Original Article

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TANAFFOS

The miR-196a SNP Rs11614913 but not the miR-499 rs37464444 SNP is a Risk Factor for Non-small Cell Lung Cancer in an Iranian Population

Neda K. Dezfuli [1, 2, 3], Ian M. Adcock [4], Shamila D. Alipoor [5], Babak Salimi [6], Sharareh Seifi [6], Mohammad Chehrazi [7], Mohammad Varahram [2], Esmaeil Mortaz [2,3,6]

[1] Department of Immunology and Laboratory Sciences, School of Allied Medical sciences, Dezful University of Medical Sciences, Dezful, Iran, [2] Clinical Tuberculosis and Epidemiology Research Center, National Research Institute of Tuberculosis and Lung Diseases (NRITLD), Shahid Beheshti University of Medical Sciences, Tehran, Iran, [3] Department of Immunology, School of Medicine, Shahid Beheshti University of Medical Sciences, Tehran, Iran, [4] Airways Section, National Heart and Lung Institute, Imperial College London, London, United Kingdom, [5] Molecular Medicine Department, Institute of Medical Biotechnology, National Institute of Genetic Engineering and Biotechnology, Tehran, Iran, [6] Chronic Respiratory Diseases Research Center, NRITLD, Shahid Beheshti University of Medical Sciences, Tehran, Iran, [7] Department of Epidemiology and Reproductive Health, Reproductive Epidemiology Research Center, Royan Institute for Reproductive Biomedicine, Tehran, Iran.

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Correspondence to: Mortaz E
Address: Clinical Tuberculosis and Epidemiology Research Center, NRITLD, Shahid Beheshti University of Medical Sciences, Tehran, Iran
Email address: emortaz@gmail.com

Background: Globally, lung cancer represents a major cause of cancer-related deaths. The regulation of gene expression is modulated by small noncoding RNAs called miRNAs that can act as both tumor suppressors and oncogenes. The maturation, expression and binding to target mRNAs is affected by single nucleotide polymorphisms (SNPs) in miRNA genomic regions thereby contributing to cancer susceptibility. SNPs Rs11614913 in miR196a and Rs3746444 in miR-499 are implicated in the development of cancers such as non-small cell lung cancer (NSCLC) in non-Arabic subjects.

Materials and Methods: A small cohort of 204 participants including 104 lung cancer patients and 100 non-cancer controls subjects were enrolled into the study. The allele frequencies were determined by Polymerase Chain Reaction–Restriction Fragment Length Polymorphism (PCR-RFLP) and their correlation with lung cancer risk was determined.

Results: The miR-196a rs11614913 polymorphism increased the risk of NSCLC (CC vs. TT+TC: OR= 2.26, 95%CI= 1.28 – 3.98, P= 0.0046) in a dominant genetic model. No statistically significant association was found between the miR-499 rs37464444 polymorphism and NSCLC.

Conclusion: The rs11614913 polymorphism in miR-196a, but not the miR-499 rs37464444 polymorphism, increased the risk of NSCLC. Further studies with larger sample sizes in correlation with functional outcomes at the cellular level should be undertaken.

Key words: rs11614913, miR196a; rs3746444; miR-499; Lung Cancer; NSCLC

INTRODUCTION

Lung cancer is currently the commonest cancer worldwide and has a poor prognosis being associated with very high mortality rates. Lung cancer is divided broadly into two main subtypes: small-cell (SCLC) and non-small-cell lung carcinoma (NSCLC). NSCLC accounts for 80-85% of all lung cancer cases whilst SCLC includes 12% of lung cancer cases particularly those with a high mortality (1, 2). Despite recent advances in the diagnosis and therapies available, lung cancer is still a major cause of death

worldwide (3). Understanding the molecular pathology of lung cancer is critical for obtaining early diagnosis and thereby enabling initiation of timely and effective therapies.

Although smoking is recognized as the major risk factor for the development of lung cancer, the disease also occurs in nonsmokers (4). Thus, genetics and lifestyle characteristics including diet, smoking and exposure to other environmental pollutants are important factors in the susceptibility and development of lung cancer (5). Inherited familiar gene changes in P53, Myc and breast cancer gene (BRCA)1 have been described in lung cancer (1, 6).

Noncoding small RNAs (ncRNA) are key factors in the development and progression of lung cancer (7). MicroRNAs are members of the small ncRNA family and act post-transcriptionally to modulate gene expression (8). MicroRNAs regulate various biological functions and may act to control the expression of oncogenes and/or tumor suppressors (8). Dysregulation of miRNA expression provokes cancer invasion, metastasis and angiogenesis (8). MicroRNA networks coordinately modulate numerous genes in the body (2, 7). A SNP occurs in just under every 300bp of the genome including the coding and non-coding regions (9). However, most (93%) of SNPs that affect miRNA function are distributed within non-coding regions (10) and it is well known that SNPs associated with cancer susceptibility (2). The induction of the single nucleotide polymorphisms (SNPs) at a specific site, especially in non-coding regions, affects the induction and maturation of miRNAs in cancer (8). For example, miRNA 196a rs11614913 T/C and the miR-499 rs3746444 A/G polymorphisms are associated with the development of breast (11,12), lung (13-15), gastric (16), esophageal (17), hepatocellular (18), head and neck (19) and colorectal (20, 21) cancers.

We hypothesize that polymorphisms within these miRNAs varies according to the ethnicity and geographical area of the patient. Thus, in this study we aimed to assess the possible association between miR-499 rs3746444, miR-196a rs11614913SNP in Iranian NSCLC patients.

MATERIALS AND METHODS

Patients

One hundred and four patients with newly diagnosed based on pathology and clinical manifestation of NSCLC at age 58.1 ± 8.0 years old (mean ± SD) were recruited at the Masih Daneshvari hospital (Tehran, Iran) between April 2015 and September 2019. One hundred age- and gender-matched healthy controls subjects with a negative history of cancer and other inflammatory diseases were also enrolled in the study. The Ethics Committee of the Dr. Masih Daneshvari Hospital approved the study and all subjects gave written informed consent (Ethics committee approval number: IR.SBMU.MSP.REC.1397.525).

Genotyping

2 ml whole blood was collected into EDTA-containing tubes from all participants and genomic DNA isolated using a DNA High Pure PCR Template Preparation Kit, (Mannheim, Roche, Germany, Version 20, Cat.No.11796828001) as described by the manufacturer. The DNA concentration was measured by Nanodrop 2000 (Thermo Fisher, MA, USA). Specific SNPs were genotyped using polymerase chain reaction–restriction fragment length polymorphism (PCR-RFLP) with the PCR reaction performed using Taq DNA polymerase master mix (Invitrogen, Massachusetts USA), in a thermal cycler (Bio-Rad, California, USA). The primer sequences for each PCR reaction are shown in Table 1. PCR cycles were as follows: initial denaturation at 95°C for 5 min, 35 cycles of denaturation at 94°C for 30 sec, annealing at 58°C for 1min and extension at 72°C for 1 min and a final extension at 72°C for 10 min.

To identify the mir-196 C/T polymorphism, the PCR product was digested with the restriction enzyme Taal (Thermo Fisher, USA) by incubating the samples at 65°C for 4h. The mir-499 T/C polymorphism PCR product was incubated at 37°C for 4h with the restriction enzyme

TSP45I (Thermo Fisher, USA) and the digestion products were detected by 3% agarose gel electrophoresis.

Statistical Analysis

The differences in genotype distribution for the two analyzed SNPs between patients and healthy subjects were analyzed using Chi-square test. All statistical analyses were carried out using SPSS-25 software (SPSS, Inc.). P values <0.05 were considered statistically significant.

RESULTS

The demographic information of participants including histological subtype, stage and smoking status are demonstrated in Table 2. 104 NSCLC patients and 100 healthy controls were enrolled in this study with mean age of 58.1 and 51.7 years, respectively (Table 2).

For rs11614913 of miR196 the uncut PCR product size was 431bp and digested products shows bands at 281 and 150bp (Table 1, Figure 1A). The PCR product size for rs3746444 of miR499 was 302bp and the digested products showed bands of 111 and 191bp (Table 1, Figure 1B). The allele frequencies for rs11614913 and rs3746444 in patient and control groups are indicated in Table 3. The CC genotype of mir-196a rs11614913 was associated with an increased risk of lung cancer using a dominant genotype model (CC vs. TT+TC: OR= 2.26, 95%CI= 1.28 – 3.98, P= 0.0046). In contrast, the mir-499 rs3746444 variant was not associated with NSCLC in any inheritance model tested (Table 3).

Table 1. PCR primer sequences and expected fragment sizes.

Polymorphism	Primer sequence	Restriction Enzyme	Product size (bp)
Rs11614913	F: 5'-CGGGGCTGAATTTCTTCCTTC -3' R: 5'-GCTGGACCCTCTTTGTCTGT -3'	TaaI	Uncut product: 431 C Allele: 431 T Allele: 150 + 281
Rs3746444	F: 5'-GTCTTCACTTCCCTGCCAAAT -3' R: 5'-GAAGCGTAAGAAGGCAGCATC -3'	TSP451	Uncut product: 302 T Allele: 302 C Allele: 111 + 191

Table 2. Demographic details of study participants.

Factors	Lung cancer patients (104 subjects) n (%)	Control subjects (100 subjects) n (%)
Age		
Mean±SD (Years)	58.1 ± 8.0	51.7 ± 8.4
Gender		
Male	81 (77.8)	80 (80)
Histological subtype		
ADC	87 (83.6)	
LCC	3 (2.8)	
SCC	14 (13.4)	
Stage		
I	3 (2.8)	
II	13 (12.5)	
III	20 (19.2)	
IV	68 (65.3)	
Smoking status		
Smoker	64 (61.5)	64 (64)
Non-Smoker	40 (38.4)	36 (36)

Table 3. Genotypic and allelic frequencies of miR-499 rs3746444, miR-196a2 rs11614913 polymorphisms in participants.

Polymorphism	NSCLC (104 subjects) n (%)	Control (100 subjects) n (%)	OR (95% CI)	P-value
miR-196a2 rs11614913 C > T				
Allele				
T	64 (30.7)	82 (41)	1 (reference)	
C	144 (69.2)	118 (59)	1.56 (1.04- 2.35)	0.031
Codominant				
TT	16 (15.3)	16 (16)	1 (reference)	
CT	32 (30.7)	50 (50)	0.64 (0.28 -1.45)	0.28
CC	56 (53.8)	34 (34)	1.64 (0.73 -3.71)	0.22
Dominant				
CT+TT	48 (46.1)	66 (66)	1 (reference)	
CC	56 (53.8)	34 (34)	2.26 (1.28 -3.98)	0.0046
Recessive				
TT	16 (15.3)	16 (16)	1 (reference)	
CC+CT	88 (84.6)	84 (84)	1.04 (0.49 -2.22)	0.9
miR-499 rs3746444 T > C				
Allele				
T	150 (72.1)	148 (74)	1 (reference)	
C	58 (27.8)	52 (26)	1.10 (0.71- 1.70)	0.66
Codominant				
TT	54 (51.9)	54 (54)	1 (reference)	
TC	42 (40.3)	40 (40)	1.05 (0.59 -1.86)	0.86
CC	8 (7.6)	6 (6)	1.33 (0.43 - 4.1)	0.61
Dominant				
TT	54 (51.9)	54 (54)	1 (reference)	
TC+CC	50 (48.07)	46 (46)	1.08 (0.62 -1.88)	0.76
Recessive				
TT+TC	96 (92.3)	94 (94)	1 (reference)	
CC	8 (7.6)	6 (6)	1.3 (0.43 -3.9)	0.63

OR = Odds Ratio.

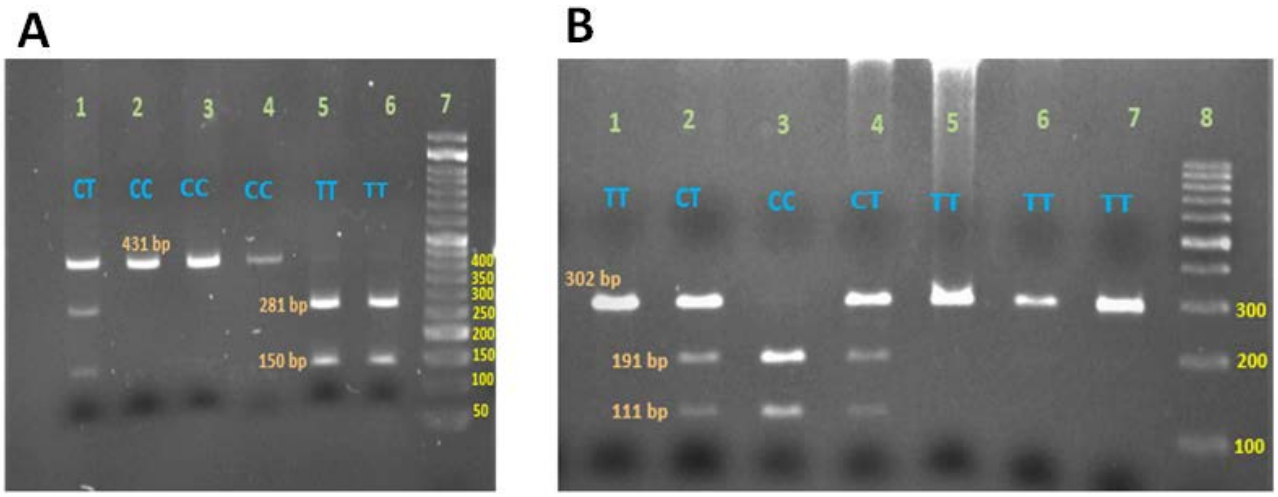

Figure 1. Representative PCR-RFLP gels. (A) miR-196 PCR products and genotypes. Lane 1 shows the CT genotype (bands at 431, 281 and 150bp); lanes 2, 3 and 4 show the CC genotype (band at 431bp); Lanes 5 and 6 show the TT genotype (bands at 281 and 150bp) and lane 7 is the 50bp DNA ladder. (B) miR-499 PCR products and genotypes. Lanes 1, 5, 6 and 7 show the TT genotype (band at 302bp); Lanes 2 and 4 show the CT genotype (bands at 302, 191 and 111bp); lane 3 shows the CC genotype (bands at 191 and 111bp) and Lane 8 is the 100bp DNA ladder with specific size markers labelled.

Rs3746444 variants were not associated with smoking status (Table 4) whereas the CC genotype frequency in smoking patients was higher than in smoking control subjects with the rs11614913 variant (OR=2.82, 95%CI=1.01-7.83, P=0.045) (Table 4).

In addition, the C allele frequency of the rs11614913 variant correlated with stage II and III NSCLC (OR=0.09, 95%CI=0.012-0.67, P=0.019 and OR=0.14, 95%CI=0.02-0.92, P=0.041, respectively). There was no association between the rs3746444 SNP and disease stage (Table 5).

Moreover, the rs11614913 SNP in miR196 was not associated with types of NSCLC disease, however, the C allele frequency in rs3746444 of miR499 variant was higher in the large cell carcinoma (LCC) subtype (OR=0.06, 95%CI=0.007-0.59, p=0.015) (Table 5).

Table 4. The association between SNPs and the risk of NSCLC stratified by smoking

Gene	SNP	Non-Smokers				Smokers			
		Control (n)	Case (n)	OR (95%CI)	P Value	Control (n)	Case (n)	OR (95%CI)	P Value
					rs11614913				
	TT	3	8	1		13	8	1	
miR-196a	TC	22	16	0.27 (0.06- 1.19)	0.08	28	16	0.92 (0.31-2.71)	0.89
	CC	11	16	0.54 (0.11-2.52)	0.43	23	40	2.82 (1.01-7.83)	0.045
					rs3746444				
	TT	18	23	1		36	31	1	
miR-499	TC	16	12	0.58 (0.22-1.54)	0.28	24	30	1.45 (0.70-2.98)	0.31
	CC	2	5	1.95 (0.33-11.2)	0.45	4	3	0.87 (0.18-4.19)	0.86

Table 5. Association between SNPs with stage and subtypes of lung cancer

Variable	rs11614913 T/C						
MiR-196a	TT	TC	CC	Allele T frequency	Allele C frequency	Adjusted OR (95%CI)	P Value
Stage I (n=3)	2	0	1	4	2		
Stage II (n=13)	1	2	10	4	22	0.09 (0.012-0.67)	0.019
Stage III (n=20)	0	9	11	9	31	0.14 (0.02-0.92)	0.041
Stage IV (n=68)	13	21	34	47	89	0.26 (0.04-1.49)	0.13
ADC Type (n=87)	12	28	47	52	122		
SCC Type (n=14)	3	4	7	10	18	1.3 (0.56-3.01)	0.53
LCC Type (n=3)	1	0	2	2	4	1.17 (0.20-6.60)	0.85
	rs3746444 T/C						
MiR-499	TT	TC	CC	Allele T frequency	Allele C frequency	Adjusted OR (95%CI)	P Value
Stage I (n=3)	1	2	0	4	2		
Stage II (n=13)	5	8	0	18	8	1.12 (0.16-7.45)	0.9
Stage III (n=20)	8	8	4	24	16	0.75 (0.12-4.58)	0.75
Stage IV (n=68)	40	24	4	104	32	1.62 (0.28-9.28)	0.58
ADC Type (n=87)	49	32	6	130	44		
SCC Type (n=14)	5	9	0	19	9	0.70 (0.30-1.69)	0.44
LCC Type (n=3)	0	1	2	1	5	0.06 (0.007-0.59)	0.015

DISCUSSION

The current study reports that the miR-196a2 rs11614913 polymorphism, but not the miR-499 rs3746444 polymorphism, was significantly associated with Iranian NSCLC patients. In addition, there was an association of the rs11614913 polymorphism with the CC genotype in smoking NSCLC patients and of the rs11614913 C allele frequency with stage II and III disease. There was also a higher frequency of the rs3746444 C allele in LCC patients.

Previous studies reported that the rs11614913 polymorphism is associated with increased risk of lung cancer (13, 15). Meta-analysis of published studies reported that the rs11614913 polymorphism was associated with an increased risk of lung cancer particularly in Asian populations (15, 22). In our study, the rs11614913 polymorphism did not show any association with the types of NSCLC disease. Little information is available in Iranian NSCLC patients regarding miRNA polymorphisms although one study from North-East Iran also failed to find any significant association between the miR-196a2 rs11614913 polymorphism and lung cancer (23). This discrepancy between our study and the one from North-East Iran of similar size may be due to practical reasons such as usage of different enzymes, different participants from the two regions and type of NSCLC. Thus, larger, multi-centred studies across Iran using the same standardised methodology are required to confirm the role of the rs11614913 polymorphism in NSCLC patients in Iran.

The CC genotype of rs11614913, which significantly increased the expression of the mature miR-196a, was associated with decreased survival of NSCLC patients (13). Furthermore, individuals carrying the TC or CC genotype of rs11614913 had an increased risk of lung cancer compared to those possessing the TT genotype among Chinese non-smoking females (14). In addition, miR-196a2 rs11614913 variant homozygote CC was associated with approximately 25% significantly increased risk of lung cancer in the Chinese population (24). Yoon and et al. reported that the rs11614913 genetic variant positively correlated with a better recurrence-free survival (RFS) in stage II and stage III of lung cancer. Overall, these findings indicated that the rs11614913 polymorphism is strongly associated with prognosis in NSCLC patients who undergo lung resection (25).

Moreover, the rs11614913 genotypes were significantly associated with overall survival (OS) and disease-free survival (DFS) in women and in patients with stage II+IIIA disease, but not in men and patients with stage I disease (26). However, there was no difference in genotype-related adjusted hazard ratios (aHR) between the different subgroups of NSCLC (26). In contrast, the CC genotype in rs11614913 was associated with lower survival compared with TT/CT genotypes in NSCLC patients (13).

In addition, the C allele frequency in rs11614913 was higher in stage II and III NSCLC in the current study. Importantly, a previous study has shown that the TC vs. TT genotype of rs11614913 is protective for NSCLC and may reduce the risk of NSCLC in the non-SCC subgroup (27). Furthermore, an earlier study reported a significant association between the miR-196a2 rs11614913 (CT/TT) genotype with NSCLC patients who are active smokers in a Korean population (28). Our data also showed that the rs11614913 polymorphism was associated with the CC genotype in smoker patients.

The rs11614913 polymorphism is also associated with other cancers such as head and neck (19), hepatocellular (22) and breast cancers (29). The presence of any variant allele was associated with a significantly reduced risk of HNSCC but homozygous variant allele carriers with pharyngeal tumors had significantly reduced survival compared to wild-type and heterozygous forms (19). Moreover, the CC polymorphic genotype demonstrated associated with a decreased risk of breast cancer and the presence of the T allele was significantly associated with an increased risk of breast cancer (29). The functional SNP rs3746444 T/C within the miR499 gene causes an A/G transition in the mature miR-499 (13).

The ethnic background of patients with the miR-499 rs3746444 polymorphisms may affect its impact on the

susceptibility to lung cancer. The rs3746444 polymorphism is associated with decreased expression of miR-499 and poor survival in Chinese lung cancer patients (30). In contrast, Hau Qiu and co-workers found that the *miR-499a* rs3746444 genotypic distribution was not different in NSCLC cases and controls but this polymorphism elevated the susceptibility of NSCLC in a never smoking subgroup (adjusted *P*=0.035 for GG vs. AA genetic model and adjusted *P*=0.049 for GG vs. AA/AG genetic model) (27). Furthermore, Serena Vinci and colleagues did not find any association between miR-499 genotype and risk of NSCLC in 101 Italian NSCLC patients (31). A meta-analysis showed no association of the rs3746444 polymorphism and lung cancer in East Asian populations (32). However, another meta-analysis has shown an association between the microRNA-499 rs3746444 A/G polymorphism and cancer susceptibility in Asians, but not in Caucasians (33).

Subgroup analysis of other cancer types, demonstrated no risk of breast, liver, or lung cancers with the microRNA-499 rs3746444 A/G polymorphism (33). An association between miR-499 rs3746444 and the susceptibility to cervical squamous cell carcinoma, prostate cancer, hepatocellular carcinoma (34), chronic obstructive pulmonary disease (35) and colorectal cancer has been reported (36). The G Allele of rs3746444 was also shown to be associated with the decreased risk of prostate cancer progression in a Serbian population (37). In addition, miR-499 rs3746444 increased the risk of cancer (38) but not for breast cancer (20, 39, 40). In another study, the rs3746444 G allele was associated with an increased cancer risk factor in Chinese subjects especially for breast cancer (41). This discrepancy may be due to difference in ethnic background, since, there was a significant association of rs3746444 with the susceptibility to cancers in Asians (12, 42, 43) but not in Caucasians (44). Hashemi and colleagues have shown that the miR-499 rs3746444 polymorphism increased the risk of prostate cancer in an Iranian population (45) whilst the rs3746444 T > C polymorphism was associated with high prevalence of cancer in Iranian and Chinese populations but low prevalence with esophageal cancer (44).

There are some limitations in our study. First, this is a single center retrospective study. Due to the untimely COVID-19 epidemic, the sample size was small and lacked longitudinal samples. Future studies should include multi-centre studies across Iran and other middle eastern countries with different stages of NSCLC stages to verify these results. In addition, it will be important examine the functional impacts on the survival of NSCLC patients.

CONCLUSION

In conclusion, the current study data suggests an ethnic difference in the impact of rs3746444 T/C polymorphism in NSCLC lung cancer incidence. Our findings proposed that miR-196a2 rs11614913 polymorphism increased the risk of NSCLC lung cancer. In addition, the results do not support an association between the genetic variant of miR-499 rs37464444 and the risk of developing lung cancer. Additional larger clinical studies together with an analysis of the related cell functional outcomes are required.

Competing Interests

The authors declare that they have no competing interests.

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