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Viral Markers Inside Neoplastic Tissues in a Set of 68 Samples from 57 Upper Airways Cancer Cases

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Abstract: A contribution of Epstein-Barr Virus (EBV), Human Cytomegalovirus (HCMV), and certain types of Human Papilloma Virus (HPV) to the overall pathogenesis of upper airways cancers seems a priori reasonably conceivable. The existing evidence mostly relates them to nasopharyngeal squamous and undifferentiated carcinomas; indications emerge about their involvement in other upper airways cancers too. Markers of EBV, HCMV, HPV16, HPV18, (and Mycoplasma pneumoniae as a negative control) were searched in a series of 68 samples of neoplastic tissues from 57 patients diagnosed with different upper airways cancers. The results provide clues in favor of an actual role of EBV, HCMV, and HPV18, in the web of causation of upper airways squamous and undifferentiated carcinomas, possibly of upper airways adenocarcinomas too.

Keywords: sinonasal cancer (SNC); nasopharingeal cancer (NPC); adenocarcinoma; squamous cell carcinoma; squamocellular carcinoma; undifferentiated carcinoma; Human Papilloma Virus (HPV); Human Papilloma Virus 16 (HPV16); Human Papilloma Virus 18 (HPV18); Epstein-Barr Virus (EBV); Human Cytomegalovirus (HCMV)

1. Introduction

In 2023 IARC published an updated synoptic table summarizing the available evidence, emerging from its Monographs from 1 to 135 included, about the causal relationships between human cancers arising from specific sites and properly studied agents that the Institute classified as carcinogenic to humans (Group 1) or probably carcinogenic to humans (Group 2A) [1].

This IARC synopsis states that in humans:

sufficient evidence exists in favor of a causal relationship of Epstein-Barr Virus (EBV), formaldehyde, Chinese-style salted fish, and wood dust to nasopharyngeal cancer (NPC);

limited evidence exists in favor of a causal relationship of traditional Asian pickled vegetables and nasopharyngeal cancer (NPC);

sufficient evidence exists in favor of a causal relationship of isopropyl alcohol manufacture using strong acids, leather dust, Nickel compounds, Radium-226 and its decay products, Radium-228 and its decay products, tobacco smoking, and wood dust to sinonasal cancer (SNC);

limited evidence exists in favor of a causal relationship of work in carpentry and joinery, Chromium(VI) compounds, formaldehyde, and work in textile manufacturing industry to sinonasal cancer (SNC).

It is worth highlighting that the IARC evaluations on the basis of which the synopsis was drawn up were conducted and published in different periods, thus the considered evidence is differently updated from one agent to another.

In 2007 IARC dedicated its entire Monograph 90 to Human Papilloma Virus (HPV), stating that, at the date, multiple serotypes of the agent resulted as established or probable carcinogens to humans at multiple sites, but that an inadequate evidence existed about the carcinogenicity of HPV relating

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to the sinonasal tract neoplasms; the nasopharyngeal tract was not separately considered [2]. No subsequent IARC evaluation of the HPV carcinogenicity is currently available.

In 2014 IARC assigned to Human Cytomegalovirus (HCMV) a high priority for an overall evaluation, in light of both studies in human pointing to its potential role in glioblastoma, and of a strong evidence in favor of its carcinogenic potential emerging from animal models and mechanistic data [3]. No subsequent IARC evaluation of HCMV carcinogenicity is currently available, but the Institute announced IARC Monographs Meeting 139: Hepatitis D virus, human cytomegalovirus, and Merkel cell polyomavirus, which will be held on 3–10 June 2025.

Studies have been progressively piling up, positively supporting a role of HPV – in particular 16 (HPV16) and 18 (HPV18) serotypes, EBV, and HCMV in the pathogenesis of upper airways neoplasms [4-17]. Clearer evidence emerges about the contribution of HPV16, HPV18, EBV, and HCMV to the genesis of nasopharingeal squamous and undifferentiated carcinomas; to date, an equally clear connection has not been delineated relating to other upper airways neoplasms.

The continuity between the nasopharyngeal and sinonasal mucosal membranes, and the substantially shared dynamic of airborne particles and vapours impacting on the surfaces of both tracts could actually sustain the possibility that the aforementioned viruses could be in a position to exert tantamount transformative actions upon multiple cell lines of both districts.

Certainly, many kinds of viruses can affect the human upper airways without exerting any carcinogenic action upon them, but the local persistence and the local continued replication of particular viruses could constitute a premise of chronical diseases, and of some steps of the carcinogenic processes too.

Just as certainly, selected viral markers testing positive or negative inside neoplastic tissues doesn't necessarily testify or rule out that the corresponding viruses played a role in the carcinogenetic process; those viruses could have been present just as "passengers" along the carcinogenetic pathways and, on the contrary, those viruses could have contributed to the early stages of the carcinogenesis, otherwise going to fade during the following passages.

Under these prudential considerations, the distribution of positive and negative results for selected viral markers tested in neoplastic tissues could provide clues about the involvement of specific viruses in the pathway of specific neoplasms.

Referring to the overall upper airways cancers, studies being available by means of PubMed result to have been conducted predominantly searching viral markers for one agent at a time; the combined presence of markers of more than one virus at a time could offer a wider vision upon the viral carcinogenic processes in these sites.

2. Materials and Methods

The research of HPV16, HPV18, HCMV, EBV, and Mycoplasma Pneumoniae (MP), the last agent merely assumed as a negative control, was performed in a set of 68 samples of neoplastic tissues, fixed in formaldehyde and included in paraffin, from 57 patients diagnosed with an upper airways cancer at the Unit of Pathological Histology and Cytology of the Macerata General Hospital (Central Italy).

All the upper airways cancer cases in the records of the aforesaid Unit of Pathological Histology and Cytology, for whom one or more cancer tissue samples resulted available, were considered, in the first instance, as possibly eligible for the study.

Just 57 patients, born between 1910 and 1955 and diagnosed with an upper airway cancer between 1991 and 2012, were actually enrolled in the study; the selection was made for the purpose of excluding any possibility of destroying samples hypothetically yet susceptible to be used for clinical investigations in the future.

The 57 enrolled patients (46 males, 11 females) were diagnosed with the following cancer types:

15 intestinal-type adenocarcinomas (ITAC), arising in the sinonasal cavities (14 male patients, 1 female patient);

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16 adenocarcinomas of non-intestinal type (among which 3 cystic adenoidal carcinomas: two male patients, one female patient) or not otherwise specified, arising in the sinonasal cavities (on the whole, 14 male patients and 2 female patients);

16 squamous and undifferentiated carcinomas, arising in the sinonasal cavities (9 male patients, 6 female patients);

8 squamous and undifferentiated carcinomas, arising in the nasopharynx (7 male patients, 1 female patient);

2 melanomas arising one in a nasal cavity and the other in a maxillary sinus (both male patients).

All the patients were Italians of Italian ancestry, with the sole exception of a patient born in Morocco, now living in Italy, diagnosed with an undifferentiated nasopharyngeal carcinoma

One enrolled patient (diagnosed with an adenocarcinoma, not otherwise specified, arising in the ethmoid) contributed three samples, nine enrolled patients (contributed two samples for each, 47 enrolled patients contributed one sample for each.

Any selected cancer tissue sample was identified by means of the reference number stamped on the including paraffin block, as at the time coded at the Unit of Pathological Histology and Cytology. When more than one sample was available from a same enrolled patient, each sample remained univocally linked to the patient by means of an identical reference number followed by a dash and the number "1", "2", or "3".

The most relevant data about each one of the 57 studied cancer cases are synthesized in Table 1; the anatomical site from where any examined sample was collected and the histopathological descriptive classification of any studied neoplasm are separately shown. The cancer sites are coded as in ICD 10: C11 Malignant neoplasm of nasopharynx; C30.0 Malignant neoplasm of nasal cavity; C31Malignant neoplasm of accessory sinuses. The histopathological diagnoses are presented as resulting from the best overall available evidence**.**

Table 1. Most relevant data about each one of the 57 studied cancer cases.

REFERENCE NUMBER OF THE SAMPLE	YEAR OF BIRTH OF THE PATIENT	SEX OF THE PATIENT (M / F)	YEAR OF THE CANCER DIAGNOSIS	SITE OF OCCURRENCE OF THE CANCER (ICD-10)	PRIMARY CANCER? (YES/NO)	CANCER HISTOTYPE
230-1	1927	M	2001	ETHMOID SINUS / CELLS (C31)	YES	ADENOCARCINOMA OF NON-INTESTINAL TYPE OR NOT OTHERWISE SPECIFIED
230-2	1927	M	2001	ETHMOID SINUS / CELLS (C31)	YES	ADENOCARCINOMA OF NON-INTESTINAL TYPE OR NOT OTHERWISE SPECIFIED
230-3	1927	M	2001	ETHMOID SINUS / CELLS (C31)	YES	ADENOCARCINOMA OF NON-INTESTINAL TYPE OR NOT OTHERWISE SPECIFIED
305	1945	M	2010	NASAL CAVITY (C30.0)	YES	INTESTINAL-TYPE ADENOCARCINOMA (ITAC)
797	1929	F	2012	MAXILLARY SINUS (C31)	NO (PRIMARY ORAL CANCER)	SQUAMOUS OR UNDIFFERENTIATED CARCINOMA (SQUAMOUS)
426	1934	M	2012	NASAL CAVITY (C30.0)	YES	SQUAMOUS OR UNDIFFERENTIATED CARCINOMA (SQUAMOUS)
706	1942	M	1997	NASAL CAVITY	YES	INTESTINAL-TYPE

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				(C30.0)		ADENOCARCINOMA (ITAC)
836-1	1938	M	1995	NASAL CAVITY (C30.0)	YES	ADENOCARCINOMA OF NON-INTESTINAL TYPE OR NOT OTHERWISE SPECIFIED
836-2	1938	M	1995	NASAL CAVITY (C30.0)	YES	ADENOCARCINOMA OF NON-INTESTINAL TYPE OR NOT OTHERWISE SPECIFIED
1083	1957	M	1997	NASOPHARYNX (C11)	YES	SQUAMOUS OR UNDIFFERENTIATED CARCINOMA (UNDIFFERENTIATED)
1275	1926	M	2003	NASAL CAVITY (C30.0)	YES	MALIGNANT MELANOMA
1469	1942	M	2004	ETHMOID SINUS / CELLS (C31)	YES	INTESTINAL-TYPE ADENOCARCINOMA (ITAC)
1678	1927	M	2002	NASOPHARYNX (C11)	YES	SQUAMOUS OR UNDIFFERENTIATED CARCINOMA (SQUAMOUS)
1828	1946	M	2003	ETHMOID SINUS / CELLS (C31)	YES	INTESTINAL-TYPE ADENOCARCINOMA (ITAC)
1999	1935	M	1993	NASAL CAVITY (C30.0)	YES	ADENOCARCINOMA OF NON-INTESTINAL TYPE OR NOT OTHERWISE SPECIFIED
2611	1937	F	1998	NASAL CAVITY (C30.0)	YES	INTESTINAL-TYPE ADENOCARCINOMA (ITAC)
9702652	1934	M	1997	NASOPHARYNX (C11)	YES	SQUAMOUS OR UNDIFFERENTIATED CARCINOMA (SQUAMOUS)
2761	1930	M	1998	ETHMOID SINUS / CELLS (C31)	YES	INTESTINAL-TYPE ADENOCARCINOMA (ITAC)
3578-1	1950	M	2009	ETHMOID SINUS / CELLS (C31)	YES	INTESTINAL-TYPE ADENOCARCINOMA (ITAC)
3578-2	1950	M	2009	ETHMOID SINUS / CELLS (C31)	YES	INTESTINAL-TYPE ADENOCARCINOMA (ITAC)
3629	1941	M	1997	ETHMOID SINUS / CELLS (C31)E	YES	INTESTINAL-TYPE ADENOCARCINOMA (ITAC)
4011	1910	M	1994	NASAL CAVITY (C30.0)	YES	SQUAMOUS OR UNDIFFERENTIATED CARCINOMA (SQUAMOUS)
4054	1934	M	2010	ETHMOID SINUS / CELLS (C31)	YES	INTESTINAL-TYPE ADENOCARCINOMA (ITAC)
4106	1930	F	2004	NASAL CAVITY (C30.0)	YES	SQUAMOUS OR UNDIFFERENTIATED CARCINOMA (UNDIFFERENTIATED WITH NEUROENDOCRINE FEATURES)
4128	1924	F	2000	NASAL CAVITY (C30.0)	UNDEFINED	SQUAMOUS OR UNDIFFERENTIATED CARCINOMA (“CLEAR CELLS” NEOPLASM)
4565-1	1942	M	1999	NASAL CAVITY (C30.0)	YES	INTESTINAL-TYPE ADENOCARCINOMA (ITAC)
4565-2	1942	M	1999	NASAL CAVITY (C30.0)	YES	INTESTINAL-TYPE ADENOCARCINOMA (ITAC)
4575	1949	F	2010	FRONTAL SINUS	YES	ADENOCARCINOMA OF NON-

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				(C31)		INTESTINAL TYPE OR NOT OTHERWISE SPECIFIED
5830-1	1927	M	1997	NASAL CAVITY (C30.0)	YES	SQUAMOUS OR UNDIFFERENTIATED CARCINOMA (PAPILLARY, NOT OTHERWISE SPECIFIED)
5830-2	1927	M	1997	NASAL CAVITY (C30.0)	YES	SQUAMOUS OR UNDIFFERENTIATED CARCINOMA (PAPILLARY, NOT OTHERWISE SPECIFIED)
2575	1920	M	1994	UPPER AIRWAYS NOT OTHERWISE SPECIFIED	YES	SQUAMOUS OR UNDIFFERENTIATED CARCINOMA (SQUAMOUS)
6739	1944	M	2011	ETHMOID SINUS / CELLS (C31)	YES	INTESTINAL-TYPE ADENOCARCINOMA (ITAC)
7822	1921	F	2004	NASAL CAVITY (C30.0)	YES	ADENOCARCINOMA OF NON-INTESTINAL TYPE OR NOT OTHERWISE SPECIFIED (CYSTIC ADENOIDAL CARCINOMA)
8718-1	1930	M	2004	ETHMOID SINUS / CELLS (C31)	YES	INTESTINAL-TYPE ADENOCARCINOMA (ITAC)
8718-2	1930	M	2004	ETHMOID SINUS / CELLS (C31)	YES	INTESTINAL-TYPE ADENOCARCINOMA (ITAC)
8890	1923	M	1991	MAXILLARY SINUS (C31) DESTRO	YES	ADENOCARCINOMA OF NON-INTESTINAL TYPE OR NOT OTHERWISE SPECIFIED
9056	1926	M	2003	NASAL CAVITY (C30.0)	YES	ADENOCARCINOMA OF NON-INTESTINAL TYPE OR NOT OTHERWISE SPECIFIED (CYSTIC ADENOIDAL CARCINOMA)
540	1920	M	1996	UPPER AIRWAYS NOT OTHERWISE SPECIFIED	YES	SQUAMOUS OR UNDIFFERENTIATED CARCINOMA (SQUAMOUS)
10554	1948	M	2010	NASOPHARYNX (C11)	YES	SQUAMOUS OR UNDIFFERENTIATED CARCINOMA (UNDIFFERENTIATED)
10784	1953	M	1999	NASOPHARYNX (C11)	YES	ADENOCARCINOMA OF NON-INTESTINAL TYPE OR NOT OTHERWISE SPECIFIED (POORLY DIFFERENTIATED)
10860	1919	F	2004	NASAL CAVITY (C30.0)	YES	SQUAMOUS OR UNDIFFERENTIATED CARCINOMA (UNDIFFERENTIATED)
11431	1910	M	1991	MAXILLARY SINUS (C31)	YES	MALIGNANT MELANOMA
11851	1936	M	2002	ETHMOID SINUS / CELLS (C31)	YES	ADENOCARCINOMA OF NON-INTESTINAL TYPE OR NOT OTHERWISE SPECIFIED
11731	1931	M	2006	NASAL CAVITY (C30.0)	YES	ADENOCARCINOMA OF NON-INTESTINAL TYPE OR NOT OTHERWISE SPECIFIED
12195	1942	M	1997	NASAL CAVITY	YES	ADENOCARCINOMA OF NON-

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				(C30.0)		INTESTINAL TYPE OR NOT OTHERWISE SPECIFIED
20220	1917	M	2009	NASAL CAVITY (C30.0)	YES	SQUAMOUS OR UNDIFFERENTIATED CARCINOMA (SQUAMOUS)
12457	1919	M	1995	NASAL CAVITY (C30.0)	YES	ADENOCARCINOMA OF NON-INTESTINAL TYPE OR NOT OTHERWISE SPECIFIED
12680	1946	M	2004	NASAL CAVITY (C30.0)	YES	ADENOCARCINOMA OF NON-INTESTINAL TYPE OR NOT OTHERWISE SPECIFIED
12754	1951	M	2004	ETHMOID SINUS / CELLS (C31)	YES	INTESTINAL-TYPE ADENOCARCINOMA (ITAC)
12757-1	1939	M	2000	NASOPHARYNX (C11)	YES	SQUAMOUS OR UNDIFFERENTIATED CARCINOMA (POORLY DIFFERENTIATED)
12757-2	1939	M	2000	NASOPHARYNX (C11)	YES	SQUAMOUS OR UNDIFFERENTIATED CARCINOMA (POORLY DIFFERENTIATED)
13151	1937	M	1994	ETHMOID SINUS / CELLS (C31)	YES	INTESTINAL-TYPE ADENOCARCINOMA (ITAC)
13343	1928	M	2002	UPPER AIRWAYS NOT OTHERWISE SPECIFIED	YES	ADENOCARCINOMA OF NON-INTESTINAL TYPE OR NOT OTHERWISE SPECIFIED (CYSTIC ADENOIDAL CARCINOMA)
14196	1936	M	2004	NASOPHARYNX (C11)	YES	SQUAMOUS OR UNDIFFERENTIATED CARCINOMA (UNDIFFERENTIATED)
14198	1928	M	2000	NASAL CAVITY (C30.0)	YES	INTESTINAL-TYPE ADENOCARCINOMA (ITAC)
14328	1928	F	2012	NASAL CAVITY (C30.0)	YES	SQUAMOUS OR UNDIFFERENTIATED CARCINOMA (UNDIFFERENTIATED
14705-1	1924	M	2003	NASOPHARYNX (C11)	YES	SQUAMOUS OR UNDIFFERENTIATED CARCINOMA (UNDIFFERENTIATED)
14705-2	1924	M	2003	NASOPHARYNX (C11)	YES	SQUAMOUS OR UNDIFFERENTIATED CARCINOMA (UNDIFFERENTIATED)
15147-1	1939	F	2011	NASOPHARYNX (C11)	YES	SQUAMOUS OR UNDIFFERENTIATED CARCINOMA (SQUAMOUS)
15147-2	1939	F	2011	NASOPHARYNX (C11)	YES	SQUAMOUS OR UNDIFFERENTIATED CARCINOMA (SQUAMOUS)
15203-1	1936	M	2003	ETHMOID SINUS / CELLS (C31)	YES	ADENOCARCINOMA OF NON-INTESTINAL TYPE OR NOT OTHERWISE SPECIFIED
15203-2	1936	M	2003	ETHMOID SINUS / CELLS (C31)	YES	ADENOCARCINOMA OF NON-INTESTINAL TYPE OR NOT OTHERWISE SPECIFIED
15269	1926	F	1998	NASAL CAVITY	YES	SQUAMOUS OR

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				(C30.0)		UNDIFFERENTIATED CARCINOMA (SQUAMOUS)
15559	1946	M	2005	NASAL CAVITY (C30.0)	YES	SQUAMOUS OR UNDIFFERENTIATED CARCINOMA (UNDIFFERENTIATED
15680	1929	M	1991	NASAL CAVITY (C30.0)	YES	ADENOCARCINOMA OF NON-INTESTINAL TYPE OR NOT OTHERWISE SPECIFIED
15901	1931	M	2004	NASAL CAVITY (C30.0)	YES	SQUAMOUS OR UNDIFFERENTIATED CARCINOMA (SQUAMOUS)
17452	1947	F	2009	ETHMOID SINUS / CELLS (C31)	YES	SQUAMOUS OR UNDIFFERENTIATED CARCINOMA (NOT OTHERWISE SPECIFIED)
18903	1950	M	2012	NASOPHARYNX (C11)	YES	SQUAMOUS OR UNDIFFERENTIATED CARCINOMA (UNDIFFERENTIATED)

The overall set of 68 samples was processed between 2023 and 2024 by the diagnostic laboratory of the IZSUM - Istituto Zooprofilattico Sperimentale Umbria e Marche "Togo Rosati" in Fermo (Central Italy).

Both the Authors from AST Macerata and the ones from IZSUM have remained unaware of the patient's individual diagnosis till the end of the laboratory tests and the beginning of the evaluation of the results.

DNA was extracted from paraffin-embedded samples by using QIAamp DNA Mini Kit (QIAGEN, https://www.qiagen.com) following the manufacturer's instructions for DNA FFPE Tissue. Excess paraffin was removed from the samples using a scalpel, the tissues were cut into small portions and then the sections were immersed in 1 ml of xylene and rehydrated by ethanol. After the washing step with ethanol, proteins and harmful enzymes such as nucleases were digested by proteinase K and tissues were lysed adding a lysis buffers and heating the samples. The lysates were loaded onto the Spin Columns. The DNA selectively bound to the membrane and the contaminants passed through. Remaining contaminants and enzyme inhibitors were then removed in a second wash step, and DNA was eluted in TE buffer. Purified DNA was subsequently stored at −20°C and used in applications of polymerase chain reaction.

The presence of EBV, HCMV, HPV16, HPV18 and MP DNA was detected in this study performing two multiplex Real-time PCR and two simplex Real-time PCR: a EBNA-1/GAPDH multiplex Real-time PCR to detect EBV [18], and the human housekeeping gene GAPDH used as an internal control; a L1 and L2 multiplex Real-time PCR to detect respectively HPV18 and HPV16 [19], a glycoprotein B Real-time PCR to detect HCMV [20], and a ADP-ribosylating toxin Real-time PCR to detect MP [21].

Table 2 synthetically presents the primers and the probes being used for Real-time reactions.

Table 2. Primers and probes used for real-time PCR detection of EBV, HCMV, MP, GAPDH and HPV16-18.

)etection item	**Target gene**	**Oligo**	**Sequence**	**Reference**
EBV	EBNA-1	Forward	CCGCTCCTACCTGCAATATCA 3′	ıer et al., 2014
		Reverse	GGAAACCAGGGAGGCAAATC 3′	
		Probe	M-TGCAGCTTTGACGATGG-BHQ1 3′	
HCMV	ycoprotein B	Forward	′ GGCGAGGACAACGAAATCC 3′	ıng et al., 2018

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		Reverse	TGAGGCTGGGAAGCTGACAT 3′	
		Probe	M-TTGGGCAACCACCGCACTGAGG-BHQ1 3′	
		Forward	5′ CACAAACCCTAACACAGTAACTAG TAGCA 3′	
HPV 16	L2 protein	Reverse	TAACTTGTTGTGTTGTGCGACTAT 3′	ıo et al., 2012
		Probe	5′ FAM-CACAAACCCTAACACAGTAACTAG TAGCA-QSY 3′	
		Forward	5′ TTGGTTCAGGCTGGATTGC 3′	
HPV 18	L1 protein	Reverse	GGCAGATGGAGCAGAACGTTT 3′	ıo et al., 2012
		Probe	C-TTGGTTCAGGCTGGATTGC-QSY 3′	
		Forward	TTGGTAGCTGGTTACGGGAAT 3′	
		Reverse	GTCGGCACGAATTTCATATAAG 3′	
MP	ribosylating toxin	Probe	5′ FAM-TGTACCAGAGCACCCCAGAAGGGC T- BHQ1 3′	hell et al., 2008
		Forward	GGTGGTCTCCTCTGACTTCAACA 3′	
GAPDH	ceraldehyde 3-phosphate dehydrogenase	Reverse	5′ GTGGTCGTTGAGGGCAATG 3′	ıer et al., 2014
		Probe	C-CCACTCCTCCACCTTTGACGCTGG-BHQ1 3′	

Real-time PCR reactions for EBV, HCMV and MP were performed using a QS7 system (Applied Biosystems) under the following conditions: initial activation of 95°C for 20 seconds, followed by 45 cycles of 95°C for 1 sec and 60°C for 20 seconds.

Real-time PCR reaction for HPV16 and HPV18 was performed using a QS7 system (Applied Biosystems) under the following conditions: initial activation of 95°C for 20 seconds, followed by 45 cycles of 95°C for 1 sec and 58°C for 20 seconds.

3. Results

The analytical results are synthetically presented in Table 3; MP always tested negative and GAPDH always tested positive, as expected.

Table 3. Results of the Real Time PCR tests for MP, EBV, HCMV, GAPDH, HPV 16, and HPV 18) (each sample is identified by three-to-seven digit reference number, followed by a dash and the number "1", "2", or "3" when single patients contributed two or three samples for each; N= Negative, P=Positive).

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ID	MP	EBV	HCMV	GAPDH	HPV16	HPV18
230-1	N	N	N	P	N	N
230-2	N	N	N	P	N	N
230-3	N	N	N	P	N	N
305	N	P	N	P	N	N
797	N	N	N	P	N	N
426	N	P	N	P	N	N
706	N	N	N	P	N	N
836-1	N	N	P	P	N	N
836-2	N	N	N	P	N	N
1083	N	P	N	P	N	N
1275	N	N	N	P	N	N
1469	N	N	N	P	N	P
1678	N	P	N	P	N	N
1828	N	N	N	P	N	N
1999	N	N	N	P	N	N
2611	N	N	N	P	N	P
9702652	N	P	N	P	N	N
2761	N	N	N	P	N	N
3578-1	N	N	N	P	N	N
3578-2	N	N	N	P	N	N
3629	N	N	N	P	N	N
4011	N	N	N	P	N	N
4054	N	P	N	P	N	N
4106	N	N	N	P	N	N
4128	N	N	N	P	N	N
4565-1	N	N	N	P	N	N
4565-2	N	N	N	P	N	N
4575	N	N	N	P	N	P
5830-1	N	N	N	P	N	N
5830-2	N	N	N	P	N	N
2575	N	P	N	P	N	N
6739	N	N	N	P	N	N
7822	N	N	N	P	N	N
8718-1	N	N	N	P	N	N
8718-2	N	N	N	P	N	N
8890	N	N	N	P	N	N
9056	N	N	N	P	N	N
540	N	N	N	P	N	N
10554	N	P	N	P	N	N
10784	N	P	N	P	N	N
10860	N	N	N	P	N	N
11731	N	N	N	P	N	N
11851	N	P	N	P	N	N
11431	N	N	N	P	N	N
12195	N	N	P	P	N	N
12220	N	N	N	P	N	N
12457	N	N	N	P	N	N
12680	N	N	N	P	N	N
12754	N	N	N	P	N	N
12757-1	N	P	N	P	N	N
12757-2	N	P	N	P	N	N

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13151	N	N	N	P	N	N
13343	N	N	N	P	N	N
14196	N	P	N	P	N	N
14198	N	N	N	P	N	N
14328	N	N	N	P	N	N
14705-1	N	N	N	P	N	N
14705-2	N	N	N	P	N	N
15147-1	N	N	N	P	N	N
15147-2	N	P	N	P	N	N
15203-1	N	N	N	P	N	N
15203-2	N	N	N	P	N	N
15269	N	N	N	P	N	N
15559	N	N	P	P	N	N
15680	N	N	N	P	N	N
15901	N	N	N	P	N	N
17452	N	N	N	P	N	N
18903	N	P	N	P	N	N

An overall pattern of 21 positive tests was found:

15 samples which tested positive for EBV (22.06% of the samples) came from 14 different patients (24,6% of the patients), respectively diagnosed with:

- 2 intestinal type adenocarcinomas (ITAC), arising one in a nasal cavity (ICD-10 C30.0), the other in an ethmoid sinus / ethmoid cells (ICD-10 C31);
- 1 papillary adenocarcinoma of non-intestinal type, not otherwise specified, arising an ethmoid sinus / ethmoid cells (ICD-10 C31);
- 1 poorly differentiated adenocarcinoma of non-intestinal type, arising in the nasopharynx (ICD-10 C11);
- 1 squamous carcinoma, arising in a nasal cavity (ICD-10 C30.0);
- 9 squamous or undifferentiated nasopharyngeal squamous carcinomas (ICD-10 C11);
- 1 squamous carcinoma, arising in not-otherwise specified upper airways.
- EBV largely resulted the most prevalent virus in the examined case series.

It is worth noting that two samples from the same patient, diagnosed with a poorly differentiated carcinoma, arising in the nasopharynx (ICD-10 C11), both tested positive for EBV (reference numbers 12757-1 and 12757-2), and that two other samples from another patient, diagnosed with a squamous carcinoma, arising in the nasopharynx (ICD-10 C11), tested the one positive and the other negative for EBV (reference numbers 15147-1 and 15147-2).

3 samples which tested positive for HCMV (4,41% of the samples) came from as many patients (5.26% of the patients), respectively diagnosed with sinonasal adenocarcinoma not otherwise specified (2 cases) and sinonasal undifferentiated carcinoma (one case).

It is worth noting that two samples from the same patient, diagnosed with one of the two aforementioned adenocarcinomas, tested one positive and the other negative for HCMV (reference numbers 836-1 and 836-2).

3 samples which tested positive for HPV18 (4,41% of the samples) came from as many patients (5.26% of the patients), respectively diagnosed with sinonasal intestinal-type adenocarcinoma (ITAC) (2 cases) and sinonasal adenocarcinoma not otherwise specified (one case).

All the 68 examined samples tested negative for HPV16.

The two sinonasal melanoma samples (reference numbers 1275 and 11431) both tested negative for all the searched viruses.

No multiple positivity was observed for any combination of the searched viruses.

The most relevant analytical results are plotted with the clinical ones in Table 4.

Table 4. Main analytical results plotted with the clinic ones. .

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REFERENCE NUMBER OF THE SAMPLE	YEAR OF BIRTH OF THE PATIENT	SEX OF THE PATIENT (M / F)	YEAR OF THE CANCER DIAGNOSIS	SITE OF OCCURRENCE OF THE CANCER (ICD-10)	PRIMARY CANCER? (YES/NO)	HISTOLOGICAL TYPE OF THE CANCER	RELEVANT RESULTS (FOR AGENT)
305	1945	M	2010	NASAL CAVITY (C30.0)	YES	INTESTINAL-TYPE ADENOCARCINOMA (ITAC)	**EBV +**
426	1934	M	2012	NASAL CAVITY (C30.0)	YES	SQUAMOUS OR UNDIFFERENTIATED CARCINOMA (SQUAMOUS)	**EBV +**
836-1	1938	M	1995	NASAL CAVITY (C30.0)	YES	ADENOCARCINOMA OF NON-INTESTINAL TYPE OR NOT OTHERWISE SPECIFIED	**HCMV+**
836-2	1938	M	1995	NASAL CAVITY (C30.0)	YES	ADENOCARCINOMA OF NON-INTESTINAL TYPE OR NOT OTHERWISE SPECIFIED	**HCMV-**
1083	1957	M	1997	NASOPHARYNX (C11)	YES	SQUAMOUS OR UNDIFFERENTIATED CARCINOMA (UNDIFFERENTIATED)	**EBV +**
1469	1942	M	2004	ETHMOID SINUS / CELLS (C31)	YES	INTESTINAL-TYPE ADENOCARCINOMA (ITAC)	**HPV18+**
1678	1927	M	2002	NASOPHARYNX (C11)	YES	SQUAMOUS OR UNDIFFERENTIATED CARCINOMA (SQUAMOUS)	**EBV+**
2611	1937	F	1998	NASAL CAVITY (C30.0)	YES	INTESTINAL-TYPE ADENOCARCINOMA (ITAC)	**HPV18+**
9702652	1934	M	1997	NASOPHARYNX (C11)	YES	SQUAMOUS OR UNDIFFERENTIATED CARCINOMA (SQUAMOUS)	**EBV+**
4054	1934	M	2010	ETHMOID SINUS / CELLS (C31)	YES	INTESTINAL-TYPE ADENOCARCINOMA (ITAC)	**EBV+**
4575	1949	F	2010	FRONTAL SINUS (C31)	YES	ADENOCARCINOMA OF NON-INTESTINAL TYPE OR NOT	**HPV18+**

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						OTHERWISE SPECIFIED	
2575	1920	M	1994	UPPER AIRWAYS NOT OTHERWISE SPECIFIED	YES	SQUAMOUS OR UNDIFFERENTIATED CARCINOMA (SQUAMOUS)	**EBV+**
10554	1948	M	2010	NASOPHARYNX (C11)	YES	SQUAMOUS OR UNDIFFERENTIATED CARCINOMA (UNDIFFERENTIATED)	**EBV+**
10784	1953	M	1999	NASOPHARYNX (C11)	YES	ADENOCARCINOMA OF NON-INTESTINAL TYPE OR NOT OTHERWISE SPECIFIED (POORLY DIFFERENTIATED)	**EBV+**
11851	1936	M	2002	ETHMOID SINUS / CELLS (C31)	YES	ADENOCARCINOMA OF NON-INTESTINAL TYPE OR NOT OTHERWISE SPECIFIED (PAPILLARY)	**EBV+**
12195	1942	M	1997	NASAL CAVITY (C30.0)	YES	ADENOCARCINOMA OF NON-INTESTINAL TYPE OR NOT OTHERWISE SPECIFIED	**HCMV+**
12757-1	1939	M	2000	NASOPHARYNX (C11)	YES	SQUAMOUS OR UNDIFFERENTIATED CARCINOMA (POORLY DIFFERENTIATED)	**EBV+**
12757-2	1939	M	2000	NASOPHARYNX (C11)	YES	SQUAMOUS OR UNDIFFERENTIATED CARCINOMA (POORLY DIFFERENTIATED)	**EBV+**
14196	1936	M	2004	NASOPHARYNX (C11)	YES	SQUAMOUS OR UNDIFFERENTIATED CARCINOMA (UNDIFFERENTIATED)	**EBV+**
15147-1	1939	F	2011	NASOPHARYNX (C11)	YES	SQUAMOUS OR UNDIFFERENTIATED CARCINOMA (SQUAMOUS)	**EBV-**
15147-2	1939	F	2011	NASOPHARYNX	YES	SQUAMOUS OR	**EBV+**

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				(C11)		UNDIFFERENTIATED CARCINOMA (SQUAMOUS)	
15559	1946	M	2005	NASAL CAVITY (C30.0)	YES	SQUAMOUS OR UNDIFFERENTIATED CARCINOMA (UNDIFFERENTIATED	**HCMV+**
18903	1950	M	2012	NASOPHARYNX (C11)	YES	SQUAMOUS OR UNDIFFERENTIATED CARCINOMA (UNDIFFERENTIATED)	**EBV+**

4. Discussion

The small dimension of the set of 68 samples from 57 patients that underwent analytical testing certainly does not allow anything else that reasonable pathogenic suggestions.

The oldness of the analysed samples constitutes a further criticality for the study, for the long time elapsed from the tissues collection and the testing probably entailed some degradation of the viral genetic material, so complicating its extraction and processing. This issue could have negatively affected the sensibility of the tests and could be also the basis of the inconsistencies between two couples of samples from a same patient who tested one positive and the other negative, respectively for EBV and HCMV.

No discernible source of false positive testing was conversely recognized, so that a high specificity of the results can be reasonably assumed.

Certainly, the mere presence of viral genetic material inside cancer cells is not a direct evidence of a real involvement of the agent in the carcinogenetic process, for it could have affected the involved tissue just as a passenger, without giving a real contribution to the neoplastic transformation.

Just as certainly, even apart from the aforementioned possibility of an after-sampling degradation, the mere absence of viral genetic material inside cancer cells not necessarily means that the agent never played a role in the carcinogenetic process, for it could underwent a clearance after having contributed, as a driver, to some steps of the tissue transformation.

Given the above, the detection of viral genetic material in a not negligible fraction of the analyzed cancer tissue samples gives clues in the direction of some involvement of EBV, HCMV, and HPV18 the carcinogenetic process, alleging the opportunity of further research.

The positivity for EBV observed in 15/68 samples (22.06%) from 14 patients (24,6%) constitutes a quantitatively unexpected result relating to an Italian population; both the sites of occurrence (4 sinonasal cancers, 10 nasopharyngeal cancers, 1 cancer arising in a not otherwise specified site of the upper airways) and the histotype pattern (4 adenocarcinomas; 11 squamocellular or undifferentiated carcinomas) result of interest too. EBV-related nasopharyngeal and sinonasal squamous carcinomas are reportedly endemic in East and Southeast Asia [22,23], but an equivalent evidence is not currently available for Italy. The association between EBV and both sinonasal and nasopharyngeal adenocarcinomas are exceptionally reported [24,25].

Unexpectedly, 2 out of 3 cancer cases which tested positive for HMCV were sinonasal adenocarcinomas; no previous report has been found about an association between HCMV and sinonasal adenocarcinomas.

Just as unexpectedly, all the 3 samples which tested positive for HPV18 referred to sinonasal adenocarcinomas, 2 classified as intestinal-type adenocarcinomas (ITAC), the other classified as adenocarcinoma not otherwise specified; no previous report has been found about an association between HCMV and sinonasal adenocarcinomas.

The absence of any positivity for HPV16 in all the 68 tested samples seems to point towards the absence of an involvement of the agent in the pathogenesis of upper airways cancers, at the very least

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relating to an Italian population. It is worth noting that an Italian study reported a high prevalence of HPV16 in sinonasal inverted papillomas presenting a high risk of transformation to sinonasal squamocellular carcinomas [26].

The negativity of both sinonasal melanoma samples for all the searched viruses is consistent with the current lack of evidence of an involvement of these agents in the pathogenesis of these non-epithelial cancers.

5. Conclusions

The observed distribution of positive and negative testing in the study group provides clues (some expected, some unexpected) about the involvement of HPV18, HCMV, and particularly EBV, in the pathways of specific epithelial upper airways cancers.

These findings suggest the opportunity to take in consideration a possible carcinogenetic contribution from these viruses to the overall set of the upper airways cancers (adenocarcinomas certainly not excluded), particularly when Public Health strategies are drawn up to contrast these cancers, both on the preventive side and on the side of focused programs for health surveillance of high risk groups.

An appropriate identification of population groups at high risk for upper airways cancers should reasonably consider any reasonable putative interaction between the considered viruses and the agents that IARC classified as certainly or probably related to sinonasal and nasopharyngeal cancers, namely leather dust, wood dust, and Nickel compounds for their prevalence in large groups of workers, and tobacco smoke for its diffusion in the general population [1].

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Informed Consent Statement: Patient consent was waived due the consideration that the analyzed samples referred to old and already completely diagnosed cases, and that the study results in no way could affect the therapeutic choices and the prognosis performed at that time, so that no patients interest subsists about both the treatment of the samples, and the study results.

Data Availability Statement: A dataset containing copies of all the original histological reports is available at AST Macerata - Occupational Safety and Health – Occupational Epidemiology Unit - Civitanova Marche (Italy); A dataset containing original reports of all the analytic results is available at IZSUM - Istituto Zooprofilattico Sperimentale Umbria e Marche "Togo Rosati" in Fermo (Italy).

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