sinus, depending on the involved site; scraping for cytological samples was performed on inferior/middle turbinates. Due to missing sending or inadequacy, only 31, 29, and 29 turbinates, pre- and post-operative scraping specimens were available, respectively, for an overall number of 124 samples for molecular testing.

For processing of mucosa tissues, specimens were incubated with 200 μl lysis buffer (Tissue Lysis Buffer, Qiagen, Milan, Italy) by brief vortexing and heating at 100°C for 5 min twice, vortexed...
briefly again, then centrifuged for 1 min at 13,000 rpm at room temperature. Subsequently, a mechanical lysis step with the rotor-stator homogenizer Tissue Ruptor was performed. A 200 µl aliquot of supernatant, as well as of nasal scraping, was subjected to nucleic acid extraction using the Nuclisens EasyMAG platform (Biomerieux, Marcy l’Etoile, France).

For herpesviruses 1-6 detection and quantification, real-time PCR was performed, using commercially available kits (Q-PCR Complete Kit [ELItech group, Milan, Italy]) following the manufacturer’s instructions, and the 7500 Real-Time PCR System (Applied Biosystems, Monza, Italy). Target regions were glycoprotein D and G for HSV-1 and HSV-2, respectively; ORF 29 for VZV; EBNA 1 for EBV; exon 4 and UL 123 for CMV; and ORF 13R for HHV6.

The occurrence of CARVs was investigated using a commercially available multiplex PCR assay according to the manufacturer’s instructions (RV15 OneStep ACE Detection [Seeplex®; Seegene, Seoul, Korea]), targeting sequences of Influenza A and B viruses, RSV type A and B, adenovirus, metapneumovirus, coronaviruses 229E/NL63 and OC43, parainfluenza viruses 1-4, rhinoviruses A/B/C, enteroviruses, and bocaviruses 1/2/3/4 and the MultiNA System (Shimadzu Corporation Italia, Milan, Italy).

For statistical analysis, the chi square and Fisher’s exact tests were applied, as appropriate. A p-value <0.05 was considered statistically significant.
RESULTS

Results are summarized in Tables 2. Overall, 21 patients (60%) were positive to at least one virus in at least one specimen. As regards herpesviruses 1-6, the highest prevalence was found for HHV-6 (15/124; 12.1%; mean viral load $1620\pm1837$ copies/$10^4$ cells; median, 820) and EBV (13/124; 10.5%; mean viral load $88\pm140$; median, 25), followed by CMV and HSV-1 (both 2/124; 1.6%). No specimen was positive to HSV-2 and VZV. In Table 2, prevalence and viral load in different sites for each virus are reported. Viral load was $\leq3\times10^3$ copies/$10^4$ cells in all the cases, except for HHV-6 on a polyp specimen (see below). Considering the type of specimen, EBV was found in eight, three, and two polyp, turbinate and cytological (one $T_0$ and one $T_2$) specimens, respectively. In one patient, EBV was positive in both polyp and turbinate specimens; in another individual in polyp, turbinate and post-operative cytological samples. In both cases, the highest viral load was found in polyp specimens. HHV-6 was positive in three polyps, 11 turbinates, and one $T_0$ scraping specimen. In two patients, both polyp and turbinate specimens were positive to HHV-6, with the highest viral load being detected in the polyp sample (49000 copies/$10^4$ cells). HSV-1 was found in two polyp samples, one also positive to EBV. CMV was found in one polyp (also EBV-positive) and in a $T_2$ scraping (other samples from the same patient were negative to herpesviruses 1-6). Although EBV-positivity tended to be higher in polyps in comparison to other specimens, as well as HHV-6 in turbinate mucosa in comparison to other samples, no statistically significant association was found. Similarly, no significant association between EBV and HHV-6 positivity on polyps and turbinate mucosa, respectively, and clinical features of allergy and asthma was found. As regards CARVs, only one pre-operative cytological specimen was positive to parainfluenza virus 1.
DISCUSSION

In this study, the prevalence of herpesviruses 1-6 and CARVs in polyp, turbinate mucosa and nasal cytological specimens from patients undergoing FESS for CRSwNP was investigated.

Considering herpesviruses, the highest prevalence was found for HHV-6 (12.1%, irrespective of the type of specimen), followed by EBV (10.5%). CMV and HSV-1 prevalence was very low, while no specimen resulted positive for other herpesviruses. These results are different from those reported in previous studies. As regards EBV, old studies reported EBV-DNA qualitative detection by PCR in 80% of normal nasopharyngeal mucosa from Chinese subjects [16,17]. In another study on 13 nasal polyps, the same Authors found EBV prevalence of 15%, 69%, and 85% using southern blot hybridization, qualitative PCR, and in situ hybridization, respectively [18]; this study evidenced a highly different sensitivity with these different methods and lead the author to hypothesized that nasal mucosa is a site where EBV persists through a low replicative level in resident lymphocytes. More recently, a 35% EBV positivity in 23 nasal polyps was found by qualitative PCR [19]; whereas in a study on nasal polyps and hypertrophied turbinates from Hong Kong patients, no specimen was positive to EBV in situ hybridization [20]. Taking into account also the different methods and particularly the absence of quantitative molecular data in previous studies, it could be argued that EBV-positivity in polyps represents its presence in the inflammatory lymphoid tissue. This hypothesis could be further supported by the fact that EBV was detected at lower rate in healthy tissue (turbinate mucosa) than in polyps, although the difference was not significant, and by the fact that viral load was always within an order of magnitude of $10^2$ copies/10^4 cells. Although EBV can persist in that lymphocytes can be infected by virus released from a lytic EBV infection in the nasal mucosa, the fact that EBV is detected in high rate in normal nasopharyngeal mucosa tissue (up to 88% in some studies [18]), whereas nasal polyps are much rarer, argues against an EBV contribution to polyp development. This is further supported by data on viral load of the present
study and the low number of EBV+ cells in each positive case described by Tao and colleagues [18].

Only one study investigated HHV-6 prevalence in polyps and inferior turbinates without finding any positive specimen [19]. This is in contrast to the present study in which HHV-6 was detected in >35% of turbinate mucosa specimens and >8% of nasal polyps. This difference could be due to the different methods: quantitative real-time PCR with high specificity and sensibility in the present study, traditional PCR with 70 bp amplicon length in the study by Zaravinos. The relatively high HHV-6-prevalence in healthy tissues found in our study could be due to its frequent occurrence and diffusion in different tissues. HHV-6 seroprevalence is the highest amongst herpesviruses; furthermore HHV-6 is the only herpesvirus which is able to integrate its DNA in human genome, as it can be detectable in chromosomically integrated status in 0.2-0.8% of general population. Persistence of HHV-6 involves both a true latent state and a low-level chronic replication, each occurring at different anatomic sites, including nasal mucosa. It is to note that a possible limitation of this study is the lack of healthy controls, as well as of normal sinonasal specimens from patients with other underlying pathologies.

Furthermore, we evaluated the presence of CARVs using a multiplex-PCR and found only one cytological sample positive to parainfluenza virus-1. These results are in accordance with a previous study on 13 sinonasal mucosa specimens from chronic rhinosinusitis patients and two from healthy subjects, that resulted negative to a panel of 12 CARVs [21]. Other Authors evaluated the presence of picornaviruses in nasal washing and turbinate mucosa from 39 patients affected by chronic rhinosinusitis and 27 healthy people and found a 21% rhinovirus positivity in patients, while no virus was found in controls [22]. A more recent study investigated CARVs in paranasal sinus mucosa and polyps by multiplex-PCR and found a 18% positivity to bocavirus and <2% positivity to rhinovirus in 102 tissue samples from 88 patients [23]. In the present study, the lack of CARVs detection in any specimen (but the cytological sample) seems to argue against a potential involvement of these viruses in this clinical context.
In conclusion, only EBV and HHV-6 were detected at certain frequency in nasal polyps and adjacent turbinate mucosa specimens, respectively, although with no statistical significance. To date, the data obtained by the present and other studies seem to argue against a definite role for herpesviruses and CARVs in the development of nasal polyps. Future studies should take into account the relatively higher frequency of EBV detection in polyps, that could suggest a causative role in the formation of nasal polyps, as previously suggested by others, or EBV persistence in the inflammatory lymphoid tissue which characterizes these lesions.
Acknowledgements

Conflict of interests: none.
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Table 1. Demographic and clinical features of study population.

<table>
<thead>
<tr>
<th>Patients characteristics</th>
<th>N</th>
<th>(%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Gender (M/F)</td>
<td>20/15</td>
<td>-</td>
</tr>
<tr>
<td>Age (mean ± SD; range)</td>
<td>50.3 ± 15.4</td>
<td>-</td>
</tr>
<tr>
<td>Allergy</td>
<td>15</td>
<td>42.9</td>
</tr>
<tr>
<td>Asthma</td>
<td>8</td>
<td>22.9</td>
</tr>
<tr>
<td>Family anamnesis</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Asthma/allergy</td>
<td>10</td>
<td>28.6</td>
</tr>
<tr>
<td>Nasal polyposis</td>
<td>3</td>
<td>8.6</td>
</tr>
<tr>
<td>Previous sinusal surgery</td>
<td>10</td>
<td>28.6</td>
</tr>
<tr>
<td>Nasal obstruction (visual analog scale 0-10)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>0-4</td>
<td>5</td>
<td>14.4</td>
</tr>
<tr>
<td>5-8</td>
<td>15</td>
<td>42.8</td>
</tr>
<tr>
<td>9-10</td>
<td>15</td>
<td>42.8</td>
</tr>
<tr>
<td>Anterior discharge</td>
<td>20</td>
<td>57.1</td>
</tr>
<tr>
<td>Posterior discharge</td>
<td>13</td>
<td>37.1</td>
</tr>
<tr>
<td>Loss of smell (any grade)</td>
<td>32</td>
<td>91.4</td>
</tr>
<tr>
<td>Facial pain</td>
<td>11</td>
<td>31.4</td>
</tr>
</tbody>
</table>
Table 2. Herpesviruses 1-6 detection and viral load on polyp, turbinate mucosa, pre- and post-operative scraping specimens from patients with chronic rhinosinusitis with nasal polyps undergoing functional endoscopic sinus surgery. For each virus the following data are reported: raw number and percentage; viral load as mean±standard deviation and median when more than two specimens are positive; otherwise, single results. Viral load is expressed as copies/10^4 cells.

<table>
<thead>
<tr>
<th>Samples</th>
<th>HSV-1 (N=124)</th>
<th>HSV-2</th>
<th>VZV</th>
<th>CMV</th>
<th>EBV</th>
<th>HHV-6</th>
</tr>
</thead>
<tbody>
<tr>
<td>Polyps (N=35)</td>
<td>2 (5.7%)</td>
<td>0</td>
<td>0</td>
<td>1 (2.9%)</td>
<td>8 (22.9%)</td>
<td>3 (8.6%)</td>
</tr>
<tr>
<td></td>
<td>25; 19</td>
<td>35</td>
<td></td>
<td>157±197</td>
<td>16580±28076</td>
<td></td>
</tr>
<tr>
<td>Turbinates (N=31)</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>70</td>
<td>440</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td>3 (9.7%)</td>
<td>11 (35.5%)</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td>23±3</td>
<td>705±1264</td>
<td></td>
</tr>
<tr>
<td>Pre-surgical scraping (N=29)</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>25</td>
<td>37</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td>1 (3.4%)</td>
<td>1 (3.4%)</td>
<td></td>
</tr>
<tr>
<td>Post-surgical scraping (N=29)</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>1 (3.4%)</td>
<td>12</td>
<td>4400</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td>1 (3.4%)</td>
<td>0</td>
<td></td>
</tr>
<tr>
<td></td>
<td>2 (1.6%)</td>
<td>0</td>
<td>0</td>
<td>2 (1.6%)</td>
<td>20</td>
<td>13 (10.5%)</td>
</tr>
</tbody>
</table>
Figure 1. Synopsis of specimen collection in study population. FESS, functional endoscopic sinus surgery.