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Original Article

Internal Medicine Section

Interplay among the Variants of One Carbon Metabolism, Methylenetetrahydrofolate Reductase Polymorphisms and Lung Cancer

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ABSTRACT

Introduction: Folates perform an integral task in Deoxyribonucleic Acid (DNA) synthesis, methylation and repair. Methylenetetrahydrofolate Reductase (MTHFR) potrays a key part in the metabolism of folate and regulates the equilibrium between the various forms of folate for DNA synthesis and DNA methylation. The MTHFR irrevocably transforms 5,10 methylenetetrahydrofolate to 5-methyltetrahydrofolate, the principal circulating folate and the carbon donor for remethylation processes. It is vastly polymorphic in the general population.

Aim: To study the association, if any, between the variants of one carbon metabolism, MTHFR polymorphisms and risk of lung cancer.

Materials and Methods: It was a case-control study conducted during March 2010 to September 2011 in the Department of Pulmonary Medicine in collaboration with the Department of Biochemistry at Government Medical College and Hospital, Chandigarh, to see whether any association exists between the variants of one carbon metabolism, MTHFR polymorphisms (C677T and A1298C), and lung cancer. Twenty biopsy proven lung cancer patients and 20 age and sex matched cancer-free controls were selected.

Results: The mean serum folate in cases was higher (12.84 ng/mL±7.527 ng/mL) as compared to controls (4.46 ng/mL±1.346 ng/mL), suggesting that high levels of serum folate are associated with lung cancer. There was no significant variance in the levels of vitamin B12 and plasma homocysteine between cases and controls. No MTHFR polymorphism C677T was seen in the blood and the bronchial biopsy samples of all cases as well as blood samples of all the controls. The MTHFR polymorphism A1298C was present in the blood as well as bronchial biopsy samples of cases as well as blood of controls. Thus, in the present study, there was no relation of this polymorphism with lung cancer.

Conclusion: Polymorphisms in MTHFR may contribute to lung cancer. More research on the basis of cellular and molecular mechanisms of lung cancer is urgently needed to aid in understanding of pathogenesis of the disease.

Keywords: Carcinogenesis, Folates, Smokers, Vitamin B12

INTRODUCTION

Lung cancer constitutes a major cause of cancer related mortality in men and women adding up to 27% of all cancer deaths in men and 25% in women [1,2]. Nearby 80-90% of lung cancers are attributable to cigarette smoking [3].

Genetic determinants also play an important role in disease initiation and progression [4]. Methylation of DNA is the chief epigenetic alteration subsequent to replication in humans [5]. The epigenetic alteration of DNA by DNA methylation has been demonstrated to have quite a lot of purposeful functions including the suppression of gene expression, and the augmentation of genomic stability [1]. Alterations or disruption to DNA methylation may accelerate malignant transformation [6].

An important epigenetic process playing a critical task in gene regulation is reduction in the predominant active circulating form of folates i.e., 5 methyltetrahydrofolate [7]. It performs an eminent part in synthesis, methylation and repair of DNA. Other B vitamins (vitamin B2, vitamin B6 and vitamin B12) are chief cofactors in the folate metabolism pathway.

Methylenetetrahydrofolate reductase (MTHFR) enzyme performs a fundamental role in folate metabolism by irrevocably converting 5,10-methylenetetrahydrofolate to 5-methyltetrahydrofolate. This process plays a key role in sustaining ample levels of S-adenosylmethionine, which is the key contributor of methyl groups for all lipid, protein and DNA methylation reactions and helps to regulate levels of intracellular homocysteine as well [8].

The MTHFR is highly polymorphic in the general population [9]. The gene for MTHFR is found at 1p36.3 and numerous MTHFR gene polymorphisms have been characterised [8].

The C677T variant is sited on exon 4 at the folate attaching spot of the MTHFR gene and its presence leads to replacement of an alanine by a valine (Ala222Val) residue, consequently causing lessened functionality of MTHFR thus leading upon to lower levels of 5-methyltetrahydrofolate, an accretion of 5,10-methylenetetrahydrofolate and escalated levels of plasma homocysteine [10].

The A1298C variant (Glu429Ala) is situated on exon 7, resulting in a diminished activity of MTHFR enzyme that is more evident in homozygotes (C/C) than heterozygotes (A/C) [11]. Factors such as demography and environmental elements (folate status, age, smoking and alcohol intake) remarkably shape the physiopathological consequence of genetic variants of MTHFR, especially the C677T polymorphism. These factors may furthermore modify the fine equilibrium of one-carbon metabolism [12].

Enhanced intake of fruits and vegetables is linked with a reduced risk of lung cancer. Vegetables and fruits are rich in folate and dietary folate may perhaps be one of the micronutrients that offer safety against lung carcinogenesis [13]. Nutrients such as vitamin B6 and B12 are also present in vegetables and fruits and their deficiency could promote DNA damage, and oxidative lesions are also induced by tobacco carcinogens [14]. Thus, dietary nutrient intake may have a tenable association with susceptibility to lung cancer [15].

Various studies [9,15-22] have been conducted in the past to see the association of the MTHFR polymorphisms in various cancers. Genetic variants in genes of one carbon metabolism could modulate the risk of lung cancer. Most of the studies

reported till date have been carried out in non Hispanic whites, Japanese, and Chinese populations. No study has so far been carried out in the Indian population. That is why we chose to study the MTHFR polymorphisms A1298C and C677T in North Indian population.

Objectives

- To study the levels of serum folic acid, B12 and homocysteine in the patients of lung cancer to know whether there exists altered one-carbon metabolism.
- To study the polymorphisms in MTHFR gene (C677T and 1298C) in lung cancer patients.
- To study the association, if any, between the variants of one carbon metabolism, MTHFR polymorphisms and risk of lung cancer.

MATERIALS AND METHODS

It was a case-control study conducted between March 2010 to September 2011 in the Department of Pulmonary Medicine in collaboration with the Department of Biochemistry at Government Medical College and Hospital, Chandigarh, India. Twenty biopsy proven lung cancer patients and 20 age and sex matched healthy controls were selected [16].

Inclusion and Exclusion criteria: Eligibility criteria included subjects with subsequently histologically proven lung cancer. Patients with metastatic cancer, having received previous radiotherapy or chemotherapy, diagnosed tuberculosis/asthma/interstitial lung disease/other lung diseases were excluded. An informed consent was taken from all subjects. A detailed history was taken and physical examination was done.

Bronchoscopy

- All cases underwent bronchoscopy performed with flexible bronchoscope XT 40 (Olympus). Biopsy specimens were taken from suspected area with biopsy forceps (Olympus) FB-35 C-1.
- Besides for histopathology, the biopsy tissue was also collected in the autoclave tubes in Phosphate-Buffered Saline (PBS) and preserved at -20°C till it was used for DNA isolation.

Blood Collection

- Five mL blood samples were gathered from cases and controls for analysis of serum folic acid, vitamin B12 and plasma homocysteine on Advia-Centaur CP by chemiluminescence assay.
- Blood from cases and controls was collected separately for DNA extraction in Ethylenediaminetetraacetic Acid (EDTA) coated vials and immediately kept at -20°C.

Chemiluminescence

These assays were done by competitive immunoassay using direct, chemiluminescent technology, where the relative light units were detected by the system.

DNA Isolation

The DNA isolation in cases was done from both blood (from buffy coat) as well as the biopsy tissue obtained by bronchoscopy, while DNA isolation in controls was done only from blood (from buffy coat) and was subjected to the Polymerase Chain Reaction (PCR).

PCR-RFLP Analysis

Genotyping for the MTHFR C677T and A1298C polymorphisms was accomplished by means of PCR and Restriction Fragment Length Polymorphism (RFLP) methods.

A 100 ng of genomic DNA was amplified in a 25 µL final volume reaction mixture comprising 10X KCl buffer (2.5 µL), 25 mM $MgCl_2$ (2 µL), 10 mM of each dNTP (0.6 µL), 10 µM of each primer (0.5 µL), 5U/1 µL T*aq* DNA polymerase (0.5 µL).

PCR products were visualised and checked on 2% agarose gel. PCR reactions were carried out in a Bio-Rad Thermocycler (USA) using the conditions given in [Table/Fig-1].

Gene	PCR Program	Size (bp)	Cycles
A1298C	Initial denaturation-95°C Denaturation-95°C Annealing-56°C Extension-72°C Final extension-72°C	160	35
C677T	Initial denaturation-95°C Denaturation-95°C Annealing-58°C Extension-72°C Final extension-72°C	198	35

[Table/Fig-1]: Thermocycler program during PCR.

The PCR products were eluted using YDF 100 High Yield PCR DNA minikit (Real Genomics, Belgium). Eluted products were subjected to restriction digestion.

Restriction digestion was carried out with Mbo11, Hinf1 endonucleases to confirm the polymorphism within amplified gene product. Reaction mixtures were incubated at 37°C for 3 hours in water bath.

STATISTICAL ANALYSIS

The statistical analysis was done by means of Statistical Package for the Social Sciences (SPSS Inc, Chicago, IL, version 15.0 for Windows). The associations between variants of one carbon metabolism, lung cancer and MTHFR genotypes (C677T and A1298C) were determined. Chi-square test and Fisher's-Exact test were utilised. For all quantitative variables, mean and standard deviation were calculated. Means were compared using student's t-test for two groups. The study was conducted strictly in accordance with the ethical guidelines as approved by the Institutional Ethics Committee (IEC).

RESULTS

Cases and controls were well matched with regard to age, gender and demographic characteristics. Body Mass Index (BMI) among cases was significantly lower than that in controls. There was no significant disparity in alcohol intake among cases (40%) and controls (15%) (p=0.157). Cases and controls were well matched for diabetes mellitus (p=1.000) and hypertension (p=1.000). Smoking habit and the number of pack years of smoking was significantly more in cases as compared to controls (p<0.001). Cough was the most common complaint among cases (90%), followed by dyspnoea (70%), fever (45%), chest pain (30%), haemoptysis (25%) and hoarseness of voice (15%), whereas there were no complaints among the control group.

The mean serum folate in cases was higher (12.84±7.527 ng/mL) as compared to controls (4.46±1.346 ng/mL), suggesting that high levels of serum folate are associated with lung cancer (p<0.001) [Table/Fig-2].

Serum folate	Cases n=20	Control n=20	Total N=40
Mean (ng/mL)	12.84	4.46	8.65
Std. Deviation	7.527	1.346	6.817
Minimum (ng/mL)	3	2	2
Maximum (ng/mL)	24	7	24

[Table/Fig-2]: Serum folate levels in cases and controls.

No significant difference in the levels of vitamin B12 between cases (541.5±379.919 pg/mL) and controls (287.50±94.031 pg/mL) (p=0.055) was seen. There was no significant difference in the levels of plasma homocysteine among cases (11.6670±8.43753) and

controls (15.7800±9.17040) (p=0.148). The MTHFR polymorphism C677T was not seen in the blood and the bronchial biopsy samples of all cases as well as blood samples of all the controls, inferring that there is no relation of this polymorphism with lung cancer [Table/Fig-3,4].

Blood C677T	Cases n (%)	Control n (%)	Total n (%)
Absent	20 (100)	20 (100)	40 (100)
Total	20 (100)	20 (100)	40 (100)

[Table/Fig-3]: MTHFR polymorphism C677T in blood samples.

Biopsy C677T	Cases n (%)	Control n (%)	Total n (%)
Absent	0 (0)	20 (100)	20 (50)
	20 (100)	0 (0)	20 (50)
Total	20 (100)	20 (100)	40 (100)

[Table/Fig-4]: MTHFR polymorphism C677T in biopsy samples.

The MTHFR polymorphism A1298C was present in the blood as well as bronchial biopsy samples of cases as well as blood of controls. The study concluded that there is no relation between MTHFR polymorphism A1298C and lung cancer [Table/Fig-5,6].

Blood A1298C	Cases n (%)	Control n (%)	Total n (%)
Present	0 (0)	20 (100)	20 (50)
	20 (100)	0 (0)	20 (50)
Total	20 (100)	20 (100)	40 (100)

[Table/Fig-5]: MTHFR polymorphism A1298C in blood samples.

Biopsy A1298C	Cases n (%)	Control n (%)	Total n (%)
Present	0 (0)	20 (100)	20 (50)
	20 (100)	0 (0)	20 (50)
Total	20 (100)	20 (100)	40 (100)

[Table/Fig-6]: MTHFR polymorphism A1298C in biopsy samples.

DISCUSSION

The present study was conducted at a tertiary care academic hospital in North India. Cases and controls were well matched for gender and age. Males were more affected than females. Similar observations were made by other investigators [16]. The mean BMI of cases was significantly less than the controls as observed by other studies [17].

The cases and controls were aptly matched for demographic characteristics and there was no significant association of any occupation with lung cancer.

Active smoking has been associated with development of lung cancer with risk increasing with period of smoking and the quantity of cigarettes smoked per day [18]. In the present study, 85% subjects were smokers among cases, whereas there were 20% smokers among controls. The number of pack years of smoking were significantly greater in cases as compared to controls, implying that smoking may be a risk factor for lung cancer.

A statistically significant increase was observed in the levels of serum folatein cases (12.84 ng/mL±7.527 ng/mL) as compared to the controls (4.46 ng/mL±1.346 ng/mL) alluding that high levels of serum folate may be associated with lung cancer.

Former studies have substantiated the linkage between elevated folate intake and dwindled risk of colorectal, pancreatic, and oesophageal malignancies [19]. Several studies have also shown a counter association between folate intake and risk of breast [20], lung [13], and stomach cancers [21]. However, recently, there has been increased scrutiny regarding the safety of folic acid, in particular with respect to risk of malignancy [22].

The timing of folate supplementation during cancer progression may modify outcomes. Administration of folate prior to the existence of preneoplastic lesions can prevent tumour development, whereas provision of folate once early lesions are established appears to increase tumourigenesis [23].

A study conducted in Norway [24] concluded that therapy with folic acid plus vitamin B12 was related to escalated cancer outcomes and all-cause mortality in patients with ischemic heart disease, where folic acid fortification of foods is not done. A bell-shaped association was observed between plasma folate concentrations and Colorectal Cancer (CRC) risk in a previous study [25]. Their findings suggested a decreased CRC risk in subjects with low folate status.

The present study revealed somewhat similar findings as the above two studies. Rationale for such findings could be that in preneoplastic and neoplastic cells where DNA replication and cell division are occurring at an accelerated rate, folate depletion causes ineffective DNA synthesis, resulting in inhibition of tumour growth and progression, which is the basis for cancer chemotherapy using antifolate agents (e.g., methotrexate) and 5-fluorouracil [26,27]. Therefore, this is the most plausible explanation as to how folate deficit impedes the development of the established preneoplastic and neoplastic foci in the colorectum. Another possible mechanism is that folate deficiency may reverse CpG promoter methylation of tumour suppressor and other anticancer genes involved, thereby reactivating these genes. The most likely mechanism by which folic acid supplementation may promote the progression of established preneoplastic and neoplastic lesions in CRC is provision of nucleotide precursors to rapidly replicate neoplastic cells for accelerated proliferation and growth [26,27].

In the present study, there was no significant difference in the levels of vitamin B12 and plasma homocysteine between cases and controls. This finding is similar to the study conducted by Hartman TJ et al., in which no significant associations were seen between serum folate, vitamin B12, or homocysteine and lung cancer risk, but significantly lower risk of lung cancer was seen among males who had elevated serum vitamin B6 levels [28]. Two most common polymorphisms of MTHFR which have been delineated are C667T and A1298C [8]. In the present study, MTHFR polymorphism C677T was not detected in the blood and the bronchial biopsy samples of all cases as well as blood samples of all the controls. The MTHFR polymorphism A1298C on the other hand was present in the blood as well as bronchial biopsy samples of cases as well as blood of controls.

Positive association between MTHFR polymorphisms and cancer has been seen in previous studies on bladder cancer [29], liver cancer [30], and CRC [31]. In studies conducted on lung cancer, positive associations were seen in a few studies [15-17,32,33]. A recent study performed in a female Chinese population evinced that the MTHFR C677T polymorphism may contribute to the development of lung cancer and lung adenocarcinoma in this population. On the other hand, the MTHFR A1298C polymorphism may be associated with the decreasing risk of lung cancer [34].

A case-control study conducted on the polymorphisms of MTHFR and the risk of lung cancer found no substantiation for an association between the MTHFR C677T and A1298C polymorphisms and risk of lung cancer in either all of the subjects or the low folate intake subgroup; nor did it find evidence for an interaction between these two MTHFR polymorphisms and dietary folate intake or alcohol use [9]. It concluded that the MTHFR C677T and A1298C

polymorphisms by themselves do not play an important role in the aetiology of lung cancer.

The findings of the present study are comparable to the study done by Shen H et al., as no association could be observed between the MTHFR polymorphisms and lung cancer [13]. The lack of association between the MTHFR C677T and A1298C genotypes and risk of lung cancer in the present study suggests that the MTHFR variant genotype did not have an effect on lung cancer or that other molecular mechanisms such as DNA damage and repair may play a major role in the aetiology of lung cancer in this study population [35].

Limitation(s)

The main limitation of this study is the number of cases, leading to limited preliminary evidence showing association amongst these polymorphisms. However, this is one study where bronchial biopsy samples have been analysed for polymorphism.

CONCLUSION(S)

It may be concluded that high levels of serum folate may be associated with lung cancer. Moreover, MTHFR C677T and A1298C polymorphisms by themselves do not play an important role in the aetiology of lung cancer. The results imply that different tumours progress by diverse pathological mechanisms. Other processes, such as DNA damage and repair may be implicated in the aetiology and progression of lung cancer. Large studies are required to determine which genes show polymorphism in only lung cancer, so as to aid in the development of surrogate markers. Besides this, the present study acts as a platform for the future polymorphic studies to be conducted on lung cancer.

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