Increased risks between TLR2 (-196 to -174 ins/del) and TLR3 1377C>T variants and head and neck cancers in Tunisia

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Abstract

Introduction: Previous studies have highlighted the importance of polymorphisms of toll-like receptors (TLRs) in the pathogenesis of certain cancers, including head and neck cancers (HNC).

Aim of the study: The aim of this study was to evaluate the association of TLR2 (-196 to -174 ins/del) and TLR3 (1377 C>T) as potential risk factors for HNC in Tunisians.

Material and methods: A case-control study including 246 HNC patients (174 nasopharyngeal carcinoma – NPC and 72 laryngeal cancer – LC) and 250 healthy controls. Genotyping was done by using PCR and PCR-RFLP methods.

Results: Higher minor allele frequencies of TLR2 (-196 to -174 ins/del) and TLR3 1377 C>T polymorphisms were seen in HNC, NPC, and LC compared to controls. In addition, higher increased HNC, NPC, and LC risk was associated with TLR2 ins/del and TLR2 del/del genotypes (p < 0.0001). Positive association with HNC, NPC, and LC risk was seen with TLR2 del-containing genotypes (ins/del + del/del) (p < 0.0001). The T/T genotype of TLR3 is associated with HNC, NPC, and LC susceptibility (p < 0.0001). Positive association with HNC and NPC risk was seen with TLR3 T allele carriers (C/T + T/T) (p < 0.0001). Increased frequency of T-ins, C-del, and T-del haplotypes was revealed in HNC and NPC cases than healthy controls; however, T-del was significantly higher in LC cases.

Conclusions: Our results demonstrate an increased risk of HNC, NPC, and LC with TLR2 ins/del, TLR2 del/del, and TLR3 T/T genotype. And positive association with T-ins, C-del, and T-del haplotypes with HNC and NPC and T-del haplotype with LC.

Key words: toll-like receptors, polymorphisms, laryngeal cancer, head and neck cancer, Tunisia, nasopharyngeal cancer.

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Material and methods

Study subjects

Between November 2012 and August 2016, 246 HNC cases including 174 NPC and 72 LC, were recruited from the Salah Azeiz Oncology Institute (SAI, Tunisia). The diagnosis was established by clinical examination and histopathology.

The control group comprised 250 unrelated blood donors who were free of chronic disease, history of malignancy, drug allergies, hypertension, diabetes, or cardiovascular disease and were recruited from the Tunisian Centre of Maternity and Neonatology, and Dispenser of Ettadhamen City. Demographic and clinical data were collected from cases and controls using a unified questionnaire. The patients were considered to be smokers if they had smoked at least one cigarette daily for at least one year during their lifetime and were classified as drinkers if they had consumed alcoholic beverages at least once a month on a regular basis.

All subjects were asked to sign a consent form, agreeing to participate in the study, after all institutional ethical requirements were met.

The TLR family includes five extracellular (TLR1, 2, 5, 6, and 10) and four intracellular (TLR3, 7, 8, and 9), and TLR4 is localised to both the plasma membrane and endosomal vesicles [10]. TLR2 and TLR3 have been particularly investigated regarding inflammation and cancer [11, 12].

Genetic studies have identified a 22-bp nucleotide deletion (-196 to -174 ins/del) in the promoter region of TLR2 gene located on chromosome 4q32. This variant may alter the promoter activity of TLR2 and thus is related to its expressivity [13] and a non-synonymous 1377C/T polymorphism, present in exon 4 of TLR3 gene on chromosome 4 that affects the receptor-ligand interaction by altering the TLR3 ectodomain and thereby functionally impairing the receptor [14]. However, published results of the TLR2 ins/del and TLR3 1377C/T polymorphisms have been conflicting, and their relationship to cancer risk remains unclear [15, 16].

The present study aimed to analyse the association of TLR2 (-196 to -174 ins/del) and TLR3 (1377 C>T; rs3775290) variants as potential risk factors for HNC and especially for NPC compared to LC in Tunisians, in view of their potential use as biomarkers to evaluate cancer risk.

TLR genotyping

Total genomic DNA was extracted from peripheral blood of study participants using the QIAamp DNA Blood Mini Kit (Qiagen, Hilden, Germany). Determination of TLR2-196 to -174 ins/del polymorphism was performed by PCR, as previously described [17], while TLR3 (1377C>T; rs3775290) genotyping was determined by PCR-restriction fragment length polymorphism (RFLP) analysis, as previously described [18].

Statistical analysis

Statistical analysis was performed using SPSS version 20.0 software (IBM, Armonk, NY), SNPstats software (www.bioinfo.iconcologia.net/snpstats/), and Haplovie version 4.2 (http://www.broad.mit.edu/mpg/haplovie). Hardy-Weinberg equilibrium (HWE) was calculated for testing variants in cases and controls. Data were expressed as percentages of total (categorical variables) or as mean ±SD (continuous variables). Student’s t-test was used for variables with a normal distribution (mean ±SD), while Pearson’s χ² and Fisher’s exact test were used to assess inter-group significance. Allele frequencies were calculated using Haplovie. Logistic regression analysis was performed in order to determine the odds ratios (OR) and 95% confidence intervals (95% CI) associated with the HNC risk, taking the control as the reference group. Statistical significance was set at p < 0.05; statistically significant differences being designated as boldface in the tables. Haplotype reconstructions were performed using SNPstats software.

Results

Clinical parameters of head and neck cancer cases

The demographic and clinical characteristics of HNC patients and controls are summarised in Table 1. The median age was 49.48 ±14.15 years for patients with HNC and 47.36 ±10.30 years for healthy controls. No statistical difference was observed between cases and controls in the distribution of age (p = 0.057). The prevalence of smoking and alcohol consumption were significantly higher among HNC patients compared to healthy controls (65.04% vs. 16.80%, p < 0.001 and 39.43% vs. 10%, p < 0.001, respectively). All NPC patients had an undifferentiated carcinoma nasopharynx tumour (UCNT). However, all LC cases had squamous cell carcinoma (SCC).

Association studies of TLR2 (-196 to -174 del) and TLR3-1377 C>T alleles, genotypes, and haplotypes

The distribution of minor allele for TLR2 (-196 to -174 ins/del) and TLR3-1377 C>T polymorphisms between...
HNC, NPC, and LC patients and controls are shown in Table 2. Higher minor allele frequencies (MAF) of TLR2 (-196 to -174 ins/del) and TLR3-1377 C>T polymorphisms were seen in HNC, NPC, and LC compared to controls \( (p = 3.92 \times 10^{-9}, p = 2.67 \times 10^{-6}, p = 7.018 \times 10^{-8}, p = 1.23 \times 10^{-6}, p = 1.99 \times 10^{-5}, p = 0.031) \), respectively.

We examined the genotype distribution of the tested polymorphisms in HNC, NPC, and LC patients and healthy controls (Table 3). The frequencies of TLR2 ins/del and TLR2 del/del genotypes were significantly higher in HNC, NPC, and LC cases compared to healthy controls \( (p < 0.0001) \). These genotypes were positively associated with HNC, NPC, and LC risk, respectively.

Positive association with HNC, NPC, and LC risk was seen when comparison was made between cases and controls with TLR2 del-containing genotypes (ins/del + del/del) vs. non-carriers (ins/ins) \( (p = 0.022, p < 0.0001, and p < 0.0001) \), respectively.

Taking a homozygous major genotype as a reference \( (OR = 1.00) \), the distribution of TLR3 T/T genotype frequency was significantly different between HNC, NPC, and LC cases and controls \( (p < 0.0001, p < 0.0001, and p = 0.054) \), respectively.

Positive association with HNC and NPC risk was seen when the comparison was made between cases and controls considering TLR3 T allele carriers (C/T + T/T) vs. non-carriers (C/C) \( (p = 0.0037 and p = 0.0023) \), respectively.

Haplotype analysis revealed a significant positive association between T-ins, C-del, and T-del haplotypes and HNC and NPC risk \( (p < 0.0001, p = 1 \times 10^{-4}, p < 0.0001, p < 0.0001, p = 1 \times 10^{-4}, p < 0.0001) \), respectively. However, of the four possible haplotypes, the frequency of T-del was significantly higher in LC cases than in healthy controls \( (p = 2 \times 10^{-4}) \) (Table 4).

### Discussion

Head and neck cancer arises from the upper aerodigestive tract that is chronically exposed to pathogens or a toxic irritant that may induce chronic inflammation where inflammatory cells regulate the tumoural micro-environment. Because TLRs are potent immune modulators and are involved in regulating cell proliferation, and survival and removal of cancer debris [8, 9], increasing

| Table 1. Characteristics of head and neck cancer cases and controls |
|---------------------|---------------------|---------------------|---------------------|
| Characteristics     | Cases | Controls | \( p \) |
| Age (mean ±SD)      | 49.4 (14.1) | 47.3 (10.3) | 0.057 |
| Gender, n (%)       | Male 187 (76) | 126 (50.4) | < 0.001 |
| Tobacco consumption, n (%) | Yes 160 (65) | 42 (16.8) | < 0.001 |
| Alcohol consumption, n (%) | Yes 97 (39.4) | 25 (10) | < 0.001 |
| HNC type            | NPC 174 – |
|                     | LC 72 – |
| Histology, n (%)    | UCNT 174 (100) | NA – |
|                     | SCC 72 (100) | NA – |

HNC – head and neck cancer, NPC – nasopharyngeal carcinoma, LC – laryngeal cancer, UCNT – undifferentiated carcinoma nasopharynx tumour, SCC – squamous cell carcinoma, NA – not applicable

| Table 2. TLR2 and TLR3 allelic distribution in cases and controls |
|---------------------|---------------------|---------------------|---------------------|
| Polymorphisms       | MA HWE MAF \( \chi^2 \) | OR (95% CI) |
|---------------------|---------------------|---------------------|---------------------|---------------------|---------------------|
| Head and neck cancer | del < 0.05 0.378 0.208 34.662 3.92 \times 10^{+} 2.31 (1.74-3.07) | |
| TLR2 (-196 to 174 del) TLR3 (1377T>T) T 0.157 0.535 0.386 22.033 2.67 \times 10^{+} 1.82 (1.41-2.35) |
| Nasopharyngeal carcinoma | del < 0.05 0.376 0.208 29.06 7.018 \times 10^{5} 2.29 (1.69-3.12) |
| TLR2 (-196 to 174 del) TLR3 (1377T>T) T 0.258 0.555 0.386 23.519 1.23 \times 10^{+} 1.98 (1.50-2.61) |
| Laryngeal cancer     | del < 0.05 0.382 0.208 18.193 1.99 \times 10^{+} 2.25 (1.57-3.50) |
| TLR2 (-196 to 174 del) TLR3 (1377T>T) T 1.0 0.486 0.386 4.638 0.031 1.50 (1.03-2.18) |

\( \ast \) study subjects included 246 HNC and 250 controls, MA – minor allele, HWE – Hardy-Weinberg equilibrium, MAF – minor allele frequency, OR – odds ratio, CI – confidence interval
Evidence implicates them in tumour development [19], of which TLR2 and TLR3 have been actively investigated in carcinogenesis. Positive TLR2 expression was revealed in the tumour microenvironment, suggesting activation of the immune surveillance against the altered epithelial cells [20]. Genetic studies have investigated the analysis of TLR gene polymorphisms as potential biomarkers of cancer risk, thus underscoring the contribution of specific gene variants to the susceptibility to various cancers. However, to the best of our knowledge, few reports have included HNC. The present study examined the association of two common polymorphisms in TLR2 and TLR3 with the susceptibility to HNC.

TLR2 ins/del polymorphism alters TLR2 promoter activity, leading to decreased expression of the TLR2 gene. Our results revealed that the carriage of the minor TLR2 del allele and TLR3 T allele are significantly associated with increased risk of HNC and its two types: NPC and LC, and thus raising may represent candidate biomarkers for evaluating these HNC risks. Consistent with our findings, it was previously shown that TLR2 ins/del polymorphism was associated with heightened risk of some cancers, such as breast cancer, gastric cancer, prostate cancer, hepatocellular cancer, and cervical cancer [21-25], but not all, such as cervical cancer [26]. In the future, controlled studies involving larger sample sizes and molecular approaches are required to clarify the exact contribution of this variant (if any) to the development and/or evolution of different cancer types, including HNC.

Haplotype analysis between TLR2 and TLR3 polymorphisms identified T-ins, C-del, and T-del haplotypes to be positively associated with HNC and NPC. However, only the minor genotype T-del is associated with a higher LC risk. To the best of our knowledge, this is the first study that investigated the possible linkage between these two polymorphisms and the risk of HNC, and as such we cannot compare our results to related studies. Additional studies investigating the linkage of these two variants and possibly others are needed to clarify the contribution of this variant to the development of HNC.

Our study has some strengths, namely that HNC patients and controls have a similar ethnic background, because they originated from North Tunisia, thus minimizing selection bias. Our study also has some limitations, including the relatively small sample size and the lack of information on tumour stage and grading.

Table 3. Distribution of TLR2 and TLR3 genotypes in head and neck cancer (HNC), nasopharyngeal carcinoma (NPC), and laryngeal cancer (LC) cases and healthy controls

| Polymorphism genotype | HNC (n = 246) (%) | OR (95% CI) | p | Controls (n = 250) (%) | OR (95% CI) | p | NPC (n = 174) (%) | OR (95% CI) | p | LC (n = 72) (%) | OR (95% CI) | p |
|------------------------|------------------|-------------|---|-----------------------|-------------|---|------------------|-------------|---|----------------|-------------|---|
| TLR2 (-196 to -174 del) |                 |             |   |                       |             |   |                  |             |   |                  |             |   |
| ins/ins                | 117 (47.6)       | 1.00 (reference) |   | 85 (48.9)           | 1.00 (reference) |   |                  |             |   |                  |             |   |
| ins/del                | 72 (29.3)        | 3.18 (1.87-5.39) | < 0.0001 | 47 (27)          | 2.33 (1.43-3.80) | < 0.0001 |                  |             |   |                  |             |   |
| del/del                | 57 (23.2)        | 2.68 (1.47-4.88) | < 0.0001 | 42 (24.1)        | 2.282 (1.66-4.80) | < 0.0001 |                  |             |   |                  |             |   |
| ins/del + del/del      | 129 (52.4)       | 2.96 (1.90-4.60) | 0.022 | 89 (51.1)        | 2.54 (1.70-3.80) | < 0.0001 |                  |             |   |                  |             |   |
| TLR3 (1377C>T)         |                 |             |   |                       |             |   |                  |             |   |                  |             |   |
| C/C                    | 61 (24.8)        | 1.00 (reference) |   | 40 (23)           | 1.00 (reference) |   |                  |             |   |                  |             |   |
| C/T                    | 107 (43.5)       | 1.31 (0.87-1.99) | < 0.0001 | 75 (43.1)       | 1.40 (0.88-2.24) | < 0.0001 |                  |             |   |                  |             |   |
| T/T                    | 78 (31.7)        | 3.36 (2.01-5.62) | < 0.0001 | 59 (33.9)       | 3.88 (2.22-6.78) | < 0.0001 |                  |             |   |                  |             |   |
| C/T + T/T              | 185 (75.2)       | 1.77 (1.20-2.60) | 0.0037 | 134 (77)        | 1.95 (1.26-3.02) | < 0.0001 |                  |             |   |                  |             |   |
ing the contribution of racial/ethnic differences inherent in genetic association studies. In addition, NPC assessment involved questionnaire-based interviews, and laboratory assessment, including histology screening, hence cancer was ascertained. However, our study had several limitations, namely that the sample size was relatively small with only two HNC types, thereby necessitating future studies involving a larger number of cases and controls and other types of HNCs, so as to fully understand the contribution of \textit{tLr2} and \textit{tLr3} gene polymorphisms on HNC as well as related malignancies.

Conclusions

In conclusion, this is the first study to evaluate the effect of two common polymorphisms in \textit{tLr2} and \textit{tLr3} on susceptibility to HNC and its LC and NPC types in Tunisia. Our results revealed that the carriage of minor \textit{TLR2} (-196 to -174) del and \textit{TLR3} -1377 T alleles appears to exert a significant influence on HNC risk, thus providing evidence of the involvement of these polymorphisms and their haplotypes as risk factors of HNC. The identification of such genetic risk predictors for NPC and LC, the two major HNC types, may lead to improving diagnosis, risk prediction, and clinical care.

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The authors declare no conflict of interest.

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