Clinical Relevance of p16 and p53 Expression in HPV-negative Sinonasal Inverted Papillomas (SIPs)

Shin, Dong Hyun

Department of Medicine

The Graduate School, Yonsei University

Clinical Relevance of p16 and p53 Expression in HPV-negative Sinonasal Inverted Papillomas (SIPs)

Directed by Professor Kim, Se-Heon

The Master's Thesis
submitted to the Department of Medicine,
the Graduate School of Yonsei University
in partial fulfillment of the requirements for the degree
of Master of Medicine

Shin, Dong Hyun

June 2010

This certifies that the Master's Thesis
of Shin, Dong Hyun is approved.

Thesis Supervisor : Professor Kim, Se-Heon

Professor Park, Jeon Han

Professor Cho, Nam Hoon

The Graduate School
Yonsei University

June 2010

ACKNOWLEDGEMENTS

이 논문이 완성되기까지 지대한 관심과 세심한 배려, 그리고 사랑과 격려를 베풀어 주신 김세헌 교수님께 진심으로 감사 드립니다. 또한 논문의 작성과 심사에 많은 조언과 지도편달을 해주신 박전한 교수님, 조남훈 교수님께도 진심으로 감사 드립니다.

항상 저에게 귀감을 보여주시고 힘들 때마다 다독여 주시는 아버님, 무한한 사랑으로 걱정해 주시는 어머님, 그리고 옆에서 힘들 때마다 채찍과 당근으로 저를 이끌어 준 이비인후과 교실 교수님 및 동료 여러분에게 이 기회를 통해 사랑한다는 말을 전하고 싶습니다.

마지막으로 물심양면으로 도움을 준 영원한 벗 류지원 양에게 이 논문을 바치고 싶습니다.

모두 감사드립니다.

2010년 6월

신동현 올림

<TABLE OF CONTENTS>

ABSTRACT ····· 1

I. INTRODUCTION ····· 3

II. MATERIALS AND METHODS ····· 6

1. Patients selection ····· 6

2. Staging of SIPs ····· 7

3. HPV identification ····· 8

4. Immunohistochemistry ····· 9

5. Statistical Analysis ····· 10

III. RESULTS ····· 10

1. Clinical factors related to the course of SIPs ····· 10

2. HPV screening test of SIPs ····· 12

3. Immunohistochemical stain related to the course of SIPs ····· 13

4. Analysis of clinical factors among immunohistochemically classified groups ····· 15

IV. DISCUSSION ····· 20

V. CONCLUSION ····· 25

REFERENCES ····· 25

FIGURE LEGENDS ····· 32

ABSTRACT (IN KOREAN) ····· 34

LIST OF FIGURES

Figure 1. Results of HPV screening test. 13

Figure 2. Comparing of immunohistochemical staining score of p16 and p53. 14

Figure 3. Separate survival function curves for p16/p53-pho groups. 16

Figure 4. Separate survival function curves for p16/p53-do7 groups. 19

LIST OF TABLES

Table 1. Krouse staging system for inverted papilloma 7

Table 2. Differences of clinical factors among the control, recurrence and progression groups. 11

Table 3. Differences of immunohistochemical stains among the control, recurrence and progression groups. 15

Table 4. Differences of clinical factors among the alternative p16/p53-pho groups. 16

Table 5. Differences of clinical factors among the alternative p16/p53-do7 groups. 18

Table 6. Multivariate analysis of risk factors regarding recurrence free survival using cox regression model. 20

<ABSTRACT>

Clinical Relevance of p16 and p53 Expression in HPV-negative Sinonasal Inverted Papillomas (SIPs)

Shin, Dong Hyun

Department of Medicine
The Graduate School, Yonsei University

(Directed by Professor Kim, Se Heon)

Sinonasal inverted papillomas (SIPs) are recurrent and progressive unusual tumors of the nasal and paranasal sinus epithelium. Many reports have suggested human papilloma virus (HPV) infection is one of the causes of SIPs. However, the infection rates differ considerably among reporters, and pRb and p53 which are considered to interact with HPV viral genome were also affected in HPV-negative SIPs. p16 plays a critical role in the tumorigenesis of p16/cyclin D1/pRb pathway, and previous studies reported that p16 expression shows inverse relation with pRb which is usually inactivated due to HPV viral genome. Also, It has been reported that p53 shows reciprocal expression with HPV infection. However, p16 and p53 expressions according to disease courses such as recurrence or progression have not been studied along with HPV infection in SIPs. In this study, the clinical factors and representative proteins of cell cycle regulators, p16 and p53 were evaluated in patients with SIPs. HPV screening test were performed with 62 SIPs and they were sorted to control, recurrence and progression (to malignancy) groups. The semiquantitive scores for immunohistochemical stains of p16 and p53 (phosphorus and mutant form) and clinical factors such as sex, age, smoking

history, operation methods, combined polyps, size of specimen, Krouse stage and time to recurrence and were compared between groups. The whole subjects were classified into four groups, p16+/p53-, p16-/p53-, p16+/p53+ and p16-/p53+, of which clinical factors also were compared. All SIPs showed HPV-negative, and endosopic surgery and advanced Krouse stage (p = 0.01 and p = 0.001, respectively) were associated with recurrence or progression. The p16 scored low (p = 0.004) and phosphorus and mutant forms of p53 (p = 0.001 and p = 0.014, respectively) scored highly in the recurrent or progression group significantly. There was difference of recurrence free survival (RFS) rate among alternative p16/p53 (phosphorus and mutant forms) groups significantly (p = 0.015 and p = 0.012, respectively), showing that the highest RFS rate was 82.4% in p16+/phosphorus form of p53- group and the lowest RFS rate was 33.3% in p16-/mutant form of p53+. In Kaplan-Meier analysis, the poorest disease courses were observed (p = 0.0003), demonstrating high possibility of recurrence in p16-/phosphorus form of p53+ group (p = 0.0009 *vs* p16-/mutant form of p53+ group).

Thus, negativitiy of p16 and positivity of p53 might be related factors to poor clinical courses concerned with recurrence or progression and might be useful to predict disease course of HPV-negative SIPs at the first time of operation.

Keywords : Sinonasal inverted papilloma, p16, p53, Recurrence, Progression

Clinical Relevance of p16 and p53 Expression in HPV-negative Sinonasal Inverted Papillomas (SIPs)

Shin, Dong Hyun

Department of Medicine
The Graduate School, Yonsei University

(Directed by Professor Kim, Se Heon)

I. INTRODUCTION

Sinonasal inverted papillomas (SIPs) are locally aggressive unusual tumors of the nasal and paranasal sinus epithelium. The etiology of inverted papilloma has not been known completely. However, since human papilloma virus (HPV) DNA was first demonstrated for SIPs in 1987 [1], many reports have shown detection of HPV DNA [1-7], suggesting that HPV is one of the etiologies in the pathogenesis of SIPs [1,3], as well as in the progression from benign SIPs to malignancy [2,4,6,7]. The presence of HPV infection has been also associated with recurrence after surgical resection of SIPs [7,8]. However, the infection rates differ considerably among

reporters, ranging from 0% to 100% [9], suggesting that HPV infection is not a certain cause of disease course in SIPs.

Previous studies showed that the upstream genes of HPV DNA, E7 and E6 are proto-oncogenes that interact with host tumor suppressor genes, pRb and p53, respectively [6]. Recent studies focuses on the analysis of 2 main cell cycle pathways, Rb pathway [$p16^{INK4a}$(p16)/cyclin D1/pRb] and p53 pathway [$p14^{ARF}$(p14)/mdm2/p53/ $p21^{WAF1}$(p21)] in the HPV-infected tissues to determine its association with malignant transformation in SIPs [9-12]. Since the p16/cyclin D1/pRb pathway plays a critical role in the cell cycle control, it is strongly believed that each component of this pathway may be affected in various malignancies. Notably, p16 is coded at the one of the most frequently disrupted tumor suppressor loci, INK4a gene in human cancer, and inactivates cyclin dependent kinase (CDK) 4/6 at the G1 to S check point of cell cycle [13,14]. CDK 4/6 combines with cyclin D1 and phosphorylates tumor suppressor protein, pRb, which results in releasing transcription factors such as E2Fs which then activate a series of events that allow entry into S phase and cell division [15]. Recent studies have revealed that pRb inactivation is usually reciprocal with p16 expression in head and neck cancers as well as various cancers [16,17]. In cervical cancers, previous studies reported overexpression of p16 owing to inactivation of pRb by HPV E7 protein [18]. However, in the previous literature, there were only two studies which insignificantly reported expressions of p16 in SIPs [19,20] and a study of association between p16 and clnical courses such as recurrence or malignant transformation

was still unprecedented.

The other representative gene, p53 is a tumor suppressor gene which activates expression of several genes including WAF1/CIP1 encoding for p21. p21 binds to the CDK 2 and cyclin E complexes for the G1 to S phase inhibiting their activity [21]. When p21 is complexed with CDK 2, the cell cannot continue to the next stage of cell division.

In human cancers comprising head and neck malignancies, p53 is commonly mutated [22], and as a consequence, the p21 protein is not available to act as the stop signal for cell division. Usually, the wild type p53 protein is present with a half-life sometimes as short as a few minutes, in contrast with the mutated p53 gene product, which often accumulates in the cells. In previous studies, there was a trend of higher p53 expression in inverted papillomas compared with the adjacent control mucosa in SIPs [20]. Furthermore, carcinoma arising in SIPs showed higher immunoreactivity than SIPs alone [11,23]. But, when considering the recurrence, there has been no report that shows any relation between recurrence and p53 in SIPs, in contrast to showing association between recurrence and HPV or Ki67 in SIPs [24]. Although previous reports showed that the expression of p53 seems to have a inverse relation with HPV in SIPs alone or carcinoma arising from SIPs [9,12], the association between p53 and disease course in SIPs was not discovered.

In this study, p16 and p53 expression status was analyzed and HPV screening were performed in SIPs, to investigate the association of clinical factors

related to disease course of SIPs among the control, recurrence and progression (to malignancy) groups and to predict prognostic factors in relation to recurrence or progression.

II. MATERIALS AND METHODS

1. Patients selection

This study analyzed a total of 171 patients who were diagnosed as SIPs histologically after biopsy or surgery between November 1993 and September 2008 at the Otolaryngology-Head and Neck Surgery Department of Yonsei University Medical Center. The excised tumors had been fixed in 10% buffered formalin and fragments were paraffin-embedded and processed for diagnostic light microscopic analysis. Clinical data using a retrospective review of charts for patients were collected and analyzed: age, sex, operation method, smoking history, combined polyps, size of specimen, staging, time to recurrence (TTS) and course of disease (control, recurrence and progression).

Patients categorized as more than 18 months of follow-up period and no history of surgery including excision with or without endoscopy in order to extirpate SIPs, except intranasal biopsy to diagnose them were included. Control group included subjects having no recurrence or progression using postoperative osteomeatal unit computed tomography (OMU CT) or endoscopy of nasal approach in the follow-up period after first operation. The recurrence group

included patients of SIPs, who underwent second operation because of recurrence indentified by suspicious lesion postoperative CT and endoscopic findings. In the recurrence group, patients diagnosed as SIPs with squamous cell carcinoma or dysplasia arising from inverted papilloma based on the pathological reports were classified to progression group separately. According to these criteria, a total 62 patients were included, consisting of 38 in control group, 18 in recurrence group and 6 in progression group, respectively. The histologic files of those patients were reviewed and tissues were analyzed from specimens of first operations. The hematoxylin and eosin-stained slides of SIPs were re-examed microscopically to select proper paraffin-embedded blocks. The selected blocks processed for HPV screening test and immunohistochemical staining of p16, phosphorus and mutant form of p53.

2. Staging of SIPs

The clinical stages of patients were determined based on Krouse staging system [25] by evaluating preoperative OMU CT, endoscopic findings and operative findings (Table 1.).

Table 1. Krouse staging system for inverted papilloma

T1	Confined to the nasal cavity
T2	Ostiomeatal complex region, ethmoid, or medial maxillary involvement (with or without nasal cavity involvement)
T3	Any wall of maxillary sinus but medial, frontal sinus, or sphenoid with or without T2 criteria
T4	Any extrasinus involvement or malignancy

3. HPV identification

Pretreatment and DNA extraction

HPV DNA detection were carried out using by Seeplex® HPV4A ACE Screening kit (Seegene, Rockville, MD, USA). DNA was purified from these tissue samples using the QIAamp DNA Mini Kit (Qiagen, Hilden, Germany) according to the manufacturers' instructions. The quality and quantity of purified DNA was measured by spectrophotometry.

Multiplex-PCR

The HPV 4A ACE screening kit screens high-risk HPV common types (26,31,33,35,39,45,51,52,53,56, 58,59,66,68,73 and 82) and two low-risk HPV types (6 and 11) in addition to individual genotyping of HPV (16 and 18) with different primer sets.

Multiplex PCR mixtures which were used to detect HPV was composed of two typing (HPV-16 and -18), and two grouping (high-risk and low-risk). The results were compared with HC2 (Digene, Gaithersburg, MD, USA) and the DNA chip assay (BioCore, Seoul, South Korea).

4. Immunohistochemistry

The 4-μm tissue sections were placed on silane-coated slides, deparaffinized, immersed in phosphate-buffered saline (PBS) containing 0.3% (v/v) hydrogen peroxide, and then processed in a microwave oven (in a 10 mM sodium citrate buffer, pH 6.5, for 15 min at 700w). After blocking with 1% (w/v) bovine serum albumin in PBS containing 0.05% (v/v) Tween-20 for 30 minutes, they were incubated with biotin-labeled rabbit anti-p53 phosphorous form (p53-pho) and mutant form (p53-do7) antibodies (dilution, 1:200; Dako, Carpinteria, Denmark) and p16 kit (prediluted; Dako) at 4°C for 16 hours. Biotinylation of the antibodies was performed with an antibody biotin-labeling kit (Dako, Envision, Glustrup, Denmark). Streptavidin-conjugated peroxidase was used as a secondary antibody (1:10000). Normal goat serum and subtype-matched normal mouse IgG were used as negative controls. The final reaction product was visualized with the addition of 0.03% (w/v) of 3, 3 -diaminobenzidine tetrachloride (DAB) for 5 to 20 minutes.

Score assessment

Expression scores were assigned quantitatively according to the number of cells nuclear-stained in the 10 high magnified fields (0.33 mm^2). In addition, semiqunatitative scores were calculated as multiplied percentage of cells stained with the intensity of the staining (0, no; 1, weak to moderate; 2, strong). After statistical analysis of those scores was done, cut-off value of each

immunohistochemical stain (p16, p53-pho and p53-do7) based on receiver operating characteristic (ROC) curve was set. After plotting ROC curve, each cut-off value was set showing best sensitivity and specificity. When the score of the stain was less than each cut-off value, the expression was categorized as negative. And, if the score of the stain was more than each cut-off value, the expression was categorized as positive.

5. Statistical Analysis

The difference of clinical factors and immunohistochemical staining results among control, recurrence and progression groups were analyzed by independent sample *t*-test, one-way analysis of variance and Fisher's exact test using the SPSS software program (SPSS Inc., Chicago, IL, USA), and p values < 0.05 were considered as statistically significant. Disease courses related to recurrence and progression were regarded as events and plotted by Kaplan-Meier curve verified with log rank test using the same software. Further analysis of all potential and confounding risk factors which especially showed significant differences among control, recurrence and progression groups and immunohistochemically classified groups, was conducted using Cox regression model.

III. RESULTS

1. Clinical factors related to the course of SIPs

During the study, 62 patients consisting of 38 males and 24 females were scheduled for excision of SIPs. The mean age of the patients was 50.8 years (Standard deviation, SD ± 12.1 years). Sex ratio and mean age were different among the three groups slightly, but there were no significant difference among the groups (p = 0.959, p = 0.307 respectively). Patients in progression group had more experiences of smoking than those in other groups (66.7%), and also patients in recurrence group had more smoking history than those in control group (33.3%) showing no significant difference statistically (p = 0.103).

Combined polyps were observed most frequently in control group (15.8%) comparing recurrence and progression group. But, difference was not significant statistically (p = 0.473). Also, mean size of excised specimen didn't show any difference among the groups (p = 0.667) reporting 10.1 cm^3 (SD ± 11.8 cm^3) in total patients. On the other hand, operation method influenced the disease courses. Endoscopic surgery was most common in the recurrence group (94.4%), and the difference of each group was statistically significant (p = 0.010). Krouse stage showed difference among the groups statistically (p = 0.001). The stages of patients increased from control to progression group, implying that the larger extent of disease tends to make SIPs to recur or progress. And mean TTS was 30.3 months (SD ± 25.9 months) in recurrent SIPs and shortened in the progression group (23.1 months) showing no significant difference (p = 0.532) (Table 2.).

Table 2. Differences of clinical factors among the control, recurrence and

progression groups.

Factors	Control group (*n*=38), No. (%)	Recurrence group (*n*=18), No. (%)	Progression group (*n*=6), No. (%)	*p* value	Total (*N*=62), No. (%)
Sex					
Male	23 (60.5)	11 (61.1)	4 (66.7)	0.959	38 (61.3)
Female	15 (39.5)	7 (38.9)	2 (33.3)		24 (38.7)
Age					
Mean, years	52.6 (± 12.8)	48.4 (± 10.0)	46.2 (± 12.9)	0.307	50.8 (± 12.1)
Smoking history					
Yes	9 (23.7)	6 (33.3)	4 (66.7)	0.103	19 (30.6)
No	29 (76.3)	12 (66.7)	2 (33.3)		42 (69.4)
Operation method					
Endoscopic	27 (71.1)	17 (94.4)	2 (33.3)	0.01	46 (74.2)
Others	11 (28.9)	1 (5.6)	4 (66.7)		16 (25.8)
Combined polyps					
Yes	6 (15.8)	1 (5.6)	0 (0)	0.473	7 (11.3)
No	32 (84.2)	17 (94.4)	6 (100)		55 (88.7)
Size of specimen					
Mean, cm^3	10.4 (± 12.6)	8.4 (± 6.5)	13.3 (± 18.6)	0.667	10.1 (± 11.8)
Krouse stage					
I	6 (15.8)	0 (0)	0 (0)	0.001	6 (9.7)
II	15 (39.5)	8 (44.4)	0 (0)		23 (37.1)
III	17 (44.7)	10 (55.6)	2 (33.3)		29 (46.8)
IV	0 (0)	0 (0)	4 (66.7)		4 (6.5)
Mean TTR, months		30.3 (± 25.9)	23.1 (± 16.8)	0.532	

Factors were compared using independent sample *t*-test, ANOVA and Fisher's exact test.

Abbreviation: TTS, time to recurrence

2. HPV screening test of SIPs

In HPV screening test using manufactured kit, PCR results from all SIPs patients showed negativities (Fig. 1.).

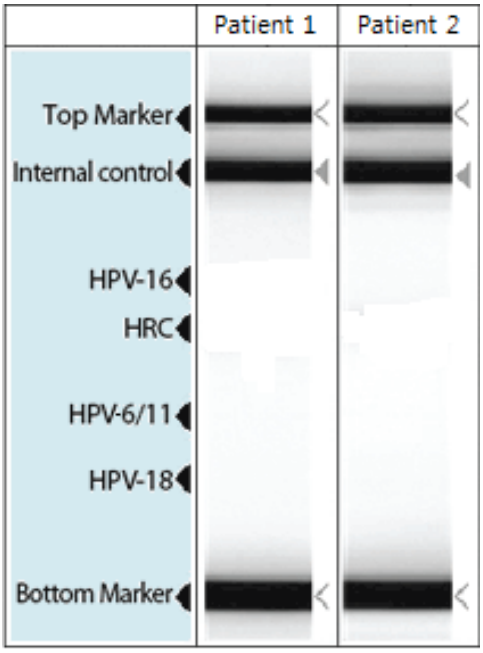

Figure 1. Results of HPV DNA screening test using multiplex PCR.

3. Immunohistochemical stain related to the course of SIPs

After score assessments of immunohistochemical stains of p16, p53-pho, p53-do7, mean score of each group was related to disease courses significantly. The mean scores of p16 showed highest in the control group (42.18) rather than recurrence group (14.44) and progression group (17.67) significantly (p = 0.004). The scores of p53-pho and p53-do7 also showed difference among groups significantly (p = 0.001 and p = 0.014, respectively). The mean scores of p53-pho and p53-do7 in control group were 3.42 and 18.45. In recurrence group, the scores increased showing 10.56 and 40.33, respectively. In progression group, the scores reported 44 and 61.83 showing the highest scores. The results of p53-pho and

p53-do7 had tendency of increase from control to progression groups (Fig. 2.).

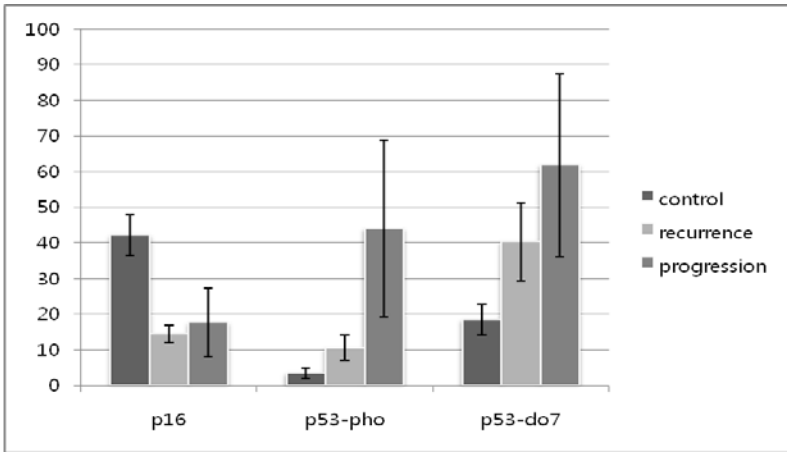

Figure 2. Comparing of immunohistochemical staining score of p16 and p53.

After setting cut-off value of each stain, patients of each group were divided according to positive and negative stains. The positivity of immunohistochemical stains for p16, p53-pho and p53-do7 were different among the control, recurrence and progression group significantly (p = 0.019 in p16, p = 0.008 in p53-pho) except one of p53-do7 stain (p = 0.111 in p53-do7) (Table 3.).

Table 3. Differences of immunohistochemical stains among the control, recurrence and progression groups.

	Control group (*n*=38), No. (%)	Recurrence group (*n*=18), No. (%)	Progression group (*n*=6), No. (%)	*p* value	Total (*N*=62), No. (%)
P16					
+	10 (26.3)	0 (0)	0 (0)	0.019	10 (16.1)
-	28 (73.7)	18 (100)	6 (100)		52 (83.9)
P53-pho					
+	5 (13.2)	8 (44.4)	3 (50.0)	0.008	16 (25.8)
-	33 (86.8)	10 (55.6)	3 (50.0)		46 (74.2)
P53-do7					
+	11 (28.9)	9 (50.0)	4 (66.7)	0.111	24 (38.7)
-	27 (71.1)	9 (50.0)	2 (33.3)		38 (61.3)

Factors were compared using Fisher's exact test.

Abbreviations: +, positive stain; -, negative stain

4. Analysis of clinical factors among immunohistochemically classified groups

The total patients were classified into four groups according to the positivity and negativity of p16 and either p53-pho or p53-do7. First, in the alternative groups of p16 and p53-pho, clinical factors such as sex, mean age, smoking history, operation methods, combined polyps, size of specimen, and Krouse stage were not different among four groups significantly. However, there was difference in recurrence free survival (RFS) rate among the groups significantly (p = 0.015) showing that the highest RFS rate was 82.4% in p16+/p53-pho- group and the lowest RFS rate was 33.3% in p16-/p53-pho+ (Table 4.). Furthermore, in Kaplan-Meier curve, similar disease course was observed (p = 0.0003), demonstrating poor prognosis of p16-/p53-pho+ group (Fig. 3.).

Table 4. Differences of clinical factors among the alternative p16/p53-pho groups.

Factors	P16+/p53-(*n*=17), No. (%)	P16-/p53-(*n*=20), No. (%)	P16+/p53+ (*n*=13), No. (%)	P16-/p53+ (*n*=12), No. (%)	*p* value
Sex					
Male	13 (76.5)	10 (50.0)	7 (53.8)	8 (66.7)	0.381
Female	4 (23.5)	10 (50.0)	6 (46.2)	4 (33.3)	
Age					
Mean, years	50.8 (± 12.5)	49.1 (± 12.1)	54.4 (± 13.6)	49.8 (± 10.6)	0.661
Smoking history					
Yes	6 (35.3)	4 (20.0)	4 (30.8)	5 (41.7)	0.558
No	11 (64.7)	16 (80.0)	9 (69.2)	7 (58.3)	
Operation method					
Endoscopic	11 (64.7)	16 (80.0)	10 (76.9)	9 (75.0)	0.789
Others	6 (35.3)	4 (20.0)	3 (23.1)	3 (25.0)	
Combined polyps					
Yes	3 (17.6)	1 (5.0)	1 (7.7)	2 (16.7)	0.606
No	14 (82.4)	19 (95.0)	12 (92.3)	10 (83.3)	
Size of specimen					
Mean, cm^3	12.3 (± 8.5)	8.7 (± 9.2)	6.0 (± 5.8)	13.7 (± 21.0)	0.318
Krouse stage					
I	2 (11.8)	4 (20.0)	0 (0)	0 (0)	0.179
II	5 (29.4)	8 (40.0)	6 (46.2)	4 (33.3)	
III	10 (58.8)	8 (40.0)	6 (46.2)	5 (41.7)	
IV	0 (0)	0 (0)	1 (7.7)	3 (25.0)	
2 year-RFS rate, %	82.4	60.0	61.5	33.3	0.015

Factors were compared using ANOVA and Fisher's exact test.

Abbreviation: RFS, recurrence free survival

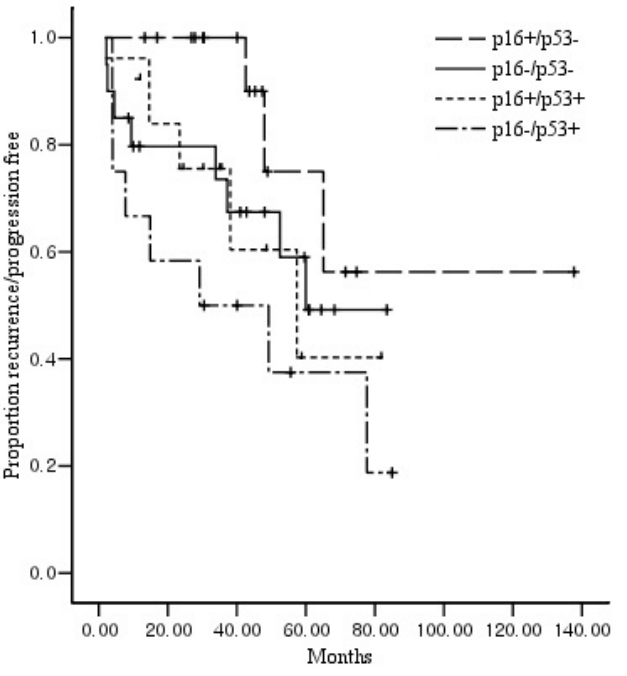

Figure 3. Separate survival function curves for p16/p53-pho groups.

In the alternative groups of p16 and p53-do7, clinical factors also didn't show any difference among the groups, whereas RFS rate was different among immunohistochemically classified groups, showing that the lowest RFS rate was 23.1% in p16-/p53-do7+ group and RFS rate in other groups were similar rate (Table 5.). Equally, Kaplan-Meier curve showed poor disease course of p16-/p53-do7+ group significantly (p = 0.0009) (Fig. 4.).

Table 5. Differences of clinical factors among the alternative p16/p53-do7 groups.

Factors	P16+/p53-(*n*=14), No. (%)	P16-/p53-(*n*=19), No. (%)	P16+/p53+ (*n*=16), No. (%)	P16-/p53+ (*n*=13), No. (%)	*p* value
Sex					
Male	9 (64.3)	10 (52.6)	11 (68.8)	8 (61.5)	0.805
Female	5 (35.7)	9 (47.4)	5 (31.3)	5 (38.5)	
Age					
Mean, years	54.3 (± 11.0)	48.6 (± 12.3)	50.6 (± 14.5)	50.3 (± 10.4)	0.629
Smoking history					
Yes	4 (28.6)	4 (21.1)	6 (37.5)	5 (38.5)	0.673
No	10 (71.4)	15 (78.9)	10 (62.5)	8 (61.5)	
Operation method					
Endoscopic	11 (78.6)	13 (68.4)	10 (62.5)	12 (92.3)	0.291
Others	3 (21.4)	6 (31.6)	6 (37.5)	1 (7.7)	
Combined polyps					
Yes	1 (7.1)	2 (10.5)	3 (18.8)	1 (7.7)	0.785
No	13 (92.9)	17 (89.5)	13 (81.3)	12 (92.3)	
Size of specimen					
Mean, cm^3	11.0 (± 7.6)	12.0 (± 16.0)	8.3 (± 8.4)	8.4 (± 12.7)	0.758
Krouse stage					
I	1 (7.1)	4 (21.1)	1 (6.3)	0 (0)	0.315
II	6 (42.9)	8 (42.1)	5 (31.3)	4 (30.8)	
III	7 (50.0)	7 (36.8)	9 (56.3)	6 (46.2)	
IV	0 (0)	0 (0)	1 (6.3)	3 (23.1)	
2 year-RFS rate, %	81.3	68.4	64.3	23.1	0.012

Factors were compared using ANOVA and Fisher's exact test.

Abbreviation: RFS, recurrence free survival

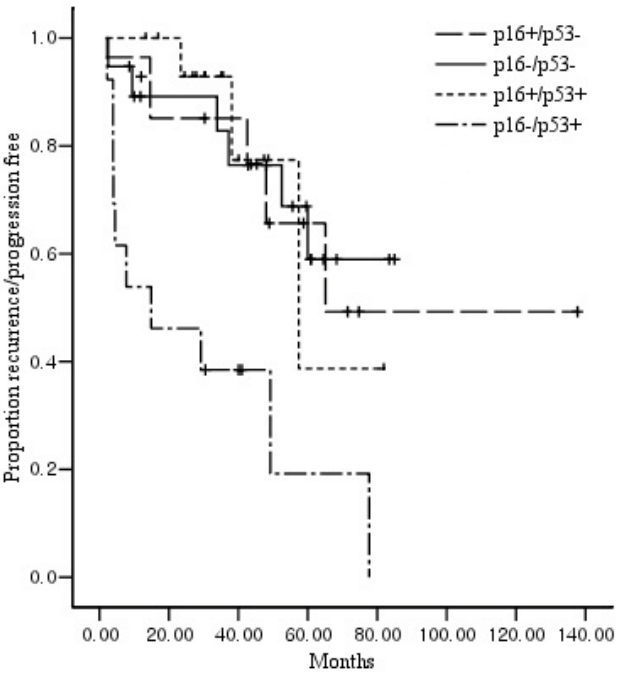

Figure 4. Separate survival function curves for p16/p53-do7 groups.

In the multivariate analysis related to RFS, p16-/p53-pho+ and p16-/p53-do7+ have a significant risk to recur or progress (p = 0.036 and p = 0.001, respectively). Also, high Krouse stage increases risk to recur or progress (p = 0.037), while endoscopic surgery decreases the risk (p = 0.042) in only the p16 and p53-pho analysis.

Table 6. Multivariate analysis of risk factors regarding recurrence free survival using cox regression model.

Factors	Analysis of alternative p16/p53-pho groups		Analysis of alternative p16/p53-do7 groups	
	HR (95% CI)	*p* value	HR (95% CI)	*p* value
Age	0.98 (0.95 to 1.02)	0.332	0.98 (0.95 to 1.02)	0.314
Sex (female vs male)	0.79 (0.28 to 2.22)	0.649	0.93 (0.31 to 2.77)	0.899
Smoking history (Yes vs No)	0.83 (0.34 to 2.05)	0.692	0.92 (0.36 to 2.32)	0.916
Operation methods (others vs endoscopic)	0.31 (0.1 to 0.96)	0.042	0.57 (0.17 to 1.86)	0.348
Krouse stage (III, IV vs I, II)	2.97 (1.07 to 8.26)	0.037	2.68 (0.95 to 7.53)	0.062
Subclassified group (p16-/p53+ group vs other group)	2.6 (1.06 to 6.38)	0.036	4.83 (1.87 to 12.5)	0.001

Abbrebivations: HR, harzard ratio; CI, confidence interval

IV. DISCUSSION

SIPs show characteristics of high recurrence rate and unpredictable nature after treatment such as surgical approach. Additionally, inverted papilloma usually showed multicentricity, local invasiveness and association with squamous cell carcinoma in about 10% of cases [26]. The tendency toward recurrence, and malignant transformation continue to make the treatment of inverted papilloma a significant challenge.

The cause of this inclination is unknown. Possible theories include proliferation of nasal polyps, allergy, rhinosinusitis, environmental carcinogens, and viral infection [27]. Using the techniques of Southern blot molecular hybridization. According to the Weber's study in 1988 [28], all recurrent inverted

papillomas were HPV DNA-positive, suggesting that the presence of the virus may affect the biological behavior of these epithelial proliferations. So far, studies have reported that HPV infection affect tumor formation and proliferation in SIPs [7]. But, in this study, all SIPs showed negative-HPV results. In the review of literature, there was a report that it was failed to identify HPV genome in any of the inverted papillomas [29]. Several reports showed low incidences of HPV infection, ranging from 6% to 6.8% [30,31]. Also, even in the studies of key molecules interactive with HPV DNA, cases were reported that HPV DNA were not always detected in recurrent or progressive course of SIPs.

Because a bio-marker in this study, p16 showed reciprocal relation with pRb inactivation [16-18], probably as a result of pRB targeting by the viral E7 protein, SIPs in this study which were all HPV-negative showed much losses of p16 expression (83.9%). It has been reported that HPV-negative C33A cervical cancer cell line was p16 positive, which indicates that a non-HPV dependent p16 expression pathway may also exist [32]. On the other hands, p53 expression showed inverse relation with HPV [9,12], of which viral E6 protein binds to and degrades p53 protein [33-35]. This explains the rarity of p53 mutations in cervical cancers which are often infected with HPV [36]. But, HPV-negative p53 in all SIPs showed more positivity relatively than other studies (38.7% in p53-do7).

Among the investigated clinical factors, operation method influenced the disease course of SIPs in this study. Especially, patients underwent endoscopic surgery had higher chances of recurrence. Although Waitz *et al.* reported [37] that

there was no difference of recurrence showing most of their patients with inverted papillomas underwent surgery by an intranasal endoscopic approach with the recurrence rate of 17% (6 out of 35), as compared to 19% (3 out of 16) after external operations, the possibilities of recurrence and progression increases according to operation methods in this study. Usually, the patients in the endonasal surgery group should have limited lesions at non-peripheral locations without signs of infiltrative growth. For this reason, instead of conservative endoscopic surgery, some authors insisted that en bloc medial maxillectomy and/or ethmoidectomy via midfacial degloving approach or lateral rhinotomy approach has become important in advanced stage of SIPs [38]. But, since there have been controversies about treatments and approach methods according to the extent and stages of SIPs, It is necessary to perform future studies for consents about operation methods based on diagnostic imaging studies and consistent staging system.

The staging system of SIPs have been suggested by Krouse [25] and Han [39] according to the extent of tumor in relation to paranasal sinus. Recently, based on the extent of surgery required for complete excision, Kamel [40] reported new staging system. Even if staging systems allow comparisons for outcomes of each stage-based surgical technique, outstanding standard or criterion is not established yet. In this study, to avoid effect on surgical decision based on selected staging system, multivariate analysis was performed. As a result, advanced stages was observed in the patients with recurrence or progression. Due to advanced stage

means multiplicity, large extent and malignant transformation of SIPs, staging system of this study is supposed to reflect recurrence or progression significantly.

In the immunohistochemical stain of p16, mean score and positive rate were lower in recurrence or progression groups than in control group. With respect to cell cycle mechanism as mentioned above, functional loss of p16 will affect cell proliferation and transformation, which may result in recurrence or progression of SIPs. Furthermore, deletions, mutations, or methylation of the p16 gene has been implicated in the development of a variety of human malignancies, including head and neck cancer [41]. In previous studies against other tumors, loss of p16 expression was significantly associated with the reduced recurrence-free in superficial bladder cancers [42]. Also, there is a significant correlation between loss of p16 expression and tumor progression in patients with minimally-invasive bladder cancer [43]. But, relationship between recurrence of benign tumor, SIPs and loss of p16 expression was not yet reported showing this study meaningful.

As to p53, in head and neck cancer, there is the report that cancer is more invasive with its expression [22]. In this way, expression of p53 is considered to influence the course of disease. Therefore, to find out mutated or phosphorylated p53 which is characterized with accumulation in the cells is significant. In this study, stain results for phosphorus and mutant form of p53 scored higher in recurrence and progression group than in control group. In order to establish standard capable of applying to clinical management, cut-off values were set. According to previous reports, p53 overexpression in HPV-negative group was

associated with a worse prognosis in hypopharyngeal cancers [44]. In the HPV-negative SIPs, based on results of this study, p53 expression is considered to have a relation with the disease course such as recurrence or progression.

Phosphorus form rather than mutant form of p53 showed more significant positivity in recurrent or progressive SIPs. The reason why phosphorus form of p53 showed strongly positivity in recurrence or progression rather than mutant form of p53 may be that cellular pathways preferentially increase molecular activity to prevent changes of genes by DNA damage or stress before genetic mutation occurs. But, it should be kept in mind that mutations of the p53 gene are not necessarily associated with immunopositivity for p53 [45], and thus, results of this study do not allow for drawing definite conclusions concerning alterations of the p53 gene in SIPs - an interesting target to be addressed in future studies.

In a previous study, p16 gene was evaluated in company with p53, particularly, in head and neck cancer reporting those markers have relation with cancer site and staging [46]. And recent researches have also linked the p53 and p16/Rb pathways, via p14, raising the possibility that the pathways may regulate each other [47,48]. Because p16 and p53 showed opposed results each other in this study, classifying groups alternately in accordance with positivity or negativity of p16 and p53 elucidates prognostic factor such as RFS much clearer. While other clinical factors were not significant among alternately classified groups, RFS rates and Kaplan-Meier curves explained that SIPs for p16+ and (phosphorus and mutant form of) p53- have the lowest recurrent probability and p16- and p53+

have the highest recurrent possibility. Meanwhile, for all patients in progression group experienced recurrence as stated above, the event of Kaplan-Meier curve and RFS means recurrence. For these reasons, comparison of recurrence and progression was conducted separately. But, in the presumption of the event was progression, plotting curves did not showed difference between recurrence and progression groups significantly (p = 0.346).

Based on these data, patients with the most chance of recurrence or progression for SIPs appear to be those with: 1) negativity of p16, 2) positivity of p53. And proper operation method according to Krouse stage is necessary for SIPs to prevent recurrence or progression. This information will guide clinicians and patients in determining prognostic factors at the time of first operation.

V. CONCLUSION

Thus, negativitiy of p16 and positivity of p53 might be related factors to poor clinical courses concerned with recurrence or progression and might be useful to predict disease course of HPV-negative SIPs at the first time of operation.

REFERENCES

1. Syrjanen S, Happonen RP, Virolainen E, Siivonen L, Syrjanen K. Detection of human papillomavirus (HPV) structural antigens and DNA

types in inverted papillomas and squamous cell carcinomas of the nasal cavities and paranasal sinuses. Acta Otolaryngol 1987;104:334-41.

2. Beck JC, McClatchey KD, Lesperance MM, Esclamado RM, Carey TE, Bradford CR. Human papillomavirus types important in progression of inverted papilloma. Otolaryngol Head Neck Surg 1995;113:558-63.

3. Shen J, Tate JE, Crum CP, Goodman ML. Prevalence of human papillomaviruses (HPV) in benign and malignant tumors of the upper respiratory tract. Mod Pathol 1996;9:15-20.

4. Hwang CS, Yang HS, Hong MK. Detection of human papillomavirus (HPV) in sinonasal inverted papillomas using polymerase chain reaction (PCR). Am J Rhinol 1998;12:363-6.

5. Kraft M, Simmen D, Casas R, Pfaltz M. Significance of human papillomavirus in sinonasal papillomas. J Laryngol Otol 2001;115:709-14.

6. McKay SP, Gregoire L, Lonardo F, Reidy P, Mathog RH, Lancaster WD. Human papillomavirus (HPV) transcripts in malignant inverted papilloma are from integrated HPV DNA. Laryngoscope 2005;115:1428-31.

7. Hoffmann M, Klose N, Gottschlich S, Gorogh T, Fazel A, Lohrey C, et al. Detection of human papillomavirus DNA in benign and malignant sinonasal neoplasms. Cancer Lett 2006;239:64-70.

8. Beck JC, McClatchey KD, Lesperance MM, Esclamado RM, Carey TE, Bradford CR. Presence of human papillomavirus predicts recurrence of

inverted papilloma. Otolaryngol Head Neck Surg 1995;113:49-55.

9. Buchwald C, Lindeberg H, Pedersen BL, Franzmann MB. Human papilloma virus and p53 expression in carcinomas associated with sinonasal papillomas: a Danish Epidemiological study 1980-1998. Laryngoscope 2001;111:1104-10.

10. Altavilla G, Staffieri A, Busatto G, Canesso A, Giacomelli L, Marioni G. Expression of p53, p16INK4A, pRb, p21WAF1/CIP1, p27KIP1, cyclin D1, Ki-67 and HPV DNA in sinonasal endophytic Schneiderian (inverted) papilloma. Acta Otolaryngol 2009;129:1242-9.

11. Franzmann MB, Buchwald C, Jacobsen GK, Lindeberg H. Expression of p53 in normal nasal mucosa and in sinonasal papillomas with and without associated carcinoma and the relation to human papillomavirus (HPV). Cancer Lett 1998;128:161-4.

12. Mirza N, Montone K, Sato Y, Kroger H, Kennedy DW. Identification of p53 and human papilloma virus in Schneiderian papillomas. Laryngoscope 1998;108:497-501.

13. Serrano M, Hannon GJ, Beach D. A new regulatory motif in cell-cycle control causing specific inhibition of cyclin D/CDK4. Nature 1993;366:704-7.

14. Koh J, Enders GH, Dynlacht BD, Harlow E. Tumour-derived p16 alleles encoding proteins defective in cell-cycle inhibition. Nature 1995;375:506-10.

15. Sano T, Oyama T, Kashiwabara K, Fukuda T, Nakajima T. Expression status of p16 protein is associated with human papillomavirus oncogenic potential in cervical and genital lesions. Am J Pathol 1998;153:1741-8.

16. Reed AL, Califano J, Cairns P, Westra WH, Jones RM, Koch W, et al. High frequency of p16 (CDKN2/MTS-1/INK4A) inactivation in head and neck squamous cell carcinoma. Cancer Res 1996;56:3630-3.

17. Papadimitrakopoulou V, Izzo J, Lippman SM, Lee JS, Fan YH, Clayman G, et al. Frequent inactivation of p16INK4a in oral premalignant lesions. Oncogene 1997;14:1799-803.

18. Tam SW, Shay JW, Pagano M. Differential expression and cell cycle regulation of the cyclin-dependent kinase 4 inhibitor p16Ink4. Cancer Res 1994;54:5816-20.

19. Zhao J, Wang Y, Kong W. [The expression of PTEN and P16 protein in nasal inverted papilloma]. Lin Chung Er Bi Yan Hou Tou Jing Wai Ke Za Zhi 2009;23:197-200.

20. Gunia S, Fritzsche F, May M, Liebe D, Koch S. Do different disorders in cell cycle regulation account for different biological behavior of urothelial and sinonasal inverted papillomas? Pathobiology 2008;75:34-41.

21. Dulic V, Kaufmann WK, Wilson SJ, Tlsty TD, Lees E, Harper JW, et al. p53-dependent inhibition of cyclin-dependent kinase activities in human fibroblasts during radiation-induced G1 arrest. Cell 1994;76:1013-23.

22. Boyle JO, Hakim J, Koch W, van der Riet P, Hruban RH, Roa RA, et al.

The incidence of p53 mutations increases with progression of head and neck cancer. Cancer Res 1993;53:4477-80.

23. Fang SY, Yan JJ, Ohyama M. Immunohistochemistry of p53 in sinonasal inverted papilloma and associated squamous cell carcinoma. Am J Rhinol 1998;12:119-24.

24. Bura M, Seiwerth S, Vladika I, Perovic D, Nagy P, Coric M, et al. Possible prognostic significance of p53 and Ki 67 in inverted sinonasal papilloma. Coll Antropol 2007;31:545-9.

25. Krouse JH. Development of a staging system for inverted papilloma. Laryngoscope 2000;110:965-8.

26. Vrabec DP. The inverted Schneiderian papilloma: a clinical and pathological study. Laryngoscope 1975;85:186-220.

27. Melroy CT, Senior BA. Benign sinonasal neoplasms: a focus on inverting papilloma. Otolaryngol Clin North Am 2006;39:601-17, x.

28. Weber RS, Shillitoe EJ, Robbins KT, Luna MA, Batsakis JG, Donovan DT, et al. Prevalence of human papillomavirus in inverted nasal papillomas. Arch Otolaryngol Head Neck Surg 1988;114:23-6.

29. Tang AC, Grignon DJ, MacRae DL. The association of human papillomavirus with Schneiderian papillomas: a DNA in situ hybridization study. J Otolaryngol 1994;23:292-7.

30. Buchwald C, Franzmann MB, Jacobsen GK, Lindeberg H. Human papillomavirus (HPV) in sinonasal papillomas: a study of 78 cases using

in situ hybridization and polymerase chain reaction. Laryngoscope 1995;105:66-71.

31. Weiner JS, Sherris D, Kasperbauer J, Lewis J, Li H, Persing D. Relationship of human papillomavirus to Schneiderian papillomas. Laryngoscope 1999;109:21-6.

32. Murphy N, Ring M, Killalea AG, Uhlmann V, O'Donovan M, Mulcahy F, et al. p16INK4A as a marker for cervical dyskaryosis: CIN and cGIN in cervical biopsies and ThinPrep smears. J Clin Pathol 2003;56:56-63.

33. Lane DP. p53 and human cancers. Br Med Bull 1994;50:582-99.

34. Mukhopadhyay D, Chatterjee R, Chakraborty RN. Association of p53 expression with cytokinetics and HPV capsid antigen prevalence in oral carcinomas. Cancer Lett 1994;87:99-105.

35. Nadal A, Campo E, Pinto J, Mallofre C, Palacin A, Arias C, et al. p53 expression in normal, dysplastic, and neoplastic laryngeal epithelium. Absence of a correlation with prognostic factors. J Pathol 1995;175:181-8.

36. Crook T, Vousden KH. Properties of p53 mutations detected in primary and secondary cervical cancers suggest mechanisms of metastasis and involvement of environmental carcinogens. EMBO J 1992;11:3935-40.

37. Waitz G, Wigand ME. Results of endoscopic sinus surgery for the treatment of inverted papillomas. Laryngoscope 1992;102:917-22.

38. Kim WS, Hyun DW, Kim CH, Yoon JH. Treatment outcomes of

sinonasal inverted papillomas according to surgical approaches. Acta Otolaryngol 2009.

39. Han JK, Smith TL, Loehrl T, Toohill RJ, Smith MM. An evolution in the management of sinonasal inverting papilloma. Laryngoscope 2001;111:1395-400.

40. Kamel R, Khaled A, Kandil T. Inverted papilloma: new classification and guidelines for endoscopic surgery. Am J Rhinol 2005;19:358-64.

41. Lai S, El-Naggar AK. Differential expression of key cell cycle genes (p16/cyclin D1/pRb) in head and neck squamous carcinomas. Lab Invest 1999;79:255-60.

42. Bartoletti R, Cai T, Nesi G, Roberta Girardi L, Baroni G, Dal Canto M. Loss of P16 expression and chromosome 9p21 LOH in predicting outcome of patients affected by superficial bladder cancer. J Surg Res 2007;143:422-7.

43. Kruger S, Mahnken A, Kausch I, Feller AC. P16 immunoreactivity is an independent predictor of tumor progression in minimally invasive urothelial bladder carcinoma. Eur Urol 2005;47:463-7.

44. Ernoux-Neufcoeur P, Arafa M, Decaestecker C, Duray A, Remmelink M, Leroy X, et al. Combined analysis of HPV DNA, p16, p21 and p53 to predict prognosis in patients with stage IV hypopharyngeal carcinoma. J Cancer Res Clin Oncol 2010.

45. Xu L, Chen YT, Huvos AG, Zlotolow IM, Rettig WJ, Old LJ, et al.

Overexpression of p53 protein in squamous cell carcinomas of head and neck without apparent gene mutations. Diagn Mol Pathol 1994;3:83-92.

46. Karsai S, Abel U, Roesch-Ely M, Affolter A, Hofele C, Joos S, et al. Comparison of p16(INK4a) expression with p53 alterations in head and neck cancer by tissue microarray analysis. J Pathol 2007;211:314-22.

47. Bates S, Phillips AC, Clark PA, Stott F, Peters G, Ludwig RL, et al. p14ARF links the tumour suppressors RB and p53. Nature 1998;395:124-5.

48. Weber A, Bellmann U, Bootz F, Wittekind C, Tannapfel A. INK4a-ARF alterations and p53 mutations in primary and consecutive squamous cell carcinoma of the head and neck. Virchows Arch 2002;441:133-42.

FIGURE LEGENDS

Figure 1. Results of HPV DNA screening test using multiplex PCR. A representative result in control group showed negative HPV infection in Patient 1. Also, no HPV detection was found typically in the patient 2 of recurrent group.

Figure 2. Comparing of immunohistochemical staining score of p16 and p53. The mean p16 score showed highest in control group while p53 showed lowest in progression group.

Figure 3. The separate survival function curves, based on Kaplan-Meier analysis for p16/p53-pho groups graphically demonstrated the difference of their recurrence/progression rates (log-rank test, p = 0.0003).

Figure 4. The demonstrated separate survival function curves among p16/p53-do7 groups showed poor prognosis of p16-/p53+ group. The curves was significantly different (log-rank test, p = 0.0009), but the group showing the highest recurrence/progression rates is different at the measured periods.

ABSTRACT (IN KOREAN)

비강 및 부비동 반전성 유두종의 재발 및 발암원인으로 인간유두종바이러스 음성군에서 p53, p16의 발현에 관한 연구

<지도교수 김세헌>

연세대학교 대학원 의학과

신 동 현

비강 및 부비동 반전성 유두종은 상피세포에서 발현하는 종양으로서 재발이 흔하고 암으로 변화하는 양상을 가진다. 많은 보고들이 인간 유두종 바이러스가 이러한 반전성 유두종의 병인에 원인일 것이라고 보고하고 있다. 그러나 인간 유두종 바이러스의 감염율은 보고자마다 다르고, 인간 유두종 바이러스와 관련된 유전자인 pRb와 p53 유전자 또한 인간 유두종 바이러스가 감염되지 않은 군에서도 발견된다. p16은 p16/cyclin D1/pRb 경로에서 종양생성에 중요한 역할을 하는 단백질로, 인간 유두종 바이러스의 게놈으로 인해 쉽게 비활성화되는 pRb와 역발현 관계를 가진 것으로 보고되어 왔다. 마찬가지로, p53은 HPV 감염과 역발현 관계를 가지고 있다. 하지만, 비강 및 부비동 반전성 유두종에 있어서 p16과 p53의 발현을 동시에, 그리고 HPV 발현과 함께 조사하여 재발이나 진행과 같은 질환의 경과에 따라 어떻게 다른지에 대한 연구는 없는 상황이다. 본 연구에서는 비강 및 부비동 반전성 유두종 환자에서 세포 주기에 관련된 대표적인 유전자인 p16과 p53 및 관련된 임상적인 요인들을 분석하였고 전체 환자에서 인간

유두종 바이러스의 DNA를 분석하였다. 총 62명의 환자를 대상으로 대조군, 재발군, 진행군으로 전체 환자를 나누었다. p16 및 p53 (활성형 및 변이형)의 면역화학염색을 전체 환자에서 시행한 뒤 반정량적인 방법을 통해 점수화하였고, 성별, 나이, 흡연력, 수술방법, 동반된 용종의 유무, 조직의 크기, Krouse 병기, 재발까지의 시간을 조사하여 각 군에서 비교하였다. 염색 점수에 대한 기준점을 적용하여 각 염색은 양성 및 음성으로 분류되었으며, 각 염색의 양성성 및 음성성을 교차하여 전체 환자를 p16+/p53-, p16-/p53-, p16+/p53+, p16-/p53+ 으로 분류하였다. 또한 이 군들에서 각 임상 요인 및 무재발 생존율을 비교하였고, 카플란-마이어 곡선을 이용해 재발율을 비교하였다. 전체 환자는 인간 유두종 바이러스 DNA 가 음성이었다. 내시경적 수술을 시행한 경우나 Krouse 병기가 높을 경우 재발이나 진행의 가능성이 증가하였다 (각각 p = 0.01, p = 0.001). 대조군보다 재발이나 진행된 군에서 p16 유전자는 낮은 점수가 보였고 (p = 0.004), p53 활성형 (p = 0.001) 및 변이형 (p = 0.014) 에서는 높은 점수가 관찰되었다. p16 및 p53으로 교차하여 나누어진 군에서는 무재발 생존율이 각기 다르게 보고되었으며, 통계적으로 유의한 차이를 보였고 (p16 및 활성형 p53 교차시 p = 0.015, p16 및 변이형 p53 교차시 p = 0.012), p16+/p53-에서 가장 높은 무재발 생존율을, p16-/p53+ 에서 가장 낮은 무재발생존율을 보였다. 카플란-마이어 곡선에서는 p16-/p53+ 의 예후가 가장 좋지 않았다 (p16 및 활성형 p53 교차시 p = 0.0003, p16 및 변이형 p53 교차시 p = 0.0009). 따라서, p16의 음성, p53의 양성이 재발이나

진행에 관련된 나쁜 예후인자로 생각되며, 첫 수술 시 수술 방법, Krouse 병기와 함께 이러한 요인을 분석하면 반전정 유두종의 경과를 예측할 수 있을 것이다.

핵심되는 말 : 비부비동 반전성 유두종, p16 유전자, p53 유전자, 재발, 진행