APMIS JOURNAL OF PATHOLOGY, MICROBIOLOGY AND IMMUNOLOGY

APMIS 133: e13505

DOI 10.1111/apm.13505

Expression of toll-like receptors in recurrent pleomorphic adenoma and carcinoma ex pleomorphic adenoma

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Jauhiainen MK, Markkanen A, Atula T, Aro K, Haglund C, Söderlund-Venermo M, Mäkitie AA, Sinkkonen ST, Hagström J. Expression of toll-like receptors in recurrent pleomorphic adenoma and carcinoma ex pleomorphic adenoma. APMIS. 2025; 133: e13505.

Pleomorphic adenoma (PA) is a benign salivary gland tumour that may recur or undergo malignant transformation (CXPA). Toll-like receptors (TLR) mediate immune responses triggered by various agents such as viruses and are related to tumour formation either by stimulating or suppressing their growth, with variation across different tumour entities. We compared TLR immunohistochemical expression in PA, its recurrent counterparts and CXPA and evaluated the effect of virus presence in these tumours. We studied the expression of TLR-2, -3, -5, -7 and -9 in 25 PA, 34 recurrent PA and 15 CXPA tumour samples. In addition, we examined the TLR expression levels in the presence and absence of herpes-, polyoma- and parvovirus DNA in a subset of tumours (n = 20). CXPA expressed significantly more TLR-5 and TLR-9 in the nucleus, cytoplasm and cell membrane compared with benign PA. The presence of virus DNA did not notably affect the TLR expression. TLR expression patterns seem to reflect tumour behaviour but are independent of the presence of viruses tested in this study.

Key words: Salivary gland; virus; immunity; parotid gland; immunohistochemistry.

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Toll-like receptors (TLRs) belong to a highly conserved family of pattern-recognition proteins, predominantly expressed in the innate cells of the immune system, serving its activation at the earliest phases (1). They have toll/interleukin-1 receptor domains, which activate specific intracellular signalling cascades when the characteristic extracellular leucine-rich repeat (LRR) domains recognize pathogen-associated molecular patterns (PAMPs) derived from microbes. Specific TLRs recognize and react to proteins of necrotic cells (damage-associated molecular patterns, DAMPs), leading to the release of additional cytokines and chemokines. PAMP and DAMP activation-derived inflammatory mediators maintain tissue homeostasis, work as a defence system and control; for example, wound healing and eliminate transformed cells (2). The 10 members of TLRs in humans (3) situate either on the cell membrane (TLR 1, 2, 4–6, 10), identifying mainly bacterial antigens or within

Received 5 March 2024. Accepted 28 November 2024

the intracellular structures (TLR 3, 7–9), recognising nucleic acids mainly from viruses, other pathogens and the host itself (4).

Viruses can cause direct damage to the host cell by various mechanisms; for example, inducing cell death, expressing viral oncogenes or via DNA integration altering oncogenes and tumour suppressor genes or other components of the signal transduction pathways. Further, the inflammatory response and pathways are activated to eliminate the pathogens, which may result in a prolonged inflammatory state (5). Many of these signalling cascades are mediated via TLRs, and the events contribute to a tumour-favouring environment (6). Hence, TLR stimulation seems to have both tumour stimulating and suppressing effects (2).

Pleomorphic adenoma (PA) is a benign salivary gland tumour, which may recur and undergo malignant transformation (7). After surgery recurrencies occur in about 1–4% of PA cases, which may be caused by incomplete excision or capsule rupture during the primary surgery but may occur also after complete excision (8). Approximately 1% of PAs and around 3% of recurrent PAs (9,10) may develop into malignancy. The causes and predictors for malignant transformation are unknown.

Due to the unknown aetiology of PAs and its derivatives and the potentially rich microbiome in the salivary glands, we previously investigated the presence of a broad range of viral DNAs in PA, recurrent PA and carcinoma ex PA (CXPA) (11). We detected virus DNA more often in CXPA (58%) than in recurrent PA (13%) and PA (32%). The cause and impact of this difference of virus prevalence on the surrounding tissue homeostasis and TLR expression is unknown.

In this report, we studied the expression of several TLRs (-2, -3, -5, -7 and -9) in PA, recurrent PA and CXPA. In addition, as TLRs recognize and initiate the defence system against viruses, we explored the TLR expression in virus DNA-positive and virus DNA-negative tumours.

MATERIALS AND METHODS

Ethical aspects

This study was approved by the Helsinki University Hospital (HUS) Ethics Committee (Authorisation number: HUS/967/2017), and an institutional study permission was granted (§41/2017 and §45/2022). All methods were performed in accordance with the relevant guidelines and regulations. Biobank Act took effect in 2021 and a portion of the samples were collected before that, and for this we had a permit from the National Supervisory Authority of Welfare and Health. Majority of our samples were collected from the biobank, and for these samples an informed consent is implicit for these individuals as a part of the Biobank act. No informed consent was specifically required for these individuals as the results of this retrospective study did not affect the treatment of any patients (Valvira Dnro 1004/06.01.03.01/2012; FIMEA 2021/006901).

Patient and sample selection and source of data

The electronic pathology archives and hospital patient records of the Helsinki University Hospital (Helsinki, Finland) served as the source of data. We compared, by immunohistochemistry of FFPE (formalin-fixed paraffin-embedded) tumour tissues, the TLR expression levels in four different tumour types: 'conventional' PA (n = 25), recurrent PA (primary event: RPA-prim, n = 11, recurrent events: RPA-rec, n = 23) and malignant PA (carcinoma ex PA-CXPA; n = 15). In the RPA-rec and CXPA groups, we included patients who had tumours in any major or minor salivary glands during the period 2000–2018. The "conventional" PA-group contained PAs that were treated during the period 2005–2006 and showed no recurrence within 12 years after surgery. Recurrent PA tumours were treated adequately (parotid gland tumours with partial or superficial parotidectomy) with no report of capsule rupture. The RPA-prim group consisted of primary tumours of the RPA-rec group. The patient and tumour data were described in detail in our previous study (12).

To analyse the effect of virus presence vs. absence of TLR immunoexpression in PA and CXPA, we utilised the virus presence results of our previous study of the same patient population as described above (11). We included altogether 20 PA and CXPA tumours (12 virus DNA-positive and 8 virus DNA-negative), previously analysed by qPCR for viral DNA. Since the virus findings concentrated in CXPA and were scarce in PA, the comparison groups were created equal in sample size, matching the cell count number but otherwise chosen randomly. The tumours were originally screened for 30 DNA viruses belonging to the herpesvirus family (herpes simplex -1 and -2, varicella zoster virus, cytomegalovirus, Epstein–Barr virus, human herpesviruses-6A, -6B and -7 and Kaposi's sarcoma-associated herpesvirus); the polyomavirus family (BK, JC, KI, WU, Merkel cell polyomavirus, trichodysplasia spinulosa, Malawi, St Louis and New Jersey polyomaviruses and human polyomavirus 6, 7, 9 and 12); and the parvovirus family (B19 virus, human bocaviruses 1–4, bufavirus, cutavirus and tusavirus).

Immunohistochemistry

We cut 4 μm thick tissue sections from each FFPE block onto glass slides for staining of TLR-2, -3, -5, -7 and -9. For antigen retrieval, the slides were heated in a pre-treatment module (Agilent Dako, Ca, USA) in retrieval solution pH 9.0, at 98°C, for 15 min (EnVision Flex target retrieval solution, high pH, DM828), and in retrieval solution pH 6.0, at 98 degrees, for 30 min (EnVision Flex target retrieval solution, low pH, DM829). These steps also removed the paraffin from the tissues. We continued by blocking any endogenous peroxidase activity with EnVision Flex peroxidase-blocking reagent SM801 for 5, 10, 15 or 25 min, and added the primary antibody dilutions for each antibody (Dako REAL Antibody Diluent S2022) for 30 or 60 min or overnight, depending on

the antibody, at 5°C. We incubated the HRP-labelled secondary antibodies (EnVision Flex/HRP SM802) for 30 min in the Rabbit/Mouse (ENV) reagent. To visualize the stains, we added the diaminobenzidine (DAB) chromogen (EnVision Flex DAB DM827) for 10 min and then washed the sections with PBS with 0.04% Tween-20 between each step. We performed the counterstaining with Dako Mayer's haematoxylin (S3309) for 1 min with Autostainer 480S (LabVision Corp, Fremont, Ca, USA). The slides were mounted with Pertex Histolab (Reagecon Diagnostics Ltd, Ireland) mounting media. For immunohistochemical staining's, the following antibodies were used: TLR-2 (Novus biological, rabbit, polyclonal, 1:300), TLR-3 (Aviva systems biology, rabbit, polyclonal, 1:800), TLR-5 (Novus biologicals, mouse, monoclonal, 1:300), TLR-7 (Novus biologicals, rabbit, polyclonal, 1:300) and TLR-9 (Novus biologicals, mouse, monoclonal, 1:500).

TLR expression level scoring

A senior pathologist and the two first authors scored the staining patterns of TLR-2, -5, -7 and -9 for nuclear, nuclear membrane, cytoplasmic and cell membrane intensity, respectively, for the whole cohort in a blinded manner without knowing the pathological information. Additionally, the virus-related TLR-3 was included for the virus study. The two first authors evaluated the staining simultaneously, and the senior pathologist thereafter. Agreement for consensus score was reached upon discussion.

The expression intensity was evaluated separately for nucleus, nuclear membrane, cytoplasm and cell membrane on a scale from 0 to 3 (0 = no positive cells, 1 = low expression, under 25% positivity, 2 = medium expression, 25–75% positivity and 3 = high expression, over 75% positivity).

Statistical analysis

We carried out the statistical analysis using SPSS (IBM, software version 28) for the main cohort. However, for the virus-related TLR group we did not perform statistical analysis due to the low number of cases. For variance analysis, we performed Kruskal–Wallis test and Bonferroni corrected pairwise Mann–Whitney U comparison as a post hoc test. p-Values ≤ 0.05 were considered statistically significant.

RESULTS

TLR expression in the investigated tumour types

We evaluated TLR-2, -5, -7 and -9 immunoexpression (scale 0–3, Tables 1 and 2) in all tumours (n = 74). In addition to comparison between tumour types, we analysed the expression rates of TLR-2, -3, -5, -7 and -9 to the viral DNA presence results from our previous work (n = 20) (11). The TLR-staining pattern was most intense within the cytoplasm (TLR-2 mean 1.4, TLR-5 mean 2.4, TLR-7 mean 2.7 and TLR-9 mean 2.5). Overall, all TLRs were expressed in all tumour types to some extent with variation in pattern.

TLR-2

The TLR-2 expression pattern was similar in the different tumour groups (Table 1). Figure 1A shows the expression of TLR-2 in healthy parotid tissue and Fig. 1B shows the expression in PA. The TLR-2 expression pattern in virus positive and negative tumours is shown in Table 2.

TLR-3

The TLR-3 expression pattern in virus-positive and -negative tumours is shown in Table 2. The TLR-3 expression level was not analysed for the whole cohort.

TLR-5

The nuclear TLR-5 expression was higher in CXPA than in RPA-prim and RPA-rec (Table 1). Higher cytoplasmic TLR-5 expression was shown in CXPA tumours compared with both RPA-rec ($p < 0.001$) and RPA-prim ($p = 0.028$). The PA group showed higher cytoplasmic TLR-5 expression compared with RPA-rec ($p = 0.005$). The nuclear and nuclear membrane TLR-5 staining was emphasised in the virus DNA-positive CXPA in contrast to the virus DNA-positive PA (Table 2).

TLR-7

TLR-7 expression variance analysis showed statistically significant differences between tumour groups in the TLR-7 cytoplasmic expression ($p = 0.047$), but further pairwise comparison revealed no differences. Figure 2A shows low expression of TLR-7 in healthy parotid gland tissue. Figure 2B shows strong nuclear expression of TLR-7 in CXPA. Cytoplasmic TLR-7 revealed strong staining pattern in both virus DNA-positive and virus DNA-negative tumours (Table 2). The overall staining was strongest within the virus DNA-positive PAs, in all patterns; however, the differences between tumour types were modest.

TLR-9

TLR-9 expression in variance analysis revealed statistically significant differences in TLR-9 nuclear membrane ($p = 0.041$) and cell membrane ($p = 0.008$) between groups (Table 1). Further investigation showed increased cell membrane expression in the CXPA group compared with the PA group ($p = 0.013$) and RPA-rec group ($p = 0.020$). Within the virus-related subset analysis, TLR-9 staining displayed medium expression within the nucleus in PAs in comparison to low

Table 1. The expression of TLR-2, -5, -7 and -9 in the different tumour types

	Mean (Md, SD)			
TLR-2	**PA (n = 25)**	**RPA-prim (n = 11)**	**RPA-rec (n = 23)**	**CXPA (n = 15)**
TLR-2, nuclear	0.5 (0, 0.7)	0.5 (0, 0.8)	0.6 (0, 0.7)	0.5 (0, 0.7)
TLR-2, nuclear membrane	0.4 (0, 0.6)	0.2 (0, 0.6)	0.6 (0, 0.9)	0.3 (0, 0.5)
TLR-2, cytoplasm	1.5 (2.0, 0.9)	1.4 (1.0, 1.0)	1.7 (2.0, 0.8)	1.9 (2.0, 1.0)
TLR-2, cell membrane	0.2 (0, 0.6)	0.1 (0, 0.3)	0.5 (0, 0.7)	0.3 (0, 0.6)
TLR-5	**PA (n = 26)**	**RPA-prim (n = 12)**	**RPA-rec (n = 23)**	**CXPA (n = 15)**
TLR-5, nuclear	2.0 (2.0, 0.5)	1.5 (2.0, 0.9)	1.7 (2.0, 0.6)	2.3 (2.0, 0.6) p = 0.015[1] p = 0.020[2]
TLR-5, nuclear membrane	0.8 (1.0, 0.9)	0.5 (0, 0.8)	0.6 (0, 0.9)	0.9 (1.0, 0.7)
TLR-5, cytoplasm	1.7 (2.0, 0.8)	1.1 (1.0, 0.9)	0.7 (0, 1.0) p = 0.005[3]	2.3 (2.0, 0.8) p = 0.001[2] p = 0.028[1]
TLR-5, cell membrane	0.4 (0, 0.8)	0.3 (0, 0.6)	0.5 (0, 0.7)	0.7 (1.0, 0.8)
TLR-7	**PA (n = 26)**	**RPA-prim (n = 12)**	**RPA-rec (n = 22)**	**CXPA (n = 15)**
TLR-7, nuclear	1.7 (2.0, 1.1)	2.2 (3.0, 1.2)	2.4 (3.0, 0.8)	1.7 (2.0, 1.2)
TLR-7, nuclear membrane	1.7 (2.0, 1.1)	1.9 (2.0, 1.0)	1.6 (2.0, 1.1)	2.1 (2.0, 0.9)
TLR-7, cytoplasm	2.0 (2.0, 0.7)	2.5 (3.0, 0.8)	2.4 (3.0, 0.8)	2.5 (3.0, 0.5)
TLR-7, cell membrane	0 (0, 0.2)	0 (0, 0)	0 (0, 0.2)	0.1 (0, 0.5)
TLR-9	**PA (n = 26)**	**RPA-prim (n = 11)**	**RPA-rec (n = 21)**	**CXPA (n = 15)**
TLR-9, nuclear	0.2 (0, 0.5)	0.7 (0, 1.2)	0.6 (0, 1.1)	0.3 (0, 0.5)
TLR-9, nuclear membrane	0.4 (0, 0.8)	0 (0, 0)	0.5 (0, 0.9)	0 (0, 0)
TLR-9, cytoplasm	2.6 (3.0, 0.6)	2.6 (3.0, 0.5)	2.5 (3.0, 0.7)	2.7 (3.0, 0.6)
TLR-9, cell membrane	0 (0, 0)	0 (0, 0)	0 (0, 0)	0.2 (0, 0.4) p = 0.020[2] p = 0.013[4]

TLR scores as mean and median (Md) with standard deviation (SD); scores of expression intensity: 0–3. Presented statistically significant p-values indicate pairwise comparisons. Values were calculated with Bonferroni corrected Mann–Whitney U, statistically significant p-value $p < 0.05$. Exact pairwise combination is marked from (1) to (4). Differences in population sizes in different TLR receptor groups were caused by insufficient tumour tissue available for the immunohistochemical staining.

CXPA, carcinoma ex pleomorphic adenoma; PA, conventional PA; RPA-prim, primary events of RPA; RPA-rec, recurrent events of RPA.

[1]RPA-prim vs. CXPA pairwise comparison.

[2]RPA-rec vs. CXPA pairwise comparison.

[3]PA vs. RPA-rec pairwise comparison.

[4]PA vs. CXPA pairwise comparison.

expression in the CXPA and no other differences between the types were observed (Table 2).

DISCUSSION

We investigated the TLR-expression profiles of TLR-2, -5, -7 and -9 in PA, recurrent PA (RPA-prim and RPA-rec) and CXPA. CXPA expressed significantly more TLR-5 and TLR-9 in the nucleus, cytoplasm and cell membrane compared with benign PA. Previously, we analysed the presence of viral DNA in PA, RPA-prim, RPA-rec and CXPA and detected herpesvirus DNA (HHV-7, HHV6B and EBV) with a predominance in CXPA (7/12, 58%), when compared to PA (2/15, 13%) or recurrent PA (2/23, 9%) (11). Hence, we examined also whether TLR expressions differed between virus-positive and virus-negative tumours.

TLR-2 located in the cell surface detects a wide variety of pathogens derived from parasites, bacteria and viruses (13). It has been suggested that activation of TLR-2, caused by pathogens and DAMPs, would correlate with more severe disease (14). Our results, related to salivary gland PA, are somewhat controversial as we observed TLR-2 expression in all tumour types, without significant variation between them and with no relation to malignant transformation. TLR-2 nuclear and nuclear membrane expression was low in all groups. No relevant difference was observed concerning the viral status. This is in line with Jouhi et al. (15), who discovered no correlation between Merkel cell polyomavirus status and TLR-2 expression in Merkel cell carcinoma.

We found moderately increased cytoplasmic TLR-3 expression in the malignant virus-positive tumours. Altered TLR-3 expression has been

Table 2. Expression of TLRs in virus-positive and -negative PA and CXPA tumours

Tumour type	Virus DNA-positive tumours; Mean (Md, SD)		Virus DNA-negative tumours; Mean (Md, SD)	
	PA (n = 6)	CXPA (n = 6)	PA (n = 4)	CXPA (n = 4)
TLR				
Total virus hits in group (n)[1]	15	17	0	0
Viruses present	HHV-7 (n = 6) HHV- 6B (n = 4) B19V (n = 3) HPyV10 (n = 1) CuV (n = 1)	HHV-7 (n = 6) HHV-6B (n = 5) EBV (n = 3) MCPyV (n = 2) B19V (n = 1)	–	–
TLR-2				
Nuclear	0.5 (0, 0.8)	0 (0, 0)	0 (0, 0)	0.2 (0, 0.4)
Nuclear membrane	0.3 (0, 0.5)	0.2 (0, 0.4)	0 (0, 0)	0.5 (0.5, 0.6)
Cytoplasm	1.5 (1.5, 1.1)	1.8 (2.0, 0.8)	0.8 (1.0, 0.5)	1.5 (1.5, 1.3)
Cell membrane	0.3 (1.0, 0.5)	0.7 (0.5, 0.8)	1.5 (0.5, 1.0)	0 (0, 0)
TLR-3				
Nuclear	2.2 (2.0, 0.4)	2.3 (2.0, 0.5)	2.8 (3.0, 0.5)	2.5 (2.5, 0.6)
Nuclear membrane	1.2 (1.0, 0.8)	1.3 (1.0, 1.0)	0.5 (0, 1)	0.8 (0.5, 1.0)
Cytoplasm	2.0 (2.0, 0)	2.7 (3.0, 0.5)	1.8 (1.5, 1.0)	2.0 (2.0, 0)
Cell membrane	0 (0, 0)	0.2 (0, 0.4)	0.7 (1.0, 0.8)	0.3 (0, 0.5)
TLR-5				
Nuclear	0.4 (0, 0.6)	1.7 (2.0, 0.8)	0.8 (1.0, 0.5)	0.8 (0, 1.5)
Nuclear membrane	0.4 (0, 0.9)	2.0 (2.0, 0.8)	2.0 (2.0, 1.2)	2.8 (3.0, 0.5)
Cytoplasm	3.0 (3.0, 0)	2.7 (3.0, 0.5)	1.3 (1.5, 1.0)	2.5 (2.5, 0.6)
Cell membrane	0 (0, 0)	0.8 (0.5, 1.2)	0.2 (0, 0.4)	0.5 (0.5, 0.6)
TLR-7				
Nuclear	0.7 (0.5, 0.8)	0.2 (0, 0.4)	0.5 (0, 1.0)	0.3 (0, 0.5)
Nuclear membrane	0.5 (0, 0.8)	0 (0, 0)	0 (0, 0)	0 (0, 0)
Cytoplasm	3.0 (3.0, 0)	2.8 (3.0, 0.4)	2.3 (2.5, 1.0)	2.5 (3.0, 1.0)
Cell membrane	0 (0, 0)	0 (0, 0)	0 (0, 0)	0 (0, 0)
TLR-9				
Nuclear	1.7 (2.0, 1.0)	0.2 (0, 0.4)	1.7 (2.0, 0.6)	0.8 (0.5, 1.0)
Nuclear membrane	0.2 (0, 0.4)	0 (0, 0)	0.3 (0, 0.6)	0.3 (0, 0.5)
Cytoplasm	2.7 (3.0, 0.8)	2.8 (3.0, 0.4)	2.3 (3.0, 1.2)	2.0 (2.0, 0.8)
Cell membrane	0 (0, 0)	0 (0, 0)	0 (0, 0)	0.3 (0, 0.5)

TLR scores as mean and median (Md) with standard deviation (SD); scores of expression intensity: 0–3.
B19V, parvovirus B19; CuV, cutavirus; EBV, Epstein–Barr virus; HHV, human herpesvirus; HPyV10, human polyomavirus 10; MCPyV, Merkel cell polyomavirus.
[1]Jauhiainen et al. (11).

associated with clinical outcome in various malignancies (16), depending on the anatomical site of the cancer. Overexpression of TLR-3 is associated with a good prognosis in hepatocellular carcinoma and lung carcinoma. However, within oral and oesophageal cancer TLR-3 expression appears to be associated with poor prognosis (17).

The TLR-5 activation includes the downstream events of MyD88-dependent activation of regulatory subunit of PI3K and p85, pathways involved in cell growth and survival (18,19). The presence of TLR-5 has been described both in normal salivary gland tissue and in salivary gland adenocarcinomas (15,20). In our cohort, we detected TLR-5 in all tumour groups. CXPA expressed significantly more TLR-5 than PA-prim and PA-rec, both within the nucleus and within the cytoplasm. These findings support the results of Park et al. (20) who suggested that promotion of invasion and migration in salivary gland carcinomas occurs via the TLR-5-induced pathway. In our study, the presence of viruses did not affect these results in a notable manner.

Within the head and neck region, high TLR-5 expression has been associated with higher mortality of tongue carcinoma, with more aggressive behaviour for oral squamous cell carcinoma (SCC) when compared to cutaneous SCC and with poor disease-specific survival in oropharyngeal SCC (15,21,22). Further, a meta-analysis, comprising six studies assessing the prognostic value of TLR-5 immunoexpression in head and neck SCC, predicted a shorter, yet non-significant, patient survival outcome for strongly TLR-5-expressing tumours (23). Our observation of the highest TLR-5 expression levels in CXPA relates to the previous findings

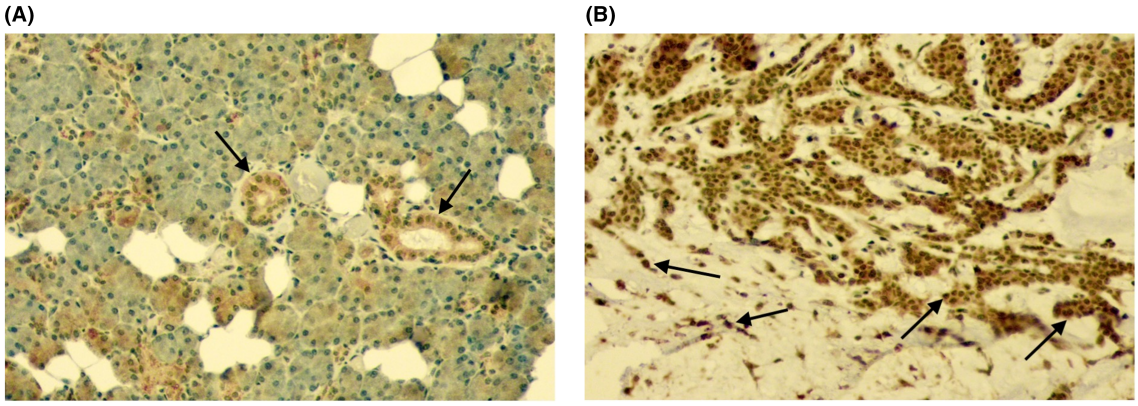

Fig. 1. Toll-like receptor-2 (TLR-2) expression in healthy parotid gland tissue and in pleomorphic adenoma (PA). (A) Healthy parotid gland tissue with toll-like receptor-2 (TLR-2) expressed in ductal and acini tissue (×200). (B) Pleomorphic adenoma (PA) tumour tissue with multicellular expression of toll-like receptor-2 (TLR-2) Expression in epithelial and myoepithelial cells (×200).

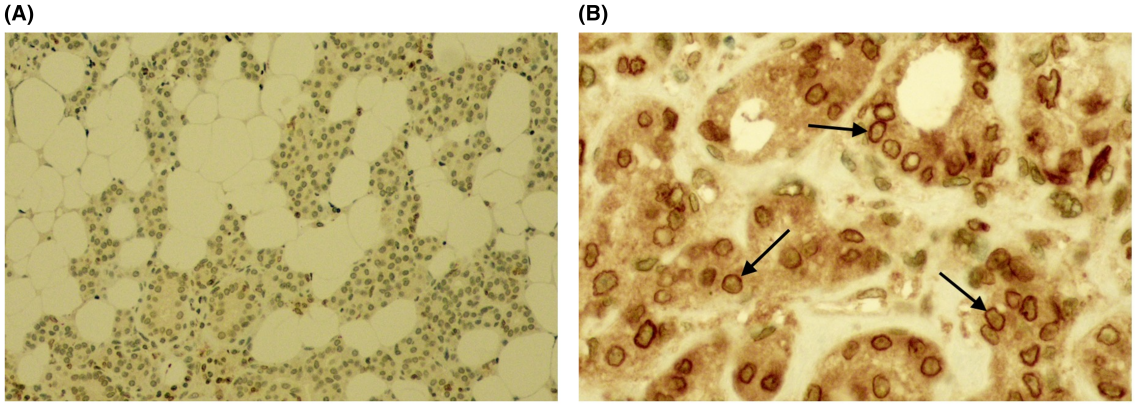

Fig. 2. Toll-like receptor-7 (TLR-7) expression in healthy parotid gland tissue and CXPA tumour with strong nuclear membrane TLR-7 expression. (A) Healthy parotid gland tissue with low toll-like receptor-7 (TLR-7) expression. (×200). (B) Carcinoma ex pleomorphic adenoma (CXPA) tumour tissue with strong nuclear membrane toll-like receptor-7 (TLR-7) expression (×400).

of high TLR-5 levels and malignant potential. However, Hirvonen et al. found no correlation between TLR-5 levels and survival in salivary gland adenocarcinomas (15), which could relate to the varying role of TLRs in different salivary gland tumour types.

Interestingly, the cytoplasm of PAs exhibited higher TLR-5 levels when compared to that of PA-rec. Comparing PA with PA-prim did not reach statistical significance, but a similar tendency was observed. This suggests that the recurrent tumour could have its own biological behaviour distinct from the 'normal' PA.

TLR-7, located in the intracellular endosomes, detects single-stranded viral RNA. TLR-7 stimulation may have protective effects. T his phenomenon is utilised in clinical practice an TLR-7 agonists are studied for oncological indications in clinical trials with hitherto inconclusive results (24). Within our cohort, the cytoplasmic expression varied within the whole study groups ($p = 0.032$), even though pairwise comparison did not reach significance. We noticed elevated TLR-7 expression levels within the RPA-rec group, especially when compared with RPA-prim tumours, which suggests that the TLR-7 may play a role in recurrence. The modest TLR-7 expression both in PA and CXPA may indicate a disturbance in the physiological response mediated by TLR-7, and TLR-7 seems not to have effect on the malignant transformation. We

observed nearly similar TLR-7 expression in virus DNA-positive and virus DNA-negative groups. This indicates irrelevance of the virus presence as regards the expression of TLR-7 in PA and CXPA. A study by Jouhi et al. reported that the expression of TLR-5, -7 and -9 in oropharyngeal SCC is associated with the presence of human papilloma virus (HPV) DNA in the tumour tissue. Our results are not in line with these findings of the viral presence. However, this is probably explained by the fact that both the specific viruses and the tumours in these studies were different.

The cell membrane TLR-9 expression was significantly higher in CXPA than in PA and RPA-rec. Our result differs from a previous study of mucoepidermoid carcinomas where high levels of TLR-9 indicated a better prognosis (25). Another study of 120 oral SCC samples compared TLR 9 expression to healthy samples and the variation in TLR-9 genotypes. The authors concluded that TLR-9 overexpression was increased in the OSCC group, and the overexpression could relate to invasiveness as well, however variation across different TLR-9 genotypes was observed indicating the effect of polymorphism (26). The higher presence of membranous TLR-9 in CXPA in our study may indicate a role for TLR-9 in malignant transformation of PAs. Hirsch et al. reported that loss of TLR-9 function could be associated with the presence of viruses. We did not observe significant differences between virus DNA-positive and virus DNA-negative tumours. Therefore, rather than viral activation, it could be cell death, present in rapidly growing malignant tumours, stimulating TLR-9 expression as TLRs are known to recognize both PAMPs and DAMPs (27).

The limitations of this study include limited sample size in each tumour group due to the rarity of recurrent tumours and CXPA. The histology of PA is heterogeneous, which poses challenges in the evaluation of the samples.

In conclusion, distinctive patterns of TLR expression within PA, RPA-prim, RPA-rec and CXPA are evident. We observed a change of TLR expression especially in the malignant transformation: both TLR-5 and TLR-9 showed significantly elevated expression levels in CXPA. However, it seems that benign tumours have their characteristic expression as well: within TLR-5, the expression levels of PA were high, in contrast to those of RPA-prim and RPA-rec. However, the presence of DNA viruses in the tumours did not seem to substantially affect the expression levels of any TLR. Inflammation-induced cancer and cancer-induced inflammation mimic each other in many ways, which may cause confusion. TLRs have an important role in mediating these microenvironmental pathways in both situations necessitating further studies.

The assistance provided by Pia Saarinen concerning immunohistochemical staining is greatly appreciated.

FUNDING

The study was supported by HusLab, and by State Funding for the Helsinki University Hospital (Y780023053, 2021338, and 2021220), and the University of Helsinki Doctoral Programme of Clinical Research.

CONFLICT OF INTEREST

The authors declare no conflicts of interest.

DATA AVAILABILITY STATEMENT

The datasets generated during and/or analyzed during the current study are not publicly available due to the sample size and rarity of these tumors. Data are available from the corresponding author on reasonable request.

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