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Molecular Genotyping of Human Papillomavirus (HPV) in Hypopharyngeal Cancer

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Abstract

Background: In recent years, head and neck cancers have become common worldwide, ranking sixth in incidence. In 2007, in France the incidence increased by 14,697 including 11,158 among men, which places them in fourth place. The same year, 32,268 patients were hospitalized for this pathology, but 95% are associated with alcohol and tobacco poisoning. Few data exist on these cancers in Africa and Senegal. In recent years, many studies have hypothesized that about 25% of head and neck cancers are associated with high-risk oncogenic human papillomaviruses (HPV) whose role in cervical cancer was already widely established. **Objective:** To know the prevalence and genotypes of HPV in head and neck cancers, particularly hypopharyngeal cancer. **Material and method:** This study was carried out on samples of biopsies of hypopharynx cancerous tissue (ulcerative-budding lesion) and healthy oropharyngeal tissue obtained from the ENT department of the Fann hospital, then sent to the Molecular Biology Unit of the Ouakam military hospital (HMO). The nucleic acids extraction was carried out using the standard method of the Zymo research kit "Quick-DNA™ Miniprep Plus kit" https://www.zymoresearch.com/. Molecular HPV detection and genotyping were performed by multiplex RT-PCR with the Seegene Anyplex™ II HPV28 kit Detection on a Biorad CFX96 automaton according to the manufacturer's protocol for the simultaneous genotyping of 28 types of HPV including 19 at High Risk (HR) and 9 low risk

(LR). **Results:** 156 patients were sampled, 61 Hypopharynx cancer biopsies and 95 healthy tissues. The median age of the general population was 36.5 years [12, 73]; the median age of the population with hypopharyngeal cancer of 40 years. Of the general study population 24.36% (38/156) was infected with HPV. In populations with hypopharyngeal cancer, HPV prevalence was 19.67% (12/61), 17.84% (5/28) in men and 21.21% (7/33) in women. HPV6 was the most frequently encountered genotype in the cancer population. Multiple infections have also been noted in cancer patients: HPV6+HPV18, HPV6+HPV56. For patients without hypopharyngeal cancer, the HPV prevalence was 27.36% (26/95), 9.59% (7/73) in women and 89.36% (19/22) in men. Several types of HPV-HR genotypes (HPV18, HPV26, HPV69), and HPV-LR genotypes (HPV42, HPV43, HPV70, HPV6) have been detected in healthy patients but also cases of co-infections (HPV6+HPV69; HPV56+HPV44; HPV58+HPV18). **Conclusion:** Our results showed a higher prevalence of HPV in non-cancer patients compared to hypopharyngeal cancer patients. The genotypes (HPV 6, 18 and 56) were observed in the study population. Molecular genotyping does not show a significant involvement of HPV in hypopharyngeal cancer.

Keywords

HPV, Genotypes, RT-PCR, ENT Cancer, Hypopharynx

1. Introduction

Head and neck cancers are essentially upper aerodigestive tract cancers developed at the expense of the oral cavity, pharynx, larynx and nasal sinus cavities [1]. Cancer of the hypopharynx is a neoformation made of anarchic and uncontrollable, primary or secondary cell multiplication developed at the expense of the component tissues of the hypopharynx [2].

The main risk factors for ENT cancer are attributed to alcohol and tobacco.

Around the world, these cancers are estimated at about 500,000 new cases every year [1].

Hypopharyngeal cancer is the most common cancer in emerging countries. It is the 7th most frequently diagnosed cancer in developing countries and the 10th worldwide [3]. France and India are the countries most affected by this cancer [4]. In 2007, in France, the incidence increased by 14,697 including 11,158 among men, which places them in fourth place worldwide [5]. That same year, 32,268 patients were hospitalized for this pathology, but 95% were associated with alcohol and tobacco intoxication [5] [6]. The risk of developing head and neck cancer increases with the number of cigarettes smoked and with the duration of smoking [7]. Tobacco is involved in 54% to 87% of head and neck cancers [8]. The combined consumption of alcohol and tobacco constitutes a multiplicative risk in the occurrence of head and neck cancer [9]. Vitamin deficiencies, especially vitamins A and C linked to an unbalanced diet would contribute to the occurrence of cancers [10]. An epidemiological study conducted now confirms that GERD is a risk

factor in laryngopharyngeal cancer [11]. Gastric acidity rising in the esophagus and in the hypopharynx leads to irritation of the pharyngeal mucosa which can be a risk of cancer of the hypopharynx [12]. In Senegal, it is the most common head and neck cancer in the ENT department of the CHU de Fann [13] [14]. Studies have shown a different epidemiological profile with a predominance of young women not consuming alcohol or tobacco [13]. These findings point to the existence of other risk factors [15]. This hypothesis has been corroborated by other studies carried out mainly in the white race [16] [17]. HPV-associated carcinomas have a better therapeutic response and a much better prognosis than other head and neck carcinomas [16]. In 1983, Syrjänen *et al.* were the first to suggest a possible role for human papillomavirus (HPV) in the genesis of a number of carcinomas of the oral cavity [18]. In recent years, numerous studies have shown that approximately 25% of head and neck cancers are associated with high-risk oncogenic human papillomaviruses (HPV), the role of which in cervical cancer has already been widely established [5]. A recent study showed that HPV infection in patients with cancer of the oral cavity, larynx and hypopharynx is at least five times lower than that of oropharyngeal cancer [19]. Specific therapeutic management of these virus-induced cancers is recommended by some authors [16].

HPVs are bicentennial naked DNA viruses belonging to the family of papillomaviridae. Currently, more than 200 different genotypes have been identified [20]. According to the International Agency for Research on Cancer (AIRC) classifies HPV genotypes 16 and 18 as human carcinogens [21]. These types of HPV-induced cancers constitute a major public health problem due to their high incidence of high morbidity and mortality [12].

The objective of this study was to assess the prevalence of HPV and identify HPV genotypes in hypopharyngeal cancers and determine the contribution of sociodemographic factors in this type of cancer.

2. Materials and Methods

2.1. Study Type and Population

This retrospective, descriptive and comparative study was carried out on samples of biopsies of cancerous tissues of the hypopharynx (ulcers-budding lesion) carried out by the team of the ENT clinic of the Fann hospital in Dakar and oropharyngeal samples in patients without hypopharyngeal cancer at the Molecular Biology Laboratory of the Armed Forces AIDS Control Program at Ouakam Military Hospital (HMO) Dakar, Senegal in the period from April 2020 to August 2021. The total population consisted of 156 patients, 61 of whom were suffering from hypopharyngeal cancer and 95 patients without hypopharyngeal cancer. Each patient had given his consent and had answered a questionnaire collecting information on his identity and socio-demographic parameters.

2.2. DNA Extraction and Nanodrop Lite Assay

DNA extraction was done from cancerous tissue biopsies from the hypopharynx

and healthy oropharyngeal tissue samples using the Zymo DNA kitTM Research, USA, (https://www.zymoresearch.com/) according to the manufacturer's protocol. DNA purity was checked by Nanodrop™ Lite reading assessing its quality and quantity, and the DNA extract was stored at −20˚C until PCR.

2.3. Molecular Genotyping Test (Multiplex PCR)

Molecular analysis was performed on a Biorad CFX96 machine (https://www.bio-rad.com/) according to the manufacturer's protocol (Hercules, California) using the Seegene Anyplex™ II HPV28 Detection kit (https://www.seegene.com/) which is a real-time multiplex PCR technique for the simultaneous genotyping of 28 types of HPV including 19 HR-HPV (16, 18, 26, 31, 33, 35, 39, 45, 51, 52, 53, 56, 58, 59, 66, 68, 69, 73, 82) and 9 to LR-HPV (11, 40, 42, 43, 44, 54, 6, 61, 70). This PCR uses DPO (double priming oligonucleotide) and TOCETM (Tagging Oligonucleotide Cleavage and Extension) technology [22].

2.4. Statistical Analysis

The data collected from the questionnaires of the patient survey form and the PCR register were entered into the Excel 2016 software and the data analysis was carried out using the R Studio software version (3.6.0). The qualitative variables were described with an absolute frequency and a relative frequency in percentage. Pearson's chi-square test or Fisher's exact test was used to compare statistical differences with a significance level set at $p < 0.05$.

3. Results

3.1. Prevalence of HPV

Our results showed HPV prevalences of 24.4%, 19.67% and 27.37% respectively in the total population, cancer patients and non-cancer populations (**Table 1**, $p = 0.367$). The positivity rate was lower in cancer patients.

3.1.1. HPV Prevalence by Age

The median age of patients with HPV+ hypopharyngeal cancer was 36 years with extremes of 12 to 57 years. In the cancer population, this prevalence was 11.48% and 8.20% respectively in the 0 to 40 and 40 to 80 age groups. The prevalence of HPV was respectively in the age groups from 0 to 40 years and 40 to 80 years, 16.03% and 8.33% in the general population and, 18.95% and 8.42% in the control population without hypopharyngeal cancer. Patients aged between 0 and 40 years were more exposed to HPV infection (**Table 2**).

3.1.2. HPV Prevalence by Gender

In the study, men were more affected by HPV infection (15.38%) than women (8.97%) in the general population. HPV prevalence in women and men was 11.47% and 8.20%, respectively, in the cancer population; 77.7% and 20% in the control population without cancer of the hypopharynx. In both types of populations, women had a higher prevalence. The cancer population was less affected

than the control population (Table 2).

According to marital status, the prevalence of HPV was 12.82% and 11.54% among married and unmarried (widowed, divorced, single) respectively in the general population.

Table 1. Prevalence of HPV genotypes in the general population.

Genotypes		Prevalence (%)		
		Overall population (N = 156) n (%)	Cancer population (N = 61) n (%)	Control population (N = 95) n (%)
	HPV	38 (24.4)	12 (19.67)	26 (27.36) (p = 0.367)
HPV high risks (HR-HPV)	**HR-HPV**	**6 (3.85)**	**0**	**6 (6.32)**
	HPV18	2 (1.28)	0	2 (2.11)
	HPV26	3 (1.92)	0	3 (3.16)
	HPV69	1 (0.64)	0	1 (1.05)
HPV Low risks (LR-HPV)	**LR-HPV**	**26 (16.67)**	**9 (14.75)**	**17 (17.89)**
	HPV6	23 (14.74)	9 (14.75)	14 (14.74)
	HPV42	1 (0.64)	0	1 (1.05)
	HPV43	1 (0.64)	0	1 (1.05)
	HPV70	1 (0.64)	0	1 (1.05)
Co-infection	**Co-infection**	**6 (3.85)**	**3 (9.84)**	**3 (3.16)**
	HPV6+18	1 (0.64)	1 (0.02)	0
	HPV6+56	2 (1.28)	2 (0.33)	0
	HPV6+HPV69	1 (0.64)	0	1 (1.05)
	HPV56+44	1 (0.64)	0	1 (1.05)
	HPV58+18	1 (0.64)	0	1 (1.05)

Table 2. Prevalence of HPV by age, sex, marital status and region.

Variables		HPV prevalence (%)		
		Population globale (N = 156)	Population cancéreuse (N = 61)	Population témoin (N = 95)
Sex	F	8.90%	11.47%	77.7%
	M	15.38%	8.20%	20%
Age	<40 ans	16.03%	11.48%	18.95%
	40 ans et plus	8.33%	8.20%	8.42%
Marital status	Marié	12.82%	3.85%	14.74%
	Non marié	11.54%	3.85%	12.64%
Region	Dakar	23.08%	100%	25.26%
	Saint-Louis	0.64%	0%	1.05%
	Thiès	0.64%	0%	1.05%

In cancer patients, the HPV prevalence was 3.85%, similar in married and unmarried (Table 2), as well as in the control group 14.74% versus 12.64%. Marital status was not related to HPV infection in our study population

3.1.3. HPV Prevalence by Region

The evaluation of HPV carriage according to the region showed an HPV prevalence of 23.07% for patients from Dakar and 1.28% for patients from Saint-Louis and Thiès (Table 2).

3.2. HPV Genotypes Found in the Study Population

HR-HPV had respective prevalences of 3.85% and 6.32% in the general and control populations and was not found in the cancer population.

LR-HPV showed prevalences of 16.67%, 14.75% and 17.89% respectively in the general, cancerous and control populations (Table 1).

Of all the genotypes studied, HPV6 was the most frequently encountered genotype in the cancer population. Multiple infections were also noted in cancer patients: HPV6+HPV18, and HPV6+HPV56 (Table 1). Several genotypes of HPV-HR (HPV18, HPV26, HPV69), and HPV-LR (HPV42, HPV43, HPV70, HPV6) have been detected in healthy patients but also cases of co-infections such as (HPV6+HPV69; HPV56+HPVs44; HPV58+HPV18).

3.3. Socio-Demographic Characteristics and Risk Factors

Since the difference in HPV prevalence was not significant between the control group and that of cancer patients ($p = 0.367$; Table 3), we were interested in other sociodemographic factors likely to be linked to hypopharyngeal cancer.

In our population with hypopharyngeal cancer, 54.1% were over 40 years old and 45.9% for under 40 years old ($p = 0.09$; OR = 2.24; Table 3).

Gender had a link with the occurrence of hypopharyngeal cancer, women were more affected than men; 54.1% versus 45.9% ($p = 0.001$; OR = 5.51; Table 3).

In addition, the data showed the occurrence of hypopharyngeal cancer was greater in non-alcoholics compared to alcoholics, 86.9% vs. 13.1% ($p = 002$; Table 3).

Smoking, marital status and region of residence were not related to the occurrence of cancer (Table 3). We were interested in the reasons for consultation, that is to say, the symptomatology.

3.4. Breakdown of Patients by Reason for Consultation

The patients came to consult for several reasons (Table 4). The following symptoms such as dysphagia, odynophagia, dysphonia, laryngeal dyspnea and reflex otalgia were significantly more present in cancer patients than in non-cancer subjects ($p < 0.001$; Table 4). The data showed a link between the occurrence of hypopharyngeal cancer and these symptoms. Hypersialorrhoea and gastroesophageal reflux were not related to the occurrence of hypopharyngeal cancer ($p > 0.05$; Table 4).

Table 3. Socio-demographic and risk factors for occurrence of hypopharyngeal cancer.

Variables		Cancer		p-value	OR
		Yes	No		
		N = 61	N = 95		
Sex	F	33 (54.1%)	22 (23.2%)	<0.001	5.51
	M	28 (45.9%)	73 (76.8%)		
Age	Under 40 years	28 (45.9%)	65 (68.4%)	0.009	2.24
	Over 40 years	33 (54.1%)	30 (31.6%)		
Alcohol	No	53 (86.9%)	94 (98.9%)	0.002	15.22
	Yes	8 (13.1%)	1 (1.1%)		
Marital status	Married	31 (50.8%)	49 (51.6%)	0.999	
	Not Married	30 (49.2%)	46 (48.4%)		
Region	Dakar	55 (90.2%)	89 (93.7%)	0.258	
	Saint-Louis	0 (0.00%)	2 (2.1%)		
	Thiès	6 (9.8%)	4 (4.2%)		
Tobacco	No	41 (67.2%)	75 (78.9%)	0.147	
	Yes	20 (32.8%)	20 (21.1%)		
HPV	Positive	12 (19.7%)	26 (27.4%)	0.367	

Table 4. Distribution of symptoms leading to consultation in cancer patients.

Reason for consultation	Tissue diagnostics		p-value
	TC (N = 61)	TS (N = 95)	
Dysphagia (%)	61 (100%)	8 (8.4%)	**<0.001**
Odynophagia (%)	41 (67.2%)	9 (9.5%)	**<0.001**
Dysphonia (%)	17 (27.9%)	4 (4.2%)	**<0.001**
Laryngeal dyspnea (%)	13 (21.3%)	3 (3.2%)	**0.001**
Reflex earache (%)	23 (37.7%)	5 (5.3%)	**<0.001**
Drooling (%)	3 (4.9%)	1 (1.1%)	0.3
GERD (%)	4 (6.6%)	4 (4.2%)	0.712

4. Discussion

The prevalence of HPV in our general population is 24.4%. Studies by Smith *et al.* in the USA (2010) and Heath *et al.* in the United Kingdom (2012) described higher prevalences, respectively of 27.8% and 30% [23] [24]. This rate was much lower than those described in Senegal (13%) [15].

HNSCC is characterized by a multifactorial etiology: known risk factors include

tobacco smoking, alcohol consumption, infection with Epstein Barr virus (EBV) and Human Papillomavirus (HPV) [25].

Few studies have addressed the relationship of HPV with hypopharyngeal carcinomas, and a wide variation (from 3% to 74%) in HPV prevalence has been reported in these cancers [26].

In our study, the prevalence of HPV in patients with hypopharynx cancer was found in 19.67% against 27.36% for the control population (p = 0.367). Our results also agree with those of Woto-Gaye *et al.* in Dakar which had found a prevalence of 19.2% [15]. This presence of HPV in the hypopharynx could be explained by the existence of squamous cells and lymphatic tissue similar in structure to that of the cervix [27]. The work of Quintero *et al.* (2013) in Colombia and Snijders *et al.* (1996) in Great Britain found similar trends of 18.9% and 20.6% respectively [28] [29]. In the southern United States, a high-risk HPV prevalence of 13% was found in oropharyngeal squamous cell carcinoma (SCC) [30]

In hypopharynx cancer 1.6% was found positive for HPV DNA by PCR, HPV is only occasionally involved in laryngeal and hypopharyngeal SCC patients in northern Spain [26]. There are various possible reasons to explain this disparity, such as geographical differences, the prevalence of smoking and drinking in the studied populations, anatomical subsite of the tumor, sample selection, time period of the study and/or distinct methods employed for HPV detection. The most informative test for a biologically relevant association between HPV infection and cancer is the analysis of viral E6/E7 oncogene transcripts in tumor specimens, but this is technically challenging [26].

By age, the 0 - 40 years range had an HPV prevalence of 11.48% in the cancer population. Patients with head and neck and HPV-positive carcinomas were between 35 and 66 years old, with a median of 42 years [15]. Contrary to the prevalences found in our series, the study by Ndiaye *et al.* showed a much lower prevalence in the age group under 40 (3.6%) [31]. This difference in prevalence can be explained by the distribution of the series which differs from one study to another. That being said, all age groups can harbor HPV. According to gender, there was a predominance of HPV in women (11.47%) in the cancer population. In cancer subjects, the prevalence of HPV was identical regardless of marital status, the status was not related to HPV infection. As far as the region is concerned, all the patients with head and neck cancer and HPV-positive came from the Dakar region.

Of all the genotypes studied, HPV 6 was the most common genotype in head and neck tumors. Consistent with our results, HPV 6 (21.7%) was also the most prevalent genotype in the study of Roncin *et al.* [32] in addition to HPV 11. However, our results differ from previous studies. Indeed, Jung *et al.*; found HPV16 and HPV33 predominantly [33]. In addition, another study conducted by Kreimer *et al.*, found the HPV16 genotype to be the most prevalent [34]. As reported by Wang *et al.*, high-risk types 16, 31, and 33 are linked biologically to the development of oropharyngeal squamous cell carcinoma (SCC) [30]. Genotypes responsible and the site of the cancer in the ORL sphere remain questions

that it is important to elucidate. There is variability in the information reported by the studies.

Hypopharyngeal cancers occupy a "privileged" place among all head and neck cancers.

The median and average age of cancer patients were 40 and 42 years, respectively, with extremes of 12 and 73 years. Many studies carried out in Senegal had similar results but remained relatively superior. Indeed, a study conducted in Senegal in 2013 by Bouchra describes similar results [35]. In 2006, Diop reported an average age of 36 and extremes of 10 and 76 years [36]. However, our results differ from those of other studies conducted in other countries. Indeed, a study carried out in Mali by Traoré *et al.* (2008) out of 18 patients with laryngopharyngeal cancer found an average age of 58.7 years [37]. These same trends have been observed in several studies in different countries such as Taiwan where the average age was 56 years old [38], in the USA, 62 years old [39] and in Egypt 59.5 years [40]. The average age of our study population being lower, a study with a larger sample would be useful to study the why and the impact of socio-demographic and environmental factors in the precocity of the occurrence of cancers in our population.

With regard to sex, a female predominance was noted (54.10%) of cancer against 45.9% in men, *i.e.* a sex ratio M/F of 0.85. In recent years, there has been a decrease in hypopharyngeal cancer in men and an increase in women in France [41]. Our results are consistent with many previous studies reported in the literature in Senegal. Studies conducted by Diop (2006), by Ndiaye *et al.* (2009) and Bouchra, (2013) describe similar results showing a female predominance [13] [32] [33]. However, in other countries, studies show results that disagree with ours. Hypopharyngeal cancer has long been considered predominant in men worldwide with 95% of cases [42]. Similarly, studies reported in 2008 in Mali, in 2010 in Taiwan and in 2019 in Mauritania respectively by Traoré *et al.*; Chang *et al.*; and El Waled had found a male predominance of hypopharyngeal cancer [37] [38] [43]. This trend of feminization of hypopharyngeal cancer could be explained by a combination of factors such as women's exposure to smoke, dietary factors such as the poverty of vitamins in meals [35], viral infections such as HPV, and the use of certain toxic products in the home.

In Senegal, all regions are affected by cancers of the hypopharynx and particularly the Dakar region with a percentage of 90.2%. The regions of Thiès and Saint-Louis are less affected.

The HPV prevalences were 24.4%, 19.67% and 27.36% respectively in the general, cancerous and control population ($p = 0.367$). Our results do not allow us to confirm the involvement of HPV in the occurrence of hypopharyngeal cancer, so we will focus on other factors that may be linked to cancer such as alcohol, tobacco, age, sex, etc.

It is well known that prolonged exposure to tobacco, tobacco-like products and alcohol increases the risk of developing head and neck cancers. Garneau *et al.* state in their study, that patients with hypopharyngeal squamous cell carci-

noma usually have a history of smoking and excessive alcohol consumption [44]. In Senegal, the role of smoking and alcohol in the occurrence of hypopharyngeal cancer is not clearly elucidated.

According to our results, 32.8% of cancer patients were smokers against 67.2% of non-smokers ($p = 0.147$), which does not confirm a direct link between hypopharyngeal cancer and tobacco. The rate of non-alcoholic cancer patients (86.9%) is higher than alcoholic cancer patients ($p = 0.002$). The role of tobacco and alcohol is difficult to discuss in our context, especially since some patients could hide their true status because of religious judgments. Indeed, the studies of Bouchra (2013) and Diop (2006) confirm our results [35] [36]. Our results disagree with those of Chang *et al.* (2010) in Taiwan who found 86.6% of patients were smokers and 69.6% were alcoholics [38].

In this study, the risk of being cancerous is 5.51 times higher in women than men with a ($p < 0.001$) so there is an association between the onset of cancer and age but also between cancer and gender ($p < 0.001$). The risk of developing cancer is ($R = 2.24$) higher in the age group of 40 years and over than in those under 40 ($p = 0.009$).

The first most common reason for consultation remains dysphagia (100%). Our results are similar to the results of the work of Touré (2012) and Diop (2006), whose patients presented dysphagia of 100% and 98.6% respectively [12] [36]. Our results also join those of El Waled in 2019 who in his study had found 100% dysphagia which was the most frequent symptom.

5. Conclusion

Our results showed a lower prevalence of HPV in the cancer population than in the control population, which does not allow us to confirm a direct link between hypopharyngeal cancer and HPV infection. The study of other socio-demographic parameters showed that age, sex and alcohol were linked to hypopharyngeal cancer in our population. HPV genotypes were found in cancer patients, HPV6, HPV18 and HPV56 and the HPV6 genotype was the most represented in this study. All patients (100%) presented dysphagia as the most common symptom and present in all patients. A study with a larger sample of cancer patients would be necessary to invalidate or confirm our results.

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Conflicts of Interest

The authors declare no conflicts of interest regarding the publication of this paper.

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