A FUNCTIONAL POLYMORPHISM RS4938723 IN THE PROMOTER OF MIR-34B/C IS ASSOCIATED WITH AN INCREASED RISK OF LUNG CANCER

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ABSTRACT

Background: The expression levels of some microRNAs (miRNAs) in lung cancer have been associated with an increased risk of cancer. miRNAs play a significant role in the pathogenesis of human cancers. Because of this, miRNA polymorphisms can be important for carcinogenesis. MiR-34 is a family of miRNAs known to have reduced levels of expression in lung cancer and other human cancers (pancreas, colon). It functions as a tumor suppressor and targets oncogenes such as MET, RET, and RAB43. Additionally, the miR-125 family is related to many cancer types and targets P53, BCL2, VEGF, and EGFR.
Methods: In this study, we investigated three polymorphisms (rs35301225 C/A, T, rs4938723 T/C, and rs12976445 C/T) in respectively miR-34a, miR-34b/c, and miR-125a. The study population consisted of 100 patients with lung cancer and 100 healthy controls. Blood samples was collected into EDTA-containing tubes and genomic DNA was extracted. Genetic polymorphisms were identified using the polymerase chain reaction-based restriction fragment length polymorphism (PCR-RFLP).
Results: We observed that individuals carrying the miR-34b/c rs4938723 variant heterozygote CT exhibited a significant increase in the risk of lung cancer when compared to those with the wild-type homozygote TT ($p<0.01$). Moreover, the presence of the C allele in this polymorphism was significantly associated with an elevatede risk of lung cancer ($p<0.001$). Additionally, the T/T haplotype of miR-34b/c and miR125a polymorphisms was found to confer a protective effect against lung cancer in our haplotype analysis ($p<0.001$).
Conclusions: The rs4938723 polymorphism in miR-34b/c is thought to play an essential role in the pathogenesis of lung cancer. The frequency of the T/T haplotype for rs4938723/rs12976445 polymorphisms was significantly higher in the control group compared to the group of lung cancer patients.

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INTRODUCTION

Lung carcinoma is the leading cause of cancer-related deaths for both men and women in Turkey and worldwide. According to GLOBOCAN 2020, there were an estimated 2.2 million new cases of lung cancer (comprising 11.4% of all cancer cases) and nearly 1.8 million lung cancer-related deaths (18.0% of all cancer-related deaths) in 2020 (Sung et al., 2021). Several factors, such as diet, environmental factors (e.g. carcinogenes, smoking, air pollution) and genetic factors, can contribute to the development of lung carcinoma (Leiter et al., 2023). Genetic factors, including changes in the DNA sequence and mutations in oncogenes, tumor suppressor genes, and other related genes have been found to play roles in cancer development (Zabransky et al., 2022). Furthermore single nucleotide polymorphisms (SNPs) are an important factor in increasing susceptibility to cancer (Shastry, 2009).

MicroRNAs (miRNAs) are small non-coding RNAs, typically 21-24 nucleotides in length. miRNAs are transcribed by RNA polymerase II and function as post-transcriptional regulators that can cleavage or bind to mRNA, thereby inhibit translational process. miRNA genes are located within cancer-associated genomic regions (Rani et al., 2022). Any genomic changes such as single nucleotide polymorphisms in miRNA genes; have the potential to alter the structure of miRNA or miRNA expression levels. Therefore, these changes can influence targets in cancer-related pathways (Tian et al., 2022).

miR-34 is a family of miRNAs known to exhibit reduced levels of expression in lung cancer and other human cancers, such as pancreas and colon. It functions as a tumor suppressors and targets oncogenes like MET, P53 and RAB43. The miR-34 family consists of three mature miRNAs; miR-34a, miR-34b, and miR-34c (Zhang et al., 2019, Li et al., 2021). The rs35301225 polymorphism in miR-34a (C>A,T) can induce structural changes in pre-miRNA (Jiang et al., 2017). Therefore, polymorphism can be significant in influencing the tumor suppressor role of miR-34a.

The rs4938723 (T>C) polymorphism in the promoter region of pri-miR-34b/c may be significant because it can affect the binding of GATA-X and alter the transcription activities of the promoter (Xu et al., 2011). However, these polymorphisms have not been investigated in the context of lung cancer.

miR-125 is a family of miRNAs that implicated in a variety of carcinomas and other diseases. The miR-125 family is composed of miR-125a, miR-125b-1 and miR-125b-2 (Wang et al., 2019). miR-125 targets several important functional genes, including P53, BCL2, VEGF, LIN28A and others (Wang et al., 2023).

miR-125 is typically found to be down-regulated in various cancers, including lung cancer and functions as a tumor suppressor (Wang et al., 2019). The rs12976445 (C>T) is a polymorphism belong to mature miR-125a. This polymorphism has been reported to play a role in the carcinogenesis of breast cancer (Li et al., 2009). In a study on lung cancer, researchers found that the rs12976445 polymorphism had a significant impact on 18FDG uptake in patients with non-small cell lung cancer (Zang et al., 2016). Another study on rs12976445 forint he context of lung cancer suggested that T allele of this polymorphism increases the risk of radiation-induced pneumonitis in patients with non-small-cell lung cancer (Quan et al., 2018).

Therefore, we hypothesized that these polymorphisms might be related to the risk of lung cancer. In this study, we performed a case –control study to inverstigate the prevalence of these polymorphisms in the Black Sea Region of Turkey.

MATERIALS AND METHODS

The study included 100 patients diagnosed with lung cancer and 100 healthy individuals as the control group. Informed consent was obtained from all of the participants, and the study was approved by Ethics Committee for Clinical Research of Zonguldak Bulent Ecevit University, Faculty of Medicine (Zonguldak, Turkey).

2 ml EDTA-blood samples were collected from patients and healthy individuals. Immediately after collection, whole samples were stored at -20 ºC until use. Genomic DNA was extracted from 250 µl blood using E.Z.N.A.® Blood DNA Extraction Kit (Omega Bio-tek).

A PCR-based restriction fragment length polymorphism method (PCR-RFLP) was used to detect genotype miR-34a rs35301225, miR-34b/c rs4938723 and miR-125a rs12976445 polymorphisms. The PCR was performed in a 25 µl volume containing 20 ng genomic DNA, 10X PCR buffer with 1.5 mM MgCl2, 0.25 mM dNTPs, 10% dimethylsulphoxide, 0.5 units of Taq polymerase (Fermantas, MBI), and 5 pmol of each primer. The PCR thermal cycling conditions were as following; an initial melting period at 95 ºC for 3 min, 35 amplification cycles at 95 ºC for 45 s, annealing temperature (55 °C for miR-34a , 57 °C for miR-34b/c and 58 °C for miR-125a) for 45 s, 72 ºC for 45 s and a final elongation 7 min at 72 ºC. The PCR products were analyzed on a 2% agarose gel. Then the PCR products digested with specific restriction enzymes (Table 1). The digestion products were electrophoresed on 3% agarose gel and visualized by staining with ethidium bromide and evaluated using the gel documentation system (Vilber-Lourmat, Cedex, France)).

A case –control study was performed, and the $\chi 2$ test was used to compare the genotype frequency of polymorphisms between the patients with lung cancer and controls. The association between polymorphisms and lung cancer patients was modeled through binary logistic regression analysis. The OR value and 95% confidence interval (CI) were calculated to compare lung cancer risk around genotypes. The characteristics of the individuals in both groups were compared with Student's t-test. All values are represented as a mean and standard deviation. $p<0.05$ was considered as significantly different. The software used for the calculation was the SPSS version 18.0 (SPSS Inc., Chicago, IL).

Table 1: PCR - RFLP details for polymorphisms.

Gene	SNP ID	Primers	Annealing Temp.	Restriction enzyme	Restriction conditions	Restriction products
miR-34a	rs35301225 C/T,A	F: *5' CAA ACA CTG GCT ACT ATT CTC CCT A 3'* R: *5' ATC CTT TCT TTC CTC CCC ACA TTT C 3'*	55 ^0C	DdeI and MseI (Thermo Scientific)	37 and 65 ^0C	$\underline{DdeI}$ C: 300, 131 bp T: 431 bp A: 431 bp $\underline{MseI}$ C: 431 bp T: 300, 131bp A: 431 bp

miR-34b/c	rs4938723 T/C	F: *5' CCT CTG GGA ACC TTC TTT GAC CCA T 3'* R: *5' CTC TGT TGG GGA CTT GGC CTT ATA 3'*	57 ^0C	BccI (New England BioLabs)	37 ^0C	T: 265 bp C: 235, 30 bp
miR-125a	rs12976445 C/T	F: *5' ATC CCC TCC TTC CCC TGA A 3'* R: *5' ATC GTG TGG GTC TCA AGG C 3'*	58 ^0C	BseSI (Thermo Scientific)	55 ^0C	C: 210, 329 bp T: 539 bp

RESULTS

The study included 100 patients (11 women and 89 men) who were diagnosed with lung cancer and a control group of 100 healthy individuals (12 women and 88 men). There were no significant differences in gender between two groups (p=0.825). Additionally, there were no significant differences in age between the patient group (mean age 63.4 ± 9.6 years) and the control group (mean age 62.1 ± 9.3 years) (p=0.331).

According to our results, the miR-34a polymorphism was not statistically significant for the risk of lung cancer. Both controls and patients have homozigous major alleles (Figure 1a, Figure 2a). Therefore, relative risk analysis couldn't be done. This polymorphism does not appear to be associated with human lung cancer.

However, concerning the miR-34b/c polymorphism, a significant association with the risk of lung cancer was observed. 42 patients with lung carcinoma have CT genotype of rs4938723 while 58 patients have TT genotype. Interestingly all of the control group have TT genotype. Not any CC genotype was observed in both patients and controls (Figure 1b, Figure 2b). Consequently, we found that CT genotype of rs4938723 is significantly associated with an increased risk of lung cancer (p<0.001). However, the odds ratio (OR) could not be calculated. Furthermore, C allele of rs4938723 (p<0.001) is statistically significant risk factor for lung cancer compared to T allele.

The rs12976445 polymorphism of miR-125a did not show a significant association with human lung cancer. 58 patients have CT genotype of this SNP while 42 patients have TT genotype. Although there were 54 CT and 46 TT genotypes in the control group, both of patients and controls have not homozygously normal genotype (CC) of rs12976445 (Figure 1c, Figure 2c). TT and CT genotypes were not significantly different between the patients and the control group (p=0.569 and OR=0.850 (0.486-1.487)). Also, C and T alleles of this polymorphism were not associated with the risk of lung cancer (p=0.656 and OR=0.906 (0.585-1.401)).

Additionally, haplotype frequency and association analyses between miR-34b/c rs4938723 and miR-125a rs12976445 were also performed (Figure 2d). T/C haplotype is the reference haplotype as it consists of non-polymorphic alleles. While C/T haplotypes was not observed in the controls, C/C haplotype was not observed in both groups. The frequency of T/T haplotype were significantly higher in controls (p<0.001 and OR=0.606 (0.386-0.952)). T/T haplotype appears to be protective against lung cancer.

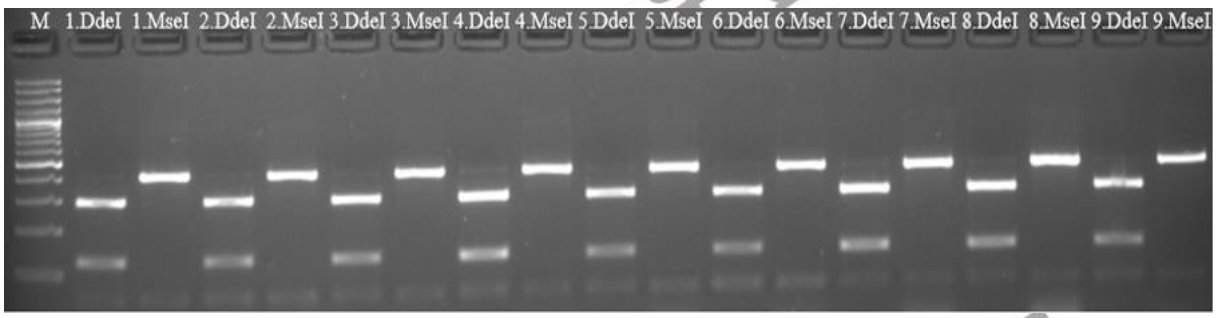

Figure 1a: Digestion results of miR-34a gene polymorphism: The first well contains 100 bp marker (M). DdeI and MseI enzyme digest products of a sample were loaded on gel consecutively. DdeI enzyme digests C allele of the gene and MseI enzyme digests T allele. The figure has results of 9 patients and all of them have CC genotype. Also, both of patients and controls have CC genotype of this polymorphism.

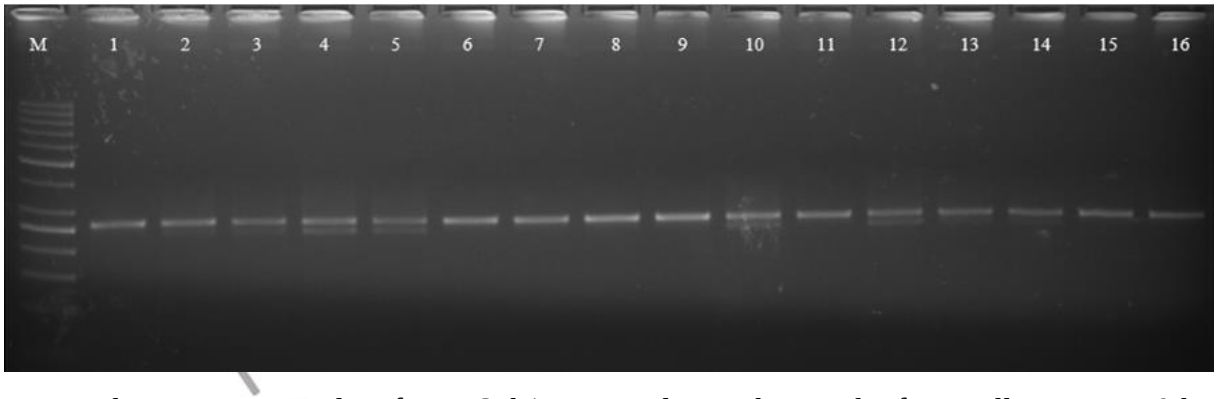

Figure 1b: Digestion results of miR-34b/c gene polymorphism: The first well contains 50 bp marker (M). BccI enzyme digests 265 bp product to 235 bp and 30 bp when has C allele. 30 bp product cannot be seen in the figure. Wells that contain 265 bp product have TT genotype (i.e. 6. patient) and wells that contain 265 bp and 235 bp products have CT genotype (i.e. 4. patient). Results have not CC genotype in both patients and controls.

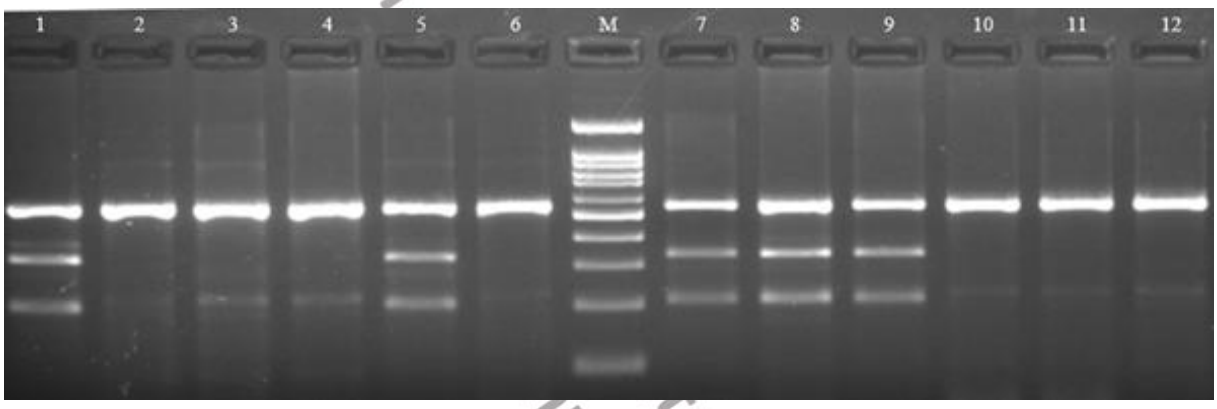

Figure 1c: Digestion results of miR-125a gene polymorphism: The well in the middle contains 100 bp marker (M). BseSI enzyme digests 539 bp product to 329 bp and 210 bp when has C allele. Wells that contain 539 bp product have TT genotype (i.e. 2. patient) and wells that contain 539 bp, 329 bp and 210 bp products have CT genotype (i.e. 1. patient). Results have not CC genotype in both patients and controls.

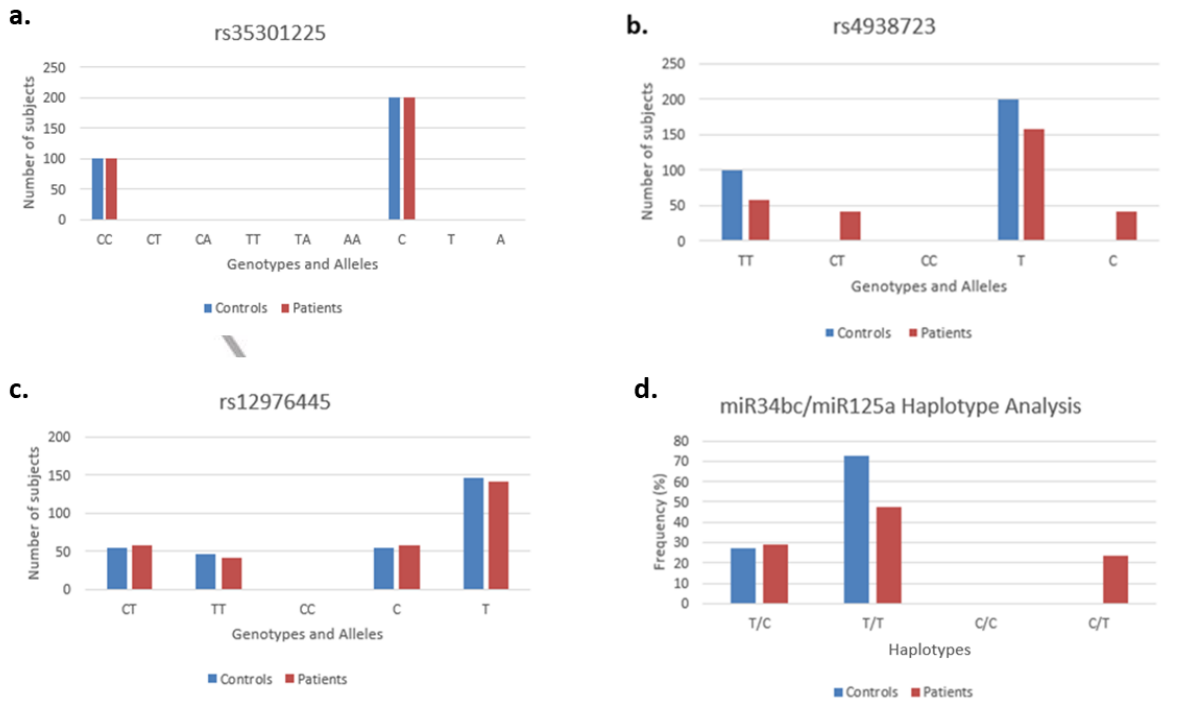

Figure 2: Bar charts of polymorphisms for lung cancer patients and controls a. Number of rs35301225 polymorphism genotypes and alleles in lung cancer b. Numbers of 4938723 polymorphism genotypes and alleles in lung cancer CT genotype and C allele of rs4938723 were significantly higher in patients ($p<0.001$). c. Numbers of rs12976445 polymorphism genotypes and alleles in lung cancer d. Haplotype frequencies of miR125a/miR34b/c polymorphisms in patients with lung cancer and controls. The frequency of T/T haplotype were significantly higher in controls ($p<0.001$).

DISCUSSION

In this study, we investigated the association between three gene polymorphisms and the risk of lung cancer in the Black Sea Region of Turkey. These polymorphisms were located in miR-34a, miR-34b/c and miR-125a genes. We found that rs4938723 in pri-miR-34b/c was associated with lung cancer. This polymorphism has provided an increased risk for lung cancer in individuals from Black Sea Region of Turkey.

Previously, it was reported that miRNAs could target oncogenes and tumor suppressor genes, altered miRNA expression levels that ultimately influence the development of human cancers. Both of these called as oncomiRs due to their significant roles in carcinogenesis (Esquela-Kerscher and Slack, 2016). Genetic alterations in miRNA genes may be associated with various cancers by altering the expression level or function of miRNAs and their target genes. Therefore, changes in these genes play an important role in cancer. As a result, numerous researchers have investigated miRNA polymorphisms and their associations with miRNA target regions and cancer-related genes (Iwai and Naraba, 2015).

The miR-34 family consists of tumor-suppressive miRNAs that are thought to target important genes such as BCL-2, CDK4, CDK6, E2F3, MET, c-MYC, SIRT1 in various cancer types (Wang et al., 2022). These miRNAs are regulated by p53 and play a role in gene expression, cell senescence and apoptosis (Li et al., 2021). miR-34a has been found to be down-regulated in different types of cancer including pancreas cancer (Chang et al., 2007), neuroblastoma (Welch et al., 2007), melanoma (Yan et al., 2008) and lung cancer (Gallardo et al., 2009).

Gong et al. (2012) showed that rs35301225's variant allele (located in miR-34a mature sequence) resulted from the loss of interaction between the miRNA and its related target by using bioinformatics analysis. Therefore, this polymorphism is believed to play an important role in various diseases, despite not being reported in large sequencing projects. Jiang et al. (2012) reported a strong association between this polymorphism and a decreased risk of colon cancer within the Chinese population. However, in our study, we did not find any significant association between the rs35301225 polymorphism and lung cancer.

In previous studies, down-regulation of miR-34b/c (with DNA methylation) was found in human cancers such as colorectal cancer (Toyota et al., 2008) and oral cancer (Kozaki et al., 2008). The SNP rs4938723, located in the CpG island of pri-miR-34b/c, can create a predicted transcription factor GATA binding site. Consequently, it may affect miR-34b/c expression and also carcinogenesis. In 2011, Xu et al. reported that the CT and CC genotypes of this polymorphism were associated with a significantly increased risk of hepatocellular carcinoma in the Chinese population (Xu et al., 2011). Son et al. (2013) found similar results for the Korean population. Li et al. (2013) also found a significantly increased risk of nasopharyngeal carcinoma for this polymorphism. However Yin et al. (2013) found that CC genotype of rs4938723 was associated with a decreased risk of esophageal cancer in Chinese population. In 2015, Pan et al. identified the SNP associated with decreased risk of gastric cancer, whereas Yuan et al. (2016) reported that CT genotype was significantly associated with an increased risk of developing cervical carcinogenesis. In the present study we found an increased risk of lung cancer associated with the CT genotype of rs4938723.

The miR-125 family has three members; miR-125a, miR-125b1 and miR-125b2. Researchers report that the miR-125 family is associated with various diseases, including carcinomas (Wang et al., 2019). This family plays crucial roles in apoptosis, cell differentiation and proliferation by targeting transcription factors and growth factors. According to miRNA target prediction tools, the miR-125 family targets P53, BCL2, VEGF, EGFR and LIN28A (Wang et al., 2023). VEGF plays critical roles in angiogenesis and vascular growth and exhibits high expression levels in most malignancies including lung cancer. Additionally, EGFR is a growth factor receptor that has important roles and its mutations identified as a potential target at patients with lung cancer (Zang et al., 2016, Na et al., 2010]. Targeted miR-125a has been found to be down-regulated in various cancers including breast, pancreatic, ovarian, colon, gastric and lung cancer (Wang et al., 2019). However, in 2015, Fu et al. reported upregulation in prostate cancer and Wang et al. found upregulation in non-small cell lung cancer (Fu et al., 2015; Wang et al., 2015). For the rs12976445 polymorphism, Lehmann et al. (2013) have found that CT and CC variants were associated with a lower level of miR-125a in comparison with the TT variant. Jiao et al. (2014) reported that the TT variant of the SNP was associated with increased risk of mortality in breast cancer.

Zang et al. (2016) showed that the rs12976445 TT genotype was implicated with a lower uptake rate of FDG. Quan et al. (2018) showed that the presence of the minor allele (T) of the rs12976445 polymorphism increased the expression levels of TGFβ by decreasing the expression levels of miR-125a. They thought that this might be associated with the development of pneumonitis in patients with lung cancer receiving radio therapy. In both studies, the CC genotype was significantly higher in lung cancer individuals, but the sample size was small and there was no comparison with control groups. In our study we did not find any significant difference between patients and controls in terms of lung cancer risk for this polymorphism. However, in the relationship analysis between miR-34b/c rs4938723 and miR-125a rs12976445 polymorphisms, the frequency of the T/T haplotype was found to be significantly higher in the control group, whereas the C/T haplotype was observed exclusively in patients. Based on these results, the T/T haplotype was associated with a decreased risk of lung cancer, whereas the C/T haplotype was associated with an increased risk.

In conclusion, our results indicate that the rs4938723 polymorphism of miR-34b/c is associated with the risk of lung cancer in the Turkish population from the Black Sea Region. Additionally, haplotype frequency and association analyses between miR-34b/c rs4938723 and miR-125a rs12976445 polymorphisms reveal that the T/T haplotype may confer a protective effect against lung cancer, while the C/T haplotype is associated with increased susceptibility to lung cancer. However, it's important to note that these findings may be specific to the province of Zonguldak, where the air pollution levels are elevated. We recommend that this study be conducted with different ethnicities and larger study groups. Additionally, we suggest analyzing the gene expression of miR-34b/c, as it is a potential factor associated with lung cancer.

FUNDING

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COMPLIANCE WITH ETHICAL STANDARDS

This study was in accordance with the ethical standards of Ethics Committee for Clinical Research of Zonguldak Bulent Ecevit University, Faculty of Medicine (Zonguldak, Turkey) and with the 1964 Helsinki declaration and its later amendments or comparable ethical standards. Ethical approval was obtained from Ethics Committee for Clinical Research of Zonguldak Bulent Ecevit University, Faculty of Medicine (Protocol no: 2017-116-20/12).

CONFLICT OF INTEREST

There are no conflicts of interest in connection with this paper.

ADDITIONAL INFORMATION

Datasets generated and/or analyzed during the current study are available from the corresponding author upon reasonable request.

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