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RESEARCH ARTICLE

IMMUNOHISTOCHEMISTRY OPTIMISATION OF SOME TUMOUR SUPPRESSOR GENES IN CHRONIC OBSTRUCTIVE PULMONARY DISEASE (COPD) ON FORMALIN FIXED PARAFFIN EMBEDDED (FFPE) TISSUE SECTIONS.

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ABSTRACT: **Background:** Lung diseases has been shown to be among the most occurring diseases globally with diseases ranging from self-limiting flues to life threatening chronic diseases such as chronic obstructive pulmonary disease (COPD) and lung malignancies. Tobacco has been identified as the major singular risk factor for the pathogenesis of chronic pulmonary diseases. The incidence of primary lung carcinoma (PLC) is increased in COPD Patients due to an existing impairment in normal lung function. This study was aimed at carrying out preliminary IHC optimisation for p53, TTC5, Bax and p21 tumour suppression genes on COPD tissue sections. **Method:** Several IHC staining using the avidin- biotin complex kit (ABC) were carried out to express the four genes using different staining conditions and different primary antibody dilutions. **Results:** Our study revealed high-level expression of the four tumour suppression genes on COPD tissue sections with 1:250, 1:500 1: 500 and 1:100 optimum primary antibody dilutions for p53, TTC5, Bax and p21 genes respectively. All experiments for optimum results were carried out under 21°C room temperature. **Conclusions:** These findings revealed an active DNA - 8damage response, even though we are still far from understanding the current function of p53 and its downstream genes in this particular tissue. Further investigations are required and the application of more techniques for an expanded study with more access to lung cancer tissue sections from different cohorts, in order to fully understand the role of p53 and the other genes that respond to DNA damage in lung cancer therapy.

KEYWORDS: IHC, Optimisation, COPD, Lung cancer, DNA damage response, P53, Laboratory research

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INTRODUCTION:

Lung diseases are among the most common pathologies globally. The major risk factor for these diseases includes smoking, infection and genetic predispositions. Several types of disease occur because of these underlining causes, ranging from self-limiting flus to life-threatening diseases such as chronic obstructive pulmonary disease (COPD) and lung cancer.

COPD is characterize by prolong obstruction of the pulmonary airway. It is also referred to as chronic bronchitis or emphysema depending on different forms of presentation of the disease. Tobacco smoke is responsible for causing up 90% of COPD, other causes include prolong inhalation of toxic-polluted air and around 1% of person with the disease might have inherited a condition referred to as alpha-1-antitrypsin deficiency[1].The incidence of primary lung carcinoma (PLC) is increased in COPD Patients due to an existing impairment in normal lung function. This functional impairment is also responsible for the low response in management and treatment of lung cancer. There is also evidence that the risk of developing lung cancer in COPD patient is up to 2-5 times greater, than in non-COPD smokers [2-4].

Genetically, two categories of genes are usually responsible for the pathogenesis of chronic diseases. Oncogenes are genes involve in upregulation of cell growth and reproduction whereas, Tumor suppressor genes (TSGs) are the second category of genes that function to down regulate the process of cell growth and reproduction. Both categories of genes if mutated are capable of fostering malignant progression [5]. A highly potent network of DNA damage response signals via the cell cycle checkpoint regulates cell genomic integrity by tumour suppressor genes on one hand, and oncogenes on the other hand. These genes become activated due to multiple accumulation of genetic alteration to give growth and survival advantage for uncontrolled proliferation of cells[6]. Mutation usually causes a reduction or impairment of TSGs normal function in cells. This, in combination with other genetic alterations leads to the development of chronic diseases. Mutation in this category of genes is likely to be more valuable in the progression to cancer than mutation in oncogenes [7].TSGs function mainly to suppress cell growth and division. They do this by one or more of the following ways; down regulating genes that are responsible for continues cell cycle (oncogenes) resulting to cell cycle arrest, coupling of cell cycle long enough to ensure repair before allowing the cycle to continue, and initiating apoptosis (programme cell death) if damage cannot be repaired[8]. They are capable of preventing metastasis by adhering to tumour cells [9, 10], and are involved in DNA repair because this group of genes if mutated cancer risk is likely to increase[11].

The P53 gene is an important member of the tumour suppressor gene category. It is nicknamed as the "guardian of the genome" by Lane, [12] and as the "cellular gatekeeper for growth and division" by Levine, [13] due to its role in transmitting various stress-induced signals to diverse non-proliferative cellular responses by acting as a transcription factor to either activate or inhibit other genes in order to regulate their functions. These activities result to either of the following outcomes; DNA repair, cell-cycle arrest, activation of apoptosis, activation of cellular senescence and inhibition of autophagy [14-16], hence the necessity of p53 to be a key gene involved instudies relating to DNA damage response.TTC5 (tetratricopeptide repeat domain 5), also referred to as STRAP (stress responsive activator of p300), is a cofactor for p53 activation. TTC5 achieves this through mobilization of coactivators of p53, likep300, JMY (Junction Mediating and Regulatory protein) and PRMT5 (Protein arginine Methyltransferase 5) leading to p53 dependant transcriptional function. TTC5 also achieve this by down regulating the levels of Mdm2 in order to inhibit its down regulation of p53 to allow p53 up-regulation[17]. TTC5 remains stable and accumulates in the nucleus of DNA damaged cells due to phosphorylation by protein kinases ATM and Chek2[18]. This accumulation of TTC5 results to the mobilisation of stress responsive coactivator complex of p53, which include p300, JMY and PRMT5[19-21]. The cyclin dependant kinases (CDKs) and their inhibitors CDKIs play a vital role in the regulation of cell cycle check points. One of the CDKIs is the $p21^{CIP1}$protein that function to regulate the progression of G1 to S phase and mediate cellular senescence. The expression of

this protein is closely dependent on p53 in association with p53 mediated cell cycle arrest at G1 in response to stress[22, 23].Bax protein, also referred to as bcl-2-like protein, is an apoptosis regulator coded as 'BAX' gene in humans. This gene belongs to the Bcl-2 gene family, a group of proteins that form either homodimers or heterodimers and function principally to regulate the mitochondrial apoptotic pathway[24,25]. Bax function to activate apoptosis by forming a heterodimer with BCL2 and interacting with mitochondrial voltage-dependant anion channel (VDAC), thus increases the opening of VDAC that brings about the lost in membrane potential and the release of cytochrome C into the cytoplasm. This initiate apoptosis by cleaving to apoptotic protease activating factor-1 (Apaf-1). The expression of Bax gene is upregulated by p53 and is involved in p53-mediated apoptosis[26, 27].

Immunohistochemistry (IHC) technique is the combination of anatomy, physiology, immunology and biochemistry techniques to detect the expression and semi-quantify antigens or proteins in tissue section. Unlike other antigen-antibody technique like immunoprecipitation and western blot, it has an added advantage of providing an on-site information in an intact tissue, thus its preferred adoption in the management and treatment of chronic diseases. This study was aimed at carrying out preliminary IHC optimisation forp53, TTC5, Bax and p21 genes on COPD tissue sections, in order to arrive at optimum IHC staining condition and antibody concentrations for further studies to understand the role of these genes in lung cancer pathogenesis as part of a larger study in finding better therapy for lung cancer.

MATERIALS AND METHODS:

Tissue Block and Sectioning

The COPD tissue blocks (labelled 634) was provided under the ethical cover of Dr. Lucy Smyth (School of environment and life Sciences, University of Salford, Manchester UK). This tissue blocks was then sectioned with a rotary microtome (LEICA RM2125RT) at 5 µm thickness and attached on pre-treated superfrost microscope slides with the aid of a floating out water bath (LEICA HI 1210) and allowed to drain dry on the hot plate (LEICA HI 1220).

IHC optimisation and Staining Protocol

Tissue sections were de-waxed in two changes of histoclear for 5 minutes each, followed by re-hydration with graded concentrations of ethanol and brought down to distilled water. Tissues were exposed to an optimum blocking period of 1-hour treatment with 3% hydrogen peroxide in methanol immediately after bringing them down to water. The heat induced epitope retrieval (HIER) methods was adopted for antigen retrieval. Sections were exposed to treatment with tri-sodium citrate buffer pH 6.5 by placing slides in a coplin jar containing the buffer and then immersing the coplin jar containing the slides in a beaker containing the same buffer to completely cover the coplin jar to ensure the slides remain completely immersed in buffer during the process. The coplin jar-beaker preparation was then transferred into an autoclave and allowed for the entire duration of autoclaving (usually within 60 minutes). After ensuring the pressure of the autoclave is completely released and the chamber safe to open, the beaker containing the coplin jar and the slides were retrieved from the autoclave and place under running tap water to cool off. Sections were then treated with 1.5% normal serum in 1% TBS-T buffer for an optimum period of 1 hour, in order to block endogenous enzyme activities.

Antibodies

The Vectastin mouse and rabbit ABC Kits (PK-6100 series) were both used for optimising the antibodies, as the antibodies used were produced from different animal species. The choice of the kit used depended on the species the primary antibody was raised in. For our experiment, the mouse ABC kit was used for DO-7 antibody whereas the rabbit ABC kit was used for TTC5, Bax and p21. This stage is very crucial and it required several optimizations with different primary antibody dilutions in order to generate a genuine result. Four different antibodies for different target antigens or proteins were used to perform this experiment. The first antibody is DO-7; this was used to detect the expression of total p53. It was used at 1:25, 1:50, 1:100, 1:200, 1:250, 1:500 and 1:1000 dilutions. The second antibody

was TTC5, to detect the expression of TTC5 in tissue. It was used at 1:50, 1:100, 1:250, 1:500, and 1:1000 dilutions. The third and fourth antibodies were Bax and p21,for the detection of the expression of their corresponding proteins in tissue. They were both used at dilutions, 1:50, 1:100, 1:250 and 1:500. These antibodies were all diluted with the prepared normal blocking serum used for blocking endogenous immunoglobulins and applied for 1 hour at room temperature.For each optimisation group, a negative control section was run alongside (this has the same treatment with the group except the omission of the primary antibody step, where only the diluent was used instead).The secondary antibody (biotinylated antibody) was prepared and applied base on manufacturer instruction. This is the same for all cases.

Avidin-biotin complex (ABC) activity

This complex was formed separately by mixing the two compounds in a ratio that leaves some of the binding sites on avidin unbound and allow to react for 30 minutes before transferring to the tissue. This unbound sites on the avidin-biotin complex in turn binds to the secondary biotinylated antibody resulting in a more prominent enzyme target attachment than using only an enzyme-conjugated secondary antibody or only a primary antibody.

Immunochromogenic staining withDAB (3, 3-diaminobenzene) solution

Tissue sections were "DABed"separately and observed for optimum colour development for a duration of 2-10 minutes. In every step, we ensure a maximum treatment of up to 10 minutes for the negative control to serve as a benchmark. Tap water was used to stop the DAB process, followed by applying Harri's haematoxylin counter stain then sections were de-hydrated with graded alcohol, cleared in histoclear and then finally mounted with DPX Mountant.Two individual Scientist examined the stained slides microscopically with a Leica microscope (DM 500) for positivity, intensity and histological distribution. Optimum images for each antibody were captured using the same microscope.

RESULTS:

Optimisation of primary antibody and other staining conditions are vital to achieving reliable and reproducible IHC results. Sections from both tissue blocks were exposed to varying concentrations of the four different antibodies and varying staining conditions like room temperature, duration of pre-treatment with hydrogen peroxide, duration in blocking serum and DAB periods were considered. In all cases, a positive staining with the different antibodies was achieved. However, getting reliable result with high specificity staining was not successful at the first instance due to heavy background staining and inappropriate antibody dilutions. This led to several optimisation experiments in all the antibodies.

In order to analyse the expression of the p53, TTC5, Bax and p21 in COPD tissue, their corresponding primary antibodies were used to perform IHC staining on the tissue block provided. Three separate sets of optimisation experiments were carried out for each antibody. The first protocol was carried out under 27°C room temperature and had only 30min pre-treatment with peroxidase; this yielded a poor outcome as showed in figure 1. We also noticed that "Dabing" period varies for different antibodies at varying concentrations. So the best optimum condition and antibody dilution for the four genes are summarized on table 1. In order to establish the optimum staining conditions and antibody dilutions for IHC on COPD FFPE tissue, Slides with best IHC imagery were selected as indicated in figure 2.

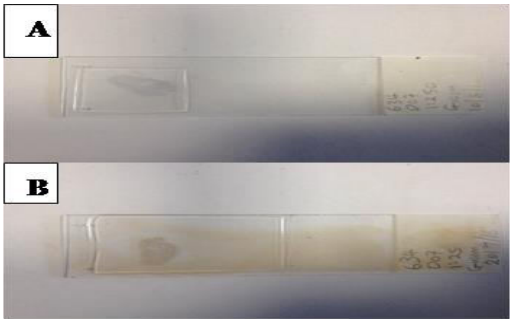

Figure 1. IHC macroscopy, showing stained slides at different room temperatures. 'A' shows the image of an IHC stained slide with DO-7 at a higher room temperature of 27^0C, whereas 'B' shows a contrast in slide background when IHC staining with DO-7 was performed at lower room temperature of 21^0C.

Table 1 Optimisation table

Primary antibody/Specie raised	Manufacturer	Product log No.	Antibody dilution	Blocking serum time	Pre-treatment with H_2O_2 time	DAB time
Negative control	NA	NA	Blocking serum	1hr	1hr	10min
DO-7 /mouse monoclonal antibody	Dako	M 7001	0.2152778	1hr	1hr	2.20min
TTC5 /rabbit polyclonal antibody	Abcam	Ab36855	0.3888889	1hr	1hr	4min
Bax (Δ 21) /rabbit polyclonal antibody	Santa Cruz Bio technology, INC.	Sc-6236	0.3888889	1hr	1hr	4min
P21 (C-19) /rabbit polyclonal antibody	Santa Cruz Bio technology, INC.	Sc-397	0.1111111	1hr	1hr	3.30min

Key: NA – not applicable

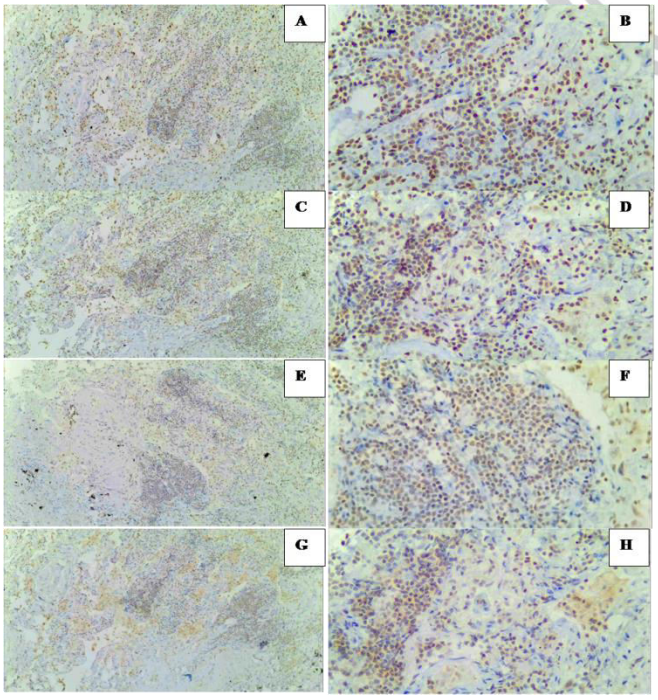

Figure 2.Optimum images for the expression of p53, TTC5, Bax and p21 proteins in COPD tissue. Microscopic images A and B are the optimum IHC optimisation results for DO-7 at X100 and X400 magnification. Microscopic images C and D are the optimum IHC optimisation results for TTC5 at X100 and X400 magnifications respectively. Microscopic images E and F are the optimum IHC optimisation results for Bax at X100 and X400 magnification. Microscopic images G and H are the optimum IHC optimisation results for p21 at X100 and X400 magnification. The brown stained cells are positive for the expression of their corresponding proteins, whereas the blue stained cells are negative for the expression of the proteins.

DISCUSSION:

Our study was aimed at carrying out a preliminary investigation to commence an IHC optimisation process for the detection of the expression of some key tumour suppressors using COPD tissue as a model, in order to contribute to our understanding for future approaches on investigations towards finding better therapy for lung cancer. Immunohistochemistry technique plays a pivotal role in the diagnosis and therapeutic studies of cancer and other chronic diseases. It has a wide range of application, due to its advantage to identify more proteins in tissue over the traditional histochemistry technique[28]. Some of the key applications of IHC in pathological researches include; investigations on prognostic markers in cancer, tumours of uncertain histogenesis, prediction of response to therapy[29], infection, neurodegenerative disorders[30], brain trauma, muscle diseases and in genetics[31].

Our study adopted the genetic application of IHC where both monoclonal and polyclonal antibodies were used to detect the expression of p53, TTC5, Bax and p21 antigens in COPD tissue sections. The positive expression of these proteins signifies a progressive DNA damage response as revealed by the expression of both upstream p53 activation marker, TTC5 and downstream p53 markers, Bax and p21. The expression of Bax and p21 also indicates the possibility of both p53-mediated apoptotic pathway and cell cycle arrest respectively[26, 22]. However, our study is limited in understanding the actual role of p53 in this particular COPD tissue as other studies have also suggested an upregulation of p21 via other signals[32] because of mutations[33].

CONCLUSION:

The IHC investigation reveals the expression of all four proteins in the COPD tissue used for optimization. Although, the results revealed by these experiments is still far from achieving the ultimate goal of finding better therapy for lung cancer. Further investigations adopting the optimisation conditions we have suggested for expressing these genes and other genes of interest, and employing the use of lung cancer tissues will be required to achieve this goal. In addition, adopting other techniques such as western blot, immunofluorescence or immunoprecipitations and studying other upstream and downstream genes of the p53 pathway, will give more insight in understanding the interactions and the nature of genes involved in DNA damage response. Furthermore, an ethical approval to have access to lung cancer tissues or tissue sections will broaden the scope and understanding of the role of p53 and its upstream and downstream genes in lung cancer.

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REFERENCES:

1. NHS Choices. Causes of COPD, 2016. Available at: http://www.nhs.uk/Conditions/Chronic-obstructive-pulmonary-disease/Pages/Causes.aspx (Accessed: 19 September 2016).
2. Mayne, S.T., Buenconsejo, J. and Janerich, D.T. Previous lung disease and risk of lung cancer among men and women nonsmokers, 1999. American Journal of Epidemiology 149;1:13–20.
3. de Torres, J.P., Bastarrika, G., Wisnivesky, J.P., Alcaide, A.B., Campo, A., Seijo, L.M., Pueyo, J.C., Villanueva, A., Lozano, M.D., Montes, U., Montuenga, L. and Zulueta, J.J. Assessing the relationship between lung cancer risk and emphysema detected on low-dose CT of the chest, 2007. Chest 132;6:1932–1938.
4. Raviv, S., Hawkins, K.A., DeCamp, M.M. and Kalhan, R. Lung cancer in chronic obstructive pulmonary disease, 2011. American Journal of Respiratory and Critical Care Medicine 183;9:1138–1146.
5. Knudson, A. Two genetic hits (more or less) to cancer, 2001. Nature Reviews Cancer 1;2:157–62.
6. Broustas, C.G. and Lieberman, H.B. DNA damage response genes and the development of cancer Metastasis, 2014. Radiation Research 181;2:111–130.
7. Weinberg, R.A. The biology of cancer, 2nd edn. New York, NY: GS Garland Science, Taylor & Francis Group; 2014
8. Sherr, C.J. Principles of tumor suppression, 2004. Cell 111;2:235–246.
9. Yoshida, B.A. Metastasis-Suppressor genes: A review and perspective on an emerging field, 2000. Journal of the National Cancer Institute 92;21:1717–1730.
10. Hirohashi, S. and Kanai, Y. Cell adhesion system and human cancer morphogenesis, 2003. Cancer Science 94;7:575–581.
11. Markowitz, S. DNA repair defects inactivate tumor suppressor genes and induce hereditary and sporadic colon cancers, 2000. Journal of Clinical Oncology 18;21 Suppl:75S–80S.
12. Lane, D.P. P53, guardian of the genome, 1992. Nature 358;6381:15–16.
13. Levine, A.J. P53, the cellular Gatekeeper for growth and division, 1997. Cell 88;3:323–331.
14. Yee, K.S. and Vousden, K. Complicating the complexity of p53, 2005. Carcinogenesis 26;8:1317–1322.

15. Riley, T., Sontag, E., Chen, P. and Levine, A. Transcriptional control of human p53-regulated genes, 2008. Nature Reviews Molecular Cell Biology 9;5:402–412.
16. Green, D.R. and Kroemer, G. Cytoplasmic functions of the tumour suppressor p53, 2009. Nature 458;7242:1127–1130.
17. Demonacos, C., Krstic-Demonacos, M. and La Thangue, N.B. A TPR motif Cofactor contributes to p300 activity in the p53 response, 2001. Molecular Cell 8;1:71–84.
18. Adams, C.J., Graham, A.L., Jansson, M., Coutts, A.S., Edelmann, M., Smith, L., Kessler, B. and La Thangue, N.B ATM and Chk2 kinase target the p53 cofactor strap, . 2008. EMBO reports 9;12:1222–1229.
19. Shikama, N., Lee, C.-W., France, S., Delavaine, L., Lyon, J., Krstic-Demonacos, M. and La Thangue, N.B. A novel Cofactor for p300 that regulates the p53 response, 1999. Molecular Cell 4;3:365–376.
20. Jansson, M., Durant, S.T., Cho, E.-C., Sheahan, S., Edelmann, M., Kessler, B. and La Thangue, N.B. Arginine methylation regulates the p53 response 2008. Nature Cell Biology 10;12:1431–1439.
21. Coutts, A.S., Weston, L. and La Thangue, N.B. A transcription co-factor integrates cell adhesion and motility with the p53 response 2009. Proceedings of the National Academy of Sciences 106;47:19872–19877.
22. Gartel, A.L. Lost in transcription: P21 repression, mechanisms, and consequences, 2005. Cancer Research 65;10:3980–3985.
23. Rodriguez, R. and Meuth, M. Chk1 and p21 cooperate to prevent Apoptosis during DNA replication fork stress 2006. Molecular Biology of the Cell 17;1:402–412.
24. Willis, S.N. and Adams, J.M. Life in the balance: How BH3-only proteins induce apoptosis 2005. Current Opinion in Cell Biology 17;6:617–625.
25. Grosse, L., Wurm, C.A., Bru ser, C., Neumann, D., Jans, D.C. and Jakobs, S. Bax assembles into large ring-like structures remodeling the mitochondrial outer membrane in apoptosis 2016. The EMBO Journal 35;4:402–413.
26. Xiang, H., Kinoshita, Y., Knudson, C.M., Korsmeyer, S.J., Schwartzkroin, P.A. and Morrison, R.S. Bax Involvement in p53-Mediated Neuronal Cell Death, 1998. The Journal of Neuroscience 18;4:1363–1373.
27. NCBI BAX BCL2 associated X, apoptosis regulator [Homo sapiens (human)]'. Available at: http://www.ncbi.nlm.nih.gov/gene/581 (Accessed: 23 September 2016) 2016.
28. Rajendran, A., Sundaram, S. and Rajendran, R. Shafer's textbook of oral pathology. 6th edn. New Delhi, India: Elsevier India;2009.
29. Mohan, H. Essential pathology for dental students. 3rd edn. New Delhi: Jaypee brother's medical publishers;2005
30. Lace, G., Ince, P.G., Brayne, C., Savva, G.M., Matthews, F.E., de Silva, R., Simpson, J.E. and Wharton, S.B. Mesial temporal Astrocyte Tau pathology in the MRC-CFAS Ageing brain cohort 2012. Dementia and Geriatric Cognitive Disorders 34;1:15–24.
31. Kaliyappan, K., Palanisamy, M., Duraiyan, J. and Govindarajan, R. Applications of immunohistochemistry, 2012. Journal of Pharmacy and Bioallied Sciences 4;6:307.
32. Abbas, T. and Dutta, A. P21 in cancer: Intricate networks and multiple activities, 2009. Nature Reviews Cancer 9;6):400–414.
33. García-Cruz, R., Camats, M., Calin, G.A., Liu, C.-G., Volinia, S., Taccioli, C., Croce, C.M. and Bach-Elias, M. The role of p19 and p21 H-Ras proteins and mutants in miRNA expression in cancer and a Costello syndrome cell model, 2015. BMC Medical Genetics 16;1.

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