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The Diagnostic Role of P16ink4a in Detecting High-Risk HPV16 in a Group of Syrian Nasopharyngeal Carcinoma Patients

Research Article

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Abstract

Introduction: P16ink4a is a tumor suppressor gene, and it plays a critical role in cell cycle organizing and aging through the regulation of the Cyclin-Dependent Kinase (CDK) 4/6 and Cyclin D complexes. its inactivation corresponds with human carcinogenesis. The overexpression of p16 is considered to be a useful marker to investigate human papilloma virus (HPV) in certain cancers, but its role in nasopharyngeal cancer patients' outcomes is still unclear.

Objectives: We aimed in this study to detectHPV high risk type16 expression in a sample of 89 nasopharyngeal carcinoma Syrian patients, and to detect expression of P16ink4a in the same sample. After that, we studied the correlation between the two expressions of HPV and P16ink4a and the power ofcorrelation if existed.

Materials and Methods: Eighty n patients with nasopharyngeal carcinoma (NPC) treated in al Moasat universal hospital between 2010 to 2017 were chosen. To investigate the expression of P16ink4aand high-riskHPV 16 (HPVhr 16), we used immunohistochemistry staining. We evaluate the diagnostic role of p16 by discussing the expression profile of p16 in a group of NPC Syrian patients, especially those with HPVhr 16 positive expression .

Results: The results of this study showed that the ratio of HPVhr 16 positive expression in 89 patients was 13.5% ,and P16ink4a positive expression was 15.7%, and there was a positive, good correlation between expression of both HPVhr and P16ink4a, so it could be an alternative aid to detect HPVhr16 in paraffin embedded formalin sections. Nonetheless more studies are needed to evaluate the importance of the lack of HPVhr 16 and P16ink4aexpression when correlate with stages of the cancer and other clinical data.

Conclusions: The results of this study showed thatratio of HPVhr 16expression is 13.5 % and it is considered a weak percentage but it motivate other studies to assure the good prognosis of patients with no virus existence .Expression of P16ink4a is 15.7% and it can be a surrogate marker for HPVhr 16 in the sample of 89 NPC patients. More studies are needed to correlate prognosis with presence of P16ink4a.

Keywords: Human Papilloma Virus; Nasopharyngeal Carcinoma; P16ink4a; High Risk.

Introduction

Nasopharyngeal carcinoma(NPC) is an uncommon tumor that arises in the epithelium of the nasopharynx [1]. The wide difference in the incidence among different people and geographic areas suggests a considerable association of NPC with diverse genetic and environmental factors [2]. Its etiology is not specified enough, because of multiple complex factors that affect its emergence. One of the obstacles that make its cure harder, is its late diagnosis, because of the similarities between the presenting symptoms and other diseases [1].

Lately, there was a huge evidence of a certain involvement and a significant pathogenic role of HPV in the occurrence of oropharyngeal carcinoma, but still little is known about the prevalence and epidemiological role of this virus in NPC and other sites of head and neck sites [3, 4, 9]. Furthermore, HPV has been recently proposed to have a suspicious role in the etiology of non-endemic nasopharyngeal carcinoma [5-7]. On the contrary, a Belgium study reported that, the high incidence of positive High-Risk human-papilloma virus in oral cancers, the very worse outcomes for these tumors when it's compared with negative HPV in oral cavity cancers [8].

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In the general, there are no approved criteria that are accepted all over the world about the preferable standard for High-Risk HPV in formalin-fixed, paraffin- embedded sections, nor a sufficient vision about the role of this High-Risk virus in locations different from the oropharynx [9].

The p16 gene, an important cell cycleregulator for G1 restriction checkpoint [10], is inactivated ina remarkable ratioof primary tumors. The absence of p16 may result in Retinoblastoma phosphorylation and uninhibited proliferation of NPC cells [11]. Despite the predictive role of p16 when it's overexpressed as a surrogate for human-papilloma virusin oropharyngeal carcinoma, it's role is notcertain in nasopharyngeal carcinomaas a prognosticator biomarker [12], and that is consistent with the biological nature of p16 whose expression is ascended as a direct reaction to uncontrolled proliferation [13].

Using p16 as a predictive marker to investigate risk factors in nasopharyngeal carcinoma may confront plenty of challenges when immunohistochemical staining [14, 15], for example the criteria that determine when p16 is positive ,and when it is overexpressed [5, 6, 16].

Our study aimed to detect the presence of human papilloma virus in a group of Syrian patients having nasopharyngeal carcinoma and to ensure the correlation between p16 expression and High-RiskHPV virus existence as it is not certain to be used as a surrogate marker of this virus in nasopharyngeal carcinoma.

Objectives

✓ To detect HPVhr 16 expression in a sample of 89 patients of nasopharyngeal carcinoma.
✓ To detect P16ink4a expression in the same sample
✓ To compare the expression of HPVhr 16with expression of P16ink4a to discuss its diagnostic role.

Materials and Methods

Patients specimens

The number of samples collected from the archive of Al Moasat university hospital between the years 2010-2017 was 94 samples of nasopharyngeal carcinoma. First, collecting samples was based on the availability of both data of the patients and paraffin blocks related to his/her case. We revised the H&E chosen slides to ensure the nasopharyngeal carcinoma diagnosis. we exclude the cases that lack data, and/or paraffin blocks, so they were 89 samples after excluding five cases.

Immunohistochemical staining

The immunohistochemical stain for P16ink4a using Anti-p161nk4a (CDKN2A) antibody, and HPVhr 16 using a mouse monoclonal antibody, from BioSB was performed using BioSB histology kit.we began the staining with deparaffinization by incubation the slides for one hour at 60°C degrees , then changing of xylene and absolute alcohol 3 to 2 changes until we put them in water, after that the step of the antigen retrieval by boiling in microwave for 30 min, later we wash the sides with Distilled Water(DW) two to three times, then blocking by incubate sections at room temperature with hydrogen peroxidase for 10 minutes, then incubate in Tris Buffered Saline TBS for five minutes. Adding the primary antibody (p16 or HPV) for about an hour, then washing slides with TBS, after that incubate for 10 min. addingHRP for 45min to one hour, after that we wash slides with TBS 3-5 times, and incubate for 10 min, then adding 250ul of 1% di-amino-benzidine, later we wash the slides with DW 3-5 times, Finally counterstaining with hematoxylin for 1 min.

P16ink4a antibody Immunohistochemical staining

We used Anti-p161nk4a (CDKN2A) antibody, Rabbit monoclonal, RM267 monoclonal from Sigma-Aldrich, reacts to human P16ink4a (Cyclin-dependent kinase inhibitor 2A). The protocol of staining was performed according to instructions supplied by the kit of BIO SB company. we used 1:1000 dilution after plenty of trials to conceive the appropriate staining. We used formalin -fixed and paraffin-embedded human colon cancer sections as control samples.

HPVhr 16 immunohistochemical staining

HPV 16 we used is a mouse monoclonal antibody that is concentrated, dialyzed, filter sterilized and diluted in buffer pPH7.5. its isotype is IgG2a and had nuclear localization. according to instructions of manufacturing company BIO SB, we handled the samples with the HPVhr 16 antibody as steps mentioned in immunohistochemical staining previously, and we used oral papilloma sections as positive control samples.

Criteria of staining

For P16ink4a staining Every sample was given a score that indicate the intensity of nucleus or cytoplasmic staining (no staining = 0, weak staining = 1, moderate staining = 2, strong staining =3), and the extension of staining is measured also by numbers cells (0% = 0, 1–10% = 1, 11–50% = 2, 51–80% = 3, 81–100% = 4. We determined the final results by multiplying the intensity scores with the extent of positivity scores, so the final score we get is limited between 0 as the minimum score and 12 as a maximum score [17-19].

For HPVhr 16 antibody staining, every slide has a nuclear staining (weak and /or strong) regarded positive.

Statistical Analysis

The results we get were subjected to statistical analysis using the Chi-Square Independence Test with (p-value<0.05), Spearman correlation, and SPSS version 25 statistical program for analyzing data.

Results

Expression of HPVhr 16 and P16ink4a in NPC sections

We found that 12 cases of 89 were HPVhr 16 positive which means 13.5% of all cases studied, whereas 14 cases of 89 had positive expression of P16ink4a, which means about 15% of all cases studied, and the positivity was ranged between high, low and no expression, (Table 1) depending on the result of multiplying

density score by intensity one [17-19].

We can notice the pale nucleus staining and strong staining very obviously of HPVhr16 in (Figure 1), and the obvious brown staining of nucleus in basal layers only, (Figure 2). which both show the different expression appearance of HPVhr16 in immunohistochemical staining.

Whereas staining of P16ink4a is seen either in the whole epithelium adjacent to NPC lesion, (Figure 3).

Relation between HPVhr16 expression and P16ink4a staining

When we compared the percentage of positive expression of HPV and P16ink4a, we found that 50% of NPC patients who had positive expression of HPVhr 16, expressed positivity of P16ink4a, but patients who didn't express positivity of the virus, had positive expression of P16ink4a in 50% of cases also.

Only 9.9% of NPC patients who have the infection of HPVhr 16 expressed negative expression of P16ink4a, which means that expression of p16 didn't give the purpose it was used for in these limitedcases, whereas 90.1% of NPC patients included in this study showed negative expression of both P16ink4a and HPV (Table 2).

Discussion

HPVHR 16 Expression in nasopharyngeal carcinoma

Since HPVhuman papilloma virus is involved in the etiology of

Table 1. Expression of P16ink4a detected in 89 patients of NPC.

Percentage	Frequency	Expression
9.0%	8	High
6.7%	6	Low
84.30%	75	Non

Table 2. HPV expression *P16ink4a expression in 89 patients of NPC ur.

Negative expression (no expression)		Positive expression (high /low)		P16ink4a
HPV-	HPV+	HPV-	HPV+	expressionHPV
73	8	4	4	frequency
90.10%	9.90%	50%	50%	percentage

Figure 1. The localized staining of HPVhr16 antibody in the epithelium of NPC section, magnification ×40.

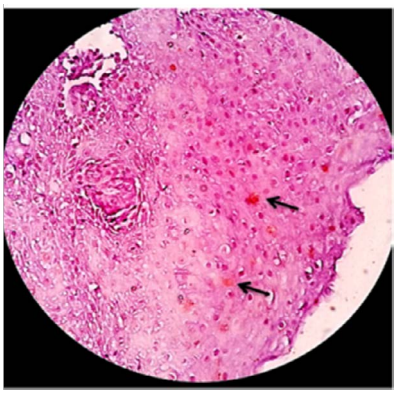


Figure 2. Immunohistochemical Staining of HPVhr16, we can notice the nucleus staining is only obvious in the basal layers of epithelium peripheral to NPC lesion. Magnification ×10.

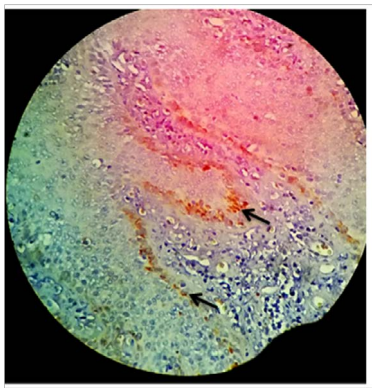
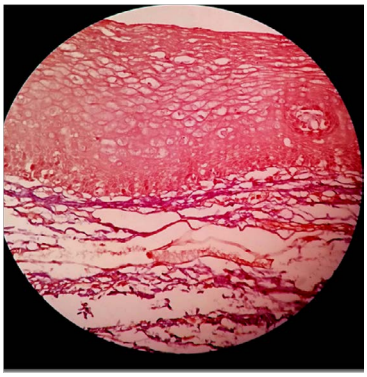


Figure 3. Expression of P16ink4a, notice the staining in the whole epithelium layers and in both nucleus and cytoplasm of cells in the epithelium peripheral to the lesion, while no staining in the connective tissue.



many head and neck cancers, they found that particularly oncogenic HPV subtype 16, has been accused to be the primary etiologic agent in squamous cell carcinoma cancers related to head and neck region, and they exhibit improved prognosis compared to patients with HPV negative tumors [20, 21].

When we investigate the HPV virus in nasopharyngeal carcinoma, we chose the high risk type (16) to be discussed and Since there are no universally accepted gold standard for High-RiskHPV assessment in formalin-fixed paraffin-embedded tissue sections [9], we followed standard in previous studies which used IHC staining to detect the virus.

HPV expression was positive in 13.5% of 85 patients of NPC, and that is less than the ratio documented in other studies like Walline and colleagues which was 5o% [9]. Also, in another study they had found the expression is 30% HPV positive, and that could be explained because of the variable methods used to detect the virus, and the differences in the study design, if the samples were collected with most possibility of having HPV infection from the beginning, or they detect the virus after collecting the sample of nasopharyngeal carcinoma patients. in a random way [22].

Because its high sensitivity for HPV, we could assert that P16ink4a a surrogate marker for HPVhr 16 in NPC and that resultis compatible with other studies like (Walline, Komarck et al., 2013, on the other hand it was not the same conclusion in studies like Bonomi and his colleagues' study [23], and our explanation is because of various reasons, like size of sample, criteria of expression and variety of methodology used in detecting both the P16ink4a marker and HPVHR 16 virus.

P16ink4a expression in Nasopharyngeal Carcinoma in general

The expression of p16 had been evaluated in many studies of nasopharyngeal carcinoma, and reported to be reduced in expression and average of reduction ranges between 40-82% [13, 24, 25], which is compatible with our study that shows absence ofexpression of P16ink4a in 84% of cases studied, as it is shown in table (1).

Absent of P16ink4al correlate with poor clinical outcome , and it could be involved in NPC development or progression but mechanism is still needs explanation, on the other hand, p16 is frequently deleted, mutated or methylated in several head and neck squamous cell carcinoma [26, 27, 13].

Expression of P16ink4a in 89 NPC Patients

The percentage of expression of P16ink4a was (15.7%), and it was similar to a UK study that showed 16% over expression [3], our percentage has gathered both low and high expression,but the UK study took the over expression of P16ink4a only. We can explain that result depending on previous studies showed that the loss of P16ink4a is increasingly common with advancing stages of various neoplasms, and in fresh non cultured primary tumors, specially that we didn't classified the samples depending on tumors' stages because of lack of clinical information and difficulties of proceeding each case after treatment.

when P16ink4a has positive expression in 50% of cases of NPC patients, there was no HPV infection detected. This result is congruent with a specific study of sinonasal undifferentiated carcinoma [28], which found overexpression of p16 in the absence of HPV expression.

We found that about 90% of cases that showed negative HPV, had also a negative expression of P16ink4a, as it is mentioned in the study of Veganzones and his colleagues, who explained the result because of loss of heterozygosity, and DNA hyper-methylation of the gene. Other researches documented that, about 90% of HPV-negative HNSCC tumors, exhibit low expression of P16ink4a, and it is compatible with our result [29, 30].

We found also 9.9% of patients who had HPV positivity, had no expression of p16, we can explain this result as a prediction of progression of the cancer, since the absence of p16 expression is related to development of neoplasm., and it needs to be correlated with stages of tumors and outcomes of treatment (Makitie, MacMillan et al. 2003).

Previous studies showed that aberrant silencing is highly associated with altered cell cycle regulation during carcinogenesis, specially silencing of CDKN2A which encodes the p16 protein [30]. Our results showed 50% of patients who had HPVhr 16 positivity, had positive expression also of p16 antibody, This result agreed with many previous studies [31-33].

After using chi-square test we found that there is positive correlation between HPV (expression) and P16 (expression) with P-Value=0.001, Chi-Square=13.105, so we tested the power of

relationship by using Spearman test, and we found positive medium relationship with{ P=0.000>(α=0.05), r=0.378}, this result is compatible with many previous studies showed an established association between HPV positivity and p16 overexpression [33], sowe suggest a potential role of p16 in investigating HPVhr 16 in nasopharyngeal carcinoma.

Although this result needs to be confirmed in wider studies,to investigate the prognostic role and its effect on outcomes of treatment, as it was confirmed in many studies [12, 34].

Conclusion

Expression of HPV16 was 13.5%, and P16ink4a was about 15% in a sample of 89 nasopharyngeal carcinoma patients. We stated in this study that P16ink4a has a moderate correlation with HPV16 and that could nominate it to be a potential marker for the virus HPV16 in nasopharyngeal carcinoma, but further studies are needed to confirm this result and to use it in routine examination to predect the prognosis and treat cancer earlier.

Our results also refer to the importance of absence of p16 in most cases and that should havean increased attention specially when it is correlated with the stage of cancer and prognosis.

To the best of our knowledge, this is the first experimental report of investigating HPVhr 16 in NPCs among Syrian patients in the period between 2010-2017.

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