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Predictive role of toll-like receptors 2, 4, and 9 in oral tongue squamous cell carcinoma

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SUMMARY

Objectives: The clinical behavior of oral tongue squamous cell carcinoma (OTSCC) can be unpredictable, and even small tumors may behave aggressively. Toll-like receptors (TLRs) are pattern-recognition molecules involved in innate immunity, and they are also expressed in many types of cancer. TLRs play an apparently pivotal role in some cancers related to tumor progression and, conversely, cancer inhibition, however their role in oral cancer is unclear. We therefore studied the expression of TLR-2, -4, -5, -7, and -9 in early-stage OTSCC.

Materials and methods: Tissue microarray technique and immunohistochemistry was employed to define the expression of TLRs from the tumors of 73 consecutive patients with Stage I-II OTSCC. Immunoexpression scores were compared with patient and tumor related characteristics and survival.

Results: All TLRs were expressed in OTSCC tissue. High/strong TLR-2, -4, and -9 expression correlated with deeper tumor invasion. Cytoplasmic TLR-2 and -4 also correlated significantly with higher tumor grade, whereas high TLR-5 expression associated with lower tumor grade. High expression of TLR-9 correlated with advanced tumor size. Negative or mild TLR-5 expression predicted poor disease-specific survival.

Conclusion: All the studied TLRs showed high expression in early-stage OTSCC. More importantly, TLR-2, -4, and -9 seemed to predict invasive tumor growth.

Introduction

The behavior of early-stage oral tongue squamous cell carcinoma (OTSCC) can be unpredictable despite improvements in diagnostic methods and treatment modalities [1,2]. Even small tumors may behave aggressively. The 5-year survival rate of early-stage (I–II) OTSCC is still modest [3]. To adjust the treatment more accurately, improved knowledge of the underlying mechanisms behind aggressiveness of the disease as well as new predictive markers are needed.

Intense research studies are currently addressing the relevance of innate immunity response in cancer initiation and progression. Cancer immunotherapy is expected to be the fourth weapon against cancer along with surgery, radiation, and chemotherapy [4]. It is well known that environmental carcinogens (tobacco, alcohol) are major contributors to the development of head and neck squamous cell carcinomas whereas tumor progression could be partly due to a failure of the innate immune response against cancer [5].

Abbreviations: DFS, disease-free survival; DSS, disease-specific survival; HNSCC, head and neck squamous cell carcinoma; HPV, human papilloma virus; LPS, lipopolysaccharide; OS, overall survival; OSCC, oral squamous cell carcinoma; OTSCC, oral tongue squamous cell carcinoma; TLR, toll-like receptor.

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Toll like receptors (TLRs) are a major class of pattern-recognition receptors that are expressed by cells of the humane immune system and epithelial cells located near host-environment boundary. In addition, they are also present in many types of cancer. Ten human TLRs have been identified, each recognizing a specific microbial component. There are two kinds of ligands for TLRs: exogenous pathogen associated molecular patterns (PAMPs), which are components of microbes, and endogenous damage-associated molecular patterns (DAMPs) released from injured or inflamed tissues [6].

TLR-2 and TLR-4 recognize lipopolysaccharide (LPS, a membrane component of Gram-negative bacteria), TLR-5 recognizes bacterial flagellin, TLR-7 recognizes double or single stranded RNA, and TLR-9 recognizes bacterial DNA [7,8]. TLRs detecting bacterial LPS and lipoproteins are located on the cell surface (TLR-1, TLR-2, TLR-4, TLR-5, and TLR-6), whereas TLR-3, -7, -8, and -9 that mainly recognize viral RNA and bacterial DNA are located in endosomes and lysosomes, where these materials are processed [6].

Thus, TLRs trigger signals activate innate and adaptive immune responses [6]. The actual role of TLRs in tumorigenesis remains unknown, and it is related to cancer progression as well as inhibition [8–10]. TLR-mediated tumor progression is considered to result from the activation of TLRs in tumor cells, whereas tumor regression is thought to derive from the activation of host immune responses [8].

The function of each TLR is complex and variable and is affected by multiple factors. Little is known about TLRs in head and neck squamous cell carcinoma (HNSCC). Various HNSCCs express TLR-2, -3, -4, -5, -7, and -9 both *in vitro* [11–18] and *in vivo* [11,13–15,19–22]. To further clarify the role of TLRs in early-stage oral cancer we studied TLR-2, -4, -5, -7, and -9 in small OTSCCs.

The surrogate marker for human papilloma virus (HPV), p16, is widely utilized in the evaluation of oropharyngeal carcinomas [23]. Thus $p16^{INK4a}$ staining of tongue carcinoma tumors was performed here, and findings compared with TLR expression.

Material and methods

Patients

The present study assesses a series of early-stage OTSCC patients with tumors clinically defined as T1N0M0 or T2N0M0 which were treated at the Helsinki University Central Hospital between 1992 and 2002. The patient material has been described in more detail previously [24,25].

Demographic data of the material is described in Table 1. Tumor samples from 73 patients were available for immunohistochemistry (36 males and 37 females, median age 59 years, range 23–95). Thirty-five tumors (48%) were clinically classified as T1 and 38 (52%) as T2. All patients had been treated with curative intent including surgical resection of the primary tumor. An experienced head and neck pathologist re-evaluated all the original histological tumor specimens. According to histopathological classification, 52 tumors (71%) were classified as pT1 and 21 (29%) as pT2. Tumor invasion depth was measured from the level of the proximate normal mucosal surface.

All patients in this study were treated according to the Finnish national guidelines for the treatment of Head and Neck cancer. Postoperative radiation therapy was given only to patients with deeply invasive tumors, and to those with neck metastases in neck dissection specimens. Consequently, 31 patients received no further primary treatment for the neck, 41 patients underwent elective neck dissection, and one patient received radiotherapy without surgery. Thus, radiotherapy had no effect on evaluation of tumor characteristics, nor on the evaluation of occult metastases, except possibly in one patient. Of all patients, 34 received post-operative radiotherapy, including the neck in 33 patients. Twenty-four patients (33%) had occult neck metastases, i.e. lymph node metastases in the elective neck dissection specimen ($n = 15$), or neck metastasis during follow up without failure at the primary site ($n = 9$). During follow-up, ten patients developed local recurrence. Only two patients were diagnosed with distant metastases, both after locoregional recurrence. All patients, but one, had a minimum follow-up time of five years or until death. Median follow-up time was 7.6 years (range, 0.3–17.2), during which 19 patients died of tongue cancer and 22 of other causes.

Dates and causes of death were provided by the national agency of population statistics, Statistics Finland. The study design was approved by the institutional Research Ethics Board.

Table 1
Demographic and clinicopathological features of 73 patients with oral tongue squamous cell carcinoma.

Clinicopathological variable	No. of patients (%)
Age, years	
⩽60	40 (55)
>60	33 (45)
Range	23–95
Median	59
Sex	
Male	36 (49)
Female	37 (51)
Grade	
I	24 (33)
II	35 (48)
III	14 (19)
Clinical T stage (mm)	
cT1 (⩽20)	35 (48)
cT2 (21–40)	38 (52)
Pathological T stage (mm)	
pT1 (⩽20)	52 (71)
pT2 (21–40)	21 (29)
Pathological node positivity[a]	
pN0	26 (36)
pN+	15 (21)
Pathological stage	
I	46 (63)
II	12 (16)
III	12 (16)
IV	3 (4)
Invasion depth (mm)	
⩽4	29 (40)
>4	44 (60)

[a] 41/73 (56%) of the patients had elective neck dissection.

Tissue array blocks

Tissue microarray (TMA) technique was used as previously described [26]. Representative tumor tissue was lacking in seven patients for TLR-2, in six patients for TLR-4 and -9, in eight patients for TLR-7, and in ten patients for TLR-5 and $p16^{INK4a}$ evaluation.

Immunohistochemistry

Tissue slides as well as TMA slides were cut into 4–5 μm thick sections. Deparaffinization was done in xylene and rehydration with graded alcohol series. Slides were treated in a PreTreatment-module (Lab Vision Corp., UK Ltd, UK) in Tris–HCl buffer (pH 8.5) at 98 °C for 20 min and with 0.3% Dako REAL Peroxidase-Blocking Solution for 5 min to block endogenous