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# Immunoexpression pattern of TLR3 and TLR7 in minor salivary gland adenoid cystic carcinoma and its role in prognosis

Aleksi Rytkönen [a,b,1], Mine Eray [c,1], Auli Suominen [d], Antti Mäkitie [e,f,g], Caj Haglund [h,i], Jaana Hagström [b,c,h,1], Hanna K. Laine [b,c,j,1,*]

[a] Department of Pathology, Oulu University Hospital, Oulu, Finland
[b] Department of Oral Pathology and Radiology, University of Turku and Turku University Hospital, Turku, Finland
[c] Department of Pathology, University of Helsinki and Helsinki University Hospital, Helsinki, Finland
[d] Department of Community Dentistry, University of Turku, Turku, Finland
[e] Department of Otorhinolaryngology – Head and Neck Surgery, University of Helsinki and Helsinki University Hospital, Helsinki, Finland
[f] Division of Ear, Nose and Throat Diseases, Department of Clinical Sciences, Intervention and Technology, Karolinska Institutet and Karolinska University Hospital, Stockholm, Sweden
[g] Research Program in Systems Oncology, Faculty of Medicine, University of Helsinki, Helsinki, Finland
[h] Research Programs Unit, Translational Cancer Biology Program, University of Helsinki, Helsinki, Finland
[i] Department of Surgery, University of Helsinki and Helsinki University Hospital, Helsinki, Finland
[j] Department of Oral and Maxillofacial Diseases, University of Helsinki, Helsinki, Finland

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ABSTRACT

*Objectives:* Adenoid cystic carcinoma (ACC) of the salivary glands has poor long-term prognosis and a high metastatic rate. Toll-like receptors (TLRs), first-line immune activators, have been associated with both tumor progression and suppression. We aimed to study TLR3 and TLR7 behavior in ACC.
*Materials and methods:* We studied TLR3 and TLR7 immunoexpression of 46 minor salivary gland ACCs diagnosed at the Department of Otorhinolaryngology – Head and Neck Surgery, Helsinki University Hospital, Helsinki, Finland over the period 1974–2012. The associations of TLR3 and TLR7 immunoexpression with clinicopathological factors were evaluated by $\chi^2$-test and Fisher's exact test.
*Results:* In the majority of samples, both TLR3 and TLR7 were immunoexpressed in cytoplasm. The immunoexpression was heterogeneous between individual tumors. Stronger TLR7 immunoexpression associated with recurrence rate and poorer disease-specific survival (DSS). TLR3 did not associate significantly with survival although we found an inverse correlation between TLR3 and TLR7 immunopositivity. Hence, when TLR3 immunoexpression was negative or mild, TLR7 immunoexpression was moderate to strong, and vice versa.
*Conclusions:* TLR3 and TLR7 are immunoexpressed in minor salivary gland ACC. TLR7 is potentially an independent prognostic marker for recurrence rate and DSS.

## Introduction

Adenoid cystic carcinoma (ACC) is the second most common malignant salivary gland tumor according to the 2017 World Health Organization (WHO) classification [1]. In the Nordic countries, for example, in Finland and Denmark, ACC has been shown to be the most common type of salivary gland cancer [2,3]. It arises in glandular structures, and about 70 % of all ACCs originate from salivary glands [2,4]. ACC progresses slowly and typically with perineural invasion and late distant metastases. Nodal involvement is rare [5,6]. Treatment for ACC includes surgery, followed by radiotherapy [7]. Due to the neural spreading and infiltrative nature of the disease, the overall prognosis remains poor [5,6]. It is crucial to add knowledge on the molecular pathogenesis of ACC in order to develop and improve treatment strategies.

Toll-like receptors (TLRs) are a family of pattern recognition receptors found in immune cells, epithelial cells, and fibroblasts [8,9]. They are the key initiators of the innate immune response, and they

* Corresponding author at: Department of Oral and Maxillofacial Diseases, Faculty of Medicine, University of Helsinki, P.O. Box 41, FI-00014 , Finland.
*E-mail address:* hanna.k.laine@helsinki.fi (H.K. Laine).
[1] Equal contribution

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**Table 1**
Patient and tumor characteristics of 46 patients with adenoid cystic carcinomas.

| | | |
|---|---|---|
| Sex | | |
| | Men | 17 |
| | Women | 29 |
| Age range | 24–83 years, (median 56 years) | |
| Tumor site | | |
| | Oral cavity | 24 |
| | Oropharynx | 3 |
| | Nasopharynx | 3 |
| | Paranasal sinuses | 3 |
| | Larynx | 2 |
| | Trachea | 6 |
| | Ear | 4 |
| | Esophagus | 1 |
| Tumor | | |
| | T1 | 13 |
| | T2 | 4 |
| | T3 | 4 |
| | T4 | 16 |
| Node | | |
| | N0 | 34 |
| | N1 | 3 |
| Stage | | |
| | I | 11 |
| | II | 5 |
| | III | 5 |
| | IV | 16 |
| | n/a | 9 |
| Neural invasion | | |
| | Yes | 26 |
| | No | 10 |
| | n/a | 10 |
| JC polyomavirus DNA positive tumor * | | |
| | Yes | 5 |
| | No | 41 |
| Tumor recurrence | | |
| | Yes, <5 years | 17 |
| | Yes, >5 years | 8 |
| | No | 21 |
| Local recurrence | | |
| | Yes | 13 |
| | No | 32 |
| | n/d | 1 |
| Regional recurrence | | |
| | Yes | 4 |
| | No | 42 |
| Distant recurrence | | |
| | Yes | 16 |
| | No | 30 |
| Status | | |
| | NED | 17 |
| | AWD | 6 |
| | DOD | 17 |
| | DOC | 6 |

Abbreviations: T, tumor; N, node; n/a, not available; NED, no evidence of disease; AWD, alive with disease; DOD, dead on disease; DOC, dead on other cause; *JC polyomavirus DNA-positive tumor detection by PCR [19].

promote adaptive immunity. In humans, the TLR family has 10 members (TLR1-TLR10), which have specific subcellular localizations and agonists [10]. TLRs can be activated by pathogen-associated molecular patterns as well as by destruction-associated molecular patterns [9,11]. TLR3 and TLR7 especially have active roles in response to viral infection [12,13].

The initiation of cancer has been associated for example with microbial infection, injury, inflammatory process, and tissue repair [14]. Therefore, the important role of TLRs has been investigated in infectious injury, in pathogenesis of different solid malignancies, and, to a lower extent, in lymphoid malignancies [15,16]. The role of TLRs in pathogenesis of malignancies is more complicated than the role in infectious injuries. The ability of TLR signaling to activate the adaptive immune system has led to several attempts to utilize TLR ligands in cancer treatment. Controversially, TLR stimulation in a variety of tumor cell lines leads to their increased survival and proliferation in vitro [11]. Previous studies on TLR3 and TLR7 have elucidated their role in oral cavity squamous cell carcinoma (SCC) [17,18]. However, the function of TLR3 and TLR7 in salivary gland cancers is still unknown.

We aimed to assess immunoexpression of TLR3 and TLR7 in ACC of minor salivary glands and to associate the results with clinicopathological factors, including presence of polyomavirus DNA in ACCs, and treatment outcome.

## Materials and methods

### *Tumor material*

The study population consisted of 46 patients with ACC of minor salivary glands treated at the Helsinki University Hospital, Helsinki, Finland. The study population is part of a cohort of 68 minor salivary gland ACC patients treated between 1974 and 2012, described in our previous study in more detail [6]. For immunohistochemistry, we had sample material available only from 46 patients forming a subcohort of the main cohort. In addition, the presence of viral DNA of oncogenic polyomavirus, JC polyomavirus, has been detected in this cohort [19]. Table 1 shows the main patient and tumor characteristics. TNM classification for tumor staging was performed according to the WHO 2017 criteria [1]. This study was performed in accordance with Finnish guidelines and legislation. The Helsinki University Hospital Research Ethics Board (HUS Regional Committee on Medical Research Ethics) approved the study concept (document no. 31/13/03/02/2010, 1 February 2010). This study was retrospective, had no effect on the treatment of patients, and the patients were not contacted. Patient consent was waived by the Finnish National Supervisory Authority for Welfare and Health (decision nos 425/05.01.00.06/2009 and 10041/06.01.03.01/2012).

### *Immunohistochemistry*

Formalin-fixed and paraffin-embedded blocks were cut into 4 μm-thick sections that were deparaffinized in xylene and rehydrated in graded ethanol and distilled water. For antigen retrieval, the slides were heated in a PreTreatment module (Agilent Dako, Santa Clara, CA, USA) in antibody-specific buffer, pH 9, at 98 °C (20 min). Endogenous peroxidase activity was blocked by incubating the slides in EnVision Flex peroxidase-blocking reagent (15 min). The primary antibody was diluted in Dako REAL Antibody Diluent. The primary antibodies were rabbit anti-TLR3 polyclonal antibody, unconjugated (OABF00700) at 1:600 (Aviva Systems Biology Corporation, San Diego, CA, USA) and rabbit polyclonal IgG TLR7 antibody (Novus 24777) at 1:800 (Novus Biologicals, Centennial, CO, USA). The incubation time for TLR3 was overnight at 4 °C and for TLR7 one hour at room temperature. This was followed by detection with the Dako REAL Detection System (Peroxidase/DAB+, Rabbit/Mouse, Dako, Glostrup, Denmark). Visualization of the slides was performed with Dako REAL DAB+ Chromogen for 10 min, and they were counterstained with hematoxylin (Mayer's Hematoxylin Dako, Glostrup, Denmark). Inflamed oral gum tissue was used as a positive control. Serving as a negative control was a slide without primary antibody.

### *Immunohistochemical scoring*

Three independent researchers (AR, HKL, and JH) performed scoring of the slides blinded to the clinical data. We analyzed the location of immunopositivity and estimated the percentage of positively stained tumor cells. For statistical analysis, we grouped the immunoscores as follows: 0 for 0–10 % (none or very low), 1 for 11–40 % (mild), 2 for 41–70 % (moderate), and 3 for 71–100 % (strong). Grouping was modified from an earlier publication [20]. We scored the co-existing normal salivary gland and apocrine gland tissue found on tumor slides

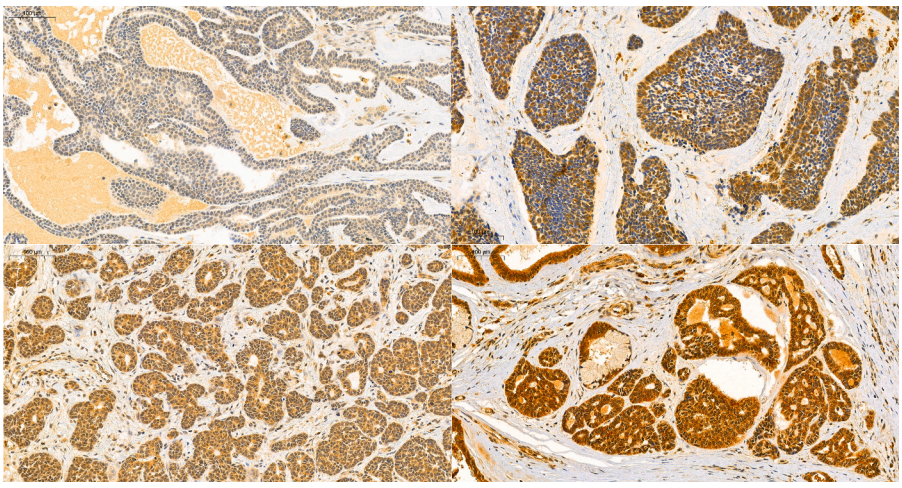

**Fig. 1.** Immunoexpression of Toll-like receptor 3 (TLR3) in minor salivary gland adenoid cystic carcinoma: Upper picture on left: Negative immunoexpression. Upper picture on right: Mild immunoexpression. Lower picture on left: Moderate immunoexpression. Lower picture on right: Strong immunoexpression.

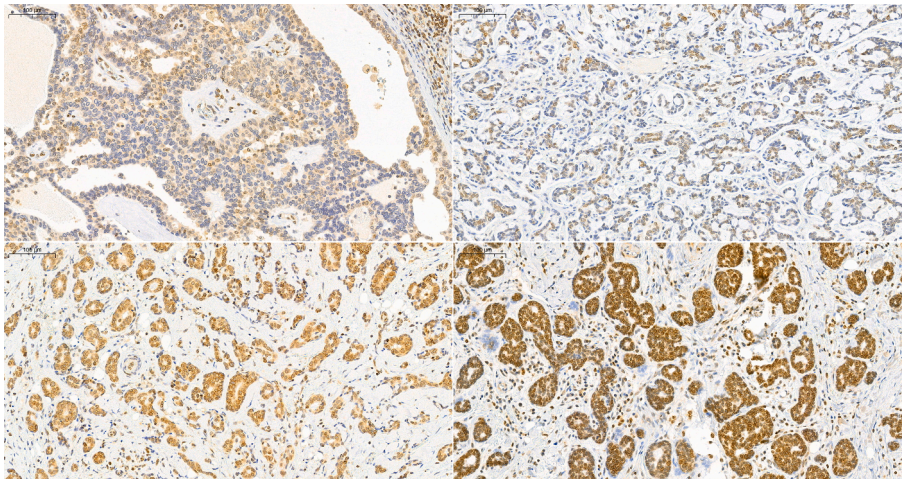

**Fig. 2.** Immunoexpression of Toll-like receptor 7 (TLR7) in minor salivary gland adenoid cystic carcinoma: Upper picture on left: Negative immunoexpression. Upper picture on right: Mild immunoexpression. Lower picture on left: Moderate immunoexpression. Lower picture on right: Strong immunoexpression.

**Table 2**
Distribution of Toll like receptor (TLR) 3 and 7 immunopositivity in minor salivary gland adenoid cystic carcinomas 0: None or very low 0–10 %; 1: mild 11–40 %; 2: moderate 41–70 %; 3: strong 71–100 %.

| Immunoscore | 0 | 1 | 2 | 3 | n/a |
|---|---|---|---|---|---|
| **TLR3 (total 44)** | 1 (2 %) | 19 (42 %) | 17 (37 %) | 7 (15 %) | 2 (4 %) |
| **TLR7 (total 46)** | 2 (4 %) | 16 (34 %) | 14 (31 %) | 14 (31 %) | 0 (0 %) |

Abbreviations: n/a, not available

to validate immunoexpression in normal tissues.

*Statistical analysis*

For statistical analysis, staining results were grouped into two categories: 0–1 (none or very low to mild) and 2–3 (moderate to strong). Overall survival (OS) was defined as dead for any reason during the follow-up and disease-specific survival (DSS) was defined as dead on disease during the follow-up. The associations of TLR3 and TLR7 immunoexpression with clinicopathological factors and survival at the end of follow up were evaluated by $\chi^2$-test and Fisher's exact test. P-values below 0.05 were considered statistically significant. Statistical analyses were performed with IBM SPSS Statistics for Windows, version 27 (IBM Corp., Armonk, NY, USA).

## Results

TLR3 and TLR7 immunoexpression was cytoplasmic in ACC tissue samples. Figs. 1 and 2 provide examples of TLR 3 and 7 scoring, respectively. In normal salivary gland tissue adjacent to a tumor, TLR3 immunopositivity was present in ductal structures and TLR7 immunopositivity in ductal and infrequently in acinar structures. Table 2 shows the distribution of TLR3 and TLR7 immunostaining in 46 ACC samples.

In statistical analysis of TLR7 immunoexpression, moderate to strong immunopositivity was associated with recurrent tumors and with poorer DSS (Table 3). TLR3 did not associate with any clinicopathological factors (Table 3). Interestingly, when comparing the TLR3 and TLR7 immunoexpression patterns, we detected an inverse association between TLR3 and TLR7 immunopositivity. When immunoexpression of TLR3 was strong, TLR7 immunoexpression was low, and vice versa ($p = 0.008$) (Fig. 3). In addition, most (60 %) of the JC polyomavirus DNA-positive ACC samples showed low TLR 3 and strong TLR 7 immunoexpression (Table 4).

**Table 3**
Associations between immunoexpressions of TLR3 and 7 and clinicopathological factors within adenoid cystic carcinoma patients evaluated by $\chi^2$-test and Fisher's exact test.

| | TLR3 immunoexpression | | | TLR7 immunoexpression | | |
|---|---|---|---|---|---|---|
| | 0–1 | 2–3 | P value | 0–1 | 2–3 | P value |
| **Age** | | | 1.000 | | | 0.486 |
| **<65 y** | 15 (75.0 %) | 18 (75.0 %) | | 15 (83.3 %) | 20 (71.4 %) | |
| **>65 y** | 5 (25.0 %) | 6 (25.0 %) | | 3 (16.7 %) | 8 (28.6 %) | |
| **Gender** | | | 0.060 | | | 1.000 |
| **Male** | 4 (20.0 %) | 12 (50.0 %) | | 7 (38.9 %) | 10 (35.7 %) | |
| **Female** | 16 (80.0 %) | 12 (50.0 %) | | 11 (61.1 %) | 18 (64.3 %) | |
| **T class** | | | 0.720 | | | 0.491 |
| **1–2** | 6 (46.2 %) | 11 (57.9 %) | | 8 (61.5 %) | 9 (47.4 %) | |
| **3–4** | 7 (53.8 %) | 8 (42.1 %) | | 5 (38.5 %) | 10 (52.6 %) | |
| **N class** | | | 0.259 | | | 1.000 |
| **N0** | 14 (100 %) | 18 (85.7 %) | | 12 (92.3 %) | 20 (90.9 %) | |
| **N+** | 0 | 3 (14.3 %) | | 1 (7.7 %) | 2 (9.1 %) | |
| **Stage** | | | 1.000 | | | 0.720 |
| **I-II** | 4 (29 %) | 7 (33 %) | | 6 (35.3 %) | 7 (46.7 %) | |
| **III-IV** | 10 (71 %) | 14 (67 %) | | 11 (64.7 %) | 8 (53.3 %) | |
| **Neural invasion** | | | 0.474 | | | 0.462 |
| **No** | 5 (35.7 %) | 5 (23.8 %) | | 5 (35.7 %) | 5 (22.7 %) | |
| **Yes** | 9 (64.3 %) | 16 (76.2 %) | | 9 (64.3 %) | 17 (77.3 %) | |
| **Primary recurrence** | | | 0.522 | | | 0.522 |
| **No** | 13 (65.0 %) | 18 (75.0 %) | | 14 (77.8 %) | 19 (67.9 %) | |
| **Yes** | 7 (35.0 %) | 6 (25.0 %) | | 4 (22.2 %) | 9 (32.1 %) | |
| **Regional recurrence** | | | 0.614 | | | 1.000 |
| **No** | 19 (95.0 %) | 21 (87.5 %) | | 17 (94.4 %) | 25 (89.3 %) | |
| **Yes** | 1 (5.0 %) | 3 (12.5 %) | | 1 (5.6 %) | 3 (10.7 %) | |
| **Distant recurrence** | | | 0.342 | | | 0.210 |
| **No** | 12 (60.0 %) | 18 (75.0 %) | | 14 (77.8 %) | 16 (57.1 %) | |
| **Yes** | 8 (40.0 %) | 6 (25.0 %) | | 4 (22.2 %) | 12 (42.9 %) | |
| **Recurrence** | | | 0.055 | | | **0.008** |
| **No** | 9 (45.0 %) | 11 (47.8 %) | | 11 (61.1 %) | 9 (33.3 %) | |
| **Early <5 y** | 5 (25.0 %) | 11 (47.8 %) | | 2 (11.1 %) | 15 (55.6 %) | |
| **Late >5 y** | 6 (30.0 %) | 1 (4.3 %) | | 5 (27.8 %) | 3 (11.1 %) | |
| **OS** | | | 1.000 | | | 0.130 |
| **Alive** | 10 (50.0 %) | 13 (54.2 %) | | 12 (66.7 %) | 11 (39.3 %) | |
| **Dead** | 10 (50.0 %) | 11 (45.8 %) | | 6 (33.3 %) | 17 (60.7 %) | |
| **DSS** | | | 0.532 | | | **0.030** |
| **Alive** | 12 (60.0 %) | 17 (70.8 %) | | 15 (83.3 %) | 14 (50.0 %) | |
| **Dead** | 8 (40.0 %) | 7 (29.2 %) | | 3 (16.7 %) | 14 (50.0 %) | |

Abbreviations: T, tumor; N, node; OS, overall survival; DSS, disease-specific survival.

## Discussion

We evaluated the immunoexpression of TLR3 and TLR7 in minor salivary gland ACC in 46 patients treated at the Department of Otorhinolaryngology – Head and Neck Surgery, Helsinki University Hospital, Helsinki, Finland. Moderate to strong TLR7 immunopositivity associated with tumor recurrence and worse DSS. Immunopositivity for TLR3 showed no association with clinical data or tumor parameters. Interestingly, we found an inverse association between TLR3 and TLR7 immunostaining patterns.

Meta-analysis by Wang et al. has previously shown similar results as our present study how abundant TLR7 expression in several cancer types predicts worse survival [21]. The material of their meta-analysis consisted of various malignancies which included carcinomas of glandular structures such as esophageal adenocarcinoma, pancreatic cancer/-ductal adenocarcinoma, colorectal carcinoma, breast cancer, ACC, and mucoepidermoid carcinoma [21]. Ruuskanen et al. have shown contrary results since TLR7-immunopositivity among nasopharyngeal carcinomas had better survival than immunonegative tumors [22]. In our earlier study (included in the meta-analysis by Wang et al.) on immunoexpression of TLR5 and TLR7 in major salivary gland ACC, the TLRs did not show any role [23]. This discrepancy could indicate differences between major and minor salivary gland ACC tumor biology. In general, acinar structures differ between minor and major salivary glands, and this fact might explain the different behavior of these tumors. In addition, minor salivary glands, e.g. in oral cavity, are located immediately under mucous membrane and might be exposed more easily to trauma, chemicals, and microbes.

Two recent review articles have suggested an important role for both TLR3 and TLR7 in oral SCC as well [17,18]. TLR3 has been shown to be highly expressed in oral SCC, and its activation inhibits tumor growth *in vivo,* suggesting a positive role in tumor progression and prognosis [17]. In accordance with this, mutated TLR3 genotype has shown to be associated with increased risk for oral SCC [17,18]. In the present study, we showed no association with clinical features or survival with TLR3 in ACC. In case of TLR7, Ni et al. have shown high immunoexpression of TLR7 relating to poorly differentiated tumors and worse prognosis [8]. Interestingly, patients with high immunoexpression of TLR7 in stroma fibroblast-like cells had low tumor stage, no lymph node metastasis, and better prognosis [8]. Their result reinforces the important role of tumor microenvironment which is recently reviewed by Egal et al. [24]. In the present study, we did not score tumor stroma since immunopositivity was mainly seen in tumor tissue as described in Figs. 1 and 2. Interestingly, in our current study, the immunoexpression patterns of TLR3 and TLR7 were controversial. Immunoexpression of TLR7 being strong, TLR3 immunoexpression was low, and vice versa. Although TLR7

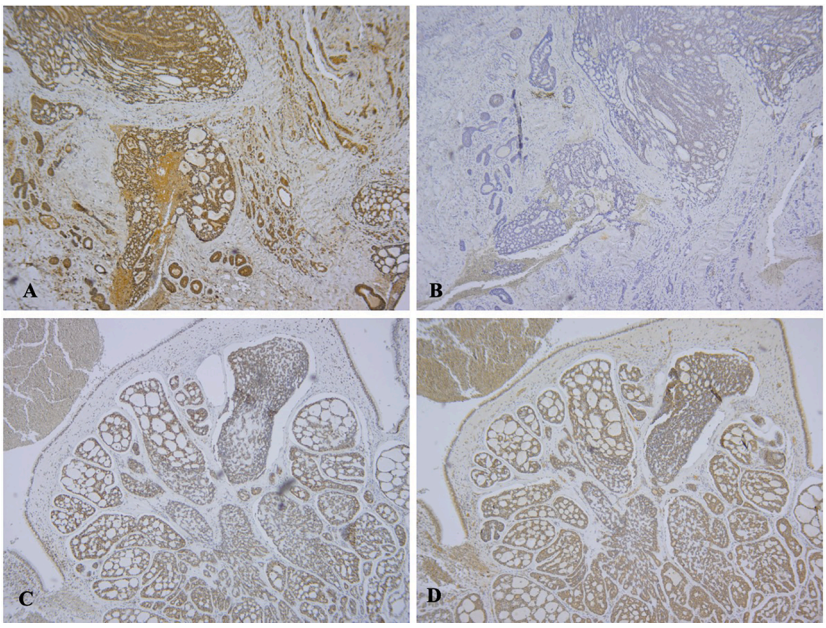


**Fig. 3.** Inverse association ($p = 0.008$) was detected between Toll-like receptor 3 (TLR3) and Toll-like receptor 7 (TLR7) immunostainings of the same adenoid cystic carcinoma (ACC) samples. Pictures A and B show strong immunoexpression of TLR3 (A) and negative immunoexpression of TLR7 (B) in oral cavity ACC. Pictures C and D show mild immunoexpression of TLR3 (C) and strong immunoexpression of TLR7 (D) in paranasal sinus ACC.

**Table 4**
Immunoexpression of Toll like receptor (TLR) 3 and 7 in JC polyomavirus DNA containing adenoid cystic carcinomas (ACC).

| Tumor | TLR3 immunoscore | TLR3 in inflammatory cells | TLR7 immunoscore |
|---|---|---|---|
| **#1 ACC in oral cavity** | 1 | No | 3 |
| **#2 ACC in trachea** | 1 | No | 3 |
| **#3 ACC in oral cavity** | 1 | No | 3 |
| **#4 ACC in paranasal sinuses** | 2 | Yes | 2 |
| **#5 ACC in trachea** | 1 | No | 1 |

immunoexpression was associated with poor prognosis, despite the inverse immunoexpression profile between TLR3 and TLR7, TLR3 did not show association to prognosis. The reason for this might be the limited series of specimens in the study. Of note, the diverse immunoexpression of TLR3 and TLR7 has not been published earlier.

Salivary gland cancer studies on TLRs are sparse [23,25–28]. TLR4 signaling has been shown to be present in head and neck SCC and in salivary gland cancer cell lines [27,28]. In salivary gland adenocarcinoma TLR5 immunoexpression might promote invasion and metastasis [26]. However, our earlier study on major salivary gland ACC showed no association of TLR5 immunoexpression with ACC progression [23]. Study on TLR9 expression in mucoepidermoid carcinoma showed TLR9 to associate with good prognosis [25]. A recent meta-analysis did not conclude any role for TLR9 in general in cancer progression [21].

These studies mentioned above on the prognostic role of TLRs are essential for future studies on discovering TLR utility in e.g. cancer treatment. We should deepen our understanding of molecular mechanism in distinct cancers in order to discover possible new treatment agents. According to immunological view, TLR agonist could act as therapeutic agents or vaccine adjuvant towards cancer and currently some TLR agonist (TLR2, 4, 5, 7, 8, and 9) have already been tested for cancer immunotherapies in clinical trials [10]. TLR7 ligand imiquimod have been studied in cancer treatment with promising results [29].

We have previously evaluated oncogenic viruses (human papilloma viruses and polyomaviruses JC, BK, and SV40) in minor salivary gland ACC [19] and detected JC polyomavirus DNA in 10 % of the ACC samples by PCR. We hypothesized that these viruses could more easily enter minor salivary glands just beneath the oral mucosa than major salivary glands, which are located more deeply in the submucosa. TLR3 and TLR7 are known to participate in recognition and induction of appropriate immune responses against viral infections [12,13]. In our virus-positive ACCs, TLR7 immunoexpression appeared to be stronger than immunoexpression of TLR3, although we lack correlations due to the small sample size.

The main strength of this study is that it comprises a remarkable cohort of minor salivary gland ACCs from a large tertiary-care institute over the long study period. Since minor and major salivary glands have differences considering structure, function, and location, we considered beneficial to study these cancers separately. Nevertheless, the number of samples of this rare tumor remains limited and tumors are from varying sites from head and neck area, although most commonly from oral cavity.

In conclusion, we found TLR7 to play a role in minor salivary gland ACC predicting poor prognosis. Its immunoexpression shows an inverse pattern to the immunoexpression of TLR3. Nevertheless, we did not find a significant effect of TLR3, the inverse immunoexpression pattern of these biomarkers warrants further investigations, preferably with a larger patient cohort.

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This study was supported by the Helsinki University Hospital Research Fund and the Cancer Foundation Finland. Open access funded by Helsinki University Library.

## Data availability statement

The datasets generated and analyzed during the study are not publicly available due privacy and ethical restrictions. Datasets that support the findings of this study are available from the corresponding author on reasonable request.

## Ethical approval

The institutional Research Ethics Board (document no. 31/13/03/02/2010, 1 February 2010) approved the study concept. Patient consent was waived by the Finnish National Supervisory Authority for Welfare and Health (decision nos 425/05.01.00.06/2009 and 10041/06.01.03.01/2012) The study was performed in accordance with the Declaration of Helsinki.

## CRediT authorship contribution statement

**Aleksi Rytkönen:** Writing – original draft, Methodology, Investigation, Formal analysis. **Mine Eray:** Writing – original draft, Formal analysis. **Auli Suominen:** Writing – original draft, Methodology. **Antti Mäkitie:** Writing – review & editing, Supervision, Resources, Project administration, Formal analysis. **Caj Haglund:** Writing – review & editing, Resources, Project administration, Methodology, Formal analysis. **Jaana Hagström:** Writing – review & editing, Supervision, Methodology, Investigation, Formal analysis. **Hanna K. Laine:** Writing – original draft, Methodology, Investigation, Formal analysis.

## Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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