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Toll-like receptor 2 rs4696480 polymorphism and risk of oral cancer and oral potentially malignant disorder

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ABSTRACT

Objectives: The aim of this study was to identify the possible association between TLR polymorphisms and an increased risk of developing head and neck cancer, including oral (OSCC) and laryngeal squamous cell carcinoma (LSCC), and oral potentially malignant disorders, such as oral lichenoid disease (OLD), including oral lichen planus (OLP) and oral lichenoid lesions (OLL).

Design: This case-control study included 40 OSCC, 35 LSCC, 175 OLD (129 OLP and 46 OLL) patients and 89 healthy controls, all of them from the Basque Country, Spain. Genetic polymorphisms in TLR1, TLR2, TLR4, TLR6, TLR9, and TLR10 were genotyped by TaqMan® assays or pyrosequencing.

Results: The chi-square analysis showed that the variant A of the SNP TLR2-rs4696480 polymorphism significantly increased the risk of OSCC ($p = 0.03$) and OLL ($p = 0.02$).

Conclusions: The TLR2-rs4696480 polymorphism may be relevant to OSCC and OLL susceptibility in this population encouraging further studies on the TLR2 pathway and its possible association with this group of oral potentially malignant disorders and oral cancer. This may also prove the use of TLR polymorphisms as risk markers for oral and laryngeal cancer.

1. Introduction

Head and neck squamous cell carcinoma (HNSCC) is a complex worldwide concern as it is the sixth most common type of cancer (Warnakulasuriya, 2009b). The etiology of HNSCC is multifactorial with chronic consumption of tobacco and alcohol as the most important risk factors (Argiris, Karamouzis, Raben, & Ferris, 2008; Warnakulasuriya, 2009b).

Immunosuppression and the inflammatory environment are other additional factors also involved in this process by enhancing tumorigenesis and/or cancer progression (Hanahan & Weinberg, 2011; Warnakulasuriya, 2009a). Cancer-related chronic inflammation may be associated with microbial infections, as occurs in cervical cancer (Chuang, Huang, Chien, & Chuang, 2012; Bergmann et al., 2011) and HNSCC, specially oropharyngeal cancer (Castellsagué et al., 2016), and the infection with the human papillomavirus.

In some cases, it may be considered as the result of an immune-mediated chronic inflammatory environment as observed in oral lichenoid disease (OLD), an oral potentially malignant disorder (Aguirre Urizar, 2008; Cort & s-Ramírez, Gainza-Cirauqui, Echebarria-Goikouria, & Aguirre-Urizar, 2009; Gonzalez-Moles, Scully, & Gil-Montoya, 2008; van der Meij, Mast, & van der Waal, 2007; Warnakulasuriya et al., 2011). According to the WHO criteria, modified by van der Meij and van der Waal (2003), oral lichen planus (OLP) is diagnosed using specific clinical and histological criteria. Furthermore, as proposed by Aguirre Urizar (2008), lesions that do not fulfil at least one of these criteria, such as: showing a non-specific pattern or presenting characteristics other than those of a lichenoid reaction, should be classified as oral lichenoid lesion (OLL), a group of lesions with a greater susceptibility for a malignant transformation. The inflammatory response can be a source of various molecules that contribute to tumor

Abbreviations: HNSCC, head and neck squamous cell carcinoma; HSP, heat shock proteins; LSCC, laryngeal squamous cell carcinoma; MAF, minor allelic frequency; OLP, oral lichen planus; OLD, oral lichenoid disease; OLL, oral lichenoid lesion; OR, odd ratio; OSCC, oral squamous cell carcinoma; PCR, polymerase chain reaction; SNP, single nucleotide polymorphisms; TLR, toll-like receptors

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development, such as growth factors, proangiogenic factors among others, or even of reactive oxygen species that may play a role in the malignant transformation process (Hanahan & Weinberg, 2011). Inflammatory molecular pathways, such as those from Toll-like receptors (TLR), might trigger this relationship (Khan, Khan, & Warnakulasuriya, 2016).

The TLRs initiate the innate immune response by recognizing molecular patterns from different microorganisms. TLR activation culminates in cytokine production and consequently, with the acute inflammatory response and with the stimulation of the adaptive immune response (Arancibia et al., 2007; Pasare & Medzhitov, 2005). Current evidence shows that TLRs can recognize constitutive proteins of the host such as heat-shock proteins (HSP) (Vabulas et al., 2002). This type of interaction is the possible link between TLRs and autoimmune and chronic inflammatory diseases (Chen et al., 2007; Kim & Karin, 2011; Lehnardt et al., 2008).

Ligand recognition by TLRs and its consequent inflammatory response is widely known and studied, yet it is known that TLRs also participate in other cell processes such as proliferation, apoptosis and angiogenesis (Kutikhin, 2011). Consequently, abnormalities in the TLR function may explain the aberrant immune response of many conditions, from immune-mediated diseases to cancer (Li, Zhou, Feng, & Su, 2009; Papadimitraki, Bertsias, & Boumpas, 2007). Finally, these functional alterations can be induced by inheritance of mutations in the TLR-encoding genes. Single nucleotide polymorphisms (SNP) are common genetic variations that can be associated with a higher susceptibility of developing complex diseases and with a variable response to treatment (Goldstein & Cavalleri, 2005).

The oral cavity is chronically exposed to a variety of pathogenic agents that are common TLR targets. These receptors also recognize toxic irritants derived from tobacco (Pace et al., 2008) and alcohol (Crews, Qin, Sheedy, Vetreno, & Zou, 2012), the main risk factors for HNSCC (Warnakulasuriya, 2009a). Furthermore, T-cell lymphocyte-mediated inflammatory oral diseases, such as OLD (Eisen, Carrozzo, Bagan Sebastian, & Thongprasom, 2005), exhibit an increase in the expression of HSP, a TLR ligand (Sugerman, Savage, Xu, Walsh, & Seymour, 1995), and in the aberrant TLR signalling, thus involving these receptors in their pathogenesis (Janardhanam et al., 2012; Li, Chen, Tan, Liu, & Liu, 2007; Ohno et al., 2011; Sinon et al., 2015; Siponen, Kauppila, Soini, & Salo, 2012; Srinivasan, Kodumudi, & Zunt, 2008; Stanimirovic et al., 2013).

Furthermore, several studies (Chuang et al., 2012; Farnebo et al., 2015; Ng et al., 2011) have identified that an altered TLR expression in HNSCC may lead to apoptosis resistance, to an accelerated tumour growth and invasion, and to poorly differentiated lesions. In addition, there is evidence that SNP are present in TLR genes in some types of cancer, including OSCC (Bergmann et al., 2011; Zeljic et al., 2014), by contributing in tumor susceptibility, in the progression of the disease and in poor survival rates. This suggests that researching the connection of TLRs in tumour immunology may help clarify cancer biology (Kutikhin, 2011).

For all this, the aim of the study was to identify the association between SNPs in TLRs and OLD, a chronic inflammatory and potentially malignant oral disorder, and malignant lesions of the head and neck region (oral and laryngeal squamous cell carcinoma – OSCC and LSCC) to further understand their pathogenesis.

2. Materials and methods

2.1. Study design, subjects and DNA samples

This case-control study included DNA samples from 40 patients with OSCC, 35 with LSCC, 177 patients with OLD, and 89 healthy controls. Cancer samples were obtained from the Otolaryngology Service of the Oncology Institute of Gipuzkoa, Spain. The OLD patients were diagnosed clinically and histopathologically and classified as oral lichen planus (OLP) or oral lichenoid lesion (OLL) as previously described (Cortés-Ramírez et al., 2009; van der Meij & van der Waal, 2003). These samples and the disease-free control samples were obtained from the Unit of Oral Medicine of the Clinical Dental Service of the University of the Basque Country/EHU between November 2009 and May 2011 after obtaining informed consent.

The data on tobacco and alcohol consumption were collected by way of interview. Regarding the tobacco consumption, subjects were categorized as smokers when reporting the habit of smoking 10 cigarettes per day in the previous 10 years, regardless if this was a current habit or if they had stopped. In relation to alcohol consumption, subjects were classified as non-drinkers or as drinkers, if consuming more than 10 units of alcohol per week (one unit of alcohol equals 10 g of ethanol).

2.2. Sample collection and DNA extraction

Mouthwash samples were collected from a 10 ml rinse containing 0.12% chlorhexidine. The DNA was extracted from the buccal cell pellet following the phenol-chloroform and ethanol precipitation method as described previously by Marichalar-Mendia et al. (2011).

2.3. Polymorphisms analysis

Eight SNPs were included in this study with the following inclusion criteria: minor allelic frequency (MAF) > 5%, linkage disequilibrium (LD) < 0.65, and missense or intronic mutations with a genetic expression alteration capacity (Table A – Supplemental material). The following SNPs included were: TLR1-rs4833095 (Assay ID: C_44103606_10), TLR1-rs5743618, TLR2-rs4696480 (Assay ID: C_27994607_10), TLR4-rs1927911 (Assay ID: C_11722141_10, TLR6-rs5743810) (Assay ID: C__1180648_20), TLR9-rs5743836 (Assay ID: C_32645383_10), TLR9-rs352139 (Assay ID: C__2301953_10) and TLR10-rs11096957 (Assay ID: C___309088_10). Six of these polymorphisms were analyzed using TaqMan™ SNP Genotyping Assays from Life Technologies (Carlsbad, CA, USA) in accordance with the manufacturer's instructions. The qPCRs where run in a Bio-Rad iCycler Thermal Cycler (Bio-Rad Laboratories Inc., California, USA) and a iQ5 Real-Time PCR Detection System (BioRad Laboratories, Hercules CA, USA). All samples were genotyped by sequencing and then run in each plate to guarantee the consistency of the assays as positive control, together with a DNA-free negative control.

The remaining two SNPs, TLR1-rs5743618 and TLR9-rs5743836, we analysed by pyrosequencing. The TLR1-rs5743618 polymorphism is located in a DNA region that shares an extended identical genomic region with the TLR6 and requires a previous nested-PCR reaction to guarantee the specific genotyping analysis in the TLR1 gene. The second case, the TLR9-rs5743836 polymorphism, is a tetra-allelic SNP in which any nucleotide is expected to be included, resulting in unattainable discriminations when using primers or probe systems.

To produce a suitable template for the pyrosequencing method, a prior conventional PCR was required. The primers were designed for the abovementioned TLR-SNPs with the PyroMark Assay Design SW 2.0 program (QIAGEN, Dusseldorf, Germany) and then synthetized by Operon (Eurofins MWG Operon, Alabama, USA). The characteristics of these primers, together with the TLR1-SNP primers of the first nested-PCR, were designed with the PerlPrimer v1.1.20 program (http://perlprimer.sourceforge.net/).

This pre-pyrosequencing PCR was performed using a C1000™ Thermal Cycler (Bio-Rad Laboratories Inc., California, USA) with the following cycling conditions: 5 min at 95 °C, followed by 40 cycles of 30 s at 94 °C, 30 s at 61 °C, 30 s at 72 °C, with a final cycle of 10 min at 72 °C. These PCR conditions are detailed in Table B (Supplemental material).

The pyrosequencing was performed with a PyroMark Q96 MD platform (QIAGEN, Dusseldorf, Germany) using the sequencing primer in accordance with the manufacturer's instructions. The results were analysed with the PyroMark MD v1.0 software (QIAGEN, Dusseldorf, Germany).