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Evaluation of CTLA-4 (+49 A/G) gene polymorphism in NSCLC (non-small cell lung carcinoma) patients among south Indian population

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Abstract

Lung carcinoma, a malignant condition characterized by uncontrolled cell growth in the tissues of lung. It is of two types *viz*, small cell lung carcinoma (SCLC) and non-small cell lung carcinoma (NSCLC). The later is the predominant carcinoma of lung constituting about 80% of lung cancer. Hence, advancements in identification of genetic variants of the immune genes (CTLA-4) that regulate the activation and proliferation of T lymphocytes examined for its susceptibility to lung cancer especially to NSCLC. The gene encoding Cytotoxic T-Lymphocyte antigen-4 (CTLA-4) is a promising candidate in the pathogenesis of NSCLC especially the polymorphism at position +49A/G (located in exon 1 leads to alanine to threonine substitution in leader sequence) of CTLA-4 gene in NSCLC. The objective of the present study is to investigate the prevalence of the +49A/G polymorphism in CTLA-4 gene and to assess the association of polymorphisms to the risk of lung cancer in and around Madurai (south Indian population). Blood samples obtained from Asirvatham specialty clinic, Madurai. Basic details like age, and smoking habit collected using a consent form. Genomic DNA isolated from blood was confirmed on agarose gel and subjected to PCR based RFLP analysis. Genotype classified by means of allele counting method. The results of the present study showed that the variant genotype of +49A/G polymorphism was lower in controls and patients. The distribution of genotypes was not in accordance to Hardy Weinberg equilibrium and the odds ratio revealed

that there is 1.25 fold risk of developing cancer due to variant genotype of +49A/G SNP and there is an association of polymorphism with NSCLC.

Keywords: Non-small cell lung carcinoma, Cytotoxic T-Lymphocyte Antigen-4, Single nucleotide polymorphism, Variant genotype

Introduction

Lung cancer has the highest morbidity and mortality rates among malignant cancers worldwide. It is the second leading cause of death globally, and is responsible for an estimated 9.6 million deaths in 2018 with a 6.45% rise in cases from 2012 to 2018 in India (GLOBOSCAN). Lung cancer is the primary cause of death in men suggesting that it results from interactions between genetic background and environmental factors (Liu *et al*., 2012) Most of 80% of primary lung cancer is NSCLC and around two-thirds of NSCLC patients are diagnosed in advanced stage (Zhang *et al*., 2011). The risk factors of NSCLC include cigarette smoking, tobacco, secondhand smoke, exposure to radon gas, asbestos, and other forms of air pollution. Smoking cannabis doubled the risk of the lung cancer (Yang *et al.*, 2013). The International agency for research on cancer says that the carcinogens include metals, ionizing radiation, toxic gases, rubber production and crystalline silica dust also increase the risk of lung cancer (Congliano *et al*., 2011).

Enormous reports on SNPs in genes are available for the past decade wherein many studies have examined and hypothesed that genetic variants of the immune genes may be relevant to the risk of variety of cancers (Clifford *et al.*, 2010). Studies on genetic variants of many genes including CTLA-4 have been explored for association with cancer and mutations in genes that induce cell proliferation like EGFR and its response to selected drugs/ inhibitors (Getitinib) for treatment of

cancer has become the focus of the research for NSCLC especially in south Indian population (Louis *et al.*, 2012).

Cytotoxic T-Lymphocyte Antigen-4 (CTLA-4) The gene encoding cytotoxic T-Lymphocyte antigen-4 (CTLA-4) reported to be a promising candidate gene in the pathogenesis of cancer (Liu *et al.*, 2001).

The CTLA-4 gene, located on chromosome 2q33, consists of four exons and has a nucleotide size of about 6.2 kb consisting 4 exons (Teft *et al.*, 2006). About 17 single nucleotide polymorphism (SNP) at different positions of CTLA-4 gene has been identified (Zheng *et al.*, 2010). CTLA-4 +49 A/G SNP in exon 1 leads to alanine to threonine substitution in leader sequence and affect the protein processing in the endoplasmic reticulum (ER), leading to lower effective glycosylation, which plays an important role in protein membrane expression. The functional role of only few of the SNPs and their association with susceptibility to diseases are well-documented (Ghaderi *et al.*, 2011). CTLA-4 (that regulate the activation and proliferation of T lymphocytes and natural killer cells may influence cancer risk (Zhang *et al.*, 2011). Reports are available for the risk of association due to variant genotype with NSCLC in Chinese, Spanish and some Asian populations (Minhas *et al.*, 2014).

Objective: The present study is to investigate the association of +49A/G SNP with NSCLC in south Indian population.

Materials and Methods

Study subjects:

Blood sample (2ml) was obtained from Asirvatham clinic from lung cancer patients as cases and healthy individuals as controls (non-smokers) in EDTA coated tubes and the samples (N=80)

were used for DNA extraction. Questionnaire framed to collect the basic details such as age, gender, and smoking habit etc. Ethical clearance obtained for the study and informed consent obtained from the study subjects.

Criteria for cases and controls

Cases includes individuals who were newly diagnosed with lung cancer and are not taking any additional nutritional supplements, and that of controls includes individuals who do not have cancer, non- smokers and are not taking any additional nutritional supplements.

Isolation of genomic DNA from human blood (Iranpur and Esmailizadeh, 2012):

DNA isolation carried out according to the phenol-chloroform method. The isolated DNA quantitatively checked through 0.7% agarose gel electrophoresis. The gel visualized under UV transilluminator.

PCR and Restriction Analysis (Ghaderi *et al.*, 2011)

Isolated Genomic DNA was subjected to PCR with a total volume of 100µl containing equal concentration of the forward and reverse primers (20picomoles) FP: 5'-AAGGCTCAGCTGAACCTGGT-3' ; RP: 5'-CTGCTGAAACAAAATGAAACCC-3',. 10mM deoxynucleotide triphosphates, 10X buffer with $MgCl_2$ and 5 units of *Taq* DNA polymerase (Sigma Aldrich).

PCR reactions were subjected to 35 cycles of amplification in a thermal cycler using the following conditions : Initial Denaturation at 94º C for 1minutes, Denaturation at 94ºC for 1 minute, Annealing at 60ºC for 1 minute, Extensions at 72ºC for 1 minute, Final extensions at 72ºC for 7 minutes. The PCR product was confirmed in 2% agarose gel electrophoresis. The amplified product was subjected to restriction fragment analysis. Restriction enzyme analysis was carried out in a total volume of 20µl containing of 10µl amplicon, 4µl buffer and 5 units of

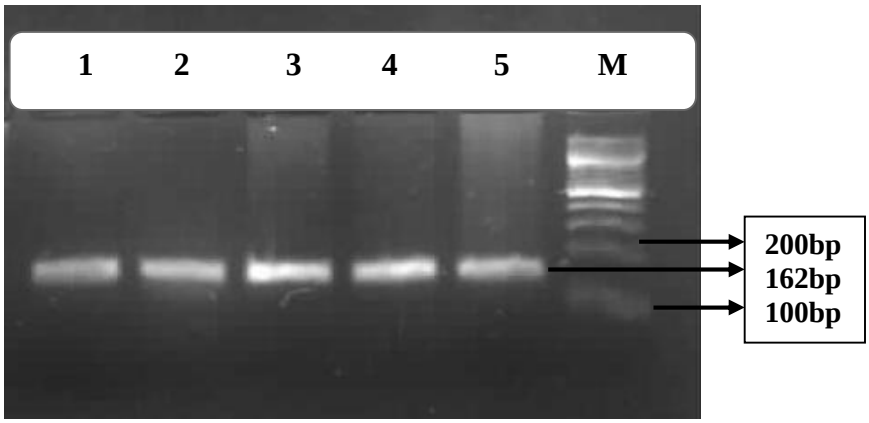
Bbv I (New England Bio labs) enzyme. Samples were incubated for 6 hours at 37ºC and the digested PCR products were visualized in ethidium bromide stained 2.5 % agarose gel electrophoresis.

Result

Table 1: Demographic profile of study subjects

Parameters		**Controls n=40 (%)**	**Patients n=40 (%)**
Age (years)	(Mean± SD)	52.2±6.3	37.8±4.3
Gender	Male	40 (100)	36 (90)
	Female	0 (0)	4 (10)
Life Style	Smokers	-	33 (82)
	Non-smokers	40 (100)	7 (18)
	Alcoholics	-	22 (55)
	Non-alcoholics	40 (100)	18 (45)

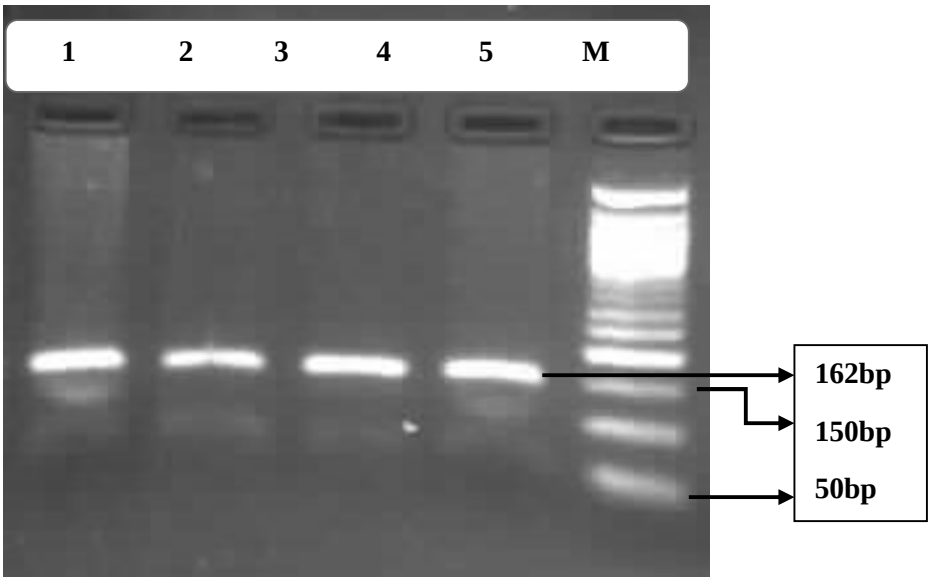
Figure 1: PCR product of *CTLA-4 (+49A/G)* gene resolved in 2.5% agarose gel



Lane 1-5: PCR Product -162 bp.

Lane M: 100bp DNA Marker.

Figure 2: Restriction analysis of *CTLA-4 (+49A/G)* gene resolved in 2.5% agarose gel



Lane M: 50bp DNA Marker .
Lane 1 & 4: 162bp, 88bp and 74bp.
Lane 2&3: 162bp (partially digested)

The expected band pattern of normal/wild type genotype (AA) is 162bp, heterozygous genotype (AG) is 162bp, 88bp and 74bp and the variant / mutant genotype (GG) is 88bp and 74bp (not obtained). The presence of variant genotype creates a restriction site for the enzyme *BbvI.*

Table 2: Genotype and allele frequency of study subjects

Genotypes	**Control n=40 (%)**	**Patients n=40 (%)**
AA	28 (70)	26 (60)
AG	12 (30)	14 (35)
GG	-	-
Alleles A (normal)	0.85	0.82
G (variant)	0.15	0.18

Odds ratio: 1.25 (95% CI: 0.4932 - 3.209) HWE- deviation observed.

Discussion

Lung cancer has the highest morbidityand mortality rate among malignant cancers worldwide (Xiaolei *et al.,* 2014). The demographic profile of the study subjects in the present study on the prevalence of non-small cell lung cancer (n=40) patients and healthy controls are tabulated (Table 1). The age of the patients ranged from 30-70 years and that of the healthy individuals (n=40) were from 30-55 years. Among the patients, 10 % were females and 9 % were males. Among study subjects smoking and alcoholism was prevalent in patients.

The frequency of normal or wild genotype in controls and patients were found to be 70% and 60%. The heterozygous genotype was found to be 30% in controls and 35% in patients. The allele frequency reveals that the variant allele was found to be lower in both controls and patients. The 'A' allele frequency in controls and patients was 0.85 and 0.82 whereas, 'G' allele frequency in controls and patients was 0.15 and 0.18 respectively (Table 2).

The genotype frequency among the study subjects reveals that the variant genotype (GG) of CTLA-4 +49A/G was completely absent among study subjects. The complete absence of homozygous variant genotype recorded in this study reveals that the distribution of the variant allele is uncommon among this population is a new finding. In contrast to the present study, the presence of variant genotype GG and allele ‘G’ lead to the increased risk of NSCLC and with AA genotype and the ‘A’ allele decreased the risk of lung cancer in Poland population (Antczak *et al.,* 2013). The presence of the ‘G’ allele in CTLA-4 +49 A/G gene increased the risk of cervical cancer by 1.16 fold as reported by Liu *et al.,* (2014). The presence of ‘G’ allele showed risk of association with other diseases, such as Systemic lupus erythematosus in Asian and European population and Graves’ disease in Chinese and Spanish population (Si *et al.,* 2012 and Devaraju *et al.,* 2014). In the present study the odds ratio revealed that there is 1.25-fold increase

in the risk of developing lung cancer among individuals with variant genotype in the study subjects and is significant at 95% (CI: 0.4932 - 3.209) (Table 2).

The result of the present study showed a deviation from Hardy-Weinberg Equilibrium and Odds ration reveals that, there is 1.25-fold increase in risk of developing lung cancer among individuals with variant genotype in heterozygous condition. As the homozygous variant genotype (MAF) was completely absent in the present study, which needs to be elucidated by increasing the sample size. Hence, to conclude from the results obtained, there is an association of variant genotype with NSCLC in south Indian population.

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References

Antczak, A., Pastuszak-Lewandoska, D., Górski, P., Domańska, D., Migdalska-Sęk, M., Czarnecka, K., and Brzeziańska, E. CTLA-4 expression and polymorphisms in lung tissue of patients with diagnosed non-small-cell lung cancer. *Biomed Res. International*. (2013).

Clifford, R. J., Zhang, J., Meerzaman, D. M., Lyu, M. S., Hu, Y., Cultraro, C. M., and Nguyen, C. V. Genetic variations at loci involved in the immune response are risk factors for hepatocellular carcinoma. *Hepatology*. (2010) 52. 6: 2034-2043.

Cogliano, V. J., Baan, R., Straif, K., Grosse, Y., Lauby-Secretan, B., El Ghissassi, F., and Galichet, L. Preventable exposures associated with human cancers. *J. of the National Cancer Institute*. (2011) 103.24:1827-1839.

Devaraju, P., Gulati, R., Singh, B. K., Mithun, C. B., and Negi, V. S. The CTLA4+ 49 A/G (rs231775) polymorphism influences susceptibility to SLE in South Indian Tamils. *Tissue Antigens*. (2014) 83.6: 418-421.

Ghaderi, A., Yeganeh, F., Kalantari, T., Talei, A. R., Pezeshki, A. M., Doroudchi, M. and Dehaghani, A. S. Cytotoxic T lymphocyte antigen-4 gene in breast cancer. *Breast Cancer Research and Treatment*. (2004) 86.1: 1-7.

Hu L, Liu J, Chen X, Zhang Y, Liu L. and Zhu J. CTLA-4 gene polymorphism +49 A/G contributes to genetic susceptibility to two infection-related cancers-hepatocellular carcinoma and cervical cancer. *Hum Immunol*. (2010) 71:888-91

Iranpur M V. and Esmailizadeh, A. K. Rapid extraction of high quality DNA from Whole blood stored at 4 ºC for long period. (2012).

Liu, M. F., Wang, C. R., Lin, L. C., and Wu, C. R. CTLA-4 gene polymorphism in promoter and exon-1 regions in Chinese patients with systemic lupus erythematosus. *Lupus.* (2001) 10.9: 647-649.

Liu, J., Liu, J., Song, B., Wang, T., Liu, Y., Hao, J. and Yu, J. Genetic variations in CTLA-4, TNF-α, and LTA and susceptibility to T-cell lymphoma in a Chinese population. *Cancer Epidemiology*. (2012) 37.6: 930-934.

Liu, P., Xu, L., and Sun, Y. The association between cytotoxic t lymphocyte-associated antigen-4 and cervical cancer. *Tumor Biol*. (2014) 35:2893-2903.

Louis, R. A., Rajenderan R, Ganesan P., SagarT. G., Krishnamurthy A. First report of upfront treatment with Gefitinib in comparison with chemotherapy in advanced non-small cell lung cancer patients from south India: Analysis of 120 patients. *Indian J. Med pediatr Oncol*. (2012) 33.3: 14-54.

Minhas, S., Bhalla, S., Shokeen, Y., Jauhri, M., Saxena, R., Verma, I. C. and Agarwal, S. Lack of any association of the CTLA-4+ 49 G/A polymorphism with breast cancer risk in a North Indian population. *Asian Pac. J. Cancer Prev.* (2014) 15.5: 2035-2038.

Si, X., Zhang, X., Tang, W., and Luo, Y. Association between the CTLA-4+ 49A/G polymorphism and Graves' disease: A meta-analysis. *Experimental and Therapeutic Medicine*. (2012) 4.3: 538-544.

Teft, W. A., Kirchhof, M. G., and Madrenas, J. A molecular perspective of CTLA-4 function. *Annu. Rev. Immunol.* (2006) 24: 65-97.

Xiaolei, L., Baohong, Y., Haipeng, R., Shuzhen, L., Jianfeng, G., Xiangpo, P. and Huanxin, W. Current evidence on the cytotoxic T-lymphocyte antigen 4+ 49G> A polymorphism and digestive system cancer risks: a meta-analysis involving 11,923 subjects. *Meta Gene*. (2015) 6: 105-108.

Yang, M., Sun, T., Zhou, Y., Wang, L., Liu, L., Zhang, X. and Li, H. The functional cytotoxic T lymphocyte–associated Protein-4 +49 G to A genetic variant and risk of pancreatic cancer. *Cancer*. (2013) 118.19: 4681-4686.

Zhang, Y., Zhang, L., Sun, D., Li, Z., Wang, L., and Liu, P. Genetic polymorphisms of superoxide dismutases, catalase, and glutathione peroxidase in age-related cataract. *Molecular Vision*. (2011) 17: 23-25.

Zheng, J., Yu, X., Jiang, L., Xiao, M., Bai, B., Lu, J. and Zhou, Y. Association between the *Cytotoxic T-Lymphocyte Antigen 4* +49G > A polymorphism and cancer risk: a meta-analysis. *BMC Cancer*. (2010) 10:522 (https://doi.org/10.1186/1471-2407-10-522)