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Quantitative Estimation of IL 8 Gene Expression in NSCLC Cell Lines by Real Time PCR and ELISA

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Abstract: **Interleukin-8 (IL-8) is a cytokine acting as an angiogenic growth factor that is over expressed in many cancers, along with Non Small Cell Lung Cancer (NSCLC). It is also recognized as an autocrine growth factor in various human cancers. In the present study, we analyzed the expression of IL-8 in H 23 and H 522 NSCLC cell lines. The quantification of IL 8 mRNA and protein expression in two human lung cancer cell lines were studied by using Real Time Polymerase Chain Reaction (RT-PCR) and Enzyme Linked Immuno Sorbent Assay (ELISA). IL-8 mRNA band was observed at 291bp and band at 593bp was observed for control glyceraldehyde-3-phosphatedehydrogenase gene. The levels of IL 8 protein in conditioned media were measured. H23 cells produced 3.97±1.2ng/ml Per10^{6}cells, 21.62±1.8ng/ml Per10^6 cells and 56.82 ± 0.9 ng/ml Per10^{6}cells, H522 cells produced 1.97±2.2ng/ml Per10^{6}cells, 12.54±2.8ng/ml Per10^{6}cells and 27.54±1.5 ng/ml Per10^{6}cells simultaneously after 24h, 48h and 72h. This represents that H 23 and H 522 cell lines constitutively secrete moderate to high levels of IL 8 protein indicating it has an important role in lung cancer occurrence and progression. This overexpression shows it could be a molecular target for gene silencing and drug invention in lung cancer therapy.**

Index Terms: **ELISA, Interleukin 8, Molecular target, Non small cell lung cancer, Real time PCR**

I. INTRODUCTION

Lung cancer is the major cause of cancer related deaths in many countries. Most of the patients with lung cancer have incurable advanced disease with poor therapeutic possibilities, so treatment approaches such as molecular targeted therapy are required. Abnormalities in growth factors may partially attribute to the malignant phenotype of lung cancer via paracrine and autocrine pathways. Therefore, understanding the over expression of such growth factors is important in finding therapeutic targets for treatment. NSCLC is one of the types of lung cancer that includes squamous cell carcinoma, adenocarcinoma, , and large cell carcinoma. Variety of growth factors has been shown to be produced in NSCLC (Woll PJ, 1996). Interleukin 8/ CXCL 8 is a cytokine, which is an important member of the CXC chemokine family categorized as a neutrophil chemoattractant that affects various human neutrophil functions and also promotes tumour angiogenesis and metastasis (Hosono M, Koma YI, 2017). In many cancers including NSCLC, the Interleukin 8 acts as a potent angiogenic factor (YM Zhu, SJ Webster, 2004) and are related with metastasis (Qian Liu, 2016, Luca M, 1996). It also promotes angiogenesis and neutrophil infiltration into the tumor (Justin M. David 2016, Xinxin Long, 2016). Angiogenesis and tumor progression in NSCLC are closely correlated with elevated levels of IL 8 (Masuya D, 2001, Orditura M, 2002). Subsequently, IL-8 has picked up consideration as a potential target gene for cancer therapy. The important methods to assess Interleukin-8 expression in a tumor tissue are qRT-PCR and immunohistochemistry. These methods are utilized to estimate separately, the distinction between the expression of IL-8 protein and mRNA (Yuan A, 2002) assessed expression of Interleukin 8 mRNA in NSCLC by qRT- PCR and inferred that IL-8 over expression correlated the metastasis, prognosis and relapse of lymph nodes. In the present work, the IL 8 protein expression and mRNA levels were investigated in two NSCLC cell lines H 522 and H 23 by ELISA and Real time PCR using GAPDH gene

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as a control gene and showed the over expression of Interleukin 8 in lung cancer. Therefore this study may be useful as a therapeutic target in non small cell lung cancer therapy.

II. MATERIALS & METHODS

A. NON SMALL CELL LUNG CANCER CELL LINES AND CELL CULTURE

Human NSCLC cell lines H 522 and H 23 (Purchased from NCCS, Pune) were cultured in RPMI 1640 culture medium (Gibco) which was supplemented with 10% FBS (Fetal Bovine Serum, Himedia) with 100 U/ml of Penicillin G sodium (Sigma) and 100 µg/ml of Streptomycin (Sigma) incubated in CO_2 incubator with humidified 5% CO_2 and 95% air at 37^0C.

B. TOTAL RNA EXTRACTION AND cDNA SYNTHESIS

Total RNA was isolated from the cell lines (1X 10^6 cells per well) grown in 12 well plate after 24h, 48h and 72h incubation using Trizol reagent (Sigma) according to the manufacturer's protocol. The total RNA obtained was determined by measuring the absorbance at 260nm in a Spectrophotometer. The recovered RNA was resuspended in DEPC water and further cDNA was synthesized as per the manufacturer protocol from 2µg of obtained RNA using RevertAid First Strand cDNA Synthesis Kit (Thermo Scientific). 2 µl of first strand cDNA was used for PCR amplification.

C. mRNA ANALYSIS BY GEL DOCUMENTATION AND QUANTITATIVE REAL TIME POLYMERASE CHAIN REACTION (QRT-PCR)

The expression of mRNA for IL 8 was analyzed by using quantitative RT-PCR. RT-PCR containing 2 µl of synthesized first strand cDNA (1:10 dilution), in triplicate, was used for PCR amplification of mRNA of Interleukin 8 and Glyceraldehyde-3-phosphate dehydrogenase- internal control (GAPDH) genes using 1 µl of primers of IL 8 - sense, 5′-ATG ACT TCC AAG CTG GCC GTG GCT-3′; antisense, 5′-TCT CAG CCC TCT TCA AAA ACT TCT-3′, the GAPDH primers are as follows: sense, 5′-CCA CCC ATG GCA AAT TCC ATG GCA-3′; antisense, 5′-TCT AGA CGG CAG GTC AGG TCC ACC-3′, 10 µl of Hi-SyBr Green I master mix (Himedia) for a reaction volume of total 20 µl. 40 PCR cycles were conducted in accordance with the below mentioned temperature and time profile. In PCR, denaturation 94^0C for 40 sec, primer annealing at 58^0C for 40 sec, primer extension at 72^0 C for 1min and a final extension of 72^0C for 6 min. Then the PCR fragments obtained were subjected to agarose gel electrophoresis on a 2% agarose gel containing Ethidium bromide (0.5 µg/ml) and images were photographed using Gel Doc imager system (BioRad). Then quantitative mRNA analysis for IL 8 and GAPDH genes were performed in 96 well plates, in triplicate samples. The gene expression quantification was performed using cycle threshold (Ct) value by qRT-PCR (Quant Studio™ 5 System). The Ct value represents the number of amplification cycles required where the fluorescent signal reaches the given detection threshold which is negatively associated with the initial mRNA amount (Li YL, Ye F, 2009, Jacob F, 2013). The mRNA quantity of IL 8 and GAPDH were calculated automatically following normalization with the GAPDH calibrator gene under the same conditions using the system software.

D. QUANTITATIVE DETERMINATION OF IL 8 BY ENZYME LINKED IMMUNOSORBENT ASSAY (ELISA)

The IL 8 protein expression was determined by ELISA kit (Invitrogen). To ELISA plate, capture antibody distributed in coating buffer (100µl) was added and incubated at 4^0C for overnight and washed. Then blocked the wells by adding 1X ELISA diluent and incubated for 1h at room temperature and washed. Then 1X ELISA diluent (100 µl) was added to the wells leaving the first wells empty. Then 200µl of standard (Human IL 8 Standard as per manufacturer protocol) concentrations were added to the first empty wells A1/A2 and then transferred 100 µl of standard from A1/A2 to wells B1/ B2 and the contents were mixed well. Now 100µl of sample was added and incubated at room temperature for 2h. After 3 washes, 100µl of diluted detection antibody (biotin-conjugated anti human IL-8 antibody) was added and at room temperature incubated for 1 h, again washed for 3 times. Then 100 µl of Avidin-horseradish peroxidase (HRP) was added, and at room temperature incubated for 30 min. Then 1X TMB (100 µl) was added (Tetramethylbenzidine) and at room temperature incubated for 15 min. Lastly, stop solution (50 µl) was added and the intensity of colour produced was estimated by taking the absorbance at 450nm in ELISA plate reader (Thermoscientific).

E. STATISTICAL ANALYSIS

The data of all experiments were indicated as mean ± Standard deviation. The association between different variables was examined by the student's t- test. A value of $p < 0.05$ was showing statistically significant.

III. RESULTS & DISCUSSION

Human NSCLC cell lines essentially produce IL-8 which shown as an important mitogenic factor. Interleukin-8 assist in human lung cancer growth by its angiogenic properties. In the present work, the NSCLC cell lines H 522 and H 23 were cultured in RPMI 1640 medium which was added with 10% FBS in humidified CO2 5%, air 95% at 37^0C as shown in fig 1. The mRNA expression of IL-8 and the protein production was assessed by using RT-PCR and ELISA in H 522 and H 23 cell lines. These cell were cultured in a time based manner. The conditioned serum free media was collected at indicated times and estimated the amount of IL-8.

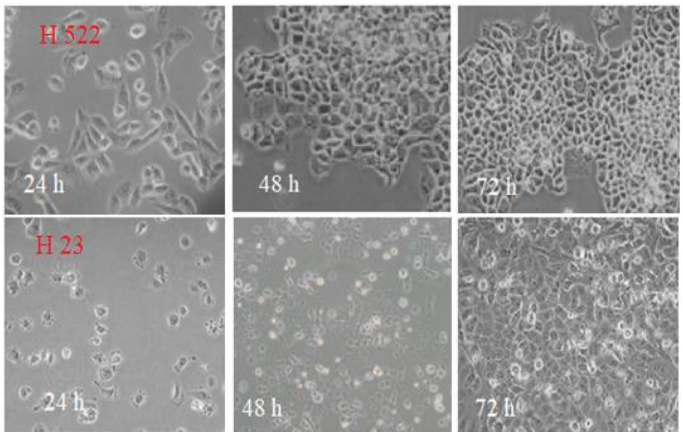

Fig 1: Cell culture images of H 522 and H 23

A. mRNA ANALYSIS BY GEL DOCUMENTATION AND QUANTITATIVE REAL TIME POLYMERASE CHAIN REACTION (qRT-PCR)

Now-a-days, RT-PCR has become a well-established procedure for quantitative estimation of the levels of gene expression (e Kok JB, 2000, Homey B, 2000, Kubo A, 2000). For the first time, absolute quantification PCR was used to detect Interleukin-8 expression in NSCLC and concluded that IL-8 mRNA expression levels were highly associated with angiogenesis. They also proved that patients with over expression levels of Interleukin 8 were prone to have metastasis, post operative relapse, and poor prognosis (Yuan A, 2002). It has the capability to detect the quantity of PCR product by fluorescence, at each and every cycle of the PCR. Many approaches are available to detect PCR products. One of the approaches is depending on the binding of cDNA template that hybridizes with SYBR Green I which is a fluorescent dye by intercalating between the DNA bases. When the dye binds to the DNA, there is a structural change in the dye which becomes less mobile, and releases energy in the form of fluorescence. The intensity of fluorescence increases with the increase in the concentration of DNA.

In the present study, after amplification the pcr product was subjected to agarose gel electrophoresis. In gel documentation, IL-8 mRNA band was observed at 289 bp and band at 593bp was observed for control GAPDH as represented in fig 2. In RT-PCR, in the exponential phase the Ct or Cq value was fixed. In the initial cycles of PCR, the fluorescent signal was usually weak to record above the background therefore the differentiation could not be observed until 15 cycles. But in the exponential phase, the fluorescence intensity was doubled at every cycle and the fluorescent signal became plateau after 35 cycles representing that the reaction reached a saturation point (Frederique Ponchel, 2003). Melting curve analysis and agarose gel electrophoresis showed a single product for IL 8 and GAPDH selected genes (Fig. 3). Primer dimers or non-specific PCR products were not identified in the controls without template. The negative result obtained using the reverse transcriptase negative control shows there was no evident genomic DNA contamination. Ct mean values for IL 8 gene were 17.1 and 18.4 was obtained for GAPDH gene. Genes with higher expression levels had lower Cq values indicating the over expression of IL 8 gene in H 23 and H 522 cell lines.

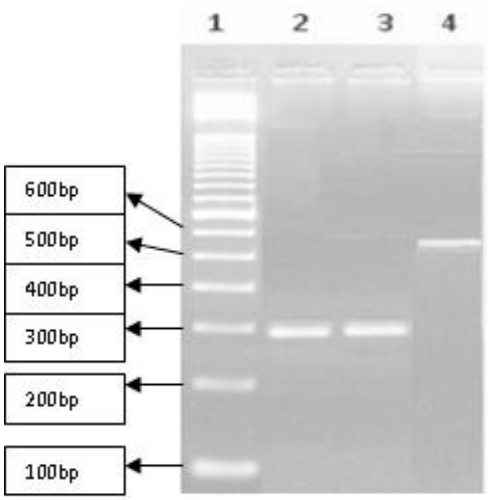

Fig: 2 Lane 1- Ladder, IL-8 mRNA band was observed at 289 bp (Lane 2 H 23 and Lane 3 H 522) and band at 593bp (Lane 4) was observed for control GAPDH.

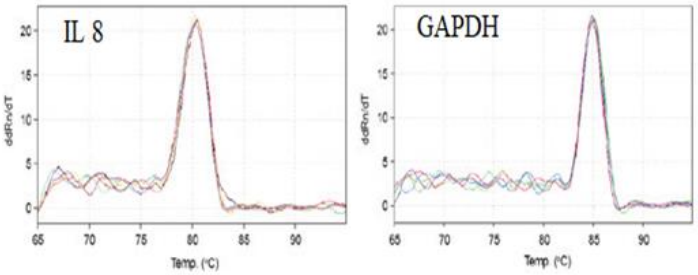

Fig 3: Melting curves of IL 8 and GAPDH mRNA H 23 and H522 cell lines

B. QUANTITATIVE DETERMINATION OF IL 8 PROTEIN BY ELISA

The protein production of IL 8 gene was quantitatively estimated by using ELISA. The IL 8 ELISA results were shown as mean± standard error of the three independent experiments as shown in table 1. IL-8 protein was not found in cell lines after 6 h of culture. It was observed after 24 h, H 23 cells produced 3.97±1.2 ng $ml^{-1}10^6cells^{-1}$and H 522 cells produced 1.97±2.2 ng ml^{-1} 10^6cells^{-1}. After 48 h, the levels were increased to 21.62±1.8 ng $ml^{-1}10^6cells^{-1}$ in H 23, and 12.54±2.8 ng $ml^{-1}10^6cells^{-1}$ in H 522 cells. After 72 h, IL-8 levels were increased to 56.82 ± 0.9 $ngml^{-1}10^6cells^{-1}$ in H 23, and 27.54±1.5 ng $ml^{-1}10^6cells^{-1}$ in H 522 cells as represented in fig 4. It was observed that the NSCLC cells produced statistically significant increased levels of IL-8. These results were in accordance with prior studies (Chen JJW, 2003).

Cell Line	Time in culture (IL 8 ng/ml Per 10^6 cells)		
	24 h	**48 h**	**72 h**
H 23	3.97±1.2	21.62±1.8	56.82 ± 0.9
H 522	1.97±2.2	12.54±2.8	27.54±1.5

Table: 1 IL 8 protein expression was analyzed after 24h, 48h and 72h by ELISA.

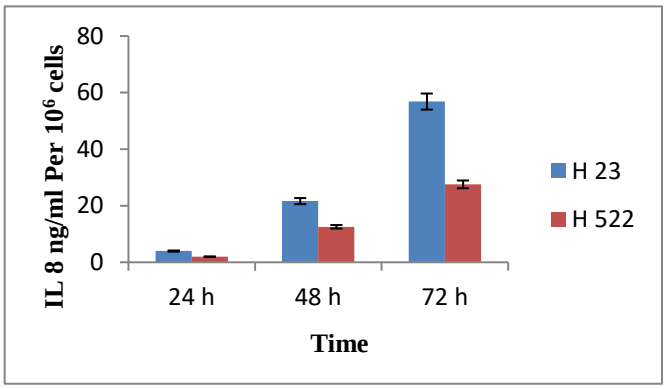

Fig: 4 Interleukin 8 protein expressions: Medium was collected after 1X 10^6 cells cultured in RPMI medium.

CONCLUSION

The expression of IL-8 in H 23 and H 522 NSCLC cell lines was quantitatively estimated by using RT-PCR and ELISA. The expression of IL-8 was significantly increased indicating the mitogenic role in lung cancer growth and the H 23 cells were found to produce higher levels of Interleukin-8 than H 522 cells. Therefore, the IL 8 production can be selected as a targeted gene to control lung cancer invasion, progression, and metastasis by gene silencing and drug invention in lung cancer therapy.

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