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RESEARCH ARTICLE

IMMUNOHISTOCHEMISTRY OPTIMISATION OF SOME TUMOUR SUPPRESSOR GENES IN CHRONIC OBSTRUCTIVE PULMONARY DISEASED FORMALIN FIXED PARAFFIN EMBEDDED TISSUE SECTIONS.

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ABSTRACT: Immunohistochemistry (IHC) technique is one of the antigen-antibody techniques that has added advantage of providing an on-site information in an intact tissue, thus its preferred adoption for diagnosis in the management and treatment of chronic diseases such as COPD and lung cancer. The use of IHC technique has been shown to be very useful in expressing tumour suppressor genes such as p53, particularly in studies for lung carcinogenesis. This study isaimed at carrying out preliminary IHC optimisation for the following tumour suppressor genes; p53, TTC5, Bax and p21 genes on COPD tissue sections, in order to arrive at optimum IHC staining condition and antibody concentrations as part of larger studies on understanding the role of these genes in lung carcinogenesis and consequently, finding better therapy for lung cancer. 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. 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, as higher background staining was seen to be associated with higher environmental staining temperature. These findings revealed an active DNA damage response, even though we are still far from understanding the current function of p53 and its downstream genes in this particular tissue.

KEYWORDS: IHC, Optimisation, Chronic lung diseases, DNA damage response, p53

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INTRODUCTION:

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 sections. Unlike other antigen-antibody techniques like immunoprecipitation and western blotting, it has an added advantage of providing an on-site information in an intact tissue, thus its preferred adoption for diagnosis in the management and treatment of chronic diseases(Duraiyan et al., 2012). The IHC technique has been shown to be very useful in expressing tumour suppressor genes such as p53, particular in ancillary studies for lung carcinogenesis, where their expressions can help in early diagnosis of lung and other cancers as they appear in pre-invasive tumours(Trivers et al., 1996). This is a preliminary study that aimed at carrying out IHC optimisation for the following tumour suppressor genes: p53, TTC5, Bax and p21 genes on chronic obstructive pulmonary disease (COPD) tissue sections, in order to arrive at optimum IHC staining condition and antibody concentrations as part of an ongoing study on understanding the role of these genes in lung carcinogenesis and consequently, finding better therapy for lung cancer.

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(Ferkol and Schraufnagel, 2014, Johnson, 2001, Mannino and Braman, 2007). COPD is characterized by prolong obstruction of the pulmonary airway. 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 (Mayne et al., 1999, de Torres et al., 2007, Raviv et al., 2011)

Tumour suppressor genes (TSGs) are genes that function to down regulate the process of cell growth and reproduction. These genes if mutated are capable of fostering malignant progression (Knudson, 2001). 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(Yokota, 2000). The P53 gene is an important member of the tumour suppressor gene category, (Lane, 1992, Levine, 1997) 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 (Haldar et al., 1994, Riley et al., 2008). TTC5 is a cofactor for p53 activation(Demonacos et al., 2001). The cyclin dependant kinases (CDKs) and their inhibitors CDKIs play a vital role in the regulation of cell cycle checkpoints. 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(Gartel and Radhakrishnan, 2005, Rodriguez and Meuth, 2006).Bax protein, also referred to as bcl-2-like protein, is an apoptosis regulator coded as 'BAX' gene in humans (Willis and Adams, 2005, Große et al., 2016). The expression of Bax gene is up regulated by p53 and is involved in p53-mediated apoptosis(Xiang et al., 1998).

MATERIALS AND METHODS:

Tissue Block and Sectioning

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

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. Tissue sections 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) method 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 that completely covered the coplin jar to ensure the slides remained completely submerged 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 was completely released and the chamber safe to open, the beaker containing the coplin jar and the slides were retrieved from the autoclave and placed under running tap water to bring down the temperature. 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, Burlingame, California,US) 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 was DO-7; this was used to detect the expression of total p53. It was used at 1:25, 1:50, 1:100, 1:200, and 1:250, 1:500 and 1:1000 dilutions. The second antibody used 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. The secondary antibody (biotinylated antibody) was prepared and applied based on the manufacturer's instructions. This was the same for all cases.

Controls

A negative control section was run for each optimization group (the control section had same treatment with other sections for each group, except that the primary antibody was omitted, the diluent was used instead).

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 then allowed 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 with DAB (3, 3-diaminobenzene) solution

Tissue sections were "DABed" separately and observed for optimum colour development for duration of 2-10 minutes. In every step, we ensured 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 dehydrated with graded alcohol, cleared in histoclear and then finally mounted with DPX Mountant. The stained slides were examined microscopically by two separate Scientistswith a Leica microscope (DM 500, Wetzlar, Germany) for positivity, intensity and histological distribution of target genes. Optimum images for each antibody were captured using the same microscope.

RESULTS:

Optimization of primary antibody and other staining conditions are vital to achieving reliable and reproducible IHC results. Sections from tissue block 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 solution were considered. In all cases, positive staining with the corresponding antibodies were achieved. However, getting reliable results with high specificity staining was not successful at the first instance due to heavy background staining and inappropriate antibody dilutions. This led to several optimization experiments with 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 sections provided. Three separate sets of optimization 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.

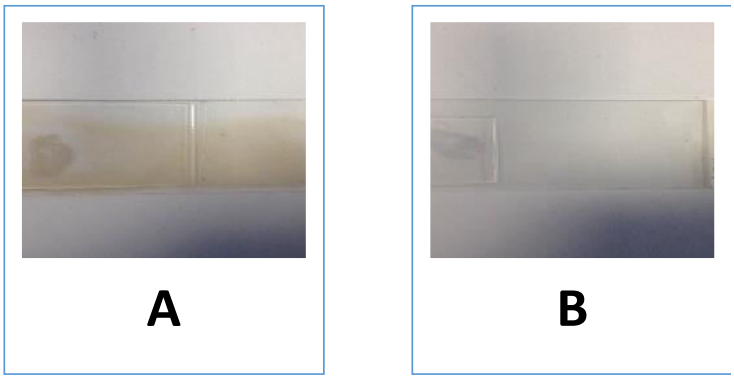

Figure 1. IHC macroscopic slides, showing stained slides at different room temperatures. 'A' shows the image of an IHC stained slide with DO-7 at a higher room temperature of 27°C, whereas 'B' shows a contrast in slide background when IHC staining with DO-7 was performed at lower room temperature of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

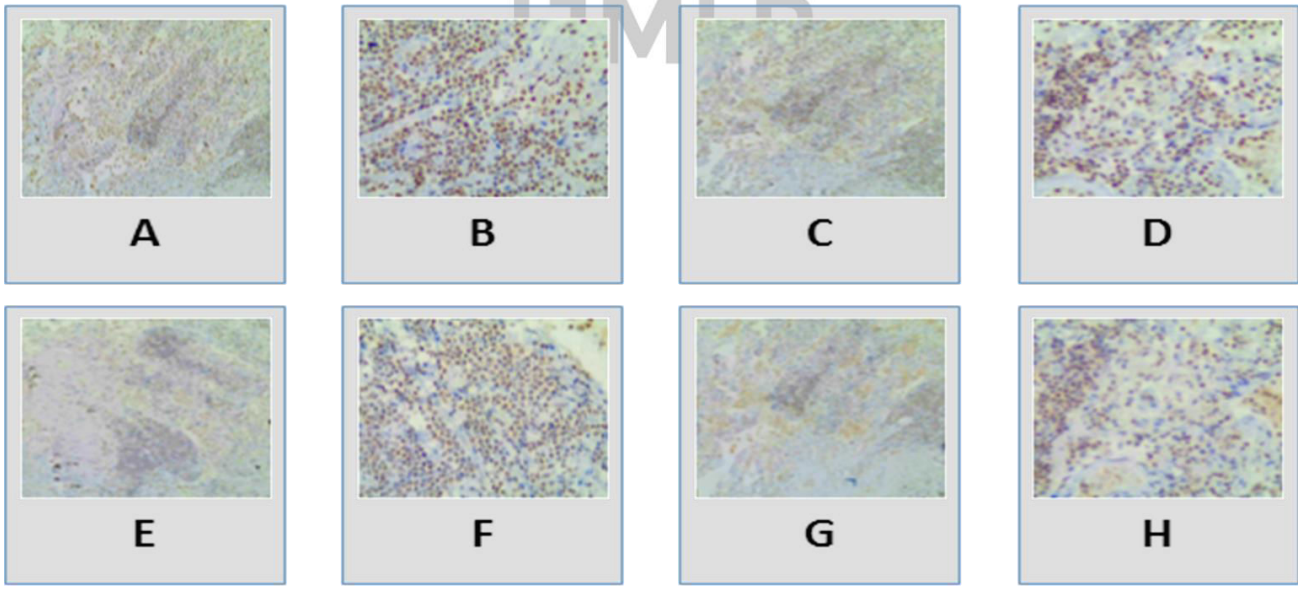

Figure 2. Optimum images for the expression of p53, TTC5, Bax and p21 proteins in COPD tissue. Microscopic images A and B are the optimum IHC optimisation results for DO-7 at X100 and X400 magnifications respectively. Microscopic images C and D are the optimum IHC optimisation results for TTC5 at X100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

Table 1 IHCOptimum Staining conditions for DO-7, TTC5, Bax and P21 Antibodies

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

DISCUSSION:

Our study was aimed at carrying out IHC optimization experiment to detection and expressed some key tumour suppressor genes using COPD tissue. This was to serve as a preliminary study for an ongoing research work on lung cancer therapy. Immunohistochemistry technique plays a pivotal role in the diagnosis and therapeutic studies of cancer and other chronic diseases. It has a wide range of applications, due to its advantage to identify more proteins in tissues over the traditional histochemistry technique (Rajendran, 2009). 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 (Mohan and Mohan, 2011), infection, neurodegenerative disorders (Lace et al., 2012), brain trauma, muscle diseases and in genetics (Duraiyan et al., 2012).

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 genes in COPD tissue sections. The positive expression of these genes 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 (Xiang et al., 1998, Gartel and Radhakrishnan, 2005).

Our optimization experiment has shown great potential for the expression of these tumour suppressor genes in lung tissue, using the optimum staining conditions we are recommending. However, there is a gap in understanding the actual role of p53 and its downstream genes in this particular COPD tissue as other studies have also suggested an up-regulation of p21 via other signals (Abbas and Dutta, 2009)due to mutations (García-Cruz et al., 2015).

The IHC optimization experiment reveals the optimum expression levels 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 chronic lung diseases, particularly lung cancer. Further investigations using our recommended optimum staining conditions for expressing these genes and other genes of interest, and also employing the use of lung cancer tissues from other patients is required. 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 for chronic lung diseases. 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 carcinogenesis.

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