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An Immunohistochemical Study on Expression of Laminin5 Gamma2 Chain and Alpha Smooth Muscle actin in Oral Squamous Cell Carcinoma

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Abstract **Original Research Article**

Background and objectives: Head and neck carcinomas are the biological heterogeneous group of cancers, of which oral cancer is the most common. Ninety percent of oral cancers are squamous cell carcinomas originating from the mucosal epithelium carcinogenesis. Oral squamous cell carcinoma (OSCC) is one of the most prevalent cancers worldwide. The transformation of the normal epithelial cell into a tumor cell depends upon certain changes that happen at the cellular and molecular level which will help in its survival and proliferation. The basement membrane will show invasion by these altered tumor cells into the connective tissue stroma and then their subsequent spread and metastasis, which is an important prognostic indicator. Laminin-5 is a protein which is found to be associated with a migratory phenotype in epithelial neoplastic cells and also the stromal myofibroblasts play a key role in tumor invasion, due to its ability to modify the extracellular matrix. ***Aim:*** Laminin-5 (Ln-5) is the major component of the basement membrane and is involved for tumour progression. Alpha-smooth muscle actin (α-SMA) is an isoform of actin, positive in myofibroblasts and is an epithelial to mesenchymal transition (EMT) marker. EMT is a process by which tumor cells develop and are able to metastasize. Tumor cells progression is always followed by alteration in the cell composition and extracellular matrix .Therefore the aim of this study is to detect and evaluate the expression of laminin5 gamma2 and α-SMA in OSCC and to evaluate the association of density of stromal myofibroblasts with tumour budding intensity. ***Materials and methods****:* Thirty paraffin embedded tissue blocks of clinically diagnosed and histopathologically confirmed cases of OSCC were evaluated .In this we have divided them into10 well differentiated OSCC,10 moderately differentiated OSCC and 10 poorly differentiated OSCC for laminin-5 and α- smooth muscle actin (SMA) using standard immunohistochemistry technique. Semi-quantitative assessment of the expression of laminin and alpha SMA was done in all the study samples. The area of staining and the staining intensity was evaluated in order to determine the staining index which was then statistically analyzed between the three groups. The study data was analysed using SPSS (Statistical Package for Social Sciences) software V.22, IBM, Corp. Statistical Analysis was done using Chi Square test .The level of significance [P-Value] was set at P<0.01. ***Results:*** There is a positive trend in high intensity expression of Lm5 γ2 increasing with the histopathological grading of OSCC. This distribution of Lm5 γ2 in varying grades of OSCC was significant (p=0.01*). Expression of laminin was observed mainly around the tumor islands in OSCC group. α- SMA was seen with increasing intensity with increasing grade of the disease. Statistical comparison of α-SMA in between the three groups using Kruskal- Wallis test showed a significant increase in the expression of α-SMA in various grades of OSCC ($p < 0.01$). ***Conclusion****:* There is no strong association between expression of Lm5 γ2 and α-SMA. Independent association of each marker with histopathological grades of OSCC was seen in the study.

Keywords: Oral squamous cell carcinoma, Myofibroblasts, Smooth muscle actin, Immunohistochemistry.

INTRODUCTION

Alpha-SMA is an actin isoform that plays a key role in process of fibrogenesis. Alpha-SMA can be found in smooth muscle cells, myofibroblasts, and in blood Vessels [1, 2] and can be correlated with activation of fibroblast to myofibroblast. The phenotype of myofibroblast in expression of α-SMA and producing extracellular matrix is controlled by transforming growth factor-beta (TGF-beta) [3, 4]. Myofibroblast are associated with α-SMA expression and are involved in inflammation, wound healing, fibrosis, and carcinogenesis [5]. Carcinoma cells that get transformed in to mesenchymal cells also express α-SMA [6, 7]. Alpha-SMA, along with vimentin, E-cadherin, and fibronectin are the markers for the epithelial to mesenchymal transition (EMT) process. The EMT is a crucial step as it is involved in normal cells to become a cancerous cell [8]. In OSCC the

extent of basement membrane (BM) defects correlates with invasive and metastatic potential [9-10]. The oral squamous cell carcinoma (OSCC) accounts for approximately 3% of all malignancies and more than 90% of cancers of the oral cavity and oropharynx [11]. OSCC is a neoplasm of epithelial origin with high prevalence rate in developing countries of the world especially in India [12]. Laminin-5 (Ln-5) is the major component of the basement membrane in most adult tissues and is a heterotrimer composed of three different laminin chains (α3, β3, and γ2 chains). Formerly called as Kalinin, nicein, epiligrin or ladsin, laminin-5 is now designated as laminin 332 using a recently introduced simplified nomenclature [13-14]. The major functions of Ln-5 include the binding of epithelial cells to the basement membrane through the formation of hemidesmosomes and the migration of epithelial cells during the process of wound repair. In addition to this, Ln-5 has been implicated in tumor progression [15]. Several literature data have also suggested the potential role of laminin- 5 in OSCC [16, 17], breast cancer [18] and cervical adenocarcinoma [19] among others. Epithelial mesenchymal transition (EMT) is the process by which epithelial cells will adopt a mesenchymal phenotype or fibroblast-like properties and this transition of the neoplastic epithelial cells to acquire fibroblasts like properties is a significant event in the metastatic potential of the tumor [20]. The Myofibroblasts are the differentiated fibroblasts that express alpha smooth muscle actin (α- SMA) and have intermediate characteristics among classic fibroblasts and smooth muscle cells. They have the ability to modify the extracellular matrix and hence actively participate in tumor invasion and metastasis [21]. Several studies have determined the role of myofibroblasts in OSCC [22-24]. Invasion of the altered tumor cells through the basement membrane into the connective tissue stroma and their subsequent spread and metastasis is an important prognostic indicator for understanding of this carcinogenesis model. Thus, the need of the present study is to evaluate the the expression of laminin5 gamma2 and α-SMA in OSCC and to evaluate association of density of stromal myofibroblasts with tumour budding intensity for the better understanding of the carcinogenesis model for any therapeutic and prognostic implications.

MATERIALS AND METHOD

Thirty formalin fixed, paraffin embedded biopsy specimens with clinically and pathologically confirmed OSCC cases and samples were obtained from the Department of Oral and maxillofacial Pathology. Insufficient tissue lacking adequate depth, tissue specimen with inconclusive diagnosis, recurrent cases, cases with other concurrent oral lesions and previous history of therapy (surgery, chemotherapy and radiotherapy) were excluded from the study. The selected samples were then immunohistochemically analyzed for the expression of laminin-5 and α- SMA using standard techniques. Semi-quantitative assessment of the expression of laminin and alpha SMA was done in all the study samples. The area of staining and the staining intensity was evaluated in order to determine the staining index. The study data was analysed using SPSS (Statistical Package for Social Sciences) software V.22, IBM, Corp. Statistical Analysis was done using Chi Square test. The level of significance [P-Value] was set at $P<0.01$.

Haematoxylin and eosin staining

Formalin fixed, paraffin embedded specimens were cut into 4µm sections and were stained with haematoxylin and eosin for histologic confirmation of clinical diagnosis of OSCC. Additional sequential sections were prepared for immunohistochemical studies.

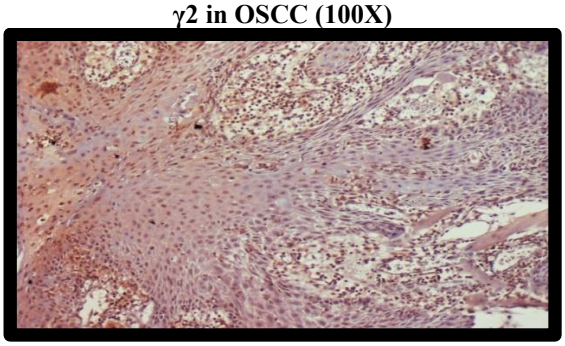
Immunohistochemistry protocol

All the 30 cases of OSCC were available for high quality immunohistochemical staining. Immunohistochemical staining was performed on 4µm thick sections. All the procedures were performed at room temperature. The sections were deparaffinised through a series of xylene baths and rehydrated in graded concentrations of alcohol. Tissue sections were treated with 3% hydrogen peroxide to block endogenous peroxidase activity. Antigen retrieval was carried out by microwave with 0.01 M sodium citrate buffer solution for tree cycles of 800 W for 5 min twice and 200W for 14 min and later was subjected to two washes of tris buffer solution for 5 min each. Sections were then incubated with ready to use primary antibody (BIORBYT and BIOCARE). After washing with tris buffer solution, the sections were then incubated for 30 min with anti mouse secondary antibody and visualized using 3, 3'-diaminobenzidine (DAB) chromogen. Sections were then visualized under microscope (4X, 10X and 40X) magnification.

The criteria used to define the Lm5 γ2 positive for the IHC staining

- Lm5 γ2 staining found localized in epithelium, tumor cells and in the tumor nests in connective tissue stroma which appeared brownish in color.
- Round/ovoid brown stained cells which must be visible completely with cytoplasmic stain.

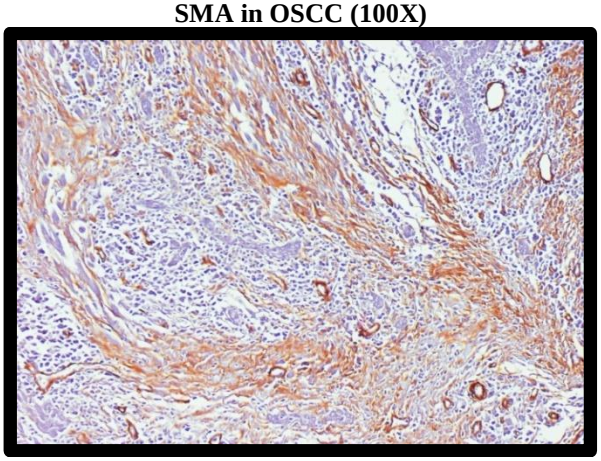
PHOTOGRAPH 1: Photomicrograph showing Lm5 γ2 in OSCC (100X)



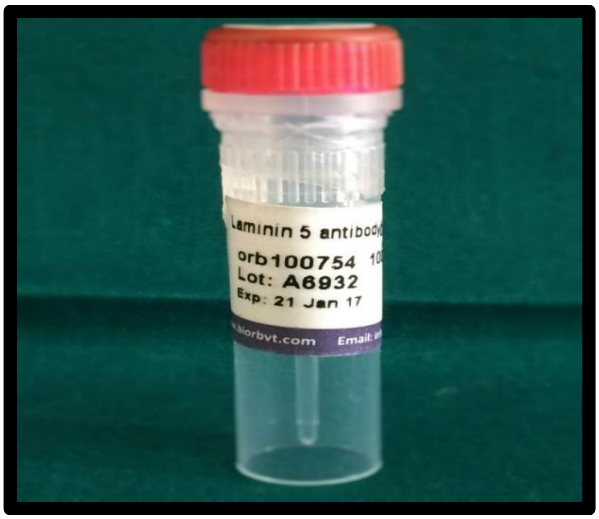
The criteria used to define the α-SMA positive for the IHC staining

- α-SMA staining found localized in large spindle-shaped cells which are long slandered resembling bundles of microfilaments and appeared brownish in color in the connective tissue stroma.

PHOTOGRAPH 2: Photomicrograph showing α-SMA in OSCC (100X)



PHOTOGRAPH 3: BIORBYT Primary antibody used in the study Lm5 γ2

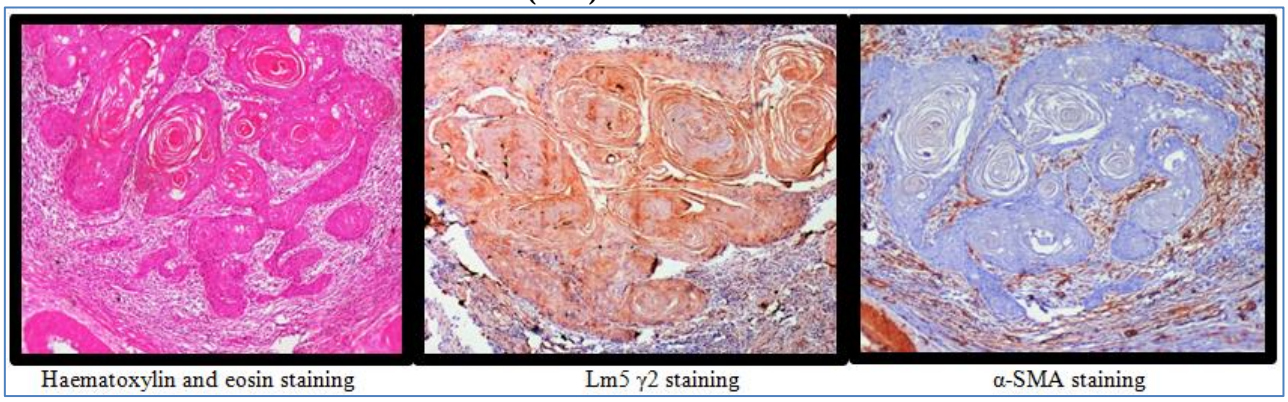


PHOTOGRAPH-4: BIOCARE primary antibody used in the study α-SMA

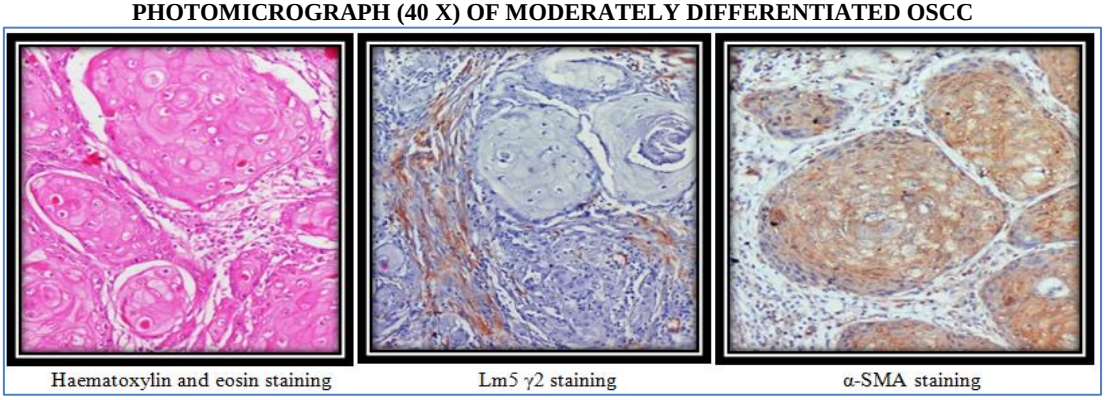
RESULTS

The present study was carried out to evaluate the expression of laminin 5 and stromal myofibroblasts in OSCC study groups. Semi-quantitative assessment of laminin and α- SMA was done in all the cases. All the cases of laminin showed cytoplasmic staining mainly around the tumor islands in OSCC group. α- SMA was seen with increasing intensity along with the increasing grade of the disease. Staining index for both was calculated by the area of staining and intensity of staining as described in methodology earlier. Grade III, Lm5 γ2 was majority in moderate intensity followed by high intensity with 30% and low intensity with 20% which shows there is a positive trend in high intensity expression of Lm5 γ2 increasing with the histopathological grading of OSCC. This distribution of Lm5 γ2 in varying grades of OSCC was significant (p=0.01*). (table 1).

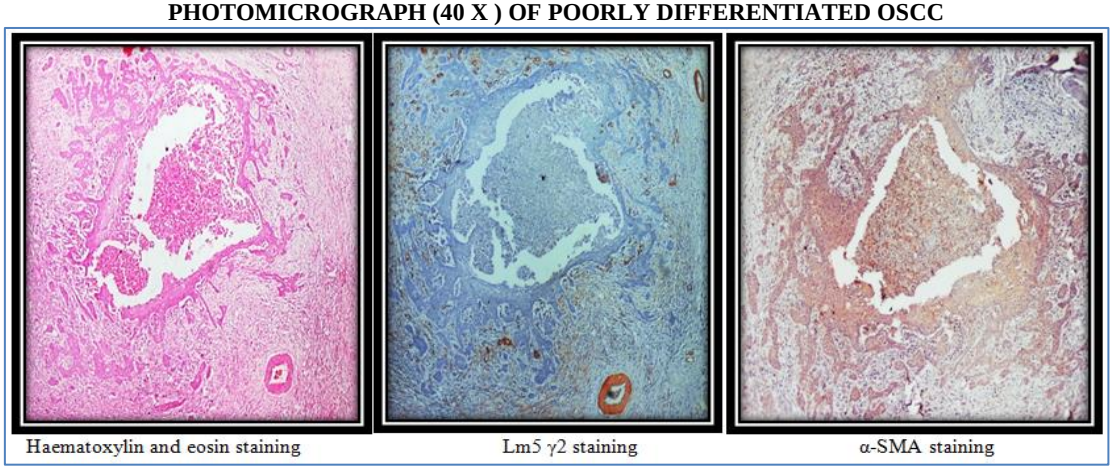
PHOTOMICROGRAPH (40 X) OF WELL DIFFERENTIATED OSCC



PHOTOMICROGRAPH (40 X) OF MODERATELY DIFFERENTIATED OSCC



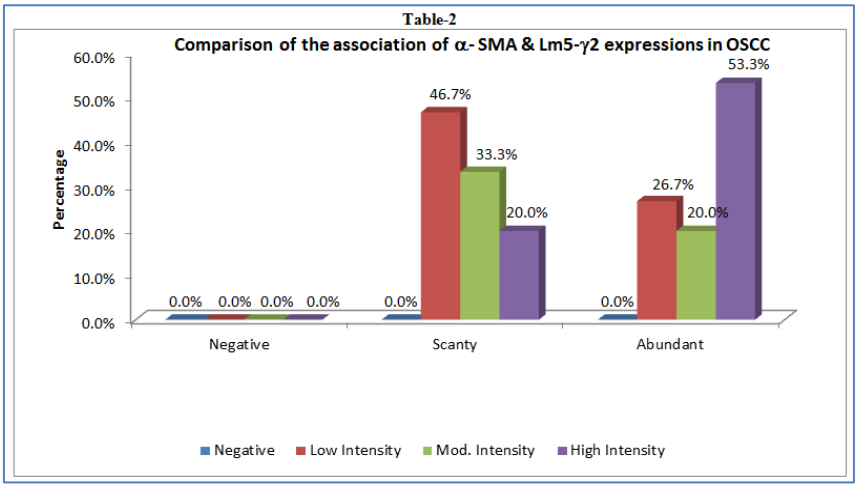
PHOTOMICROGRAPH (40 X) OF POORLY DIFFERENTIATED OSCC



Evaluation of myofibroblasts through the immunohistochemical expression of α-SMA revealed a low intensity in well differentiated OSCC, moderate intensity in moderately differentiated and intense staining in poorly differentiated OSCC especially around the tumor islands.

Table-1

Comparison of the association of a - SMA and Lm5 γ2 expressions using Chi Square test								
Lm5 γ2	**Negative**		**Scanty**		**Abundant**		**c^2 Value**	**P-Value**
	n	**%**	**n**	**%**	**n**	**%**		
Negative	0	0.0%	0	0.0%	0	0.0%	3.591	0.17
Low Intensity	0	0.0%	7	46.7%	4	26.7%		
Mod. Intensity	0	0.0%	5	33.3%	3	20.0%		
High Intensity	0	0.0%	3	20.0%	8	53.3%		



Additionally, regression analysis was done to evaluate the strength of association between α-SMA and laminin in the three groups. The analysis (table-2) revealed statistical significance in OSCC and the association was not significant (p = 0.17). This infers as the α-SMA expression increases, relative to the histopathological grading of OSCC, the Lm5 γ2 also increases correspondingly. However, the association with the expressions of α-SMA(density of stromal myofibroblasts) and Lm5 γ2 for varying histopathological gradings of OSCC was not statistically significant (p=0.17).

DISCUSSION

The study results were in accordance with several other results obtained from the literature [25-26]. Progressive decrease in laminin expression in OSCC could be attributed to the E-cadherin expression loss. The altered distribution of laminin and collagen IV are associated with the progression of OSCC [27].

In present study the α-SMA expression for myofibroblasts were found to be increasing with increased grade of OSCC and was statistically significant. This was in accordance with various studies [28, 29]. In OSCC, an increase in myofibroblasts population causes proteolytic degradation of the stromal components thereby aiding in the spread of tumor cells. Additionally, myofibroblasts may contribute to cytokine production and tumor angiogenesis. The stromal reaction to the altered overlying epithelium varies from increased inflammatory response, altered collagen fiber remodelling and vasculature and to some extent to the presence of myofibroblasts. The possible reason for an increased myofibroblasts is that that the genetically altered epithelium may have an inductive effect on the adjacent stroma to produce myofibroblasts [22]. Additionally, the changes in the composition and organization of the stromal micro-environment associated with the cytokine release may aid in the formation of myofibroblasts [30]. The exact role of these cells however is unknown.

Etemad-Moghadam *et al*. (2009) showed that the presence of myofibroblasts was significantly higher in oral squamous cell carcinomas compared to both dysplasia and normal mucosa. These findings show the presence of myofibroblasts in the stroma of oral squamous cell carcinoma but not in dysplasia and normal mucosa, suggesting further investigation to clarify the role of myofibroblasts in the carcinogenesis process [22]. Chaudhary *et al*. (2012) found an increase expression as the disease progresses from oral premalignancy to verrucous carcinoma and to invasive OSCC thereby suggesting that the proliferation of myofibroblasts may be used as a stromal marker for premalignancy and malignancy [29]. Analysis of α-SMA expression for myofibroblasts proliferation can be used as a stromal marker for predicting behaviour in oral pre-cancer and cancer.

The Trans differentiation of oral fibroblasts to myofibroblasts could occur secondary to the release of transforming growth factor-β1 that is released by OSCC cells. Analysis of myofibroblasts expression in varying grades of OSCC may indicate that the loss of cellular differentiation affects the number of myofibroblasts in the tumor stroma [23, 28]. Stromal destruction in OSCC occurs either through cancer cell-prompted destruction or due to cooperation between cancer cell and surrounding stroma. Myofibroblast appearance in invasive cancer and tumor desmoplasia are important reflection of the tumor–host interaction, especially in aggressive cancers. The Trans differentiation of myofibroblasts is generally induced during the invasive stage of SCC and further loss of tumor differentiation would not affect the number of these cells [30].

Statistical correlation of laminin-5 and alpha-SMA in oral epithelial dysplasia and OSCC showed an inverse relationship between the two wherein the decreased expression of laminin was associated with an increase in myofibroblasts population. Franz *et al*. (2010) demonstrated that increased laminin chain deposition is associated with a rising ASMA positivity suggesting a special role of stromal myofibroblast transdifferentiation for laminin matrix remodelling in OSCC. Laminin α2 chain significantly decreased while α3, α4, α5 and γ2 chains and α-smooth muscle actin (ASMA) significantly increase with rising grade of oral squamous cell carcinoma [31]. Similar kind of result was noted in our study. It was hypothesized that mediated by myofibroblasts, OSCC development is associated with a stromal upregulation of laminin isoforms possibly contributing to a migration promoting microenvironment. It is possible that mesenchymal cells contribute to the promotion of tumor cell migration as well as vessel formation in OSCC by providing and organizing pro-migratory laminin-5 fragment [32]. Epithelial-mesenchymal interaction plays a key role in oral carcinogenesis. An inverse relationship between laminin 5 and α-SMA expression probably suggest that the epithelial and mesenchymal components have a synergistic effect for tumor invasion. Terashi *et al*. in there study told that the Laminin-5 c2 expression was detected in the cytoplasm of tumor cells, predominantly in single cells or in the cells of tumor nests and epithelium. Expression of laminin-5 c2 was reported in several epithelial malignant tumors. Our study showed that Grade III, Lm5 γ2 was majority in moderate intensity followed by high intensity with 30% and low intensity with 20% which shows there is a positive trend in high intensity expression of Lm5 γ2 increasing with the histopathological grading of OSCC. This distribution of Lm5 γ2 in varying grades of OSCC was significant (p=0.01*).

CONCLUSION

There is no strong association between expression of Lm5 γ2 and α-SMA and show an independent association of each marker with

histopathological grades of OSCC. Future study should also focus on α-SMA as a prognostic marker for targeted therapy as it is one of the epithelial to mesenchymal marker. Laminin can be adopted as a useful marker in evaluating the histological differentiation and aggressiveness of oral carcinoma. The increased expression of α-SMA suggests that the proliferation of myofibroblasts may be used as a prognostic marker for malignancy. Additionally, myofibroblasts could be useful as a potential target for chemotherapeutic regimen in oral squamous cell carcinoma.

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