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ROLE OF TGF BETA AND IL-18 PROMOTER POLYMORPHISM IN ASSOCIATION WITH LUNG CANCER

Khadeer Basha Shaik[1*], Hema Prasad M[2], Lakshmi Velaga[3], G Sudhakar[3] and Swarupa R Didla[3]

[1,3]Department of Human Genetics, College of Science & Technology, Andhra University, Visakhapatnam-530003, India.
[2]Department of environmental toxicology, Institute of Genetics & Hospital for Genetic diseases. Begumpet, Osmania University, Hyderabad-500016. India.

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Abstract: Lung cancer is the number one cause of cancer-related deaths in the world. World Health Organization classified it into two main histological groups such as non-small cell lung cancer and small-cell lung cancer. Its incidence is highly correlated with cigarette smoking, among the 10% of patients who develop it without a smoking history, the environmental or inherited causes of this is usually unclear. These are most predominant as the cancer is a multistep, progressive disease and early chromosomal changes provide the cell with proliferative advantage. The role of TGF beta in association with pulmonary tumorigenesis and inhibition is a hallmark of multiple cancers including lung cancer. Restoration of this TGF beta signalling reduces tumorigenicity in human lung cancer cells. The present study undergone to assess the role of TGF beta and IL-18 promoter polymorphism in association with lung cancer by molecular and cytogenetic studies in lung cancer patients. The results were compared with the healthy population series. It was found that the recessive allele "T" in TGF Beta and A/A frequency at the position -607 of IL 18 is considerably and significantly higher in patients. There were no abnormalities derived from the cytogenetic study.

Key Words: Lung Cancer, Polymorphism, TGF Beta and IL-18, Tumorigenicity

INTRODUCTION

Lung cancer is number one cause and most common cancers worldwide. Based on current projections, cancer deaths will continue to rise, with nine million people estimated to die from the disease in 2015, and more than 11 million in 2030 [1]. This is also known as carcinoma of the lung, which derives from epithelial cells. It is a malignant lung tumor characterized by uncontrolled cell growth in tissues of the lung. This growth may lead to metastasis, which is the invasion of adjacent tissue and infiltration beyond the lungs. Its pathogenesis is closely associated with the vast majority (80–90%) of tobacco smoking [2][3][4] such as continuous exposure of smoking carcinogens [5] results in the accumulation of several alterations of tumorigenesis related genes leading to neoplastic bronchial lesions. The occurrence of lung cancer in non-smokers that is 15% of is attributed to a combination of genetic factors [6], radon gas [7], asbestos [8] and air pollution including second hand smoke [9]. According to world health organizations (WHO), Lung cancer divided into two main histological groups, non-small cell lung carcinomas (NSCLCs) and small cell lung carcinomas (SCLCs) [10]. It seems that lung tumorigenesis is a multistep process in which a number of genetic events including alterations of oncogenes and tumor suppressor genes have been occurred.

Cytogenetic abnormalities in lung cancer are very complex. During the last 30 years, significant advances have been made with regard to understanding the mechanisms of lung carcinogenesis. These advances have not been paralleled by comparable advances in its treatment and cure [11]. Over a last decade, intense interest has been focused on tumor-specific biomarker discovery and their clinical uses. This interest is accelerated by the completion of human genome project and the progress of techniques in proteomics. Protein biomarkers can be more accurate signatures of a disease state than DNA biomarkers since proteins and not transcripts are the actual functional players [12][13]. Molecular genetics of lung cancer has opened up new vistas of research in carcinogenesis. Cytogenetic and molecular studies have provided evidence that multiple genetic lesions occur during the pathogenesis of lung cancer. In fact, chromosomal abnormalities, activation of dominant oncogenes, and inactivation of tumor suppressor genes have been described [14][15]. However, the sequence of occurrence of these lesions during the multistep carcinogenetic process is still unknown. The aim of our study was to analyze changes in patients carrying either a first or a second primary lung cancer by cytogenetic and molecular studies to investigate the role of TGF beta and IL-18 promoter polymorphism in association with lung cancer.

***Corresponding Author:**
Khadeer Basha Shaik,
Department of Human Genetics,
College of Science & Technology,
Andhra University,
Visakhapatnam-530003, India.

MATERIALS AND METHODS

Cytogenetic Studies: Lymphocyte culture was setup according to the method of Moorhead et al [16]. For this, Intravenous blood from the lung cancer patient and controls was collected and placed into a heparinized tube. 0.5 ml of blood from each individual was added to 5 ml of culture medium containing75 ml of autoclaved triple distilled water and RPMI 1640 (GIBCO), PHA- phytohemagglutinin, (GIBCO), supplemented with 15 ml of fetal bovine serum (GIBCO) and 250 µl Dicrysticin-S antibiotic (Sarabhai Chemicals) then pH is adjusted to 7.2 using sodium bicarbonate. Peripheral blood cultures were placed in a 37°C incubator for 72 hrs. At the end of the incubation, Colchicine (100 µl) (Sigma) was added to the cell culture to inhibit the spindle formation. Cultures were incubated for 50-60 minutes/37°C and centrifuged at 2000 RPM for 10 minutes. The supernatant was discarded and hypotonic solution (0.75M KCl) (Qualigens) was added to the pellet incubated for 20 - 30 minutes in co_2 incubator. Centrifuged at 2000 RPM for 10 minutes. To the pellet, 5 ml of fixative solution (3:1 ratio of methanol and acetic acid (Qualigens) was added and centrifuged. 4-5 times of fixative washes were given. The fine pellet was dropped onto sterile slides using glass droppers. The slides were air dried, labelled and stained with Giemsa's stain. After staining, the slides were rinsed in distilled water and dried for observation under microscope.

Sister Chromatid Exchange assay: Culturing of peripheral blood lymphocytes was carried out in a similar way as the analysis of chromosomal aberrations except that Brdu (31.1g/m1) was added after 24 hours of culturing. The culture vials were then wrapped in dark black paper to avoid photolysis of Brdu substituted DNA and incubated at 37°c. colchicine was added at the 70th hour of incubation to arrest the spindle formation. The cultures were harvested for 72th hours. Slides were dried obtained by conventional flame drying method and were stained by FDG (fluorescence plus Glemsa) technique of Perry and Wolff [17].

Molecular Studies: Genomic DNA was isolated from peripheral blood obtained from patients and stored at -20°C. A modified DNA extraction method suggested by Iranpur et al [18] uses sucrose and chloroform as it major reagents. It yields up to 150 mg of DNA from 5 ml of blood. Here, 1ml of blood was taken and duplicates are done with 0.5 ml in each tube. This was quantified using agarose gel electrophoresis. An advantage of this method is that it avoids the use of any toxic organic solvents required for elimination of cellular proteins. Unlike other standard techniques, these proteins are removed by using saturated sodium chloride solution. PCR Amplification was performed according to the modified method of simmoni et al [19] in selected regions. Then followed by Amplification Refractory Mutation System (ARMS) test by newton et al [20] to detect promoter polymorphism in the gene was used to determine the genotypes.

RESULTS AND DISCUSSION

Genotyping of TGF-β 1 gene at -509 promoter region: A reaction was performed with volume of 15 µL which includes 12.0 µL of water 1.5 µL 10xPCR buffer,0.2µL of 10mmol dNTP, 0.1µL of 20ng genomic DNA and 0.1 µL of 3U Taq polymerase,0.1 µL of forward primer and 0.1 µL of reverse primer in thermal cycler with Initial denaturation with 94°C-5 minute, Denaturation at 94°C-60 seconds, Annealing at 57°C-60 seconds, followed by extension at 72°C-2 minutes and final extension at 72°C-5 minutes with 25 cycles respectively. Amplification was done using sequence specific primers given below.

Table 1: List of primers used for TGF-β gene at -509 region.

Primer	Primer sequence	Product size
Reverse C specific Primer	5'- AAGGGGCAACAGGACACCTGGG-3'	349 base pairs
Reverse T specific Primer	5'- AAGGGGCAACAGGACACCTGGGA-3'	
Forward primer (Common)	5'- AAGGGGCAACAGGACACCTGGG-3'	

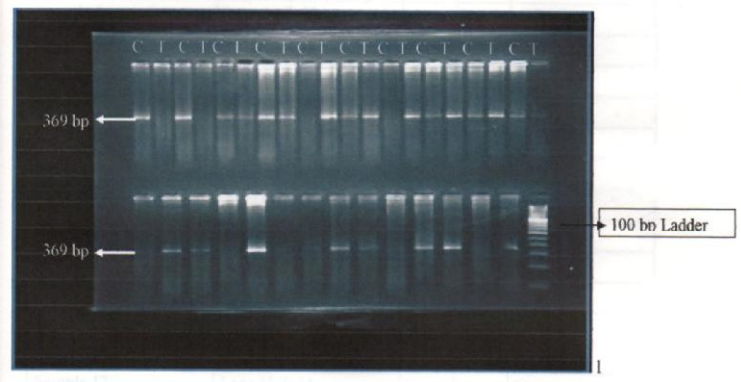


Figure 1: Genotypes resolved using Gel electrophoresis: **C**-Sample Loaded with **C** specific primer, **T**-Sample loaded with **T** specific primer**, C** and **T** specific master mixes containing DNA sample from the same source added in adjacent lanes.

Table 2: Scoring of genotypes

PATIENTS		BANDS OBSERVED		GENOTYPE
		C	T	
Sample 1	Lane 1&2	+	-	CC
Sample 2	Lane 3&4	+	-	CC
Sample 3	Lane 5&6	+	+	CT
Sample 4	Lane 7&8	+	+	CT
Sample 5	Lane 9&10	-	+	TT
Sample 6	Lane 11&12	+	+	CT
Sample 7	Lane 13&14	-	+	TT
Sample 8	Lane 15&16	+	+	CT
Sample 9	Lane 17&18	+	+	CT
Sample 10	Lane 19&20	+	+	CT
Sample 11	Lane 21&22	+	+	CT
Sample 12	Lane 23&24	+	-	CC
Sample 13	Lane 25&26	+	-	CC
Sample 14	Lane 27&28	-	+	TT
Sample 15	Lane 29&30	-	+	TT
Sample 16	Lane 31&32	+	-	CC
Sample 17	Lane 33&34	+	-	CC
Sample 18	Lane 35&36	+	+	CT
Sample 19	Lane 37&38	+	-	CC
Sample 20	Lane 39&40	+	+	CT
Sample 21	Lane 41&42	+	+	CT
Sample 22	Lane 43&44	-	+	TT
Sample 23	Lane 45&46	+	-	CC
Sample 24	Lane 47&48	+	+	CT

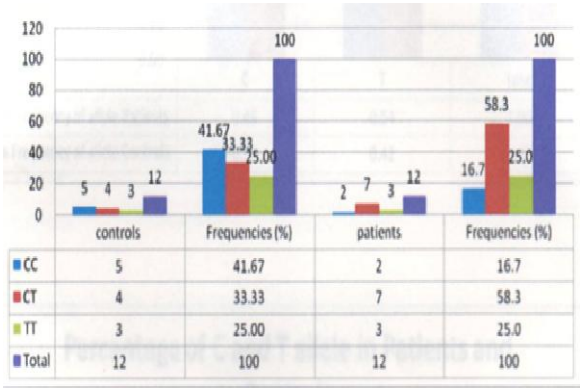
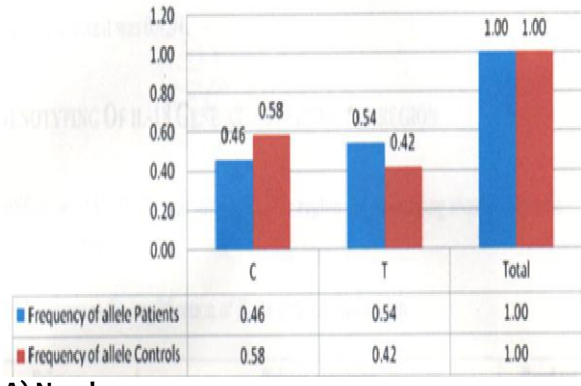
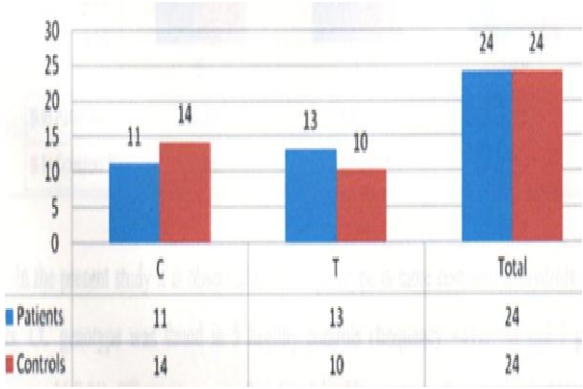


Figure 2: Frequency of genotypes in patient's and controls



A) Number



B) Frequency

Figure 3: Number and frequency of C & T alleles in controls and patients

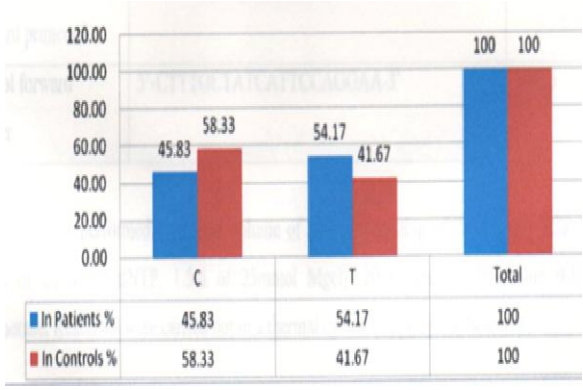


Figure 4: Percentage of C and T allele in Patients and controls

In the present study, it is observed that CC genotype is more common in controls than in patients. It was found in 5 healthy controls (frequency =41.67%) and 2 patients (frequency =16.7 %), CT genotype was found in 4 healthy controls (frequency =33.33%) and 7 patients (58.3%), whereas TT genotype was found in 3 healthy controls (frequency =25%) and 3 patients (25%).Number of C alleles was higher with 14 in controls and 11 in patients. As of T, allele it was higher in patients with 13 and 10 in healthy controls. Percentage of C allele was higher in healthy controls with a frequency of 58.33 % and in patients it was 45.83%. Whereas the frequency of T allele was higher in patients with 54.17% and lower in patients with 0.46/1 and in controls it was 0.58/1, whereas the frequency of T allele was higher in patients with 0.54/1 in patients whereas in healthy controls it was 0.42/1.

Genotyping of IL-18 gene at -607 promoter region: Genotyping of IL-18 promoter region was done by using PCR-SSP Giedraitis et al [21]. A reaction was performed with volume of 25 µL which includes 2.5 µL10xPCR buffer, 0.5 µL of 10 mmol dNTP, 1.5 ul of 25mmol $Mgcl_2$, 20ng genomic DNA and 0.5U Taq polymerase in thermal cycler with Initial denaturation

with 94° C-2 minute, Denaturation at 94°C - 20 seconds, Annealing at 57°C-40 seconds, followed by extension at 72°C-40 seconds and final extension at 72°C-7 minutes with 32 cycles respectively. Amplification was done using sequence specific primers given below.

Table 3: List of primers used for IL-18 gene at -607 promoter region.

Primer	Primer sequence	Product size
Common reverse primer	5'-TAACCTCATTCAGGACTTCC-3'	
Sequence specific forward primers 1	5'-GTTGCAGAAAGTGTAAAAATTATTAC-3'	196 bp
Sequence specific forward primers 2	5'-GTTGCAGAAAGTGTAAAAATTATTAA-3'	
Control forward primer	5'-CTTTGCTATCATTCCAGGAA-3'	301 bp

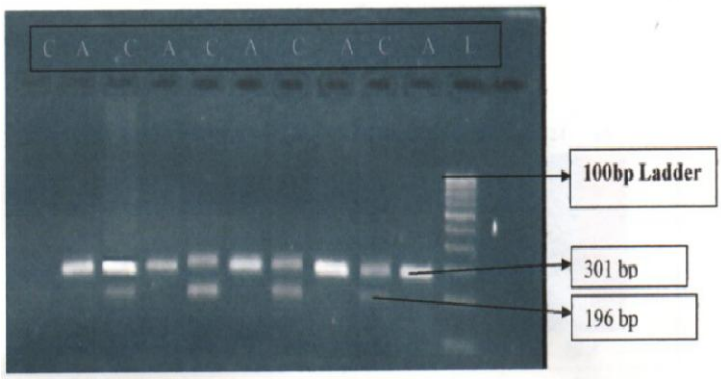


Figure 5: PCR amplification of IL-18 promoter region at -607 position.

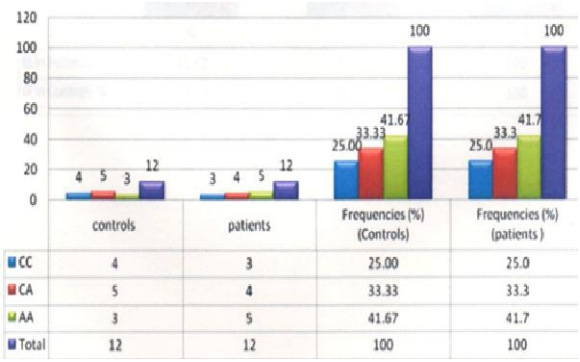


Figure 6: Number and frequency of patients and controls with given genotypes

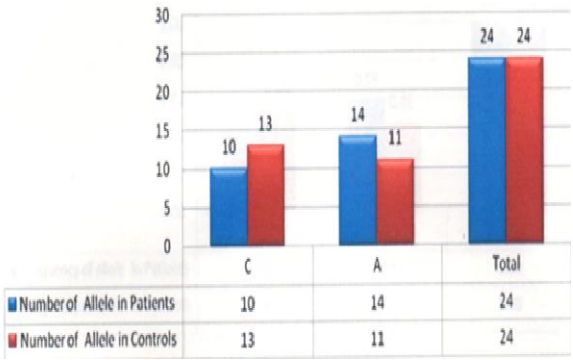


Figure 7: Number of alleles in Patients and controls

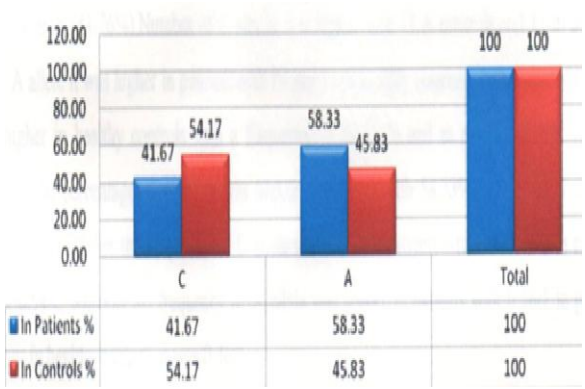


Figure 8: Percentage of C and A allele in controls and patients

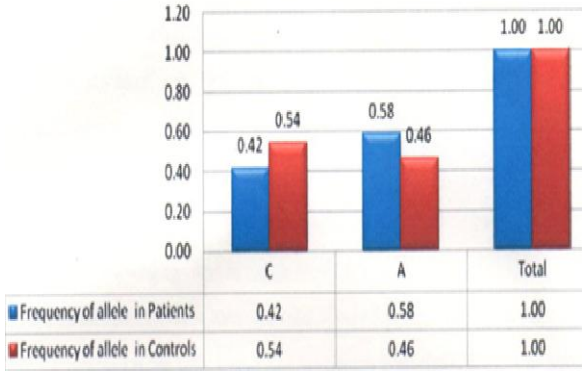


Figure 9: Frequency of alleles in Patients and controls

In the present study, it is observed that CC genotype is more common in controls than in patients. It was found in 4 healthy controls (frequency -25%) and 3 patients (frequency 25%), CA genotype was found in 5 healthy controls (frequency=33.33%) and 4 patients (33.3%), whereas AA genotype was found in 3 healthy controls (frequency = 41.67%) and 5 patients (41.76%). Number of C alleles was higher with 13 in controls and 11 in patients. As of A allele it was higher in patients with 14 and 11 in healthy controls; Percentage of C allele was higher in healthy controls with a frequency of 54.17% and in patients it was 45.83%. Whereas, the percentage of A allele was higher in patients with 58.33% and lower in controls with 45.83%. Further the frequency of C allele was lower in patients with 0.42/1 and in controls it was 0.54/1, whereas the frequency of A allele was higher in patients with 0.58/1 in patients whereas in healthy controls it was 0.46/1.

CONCLUSION

In the present study, the role of TGF beta promoter polymorphism in association with lung cancer was evaluated. it was found that the recessive allele "T" was considerably higher in patients than wild allele "C" was common in the healthy counterparts suggesting the recessive allele is a causative factor contributing to lung cancer. The role of IL-18 promoter polymorphism was found that A/A frequency at the

position -607 of IL -18 is significantly higher in patients compared to healthy controls. The polymorphism in the promoter region renders the transcription factors to bind with reduced affinity to the promoter thereby make the gene to produce diminished level transcripts, thereby manifesting as a causative factor for metastasis. The cytogenetic studies have paved good understanding in the etiology of lung cancer; Chromosomal breakages, translocations, deletions, dicentrics and duplications have been associated with cancers. In the study of chromosomal aberrations and sister chromatid exchanges in patients and healthy controls have no abnormalities derived from the cytogenetic studies in patients compared to controls. Since the sample size is small, no concrete conclusions can be drawn from the data the study needs to be investigated further.

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