■ ORIGINAL ARTICLE

Viral Infection of Herpes simplex, Epstein-Barr, Varicela Zoster, Human Papilloma, Cytomegalovirus, or Adenovirus Are Not Related to Sinonasal Adenocarcinomas

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Objective: Several types of virus have been implicated in the development of head and neck tumours. However, until now sinonasal adenocarcinomas (SNAC) have not been studied. The aim of this study is to screen a series of SNAC for the presence of a number of viruses known to play a role in cancer.
Material and method: Viral DNA sequences of Herpes simplex virus, Epstein-Barr, varicela zoster, human papilloma, cytomegalovirus, and adenovirus were analysed by PCR in 37 primary SNAC.
Results: Three tumours (8.1%) were positive for Epstein-Barr virus and 1 case (2.7%) for cytomegalovirus.
Conclusions: Viral infections do not seem to play a role in the aetiology of SNAC.

Key words: Sinonasal adenocarcinoma. Virus. Aetiology. Tumorigenesis.

Las infecciones por virus herpes simplex, Epstein-Barr, varicela zoster, papiloma humano, citomegalovirus o adenovirus no tienen relación con los adenocarcinomas nasosinusales
Objetivo: Se ha relacionado a diversos virus con el desarrollo de tumores epidermoides de cabeza y cuello. No obstante, no existen estudios previos que relacionen a los adenocarcinomas nasosinusales (ACN) con la presencia de virus. El objetivo de este estudio es determinar, en una serie de ACN, la presencia de virus que se sabe desempeñan un papel en el cáncer.
Material y método: Se estudió mediante PCR 37 ACN, para determinar la presencia de ADN de virus de papiloma humano, virus de Epstein-Barr (VEB), virus herpes simplex, virus de la varicela zoster, adenovirus y citomegalovirus.
Resultados: Se detectó ADN de VEB en 3 (8,1 %) de las 37 muestras tumorales y ADN de citomegalovirus en 1 (2,7 %) de los 37 casos analizados.
Conclusiones: Nuestros resultados indican que los virus estudiados no desempeñan papel alguno en la etiología de los ACN.

Palabras clave: Adenocarcinoma nasosinusal. Virus. Etiología. Tumorigénesis.

INTRODUCTION

It is estimated that 15% of all cancers that are diagnosed worldwide are virus-related.[1] Viruses may favour tumour formation through different mechanisms, such as immunosuppression, cellular genome alterations, or by changing the protein expression of the host cells.[2]

A virus relies on host cell machinery to reproduce its genetic material; in order to do this it encodes non-structural proteins. These viral oncoproteins target host cell proteins that intervene in regulating the cell cycle. This interaction

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is vital for cellular transformation. In this way, human papilloma virus (HPV) oncoproteins E6 and E7 bind with tumour suppressing proteins p53 and pRb (respectively) and cause them to degenerate faster. Then the antiproliferation effects of these tumour suppressors are either destroyed or decreased and the cellular cycle is then regulated abnormally.[3]

Table 1. Approaches Used

Type of Surgery	*Patients*
No surgery	1 (3%)
Nasal endoscopic surgery	1 (3%)
Paralateronasal approach	9 (24%)
Craniofacial approach	17 (46%)
Subcranial approach	9 (24%)

Table 2. Distribution of T Staging in the 37 Sinonasal Adenocarcinomas

Stage	*n (%)*
T1	10 (27)
T2	5 (13.5)
T3	13 (35.1)
T4	9 (24.3)
Total	37

Certain viruses have been linked to the development of head and neck tumours. HPV has been linked to the appearance of epidermoid carcinomas of the head and neck, mainly located in the oropharynx.[4] The Epstein-Barr virus (EBV)[5-7] has been linked to nasopharyngeal carcinoma, epidermoid carcinoma of the palatine tonsils, and supraglottic carcinoma. The Herpes simplex virus (HSV) has also been linked to epidermoid laryngeal and oral cavity carcinomas.[8] This study will focus on sinonasal adenocarcinomas (SNAC). No previous studies have been done linking these with virus presence.

Sinonasal adenocarcinomas account for between 1% and 4% of upper respiratory tract tumours.[9] The aetiology points to prolonged exposure to sawdust, which may be related to chronic inflammation of the nasal mucosa caused by dust particles.[10]

Most cases (85%) originate in the upper part of the nasal passages, on the surface of the middle and superior conchae of the ethmoid bone. The tumours have a low incidence of metastasis, but a high incidence of local recurrence, which is why the mortality rate is high. The 5-year survival rate is 50%. World Health Organization (WHO) criteria are used for histopathological classification and 3 types of SNAC are listed. The most common are muco-alveolar or mucinous, similar to those found in the intestinal tract, which is why they are also referred to as "colonic." The other 2 types, which are less frequent, are papillary and NOS (not otherwise specified).[9,10]

Little is known about SNAC tumorigenesis. Studies[11,12] with comparative genomic hybridization (CGH) show that gains in chromosomal regions 5p15, 8q24, 20q13, and 12p13

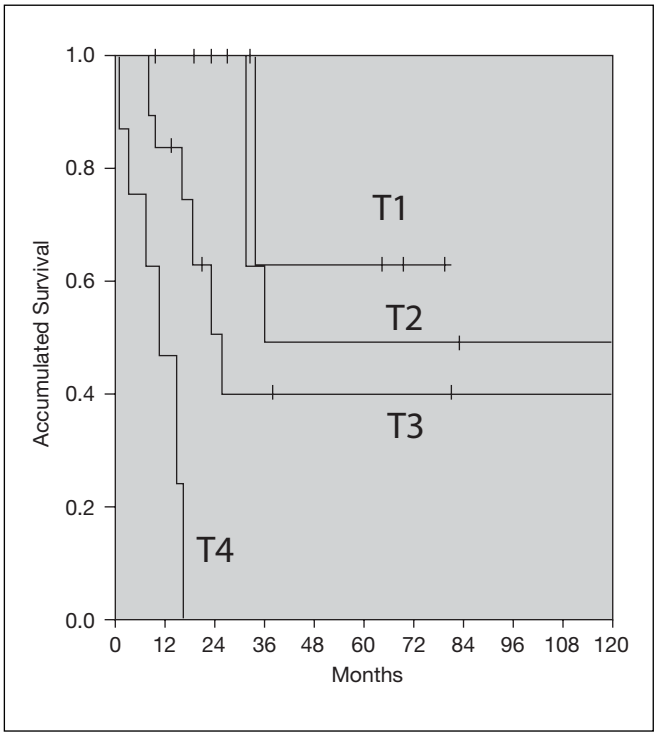

Figure 1. Kaplan-Meier survival chart in relation to the T staging of the 37 sinonasal adenocarcinomas studied.

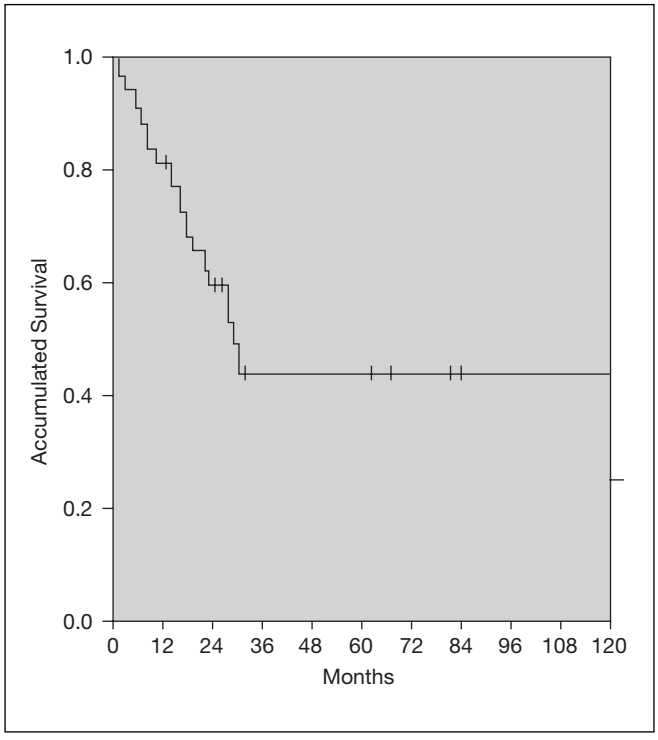

Figure 2. Kaplan-Meier general survival chart for the patients studied.

are frequent as well as losses in 4q31-qter, 8p23, 18q12-22, and 5q11-qter. The aim of this study is to try to determine the presence of HPV, EBV, HSV, varicela zoster virus (VZV), adenovirus, and cytomegalovirus (CMV) in a SNAC series in order to know whether or not these viruses are involved in SNAC tumorigenesis.

MATERIALS AND METHOD

Thirty-seven samples were studied that were taken from patients having sinonasal adenocarcinoma, and who underwent unsuccessful interventions between 1991 and 2006. All were male and the average age was 65 years old (the interval was 45-91).

From a histological point of view, 26 adenocarcinomas were included in the colonic-type group, and 11 in the papillary-type group. Of the 37 patients, 32 (86%) were exposed to wood for an average of 28 years. Except for 1 patient who received preoperative radiotherapy, the primary treatment for all patients was surgery; 23 (62%) of the patients were operated on once, 1 patient was not operated on at all, and the 13 remaining patients were operated on 2 or more times. The approaches used are shown in Table 1. Twenty-six patients (70%) received complementary radiotherapy, 10 did not receive any complementary treatment and 1 received adjuvant chemotherapy.

Complete removal of the tumour was done in 33 (89%) patients, while the rest (4 patients) were left with either a microscopically or macroscopically affected area. The average hospital stay was 15 (11.5) (4-55) days.

All the patients were N0 and M0 at the time of diagnosis. T staging is shown in Table 2 and survival rates in Figure 1. Local recurrence was seen in 20 patients (51%) and remote metastasis in 4 (13%). On average recurrence or metastasis occurred 18 months after surgery. The 5-year survival rate for the series was 44% (Figure 2).

DNA Extraction

Tumour samples were taken during surgery. These were put in RNAlater and frozen at 20ºC. They were stored under those conditions until they were processed. DNA was taken using the Qiagen DNA Mini-Extraction Kit (Qiagen® GmbH, Hilden, Germany) following the manufacturer's recommendations.

Virus Detection Through PCR

Once the samples were prepared different protocols were used for genomic amplification of the different viruses: for HSV-1, HSV-2, VZV, CMV, and EBV a 1-tube multiple nested PCR was done; for adenovirus the nested PCR was done with 2 tubes, and for HPV, for 1 gene as well as the other, only 1 round of amplification was done. The primer sequences (Invitrogen®) are listed in Table 3.

In the case of the 1-tube nested PCR, 5 µL DNA was briefly added to a 20 µL reaction mix to 0.5 IU thermostable polymerase (Bioline, USA) to a 0.2 mL tube; 200 µmols of dNTP (Promega, U.S.A.), 1X PCR plug-in, 2 mmol of $MgCl_2$ and 0.5 pmol of each external primer. The thermal profile consisted of one cycle at 94ºC for 2 min., followed by 40 cycles at 94ºC for 30 s, 55ºC for 30 s, and 72ºC for 1 min., finishing up with one cycle at 72ºC for 5 min. For the second amplification 3 µL of 0.5 IU Taq polymerase was added, along with 25 pmol of each internal primer, to the previous tube, and then a new amplification round was done: 94ºC for 5 min., followed by 30 cycles at 94ºC for 30 s, 55ºC for 30 s, and 72ºC 30 s, finishing up with 1 cycle at 72ºC for 5 min.

For the double-tube nested PCR (adenovirus) the first part is exactly the same as the previous one, the only difference being that 25 pmol of the external primers was added. For the second amplification 5 µL of the first was added with 20 µL of a similar mix that contained the internal primers, with a thermal profile of: 94ºC for 2 min., followed by 30 cycles at 94ºC for 30 s, 55ºC for 1 min., and 72ºC for 30 s, finishing up with 1 cycle at 72ºC for 5 min.

For HPV the reactive mix was similar to that of adenovirus, with the following thermal profile: 94ºC for 5 min., followed by 40 cycles at 94ºC for 30 s, 55ºC for 30 s, and 72ºC for 1 min., finishing up with 1 cycle at 72ºC for 5 min.

All the PCR tests were done with standard measures to avoid cross contamination. The amplified segments are stored at 4ºC until they were identified by agarose gel electrophoresis with ethidium bromide. Reactions were considered to be positive when amplification products were detected at 137 bp for HSV-1, 109 bp for HSV-2, 325 bp for VZV, 300 bp for CMV, or adenovirus, 200 bp for EBV, 450 bp for the L1 gene of HPV, or 250 bp for the gen *ONCO* of HPV.

RESULTS

EBV DNA was detected in 3 (8.1%) of the 37 tumour samples taken and CMV DNA in 1 (2.7%) of the 37 cases that were analyzed. In both situations the tumours were well differentiated, at initial stages (2 in stage I and 2 in stage II) and with exophytic growth. Three of the patients worked with wood. The 4 patients that tested positive for the viruses evolved favourably; 3 patients were still alive 5 years later, while one 91-year-old patient died immediately after the surgery due to heart failure. Regarding survival rates, this difference was not significant, possibly due to the small number of cases within the infected group.

No HSV, HPV, adenovirus, or VZV DNA was seen in any of the 37 samples studied.

DISCUSSION

There are no previous studies linking viral presence to SNAC carcinogenesis. It is known that prolonged exposure to sawdust is an important aetiological factor. However, the sawdust mechanism of action is still unknown, since it does not seem to have mutagenic abilities, per se. SNAC aetiology has been linked to chronic inflammation, which may be caused by sawdust, just as it is in other

Table 3. Oligonucleotides Used As Primers for the Detection of the Viruses and the Genes They Amplify

Virus	*Name*	*Sequence (5'>3')*	*Gene*
VHS-1	HSV1.1	ATCACGGTAGCCCGGCCGTGTGACA	Glycoprotein D
	HSV1.2	CATACCGGAACGCACCACACAA	
	HSV1.3	CCATACCGACCACACCGACGA	
	HSV1.4	GGTAGTTGGTCGTTCGCGCTGAA	
VHS-2	HSV2.1	TCAGCCCATCCTCCTTCGGCAGTA	Glycoprotein G
	HSV2.2	GATCTGGTACTCGAATGTCTCCG	
	HSV2.3	AGACGTGCGGGTCGTACACG	
	HSV2.4	CGCGCGGTCCCAGATCGGCA	
VZV	VZV.1	GTTTTGTACTCCGGGTTG	*IE63*
	VZV.2	TTACATCCGATGGCGTAG	
	VZV.3	GCTCGTTGAGGACATCAACCGTGTT	
	VZV.4	CATCGTCGCTATCGTCTTCACCAC	
VEB	VEB-E1	AAGGAGGGTGGTTTGGAAAG	*EBNA*
	VEB-E2	AGACAATGGACTCCCTTAGC	
	VEB-I3	ATCGTGGTCAAGGAGGTTCC	
	VEB-I4	ACTCAATGGTGTAAGACGAC	
CMV	CMV-E1	TGAGGAATGTCAGCTTC	Glycoprotein B
	CMV-E2	TCATGAGGTCGTCCAGA	
	CMV-I3	CCAGCCTCAAGATCTTCAT	
	CMV-I4	TCGTCCAGACCCTTGAGGTA	
VPH-L1	MY11(+)	GCMCAGGGWCATAAYAATGG	*L1*
	MY09(–)	CGTCCMARRGGAWACTGATC	
VPH-ONCO	ONC-1	TGTCAAAAACCGTTGTGTCC	*E6/E7*
	ONC-2	GAGCTGTCGCTTAATTGCTC	
Adenovirus	Ade1N	TGGCCACCCCCTCGATG	*Hexon*
	Ade2N	GAGTAGGGTTTAAAGCTGG	
	Ade3N	CCGCAATGGTCTTACATG	

inflammatory processes, such as ulcerative colitis or colon adenocarcinomas.

Another aetiological possibility, as with what happens in the oropharynx, is a change caused by an oncogenic viral infection, which is why this study was done.

However, in our series of 37 SNAC samples, only 4 tested positive for virus. EBV DNA was found in 3 cases, and CMV DNA was found in 1 case. A technique such as PCR does not allow us to identify which cells hold the viral genome or where it is located, since EBV as well as CMV are very common viruses in the adult population, as well as the fact that once the infected individuals get the virus, they are carriers for the rest of their lives.[13,14] The presence of EBV and CMV seems due to the prevalence of the virus within the general population. For EBV, through PCR, the region that encodes EBNA proteins is amplified, while with CMV the region that is amplified encodes a protein that coats the virus (glycoprotein B), which is why the latter, as opposed to the former, does not imply that viral DNA is found within the host's genome even if testing positive for the virus. EBV was included in the study because an undeniable aetiological link has been seen with nasopharyngeal undifferentiated carcinoma.[13] It is a herpes virus involved as a causal agent in different types of tumours, among which are Burkitt's lymphoma, Hodgkin's disease, and nasopharyngeal carcinoma, which is a tumour anatomically similar to adenocarcinomas. All these tumours are characterized by the presence of viral DNA in their tumour cells and EBV DNA encoded latent gene expression together with an important degree of inflammatory infiltration. It is also

believed that inflammation may play an important part in tumour cell proliferation.[13]

Another virus analyzed was HPV, but with negative results in our study. In the studies done of head and neck epidermoid carcinomas, HPV was seen with a varying frequency, ranging from 10% to 80%,[15-17] even though the percentage of positive cases in those series with a larger number of patients is around 20%. HPV has a wide range of phenotypes. In this study we analyzed the *L1* gene in the late region, which encodes structural capsid proteins that are highly intact and similar in all types of HPV, as well as *E6* and *E7* genes (from the early region), which encode proteins that are important for neoplastic transformation of the infected epithelial cell and that favour neoplastic proliferation. The meta-analysis subdivided into different anatomical regions of the head and neck done by Hobbs et al[18] indicates that the link between cancer and HPV-16 is very strong for tonsils, intermediate for the oropharynx and weak for the oral cavity and larynx.[18] Studies have also been done in heterogeneous nasal and paranasal sinus tumours (not just adenocarcinomas). El-Mofty et al,[19] through PCR, studied HPV presence in 21 keratinizing squamous cell carcinomas (KSCC), 8 cases of non-keratinizing squamous cell carcinoma (NKSCC) and 10 cases of undifferentiated carcinoma (UC), with a total of 39 cases of sinonasal carcinomas. HPV-16 DNA was found in 9 cases (23%): 4 (19%) KSCC out of 21, 4 (50%) NKSCC out of 8 and 1 (10%) UC out of 10.

Adenovirus results were also negative in every case and were basically studied due to the interactions that oncoproteins E1B-55k and E1A of the adenovirus present, targeting, for example, p53 or pRB.

Viral DNA of other types of herpes viruses, such as HSV-1, HSV-2 and VZV, were also analyzed in this study, all resulting negative. The link between these and tumours is even rarer, but recent studies,[20] albeit not all of them,[21] indicate that HSV-1 and HSV-2 increase the risk of head and neck carcinomas.

Conclusion: our results indicate that HPV, EBV, HSV, VZV, adenoviruses, and CMV do not play an aetiological role in SNAC carcinogenesis.

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