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Review

The Role of Toll Like Receptors (TLRs) in Oral Carcinogenesis

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Abstract. *Oral cancer accounts for 10% of head and neck tumors. Despite the recent advancements in surgical techniques, as well as in chemo- and radiotherapy therapeutic protocols, survival rates for oral cancer patients have not improved significantly in the last decades. Recently, toll like receptors (TLRs) have been described as promoters of cell proliferation, invasiveness, and angiogenesis in a variety of cancers. The aim of this review is to identify how TLRs participate in oral carcinogenesis in order to evaluate their biological relevance. Data revealed that some TLRs participate in oral carcinogenesis since TLR5 and TLR9 promote tumor growh, whereas TLR3 is closely involved to anti-cancer properties. They represent a promising alternative for oral cancer therapy. However, TLR2, TLR4 and TLR7 need further investigation, since current data are not sufficient to conclude about their role. Certainly, such data will contribute to new scientific knowledge, which will be incorporated to the preventive actions and therapeutic protocols for oral cancer patients suffering from this devasting disease.*

Oral cancer accounts for approximately 10% of all head and neck tumors (1). The most common histopathological presentation is squamous cell carcinoma. The main etiologic causes that are known to induce oral neoplasms are cigarette smoking and consumption of alcoholic beverages, especially the strongest alcoholic drinks (2). Oral cancer is more frequent in developing countries such as India, Vietnam and Brazil when compared to incidence rates of industrialized countries. This is due to the fact that cultural habits, continnous environmental exposure to chemical compounds categorized as carcinogenic and lack of public policies against the disease.

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Despite the recent advancements in surgical techniques, as well as in chemo- and radiotherapy therapeutic protocols, survival rates for oral cancer patients have not improved significantly in the last decades (3). Thus, the search for new scientific knowledge related to oral cancer pathogenesis is imperative and necessary for both professionals and patients. Among the new cellular signaling pathways extensively investigated in the scientific literature is Toll Like Receptors (TLRs) signaling pathway.

TLRs participate in the activation of innate immunity (4). Hence, TLRs are commonly expressed in immune cells such as B-lymphocytes, and monocytes. However, other cellular types such as dendritic cells and epithelial cells express TLRs as well. Recently, TLRs signaling pathway has been found to promote cell proliferation, invasiveness, and angiogenesis in a variety of cancers (5). In fact, activation of TLRs has been implicated in promoting malignant cell invasion and metastatic potential, whereas their biological revelance for oral carcinogenesis remains unclear. For example, some authors have postulated that TLRs have a dual effect of promoting tumor progression and protecting against cancer (5).

As a result and because of conflicting evidence in the scientific literature, the aim of this review is to identify how TLRs participate in oral carcinogenesis in order to evaluate their biological relevance for better understanding of the disease.

TLRs Signaling Pathway

Receptors of the innate immune system, termed as pattern recognition receptors (PRRs), that recognise molecular patterns of infectious agents, pathogen-associated molecular patterns (PAMPs) and of cell components released through tissue damage—damage-associated molecular patterns (DAMPs) have been described. PAMPs constitute the components of microbes and DAMPs are released from damaged or sore tissue (6).

The most studied PRRs in cellular and endosomal membranes are TLRs that can activate several cellular pathways following ligand stimulation (7). TLRs play a crucial role in the recognition of invading pathogens and the

activation of immune responses that protect the host, ranging from the recognition of a variety of PAMPs, among which are lipopolysaccharide (LPS), peptidoglycans and lipoproteins.

TLRs belong to a family of type I transmembrane receptors, which include at least 11 types in humans and 13 in rodents. TLRs are pattern-recognising proteins involved in innate and adaptive immune defence (8).

It is known that TLR1, TLR2, TLR4, TLR5 and TLR6 are located on the cell surface and detect lipoproteins and bacterial LPS, a membrane component of gram-negative bacteria, which generally inhabit the host intestine (9), whereas TLR3, TLR7, TLR8 and TLR9 are located in endosomes and lysosomes and mostly recognise viral RNA and bacterial DNA (6).

Low serum concentrations of LPS are detected under normal conditions. LPS is essential for immune system modulation. However, increased concentrations of circulating LPS lead to low-grade chronic inflammation, which is involved in the pathophysiology of inflammatory and metabolic diseases. At high concentrations, LPS forms a complex-containing LPS-binding protein and the CD14 co-receptor that is recognised by TLR4 and TLR2 and triggers intracellular signalling (8).

After activation, TLRs, except TLR3, homodimerise and can induce two different signalling pathways myeloid differentiation factor 88 (MyD88) dependent pathway and MyD88-independent pathway which induce the production of proinflammatory cytokines and interferon type 1, respectively. In the dependent pathway, MyD88 is recruited to activate phosphorylation of interleukin receptor-associated kinases IRAK1 and IRAK4, that in turn activate the tumor necrosis-associated factor TRAF-6 adapter protein. TRAF-6 forms a complex with enzymes involved in the ubiquitination process, activating transforming growth factor beta-activated kinase 1, which then phosphorylates the inhibitor kinase complex IKKβ, triggering the decoupling of NFκB in NFκBp50 and NFκBp65 dimers through degradation of its inhibitory protein IκB. NFκB then translocates into the nucleus and controls the expression of proinflammatory cytokines and chemokines (8).

Furthermore, the MyD88-dependent pathway triggers another transcription factor activator protein (AP)-1 mediated by mitogen-activated protein kinases (MAPKs) as well as c-Jun N-terminal kinase (JNK), p38 and extracellular signal-regulated kinase (ERK) (10). The TLR signalling pathway is demonstrated in Figure 1.

In this way, TLRs have been identified as important regulators of metabolic inflammation in many tissues (9). Among the metabolic diseases, several cancers have been studied with respect to physiological ligands and functions of TLRs, not only in progression but also in cancer inhibition (8).

TLRs and Oral Carcinogenesis

After reviewing the scientific literature, a variety of TLRs have been expressed and positively correlated with the risk of oral squamous cell carinoma. The first approach for understanding the putatite relationship between TLRs and oral carcinogenesis has been conducted on the rationale of inflammatory cells and apoptosis in human cancer patients. The findings revelated that human neutrophils expressed TLR2 and TLR6 regulating the intrinsic apoptotic pathway. Also, it has been shown that decreased expression of TLR2 and TLR6 in human neutrophils was controlled by activation of p38PAPK, resulting in downregulation of antiapoptotic Mcl-1 protein (11).

In 2010, Park and collleages (12) examined whether TLR signaling pathway is involved in tumor progression as a result of positive expression in oral squamous cell carcinoma. All tested TLRs including TLR2, TLR3, TLR4, TLR5, TLR7 and TLR9 were expressed in oral cancer cells. These findings indicated that TLRs activation may have a predictive value for tumorigenesis, without knowing the biological role of each TLR in oral carcinogenesis process.

To assess the role of TLR signaling in peripheral blood lymphocytes from oral cancer patients, the expression of TLR2, TLR3, TLR4 and TLR9 on various lymphocyte subsets were also evaluated. Results revealed an increased expression of TLRs on T cells, such as $CD4^+$, $CD8^+$ and NK cells (13). Overall, TLR2, TLR4, TLR5, TLR7 and TLR9 appear to reflect certain clinicopathological variables and prognostic markers of oral tumors (14). However, the precise biological mechanism involved in this process remains to be elucidated.

TRL9

After reviewing the literature, the most studied receptor involved in oral cancer so far is TLR9. This is a new member of the interleukin-1 receptor superfamily. It was recently found to be highly expressed in many carcinoma specimens. For example, recent results showed that TLR9 expression was higher in oral squamous cell carcinoma tissues when compared to ordinary controls (15, 16). Expression of TLR9 was high in well-differentiated squamous cell carcinoma and moderately differentiated squamous cell carcinomas, and moderate to low in poorly differentiated squamous cell carcinomas (17). The same authors as well as others purposed that the expression of TLR9 was significantly associated with tumor size, clinical stage and proliferative status (15, 16). High TLR9 expression was also correlated with deeper tumor invasion (18, 19). This was clarified by high cytoplasmic TLR9 expression in primary tumors than local recurrent counterparts (8).

High TLR9 expression was also associated with high MMP-13 expression and poor tumor differentiation (16). Others have yet mentioned that activation of TLR9 induced migration and subsequent invasion of oral cancer cells (18). An increased expression, and activity of MMP-2 were observed as well (18). It was, therefore, hypothesized that

increased expression of TLR9 may play a significant role in oral carcinogenesis, and could be used as a new target for oral cancer prevention and therapy in the near future (20).

In vitro studies suggested that the treatment of oral cancer cells with TLR9 agonist, CpG-ODN, significantly increased tumor cell proliferation as well as inflammatory host response, as depicted by upregulation of sIL-1α and IL-6 (20). In fact, blockage of the TLR-9/AP-1 pathway significantly decreased IL-6 expression and T-cell immune response (18). Moreover, some findings indicate that CpG-ODN stimulates tumor cell proliferation through TLR9-mediated AP-1-activated cyclin D1 expression in oral squamous cell carcinoma *in vitro* (15). Taken together, is seems that inhibition of TLR9 may be a means to prevent oral carcinogenesis since there is a consensus that the activation of TLR9 is closely linked to oral cancer risk and prognosis.

TLR2

The second TLR most investigated in the scientific literature for oral cancer risk is TLR2. Ng *et al*. have demonstrated that immune cells expressed TLR2 in oral carcinoma and oral dysplasia when compared to hyperplastic tissue (21). Positive TLR2 expression in the tumor environment suggests activation of an immune response against neoplastic cells, as a result of resistance to apoptosis due to pro-survival mechanisms (21). This was also demonstrated by others (22). Nevertheless, high TLR2 expression was positively correlated with deeper tumor invasion (19).

It is important to stress that nuclear TLR2 expression occurred more often in primary tumors than metastases or even recurrent tumors (8). Conversely, cytoplasmic TLR2 expression was correlated with tumor size, while nuclear TLR2 expression with larger tumors (14). Cytoplasmic TLR2 was also correlated significantly with higher tumor grade (19). Taking into consideration that cigarre smoke is the main environmental condition for inducing oral cancer in human populations so far, the genotype frequencies of TLR2 in the smoking and non-smoking population were examined. Saliva samples were collected from 177 smokers and 126 non-smokers. Results showed that TLR2 has a significant effect in short-term smokers only (23). Based on studies described above, further studies are welcomed to clarifty whether the activation of TLR2 in oral cancer cells may be important for protecting or contributing to the invasion, metastatasis and/or prognosis in human cancer patients.

TLR3

TLR3 is found highly expressed in oral squamous cell carcinoma. Activation of TLR3 stimulated the expression of HIF-1 through NF-κB. In addition, HIF-1 increased the expression of TLR3 as well (24, 25). Activation of TLR3 was responsible for inhibiting oral squamous cell carcinoma tumor growth *in vivo* (26). Significant increase in oral cancer risk was observed in individuals with mutated *TLR3* genotype when compared to wild-type (27). The heterozygous and mutated genotype of TLR3 rs5743312 polymorphism had worse survival in a group of patients with stage III tumors. Taken as a whole, the *TLR3* polymorphism could be considered as a potential predictor of worse overall survival in advanced oral cancer (27). Haplotypes in the *TLR3* gene might be associated with poor prognosis in oral cancer patients (27).

Treatment of poly I:C, a TLR agonist, inhibited the cell growth in a dose-dependent manner (25). Poly I:C effectively inhibited oral cancer cell proliferation (25). Poly(I:C)-TLR3-induced apoptosis was caspase-3-dependent in oral squamous cell carcinoma (26). Synthetic dsRNA-polyinosinic-polycytidilic acid, a TLR3 ligand, induced apoptosis of oral squamous carcinoma cells mainly *via* TLR3, through interferon-β production and activation of caspases 3 and 9 (26). Therefore, the direct proapoptotic activity of TLR3 in human oral squamous carcinoma cells may make this protein a viable therapeutic target in the treatment of oral squamous cell carcinoma (28), especially in advanced cases or even oral tumors with high index of recurrence or not sensitive to conventional anti-cancer drugs.

TLR4

A great amount of data has evidenced that TLR4 participates in oral carcinogenesis process by secretion of anti-apoptotic proteins. Hence, TLR4 was highly expressed in oral squamous cell carcinoma (29). Ren *et al*. (30) assumed that the expression of TLR4 was increased in oral squamous cell carcinoma and high levels of TLR4 are associated with a short survival rate. In particular, positive expression of TLR4 was found in all cases of well differentiated and moderately differenciated squamous cell carcinomas, whereas in the majority of poorly differenciated carcinoma showed positivity for TLR4 as well (17). High TLR4 expression was correlated with deeper tumor invasion (18). Cytoplasmic TLR4 also correlated significantly with higher tumor grade (18). Recently, Makinen *et al*. (8) demonstrated that TLR4 expression was higher in primary tumors than in local recurrent ones. Nevertheless, TLR4 and MyD88 were highly expressed in oral squamous cell carcinoma cell lines resulting in production of proinflammatory cytokines, chemokines, growth factors and thus enhance anti-cancer immunity in the early stages of disease (31).

All cases positive for TLR4 also showed positivity for HSP70 (17). Activation of TLR4 stimulated the expression of HIF-1 through NF-κB (24). In addition, HIF-1 increased the expression of TLR 4 (24).

Curiously, TLR 4 ligand lipopolysaccharide enhanced cell migration. Also, it stimulated epithelial-mesenchymal transition demonstrated by decreased E-cadherin and increased

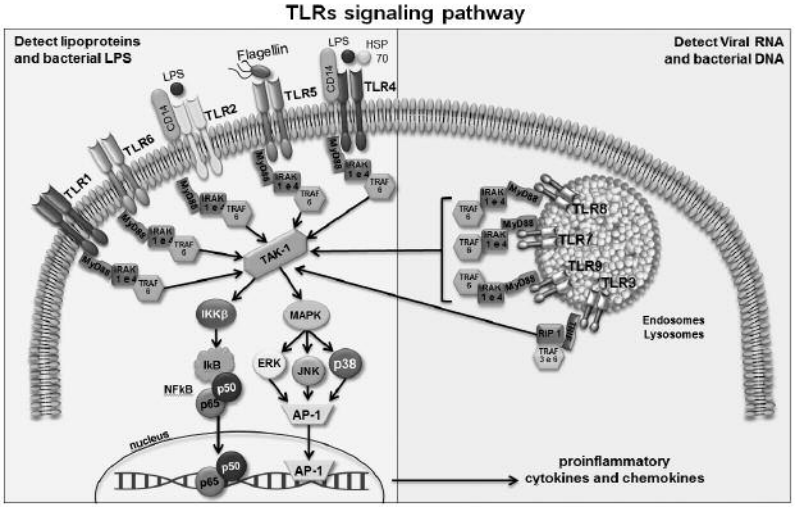

Figure 1. *TLRs signaling pathway – TLR: Toll Like Receptor; MyD88: Myeloid Differentiation Primary Response 88; IRAK: interleukin-1 receptor-associated kinase; TRAF: TNF Receptor Associated Factors; TAK-1: Transforming growth factor-β activated kinase 1; IKKβ: The IκB kinase; IkB: inhibitor of Nfkb kinase; P50: NFkB subunit 50; P65: NFkB subunit 65; MAPK: mitogen-activated protein kinase; ERK: extracellular signal–regulated kinase; JNK: c-Jun-N-terminal kinase; P38: p38 protein kinase; AP-1: activator protein 1; TRIF: TIR-domain-containing adapter-inducing interferon-β; HSP70: heat shock protein 70; LPS: lipopolysaccharide.*

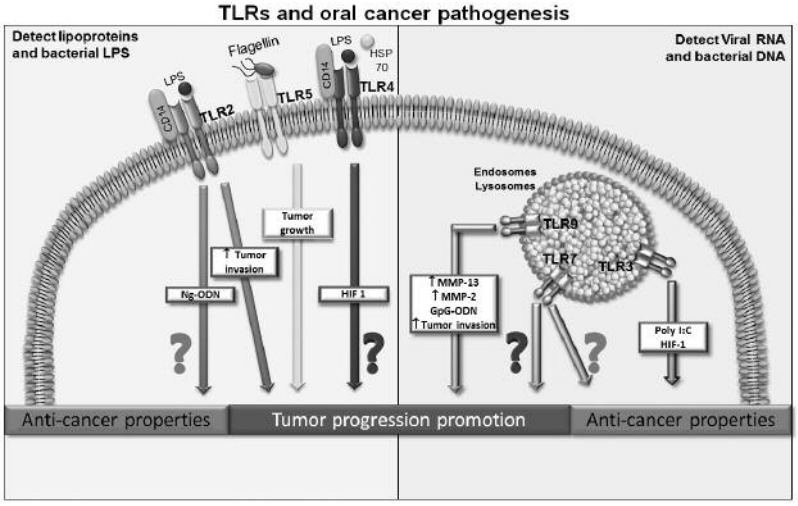

Figure 2. *TLRs and oral câncer pathogenesis – TLR: Toll Like Receptor; HSP70: heat shock protein 70; LPS: lipopolysaccharide; Ng-ODN: Ng-oligodeoxynucleotide; HIF-1: Hypoxia-inducible factor 1; MMP-13: Matrix metalloproteinase-13; MMP-2: Matrix metalloproteinase-2; GpG-ODN: GpG-oligodeoxynucleotide; Poly I:C: Polyinosinic-polycytidylic acid.*

vimentin expression (26). Lipopolysaccharide can induce TLR4-mediated epithelial-mesenchymal transition and cell migration in oral squamous cell carcinoma. Thus, such responses could further affect tumor progression by inducing tumor cell metastasis (26). In summary, it is not yet clear if suppression of TLR4 and its signaling pathway might potentially help improve the prognosis of patients with oral squamous cell carcinoma.

TLR5

TLR5 has a previously undescribed role in the pathophysiology of oral squamous cell carcinoma, even though it may represent a link between bacteria and cancer. TLR5 is involved in recognition of bacterial flagella and is thought to promote tumor growth through inflammation-dependent mechanisms in epithelial cells (32). Activation of

TLR5 results in cancer invasion and subsequent cytokine release (33). Therefore, TLR5 expression could be closely linked to pathogenic bacterial or viral compounds in the oral cavity following carcinogenesis (32).

Expression of TLR5 was increased in oral cancer when compared with normal tongue epithelium (16, 33). Some researchers have revealed that TLR5 is relevant for differentiating the clinical behavior of oral cancers (29). In fact, high TLR5 expression was associated with lower tumor grade in early stage (18). According to this finding in the field, negative or mild TLR5 expression predicted poor disease-specific survival in oral tumors (18). Higher TLR5 was associated with age of >70 years at the time of diagnosis, female gender and disease recurrence (16). Conversely, no association between TLR5 expression and tumor grade, stage, therapeutic approach or even prognosis was found by others (16, 32). Even so, some authors have suggested that TLR5 could be a useful marker for predicting recurrence or survival of oral squamous cell carcinoma patients (16).

TLR7

To the best of our knowledge, there are few studies investigating the role of TLR7 in oral tumors. Immunohistochemical staining of TLR7 showed up-regulated expression during oral carcinogenesis (34). Patients with high expression of TLR7 in tumor cells had poor differentiation and prognosis (34). Interestingly, patients with high expression of TLR7 in stroma fibroblast-like cells had low tumor stage, no lymph node metastasis and better prognosis (34).

When imiquimod, a TLR7 agonist, was administrated to oral cancer cells, the proliterative activity was drastically inhibited in oral cancer cells (35). Imiquimod induced IL-6 and IL-8 production in oral squamous cell carcinoma, suggesting the functional expression of TLR7 against oral cancer development (35).

Conclusion

In this review, we present all published results reporting the role of TLRs signaling pathway for oral cancer pathogenesis and prognosis. Such data revealed that some TLRs such as TLR3, TLR5, and TLR9 play a pivotal role in oral carcinogenesis (Figure 2). They represent a promising alternative for oral cancer therapy. However, some TLRs such as TLR2, TLR4 and TLR7 need further investigation, because current data are not sufficient for a conclusive biological response (Figure 2).

In another context, the investigation of biomarkers belonging to the TLR signaling pathway such as MyD88, TRAF, IKK and NFKB among others is crucial and mandatory to elucidate whether the cascade is effectively activated resulting in the release of pro-inflammatory cytokines and/or cell-cycle regulatory proteins. Furthermore, it is necessary to elucidate whether expression of TLRs is actually responsible for modulating the apoptotic process. This information will be very useful for assessing the biological relevance of TLR signaling pathway in carcinogenesis. Morever, the use of medium-term oral carcinogenesis assays in rodents is interesting for studying the role of TLRs at initiation, promotion and progression phases. Certainly, such data will contribute to new scientific knowledge, which will be incorporated to the preventive actions and/or therapeutic protocols for patients suffering from oral cancer.

Conflicts of Interest

None declared.

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