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Laryngology

Angiotensin-converting enzyme insertion/deletion gene polymorphism in patients with laryngeal cancer

Polimorfismo del gene di inserzione/delezione dell'enzima di conversione dell'angiotensina in pazienti con tumore della laringe

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SUMMARY

Objectives. The aim of this study was to compare the distribution of the angiotensin-converting enzyme (ACE) I/D polymorphism between patients with laryngeal cancer (LC) and a control group and to examine the distribution of this polymorphism with clinical parameters related to LC.

Methods. We enrolled 44 LC patients and 61 healthy controls. The ACE I/D polymorphism was genotyped with the PCR-RFLP method. The distribution of ACE genotypes (II, ID, and DD) and alleles (I or D) was evaluated with Pearson's chi-square test, and logistic regression analysis was performed for statistically significant parameters.

Results. There was no significant difference in ACE genotypes and alleles between LC patients and controls (p = 0.079 and p = 0.068, respectively). Among clinical parameters related to LC (extension of tumour, node metastasis, tumour stage and tumour location), only the presence of node metastasis was found to be significant in terms of ACE DD genotype (p = 0.137, p = 0.031, p = 0.147, p = 0.321 respectively). In the logistic regression analysis, the ACE DD genotype was increased 8.3 fold in nodal metastases.

Conclusions. The findings of the study suggest that ACE genotypes and alleles do not affect the prevalence of LC, but the DD genotype of ACE polymorphism may increase the risk of lymph node metastasis in LC patients.

KEY WORDS: laryngeal cancer, lymphatic metastasis, angiotensin converting enzyme, gene polymorphism

RIASSUNTO

Obiettivi. *Lo scopo di questo studio è confrontare la distribuzione del polimorfismo di ACE I/D tra i pazienti con cancro laringeo e un gruppo di controllo, e correlare la distribuzione del polimorfismo ai parametri clinici del tumore.*

Metodi. *Sono stati arruolati 44 pazienti con cancro laringeo e 61 soggetti sani. Il polimorfismo ACE I/D è stato genotipizzato con il metodo PCR-RFLP. La distribuzione dei genotipi ACE (II, ID e DD) e degli alleli (I o D) è stata valutata con il Pearson chi-square test, e analisi logistiche di regressione sono state eseguite per i parametri statisticamente significativi.*

Risultati. *Non è stata dimostrata alcuna differenza statistica tra i soggetti con cancro laringeo e i controlli per quanto concerne i genotipi di ACE e gli alleli (rispettivamente p = 0,079 e p = 0,068). Tra i parametri clinici tumorali (estensione del tumore, metastasi linfonodali, stadio e sede del tumore), solo la presenza di metastasi linfonodali è stata correlata in maniera statisticamente significativa con il genotipo ACE DD (rispettivamente, p = 0,137, p = 0,031, p = 0,147, p = 0,321). Nelle analisi di regressione logistica, il genotipo ACE DD è correlato ad un incremento del rischio di metastasi linfonodali di 8,3 volte.*

Conclusioni. *I risultati di questo studio suggeriscono che i genotipi e gli alleli di ACE non influenzano la prevalenza del cancro laringeo, mentre il genotipo DD del polimorfismo ACE potrebbe aumentare il rischio di metastasi linfonodali.*

PAROLE CHIAVE: *cancro della laringe, metastasi linfonodali, enzima di conversione dell'angiotensina, polimorfismo del gene*

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Introduction

Squamous cell carcinoma is the most common type of laryngeal cancer (LC), accounting for 85% to 95% of all malignant neoplasms [1]. In 2021, the estimated number of new cases diagnosed with LC in the US was 12,620, and LC accounted for 0.7% of all new cancer cases. Again in 2021, the estimated deaths due to LC were 3,770 individuals and LC accounted for 0.6% of all cancer deaths [2]. Despite the improvements in diagnosis and treatment, survival rates are still low. While the 5-year survival rate for LC was 66% in the 1970s, it decreased to 60.7% in 2017 [2,3]. The reason for treatment failure is mostly advanced stage disease at the time of diagnosis. Therefore, otolaryngologists, radiation oncologists and medical oncologists are constantly seeking to improve survival.

Angiotensin-converting enzyme (ACE) is a zinc metallopeptidase that converts angiotensin I (AT-1) to angiotensin II (AT-2) and reduces bradykinin; hence, it controls blood pressure and cardiovascular homeostasis [4]. An insertion/deletion (I/D) polymorphism consisting of 287 base pairs is defined in intron 16 of the ACE gene located on chromosome 17q23 [5]. The D allele is associated with higher ACE plasma concentrations than the I allele [5,6]. Thus, the DD genotype correlates with AT-2 levels. Higher AT-2 levels are also associated with neovascularisation, cell proliferation, inflammation and possibly cancer development [4,7].

In previous studies, the association of ACE gene polymorphism types (DD, ID, or II) with various cancers and clinical findings in cancer patients was investigated. Carriers of the DD genotype for the ACE gene were shown to be at risk for breast and squamous cell lung cancer, and for lymph node metastasis in those with gastric cancer [4,8,9]. A relationship between ACE gene polymorphism and colorectal, lung, and prostate cancer was not demonstrated [4,8]. As a result, data on ACE gene polymorphisms and development of cancer (regardless of cancer type) are inconsistent.

Finding a relationship between ACE gene polymorphisms and development of LC or parameters associated with LC [e.g., tumour (T), node (N)] might help prevent and slow progression of the disease, thus contributing to increased survival. Our study was conducted to reveal the frequency of ACE I/D polymorphism in LC and to investigate relationships with clinical findings. To the best of our knowledge, this is the first study to reveal a relationship between ACE I/D polymorphism and LC.

Materials and methods

The study was carried out between September 2020 and September 2021 at the Department of Otorhinolaryngology and Medical Genetics at Süleyman Demirel University Faculty of Medicine, Isparta, Turkey.

Subjects

All clinical and pathological parameters were collected by chart review. The criteria for inclusion in the LC group were as follows: 1. histopathological diagnosis of laryngeal squamous cell carcinoma; 2. regular smoker; and 3. data on clinical parameters [e.g. tumour (T), node (N), metastasis (M) classification, and location of tumour, according to the AJCC Cancer Staging Manual, 8th Edition] [10]. The criteria for inclusion in the control group were as follows: 1) no history of malignancy; 2) regular smoker; 3) undergoing septoplasty surgery at the otolaryngology clinic; 4) a normal laryngeal examination prior to surgery. In both groups, patients with a history of use of ACE inhibitors and/or AT receptor blockers and patients with genetic diseases involving predisposition to malignancy were excluded. Descriptive information was collected on the age, gender, tumour localisation, TNM classification and stage. The frequency of II, ID and DD genotypes and I/D alleles of the ACE gene in patients diagnosed with LC were compared with the control group. In addition, the clinical findings of patients diagnosed with LC were analysed in terms of genotype and allele frequency. For genetic analysis, 4 cc venous blood was taken from all patients in the study group into tubes containing EDTA during routine preoperative examinations. Samples were stored at -20°C in the medical genetics laboratory until DNA isolation.

Genotyping

DNA from venous blood samples was obtained using the High Pure Polymerase Chain Reaction (PCR) Template Preparation kit (Roche Applied Science, Germany) according to the manufacturer's protocol. The concentration and purity of the obtained DNA samples were determined by measuring with a Nanodrop 2000c spectrophotometer (Thermo Scientific, USA). The A60/280 absorbance ratio for DNA was in the range of 1.8-2.0. Polymerase chain reaction was performed of each DNA sample with ACE gene I/D polymorphism genotype allele specific primers (Forward: TGGAGACCACTCCCATCCTTTCT, Reverse: GATGTGGCCATCACATTCGTCAGAT) and FastStart High Fidelity PCR System, dNTPack (Roche Applied Science, Germany) kit. Each polymerase chain reaction contains 10 pmol of F and R primers, 1.8 mM $MgCl_2$, 10 mM dNTP mixture, 1.25 U Taq polymerase and 250 ng genomic DNA. The polymerase chain reaction was carried out under the following conditions; after 10 minutes of pre-incubation at 94°C, 35 cycles at 94°C for 2 minutes, 57°C for 30 seconds, 72°C for 1 minute, and finally at 72°C for

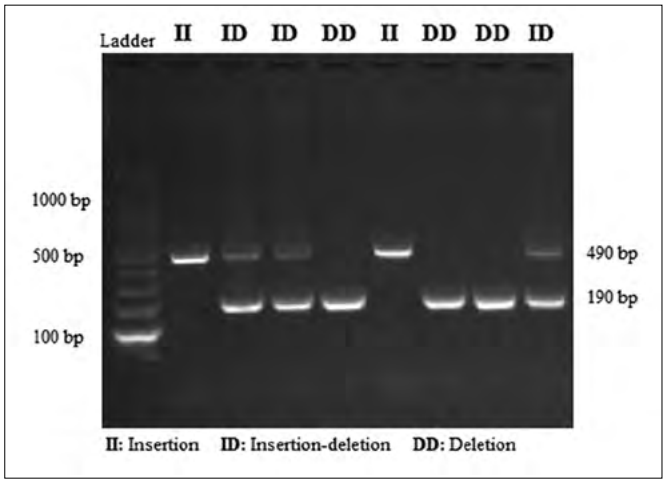

Figure 1. Gel electrophoresis of PCR-RFLP products from representative blood samples for ACE I/D polymorphism. Lane ladder represents DNA ladder marker (Fermentas). I/I columns show the 490 bp band. I/D columns show the 190 bp and 490 bp bands. D/D columns show the 190 bp bands.

7 minutes. After the PCR, 2% agarose gel electrophoresis was applied to the products and stained with GelRed Nucleic Acid Gel Stain (Biotium, USA). The gel was visualised with a UV Transilluminator. I and D polymorphism genotypes of ACE gene in the samples were determined as II genotype single band 490 bp, ID genotype double band 190 bp and 490 bp, and DD genotype single band 190 bp (Fig. 1).

Statistical analysis

Data was analysed by using SPSS Statistics for Windows, Version 23.0 (IBM SPSS. Armonk, NY: IBM Corp). In descriptive findings, categorical variables are presented as number and percentage, and continuous variables are presented as mean ± standard deviation. Whether the age variable was normally distributed or not was evaluated with the Kolmogorov-Smirnov test and compared between groups with the independent samples t test. Since the study group consisted of only males, the gender variable was not included in the analysis. Pearson chi-square test was used when examining the relationship of ACE genotypes and alleles between patients with LC and the control group. Similarly, the relationship of ACE genotypes and alleles with clinical parameters in LC patients was evaluated with the Pearson chi-square test. Variables that were statistically significant in univariate analysis were evaluated with logistic regression analysis. The ID genotype was taken as a reference. Results are presented with odds ratio (OR) and 95% confidence intervals (CI). Statistical significance was accepted as $p < 0.05$.

Table I. Characteristics of LC patients and controls.

Variables	LC patients (n = 44) n (%)	Controls (n = 61)	p^a
Age	62.73 ± 9.05	61.93 ± 7.48	0.625
Gender			
Male	44/44	61/61	
Female	-/-	-/-	
Tumour localisation			
Supraglottis	7 (15.90)		
Glottis	35 (79.54)		
Subglottis	2 (4.54)		
Tumour extension			
T^1	14 (31.8)		
T^2	20 (45.5)		
T^3	5(11.4)		
T^4	5 (11.4)		
Nodes			
N^0	34 (77.2)		
N^1	1 (2.27)		
N^2	9 (20.45)		
N^3	-		
Stage			
S^1	14 (31.8)		
S^2	16 (36.4)		
S^3	5 (11.4)		
S^4	9 (20.5)		

[a]: p-value for Independent Samples T Test.

Results

In this study, 44 patients with LC and 61 healthy controls were enrolled. There were no female patients in either group. The mean age of patients with LC was 62.73 ± 9.05 years, while it was 61.93 ± 7.48 years in the control group. There was no significant difference between groups in terms of age ($p = 0.625$). The characteristics of study subjects are shown in Table I (none of the LC patients had distant metastases at initial diagnosis). Table II shows the frequency distribution of ACE genotypes and alleles among patients with LC and controls. There was no significant difference between ACE genotypes and alleles between LC patients and controls ($p = 0.079$ and $p = 0.068$, respectively). In addition, the relationship between the distribution of ACE genotypes and alleles in patients with LC and clinical parameters is presented in Table III.

Discussion

ACE is the key molecule involved in the formation of AT-2 from AT-1 and the inactivation of bradykinin. The hypothesis that increased levels of AT-2 may play a role in the development of any cancer has been hypothesised especially for type 1 receptors of AT-2, because they can contribute

Table II. Distribution of ACE insertion/deletion polymorphism genotypes and alleles for LC patients and controls.

	LC patients (n = 44) n (%)	Controls (n = 61) n (%)	p^a
ACE genotypes			
DD	10 (22.7)	10 (16.4)	0.079
ID	28 (63.6)	31 (50.8)	
II	6 (13.6)	20 (32.8)	
ACE alleles			
D	48 (54.5)	51 (41.8)	0.068
I	40 (45.5)	71 (58.2)	

[a]: p-value for Pearson Chi-Square Test.

to cell proliferation, angiogenesis, inflammation and cell migration through various signal transduction pathways [7]. Previous studies showed that the D allele in the ACE gene polymorphism is associated with higher ACE levels [5,6], meaning also high levels of the AT-2 and AT-2 type 1 receptors. In this context, the relationship between ACE I/D gene polymorphism and many solid cancer types was investigated in several studies [4,7,9,11-13] and it is worth investigating also for LC.

A relationship was found between ACE I/D gene polymorphism and the development of prostate, breast, endometrial cancers and oral precancerous lesions. In these studies, it was shown that the presence of the D allele increases the risk of cancer. It is thought that malignant transformation is triggered after long-term exposure to the high amount of AT-2 formed in the DD genotype of the *ACE* gene in normal tissues [14-17]. It is known that AT-2, which has high levels in the malignant transformation process, activates some intracellular signal transduction pathways (e.g., transforming

Table III. Comparison of ACE genotypes/alleles with clinical parameters of LC patients.

	Clinical parameters n (%) n (%)	p^a	OR - (CI) - p^c
	Tumour extension T_1+T_2 T_3+T_4		
Genotype			
DD	7 (20.6) 3 (30.0)		
ID	24 (70.6) 4 (40.0)	*0.137*	
II	3 (8.8) 3 (30.0)		
Allele			
D	38 (55.9) 10 (50.0)		
I	30 (44.1) 10 (50.0)	*0.642*	
	Nodes N- N+		
Genotype			
DD	5 (14.7) 5 (50.0)		8.333 - (1.487-46.705) - *0.016*
ID	25 (73.5) 3 (30.0)	*0.031*	1.00[b]
II	4 (11.8) 2 (20.0)		4.167 - (0.522-33.263) - 0.178
Allele			
D	35 (51.5) 13 (65.0)		
I	33 (48.5) 7 (35.0)	*0.285*	
	Stage S_1+S_2 S_3+S_4		
Genotype			
DD	5 (16.7) 5 (35.7)		
ID	22 (73.3) 6 (42.9)	*0.147*	
II	3 (10.0) 3 (21.4)		
Allele			
D	32 (53.3) 16 (57.1)		
I	28 (46.7) 12 (42.9)	*0.738*	
	Tumour localisation Supraglottis Glottis Subglottis		
Genotype			
DD	1 (14.3) 9 (25.7) 0 (0)		
ID	4 (57.1) 23 (65.7) 1 (50.0)	*0.321*	
II	2 (28.6) 3 (8.6) 1(50.0)		
Allele			
D	6 (42.9) 41 (58.6) 1 (25.0)		
I	8 (57.1) 29 (41.4) 3 (75.0)	*0.267*	

[a]: p-value for Pearson Chi-Square test; [b]: reference category; [c]: p-value for binary logistic regression (according to enter model).

growth factor B, phosphoinositide 3-kinase, mitogen activated protein kinase/extracellular signal-regulated kinase, Janus kinase-signal transducer and activator of transcription, protein kinase C and rat sarcoma protein). This process occurs when AT-2 stimulates these pathways directly or via epidermal growth factor. Another reason thought to be related to tumourigenesis is the high reactive oxygen species formed by AT-2 in tissues [7]. In our study, no significant difference was found between LC and the control group in terms of the distribution of ACE I/D gene polymorphisms. Similarly, in our study, no relationship was found between the distribution of ACE I/D gene polymorphisms and parameters such as T stage or tumor location. We attribute this to the fact that environmental factors such as smoking are more effective than genetic factors for the development of LC.

One of the most important factors determining the prognosis in LC patients is cervical lymph node metastasis [18]. Therefore, one way to increase survival is to focus on factors that prevent lymph node metastasis or, if metastasis has occurred, to stabilise the metastasis. In a study in gastric cancer patients, there was no difference when ACE I/D polymorphisms were compared with the control group, but it was shown that the number of metastatic lymph nodes was higher with the DD genotype than with the II genotype [9]. In patients with colorectal carcinoma, it was reported that the DD genotype of the *ACE* gene increases the risk of lymph node metastasis [19]. In another study in patients with endometrial cancer, no relationship was found between lymph node metastasis and the ACE I/D gene polymorphism. However, the low number of patients with lymph node metastases in this study may have affected the statistical analysis [13]. In most of the above-mentioned studies, it was shown that the DD genotype of the *ACE* gene is associated with increased lymph node metastasis. Angiogenesis is required for an invasive tumour to metastasise and the balance between proangiogenic factors and anti-angiogenic factors should be disturbed in favour of proangiogenic factors for angiogenesis. One of the proangiogenic factors is vascular endothelial growth factor [20]. Previous studies showed that AT-2 has a stimulating effect on vascular endothelial growth factor [21]. We think that the increased risk of cervical lymph node metastasis is due to the high AT-2 levels occurring in the DD genotype of the *ACE* gene. In the current study, the risk of cervical lymph node metastasis in patients with LC with the DD genotype of the *ACE* gene was also found to be 8.3 times higher than the other genotypes.

The pathophysiological mechanism of the susceptibility of the *ACE* gene DD genotype for lymph node metastasis should be elucidated further because this intervention may enable ACE inhibitors to be included in treatment protocols to prevent lymph node metastases and/or their spread. It was shown that long-term and high-dose ACE inhibitor use in individuals carrying the DD genotype of the *ACE* gene reduces the risk of some cancers (colorectal, lung, breast, and prostate cancer) [4]. Thus, simple identification of the ACE I/D gene polymorphism can identify the population to which this treatment can be administered and might significantly contribute to survival in patients with LC.

Finally, our study has some limitations. Firstly, our sample size was relatively small. Secondly, the levels of ACE and AT-2 in tumour tissue and plasma could not be studied quantitatively. We suggest that these parameters be included in evaluations in future studies. Thirdly, we had no LC patients with distant metastasis. Therefore, we could not perform statistical analysis for this parameter.

Conclusions

The findings of the study suggest that ACE genotypes and alleles do not affect the prevalence of LC, but the DD genotype of ACE polymorphism may pose a risk for lymph node metastasis. Well-designed studies with larger sample sizes and more ethnic groups are needed to further validate these results.

Conflict of interest statement

The authors declare no conflict of interest.

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Author contributions

YÇK: conception and design, acquisition of data, analysis and interpretation of data, wrote the manuscript, critical revision of the article, final approveal of the version to be published. KHÖ: conception and design, acquisition of data, analysis and interpretation of data, wrote the manuscript, final approveal of the version to be published. HY: conception and design, wrote the manuscript, critical revision of the article, final approveal of the version to be published. VA: conception and design, acquisition of data, wrote the manuscript, critical revision of the article, final approveal of the version to be published. MES: conception and design, wrote the manuscript, critical revision of the article, final approveal of the version to be published. FC: conception and design, wrote the manuscript, critical revision of the article, final approveal of the version to be published.

Ethical consideration

This study was approved by the Institutional Ethics Committee (Ethics Committee of the Suleyman Demirel University School of Medicine) (approval number/protocol number: 04/09/2020 - 239).

The research was conducted ethically, with all study procedures being performed in accordance with the requirements of the World Medical Association's Declaration of Helsinki.

Written informed consent was obtained from each participant/patient for study participation and data publication.

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