

RESEARCH ARTICLE

Combined Germline Variations of Thrombophilic Genes Promote Genesis of Lung Cancer

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Abstract

Background: A large variety of familiar and non-familiar lung carcinomas (LC) are caused by long term exposure to chemical carcinogens that are present in tobacco smoke. We aimed to investigate the prevalence of 5 thrombophilic germ-line mutations in patients with lung carcinomas. **Materials and Methods:** A total of 52 LC patients and 212 healthy controls from same population were analyzed for *FV* Leiden, factor V *H1299R* (R2), *PAI-1*, *MTHFR* C677T, *MTHFR* A1298C, *ACE* I/D, and *Apo E* genes and compared. **Results:** Overall, heterozygous and/or homozygous point mutations in *FV* Leiden *Apo E2*, *PAI-1* and *MTHFR* C677T genes were associated with LC in the current cohort. There was no meaningful association between LC and *ACE* I/D gene markers. **Conclusions:** The current results showed that LC is related to combined thrombophilic gene mutations and individuals with homozygosity of 4G in *PAI-1* and *MTHFR* C677T genes and heterozygosity of *FV* Leiden, *Apo E4* genes have a germ-line risk for LC tumorigenesis.

Keywords: A case control study - combined thrombophilic genes - lung cancer - germ-line variations

Asian Pac J Cancer Prev, 14 (9), 5449-5454

Introduction

Lung cancer (LC) is most common cancer type that considered as a major cause of morbidity and mortality through the worldwide (Cui et al., 2011; Gao et al., 2012). LC caused by many factors such as; asbestos, ionising radiation, environmental dust, occupational chemicals exposure, outdoor air pollution, cooking oil-coal fumes, smoke and molecular genetic variations. It is well known that the somatic and/or germ-line genetic variations contribute to individual differences in cancer susceptibility, cytotoxicity and individual drug efficacy. Those factors might also contribute to the LC initiation and/or progression. In recent years there are many studies that investigated the incidence of specific thrombophilic gene mutations in lung cancer. Some showed significant association (Bennet et al., 2007; Dahlback, 2008; Nacak et al., 2010; Kiyohara et al., 2011; Su et al., 2011; Tug et al., 2011) but still some reports showed lack of any association (Liu et al., 2009; Arslan et al., 2011; Dević et al., 2012; Zhang et al., 2012). According to the some recent reports the high portion of lung cancers in never smokers may be attributable to heritable different molecular genetic and epigenetic parameters. Couraud et al have reviewed that an estimated portion of 10-25% of LC in worldwide occur in never smokers and/or undersmokers of individuals having smoked less than 100 cigarettes in their lifetime (Couraud et al., 2012). Thu et al. (2012) have claimed that there are clinical distinctions between lung tumors

in smokers and never smokers extend due to alternative gene mutations of EGFR, EML4-ALK, and KRAS (Thu et al., 2012). Germ-line mutation profiles in some suspicious genes may associate to the distinct cancers in human. Reports on common thrombophilic mutations such as; *FV* Leiden, *MTHFR*, *PAI-1*, *ACE* and *Apo E* were discussed in the current article. Five common thrombophilic mutations that considered as a cancer-related genes by International Lung Cancer Consortium (ILCCO) were selected because of their prior evidence of an association with lung cancer in the current investigation. A single nucleotide polymorphism (SNP) in codons C677T and A1298C of *MTHFR* gene cause producing a thermolabile enzyme with reducing enzyme function and eventually defects in DNA hypomethylation (Siemianowicz et al., 2003; Prasad and Wilkhoo, 2012). Apolipoprotein E (*Apo E*) gene located chromosome 19q13.2.2 encodes three alternative alleles of epsilon 2 (E2), epsilon 3 (E3), and epsilon 4 (E4) that plays a crucial role in the cholesterol-triglycerides metabolism (Lam et al., 2013). Homozygous individuals harbouring the E4 allele have a higher total cholesterol level hence cardiovascular risk (Lam et al., 2013). We have previously reported the high frequency of *ApoE4* gene polymorphisms as a risk factor for LC (Arslan et al., 2011). The low enzymatic activity of the *MTHFR* C677T genotypic variant is associated with DNA hypomethylation, which may induce genomic instability or randomly reactivates the proto-oncogenes to oncogenes. Two common SNPs (*FVL*, G1691A and *FVR2*, H1299)

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are the specific point mutations that have been reported as a risk factors for the development of venous thrombosis for Factor V gene (Zaatari et al., 2006). The plasminogen activator inhibitor-1 gene (*PAI-1*) is an essential regulator of plasminogen activation and has been linked to the pathogenesis of lung carcinomas (Pavey et al., 1996; Chorostowska-Wynimko et al., 2004). The angiotensin-converting enzyme (*ACE*) is a zinc metallopeptidase that plays an important role in the blood pressure and inflammation. The insertion/deletion polymorphism (I/D) of a 287-bp deletion in intron 16 leads to decrease enzyme activity and associate with poor prognosis in lung cancer (Bakan et al., 1988; Yaren et al., 2008; Gao et al., 2012).

This study was designed to compare the prevalence of germ-line thrombophilic gene mutations in LC patients to healthy control individuals from the same population.

Materials and Methods

Patient and biological specimens

Totally, 52 [(46/88.46%) male and (6/11.54% female)] peripheral blood samples from patients with LC were investigated for germ-line thrombophilic genes mutations among patients of 61.34±2.47 (47-81) years mean age with clinically diagnosed and treated for LC. The germ-line mutation profiles for target genes that investigated in the couples with recurrent pregnancy lose (RPL), (Yenicesu et al., 2010) and current LC cohort were compared to the healthy individuals from the same population. Peripheral blood samples were obtained during routine diagnosis from LC patients in Cumhuriyet University Training and Research Hospital by the collaboration of department of medical genetics and chest between May 2007 and January 2010. Samples were used for genotyping for point mutations of *FV*, *PAI-1*, *MTHFR*, *ACE* and *Apo E* genes.

Genes

Five thrombophilic markers of thrombophilic genes; factor V G1691A (*FV* Leiden), factor V H1299R, plasminogen activator inhibitor-1 (*PAI-1*), two polymorphic regions for *MTHFR* (C677T and A1298C), *ACE* I/D and *Apo E* (E2, E3, E4) were analysed in the current results.

Mutation analysis

Peripheral blood tissues containing EDTA from patients and control group were used for genomic DNA isolation. The total genomic DNA was extracted by the MagnaPure Compact (Roche) and Invitex kit extraction techniques (Invitex®; Invisorb spin blood, Berlin, Germany). Target genes were simultaneously amplified in a biotin-labelled single multiplex amplification reaction (ViennaLab®; PGX-HIV StripAssay, Vienna, Austria) which is based on the reverse-hybridization principle automatically and by Real Time PCR, LightCycler 2.0 methods (Roche). The multiple polymerase chain reaction (PCR) was performed in a Perkin Elmer 9600 and the profile consisted of an initial melting step of 2min at 94°C; followed by 35 cycles of 30s at 94°C, 30s at 61°C, and 30s at 72°C; and a final elongation step of 7min at 72°C for stripAssay genotyping. High portion of samples were also analysed by real-time PCR technique (LightCycler

2.0, Roche). Briefly, LightCycler FastStart DNA Master HybProbes, master mix (water, PCR-grade, MgCl₂, stock solution, Primer mix, HtbProbe Mix) and DNA template were used for real-time amplification. The protocol consisted of a denaturation step of 30 seconds at 95°C. The amplification conditions for 45 cycles were; denaturation in 95°C for 10 seconds, annealing for 5-20 seconds, extension in 72°C, melting curve step with denaturation in 95°C, annealing for 30 seconds, melting in 95°C and cooling step in 40°C for 30 seconds. Software programme (LightCycler 2.0, Roche) was used for detection of the mutated and normal genotype profiles of target genes in the current LC and control couples.

Statistical analysis

Statistical analysis was performed using SPSS version 16 (SPSS, Chicago, IL, USA). The frequencies of homozygous and heterozygous thrombophilic gene mutations, the frequencies of allelic mutations in patients of LC and controls were compared using chi-square analysis. A value of p<0.05 was considered as statistically significant. The statistically significant mutation profiles were discussed in the current report.

Results

Peripheral blood-EDTA samples from healthy controls and LC patients were examined for genotyping in the current study. In a total of 52 LC patients [(46 male (88.46%) and 6 female(11.54%)] of 15(30.0%) small cell (SCLC), 37(70.0%) non-small cell (NSCLC) and mean age 61.34±2.47 (47-81) were compared to the 212 healthy individuals. Thirtyfour patients (65.39%) were heavy smoker who use >1 pocket/per day, seven were (13.46%) smoker who use <1 pocket cigarette/per day and/or passive smoker and eleven patients (21.15%) were non-smokers in the current studied cohort. Nineteen tumours (36.53%) were in grade II that greater than 3 cm and surrounded by lung or visceral pleura, three (5.77%) were in grade III that greater than 7 cm and invade parietal plural chest wall and thirty tumours (57.7%) were in grade IV that invade mediastinum and vessels.

Results show combined germ-line variations in *PAI-1*, *MTHFR* and *FVL* genes are susceptible in the promoting tumorigenesis in lung carcinomas when compare to the control group(healthy individuals) from the same population. Some germ-line thrombophilic gene mutations have been shown to be a risk factor for LC in the current cohort.

The prevalence of allelic mutations of *PAI-1* 5G/4G gene in patients with LC (11.5% for 5G/5G, 73.1% for 5G/4G and 15.4% for 4G/4G respectively) was higher than the control group (34.0% for 5G/5G, 57.0% for 5G/4G and 9.0% for 4G/4G respectively), (Table 1). The 4G allele frequency was 0.520 for LC patients and 0.366 for health individuals in the current results. The difference of *PAI-1* 4G allele frequency was also statistically significant when compared to the control group (Table 1), (OR: 0.53, CI: 0.34-0.82), p<0.05.

The prevalence of allelic mutations of *MTHFR* gene C677T SNP in patients with LC (53.8% for CC, 30.8%

Table 1. The Genotype and Allele Frequencies of MTHFR C677T and A1298C, PAI-1 5G/4G and ACE I/D Genes SNPs in Current Patients with Lung Carcinoma and Control Individuals from the Same Population

Gene/Genotypes	Patients n=52 n(%)	Controls ²⁴ n=212 n(%)	p value	Odds ratio	95%CI
MTHFR C677T					
CC	28 (53.8)	155 (73.0)	-	1.0	-
CT	16 (30.8)	57 (27.0)	0.205	1.55	0.78-3.08
TT	8 (15.4)	0 (0)	0.00	-	4.64-9.19
Alleles	C 72 (0.692)	367 (0.870)			
	T 32 (0.308)*	57 (0.130)	0.00	0.35	0.21-0.58
MTHFR A1298C					
A/A	31 (59.62)	72 (34.0)		1.0	-
A/C	15 (28.85)	140 (66.0)	0.000	0.25	0.12-0.49
C/C	6 (11.53)	0 (0)	0.000	-	2.47-4.46
Alleles	A 77 (0.740)	284 (0.660)			
	C 27 (0.260)	140 (0.340)	0.165	1.4	0.86-2.27
PAI-1 4G/5G					
5G/5G	6 (11.5)	72 (34.0)		1.0	-
5G/4G	38 (73.1)	121 (57.0)	0.003	3.76	1.51-9.35
4G/4G	8 (15.4)	19 (9.0)	0.004	5.05	1.56-16.32
Alleles	5G 50 (0.480)	275 (0.634)			
	4G 54 (0.520)**	159 (0.366)	0.004	0.53	0.34-0.82
ACE I/D					
I/I	10 (19.23)	67 (31.7)	-	1.0	-
I/D	30 (57.70)	105 (49.5)	0.098	1.92	0.87-4.17
D/D	12 (23.07)	40 (18.8)	0.135	2.01	0.79-5.07
Alleles	I 50 (0.481)	239 (0.564)			
	D 54 (0.519)	185 (0.436)	0.128	0.71	0.46-1.10

*The T allele in MTHFR C677T SNP was significant for patient group in the current LC cohort; Odds Ratio=0.35 (0.21-0.58), p<0.05, **The 4G allele was significant for the patient group of the current LC cohort; Odds Ratio=0.53 (0.34-0.82), p<0.05

Table 2. Results Show the Genotype and Allele Frequencies of Factor V G1691A Leiden and H1299R (R2) and ApoE Genes in Current Lung Carcinomas Cohort and Control Individuals from the Same Population

Gene/Genotypes	Patients n=52 n(%)	Controls ²⁴ n=212 n(%)	p value	Odds ratio	95%CI
Factor V G1691A Leiden					
G/G	46 (88.5)	207 (97.6)	-	1.0	-
G/A	6 (11.5)	5 (2.4)	0.003	5.4	1.58-18.45
A/A	0 (0.0)	0 (0.0)	-	-	-
Alleles	G 98 (0.942)	419 (0.988)			
	A 6 (0.058)*	5 (0.012)	0.003	5.13	1.53-17.1
Faktor V A4070G(R2)					
A/A	43 (82.7)	209 (98.6)	-	1.0	-
A/G	9 (17.3)	3 (1.4)	0.000	14.58	3.79-56.09
G/G	0 (0.0)	0 (0.0)	-	-	-
Alleles	A 95 (0.913)	421 (0.993)			
	G 9 (0.087)**	3 (0.007)		13.2	3.53-50.04
APOE					
E3/E3	37 (71.15)	167 (78.7)	-	1.0	-
E3/E2	6 (11.55)	23 (10.9)	0.740	1.17	0.45-3.09
E3/E4	7 (13.45)	22 (10.4)	0.440	1.43	0.57-3.61
E2/E4	2 (3.859)	0.0	0.003	-	4.12-7.38
Alleles	E3 87 (0.836)	379 (0.894)			
	E2 8 (0.077)	23 (0.054)	0.328	0.66	0.28-1.52
	E4 9 (0.087)	22 (0.052)	0.157	0.56	0.25-1.26

*The A allele was significant for FVL, Odds Ratio=5.13 (1.53-17.1), p<0.05; **The G allele was also significant for FVR2, Odds Ratio=14.58 (3.79-56.09), p<0.05

for CT and 15.4% for TT respectively) was higher than the control group (73.0% for CC, 27.0% for CT and 0.0% for TT respectively), (Table 1). The T allele frequency was 0.308 for LC patients and 0.130 for health individuals in the current results. The difference of MTHFR C677T SNP

T allele frequency was also statistically significant when compared to the control group (Table 3), (OR: 0.35, CI: 0.21-0.58), p<0.05. Nearly similar findings were detected in the second SNP marker of MTHFR A1298C gene in patients with LC (59.62% for AA, 28.85% for AC and 11.53% for CC respectively) and control group (34.0% for AA, 66.0% for AC and 0.0% for CC respectively), (Table 1). The C allele frequency was 0.260 for LC patients and 0.340 for health individuals in the current results. In spite of 11.53% patients group have a homozygous CC allele, the difference of MTHFR A1298C SNP C allele frequency was not statistically significant when compared to the control group (Table 1), (OR: 1.40, CI: 0.86-2.27). The frequency of E3/E3, E3/E2, E3/E4 and E2/E4 profiles for ApoE were 71.15%, 11.55%, 13.45% and 3.85% in the LC patients, and 78.7%, 10.9%, 10.4% and 0.0% in control group respectively. ApoE3 allele frequency was 0.836 for LC, 0.894 for control group, the ApoE2 frequency was 0.077 for LC, 0.054 for healthy individuals and the ApoE4 frequency was 0.087 for LC, 0.052 for healthy individuals (Table 2).

Both SNP markers of FV gene were statistically significant in the presented cohort of LC patients when compared to the healthy individuals from the same population. The prevalence of genotypes of GG, GA and AA profiles for FV-Leiden gene were 88.5%, 11.5% and 0.0% in the LC patients, and 97.6%, 2.4%, and 0.0% in control group respectively. The mutated A allele frequency was 0.058 for LC patients and 0.012 for control group (Table 2). The difference of FV-Leiden SNP A allele frequency was statistically significant when compared to the control group (Table 2), (OR: 5.13, CI: 1.53-17.1), p<0.05.

The prevalence of genotypes of AA, AG and GG profiles for FVA4070G (R2) gene were 82.7%, 17.3% and 0.0% in the LC patients, and 98.6%, 1.4%, and 0.0% in control group respectively. The mutated A allele frequency was 0.087 for LC patients and 0.007 for control group (Table 2). The difference of FV-Leiden SNP A allele frequency was statistically significant when compared to the control group (Table 2), (OR: 5.13, CI: 1.53-17.1), p<0.05.

Insertion-deletion mutation is a 287-bp fragment loss in intron 16 of ACE gene and associated with enhanced conversion of angiotensin I to angiotensin II in plasma. The prevalence of genotypes of I/I, I/D and D/D profiles for ACE gene were 19.23%, 57.70% and 23.07% respectively in the LC patients, and 31.7%, 49.5%, and 18.8% respectively in control group. D allele frequency was 0.519 for LC patients and 0.436 for control group (Table 2). Similar findings were detected in ACE I/D and ApoE variations in both LC and control groups. No statistically significant differences were found in the prevalence of the both studied genes (Table 1 and Table 2).

Discussion

The current study represents small number of LC patients and healthy individuals (52 lung cancer patients and 212 controls) from the same population reported

to evaluate a possible association between germ-line thrombophilic genes markers such as; *MTHFR* C677T/A1298C, *FV* Leiden/R2, *PAI-1*, *ACE* and *ApoE* gene polymorphisms and in the susceptibility of the lung cancer in Turkish population. The *FV* Leiden, *MTHFR*, *PAI-1* and *Apo E* showed significant association but *ACE* showed miscellaneous and lack of any association in the current LC cohort. This is the first reported study focusing on the association between multiple germ-line thrombophilic gene polymorphisms and the risk of lung cancer in a Sivas/Turkey population. Results have showed that germ-line mutations in *FV* Leiden, *MTHFR*, *PAI-1* and *Apo E* genes are leading to promote the LC in the presented cohort. The small sample size may be responsible for that discrepancy for the current results.

The epigenetic processes is recognized as being a common feature of human neoplasia by the hypermethylation and/or global genomic hypomethylation that occur simultaneously in CpG island in transformed cells. Homozygous and/or heterozygous *MTHFR* mutated individuals have a risk for being different solid cancers due to DNA hypomethylation and gene reactivation in the presence of smoke and other environmental chemicals. It is well known that both SNPs (C677T and A1298C) of *MTHFR* gene may lead to a decreased activity of the enzyme. Cui et al. (2011) also suggested that *MTHFR* C677T SNP may contribute to NSCLC development in Chinese women and could influence treatment response for that patients with platinum-based chemotherapy (Cui et al., 2011). Siemianowicz et al (2003) have reported that all SCLC and NSCLC patients had statistically significantly higher percentage of *MTHFR* 677TT genotype when compared to the non-cancer controls (Siemianowicz et al., 2003; Vaissière et al., 2009). A strong association was reported between hypermethylated *MTHFR* gene and lung cancer in heavy smokers (Thu et al., 2012). Stankova et al. (2005) have generate therapy model by antisense-mediated inhibition of *MTHFR* on survival of human cancer cells (Stankova et al., 2005) As claimed by Alberola et al. (2004) the carriers and homozygous mutated for the *MTHFR* 677T allele could benefit from supplementation with folic acid and vitamin B12 for early prevention of oncogenesis (Alberola et al., 2004). The prevalence of allelic mutations of *MTHFR* gene C677T SNP in the current patients with LC (53.8% for CC, 30.8% for CT and 15.4% for TT respectively) was higher than the control group (73.0% for CC, 27.0% for CT and 0.0% for TT respectively). The T allele frequency (0.308) for LC patients was higher than the health individuals (0.130) and difference was also statistically significant (OR: 0.35, CI: 0.21-0.58), $p < 0.05$. Lee et al (2012) have claimed that the low folate consumption due to *MTHFR* polymorphisms suggesting an important role of genetic and nutritional factors in protecting DNA damage (Lee et al., 2012). International Lung Cancer Consortium (ILCCO) has suggested 10 common gene variations from different cancer-related pathways including inflammation, folate metabolism, regulatory function, cell adhesion and apoptosis that associated with LC risk (Truong et al., 2010). Carriers of the factor V Leiden and R2 mutations appear to have 12-fold increased risk for LC (Shetty and

Idell, 2000; Bloom et al., 2005; Brüggemann et al., 2008; Onitilo et al., 2013). Both SNP markers of *FV* and *Apo E4* allele in *Apo E* genes were statistically significant in the presented cohort of LC patients when compared to the healthy individuals from the same population. The current results of LC patients from Turkish population showed that combined germ-line thrombophilic gene mutations play a crucial role in LC. Su et al. (2011) have claimed that the over-expression of *Apo E* in malignant pleural cells is associated with poor survival in lung adenocarcinoma (Su et al., 2011). As claimed by Shetty et al. (2000; 2008) and Li et al (2008) *PAI-1* gene is related to the pathogenesis of lung cancer but a the molecular mechanisms that regulate its expression in human lung cancer cells are not well understood (Shetty et al., 2000; 2008; Li et al., 2008).

Results showed higher prevalence of allelic mutations of *PAI 1* 5G/4G gene in patients with LC (11.5% for 5G/5G, 73.1% for 5G/4G and 15.4% for 4G/4G respectively) than the control group (34.0% for 5G/5G, 57.0% for 5G/4G and 9.0% for 4G/4G respectively). The 4G allele frequency (0.520) for LC patients was higher than the control group (0.366) and that difference was statistically significant (OR: 0.53, CI: 0.34-0.82), $p < 0.05$. As claimed by some reports increased plasma level of *PAI-1* and plasminogen activation system plays a pivotal role in the pathogenesis of tumor growth, poor prognosis and metastatic spread in many tumors including lung cancer (Werle et al., 2004; Zekanowska et al., 2004; Offersen et al., 2007). Offersen et al. (2007) have claimed that the over *PAI-1* are prognostic markers in NSCLC and whether they are related to angiogenesis (Offersen et al., 2007). It was found that the heterozygous mutations in *FV* Leiden, *FVR2* and the homozygous mutations frequencies of *PAI-1* and *MTHFR* C677T and *Apo E* (E4) genes were higher in LC patients when compared to the health controls.

Published studies on the relationships between *ACE* I/D polymorphism and lung cancer risk have been conflicting. Nacak et al. (2010) showed decreased *ACE* enzyme activity and plasma concentrations that associated with poor prognosis in lung cancer (Nacak et al., 2010). A meta-analysis of *ACE* gene I/D polymorphism suggest that the *ACE* gene A-240T polymorphism might be a genetic marker for the development of lung cancer (Gao et al., 2012). The association between Angiotensin-converting enzyme 2 (*ACE2*) and tumor growth, angiogenesis in lung cancer were also reported (Feng et al., 2011). The second SNP marker of *MTHFR* A1298C gene and insertion-deletion mutation is a 287-bp fragment loss in intron 16 of *ACE* genes were lack of any association in the current limited LC cohort.

In conclusion, The presented case-control results showed germ-line mutated thrombophilic genes profiles mainly have a potential risk for LC most possibly have a combined effect on the promoting tumorigenesis in lung cancer. Briefly, the germ-line homozygosity of 4G in *PAI-1* and *MTHFR* C677T genes and heterozygosity of *FV* Leiden, *FVR2*, and *Apo E2/4* genes should be consider as risk factors in LC. Studied mutations of thrombophilic genes could be associated with the disease and might be clinically useful as a marker to assess the germ-line risk for LC. Further studies with larger sample sizes are required

to validate that association.

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