Original Article

To analyze the concomitant expression of human papillomavirus-16 in the pathogenetic model of p53-dependant pathway in oral squamous cell carcinoma

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Abstract

Background: Oral squamous cell carcinoma (OSCC) accounts for 90% of all primary oral malignancies. Association between human papillomavirus (HPV) as a risk factor of cervical cancer is well known; there is a need to widen the knowledge for its role in oral cancer development. The viral E6 protein of HPV binds to p53 making it nonfunctional.

Aims and Objective: To study mutated/wild type p53 expression using immunohistochemistry and detect HPV-16 presence using polymerase chain reaction (PCR), in OSCC and correlating their expression.

Materials and Methods: Immunohistochemical staining for p53 molecule in 24 sections of OSCC followed by DNA extraction of the cases using qiagen extraction kit and subsequent HPV-16 detection using PCR technique.

Statistical Analysis: The data were analysed using SPSS software version 19.

Results: Out of 24 cases of OSCC, twenty cases were positive for P 53 expression and four cases were negative for P 53 expression. Out of the four negative cases, one case was detected positive for HPV-16.

Conclusion: HPV infection along with p53 expression helps in understanding its exact pathogenesis which further helps in expanding our spectrum of therapeutic modalities

Key Words: E6 oncoprotein, human papillomavirus 16, oral squamous cell carcinoma, P 53

INTRODUCTION

Oral cancer is a disease with a complex etiology and affects approximately 31,000 persons per year and is responsible for around 8600 deaths.[1] OSCC has a multiphasic and multifactorial pathogenesis, commonly associated with the use of risk factors such as tobacco and alcohol. Recently, there has been an increase in the cases of OSCC unrelated to this risk factor, and a concept of putative virus exposure commonly human papillomavirus (HPV), in its etiopathogenesis has been evolved.

Molecular epidemiological studies have shown a strong correlation between oncogenic HPV infections and a subset of oral precancer and cancers in the Indian population.[2] Chaudhary et al. reported that in Western populations, head

and neck cancer associated with HPV infection is increasing very rapidly, it is seen commonly in younger age group with better prognosis,[3] and an improved response to treatment.[4]

HPV is a heterogeneous virus and is important in human carcinogenesis. It not only causes a vast majority of cervical cancers but also plays an important role in anogenital and oral cancers. HPVs are small viruses with double-stranded DNA of approximately 7900 base pairs. At present, more than 150 different types of HPV have been identified, most important genotypes being 6, 11, 16 and 18.[5]

p53 is the guardian of the genome or molecular policeman, located on the short arm of chromosome 17. It senses cellular stress, such as DNA damage, shortened telomeres and hypoxia. p53 protein is both in wild and mutant forms. Inactivated tumor suppressor genes that are suspected in oral cancer include Rb (retinoblastoma gene), p16 (MTS1 or CDKN2), E-cadherin, doc-1 and p53.[6]

In case of cervical cancer in the Indian population, almost all cases show the presence of >90% of high-risk (HR)-HPV types 16 and 18.[7] However, in case of oral cancer, contradictory results ranging from 0%[8] to 74%[9] have been reported. In 2005, Syrjänen suggested, after 20 years of active research on the subject, that HPV seems to be associated with at least a proportion of oral carcinomas.[10]

It has been proven that HPV oncoproteins coincide with various cell cycle regulators, chiefly p53 and Rb protein. Therefore, the study was undertaken to detect immunohistochemically the expression of mutated/wild p53 in oral squamous cell carcinoma (OSCC), the presence of HPV-16 in OSCC cases using polymerase chain reaction technique and to correlate any association between them.

MATERIALS AND METHODS

Sample collection and histopathological diagnosis
A total of 24 cases of different grades of OSCC and four controls (buccal mucosa) were taken from the archives of the Department of Oral and Maxillofacial Pathology and Microbiology. in a random manner after obtaining consent from the Institutional Ethical Committee. Patients who had no previous history of treatment for HPV infection were included in the study. All specimens were independently examined by two different histopathologists in a double-blind fashion.

Immunohistochemistry
Immunohistochemical staining was done for the p53 molecule. The deparaffinized sections were placed in 0.01 M of tris-EDTA buffer at pH 9 and were brought to boil in E7

antigen retrieval machine (biogenex) in 2 cycles; Cycle 1–85° for 5 min and Cycle 2–98° for 10 min. The peroxidase-labeled streptavidin antibody was added from the same staining kit and incubated under similar incubation conditions. Finally, 3,3'-diaminobenzidine was used as the chromogen in the presence of hydrogen peroxide. Counterstaining was done using Meyer's hematoxylin.

Quantitative and qualitative assessment of P 53
Stained sections were scanned to determine the areas that were positively stained. Three to four representative fields were selected at ×400 and a number of positive cells were counted out of thousand cells in total representative fields and immunoreactive score (labeling index) was calculated.

Labeling index = Total number of positive cells stained/1000 × 100

Qualitative analysis was also scored for all the cases [Table 1].

Diagnosis of human papillomavirus-16 in oral squamous cell carcinoma by conventional polymerase chain reaction analysis
Deparaffinization of tissue sections
The tissue sections were collected in the microcentrifuge tube; 40 μm thick paraffin embedded section was used. 1200 μl of xylene was added, pulse vortexed and centrifuged at full speed, followed by 1200 μl of 100% ethanol and incubated at 37° for 15 min.

Isolation of DNA from oral biopsies
High-molecular-weight genomic DNA was extracted from oral biopsies using the Qiagen QIAamp DNA tissue Kit (Qiagen Inc., USA). The extracted genomic DNA was quantified and checked for purity using a spectrophotometer (Spectro Ultraviolet-Visible Double Beam PC, UVD Model 2950; LABOMED, Inc., Culver City, CA, USA). Ethidium bromide-stained 1% agarose gel electrophoresis was used to confirm the presence of DNA in the samples.

Diagnosis of human papillomavirus-16 in oral squamous cell carcinoma using conventional polymerase chain reaction analysis
Polymerase chain reaction (PCR) amplification was performed for initial molecular diagnosis of HPV using consensus degenerate primers derived from highly conserved L1 open reading frame of HPV genome. Further typing of HPV-16 was done with type-specific primers [Table 2].

Table 1: Scoring criteria

Qualitative score	Staining
0	No staining
+	Mild immunostaining
++	Moderate immunostaining
+++	Intense immunostaining

Table 2: Typing of HPV-16 with type-specific primers

Primer	Primer sequence	Nucleotide position	Amplicon size (bp)
Glutamate dehydrogenase	Forward primer - 5' CTGGCTTGGCATACACAATG Reverse primer - 5 GCTGTTCTCAGGTCCAATCC	450	702
MY09/MY11	Forward primer - 5'CGTCCMARRGGAWACTGATC 3' Reverse primer - 5'GCMCAGGGWCATAAYAATGC3' (M=A + C, W=A + T, Y=C + T, R=A + G)	LI consensus	450[11]
HPV-16 E7 primer	Forward primer - 5'AGCTCAGAGGAGGAGGATGAA Reverse primer - 5'GGTTACAATATTGTAATGGGC	7763-7781 57-75	217[12]

PCR was performed in a 25 µL reaction mixture containing 50–100 ng DNA, 10 mM Tris-HCl (pH 8.4), 50 mM KCl, 1.5 mM $MgCl_2$, 100 µM of each dNTPs (dATP, dGTP, dCTP, dTTP) (Fermentas Inc., USA), 10 pmol of oligonucleotide primers and 0.5 U Taq DNA polymerase (Bangalore Genei Pvt. Ltd., India). A thermal cycler (PTC-100; MJ Research GMI, Inc., Minnesota, MN, USA) was used, After an initial denaturation for 3 min at 95°C, each of the 35 cycles would consist of 30 s of denaturation at 95°C, primer annealing for 30 s at 55°C and chain elongation for 30 s at 72°C. At the end, an extra incubation for 5 min at 72°C was carried out. Electrophoresis of PCR product was done on a 2% gel.

Polymerase chain reaction amplification of housekeeping gene
A fragment of the human glutamate dehydrogenase (Glu DH) gene was amplified with Glu DH primers. A positive Glu DH amplification proved that the sample contains enough DNA and that no PCR inhibitors were present.

Statistical analysis
The resulting data were analyzed using SPSS software version 19. Pearson's Chi-square test was carried out to determine the level of correlation or association between the groups under study. $P < 0.05$ was considered statistically significant.

RESULTS

Immunohistochemistry analysis
Twenty-four cases of varying grades of OSCC were assessed quantitatively and qualitatively for p53 expression. We observed out of 24 cases, 83% (20 cases) were positive for p53 expression with labeling index >50 and 16% (4 cases) were negative for p53 expression labeling index <10 [Graph 1].

We also observed that 50% cases (12 cases) showed moderate intensity as well as 50% (12 cases) cases showed strong staining intensity and none of the cases showing mild intensity [Table 3]. p53 expression was positive in peripheral and central cells in well- and moderately-differentiated OSCC, whereas poorly differentiated showed more positivity in sheets of cells.
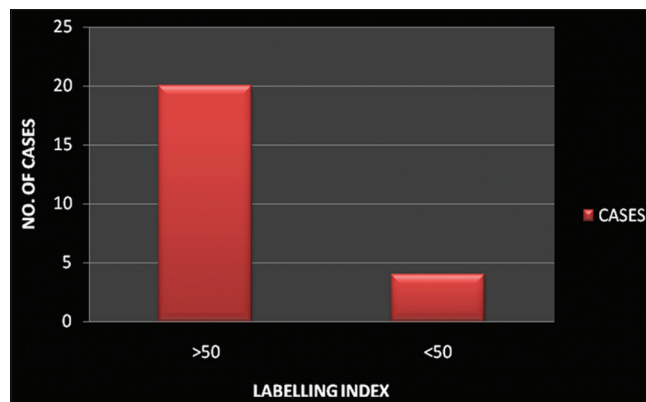
Graph 1: Labeling index for p53 expression

Polymerase chain reaction analysis
Detection of HPV in a single master mix reaction by PCR generic screening for all lesions was achieved using MY09/MY11 primers and Glu DH was used as an internal control to overcome the quality issues of isolated DNA. HPV infection screening revealed 1 out of 24 samples to be positive for HPV. Subsequent PCR-based HPV typing using type-specific primers revealed that HPV L1 positive OSCC cases were infected with HPV type 16, whereas the normal control samples were HPV negative.

HPV-16 positivity was observed in 1 out of 4 cases negative for p53 expression [Table 4].

DISCUSSION

Oral carcinogenesis represents one of the models used for the study of the multistage nature of cancer.[13] Chaudhary *et al.* reported that approximately 15% of all malignancies worldwide appear to be connected with viral infections, and several human DNA viruses are now accepted as causative factors.[14] The studies have shown HPV infection to be associated with an increased risk (up to three times) of OSCC, independent of exposure to alcohol and tobacco demonstrating that the relative risk of OSCC with HPV infection is equal to or exceeds the risk associated with tobacco and alcohol consumption.[15]

There have been numerous reports on HPV-DNA detection in head and neck (HN)-SCC with rates varying from 0% to 100% of carcinomas studied.[16,17] These differences in detection rate

Table 3: Distribution of cases according to intensity of p53 immunostaining

Staining intensity	N	Number of cases (%)	P
Mild	24	0	0.03 (significant)
Moderate		12 (50)	
Intense		12 (50)	

Table 4: Correlation of p53 and HPV16 positivity

Percentage positivity	p53 positive cases (*n*=24)	HPV-16 positive cases (*n*=4)	P
Positive (%)	20 (83.33)	1 (25)	0.67 (non-significant)
Negative (%)	4 (16.66)	3 (75)	

are due to at least two principal factors: (1) Differences in the epidemiological distribution of oncogenic HR-HPVs in the world and (2) different analytical methods used.[18,19] Certain types of HPV such as HPV1 infect cutaneous epithelial cells, whereas HPV 6, 11, 16 and 18 infect mucosal epithelial cells of the oral cavity, oropharynx, anogenital tract and uterine cervix.[20] The genomic HPV DNA has nine open reading frame sequences present on single strand of DNA. These are divided into seven early (E1–E7) and two late phase genes (L1–L2). The expression of viral oncoproteins E6 and E7 interferes with crucial cellular mechanisms such as cell cycle regulation and apoptosis.[21]

The p53 mutant forms are more stable and have an extended half-life than wild-type p53 and can be detected using immunohistochemical techniques. In the present study, immunohistochemical expression of p53 was observed to be positive in the basal layer of normal oral mucosa [Figures 1 and 2]. In OSCC, peripheral to central tumor cells were positive for p53 expression [Figures 3 and 4]. This was in accordance to the study by Gleich *et al.* (2000), where they found that 50.94% of oral and pharyngeal cancer show nuclear immunoreactivity suggesting longer half life of mutant p53 protein which results in accumulation of this phosphoprotein in the nuclei, facilitating its detection by immunohistochemical analysis with specific antibodies.[22]

In the present study, out of 24 samples of OSCC, only one case was positive for HPV-16, which was one of the four samples negative for p53 [Figures 5 and 6]. Positive HPV-16 sample of p53 negative case indicates the degradation of p53 by E6 oncoprotein by ubiquitin-mediated pathway [Figure 7]. Although PCR analysis detected HPV-16 strain subtype, nonetheless the possibility that minor HPV types or HPV DNA fragments which did not encode the L1 sequences, may have been present in the negative samples, cannot be excluded from the study. On the other hand, HPV E6 was detected in one of the OSCC samples. This could also be due to the loss of the episomal form of the virus.

It is this pathway which hampers p53 action and thus abnormal proliferation of aberrant cells takes place. With homozygous
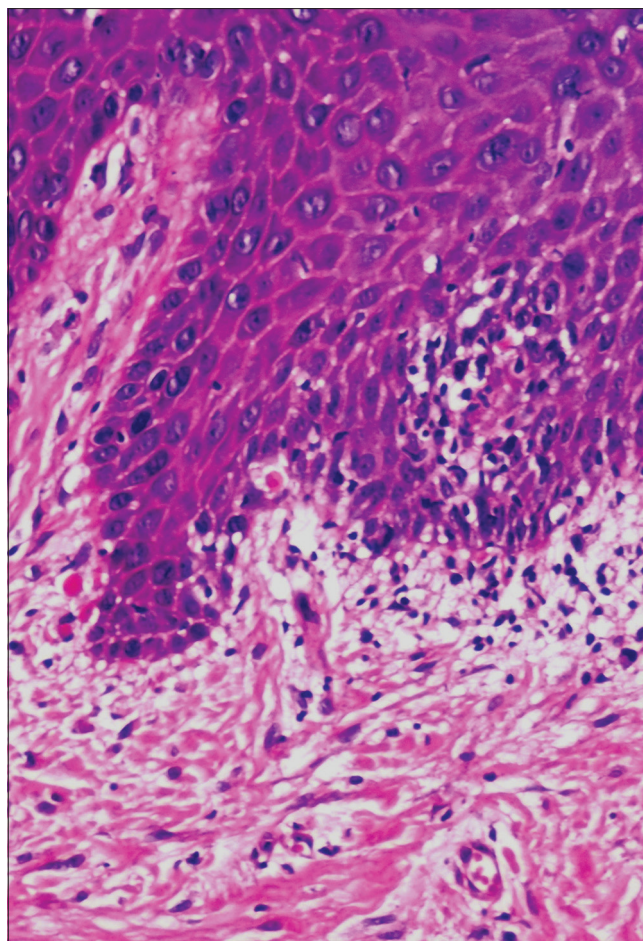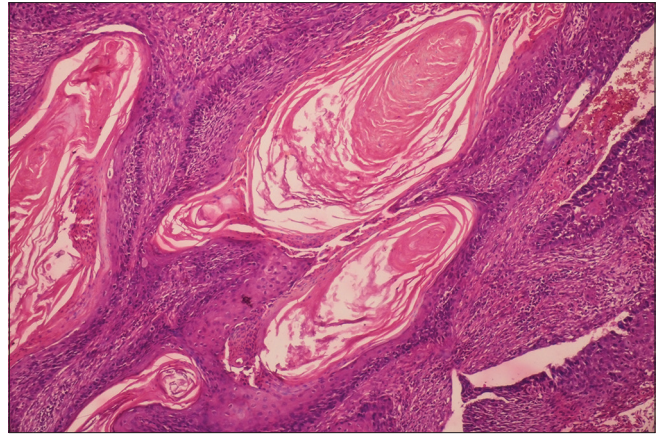

Figure 1: Normal epithelium (H&E stain, ×400)

loss of p53, DNA damage goes unrepaired, mutations become fixed in dividing cells and the cell turns onto a one-way street leading to malignant transformation. In a small study by Crook *et al.*,[23] six HPV positive lesions contained wild type p53 but the three HPV negative lesions had point mutations. A recent study on cervical cancer by the same workers also found point mutations in p53 in the three HPV negative cervical cancers in the series.[24]

The assay methods have differed in the various investigations and no study to date have p53 protein levels been quantitated in HPV negative and positive lesions or any clinicopathologic correlations made though there are many alternative methods to detect viruses such as *in situ* hybridization, Southern blot, dot blot, etc., Although point mutation appears to be the basis for the deregulation of p53 expression in human cancers, there is evidence from experimental murine epidermal carcinogenesis of novel mechanisms for p53 inactivation which do not involve point mutation.[25]
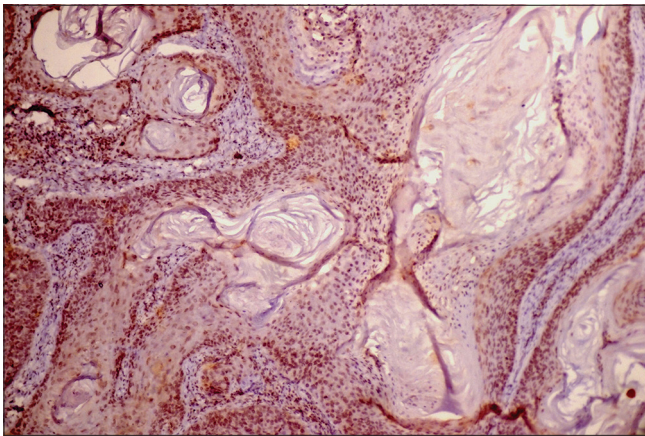
The epidemiology, diagnosis and treatment of oropharyngeal cancers are in a state of transition. New models of oncogenesis
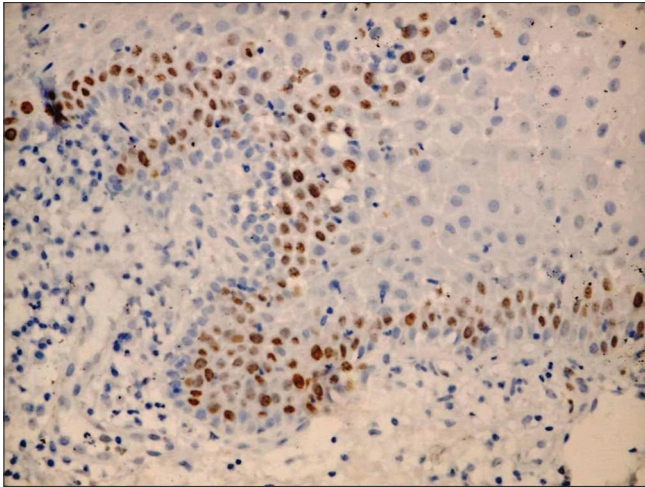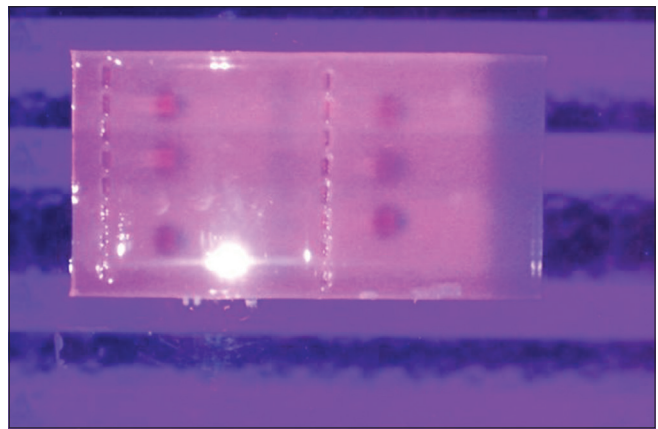
Figure 2: Positive expression of p53 in basal layer of normal mucosa (IHC stain, ×400)
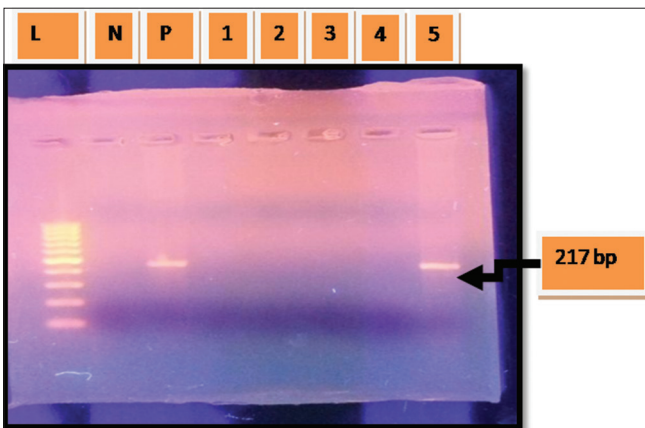

Figure 4: p53 positive cells in the periphery of the epithelial whorls (IHC stain, ×100)

Figure 6: Image of Gel electrophoresis representing Ebro staining of 2% agarose gel and quality of DNA content using transilluminator. L represents molecular weight marker; P is the positive control (HeLa cell line DNA) and N is negative control with genomic DNA from a human papillomavirus negative human cell line. Lanes 1–4 represent negativity of oral biopsy samples by DPX-polymerase chain reaction amplification. Sample 5 represents positive human papillomavirus-16

Figure 3: Well-differentiated OSCC (H&E stain, ×100)

Figure 5: Detection of DNA strands using transilluminator
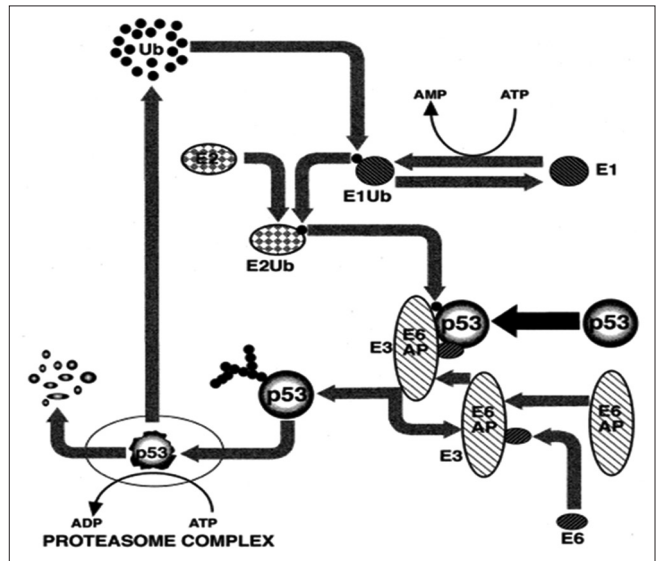
Figure 7: Flowchart depicting degradation of p53 by human papillomavirus E6. E1 ubiquitin activating enzyme activates ubiquitin, which is transferred by E2 ubiquitin-conjugating enzyme to E3 ubiquitin-protein ligase. Human papillomavirus E6 binds to E6-AP forming E3 enzyme, which specifically binds and ubiquitinates p53. The polyubiquitinated p53 is then degraded by 26S proteasome complex

are under investigation.[26] According to this present study, positive case for HPV-16 strain with p53 negative expression suggests that some strains of HPV-6, 11, 18 and not HPV-16, might be responsible for inhibition of the p53 gene and also indicates the degradation of p53 by E6 oncoprotein. As a consequence, p53's growth-arrest and apoptosis-inducing activities are abrogated. Thus, HPV may be directly involved in p53 suppression in OSCC.

CONCLUSION

Although the etiology of squamous cell carcinoma of the oral mucosa involves many different agents, viruses are important. HPV-positive oral and oropharyngeal cancer make up a distinct clinicopathological entity. The standardization of the methods for sample collection and analysis are mandatory to obtain reliable data and to compare the results obtained in different studies on the presence of HPV in variable proportions in OSCC tissues. Some tumors are associated with papillomaviruses and some with viruses of the herpes family; however, the exact role of these viruses must still be evaluated carefully. These viruses may provide targets for therapy and for diagnostic tests and may widen our understanding about the mechanisms by which the tumors develop.

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Conflicts of interest
There are no conflicts of interest.

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