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Investigation of some DNA repair genes association in non small cell lung cancer

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Abstract

Ribonucleoside-diphosphate reductase subunit M2, also known as ribonucleotide reductase small subunit, is an enzyme that in humans is encoded by the RRM2 gene and also Ribonucleoside-diphosphate reductase large subunit is an enzyme that in humans is encoded by the RRM1 gene. RRM1 is a gene important in determining tumor phenotype, but also induced the expression of PTEN tumor suppressor gene, cell migration, invasion and metastasis formation, and play a preventive role. ERCC2 DNA repair mechanism is associated in more than 20 genes involved in the NER pathway. The aim of this study is to investigate rs13181 ERCC2 (T>G) (Lys751Gln), rs12806698 RRM1 (-269C>A) and rs6759180 (located in the 5'UTR) RRM2 (10126436G>A) gene polymorphisms by using real time PCR technique in patients with NSCLC. 193 NSCLC cases and 141 healthy control cases were included in this study. A significant difference was found between rs12806698 RRM1 genotype distributions (*p: 0.034) and were determined increases the risk of disease approximately 3.044 times AA genotype having (*p: 0.014 OR: 3.044, 95%CI: 1.205-7,688). A significant difference was found between rs6759180 RRM2 genotype distributions (*p: 0.033) and were determined increases the risk of disease approximately 3.49 times GG genotype having (p: 0,009 OR: 3, 49, %95CI:1.291-9,482). It was found significant difference in serum 8-OHdG levels between patients and controls (*p: 0001).

Key words: rs13181 ERCC2 (T>G) (Lys751Gln); rs12806698 RRM1 (-269C>A), rs6759180 (located in the 5'UTR) RRM2 (10126436G>A), NSCLC, biomarker.

Introduction

Lung cancer is a multifactorial disease that is the leading cause of death after heart disease and represents 24% of deaths from cancer. It causes the highest number of cancer-related deaths with the continuous increasing of incidence and mortality worldwide (1). Non Small Cell Lung Cancer (NSCLC) represents about 80% of all lung cancers. Molecular mechanisms of the malignant transformation in NSCLC is not clear. It has been supposed that the tumorigenesis is accumulation of multiple genetic aberrations which are involved in cell cycle regulation. Recently explored that dynamical rearrangements during DNA damage is responsible for positively or negatively regulating genome organization and integrity (2). DNA repair, cell growth, differentiation and apoptosis as evidenced by cell-based studies (3,4).P53 plays a central role in the G1 phase of the cell cycle (5-7). The p53-inducible ribonucleotide reductase small subunit 2 (hRRM2) homologue plays a crucial role in supply Deoxyribonucleotides (dNTPs) for DNA repairing. It participates in p53-induced DNA repairing by supply nuclear deoxynucleotide triphosphates in response to various types of DNA damage caused by ionizing radiation, UV irradiation, and anticancer drugs (8-12). Ribonucleotide reductase is an unique enzyme which is responsible for the reduction of all four ribonucleotides to their corresponding deoxyribonucleotides (dNTPs), which are the building blocks for DNA replication and DNA repairing in all living cells (13, 14). To date, any unique gene or chromosome region responsible for the development of NSCLC has not been identified. Polymorphisms of DNA repair genes in a number of different ways to effect repairs on the properties of these genes are unknown despite the identification. Ribonucleoside-diphosphate reductase subunit M2, also known as ribonucleotide reductase small subunit, is an enzyme that in humans is encoded by the RRM2 gene and also Ribonucleoside-diphosphate reductase large subunit is an enzyme that in humans is encoded by the Ribonucleotide Reductase M1 (RRM1) gene. RRM1 is important in determining tumor phenotype, but also induced the expression of Phosphatase and Tensin homolog (PTEN) tumor suppressor gene, cell migration, invasion and metastasis formation, and play a preventive role. In lung cancer, RRM1 is located on chromosome 11p15.5 that is showing loss of heterozygosity property (15). Excision Repair Cross-Complementation group 2 (ERCC2) is a multi-protein complex; which is part of cell cycle regulation, apoptosis, Nucleotide Excision Repair (NER), transcription, etc., and Transcription Factor II H (TFIIH) transcription factor involved in the regulation of many different functions. ERCC2 DNA repair mechanism is associated in more than 20 genes involved in the NER pathway. ERCC2 gene polymorphism is associated with the DNA repair capacity and cancer risk. The absence of anemia, normal serum calcium and Lactate Dehydrogenase (LDH) levels, age, sex, and absence of smoking history can be included among good prognostic markers in the formation of NSCLC. Unfortunately, there is no unique biomarker for NSCLC in clinical practice (13, 16-22). We consider that the determining of a prognostic marker in NSCLC has predictive importance in terms of both diagnosis and prognosis.

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Table 1. Characteristics features of NSCLC patients.

	NSCLC group		Control group		
	Median	SD	Median	SD	p value
LDH (U/L)	238,074	129,551	171,992	32,907	0,001
Triglycerides (mg/dl)	133,648	72,964	180,308	95,240	0,011
Hemoglobin (g/l)	13,142	1,963	13,899	1,917	0,917
Hematocrit (%)	39,705	5,341	42,300	5,030	0,740

Materials and Methods

Study population and collection of specimens

Samples were collected from the Istanbul Yedikule Chest Diseases and Thoracic Surgery Training Hospital and the Istanbul University Cerrahpasa Medicine Faculty Department of Thoracic Surgery Clinic. The study examined 193 NSCLC patients and 141 healthy controls. The mean ages and percentage of the patients and controls are shown in Table 1. One hundred and fourty-one healthy subjects without any malignancy were selected for the control group, which comprised only individuals with a negative family history of cancer. The patient and control groups were matched for age. NSCLC patients who were diagnosed by histology or cytology. All samples recruited before treatment either surgical resection or chemoradiotherapy. All participants signed an informed consent form before enrollment, and institutional Ethics Committee approval was obtained for the study. The study protocol was approved by both the ethics committee of the Istanbul Faculty of Medicine (July 08, 2010; No. 378) and the Scientific Research Projects Coordination Unit of Istanbul University (Project No. 14807). Detailed histories and physical examinations of all patients were performed. The age, gender, smoking, height (m), and weight (kg) were recorded for each individual. Blood samples were taken after fasting for 12 hours. A 10 cc blood sample was taken using an ethylenediaminetetraacetic acid tube from patients who signed the consent form and were brought to Istanbul University, Department of Molecular Medicine of Research Institute of Experimental Medicine. Then DNA was isolated and preserved at -20°C. In the study, real time PCR was used to detect rs12806698 RRM1, rs6759180 Ribonucleotide Reductase M2 (RRM2), rs13181 ERCC2 gene polymorphisms in Turkish NSCLC patients and healthy individuals. ELISA method used to determined for 8-Hydroxy deoxyguanosine (8-OHdG) serum levels. Genomic DNA isolated according to the kit protocol (Roche kit; Roche, Manheim). LightCycler 1.5 system was used to perform Single Nucleotide Polymorphism (SNP) genotyping using hybridization probes consisting of 3'- fluorescein and a 5'- LightCycler Red labeled pair The concentration and purity of DNA were determined using an NanoDrop-1000 (ND-1000) spectrophotometer using hybridization probes consisting of 3'- fluorescein and a 5'- LightCycler Red labeled pair of oligonucleotide probes (23-26). (NanoDrop Technologies Inc., Wilmington, DE, USA) at A260 and A280. Template DNA (0.5-1.0 μg) was used in a polymerase chain reaction (PCR) under sterile conditions.

Statistical analysis

All statistical analyses were carried out using the SPSS version 17.0 statistical package for Windows. Numeric values were analyzed with the Student *t*-test.

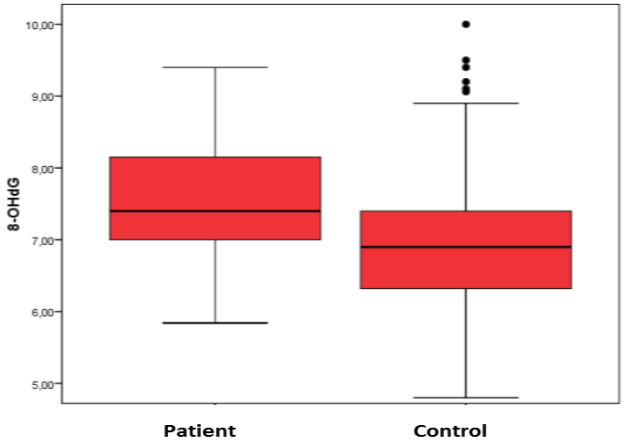

Figure 1. Dissociation of 8-OHdG serum levels.

The chi-square test was used to assess both the prevalence of the genotype of the gene region and allele differences between groups. The relative associations between NSCLC patients and controls were assessed by calculating crude Gart's odds ratios (ORs) and 95% confidence intervals (95%CIs). The threshold for significance was $P < 0.05$. Linkage disequilibrium between these rs12806698 RRM1, rs6759180 RRM2, rs13181 ERCC2 polymorphisms was assessed using D0 and r2 values obtained with the Haploview program (http://www.broadinstitute.org/scientific-community/science/programs/medical-and-population-genetics/haploview/haploview). A multivariate logistic regression model was used to investigate the effects of genotypes and alleles after adjustment for age. Values of $P < 0.05$ were considered statistically significant.

Results

Gene polymorphism distribution of rs12806698 RRM1 was found CC:41.96%; CA:46.11%; AA:11.91% in patients and CC:50.35%; CA:45.39%; AA:4.25% in controls, rs6759180 RRM2 was found AA:49,74%; AG:38,86%; GG:11,39% in patients and AA:55,31%; AG:41,13%; GG:3,54% in controls. Gene polymorphism distribution of rs13181 ERCC2 was found TT:34,71%; TG:49,22%; GG:16,06% in patients and TT:43,26%; TG:49,64%; GG:7,09% in controls. Serum levels of 8-OHdG were determined average 7,5 ng/ml in patients and 6,9 ng/ml in controls (Figure 1.). It was found significant difference in serum 8-OHdG levels between patients and controls (p: 0001). Table 1. shows the characteristics and laboratory parameters of our study groups. We found a statistically significant difference in LDH parameter but not in the hemoglobin, hematocrite (%) values between patients and controls. Genotypes and allele frequencies for rs12806698 RRM1 in NSCLC patients and controls are listed in Table 2. We found statistically significant difference in rs12806698 RRM1 genotype frequencies between NSCLC patients and controls (p=0.034). We found significant

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Table 2. Genotype and allele distributions of rs12806698 RRM1 in NSCLC patients and control groups.

	NSCLC group		Control group		
RRM1	n	%	n	%	P value
Genotype					
CC	81	41,96	71	50,35	
CA	89	46,11	64	45,39	
AA	23	11,91	6	4,25	**0,034**
Allele					
C	251	65,02	206	73,04	
A	135	34,97	76	26,95	**0,02**

Table 3. Genotype and allele distributions of rs6759180 RRM2 in NSCLC patients and control groups.

	NSCLC group		Control group		
RRM2	n	%	n	%	P value
Genotype					
AA	96	49,74	78	55,31	
AG	75	38,86	58	41,13	
GG	22	11,39	5	3,54	**0,033**
Allele					
A	267	69,17	214	75,88	
G	119	30,82	68	24,11	0,056

Table 4. Genotype and allele distributions of rs13181 ERCC2 in NSCLC patients and control groups.

	NSCLC group		Control group		
ERCC2	n	%	n	%	P value
Genotype					
TT	67	34,71	61	43,26	
TG	95	49,22	70	49,64	
GG	31	16,06	10	7,09	**0,032**
Allele					
T	229	59,32	192	68,08	
G	157	40,67	90	31,91	**0,02**

difference in rs6759180 RRM2 genotype frequencies between NSCLC patients and controls (p=0.032). We also found significant difference in rs13181 ERCC2 genotype frequencies between NSCLC patients and controls (p=0.033). Genotype and allele frequencies are listed in Table 2., Table 3., Table 4. No association was found between the rs12806698 RRM1, rs6759180 RRM2, rs13181 ERCC2 allele frequencies and tumor stage, lymph node, or metastasis status in NSCLC patients. Haplotypes were evaluated for association with NSCLC. No linkage disequilibrium was found between rs12806698 RRM1, rs6759180 RRM2, rs13181 ERCC2 (D': 0.11; r*2*: 0.001) (Table 5.). A multivariate logistic regression model was performed to investigate possible independent effects of smoking (pack/ year), gender, and rs12806698 RRM1, rs6759180 RRM2, rs13181 ERCC2 genotypes on risk of NSCLC (Table 6.).

Discussion

Lung cancer is a multifactorial disease and, at diagnosis, more than 75% of lung cancers are NSCLC. Various DNA alterations can be caused by exposure to environmental and endogenous carcinogens. A better understanding of the complex biology of NSCLC could enable us to predict for recurrence and may also be helpful to select the specific therapeutic choice for individual basis. The aim of this work is to clarify the possible role of rs12806698 RRM1, rs6759180 RRM2, rs13181 ERCC2 gene polymorphisms in NSCLC.

Failure of DNA repair which should remove adducts of DNA, and polymorphisms which affect each of these steps can be inherited (13). Ribonucleotide reductase enzyme catalyzes the rate-limiting step of the pathway for the synthesis of DNA monomers (18-20). Human

Table 5. Analysis of haplotypes.

Haplotype No	Haplotype	Frequency			Chi Square	P value
		Total	NSCLC patients	Control		
1	CAT	0,326	0,274	0,398	11,351	**8,0E-4**
2	CAG	0,184	0,192	0,174	0,362	0,5474
3	AAT	0,132	0,143	0,117	0,936	0,3332
4	CGT	0,123	0,120	0,127	0,065	0,7992
5	AAG	0,077	0,083	0,070	0,366	0,545
6	AGG	0,057	0,068	0,043	1,859	0,1728
7	CGG	0,051	0,064	0,032	3,424	0,0643
8	AGT	0,049	0,056	0,039	1,017	0,3133

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Table 6. Analysis of binary logistic regression.

Variables in the equation	B	S.E	Wald	df	Sig.	Exp(B)	95.0% C.I. for EXP(B)	
							Lower	Upper
RRM1C	-1,123	0,517	4,711	1	0,030	0,325	0,118	0,897
RRM2A	-1,170	0,549	4,546	1	0,033	0,310	0,106	0,910
ERCC2T	-1,051	0,415	6,431	1	0,011	0,349	0,155	0,788
Gender	-1,380	0,332	17,243	1	0,000	0,252	0,131	0,483
Smoking 50	-1,206	0,304	15,701	1	0,000	0,299	0,165	0,544
Constant	4,507	0,851	28,027	1	0,000	90,671		

ribonucleotide reductase enzyme is a tetramer complex, composed of two non-identical homodimers, hRRM1 and hRRM2. The large subunit hRRM1 (87 kD) harbors the catalytic site, allosteric effector-binding sites, and redox active disulfides that participate in the reduction of substrates, whereas the small subunit hRRM2 (43 kD) contains an oxygen-linked di-ferric center and one tyrosyl radical per monomer that are essential for enzymatic activity (15, 27-29). Ribonucleotide reductase is considered as an important target for cancer chemotherapy such as gemcitabine. The changes at p53R2 and hRRM1 genes may lead differences in expression of ribonucleotide reductase enzyme. Therefore, these genes can be used as biomarkers of carcinogenesis. It is known that biologic behavior of the tumor affects the survival rates. Use of biomarker especially in NSCLC is very essential to understand the genetic mechanisms of a cancer development, and determine the prognosis and the survival rate of the disease. Lewis J et al in 2008 (21), M Felicitas Lopez-Cima et al in 2007 (26) are both expressed in the work they have done, the ERCC and RRM1 gene can be used to determine the response to treatment and predict the prognosis of patients with NSCLC particularly. RRM1 and RRM2 are encoded in different genes on different chromosomes and mRNAs are expressed at different rates throughout the cell cycle (21, 26). Increased expression of RRM1 is associated with decreased metastasis formation in experimental studies (30, 31) and, prolonged survival in NSCLC (32). It is also a particular target for the chemotherapeutic agent, specifically gemcitabine. Association between RRM1 polymorphisms/ RRM1 expression and sensitivity to chemotherapeutic regimens have been detected in previous studies. (33). We found statistically differences in rs12806698 RRM1genotype frequencies between NSCLC patients and controls (p=0.034).

The ERCC2 gene is involved in the NER pathway of DNA repair to eliminate variety of damage to the human genome including oxidative damage. The ERCC2/ XPD (Xeroderma pigmentosum D) protein is a critical player in the NER pathway. The ERCC2 751 Gln/Gln genotype might have a contributory role in the risk of NSCLC as reported before in the esophageal squamous cell carcinoma in an Indian population. Although controversial results available in the literature, homozygous variant genotype ERCC2 Lys751Lys is associated with a higher risk of cancer development including lung cancer in different populations (34-36). No reports on polymorphisms in ERCC2 and susceptibility to NSCLC in Turkish population are available in the literature. We found statistically significant differences in rs13181 ERCC2 genotype frequencies between NSCLC patients and controls (p=0.033) which this result is consisted with the previous studies to show possible risk associations between lung cancer development and rs13181 ERCC2 genotyping. Topinka et al after doing their research with 729 Caucasian child, claimed that exon 6 arg156arg and exon 23 in lys751gln polymorphisms in ERCC2 gene are both individually and associated with each other, has a strong relationship and this may be relevant to cancer disposition (37). Our results consisted with the study has been evaluated in head and neck cancer to show possible associations with Lys751Lys homozygous mutant genotype and higher risk of cancer. (Frequency of GG genotyping 16.1% for patients and 7.1% for controls). We therefore evaluated the relationships between rs13181ERCC2 and both rs12806698 RRM1, and rs6759180 RRM2 polymorphism and lung cancer risk. In our study, haplotypes were evaluated for association with NSCLC. No linkage disequilibrium was found between rs12806698 RRM1, rs6759180 RRM2, rs13181 ERCC2 (D': 0.11; r*2*: 0.001). We also aimed to identify any association of these genes with smoking status of lung cancer patients in the Turkish population. The potential effect of XPD genotype on the associations between smoking-related cancers and tobacco smoking is a critical interest. Contradictory results were reported for the association of XPD polymorphisms and level of smoking both Asp312Asn and Lys751Gln polymorphisms (25, 38, 39). In our results, no associations have been identified between smoking habits and any of the polymorphisms.

Human 8-OHdG is one of the most widely used oxidative stress biomarker can be measured in serum or urine samples. Evidences have indicated that urinary 8-OHdG not only is a biomarker of generalized, cellular oxidative stress but might also be a risk factor for cancer. In the previous reports, elevated level of 8-OHdG in serum or urine has been detected in patients with various cancers including lung cancer (40). It should be addressed for increased oxidative stress, impaired antioxidant defense or inadequate repair of oxidatively damaged DNA. But, relationship between 8-OhdG and cancer is controversial (41, 42). Damage of the enzymes involved in the DNA repair mechanism causing damage to accumulate, resulting in delays in the replication stage and ultimately it may trigger the process of carcinogenesis. 8-OHdG is an important biomarker is known to have an impact on this pathway. Both in this study and in the study of many other researchers both been referred to above this situation was expressed clearly. Known role of oxidative DNA damage generating by radical oxygen metabolites in carcinogenesis process is a guiding spirit for proper therapeutic dose adjustment for patients. Determination of oxidative DNA damage, especially with the patients associated with poor prognosis and lower survival rate may also contribute to explain the molecular mechanisms of the disease. The

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relationship between symptoms and complications of NSCLC can be demonstrated by the obtained results.

The 8-OHdG plasma levels were significantly higher in controls than carcinoma patients in a study conducted by Dincer and coworkers which is consistent with our results. (43). In our study, significant difference in serum 8-OHdG levels has been detected between patients and controls (p=0.0001) (6.9 ng/ml in controls vs. 7.5 ng/ml in patients).

In the present study, reduced lung cancer risk has been observed with all rs13181 ERCC2 (T>G) (Lys-751Gln), rs12806698 RRM1 (-269C>A) and rs6759180 (located in the 5'UTR) RRM2 (10126436G>A) gene polymorphisms. We also detected that the genotype combinations of rs12806698 RRM1, rs6759180 RRM2, rs13181 ERCC2 are not related with the risk alterations against NSCLC in our population. We therefore evaluated the relationships between ERCC2 and both RRM1 and RRM2 gene polymorphism and lung cancer risk, trying also to identify any association of these genes with smoking status of lung cancer patients in the Turkish population. This study is first has been conducted in Turkish population to predict DNA repair gene polymorphisms and risk of NSCLC.

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