ORIGINAL ARTICLE

Effects of indomethacin on expression of PTEN tumour suppressor in human cancers

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ABSTRACT

Background: Previous studies reported that Non-steroidal Anti-inflammatory Drugs (NSAIDs), chemicals, and food supplements can be used to up-regulate the PTEN mRNA and protein expression, suggesting that these substances may be used in prevention and/or treatment of various human cancers like spinal, brain, colon, breast, prostate, bladder and endometrial cancers. **Aim:** This was to study expression and sub-cellular localisation of PTEN protein, and review the effect(s) of indomethacin on PTEN's expression in cultured Human Endometrial Cancer (HEC 1B) cell line, which is known to express significant amounts of the wild-type PTEN. **Materials and Methods:** This involves culture and incubation of artificial HEC 1B cells. All procedures were undertaken in the cell culture hood under the recommended sterile conditions. The cells were then incubated with different concentrations of indomethacin solution, for variable durations and finally fixed (with paraformaldehyde) and stained with fluorescein-labelled diluted secondary antibody (FITC). Immunocytochemistry (IHC) and fluorescent microscopy were then employed for the detection and localisation of the specific antigen (PTEN), using antibodies. **Results:** The HEC 1B cells, which were cultured and incubated with different concentrations of indomethacin solution, expressed the PTEN protein, most of which was localised to the nucleus with minimal cytoplasmic expression. Increased PTEN expression was observed following treatment of the cells with various concentrations of the solution for variable durations, although there was cell death at higher concentrations and longer duration. This procedure was repeated several times, in order to have consistency and to validate the results. **Conclusion:** This study agrees with previous studies in similar human cell lines and supports the idea that NSAIDs and other drugs may be used in the future for prevention of human cancers. However, more studies need to be carried out to substantiate these observations.

Key words: Cancer prevention, chemoprevention, expression, HEC 1B cells, human cancers, immunocytochemistry, indomethacin, localisations, NSAIDs, phosphatase and tensin homologue, PTEN

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INTRODUCTION

Phosphatase and tensin homolog deleted on chromosome ten (PTEN) is a tumour suppressor gene,[1] which is deleted or mutated in approximately 45% of uterine endometrial cancers,[2] 30% of glioblastomas and spinal tumours,[3] and less commonly in the cancers of the prostate,[4] bladder,[5] adrenal glands,[6] thyroid, breast,[7] skin (melanomas)[8] and colon.[9-11]

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This tumour suppressor gene (PTEN) was discovered in 1997 by three separate research groups,[12,13] who revealed that chromosome 10 was partially or completely lost in many human cancers like glioblastoma of the brain, spinal tumours, as well as bladder, prostate, colon and breast cancers.[14] The most common region of loss of heterozygosity (LOH) was later identified to be 10q23.[1]

Further evidence suggested that a tumour suppressor gene may be found at this location, and this was later confirmed by the discovery of a 403-amino acid open reading frame (ORF).[13] Sequence analysis was subsequently used which showed that the gene encoded a protein tyrosine phosphatase with similarity to chicken tensin.[15] As a result of that, the gene was named PTEN for phosphatase and tensin homologue deleted on chromosome 10.[13] Steck *et al.*, (1997)[14] later cloned a tumour suppressor gene and

named it MMAC 1 (mutated in multiple advanced cancers), and Li and Sun (1997)[12] reported the identification of TEP 1 (TGF-regulated and epithelial cell-enriched phosphatase), both of which proved to be identical to PTEN and were used as synonyms for sometime before they were dropped for the more popular PTEN.[11]

MATERIALS AND METHODS

Procedure and maintenance of cell culture

These include HEC 1B (Human Endometrial Cancer) cell line, Dulbecco's phosphate buffered saline (D-PBS) (GIBCO, Scotland, UK), McCoy's medium (GIBCO, Scotland, UK), L-glutamine (GIBCO, Scotland, UK), Penicillin/Streptomycin (GIBCO, Scotland, UK) and Trypsin/EDTA (GIBCO, Scotland, UK). The McCoy's culture medium was stored at +4°C, while the cell cultures were incubated at 5% CO_2 and 37°C. The equipments were sterile medium (175 cm^3) cell culture flasks, sterile plastic pipettes (1 ml, 5 ml, 10 ml and 20 ml), haemocytometer, chamber slides and humidity chamber. Inverted phase contrast microscope was used to monitor the cells for attachment, detachment and viability. Fluorescence microscope was used for detection of the antigen and its distribution.

All procedures were undertaken by the standard protocol in the cell culture hood under the recommended sterile conditions. Gloves were worn throughout and Barrycidal 36 was used to clean the hood and equipment before and after the procedures. Spirit-soaked swabs were used to clean the haemocytometer and cover slips. Spillages were similarly sprayed with Barrycidal 36 before being wiped and cleaned; and spent medium as well as solid wastes were appropriately disposed.

Immunocytochemistry and fluorescent microscopy

This is a technique employed for the detection and localisation of a specific antigen (PTEN), using antibodies. The primary antibodies are raised against the antigen, and can be monoclonal or polyclonal. Monoclonal antibodies are generally raised in mouse and they bind to only one epitope (antigenic determinant). On the other hand, polyclonal antibodies are usually raised in the rabbit and identify several epitopes.[16] The cells are incubated with an antibody to enable its binding to a specific antigen. The unbound antibody is removed or washed out, and this is followed by incubation with a fluorescently labelled secondary anti-mouse or anti-rabbit immunoglobulin. The secondary antibody recognises and binds to the primary antibody, and the distribution of the bound antibodies is revealed by fluorescence microscopy which reflects the distribution of the antigen. This is based on the principles of indirect immunofluorescence.[17] Materials required include 3.7% paraformaldehyde, methanol (−20°C), acetone (−20°C), monoclonal 26H9 anti-PTEN antibody (Cell signalling technology, Beverly, USA), anti-mouse immunoglobulin (Sigma, St. Louis, USA) and 4', 6-diamidino-2-phenylindole (DAPI) mounting medium.

Chamber slide preparation and incubation with indomethacin solution

Four chamber slides were filled with the cell suspension [Figure 1] and placed in the incubator until they reached a confluence of 60-80%. Two of the slides were subjected to indomethacin treatment [Figure 2] according to the agreed protocol (50 mM of indomethacin prepared in 0.2 M Na_2CO_3 and 1M NaH_2PO_4 solution), and the other two were left untreated as controls [Figure 3]. Thereafter, all cells were fixed and made ready for the immunocytochemical detection of the antigen [Figure 4]. The anti-PTEN antibody was diluted a hundred times, while the secondary antibody was diluted two hundred times. All the eight wells in each of the four chamber slides were filled with 400 µl of the diluted cell suspension and left overnight at 37°C and 5% CO_2. This allowed the cells to form a monolayer.

Figure 1 shows the treatment of the two selected slides with the indicated volumes of indomethacin solution,

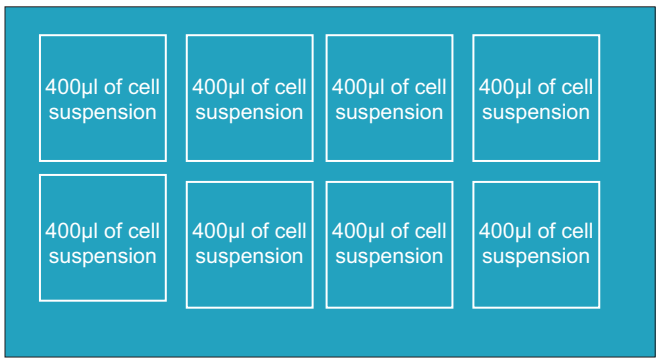

Figure 1: Chamber slide preparation

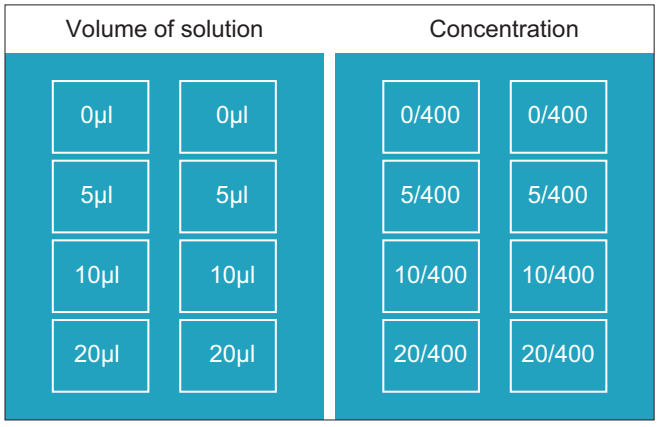

Figure 2: Treatment of cells (incubation) with different concentrations of indomethacin solution

followed by placement in the incubator to allow the drug (indomethacin) to take effect on the cells.

Cell fixing and immunocytochemical staining

All slides were washed with PBS and fixed with 3.7% paraformaldehyde. This was followed by washing with PBS and immersion in cold methanol and acetone (–20°C) for 4 and 2 minutes, respectively. The slides were then washed again, and excess PBS removed. Three slides were then incubated with 50 µl of the primary antibody (diluted ×100) into each well, and left overnight (+4°C). The 4th slide was left without primary antibody and served as control.

The following morning, all the slides were stained with the fluorescein-labelled diluted (×200) secondary antibody (FITC), for 1 hour and then allowed to incubate in the dark [Figures 3 and 4]. This was followed by washing with PBS, and mounting with DAPI, which is a dye that intercalates deoxyribose nucleic acid (DNA) and stains the nuclei blue under the fluorescence microscope. A cover slip was placed, sealed with nail varnish and allowed to dry. The slides were later examined with the fluorescence microscope which revealed positive staining as green fluorescence. Pictures were taken for analysis.

Figure 3 illustrates the two slides which were not treated with indomethacin. Slide 1 had no primary antibody added, while slide 2 had both primary and secondary antibodies added. Both slides were stained with DAPI and wrapped in foil paper for storage in the dark.

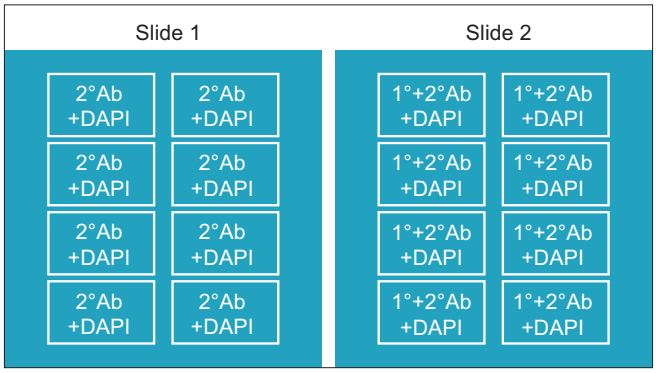

Figure 3: Pattern of antibody staining of the untreated cells in the chamber slides

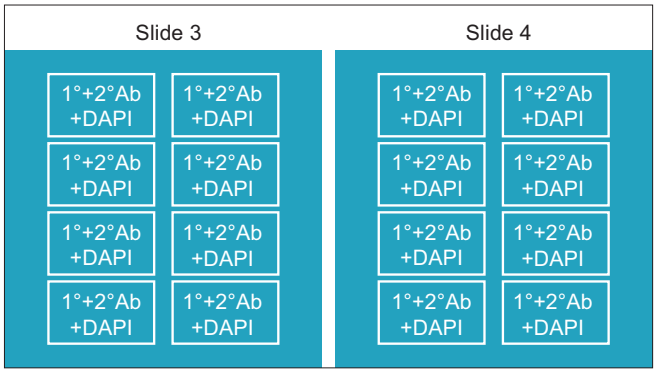

Figure 4: Pattern of antibody staining of the treated cells in the chamber slides

Figure 2 illustrates how the two treated slides were stained with both primary and secondary antibodies, as well as DAPI. Slide 3 was only treated with the drug for 1 hour, while the 4th slide received treatment overnight and 1 hour the following morning.

RESULTS

The effect of indomethacin on expression and sub-cellular localisation of PTEN in HEC 1B cells was studied by immunocytochemistry. Initially, untreated cells were analysed for comparison. When control cells which were incubated only with the secondary antibody were analysed, minimal fluorescence due to non-specific binding of the secondary antibody was detected [Figure 5]. Green fluorescence was observed when cells were incubated with an anti-PTEN monoclonal antibody followed by a secondary anti-mouse antibody labelled with FITC [Figure 6]. This indicated the presence of PTEN and was in agreement with the published data.

Two different protocols for treatment of cells with indomethacin were applied. Treatment of cells with indomethacin for 1 hour resulted in an increased fluorescence as detected with anti-PTEN antibody that was interpreted as an up-regulation of PTEN expression [Figure 7]. Cells differed in the intensity of the signal and this suggests that individual cells expressed different levels of PTEN. The staining was mostly in the nucleus, with very weak cytoplasmic fluorescence. To facilitate identification of nuclei, staining with DAPI for DNA was used. When cells were treated with indomethacin twice (resulting in a higher concentration of indomethacin and longer treatment time), the intensity of staining was even more enhanced, however, fewer cells were present on the slides, suggesting the possibility that cells were dying [Figure 8].

DISCUSSION

Cell culture technique

This study made use of the HEC 1B (Human Endometrial Cancer) cell line for the immunocytochemical detection of the PTEN protein. Various investigators have used different human cell lines in order to look at PTEN mRNA and protein expression, as well as the effects of certain drugs and other substances on the expression of PTEN.[8,11] These cell lines include colon cancer, gastric cancer, prostate cancer, breast cancer cell lines and melanoma and so we can use any of these to try and reproduce results produced by previous workers.[18-20] Normal human cells can also be cultured and used to study PTEN protein expressions, because these proteins are produced in most normal human tissues.[21]

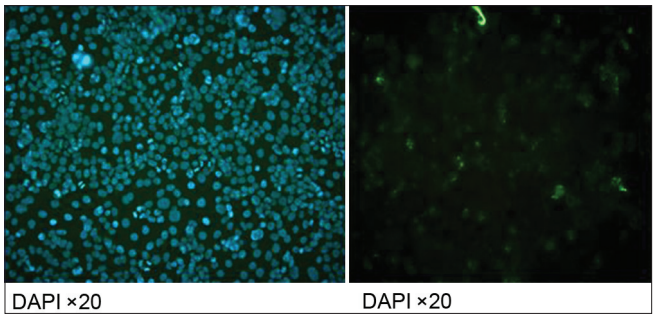

Figure 5: Pattern of DAPI and FITC staining in the control slide

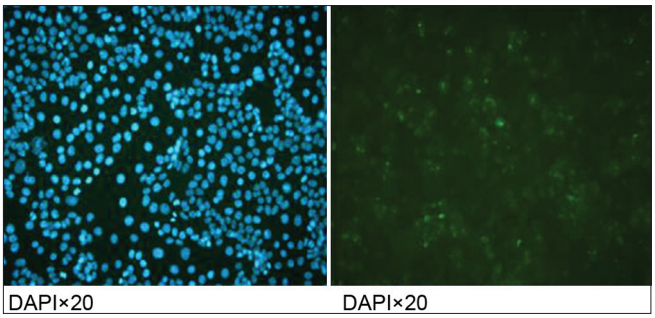

Figure 6: Pattern of DAPI and FITC staining following 1° and 2°antibody incubation

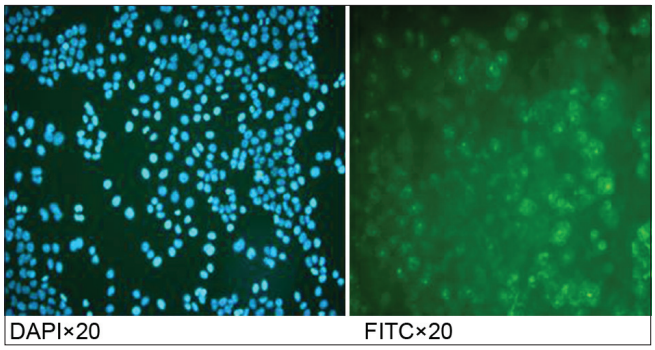

Figure 7: Pattern of DAPI and FITC staining of the slide treated with indomethacin for one hour

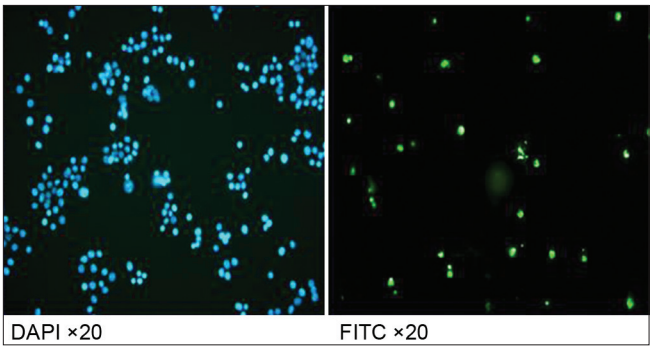

Figure 8: Pattern of DAPI and FITC staining of the cells treated twice with indomethacin stock solution

Using mammalian cell culture techniques in this work allowed the study of effects of the drug on cells without using human tissues and laboratory animals. Therefore, in this case ethical issues were not involved which could have slowed down the progress of the research while waiting for ethical approval.

Furthermore, cell cultures are important in comparative experiments for screening substances (often toxic) with similar sites and mechanisms of action, and can provide specific knowledge which can be missed in a complex animal. This is why it is possible to use cell lines for toxicology and expression studies using various drugs and chemical agents which can be harmful to the complete animal.[22] However, as cell cultures are merely collections of similar cells in a nutrient solution of culture medium, they only exhibit limited metabolism, and so quantitative data for the whole body are difficult to obtain from such studies, and it is impossible to determine the target organs of the drug being tested as well as the profile or timing of the harmful effect on not just the individual cells, but also the whole organism.[23] Also, it is difficult to simulate the effects resulting from the interactions of various organs or tissues in the whole organism using cell cultures. Lastly, physiological responses to drugs and chemicals are usually modified by age, sex and total genetic make-up of the human/laboratory animal, which cannot be seen in cell culture studies and so it may be worthwhile carrying out these experiments in whole laboratory animals to study the effects of the various drugs and substances on the expression of PTEN mRNA and protein.[24]

PTEN protein detection

It was observed from this study that the HEC 1B cells gave a positive signal for the detection of PTEN protein following incubation of the cells with the primary anti-PTEN antibody. This is in agreement with previous studies, which reported that the HEC 1B cells express abundant amounts of the wild-type PTEN protein.[25,26] The PTEN mRNA has been previously reported to be present in fairly large amounts in all normal tissues studied, such as heart, lung, liver, muscle, kidney and pancreas.[27] Stambolic *et al.,* (2001)[21] also observed that most mammalian cells with wild-type PTEN gene express significant levels of PTEN mRNA and protein under normal physiological conditions. Furthermore, several previous studies,[1] have documented that PTEN mutations lead to partial or complete inactivation of its phosphatase activity, in addition to total or partial loss of PTEN mRNA and/or protein expression.

However, Leslie and Downes (2002)[28] had pointed out that PTEN expression can be lost or remarkably decreased in a number of tumours without mutation of the coding sequence of the PTEN gene. This they attributed to mutations or methylations in the non-coding regions of PTEN required for expression, probably in the promoter regions of the gene. Such mutations have been identified in many human cancers and cancer syndromes, and

this supports the role of PTEN as a tumour suppressor in the pathogenesis of human cancers.[11] Reifenberger *et al.,* (2000)[29] reported that the PTEN protein is required for regulating cell-cycle progression, cell migration and spreading, and noted that these regulatory and anti-apoptotic functions of PTEN are intimately related to its role as a negative regulator of the protein kinase B (PKB/Akt) pathway. In support of this, Stambolic *et al.,* (2001)[21] observed that cells with PTEN mutation have activation of the PKB/Akt pathway and that this gives the cells resistance to a number of apoptotic stimuli such as ultraviolet light, osmotic shock and treatment by heat or TNFα.

This study was carried out using immunocytochemistry to detect the PTEN protein in the cultured cells. The technique has significant advantages which include high sensitivity and specificity, wide applicability as well as ease of correlation with the traditional physical parameters such as cell size, morphology, number and distribution. It has also been found to be compatible with various fixatives commonly used, and can be used along with electron microscopy.[16] However, immunocytochemistry is observer-dependent and need to be repeated several times for it to produce reliable and accurate results. In addition to that, it can produce false negative results due to the use of inappropriate, denatured or wrong concentration of the antibody, loss of antigen due to cell destruction and/or antigen diffusion, or the presence of very low level of antigen in the cell line or specimen.[17] False positive results can be produced due to antibody cross-reactivity with different antigens from the one being sought, non-specific binding of the antibody to the tissue in cells in question, release of soluble proteins from the normal cell cytoplasm, antigen sharing, as well as ectopic antigen expression by unrecognised cross reactions.[2,30]

Therefore, in order to circumvent these problems and improve the reliability of PTEN antigen detection, Western blotting can be employed. This is a technique which detects specific proteins in a protein mixture using antibodies. The proteins are initially separated using sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE), and subsequently transferred to a nitrocellulose membrane and allowed to react.[31] The secondary horse radish peroxidase-labelled anti-immunoglobulin is then allowed to react with the primary antibody, and this can be detected with a coloured substance such as diaminobenzidine. Bands will be observed on the membrane and represent the position of the antigen.[31]

In addition to that, polymerase chain reaction (PCR) can be used to determine the DNA present in the PTEN-bearing cell lines. Real-time PCR (RT-PCR) allows the determination of the number of new DNA molecules formed in the reaction after each cycle, and enables continuous observation of the reaction in real time.[32] This allows the determination of the quantity of template used at the beginning of the reaction specifically and sensitively. This is very useful as it allows for accurate determination and monitoring of the quantity of DNA produced per unit time. In addition to that, RT-PCR is very quick, reproducible, has a dynamic range and allows quantification of gene expression and so can be used to study PTEN gene expression and/or mutations.[33]

PTEN expression after indomethacin treatment

Results from this study suggest that there was up-regulation of the PTEN protein following treatment of the cells with indomethacin solution overnight and for 1 hour. This was demonstrable by the increased green fluorescence in the treated cells following staining with the primary anti-PTEN antibody, compared to the untreated cells which served as control. This is in agreement with the work of Chu *et al.,* (2004)[11] which demonstrated increased expression of the PTEN protein following treatment of human colon cancer cell lines with a 100 mM solution of indomethacin for 1 and 5 hours, at 37°C. However, this work differs slightly because a different cell line (human endometrial cell line) was used to see if their findings apply to other cell lines. In addition to that, in this study, cells were also treated twice (overnight and for one hour in the morning) with the drug, to look at its effects on cell viability and PTEN expression in relation to exposure and drug concentration.

It was observed that although the intensity of the green fluorescence increased (suggesting increased PTEN expression) following the double treatment, there were fewer cells in number and density and they were more scattered. This was thought to be as a result of cell death caused by the prolonged exposure to the drug as well as drug concentration which could not be tolerated by some of the cells. PTEN protein expression has also been reported (Leslie and Downes, 2002)[28] to be significantly up-regulated by agonists of the peroxisome proliferator-activated receptor-gamma (PPAR-gamma), which regulates PTEN gene transcription and results in increased mRNA and PTEN protein, PtdIns (3, 4, 5) P_3 3-phosphatase activity as well as decreased activity of PKB. They suggested that this may account for the anti-inflammatory and anti-cancer properties of the PPAR-gamma agonists, which are promising agents in the possible treatment of cancer.

Waite *et al.,* (2005)[18] looked at the effects of dietary phytoestrogens on the expression of PTEN in breast cancer cell lines. They investigated the effects of three dietary phytoestrogens: Genistein from soybeans, resveratrol from grapes and quercetin found in many fruits and vegetables, and observed that these substances increased the mRNA level as well as PTEN protein expression, resulting in decreased levels of phosphorylated Akt (PAkt). This they concluded was responsible for the cancer protective effects of these dietary substances. Previous studies have established that when there is PTEN mutation or deletion,

there is resultant activation of the PI3/Akt kinase cascade, and Priulla *et al.,* (2007)[4] observed that this can lead to drug resistance in prostate cancer, and so they looked at the effect of chemosensitisation and how this influences PTEN's role on the Akt pathway. They reported that there was a significant increase in the anti-tumour effects of taxol as a result of silencing the Akt pathway by this drug in a human prostate cancer cell line expressing normal PTEN.

Sub-cellular localisation of PTEN

This study revealed that most of the staining for the PTEN protein was in the nucleus, with less in the cytoplasm. This suggests predominant nuclear localisation of the protein. Gericke *et al.,* (2006)[34] observed that PTEN usually has a scanty cytoplasmic distribution with a heavy presence in the nucleus. They (Gericke *et al.,* 2006),[34] also noted that the nuclear PTEN diffused quickly and was not held down by any cellular components, whereas cytoplasmic PTEN diffused more slowly because it is transiently bound to immobile cytoplasmic structures. These observations are important because they may influence how fast PTEN can diffuse to reach its substrates on the plasma membrane within a short time, in response to stimuli. However, Leslie and Downes (2002)[28] had earlier reported that in most cell types analysed, PTEN was predominantly found in the cytoplasm, but the general agreement is that it is both cytoplasmic and nuclear.[35,36]

CONCLUSION

Our results suggest an increased expression of PTEN antigen and protein following induction of the cultured cells in different concentrations of indomethacin, in line with previous literature using similar NSAIDs like aspirin. This is promising, and may be translated in future towards developing novel therapies or ways of preventing human cancers, especially in high risk individuals. PTEN may also be used in the future as a diagnostic and prognostic tool to follow disease progression and review the outcome of treatment. However, future studies using Western blotting and PCR are required to substantiate these results.

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