

Original Article

The prevalence and distribution of HPV types in nasal inverted papilloma in Chinese Han population

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Abstract: Background: Human papillomavirus (HPV) was reported as a possible etiological agent for Nasal inverted papilloma (NIP). However, the prevalence of HPV types in NIP was not well known. Materials and Methods: 102 formalin-fixed paraffin-embedded NIP biopsy samples were obtained from patients and 20 samples were got from healthy controls. HPV DNA genotyping was achieved by a flow-through hybridization and gene-chip method and genotyping of 21 different HPV types. The incidences of HPV subtype infection in patients with NIP and its distribution across age, gender and stage were described. Results: Patients with NIP had higher incidence of HPV infection (64.7%) compared with healthy control. The positive rate was higher in patients with advanced Krouse Stage and in patients more than 59 years old. HPV 11 was the mostly-seen type in patients at Krouse Stage T1 and T2, and HPV 58 was found in 45.0% of patients with T3 NIP. There tends to be a difference in HPV infection rate between patients with NIP only and patients with NIP and dysplasia with no statistical significance. The most common HPV types in patients with NIP and dysplasia were HPV11 (40.8%) and HPV58 (27.6%) respectively. Conclusions: The prevalence of HPV in NIP was high (64.7%) and HPV infection was more seen in older patients and patients with advanced Krouse Stage. Different HPV type distribution was found across Krouse Stage and the degree of dysplasia.

Keywords: Papilloma, nasal inverted papilloma, HPV, genotype

Introduction

Nasal inverted papilloma (NIP) is one of the most common benign epithelial tumors of the nasal cavity and paranasal sinuses. It arises from the Schneiderian membrane lining of the nose and paranasal sinuses. Papilloma from the membrane is found to be growing inwards with unique history, biology and location. NIP is reported to have tendency of recurrence and associated with squamous cell carcinoma (SCC). Incidence of NIP is about 0.5% to 7.0% with recurrence rate of 0% to 80% depending on treatment types. Moreover, about 5% to 15% of benign NIP was reported to associate with SCC [1-4].

Little is known about the etiology of NIP. Human papillomavirus (HPV) was reported as a possi-

ble etiological agent for papilloma [5-8]. Clinical behavior of NIP is similar to recurrent respiratory papillomatosis (RPP) and genital warts, which were confirmed to have associations with HPV 6, 11, 16 and 18 infections. Most of the studies focused on the relationship of NIP and these four types. However, the prevalence of the HPV 6 and 11 in NIP were comparatively lower than that of the RRP which has about 100% prevalence [9].

Recently, a number of PCR-based HPV detection assays were developed, which advanced the detection and diagnosis of HPV infections. The HPV GenoArray assay (GA) utilized the PCR amplification technology followed by reverse hybridization against immobilized genotype-specific DNA probes, which were able to detect and genotype 21 different HPV types. Studies

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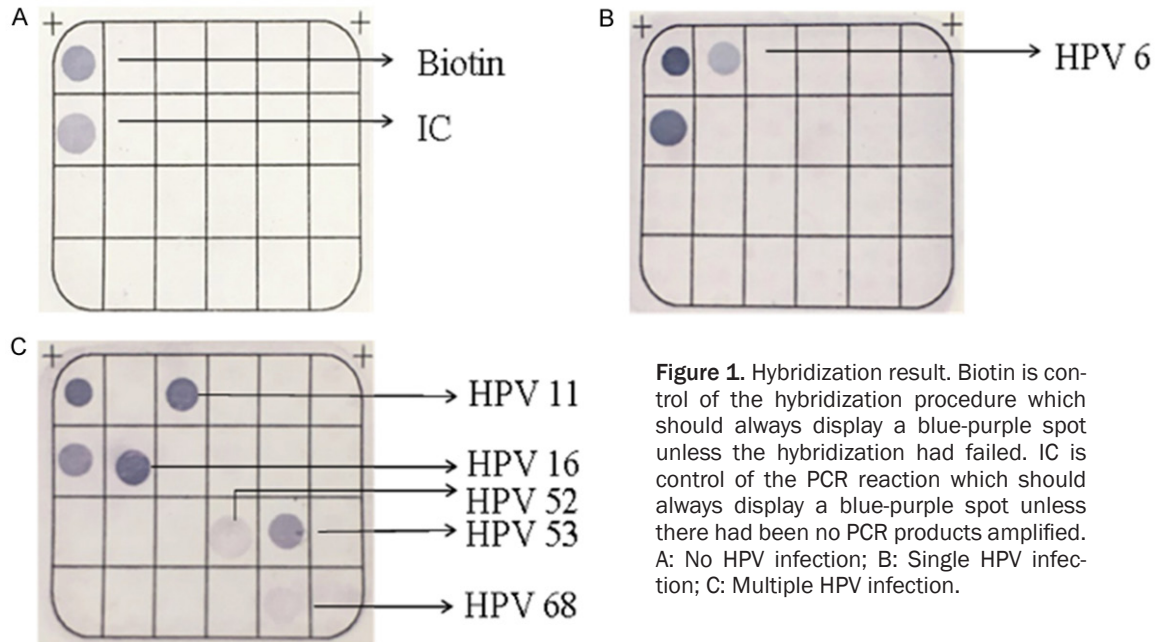


Figure 1. Hybridization result. Biotin is control of the hybridization procedure which should always display a blue-purple spot unless the hybridization had failed. IC is control of the PCR reaction which should always display a blue-purple spot unless there had been no PCR products amplified. A: No HPV infection; B: Single HPV infection; C: Multiple HPV infection.

suggested that the GA test has a high sensitivity and specificity for the detection of 21 HPVs with comparable performance with other similar PCR/hybridization-based HPV assays including Roche Linear Array HPV test and Qiagen Hybrid Capture II HPV test [10, 11]. The present study was designed to investigate the prevalence and genotype of HPV infections in NIP using paraffin-embedded NIP biopsy samples, aiming to investigate the distribution of different HPV types in NIP and its relationship with nasopharyngeal carcinoma development.

Materials and methods

Study population

In total, 102 cases of NIP tissue samples were obtained from the Department of Pathology, Shanghai Pudong New Area Gongli Hospital (Shanghai, China) between 2005 and 2014. Written informed consents from patients were obtained and ethical approval was got from local ethics committee. Turbinate mucosa samples from 20 healthy patients were obtained as control group.

Krouse stage of NIP

The patients were classified into Krouse Stage I to IV according to the Krouse staging system for NIP [12]. T1 means tumors confined to one wall of nasal cavity without sinus involvement.

T2 means tumors limited to the medial and superior maxillary sinus with or without the nasal cavity involvement. Ethmoidal sinus and nasal cavity may be also involved. T3 means tumors involves the lateral, inferior, anterior and/or posterior wall of maxillary sinus, sphenoid sinus or frontal sinus. Ethmoidal sinus and nasal cavity may be also involved. T4 means tumors extends outside the nose and/or paranasal sinus involving the adjacent structures including cranial bones and intracranial compartment.

Experimental procedures

DNA extraction: DNA was extracted from three sections of 10 μ m thick paraffin-embedded NIP samples as described elsewhere [5]. Total DNA was extracted using QIAamp DNA Blood mini-kit (Qiagen) according to manufacturer's instruction.

HPV genotyping: HPV genotyping was performed by the 21 HPV GenoArray Diagnostic Kit (Hyribio Limited, Hong Kong) as described previously [11]. The GA test is able to detect 21 types of HPV, including 13 High-risk types (HPV 16, 18, 31, 33, 35, 39, 45, 51, 52, 56, 58, 59 and 68), 6 low-risk and undetermined risk types (HPV 6, 11, 42, 43, 44 and CP 8304) and 2 probable high-risk types (HPV 53 and 66), using L1-consensus primer based PCR assay followed by reverse hybridization.

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Table 1. HPV Prevalence across gender, age and Krouse Stage in patients with NIP

	HPV		χ^2	P-value
	Positive	Negative		
Male	48	25	0.123	0.725
Female	18	11		
<40 years old	11	3	6.227	0.044
40-59 years old	34	26		
≥60 years old	21	7		
Krouse I (T1)	10	12	10.441	0.015
Krouse II (T2)	36	22		
Krouse III (T3)	18	2		
Krouse IV (T4)	2	0		
NIP only	48	32	3.597	0.058
NIP with dysplasia	18	4		

Table 2. HPV type distribution across Krouse Stage and pathological classification

Subtype	Total	Krouse Stages				Dysplasia	
		T1	T2	T3	T4	With	Without
11 [^]	33 (33.33%)	5	25	3	0	4	29
58 [▲]	19 (19.19%)	4	5	9	1	8	11
16 [▲]	13 (13.13%)	4	4	4	1	7	6
44 [^]	11 (11.11%)	1	9	1	0	2	9
52 [▲]	8 (8.08%)	2	2	4	0	3	5
33 [▲]	4 (4.04%)	1	0	3	0	2	2
6 [^]	3 (3.03%)	0	1	2	0	1	2
18 [▲]	3 (3.03%)	2	1	0	0	2	1
53 [▲]	2 (2.02%)	0	0	2	0	0	2
39 [▲]	1 (1.01%)	0	1	0	0	0	1
68 [▲]	1 (1.01%)	0	1	0	0	0	2
56 [▲]	1 (1.01%)	0	1	0	0	0	1

▲, HPV High-risk types; ^, HPV Low-risk types.

In brief, the PCR amplification was performed with a reaction volume of 25 μ l containing 1 μ l of extracted DNA template, 23.5 μ l of PCR master mix and 0.75 μ l of DNA Taq polymerase in the Verti-96-well thermocycler (Applied Biosystems). PCR amplification protocol was as follows: Initial denaturation at 95°C for 9 min, 40 amplification cycles at 95°C for 20 s (denaturation), 53°C for 30 s (annealing) and 75°C for 30 s (elongation) followed by 5 min at 72°C for final extension.

After PCR amplification, the amplicons were subjected to hybridization according to the manufacturer's protocol. The PCR amplicons were hybridized with the immobilized genotype-specific nucleotide probes embedded in the

nylon membrane. Duplex molecules were retained after stringent washes. Streptavidin-horseradish peroxidase conjugate was added which binds to the biotinylated PCR products, upon addition of the NBT/BCIP substrate, purple insoluble precipitation was formed for result visualization. Internal control (IC) dot (for monitoring of PCR) and biotin control dot (for monitoring of hybridization) should be present for each valid test. The results were then evaluated by a colorimetric change on the chip under direct visualization. Blue-purple spots were recognized as HPV positive. HPV genotypes then were determined according to the distribution of colorimetric changes on each chip. There was no change on the negative chip. Representative demonstration of HPV positive and negative results are shown in **Figure 1**. Moreover, external positive control using HPV 18 plasmid and negative control using distilled water were included in each batch of PCR.

Outcome measures

The main outcome measures are the incidence of HPV infection in patients with NIP and its distribution across gender, age, clinical stage and pathological types. Furthermore, we explored the distribution characteristics within different subtype HPV infection.

Data analysis

The results obtained by the GA test were processed and analyzed by IBM SPSS statistical software version 20.0 (Armonk, NY). Continuous data (age and duration of follow-up) were expressed as median (range) and categorical data were expressed as rate or incidence. χ^2 test was used to detect the difference in HPV and HPV subtype infection across age, gender, Krouse Stage and pathological manifestations. A P-value of <0.05 was considered statistically significant.

Results

Demographics of include patient

NIP samples were obtained from 102 patients (73 males, 29 females) with age ranged from 21-78 years old, Turbinate mucosa samples were obtained from 20 healthy patients (10 males and 10 females) with age ranged from 24-62 years old. The course of disease for the NIP group ranged of 2 months to 30 years. In

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Table 3. Pathological classification of NIP with HPV prevalence and high/low risk

	Total	Pathological classification		x ²	P-value
		NIP only	NIP with dysplasia		
High Risk infection	23	11	12	11.212	0.004
Low Risk infection	32	27	5		
Mixed infection	11	10	1		

NIP samples, 22 patients were in T1, 58 patients were in T2, 20 patients were in T3 and 2 patients were in T4. 80 patients were in NIP only group (without dysplasia) and 22 patients were in dysplasia group.

HPV types distribution

A total of 66 out of 102 (64.7%) NIP samples were detected as positive for HPV. There was no HPV DNA detected in the control group; significant difference between two groups was found (P=0.000).

There was no significant difference found between HPV positive rate of NIP in male and female patients (P=0.725, **Table 1**). HPV positive rate was found to be higher in patients more than 59 years old (**Table 1**). According to the Krouse Stage system for NIP, HPV positive detection rates in Krouse Stage I to IV samples were about 45.5% to 100%. Moreover, the HPV positive detection rate was shown to be significantly increased accordingly with the stage of disease (P=0.015, **Table 1**). HPV infection rate of NIP only and NIP with dysplasia group showed a difference but not statistically significant (P=0.058, **Table 1**).

12 different HPV types were found in NIP samples, and the distribution across Krouse Stage and pathological classification was shown in **Table 2**. HPV 11 infection was found to be the most prevalent type in T1 and T2 while HPV 58 was the most prevalent type in T3 (**Table 2**). HPV 11 was the most prevalent subtype that was found in NIP only group while HPV 58 was the most prevalent in the NIP with dysplasia group (**Table 2**). The relationship of high-risk and low-risk types HPV infections and different pathological classification of NIP was also investigated. Significant difference could be observed across the groups between high-risk and low-risk infections (P=0.009) and high-risk and mixed (high-and low-risk) infections (P=

0.041) but not for low-risk and mixed infections (**Table 3**). Importantly, a significantly higher proportion of high-risk HPV infections (66.7%, 12/18) can be observed in the NIP with dysplasia group compared to the NIP only group (22.9%, 11/48).

Single and multiple HPV infection status in NIP

A total of 62.1% (41/66) of cases were found to have single HPV subtype infection while 37.9 % displayed more than one types of HPV infections simultaneously. Of the 25 cases of two or multiple HPV infections, 72.0% (n=18), 20.0% (n=5) and 8.0% (n=2) displayed two, three and four types of HPV infections respectively.

Discussion

In the present study, HPV infection status of 102 cases of the nasal inverted papilloma was investigated retrospectively. The HPV infection status and type distribution in NIP with different Krouse stages and pathological features were also examined. HPV DNA positivity rate of NIP varied from 0% to 86% reported previously [5-7] and strongly associated with HPV 6, 11, 16, 18 and 33. The variation of the reported HPV DNA positive rate was mainly due to the detection technology adopted, involved population size and the type of papilloma [8]. Our recent findings using the PCR-based reverse hybridization technology demonstrated that there was 64.71% (66/102) of NIP showing positivity towards HPV DNA and the result was agreed with the general belief that HPV is highly associated with NIP.

There are more than 120 types of HPV identified currently and are classified into high risk, low risk and probably high risk types based on the carcinogenicity. Commonly seen high risk HPV types include HPV 16, 18, 31, 33, 35, 39, 45, 51, 52, 56, 58, 59 and 38, while HPV 6, 11, 40, 42, 43, 44, 53, 54, 66 and 70 were classified as low risk HPV types [13]. In the current study, 76.7% (76/99) of cases displayed HPV 11, 58, 16 and 44 as the main HPV infections detected in NIP. Comparing to the other similar studies, HPV 11 and 16 were reported to be associated closely with NIP [5, 6]. The different types infections could be due to geographic or

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distribution variance of HPV prevalence. The current finding with a comparatively larger sample size could provide more information on the relationship between different types of HPV and the development of NIP.

In China, HPV 16 and 18 are the most commonly observed types in cervical cancer patients followed by HPV 58, 52, 31, 33 and 45 [14]. The discrepancy of the type prevalence between cervical cancer and NIP observed could be the result of different epithelial environment of the two locations and different infection tendency of HPV types toward various epithelial tissue types. In addition, the current study is the first to report findings of high risk HPV 16 and 58 infections are associated with NIP with dysplasia while low risk HPV 11 and 44 infections are associated with NIP cases without dysplasia.

Infection and genome integration of HPV are the prerequisites for carcinogenesis [15]. Immune response of cytotoxic T-lymphocytes was shown to be important in the prevention of squamous intraepithelial lesion development especially for HPV 16 infection [16]. The HPV infection rate in NIP was found to be increased across the age group with the highest prevalence at the >60 years old age group (75.0%). The finding indicated that weakened immune response due to age increase contributes to the observed increasing trend of infection incidence across the age groups.

The relationship of HPV infection and clinical staging of NIP was investigated. Significant difference of HPV infection incidence between different Krouse stages was observed. We demonstrated the HPV infection incidence was increased across the Krouse stages which suggested that the HPV infection could increase the area and seriousness of NIP. It was suggested that HPV E6 E7 protein could promote the growth of tumor cells and also increase infection rate of the neighbor cells which lead to the increase of affected area [17]. This could explain the clinical observation of high invasiveness of HPV-associated NIP. Walboomers [18] suggested that persistence HPV infection is an essential process for carcinoma development. According to the analysis, 86.36% (19/22) of Krouse Stage III and IV cases displayed HPV infection while 72.73% of them were infected with high risk HPV with disease

course of 5 to 10 years. Moreover, studies suggested that persistence high-risk HPV infections could lead to immune evasion [19] and conferment of the protection against apoptosis [20] for the infected cells. The observation suggested the possibility of persistence high risk HPV infection could increase the affected area of NIP via immune evasion and/or protection against apoptosis. It is reasonable to consider increasing the surgical removal area of the papilloma including the adjacent neighbor normal tissue to avoid recurrence for HPV positive patient.

Recent studies suggested HPV could only infect a narrow spectrum of the host cell. HPV can only grow in differentiated skin epithelial cells and mucosal epithelial cells. The columnar epithelial and squamous cells of the nasal mucosal membrane were most sensitive to mucosal HPV type infection. Normal cell cycle was interrupted with the genome integration of HPV thus induced the dysplasia progression and/or carcinogenic progression of the cell [21]. In intraepithelial neoplasia of cervical cells, low risk type of HPV 11 was detected mostly in CIN 1 stage while high risk type HPV 16 and 18 were detected mostly in CIN 2 or CIN 3 stages [22]. This suggested that high risk HPV infection is associated with the progression of CIN. By comparing the dysplasia associated-NIP group with NIP only group, HPV infection rate showed a difference but not statistically significant ($P=0.058$). Instead, significant difference was observed between high-risk and low-risk HPV prevalence between groups. The prevalence of high-risk HPV infection in dysplasia associated-NIP was shown to be significantly higher than the NIP only group. The observation suggested that the progression of NIP is associated with the HPV infection status which high-risk HPV infection could increase the dysplasia progression rate of the disease. In contrast, a study examined the HPV infection status of 57 paraffin-embedded NIP samples showed a HPV positivity rate of 12.3% and no HPV infection was detected in all 7 cases of the dysplastic inverted papilloma. The controversy findings could be result of different detection technology used and/or the small sample number involved.

Effects of single-type or multiple-type HPV infections on the cervical neoplasia progression are controversial. Patients with multiple HPV infection were demonstrated to have an elevat-

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ed risk of CIN progression and cervical cancer development compare to the single infection of HPV [23, 24]. On the other hand, studies suggested that multiple HPV infection rate was decreased across the CIN stages and single HPV infection was observed mostly in squamous epithelial carcinoma of cervix [25]. Of the HPV-infected NIP cases included in the current study, both single-type and multiple-type HPV infections were observed. The multiple HPV infection rate of the NIP only group is significantly lower than the dysplasia-associated NIP group (20.0% vs 40.91%). An increasing tendency of multiple HPV infection rate with the pathological classification of NIP indicated the association of the dysplasia progression with multiple HPV infections. The relationship of multiple HPV infections with NIP progression remains elucidated.

In conclusion, our results suggested that the development and progression of NIP are associated with HPV infection. HPV 11, 16, 58 and 44 are mostly detected among the NIP with possible single or multiple infection status. Moreover, HPV infection could increase the area of NIP and correlated with the HPV infected, high-risk HPV 16 and 58 infection could enhance the dysplastic progression of NIP. Investigating of the HPV infection status and type distribution of the NIP case could be useful for the clinical management with prognosis value.

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Disclosure of conflict of interest

None.

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