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Assessment on the influence of TLR4 and DNA repair genes in laryngeal cancer susceptibility: a selective examination in a Romanian case control study

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Abstract

***Background**: Tumor characterization through the study of molecular biology has become an invaluable tool in understanding cancer development and evolution due to its relationship with chromosomal mutations, alterations or aberrations. The purpose of this study was to investigate the involvement of genes such as TLR-4 and DNA repair pathways (XRCC1 and XPD) in laryngeal cancer susceptibility in a Romanian population. **Method**: We performed a case-control study on 157 laryngeal cancer patients and 101 healthy controls. Genetic testing was carried out using Polymerase Chain Reaction-Restriction Fragment Length Polymorphism. **Results**: We identified the Gln allele of the XPDLys751Gln polymorphism as an individual risk factor in laryngeal cancer development (Gln vs Lys, adjusted OR=1.65, 95%CI=1.13-2.40, P=0.008). Subjects with the mutant homozygote variant (Gln/Gln) had a two fold increase in cancer risk (adjusted OR=2.18, 95%CI=1.06-4.47, p=0.028) when compared to the reference wild type genotype (Lys/Lys). Stratification by sex and age, identified males under 62 years as the most susceptible group with an almost three fold risk (adjusted OR=2.94, 95%CI=1.31-6.59, p=0.007) for the dominant model (Lys/Gln+Gln/Gln). No associations were found for TLR-4Thr399Ile, XRCC1Arg194Trp and XRCC1Arg399Gln. **Conclusion**: The results of the study show that the XPDLys751Gln polymorphism may be among other independent risk factors for developing laryngeal cancer where as TLR-4Thr399Ile, XRCC1Arg194Trp and XRCC1 Arg399Gln show no such association. However, we consider the relative small number of the subjects selected for this analyses a possible limitation towards the real influence the obtain results may pertain in laryngeal cancer evolution.*

***Keywords:** single nucleotide polymorphism, laryngeal cancer, Genetic susceptibility, TLR-4Thr399Ile, XRCC1Arg194Trp*

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Introduction

Laryngeal cancer represents one the most influential candidates for malignancy within Head and Neck cancers (HNC) with a favorable and stable 5-year survival rate (61% -67%) over the past decades (1), yet severe and lasting impact on the patients' quality of life. As one of the most susceptible regions of the human body towards chronic inflammation, the larynx is considered a prime contender towards malignancy. The risks of such exposure have been well documented over the years by identifying the triggers and hallmarks of cancer-related inflammation (2, 3), a process which tends to create a specific microenvironment whose unique composition may then foster the neoplastic process (4). Although the most common form of presentation to date is that of a squamous cell carcinoma (SCC), the particular anatomical conformation of the larynx offers the possibility of numerous tumor sites, each one with characteristic symptoms, specific treatment and prognostic (5, 6).

Toll-like receptors (TLRs) are a class of proteins belonging to the family of pattern recognition receptors (PRR) which play a significant role in innate immunity. They activate cellular signalling pathways after initial detection of the pathogenic microorganism by PRRs and induce immune response genes (7, 8). Without the signals provided by toll-like receptors, the regulation of both the innate and adaptive immune responses may be deficient. As such, developed neoplastic cells have the capability to by-pass the body's immune response by turning the immune balance in their favour (9). Ten isoforms (TLR 1-10) are found in humans, of which TLR-4 is expressed in both normal cells and in cancer cells (10). TLR-4 has been widely studied and linked as an independent carcinogenetic risk factor with overall high associations for a large variety of cancer forms (11, 12).

DNA repair genes hold a key role in maintaining genome integrity by operating on specific types of damaged DNA. The three major DNA repair pathways implicated in this process are nucleotide excision repair (NER), base excision repair (BER) and double strand break (DSB) (13, 14). The implication of DNA repair genes in the carcinogenetic process has become a widely studied subject towards understanding malignancy (15). As part of BER pathway, the x-ray repair cross-complementing group 1 (XRCC1) gene acts along with specific ligase and polymerases in order to provide DNA repair (16). Amongst the many single nucleotide polymorphisms (SNPs) of the XRCC1 gene, codons 194 (Arg>TRP), 280 (Arg> His) and 399 (Arg > Gln) are the most common and the most studied coding region polymorphisms in regard to cancer susceptibility with overall significantly increased risk towards a wide rage of neoplasms including head and neck cancers (17-20).

The Xeroderma pigmentosum Type D (XPD) protein is encoded by the ERCC2 (excision repair cross-complementation group 2) gene, a subunit of the TFIIH (Transcription factor II Human) complex implicated in the transcription of various protein-coding genes and NER-pathways (21). It holds a critical role for the cell viability by unwinding the double helix DNA in the 5'-3' direction after damage is initially recognized, thus allowing for other proteins to then remove the damaged section and replace it with the correct DNA strand (22). The Lys751Gln polymorphism is located within the carboxy-terminal domain (CTD) of XPD protein (23). Mutations in the XPD C-terminus cause binding issues of the p44 subunit of TFIIH which reduces DNA repair activity and increases cancer susceptibility (24). Several publications report on the existing link between $XPD_{Lys751Gln}$ polymorphism and cancers such as breast (25), prostate (26), lung (27) or the gastrointestinal tract (28).

Case-control studies have been widely used over the last few years in order to assess the relationship between genetics and malignancy genetic associations. However, there are still few stud-

ies to date regarding a Romanian population base. The purpose of this study was to establish whether the TLR-$4_{Thr399Ile}$, $XPD_{Lys751Gln}$, $XRCC1_{Arg194Trp}$ and $XRCC1_{Arg399Gln}$ polymorphisms are involved in laryngeal cancer susceptibility in this ethnic group.

Material and Methods

Patients and controls

A total of 258 individuals were enrolled in this case-control study. The study group included 157 laryngeal cancer patients and 101 healthy volunteers with no history of laryngeal pathology from the Departments of Otorhinolaryngology and Genetics, Cluj County Emergency Clinical Hospital, Romania. The research protocol was approved by the Ethics Committee of 'Iuliu Hatieganu' University of Medicine and Pharmacy, Cluj-Napoca and it complies with the World Medical Association Declaration of Helsinki regarding ethical conduct of research involving human subjects and/or animals (29). Informed consent was obtained from all participants regarding genetic testing.

Genotyping analysis of studied polymorphisms

TLR-$4_{Thr399Ile}$

Extraction of the genomic DNA from the *TLR-4 gene* Thr399Ile polymorphism (rs4986791) was performed on ETDA (ethylenediaminetetraacetic acid) treated venous peripheral blood samples (400 µL) with the help of the Wizzard Genomic DNA Purification Kit, Promega (Madison, Wisconsin, USA). Samples were initially stored in a frozen state (-20°C) before molecular processing. Genotyping was performed using Polymerase Chain Reaction followed by enzymatic Restriction Fragment Length Polymorphism (PCR–RFLP) according to manufacturer's instructions. Subsequent PCR methodology towards fragment amplification of the obtained 100 ng of genomic DNA required the following primers (F5′-CTACCAAGCCTTGAGTTTCTG-3′ and R3′-AAGCTCAGATCTAAATACT-5′) and specific cyling conditions: 94°C for 5 minutes; 35 cycles at 94°C for 40 seconds, 58°C for 45 seconds, 72°C for 40 seconds; followed by an additional 72°C for 10 minuets at the end. The final amplified products were digested by 5 units of restriction enzyme (Bsl1) at 37°C and then separated using gel electrophoresis (3% agarose gel). Genotype variants were detected by ethidium bromide staining which identified 3 distinct banding patterns corresponding to the 3 genotypes: wild type (AA), heterozygote (AG) and variant homozygote (GG).

$XPD_{Lys751Gln}$

The genotyping of ERCC2/XPD Lys751Gln (rs13181) polymorphism was performed using the multiplex PCR–RFLP technique on DNA initially extracted from ETDA-treated peripheral blood samples (300 µl) using Wizard Genomic DNA Purification Kit (Promega, Madison, USA). The obtained 100 ng of genomic DNA were amplified in a total of 25µl reaction mixture before PCR was carried out using a thermal cycler (Eppendorf Mastercycler Thermal Cycler) with the following steps: 1 minute denaturation at 96 °C; 30 cycles of denaturation at 94 °C for 30 seconds each, followed by primer annealing at 60 °C for 30 seconds and primer extension at 72 °C for 1 minute. The final step consisted of a 2 minutes extension at 72 °C. After PCR amplification, the obtained products were digested with 4 units of Tfi enzyme (Fermentas) for 6 hours. Subsequent detection of the resulting variants (wild type Lys/Lys, heterozygote Lys/Arg and mutant homozygote Arg/Arg) was carried out using gel electrophoresis (2% agarose gel transilluminated with ultraviolet light).

$XRCC1_{Arg194Trp, Arg399Gln}$

Genomic DNA extraction of XRCC1 gene Arg194Trp (rs1799782) and Arg399Gln (rs25487)

polymorphisms was carried out on samples of 300 μL ETDA-treated venous blood with the help of two purification kits: Wizard® genomic DNA purification kit (Promega Corporation, Fitchburg, WI, USA) and ZymoBead™ genomic DNA kit (Zymo Research Corporation, Irvine, CA, USA). Both polymorphisms of the XRCC1 gene (Arg194Trp and Arg399Gln) were genotyped using the multiplex PCR–RFLP technique. The obtained DNA, a total of 100 ng, was then amplified in a total volume of 25 μL of specific reaction mixture and put through successive thermocycling conditions (Mastercycler® Gradient; Eppendorf AG, Hamburg, Germany): 94°C for 5 minutes, 30 cycles of 94°C for 30 seconds, 62°C for 1 minute, 72°C for 45 seconds, 72°C for 5 minutes. Resulting genotypes were identified using the following criteria: for codon 194 the common allele corresponded with Arg (293 bp fragment) and the variant allele with Trp (313 bp fragment); for codon 399 the common allele corresponded with Gln (375 bp fragment) and the variant allele with Arg (240 bp fragment).

Statistical analysis

Statistical analysis was carried out using Epiinfo 7 software for the Windows operating system. Two sided chi-square test (χ^2) and Fisher's exact test were used in order to compare the two groups of categorical data. The obtained data was then subjected to comparative analysis against the reference wild type variants for specific genetic models (heterozygote, homozygote, dominant, recessive and allele-contrast). Multivariate logistical regression with adjustment for gender and age (in years) was performed in order to measure the association between exposure and outcome parameters. The genotypes of TLR-$4_{Thr399Ile}$, $XPD_{Lys751Gln}$, $XRCC1_{Arg194Trp}$ *and* $XRCC1_{Arg399Gln}$ were recorded as dummy variables for logistic regression. Results were considered statistically significant for a p-value of <0.05.

Results

Distribution of demographic characteristics in study groups

Of the total 157 laryngeal cancer subjects included, 145 were males (92.36%) and 12 females (7.64%) as shown in Table I. Although male subjects were more common in cases than in controls than females, there were no statistically significant differences between genders (P=0.1321). Age varied in patients from 21-83 with an average diagnosis age of 62 years (standard deviation 9.14 years) for cases and 61 years (standard deviation 6.59 years) for controls. The most common age group was 55-65 years with a total of 74 (47.13%) cases and 44 (43.56%) controls, the results being statistically significant (P=0.021).

Distribution of genotype frequencies in patients and controls

We analysed TLR-$4_{Thr399Ile}$, $XPD_{Lys751Gln}$, $XRCC1_{Arg194Trp}$ and $XRCC1_{Arg399Gln}$ polymorphisms in 157 laryngeal cancer patients and 101 healthy controls. The distribution of genotypes and allele frequencies are presented in Table II.

Table I. Demographic characteristics of the studied groups.

Variables	Cases (%)	Controls (%)	P-value [a]
Gender			
Male	145 (92.36)	92 (91.09)	0.1321
Female	12 (7.64)	9 (8.91)	
Age Mean	60.541	62	
Age Range	21-83		
<45	8 (5.10)	0 (0)	0.0026
46-55	32 (20.38)	23 (22.77)	
56-65	74 (47.13)	44 (43.56)	
66-75	35 (22.29)	33 (32.67)	
>75	8 (5.10)	1 (0.99)	

a: Abbreviation: Two-sided chi square test ($\chi 2$) test.
b: Statistically significant result, p<0.05 in bold.

Chi-square test was used on the control group in order to calculate Hardy–Weinberg equilibrium (HWE) according to the theory: $p^2 + 2\,pq + q^2$, where p is the frequency of the "A" allele and q is the frequency of the "a" allele in the population. Deviation from HWE was calculated using internet based programs (https://wpcalc.com/en/equilibrium-hardy-weinberg/;http://www.dr-petrek.eu/links.html (HWE.xls)). Only the observed genotype frequencies of TLR-$4_{Thr399Ile}$ in the control group showed no significant departure from HWE (P=0.21, data not shown) while $XPD_{Lys751Gln}$, $XRCC1_{Arg194Trp}$ and $XRCC1_{Arg399Gln}$ were all significantly deviated (P=0.015, P=0.000067, P=0.0011 respectively). The lack of balance may be caused due to the type of subjects we were able to obtain at the moment of this study for the purpose of performing this type of genetic analyses, the relative small number of subjects included in the study and the possible selection bias in hospital-based case-control studies, genetic drift in the control group.

The observed genotype distribution in patients and controls was not statistically significant

Table II. Distribution of *XRCC1Arg194Trp, XRCC1Arg399Gln, XPDLys751Gln* and *TLR-4Thr399Ile* polymorphisms in cases and controls.

Polymorphism	Cases N=157 (%)	Controls N=101 (%)	χ^2	P-value
$XRCC1_{Arg194Trp}$				
AA	92 (58.60)	64 (63.37)		
AT	37 (23.57)	23 (22.77)	0.843	0.655
TT	28 (17.83)	14 (13.86)		
A Allele	221 (70.38)	151 (74.75)	1.166	0.28
T Allele	93 (29.62)	51 (25.25)		
$XRCC1_{Arg399Gln}$				
Arg/Arg	97 (61.78)	66 (55.35)		
Arg/Gln	37 (23.57)	24 (23.76)	0.783	0.675
Gln/Gln	23 (14.65)	11 (10.86)		
Arg Allele	231 973.57)	156 (77.23)	0.878	0.348
Gln Allele	83 (26.43)	46 (22.77)		
$XPD_{Lys751Gln}$				
Lys/Lys	35 (22.29)	14 (13.86)		
Lys/Gln	59 (37.58)	32 (31.68)	5.665	0.588
Gln/Gln	63 (40.13)	55 (54.46)		
Lys Allele	185 (58.92)	142 (70.30)	6.857	**0 .008**[b]
Gln Allele	129 (41.80)	60 (29.70)		
$TLR\text{-}4_{Thr399Ile}$				
AA	104 (66.24)	71 (70.30)		
AG	43 (27.39)	26 (25.74)	0.868	0.647
GG	10 (6.37)	4 (39.15)		
A Allele	251 (79.94)	168 (83.17)	0.841	0.359
G Allele	63 (20.06)	34 (16.83)		

a: Abbreviation: Two-sided chi square test ($\chi 2$) test; A-Adenine; G-Guanine; T-Thymine; Arg-Arginine; Trp-Tryptophan; Gln-Glutamine; Ile-Isoleucine; Lys-Lysine; *b*: Statistically significant results compared with control group, $p<0.05$, in bold; c: Genotypes: homozygote wild-type (AA, Arg/Arg, Lys/Lys, AA), heterozygote (AT, Arg/Gln, Lys/Gln, AG), mutant homozygote (TT, Gln/Gln, Gln/Gln, GG).

for the TLR-$4_{Thr399Ile}$, $XRCC1_{Arg194Trp}$ and $XRCC1_{Arg399Gln}$ polymorphisms, as shown in Table II. For $XPD_{Lys751Gln}$, the distribution of frequency in the Lys/Lys, Lys/Gln and Gln/Gln genotypes was (22.29%, 37.58%, 40.13 respectively) in case and (13.86%, 31.68%, 54.46 respectively) in controls. Although the Gln/Gln genotype was the more common variant in cases than in controls, the difference in distribution was not statistically significant (P=0.588). Distribution by allele frequency found the Gln allele to be more common in cases than in controls, the difference being statistically significant (P=0.008)

Comparative analysis of genetic models

In order to evaluate the risk for laryngeal cancer in the study group compared with the control group, comparative analysis was performed for TLR-$4_{Thr399Ile}$, $XPD_{Lys751Gln}$, $XRCC1_{Arg194Trp}$ and $XRCC1_{Arg399Gln}$ polymorphisms based on five genetic models (heterozygote, mutant homozygote, dominant, recessive and allele contrast). Crude and adjusted OR with 95% CI was calculated using multivariate logistic regression as shown in Table III. We investigate the role innate factors such as gender and age have as modulating factors for laryngeal cancer development when assessed in the presence of the different genetic variants belonging to the studied polymorphism. Other confounding factors such as external risk factors were excluded from the investigation process.

Results for TLR-$4_{Thr399Ile}$, $XRCC1_{Arg399Gln}$ and $XRCC1_{Arg194Trp}$ genotypes showed no evidence for increased cancer risks association in any of the studied genetic models.

Adjusted values for $XPD_{Lys751Gln}$ show the Gln allele as an individual risk factor for laryngeal cancer development (Gln vs Lys, adjusted OR=1.65, 95%CI=1.13-2.40, P=0.008), while individuals carrying the Gln allele express a two fold risk (adjusted OR=2.18, 95%CI=1.06-4.47, P=0.028) in the mutant homozygote genotype (Gln/Gln) when compared to the reference wild type genotype (Lys/Lys). We also observed positive associations between $XPD_{Lys751Gln}$ and laryngeal cancer after secondary stratification based on gender and age (in years) in a logistic regression model as shown in Table IV. After controlling for sex, younger subjects (age<62 years) show a two fold risk (OR=2.37, 95%CI=1.12-4.99, P=0.021) for Gln allele carriers in the dominant model (Lys/Gln+Gln/Gln). Stratification by gender could not find positive associations in either males or females. However, subsequent stratification in a double model (gender and age) highlighted the Gln/Gln genotype as the predominant risk variant in male laryngeal cancer patients under 62 years of age with an almost four fold risk (adjusted OR=3.70, 95%CI=1.10-12.35, P=0.02) followed by the dominant model with an almost three fold risk (adjusted OR=2.94, 95%CI=1.31-6.59, P=0.007) and the heterozygote variant (Lys/Gln) with a two fold risk (OR=2.61,95%-CI= 1.04-6.51, P=0.033). No such associations were identified for TLR-$4_{Thr399Ile}$, $XRCC1_{Arg399Gln}$ and $XRCC1_{Arg194Trp}$ polymorphisms after stratification by gender and age (data not shown).

Discussion

Laryngeal cancer is one of the predominat forms of Head and Neck cancers. The contribution of multiple environmental and genetic factors plays an important role in the overall characterization of this particular malignancy (30). To date, it is one of the most studied cancers in regard to genetic susceptibility. To our knowledge, this is the first study to perform a comparative analysis of the TLR-$4_{Thr399Ile}$, $XPD_{Lys751Gln}$, $XRCC1_{Arg194Trp}$ and $XRCC1_{Arg399Gln}$ polymorphisms on patients diagnosed with laryngeal cancer in a Romanian population.

The results of our study showed that there was no association between the TLR-$4_{Thr399Ile}$ polymorphism and laryngeal cancer risk in any ge-

netic model. Studies in TLR polymorphisms and laryngeal cancer are still limited to this day in terms of genotyping specific polymorphisms towards identifying cancer risk. Literature data previously outlined the expression of TLR-4 in the laryngeal carcinoma microenvironment in

Table III. Comparative analysis models of XRCC1Arg194Trp, XRCC1Arg399Gln, XPDLys751Gln and TLR-4Thr399Ile in laryngeal cancer patients.

Genotypes	Cases N=157 (%)	Controls N=101 (%)	Crude OR (95% CI)	P-value	Adjusted OR[c] (95% CI)	P-value
XRCC1Arg194Trp						
Wild type variant (AA)	92 (71.32%)	64 (73.56%)	(ref) 1.00[b]		(ref) 1.00[b]	
Heterozygote (AT)	37 (28.68%)	23 (26.44%)	1.12 (0.58-2.17)	0.758	1.11 (0.60-2.05)	0.717
Homozygote mutant (TT)	28 (23.33%)	14 (17.95%)	1.39 (0.64-3.09)	0.477	1.39 (0.67-2.84)	0.361
Dominant (AT+TT) vs AA	65 (41.40)	37 (36.63)	1.22 (0.70-2.11)	0.514	1.22 (0.73-2.04)	0.443
Recessive TT vs (AA+AT)	129 (82.17)	87 (13.86)	1.35 (0.64-2.93)	0.492	1.34 (0.67-2.70)	0.394
Allele contrast model (Trp vs Arg)						
Allele Arg	93 (29.62%)	51 (25.25%)	(ref) 1.00[b]	0.314	1.245 (0.835-1.857)	0.278
Allele Trp	221 (70.38%)	151 (74.75%)	1.25 (0.82-1.90)			
XRCC Arg399Gln						
Wild type variant (Arg/Arg)	97 (61.78%)	66 (65.35%)	(ref) 1.00[b]		(ref) 1.00[b]	
Heterozygote (Arg/Gln)	37 (27.61%)	24 (26.67%)	1.05 (0.58-2.17)	1	1.04 (0.57-1.91)	0.876
Homozygote mutant (Gln/Gln)	23 (19.17%)	11 (14.29%)	1.42 (0.64-3.09)	0.442	1.42 (0.64-3.11)	0.371
Dominant (Arg/Gln+Gln/Gln) vs Arg/Arg	60 (38.22)	35 (34.65)	1.17 (0.70-2.11)	0.598	1.16 (0.69-1.96)	0.561
Recessive Gln/Gln vs (Arg/Arg+Arg/Gln)	134 (85.35)	90 (89.11)	1.4 (0.62-3.32)	0.453	1.40 (0.65-3.02)	0.378
Allele contrast model (Gln vs Arg)						
Allele Arg	83 (26.43%)	46 (22.77%)	(ref) 1.00[b]	0.404	1.21 (0.80-1.82)	0.346
Allele Gln	231 973.57%)	156 (77.23%)	1.22 (0.79-1.88)			
XPD Lys751Glu						
Wild type variant (Lys/Lys)	63 (51.64%)	55 (63.22%)	(ref) 1.00[b]		(ref) 1.00[b]	
Heterozygote (Lys/Gln)	59 (48.36 %)	32 (36.78%)	1.61 (0.88-2.94)	0.1195	1.60 (0.91-2.82)	0.095
Homozygote mutant (Gln/Gln)	35 (35.71%)	14 (20.29%)	2.18 (1.01-4.84)	0.0383	2.18 (1.06-4.47)	**0.028**[d]
Dominant (Lys/Gln+Gln/Gln) vs Lys/Lys	94 (59.87)	46 (45.54)	1.78 (1.04-3.05)	0.0294	1.78 (1.07-2.95)	0.024
Recessive Gln/Gln vs (Lys/Lys+Lys/Gln)	122 (77.71)	87 (86.14)	1.78 (0.87-3.80)	0.1051	1.78 (0.90-3.51)	0.086
Allele contrast model (Gln vs Lys)						
Allele Lys	129 (41.80%)	60 (29.70%)	(ref) 1.00[b]	0.0084	1.65 (1.13-2.40)	**0.008**[d]
Allele Gln	185 (58.92%)	142 (70.30%)	1.65 (1.11-2.45)			
TLRThr399Ile						
Wild type variant (AA)	104 (69.33%)	71 (24.47)	(ref) 1.00[b]		(ref) 1.00[b]	
Heterozygote (AG)	46 (30.67%)	23 (24.47%)	1.13 (0.73-2.57)	0.771	1.12 (0.63-2)	0.677
Homozygote mutant (GG)	10 (8.77%)	4 (5.33%)	1.71 (0.46-7.73)	0.571	0.58 (0.17(1.94)	0.367
Dominant (AG+GG) vs AA	53 (33.76)	30 (29.70)	1.20 (0.68-2.15)	0.585	1.20 (0.70-2.06)	0.495
Recessive GG vs (AA+AG)	147 (93.63)	97 (96.04)	1.64 (0.45-7.39)	0.575	1.64 (0.503-5.409)	0.395
Allele contrast model (G vs A)						
Allele A	63 (20.06%)	34 (16.83%)	(ref) 1.00 [b]	0.419	1.24 (0.78-1.96)	0.356
Allele G	251 (79.94%)	168 (83.17%)	1.24 (0.76-2.03)			

a: Abbreviation: OR-odds ratio; 95% CI-95% confidence interval; FDR-false discovery rate; *b*: Reference categories (OR=1): the wild type genotype and allele for each individual polymorphism; c: Adjusted OR, calculated in a logistic regression model without control for gender and age; d: Bold values as statistically significant after application of FDR correction, $p<0.05$.

Table IV. Stratification analysis of the association between the XPDLys751Gln polymorphism and laryngeal cancer

$XPD_{Lys751Gln}$	Number of cases			Number of controls			Adjusted OR[c] (95% CI)			
Variables	Lys/ Lys	Lys/ Gln	Gln/ Gln	Lys/ Lys	Lys/ Gln	Gln/ Gln	Heterozygote	Homozygote mutant	Dominant	Recessive
Age (in years)										
<62	34	30	19	28	13	4	1.90 (0.83-4.31), p=0.120	3.91 (1.19-12.83), p=0.014	2.37 (1.12-4.99), **p=0.021**[d]	3.04 (0.96-9.58), p=0.039
≥62	29	29	16	27	19	10	1.42 (0.65-3.10), p=0.376	1.48 (0.57-3.84), p=0.407	1.44 (0.71-2.91), p=0.303	1.26 (0.52-3.05), p=0.593
Gender										
Male	58	55	32	52	27	13	1.82 (1.00-3.30), p=0.0447	2.20 (1.04-4.64), p=0.0328	1.94 (1.14-3.31), p=0.0129	1.72 (0.84-3.48), p=0.1230
Female	5	4	3	3	5	1	0.48 (0.06-3.35), p=0.455	0.20 (0.01-2.91), p=0.212	0.7 (0.11-4.23), p=0.696	0.29 (0.02-3.45), p=0.303
Age-Gender										
Males <62	31	27	17	27	9	4	2.61 (1.04-6.51), **p=0.033**[d]	3.70 (1.10-12.35), **p=0.021**[d]	2.94 (1.31-6.59), **p=0.007**[d]	2.63 (0.82-8.46), p=0.081
Males ≥62	27	28	15	25	18	9	1.44 (0.64-3.21), p=0.372	1.54 (0.57-4.15), p=0.386	1.47 (0.71-3.04), p= 0.294	1.30 (0.52-3.26), p=0.569
Females <62	4	3	2	1	4	0	0.18(0.01-2.66), p=0.187	-[c]	0.31 (0.02-4.03), p=0.348	-[c]
Females ≥62	1	1	1	2	1	1	2 (0.05-78.24), p=0.709	1 (0.01-50.39), p= 1	2 (0.09-44.34), p=0.657	1.5 (0.05-40.74), p=0.809

a: Abbreviation: OR-odds ratio; 95% CI-95% confidence interval; b: Adjusted OR for covariates such as gender and age (in years) in a logistic regression model for each stratum; c: OR could not be calculated due to zero values in one category; d: Bold values as statistically significant after application of FDR correction, p<0.05.

works by Szczepański et al. (31) and Sikora et al. (32), who report on how laryngeal carcinoma cells mainly express three of the ten human isoforms: TLR2, TLR3 and TLR4. Bergman et al. found the heterozygous genotype of TLR-$4_{Thr399Ile}$ in head and neck cancer scuamous cell carcinoma (HNSCC) patients to have reduced disease-free survival rates (33). In a meta-analysis by Ding et al. TLR 4 polymorphisms were associated with decreased cancer risk in both Asian and Caucasians, but not TLR-$4_{Thr399Ile}$ who showed no such association (34).

Research on DNA repair genes has previously reported on the implication of the XRCC1 gene in laryngeal cancer development with various results throughout the years. Alimu et al. (35) identified that carriers of the heterozygote and mutant homozygote variants of both $XRCC1_{Arg194Trp}$ and $XRCC1_{Arg399Gln}$ may be associated with laryngeal cancer in an Asian population group. In a meta-analysis, Li et al. (36) could only link the $XRCC1_{Arg399Gln}$ polymorphism under the homozygote model with increased cancer risk for laryngeal cancer (OR = 1.29, 95% CI= 1.00–1.65, *P*= 0.05). Olshan et al. (37) reported a weak elevation in the risk for head and neck cancer (HNC) patients for the $XRCC1_{Arg194Trp}$ polymorphism (OR=1.3, 95% CI 0.6-2.9) and a decreased risk for the X$RCC1_{Arg399Gln}$ polymorphism (OR=0.6; 95%CI= 0.4-1.1). The same decreased cancer risk was noted for HNC in studies by Huang et al. (38) for the homozygote model of X$RCC1_{Arg399Gln}$ (OR=0.56, 95% CI= 0.32-0.94) and by Hu et al. (39) for Trp allele carriers of the $XRCC1_{Arg194Trp}$ polymorphism (OR=0.89, 95% CI= 0.81-0.98).

The results of our study showed no association between the $XRCC1_{Arg194Trp}$ and $XRCC1_{Arg399Gln}$ polymorphisms and the risk of laryngeal cancer in our Romanian population. According to the literature, the same lack of association between XRCC1 gene polymorphisms and laryngeal cancer was reported in works by Varzim et al. (40), Chen et al. (41) and Wu WQ et al. (42). In reference to HNC susceptibility, several articles also affirm no such relationship for the *XRCC1* polymorphisms (43-45).

Our analysis in the $XPD_{Lys751Gln}$ polymorphism identified a high risk association towards laryngeal cancer development in Gln allele carriers. Young subjects possessing the Gln/Gln genotype reported a two fold cancer risk. We observed a directly proportionate amplification in risk once the subjects were also distributed according to sex. The greatest magnitude towards cancer risk in the studied population was thus found in male patients, age under 62 years, in the mutant homozygote variant, the dominant model and finally the heterozygote model. This modification in risk according to individual factors such as age and gender is in accordance with the literature data as both the male-to-female incidence ratio and the age distribution in laryngeal cancer shows predominance towards male patients between 40-60 years in age (6).The obtained results regarding the genetic models of the $XPD_{Lys751Gln}$ polymorphism are in agreement with other literature data reported on this subject. Lin et al. (46) observed high risk association for both laryngeal and nasopharyngeal cancers in the same heterozygote and dominant models while Mitral et al. (25) observed an increase in risk for the mutant homozygote (OR=1.680, 95% CI=1.014-2.784), heterozygote (OR=1.531, 95% CI=1.092-2.149) and dominant (OR 1.560, 95% CI=1.128-2.158) genetic model in HNCSCC. Yuan et al. (47) found the same dominant model linked with HNC (OR= 1.12, 95%CI= 1.03-1.22, P= 0.01), while the heterozygote model was borderline significant (OR=1.08, 95%CI= 0.99-1.19, P= 0.08). As literature has also confirmed that the two XPD variant alleles may be related to decreased NER function and thus lower levels of DNA damage repair attributes as opposed to the more common wild-type alleles (48), it also highlights how the more predominant influences of the $XPD_{Lys751Gln}$ polymorphism in carcinogenesis seem to belong to Gln allele carriers in terms of development and poorer survival rates (49). Indeed, this characteristic cannot yet be considered a wholly independent factor, but it may be an additive one especially when further analyzed with other possible independent factors, innate or environmental. After all, a lack of associations between the $XPD_{Lys751Gln}$ polymorphism and cancer has also been observed in the literature and thus further analysis is required in order to correctly asses this apparent particularity of the XPD variants (50, 51).

In terms of the limits of our study, the relative small number of subjects subjected to genetic testing as well as their overall charactheristics at the moment this study took place must be considered along with the population type. We only included Caucasians of Romanian ancestry, as such the genetic characteristic of this ethinic group may present particular variations in terms of characteristics typical of Eastern Europe populations. Future studies with large-scales populations and multiple ethnic groups are still needed in order to determine the precise genetic distribution of alleles and variants of these important genes in terms of further evaluating the role genetic mutations within them have towards laryngeal cancer susceptibility.

Conclusion

Results highlight no evidence of a relationship between the $TLR\text{-}4_{Thr399Ile}$, $XRCC1_{Arg194Trp}$ and $XRCC1_{Arg399Gln}$ polymorphisms and laryngeal cancer. Gln carriers of the $XPD_{Lys751Gln}$ polymorphism

showed individual association with laryngeal cancer risk in the mutant homozygote variant as well as increased risk when patients were then stratified by gender and age. The overall results of our study support the hypothesis that the $XPD_{Lys751Gln}$ polymorphism may contribute to the risk of developing laryngeal cancer in this ethnic group. However, it is possible that our results may be caused by chance with the stratified analysis model due to the number of subjects included in the study as well as the possible selection bias of hospital based subjects. Thus, we consider our results as preliminary and in need of further validation from larger studies.

Abbreviations

TLR: toll-like receptors
DNA: deoxyribonucleic acid
HNC: head and neck cancers
SCC: squamous cell carcinoma
PRR: pattern recognition receptors
NER: nucleotide excision repair
BER: and base excision repair
DSB: double strand break
XRCC1: x-ray repair cross-complementing group 1
SNP: single nucleotide polymorphism
Arg: arginine
Trp: tryptophan
Gln: glutamine
Ile: lsoleucine
Lys: lysine
A: adenine
G: guanine
T: thymine
XPD: xeroderma pigmentosum type D
ERCC2: excision repair cross-complementation group 2
TFIIH: transcription factor II human complex
CTD: carboxy-terminal domain
ETDA: ethylenediaminetetraacetic acid
PCR–RFLP: polymerase chain reaction-restriction fragment length polymorphism
OR: odds ratio
CI: confidence interval
HNSCC: head and neck squamous cell carcinoma
FDR: false discovery rate

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Authors' contributions

CIC (Conceptualization, Acquisition of data, Analysis and Interpretation of data, Writing-original draft preparation)
VN (Writing-review and editing)
MC (Writing-review and editing)
AAM (Writing-review and editing, Visualization)
AC (Conceptualization, Methodology, Resources, DNA isolation, Genotyping, Validation)

Conflict of interest statement

The authors declare that there are no conflicts of interest.

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