

Aquaporin 5 Expression and Its Relationship to Apoptosis in Different Grades of Differentiated Non-Small Cell Lung Carcinoma

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Abstract

Aquaporin 5 has been recently found as an important oncogenic marker whose expression levels seem to be determined by the level of cellular differentiation. Despite aquaporin volume decrease (AVD) being the most conserved earliest event in apoptosis, there is still a paucity of studies exploring on aquaporin expression and its relationship with apoptosis in cancer. The aim of this study was to investigate the expression of aquaporin 5 channel protein and to explore on its relationship with apoptosis in well and poorly differentiated non-small cell lung carcinoma both *in-vivo* and *in-vitro*. Findings from the study showed that the expression of AQP5 both *in-vivo* and *in-vitro* was dependent on the type and degree of tumour differentiation. *In-vivo*, an increase in aquaporin 5 expression was associated with an increased apoptosis in both poorly and highly differentiated adenocarcinoma (AC) while there was no association between aquaporin 5 expression and apoptosis in both poorly and highly differentiated squamous cell carcinoma (SCC). *In vitro*, differentiation therapy in the form of ATRA decreased both cell proliferation and increased the expression of AQP5 in A549 cells. The cytomorphological changes, expression of differentiation markers and flow cytometry apoptotic results were dependent on the dose of ATRA treatment. In conclusion, a higher expression of aquaporin 5 was found to promote the rate of the apoptotic process in lung adenocarcinoma (AC).

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Keywords

Aquaporin 5, Non-Small Cell Carcinoma, Apoptosis, Apoptotic Index

1. Introduction

Aquaporins are integral membrane channel proteins that regulate water and small solute movement across membranes in both physiological and pathological conditions [1]-[3]. In cancer, increased aquaporin expression levels have been reported in aggressive tumours of various origins and a link between aquaporin expression and the level of histological tumour grade of cancer has been described [4]-[8]. The increased aquaporin expression in cancer cells has been implicated to play a major role in the rapid penetration of water into the growing tumour to meet the high energy demands of rapid replication and multiplication [9]. Studies where the over-expression of AQP5 was associated with an increased rate of cell migration, proliferation and invasiveness of cancer cells include those of the cancers of the colon [7], ovary [10], pancreas [11], stomach [12], bone [13] and the lungs [14].

Apoptosis is a programmed cell death process characterized by distinct morphological features and biochemical processes that was first described by Kerr *et al.*, in 1972 [15]. In cancer, apoptosis remains a complex biochemical process in which the balance between cellular proliferation and cell death has been shown to play a critical role in tumour progression [16]. Of note is that the earliest morphological event of apoptosis is water loss accompanied by subsequent volume decrease which has been reported to be aquaporin mediated in lymphocytes and ovarian cells [17] [18]. However, other studies have suggested that cell volume water losses alone are not always sufficient for the triggering and progression of cell death [19] [20]. With regards to AQP5 expression in apoptosis, current research has demonstrated that AQP5 increased expression inhibited apoptosis in chronic myelogenous leukemia while it enhanced apoptosis in differentiating bone marrow derived mesenchymal cells [13] [21]. Unfortunately, no similar study has been done for the lung cancer despite it being the leading cause of cancer related deaths in the China [22]. Therefore the aim of this study was to investigate the expression of AQP5 in cancer cells and to find its relationship to apoptosis in various grades of differentiated non-small cell lung cancer.

2. Materials and Methods

2.1. Tissue Study

The tissue specimens study included 40 specimens of non-small cell lung cancer (NSCLC) subtypes that were confirmed by histopathological analysis after surgical resection at the Jilin Province Tumor Hospital, from October 2014 to January 2015. The study protocol was approved by the Institutional Review Board of Jilin University and all participants signed an informed consent form. The patients' ages ranged from 44 to 84 years, and no radiotherapy or chemotherapy treatment had been given to these subjects. Paraffin-embedded blocks of surgically resected and adjacent non-tumor lung cancer tissues were prepared.

2.2. Tissue Immunohistochemistry and Cell Diameter Measurement

Formalin-fixed tissues were embedded in paraffin, sectioned to 5 μm , and mounted on glass slides. The sections were dewaxed and rehydrated through descending grades of alcohol to distilled water followed by heat induced antigen retrieval in 0.01 M citrate buffer. The sections were blocked with endogenous peroxidase using 3% (v/v) hydrogen peroxidase in phosphate-buffered saline (PBS). They were washed in 0.01 M PBS and blocked with sheep serum (Beijing Zhongshan Biotechnology) for 2 hours, then incubated overnight at 4 degrees celsius with a monoclonal AQP5 antibody (diluted 1:200, from Beijing Biosynthesis Biotechnology Company). Following subsequent washes in PBS, the sections were incubated with a secondary antibody (Beijing Zhongshan Biotechnology) for 20 minutes at room temperature and later the peroxidase antiperoxidase complex. Immunoreactivity was demonstrated using diaminobenzadine (Fuzhou New Biotechnology Development Company) which produced a brown insoluble precipitate at immunopositive sites. The sections were counterstained with hematoxylin and mounted on a cover glass. The negative controls were incubated with 0.01 M PBS without the primary antibody. After observation under the microscope, pictures were taken using a Nikon camera (Japan, 16 megapixels) at 200 \times magnification. The cell diameter was measured under a calibrated protocol while the integral optical density (IOD) to determine AQP5 expression was measured using Image-Pro-Plus Version 6.0.

2.3. The Tissue TUNEL Staining Method

Tissue apoptosis was detected by the terminal deoxynucleotidyl transferase-mediated deoxyuridine triphosphate-biotin nick end-labeling (TUNEL) method, which detects not only cells with typical morphologic features of apoptosis but also cells in an earlier stage of apoptosis showing no apparent morphological change. After TUNEL staining procedure, counterstaining was performed with hematoxylin. For negative controls, the terminal deoxynucleotidyl transferase was omitted. Ten fields of 100 cells each were randomly chosen from different parts of the tumor sections. The numbers of apoptotic cells and the number of malignant cells in each field were recorded and expressed as the apoptotic index (number of apoptotic cells per 1000 malignant cells).

2.4. Cell Culture Study

The human tumorigenic lung epithelial cell line A549 was obtained from American Type Culture Collection (USA). The cells were maintained in high glucose DMEM supplemented with 10% fetal bovine serum and 1% of penicillin-streptomycin at 37°C in a 5% CO₂ incubator. On reaching 75% confluency, the A549 cells were washed with 0.01M PBS and then removed from the flask by the addition of 0.25% trypsin.

2.5. ATRA Induction of Differentiation and the MTT Proliferation Study

The MTT assay (3-[4,5-dimethylthiazol-2-yl]-2, 5-diphenyltetrazolium bromide) was used to determine cell growth rates. Cells were grown in 96-well plates in 10% FBS-containing medium at an initial density of 2×10^3 cells per well. After 24 hrs, the wells were treated with various concentrations of all trans-retinoic acid 1, 2.5, 5.0, 7.5, 10, 12.5, 15, and 20 µg/ml. After 24, 48 and 72 hrs, 20 µl of MTT solution was added, and plates were incubated at 37°C for 4 hrs. Media was then aspirated, and the formazan formed was solubilized with 150 µl DMSO. Cell viabilities were measured as individual well absorbances at 570 nm using a microplate reader. The cell viability absorbance was based on the absorbance of untreated cells which was considered as 100%. The results were determined by three independent experiments.

2.6. Measurement of A549 Reversal of Phenotype and Differentiation

The morphological changes of A549 cells after 72 hrs ATRA treatments were examined and pictures taken. The aspect ratios of the cells (ratio of width to length) and area were calculated using the Image-Pro-Plus Version 6.0 Program that included 50 cells in each picture of **Figure 3**. To determine whether differentiation and reversal of malignant phenotype of A549 cells the expression of MUC5AC (and other associated mucins) and CEA in A549 cells was examined by Alcian Blue/Periodic acid Schiff staining and immunohistochemical assay respectively.

2.7. A549 Cell Immunocytochemistry and Immunofluorescence.

The protein expression of CEA in A549 cells was examined by immunohistochemistry using the anti-CEA mAb. A549 cells were seeded on sterile glass cover slides for 24 hours and then treated with ATRA at 5, 10 and 15 µg/ml for 72 hrs. Following treatment, the cells were stained using the Ultra Sensitive™ Immunohistochemical Staining Kit (Maixin Bio, Fuzhou, P.R. China) according to the manufacturer's instructions. Immunolocalization was done using DAB as a chromogen. Slides were counterstained with hematoxylin before dehydration and mounting. The intensity of the immunoreactivity was analyzed using the Image-Pro Plus (Media Cybernetics) Version 6 software. For AQP5 immunofluorescence assay, the cultured cells, after treatment with retinoic acid of varying concentrations were washed three times with PBS and fixed in 4% paraformaldehyde (PFA) for 15 mins. The fixed cells were covered with 0.01 M PBS, treated with 10% donkey serum to block any non-specific binding, and the slides labeled with the rabbit AQP5mAb (1:200). After overnight incubation at 4°C, the cells were washed in PBS and then incubated with anti-rabbit Alexa 488 as the secondary antibody. An hour later, the slides were washed in PBS at 37°C and stained with Hoechst 33342 for 15 minutes. After washing in PBS, slides were mounted in 40% glycerol and finally examined under a fluorescence microscope.

2.8. Annexin V/PI Staining and Flow Cytometry

A549 cells were incubated with varying concentrations of all trans-retinoic acid (0, 5, 10 and 15 µg/ml) for 72 h. The cells ($>1 \times 10^6$) were harvested from each group for the Annexin V-fluorescein isothiocyanate (FITC) and

PI double-staining apoptosis assay. Following centrifugation at 1000 rpm for 3 minutes of the harvested cells, the pellets were re-suspended in 100 μ l 1X binding buffer with 5 μ l Annexin V and 5 μ l PI. After incubation for 15 minutes in the dark, the samples were taken for flow cytometry. Flow cytometry data analysis was done using the Lysis software. In total, $\geq 10,000$ events were analyzed for each sample.

2.9. Statistical Analysis

SPSS version 19 software (Chicago, IL) was used for data analysis. Descriptive statistics were used to describe data. Continuous data were compared using Student's t test, if the distribution of samples followed a normal distribution, using the 0.05% level of significance. For correlation analysis the Spearman's rank correlation value was used ($p < 0.01$ was regarded as significant).

3. Results

3.1. Diameter of Cancer Cells and Mean Optical Density in Lung Squamous Cell Carcinoma (SCC) and Adenocarcinoma (AC)

In squamous cell lung carcinoma the mean optical density of the well-differentiated squamous cell carcinoma (0.283 ± 0.02), was significantly higher than that in poorly differentiated squamous cell carcinoma group (0.219 ± 0.05) ($p < 0.05$), while the diameter of well-differentiated cancer cells (27.02 ± 0.83) was significantly higher than that of poorly differentiated squamous cell carcinoma (17.85 ± 0.82) ($p < 0.05$). For lung adenocarcinoma the diameter of well-differentiated adenocarcinoma (13.60 ± 0.82) was significantly lower than that of poorly differentiated lung adenocarcinoma cells (28.01 ± 1.01) ($p < 0.05$), while the mean optical density of the well-differentiated group (0.281 ± 0.03) was significantly higher than that in poorly differentiated lung adenocarcinoma group (0.238 ± 0.02) ($p < 0.05$) (**Figure 1** & **Table 1**).

3.2. Apoptotic Indices (per 1000 Cells) for Lung Squamous Cell Carcinoma (SCC) and Adenocarcinoma (AC)

The values for apoptotic index for squamous cell carcinoma ranged from 14 to 23 apoptotic cells per 1000 cells (**Figure 2**). The apoptosis index (per 1000 cells) of well differentiated lung squamous cell carcinoma group (19.93 ± 2.37) was significantly higher than that in poorly differentiated lung squamous cell carcinoma group (17.27 ± 1.80) ($p < 0.05$). The apoptosis index (per 1000 cells) of well-differentiated adenocarcinoma group (20.20 ± 2.40) was significantly higher than that in poorly differentiated adenocarcinoma group (18.53 ± 1.73) ($p < 0.05$).

3.3. Correlations between Diameters, Mean Optical Density and Apoptotic Indices

Table 2 shows the correlation coefficients (Spearman's Rank correlation values) between MOD and AI. For correlation analysis, no significant correlation was found in both high and poorly differentiated squamous cell groups between mean optical density and the apoptosis index ($p > 0.05$). In both high and poorly differentiated adenocarcinoma tissues, significant correlation was found between MOD and AI as shown in **Table 2**.

3.4. ATRA Induction of Differentiation and the MTT Proliferation Study

Figure 3 shows a dose and time dependent effect on cell viability and proliferation of A549 cells following ATRA treatment. ATRA treatment of 2.5 μ g/ml concentration was ineffective for growth inhibition after 24, 48 and 72 hours of treatment. Significant decrease in cell proliferation started from 5 μ g/ml ATRA treatment in all the 3 groups. Cell viability was decreased to less than 50% after 20 μ g/ml ATRA treatment for 48 hours and 12.5 μ g/ml ATRA for 72 hours. The IC₅₀ values for ATRA for 48, 72 and 96 hrs were 20.96, 16.81 and 11.37 μ g/ml respectively.

3.5. A549 Cytomorphological Changes after ATRA Treatment for 72 Hours

Quantitative analysis of the cells in **Figure 4** after treatment of various concentrations of ATRA for 72 hours was done and the results are shown in **Table 3**.

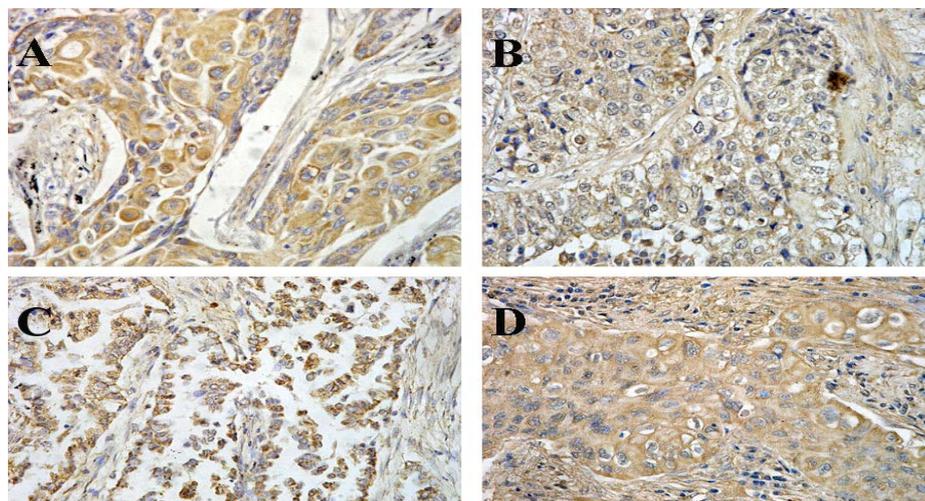


Figure 1. AQP5 tissue immunohistochemistry brown staining of lung squamous cell carcinoma and adenocarcinoma (AC). A: high differentiated squamous cell carcinoma. B: low differentiated squamous cell carcinoma. C: high differentiated adenocarcinoma D: low differentiated adenocarcinoma. $\times 200$ magnification.

Table 1. Diameters, IOD and mean optical densities for AQP5 expression in lung squamous cell carcinoma (SCC) and adenocarcinoma (AC).

Type	Diameter	Integrated Optical Density	Mean Optical Density
High differentiated SCC	27.02 ± 0.83	397,546	0.283 ± 0.02
Low differentiated SCC	$17.85 \pm 0.82^*$	381,515	$0.219 \pm 0.05^*$
High differentiated AC	$13.60 \pm 0.82^*$	337,903	$0.281 \pm 0.03^*$
Low differentiated AC	$28.01 \pm 1.01^*$	590,950	$0.238 \pm 0.02^*$

*Significant vs untreated control ($p < 0.05$).

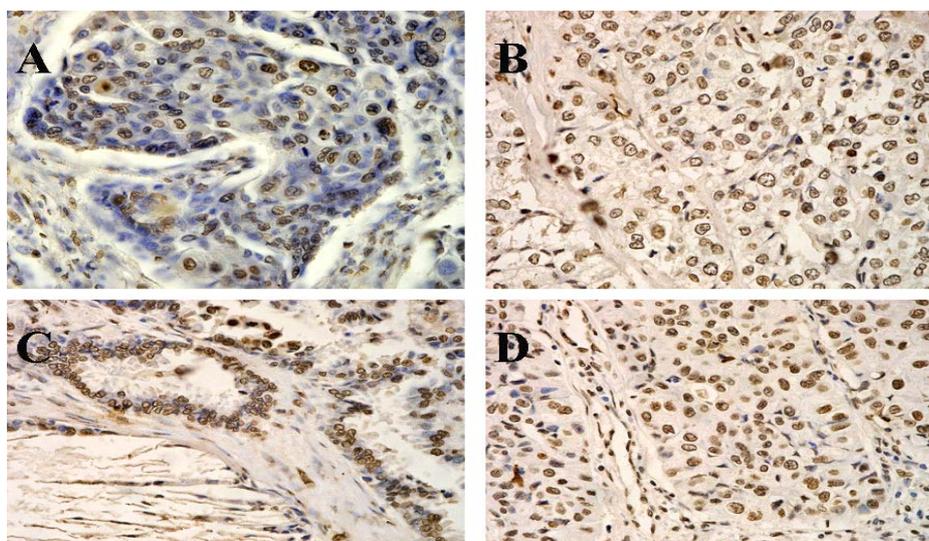


Figure 2. Tissue apoptosis TUNEL staining of lung squamous cell carcinoma and adenocarcinoma (AC). A: high differentiated squamous cell carcinoma, B: low differentiated squamous cell carcinoma, C: high differentiated adenocarcinoma, D: low differentiated adenocarcinoma. $\times 200$ magnification.

Table 2. Correlation results for mean optical density (MOD) vs. apoptotic index (AI), (per 1000 cells) in lung squamous cell carcinoma (SCC) and adenocarcinoma (AC).

Type	Spearman's Rank Correlation (r-value)	p-value
High differentiated SCC	-0.069	0.807
Low differentiated SCC	-0.299	0.279
High differentiated AC	0.524	0.045*
Low differentiated AC	0.661	0.007**

p-value: *Significant at 0.05 two tailed test; p-value: **Significant at 0.01 two tailed test.

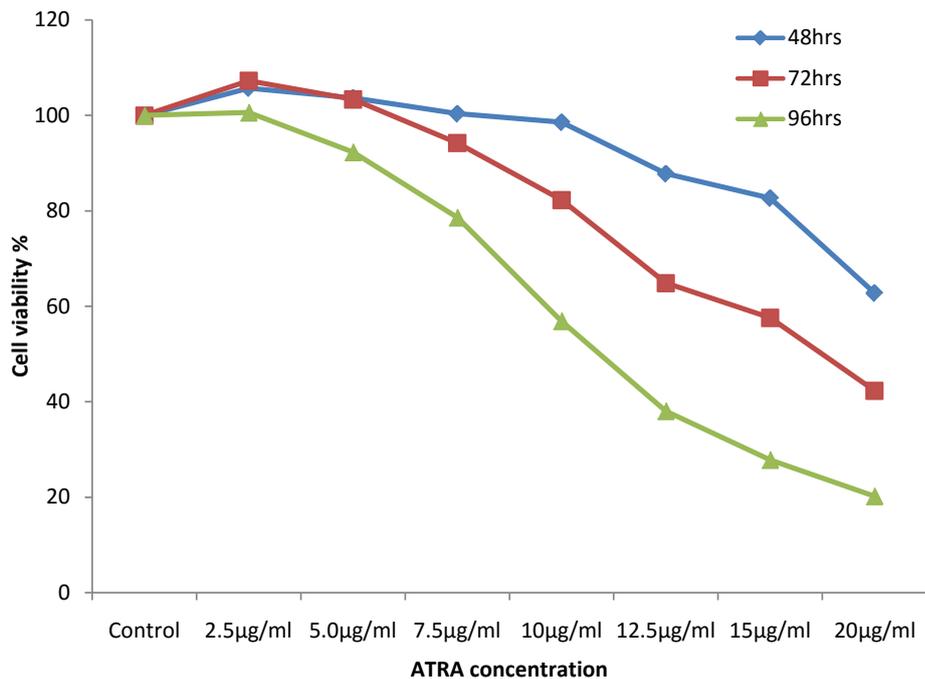


Figure 3. Cell viability following MTT cell proliferation study for A549 cells after ATRA treatment, 48 hrs (blue), 72 hrs (red) and 96 hrs (green).

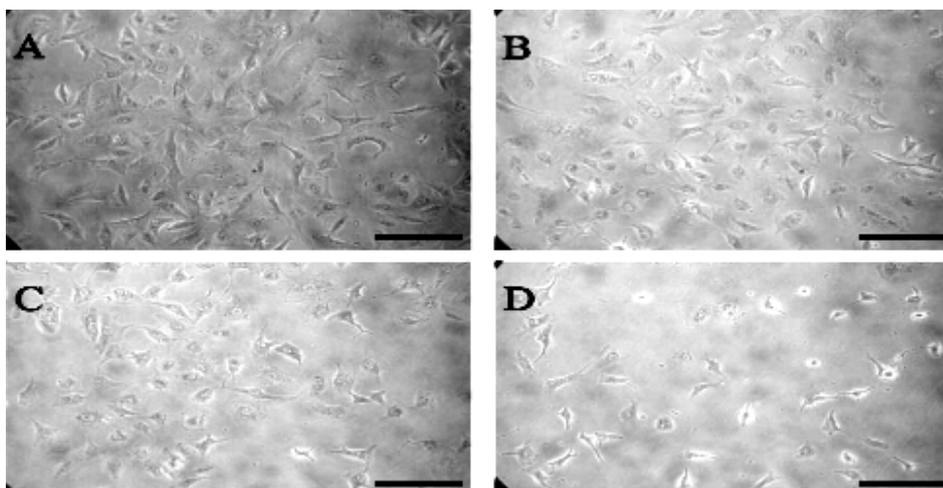


Figure 4. Morphological changes of A549 cells following ATRA treatment for 72 hours. A, no ATRA treatment; B, 5 µg/ml ATRA; C, 10 µg/ml; D, 15 µg/ml ATRA treatment. ×200 magnification.

Table 3. Quantitative analysis of the cell morphological characteristics after ATRA treatment.

Concentration of ATRA	Cell Aspect Ratio	Area (μm^2)
Control	1.62	2266.4 \pm 24
5 $\mu\text{g/ml}$	1.57	1672.5 \pm 32*
10 $\mu\text{g/ml}$	1.59	1097.5 \pm 54*
15 $\mu\text{g/ml}$	1.32	816.90 \pm 27*

*Significant vs untreated control ($p < 0.05$).

3.6. A549 Reversal of Phenotype and Differentiation

Mucins (MUC5AC and associated glycoconjugates) and CEA (carcinoembryonic antigen) were taken as markers for measuring the differentiation process of A549 cell using retinoic acid. As shown in **Figure 5**, the blue colour staining is for acidic mucins, magenta for neutral staining and a mixture of both colours is purple. The results show differences in staining for the mucins. Intense staining for acidic mucins is shown in the non-treated group, followed by the 5 $\mu\text{g/ml}$ group while there is faint staining for both acidic and neutral mucins in the 15 $\mu\text{g/ml}$ ATRA treated group. **Figure 6** shows that CEACAM 5 (commonly known as CEA) expression was higher in the untreated group than the treated groups. It can be noted that not all cells secrete CEA, as shown by the brown staining of DAB. The more the confluent the cells are, the more the expression of the carcinoembryonic antigen.

3.7. A549 Cell Immunohistochemistry and Immunofluorescence

Figure 7 shows the immunofluorescence expression of aquaporin 5 in A549 cells following ATRA treatment. The images were quantified using Image Pro Plus Version 6 Software and the Mean Optical Densities (IOD) values (per cell) were recorded (**Table 4**). The untreated cells had the lowest expression of aquaporin 5 per cell and the expression of aquaporin 5 for the treatment groups was dependent on the dose of ATRA.

3.8. Annexin V/PI Staining and Flow Cytometry

As shown in **Figure 8**, the control (no treatment) group had the highest number of dead cells (2.93%), while the highest concentration of ATRA, 15 $\mu\text{g/ml}$ group had the highest number of cells in early and late apoptosis (7.15%). The 5 $\mu\text{g/ml}$ ATRA concentration had the highest percentage of cells in late apoptosis. The mean optical densities for aquaporin 5 and the apoptotic indices of A549 cells after ATRA treatment are shown in **Table 4**.

A significant positive correlation was found between AQP5 expression (MOD) and the percentage of cells in early and late apoptosis ($r = 0.970$; $p = 0.0304$) after all trans-retinoic acid (ATRA) treatment of A549 lung adenocarcinoma cells for 72 hours (**Table 4**). A significant positive correlation was found between AQP5 expression (MOD) and the percentage of cells in early and late apoptosis ($r = 0.970$; $p = 0.0304$) after all trans-retinoic acid (ATRA) treatment of A549 lung adenocarcinoma cells for 72 hours (**Table 4**).

4. Discussion

The processes of cellular proliferation and apoptosis have each been shown to play important roles in tumor development but their contributions to tumor progression are still yet to be defined [16]. A detailed examination of surgically resected tissues in this study showed that in squamous cell carcinoma (SCC), the histological degree of differentiation of cancer cells determines the difference in the diameters of the cells and the expression of aquaporin 5. In these cells, well differentiated cells had higher diameters, higher expression of AQP5, and had a higher apoptotic index compared to poorly differentiated SCC. The finding that AQP5 expression is coupled with a lower apoptotic index (AI) in poorly differentiated SCC might explain its aggressiveness and ability to metastasize more than the well differentiated type. In adenocarcinoma (AC), although the diameter of the well differentiated AC was lower than that of low differentiated AC, the relative expression of AQP5 (measured per cell as MOD) and the apoptotic index were significantly higher than those for low differentiated AC. This finding agrees well with the literature which reported an over-expression pattern ratio to be high in well differentiated compared to poorly differentiated AC [14]. A higher expression of AQP5 in poorly differentiated AC than poorly differentiated SCC might also be related to the involvement of the AQP5 channel protein in water permeability of AC cells which are specialized for mucus secretion.

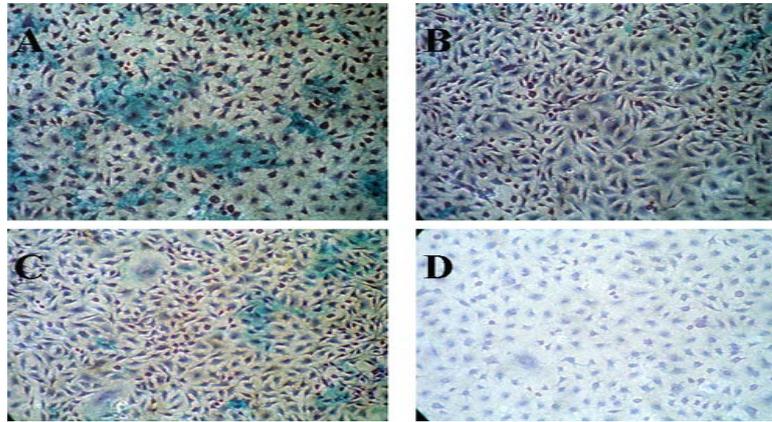


Figure 5. Measurement of A549 reversal of phenotype and differentiation. A549 cells Alcian Blue-Periodic Acid Schiff staining for mucins and other glycoconjugates after 72 hrs ATRA treatment. A, no ATRA treatment; B, 5 µg/ml ATRA; C, 10 µg/ml; D, 15 µg/ml ATRA treatment. ×200 magnification.

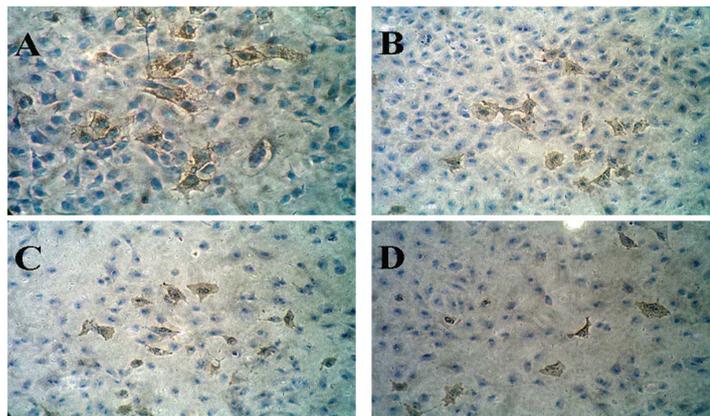


Figure 6. Measurement of A549 reversal of phenotype and differentiation. CEA immunohistochemistry staining after 72 hours ATRA treatment of A549 cells. A, no ATRA treatment; B, 5 µg/ml ATRA; C, 10 µg/ml; D, 15 µg/ml ATRA treatment. ×200 magnification.

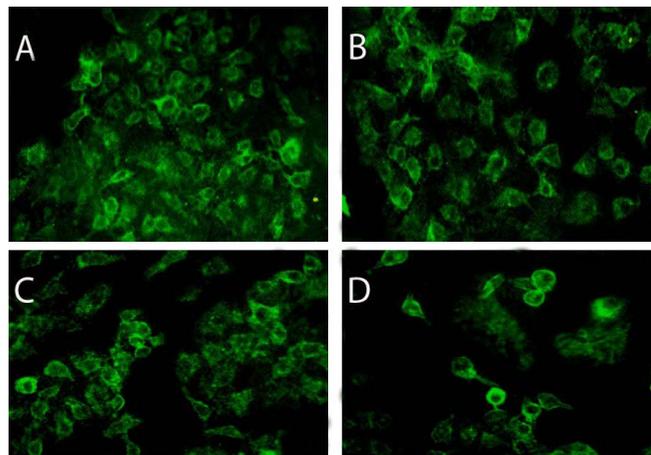
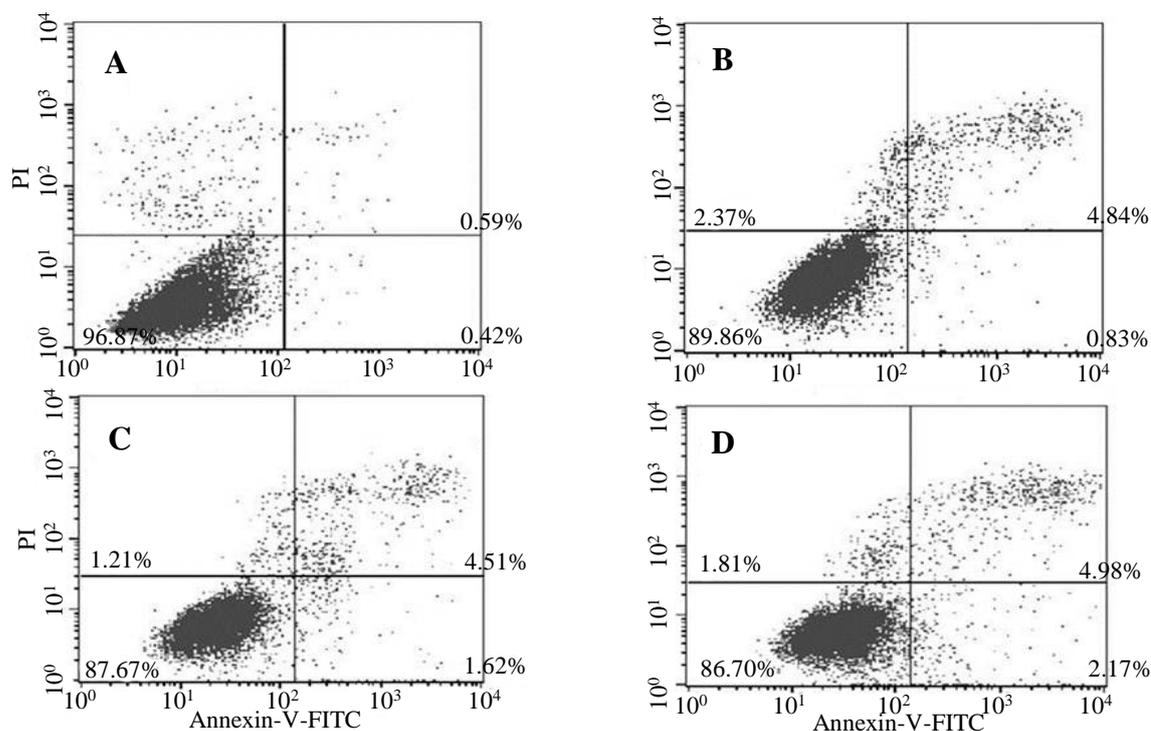


Figure 7. AQP5 immunofluorescence staining after 72 hours of ATRA treatment. A, no ATRA treatment; B, 5 µg/ml ATRA; C, 10 µg/ml; D, 15 µg/ml ATRA treatment. ×200 magnification.

Table 4. Mean optical density for aquaporin 5 and apoptotic index for A549 cells after 72 hours of ATRA treatment.

ATRA concentration	AQP5 Mean Optical Density	Flow cytometry Apoptotic index
Control	1.2851 ± 0.14	1.01%
5 µg/ml	1.3149 ± 0.15*	5.67%
10 µg/ml	1.3162 ± 0.10*	6.13%
15 µg/ml	1.3351 ± 0.12*	7.15%

*Significant vs untreated control ($p < 0.05$).**Figure 8.** Annexin-V-FITC-PI flow cytometry results for A549 cells following ATRA treatment for 72 hours. A, no ATRA treatment; B, 5 µg/ml ATRA; C, 10 µg/ml; D, 15 µg/ml ATRA treatment.

The clinical significance and mechanism of apoptosis in NSCLC has not been fully established, but various authors have reported the apoptotic rate to be an independent prognostic factor in NSCLC [23] [24]. In this study the AI of AC cells (19.36 ± 2.22) was higher than that of SCC (18.60 ± 2.47), with a similar pattern following for well differentiated compared to poorly differentiated in both subtypes (SCC and AC) of lung cancer. These results are comparable to the AI per 1000 cells reported previously which found AI in AC (19 ± 1.7), to be slightly higher than that of squamous cell carcinoma (18.3 ± 2.4). While other researchers, cautions on the interpretation of the data obtained on apoptosis between different studies [25], data from the present study sought to clearly demonstrate whether AQP5 expression, viewed in relation to apoptosis, had any effect on the apoptosis rate of cancer cells in NSCLC. A statistically significant correlation between MOD and AI was found ($p < 0.05$), for low differentiated AC ($p < 0.01$) and high differentiated AC ($p < 0.05$), while no significant relationship was found between the expression of AQP5 and the apoptotic index ($p > 0.05$) in SCC. From this finding, it can be shown that while AQP5 might be involved in development and progression of lung cancer, it does not have a direct influence on the apoptosis in all the lung cancer subtypes. It therefore implies that, apoptosis still remains a highly complex process with different morphological and biochemical features that are controlled by many different pathways in the different lung cancer subtypes.

Retinoic acid has been found to be an effective differentiation agent and inhibitor of squamous metaplasia in many studies [26]-[31]. Treatment of A549 cells in this study, with various concentrations of ATRA for 24, 48

and 72 hours showed a dose and time dependent effect on cell proliferation (**Figure 3**). Although the concentration values of ATRA used in this study seem higher than the previously reported values, these results almost concur with the 10 μ M (3 μ g/ml) concentration of ATRA which has been found to induce differentiation in H226 and A549 cells after 3 days [32]. The directed differentiation of A549 cells through ATRA treatment for 72 hours was carried out *in-vitro* using mucins and CEA as differentiation markers. The treated cells showed a dose dependent expression of these markers as shown in **Figure 5** & **Figure 6**. While CEA can be measured from the supernatant of A549 cells, attempts to measure it using electrochemiluminescence method, proved futile in this study. The reasons are still unknown, but it may be explained by the fact that A549 cells contain both tumorigenic (A549-T) and non-tumorigenic cells (A549-NT), which differ in the expression for malignancy markers hence the need to separate the different subpopulations by gradient centrifugation so as to measure the differentiation markers accurately [33].

Results from this experiment show that ATRA induced differentiation of A549 lung adenocarcinoma cells increases the expression of AQP5 per A549 cell (**Figure 7**). This demonstrates that AQP5 is a putative protein that is involved lung cancer cellular growth and transformation thereby confirming many previous reports in which this protein has been implicated in promotion of tumorigenesis [7] [10] [14] [32] [34]. AQP5 over-expression was also accompanied by some phenotypic changes of lung adenocarcinoma cellular transformation which agrees well with observations made by others researchers, which implicated ectopic AQP5 expression to play a major involvement in human lung cancer growth and metastasis [11] [32]. In this study, ATRA induced differentiation and apoptosis (of 7.15% after 15 μ g/ml ATRA treatment for 72 hours), which can point to the possibility that it might be adequate for therapy if it can be used in combination with other therapeutic agents.

In summary, the cell culture part of this study, showed an increase in the expression of AQP5 per cell (mean optical density) (**Figure 7**) with increasing dose of ATRA treatment in A549 cells which was significantly correlated to the increase in the apoptosis of A549 cells ($r = 0.970$; $p = 0.0304$) (**Figure 8** & **Table 4**). These results concur with the findings in the AC tissue part of this study which also found a statistically significant correlation between mean optical density for both high ($p < 0.01$) and poorly differentiated AC ($p < 0.05$). Based on these findings it can therefore be seen that, a higher expression of aquaporin 5 both *in-vivo* and *in-vitro* promotes the rate of the apoptosis process in lung AC which is similar to a study where over-expression of AQP-1 in Chinese hamster ovary cells was found to enhance the rate of apoptosis [35]. From this line of thinking, it may be suggested that apoptosis was a result of ATRA induced A549 growth inhibition. This agrees well with studies of apoptosis where a decrease in cell volume (AVD) of whatever cause was found to create an intracellular environment conducive to the activation of the caspase death machinery [17] [18] [20] [35]. Therefore, this means that development of effective differentiation modulators for AQP5 might go a long way in opening a less toxic therapeutic intervention for lung cancer.

In conclusion, AQP5 expression either *in-vivo* or *in-vitro* is dependent on the level of cellular differentiation. In both SCC and AC tissues, the well differentiated the cells are so is the higher expression of AQP5 and the apoptotic index. *In-vivo*, the apoptotic index of AC is higher than that of SCC, while the expression of AQP5 has a more significant effect on the increase of apoptosis in low differentiated than well differentiated AC subtypes. The more the expression of AQP5, the higher is the apoptotic rate. However, no association exists between the expression of AQP5 and apoptotic index in both high and poorly differentiated SCC, an indication that in the body, the apoptotic process, although highly conserved, its progress varies in tissues of different differentiation states. As a differentiation agent, all-trans retinoic acid (ATRA) decreased cell proliferation but increased the expression of AQP5 per cell (calculated as MOD) in A549 cells. The increase in AQP5 per cell which was associated with an increase in apoptosis was significant in A549 cells, a result similar to the tissue study. This means a higher expression of aquaporin 5 both *in-vivo* and *in-vitro* promotes the rate of apoptotic process in lung AC. Since the study did not explore in depth the underlying molecular mechanism between AQP5, ATRA and the apoptotic process, a recommendation for future studies is made to better understand this area.

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Conflict of Interest

The authors declare none.

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