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The Expression and Significance Profile of TLR-2 in the Salivary Gland Tumor and Inflammation

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This study was to research the different mRNA and protein expression of Toll-like receptors 2 (TLR-2) characteristics in saliary gland benign (SGBT), malignant tumor tissue (SGMT)and saliary gland inflammation (SGI) by the methods of PCR, qRT-PCR and immunohistochemisty. the results showed that, compared with normal tissues, the TLR-2 mRNA expression in salivary gland benign tumor , salivary gland cancer and inflammation tissues were amount to 6.628 fold , 0.432 fold and 8.583 fold respectively ($p < 0.05$). Importantly, we also found that TLR-2 in these organizations not only has a say in the cytoplasm, and the nucleus has expressed only in the SGMT, which was instant differently previous research. This study suggests that there is an variation of TLR2 association between salivary gland tumor, inflammation and NSG. This finding supports a potential role in tumor development, and provide a foundation for development of immune modulation and chemotherapy for SGT.

Key words: TLR-2, salivary gland tumor, salivary gland inflammation.

In [1]our country, the incidence of salivary gland tumors in the head and neck tumors is 2-3%(Wang, *et al.*,2012), and the salivary gland tumor is also unique second categories tumor in the oral and maxillofacial region. In this innate immunity system, Toll-like receptors-2(TLR-2) play an important role. (Lammers, *et al.*,2012) Recognition of pathogen associated molecular patterns (PAMPs) by Toll-like receptor-2 (TLR-2) on antigen presenting cells (APCs) induces the expression of various pro-inflammatory cytokines and activates the effector functions of innate immune cells. (Shakhnovich, *et al.*, 2011) TLR-2 signaling in mucosal epithelial cells looked contribute to immunosilent environment in order to keep harmonious symbiosis, which depends on variable pathways (Allam, *et al.*, 2011). Whereas polarized apical activation of TLR-2 by CpGODNs also displayed inhibition of inflammatory response and basolateral stimulation of TLR-2 led to NF-êB activation (Sen, *et al.*, 2011). The activation of all TLR-2 leads to myeloid differentiation factor 88 (MyD88)-dependent signaling pathway, (Kar, *et al.*, 2011) and induce the synthesis or release of pro- and anti-inflammatory cytokines and chemokines via the activation of transcription factors, such as NF-êB, interferon regulatory factor (IRF)-3/7, AP-1 and others. (Batbayar, *et al.*, 2011)

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Thus, On the one hand ,the exogenous activation of the immune system via TLR-2 could on one side be a strategy to fight cancer, but on the other exacerbate, a latent chronic inflammation, which in turn, would further favour carcinoma progression. The recognition of specific molecules by TLR-2 leads to the activation of the adaptive immune system. (Sayi, *et al.*, 2011) On the other hand, relatively little is known about the immunogenic versus immunomodulatory roles of the constitutive expression of TLR-2 in gland tissue cells, and it is not clear whether human salivary gland tumor and salivary gland inflammation activate by producing TLR-2 ligation. To demonstrate the interaction between the expression of TLR-2 mRNA and protein in the SGBT or SGMT, SGI and NSG tissues, and the expression situation of these different organizations.

METHODS

Patients and samples

Specimens from 2008-2010 years in shenzhen hospital of Peking University, after surgery and pathology confirmed salivary gland benign tumor specimens(SGBT) 33 cases, (male 19 cases, female 14 cases, mean age, 45.7 years), normal salivary gland specimens (NSG) in 24 cases, (13 cases male, 11 female patients, with an average age of 43.5 years), salivary gland malignant tumor specimen(SGMT) 10 cases(6 cases of male, 4 cases of female, mean age, 57.6 years), 17 cases of salivary gland inflammation specimens (SGI) in patients (10 cases of male and 7 female, mean age, 52.9 years). Among them, the pleomorphic adenoma(29 cases), basal cell tumor (3 cases), benign muscle epithelioma(1 cases), mucoepidermoid carcinoma (5 cases), adenoid cystic carcinoma (4 cases), poorly differentiated muscle epithelioma (1 cases). The selected specimen were stored in liquid nitrogen (extracting RNA); To fix others by more than 4% polyformaldehyde in 4!storage (Immunohistochemical). (The study was approved by the Ethics Committee of Peking University Shenzhen Hospital. Written informed consent was obtained from all the patients).

PCR and quantitative Real-Time PCR (qRT-PCR) analysis

The total specimens RNA were isolated using DP420-RNAprep pure Micro kit (Invitrogen, Carlsbad, CA) according to the manufacture's protocol. cDNA was synthesized using 2 ìg total RNA and SuperScript reverse transcriptase kit (Invitrogen, Carlsbad, CA). Reverse-transcriptase–polymerase chain reaction was performed according to manufacturer's protocol. The following primers (Sangon, Shanghai, China) were used: TLR-2:
forward:5'-AAACTGGAACGGTGAAGGTG-3',
reverse:5´-CATGAGGGTCTTGTATCTCCTCT-3'.
housekeeping gene(control) β-actin:
forward: 5'-AGTGGGGTGGCTTTTAGGAT-3'
reverse: 5'-CCTCCTCATGGCTTTTTGCAGC-3'.
According to a standard protocol used in our previous study(Yu SY, *et al.*, 2011), Samples were subjected to thermal cycling at 95°Cfor 5 minutes followed by 40 cycles of 94°C for 30 seconds, 62! for 30 seconds, and 72°C for 30 seconds; and with a final extension at 72°C for 5 seconds. qRT-PCR was performed using SYBR Green PCR Master Mix with 2 µl of cDNA and 200nM of the upstream and downstream primers per 20µl of reaction. Each sample was amplified in duplicate for every experiment. The gene for β-actin was amplified as an endogenous reference. Amplification was performed using 40 cycles of the following program: 94°C for 30 seconds, followed by 62! for 30 seconds, and 72°C for 30 seconds. The mean efficiency of TLR-2 and β-actin amplification was 2.04 and 2.08, respectively. Reactions were run on an ABI 7900HT instrument (Carlsbad, CA, USA). Each quantitative PCR was run in triplicate, and the experiment was repeated at least 3 times. Relative quantification of gene expression was determined by using the comparative Ct method. Results were normalized using the human β-actin housekeeping gene. Each sample was in triplicate for each quantitative PCR measurement. Melting curves were checked to ensure specificity. Relative quantification of mRNA expression was calculated using the $2^{-3\%Ct}$ method, with the endogenous housekeeping gene β-actin as normalizer and control sample calibrator.

Evaluation of TLR-2 immunostaining

Tissue sections (3 µm) were mounted on poly-L-lysine-coated slides, deparaffinized, and incubated overnight in an oven at 37°C. Endogenous peroxidase activity was inhibited with 3% hydrogen peroxide for 15 min, and the samples were processed in a microwave oven in 10 m

Mcitrate buffer (pH 6.0) to unmask the epitopes. After antigen retrieval, the sections were incubated with diluted primary antibodies against TLR-2 (1:300, Sigma–Aldrich, St. Louis, MO, USA) for 1 h, followed by washing with PBS. A horseradish peroxidase/Fab polymer conjugate was then applied to the sections for 30 min. After extensive washing, the sections were incubated for 3 min with the peroxidase substrate diaminobenzidine, counterstained with Gill's hematoxylin, and mounted with mounting medium. TLR-2 immunoreactivity was evaluated independently and in a blinded manner by 2 investigators for the specimens, To select five at high magnification vision (x 400) to judgment by random, and score tumor cells positive percentage and dyeing strength tumor cells positive, 2 score additive that is for the specimen immunohistochemical positive scoring. Negative expression or no expression means 0 to 1 point, Weak positive, 2-3 points; Positive, 4-5 points; Strong positive, 6-7 points. Dyeing strength rating: 0 is divided into colorless, 1 is divided into cell dyeing, which is light yellow, 2 is divided into the cells are yellow, 3 into the cells are brown. Positive cell proportion 1% - 25% for 1 point, 26% - 50% for 2 points, 51% - 76% for 3 points, and76% - 100% for 4 points. Provisions integral g" 3 is divided into positive, no positive cell expression is 0. All specimens with discordant scores were revaluated by the 2 point investigators using a multiheaded microscope, and the consensus score was used for further analysis.

Statistical analyses

A paired, 2-tailed Student t test was used to assess the statistical significance between the 2 groups. Among many groups (>2 groups), statistical significance was determined by analysis of variance. Comparisons between TLR-2 expression and pathologic features were evaluated by using ÷2 test and Fisher's exact test. Version 13.0 of Statistical Package of Social Sciences software (SPSS Inc.) was used for statistical analysis. A p-value of less than 0.05 is considered as statistically significant.

RESULTS AND DISCUSSION

The mRNA expression level of TLR-2

The mRNA expression level of TLR-2 in each group was analyzed with PCR and Real-Time qPCR, using β-actin as a control gene. The analysis results were shown in Figures 1 and 2. Briefly, no significant difference could be observed in mRNA with PCR, and TLR-2 are detected in the SGBT, SGMT, SGI and NSG tissues. However, the results of qRT-PCR appear obvious differences: compared with NSG group, SGBT group TLR - 2 mRNA expression quantity was up-regulated to 6.628-fold ($p < 0.05$); SGI group was also significantly up-regulated to 8.583-fold ($p < 0.05$). but the SGMT group to 0.432-fold, significantly lower than NSG group ($p < 0.05$);its smilar to the finding of Kato S, The expression of TLR2 mRNA was increased by A actinomycetemcomitans infection. Phagocytosis and apoptosis in A actinomycetemcomitans-infected THP-1 cells were inhibited by the addition of anti-TLR2 antibody.(Kato, *et al.* 2012.) These data confirmed the implication of TLR-2 signaling pathways in early inflammatory events and SGBT stages, while in the SGMT tissues the expression of TLR-2 obviously down-regulated, which suggested TLR-2 have been accumulated in bacterial amyloid fibrils, and the microscopic observation TLR-2 recognition of bacterial amyloid fibrils in the intestinal mucosa represents a novel mechanism of immunoregulation, and it contributes to the generation of inflammatory responses. (Layoun,*et al.*, 2012.)

Table 1. TLR-2 in the tissue of the immunohistochemical expression, and their pathomorphological scores

	Case (N)	Location	The expression of TLR-2(%)		
			Positive scoresg"3	Negative scores<3	P
NSG	24	cytoplasm	11 (43.48)	13(56.52)	
SGI	17	cytoplasm	11 (65.75)	6 (34.25)	<0.05
SGBT	33	cytoplasm	23 (69.71)	10(30.29)	<0.05
SGMT	10	cytoplasm, nucleus	2 (20)	8(80)	<0.05

Immunohistochemical examinations

Staining intensity for TLR-2 varied and was detected at different localizations in all SGBT, SGMT, SGI and NSG samples, But there were negative expression commonly in SGMT tissues. In SGI tissues (n = 17), SGBT tissues (n = 33) and NSG tissues (n = 24) TLR-2 was located in the cytoplasm(Fig 3), but in the SGMT tissues, TLR-2 was both located in the nucleus and the cytoplasm, In addition, in the SGMT, there was weak cytoplasmic TLR-2 staining(Fig 3 D and D1). Compared with NSG tissues, the expression of TLR-2 in SGBT tissues significantly weak; the expression of TLR-2 in SGI and SGBT tissues remarkable enhancement. And the TLR-2 negative expression and weak positive expression limited to low expression, the results showed that in the SGMT patients, the expression is obviously lower than the normal tissue ($P < 0.05$), (Tab 1) and its expression is not only confined to the cytoplasm, it is also found in the nucleus. In comparison with NSG tissues, there was significant difference between SGBT and SGI tissues ($P < 0.05$). These results similar to Hagström J. in their research about the TLR-2 in the follicular thyroid carcinoma, TLR-2 expression was stronger in adenomas than in carcinomas, And chronic inflammation tissues outside the primary tumour was stronger for carcinomas than for adenomas.(Hagström J, *et al.*, 2012.). However, besides the strength expression is different salivary glands, we also detected their expression loction change, similar to findings by Paoletti I *et al.* Keratinocytes stimulated by microbial organisms secrete not only a variety of cytokines, chemokines and growth factors, but also antimicrobial peptides such as TLR-2 induced ERK/MAPK phosphorylation in human keratinocytes, thus providing evidence that HBD-2 and HBD-3

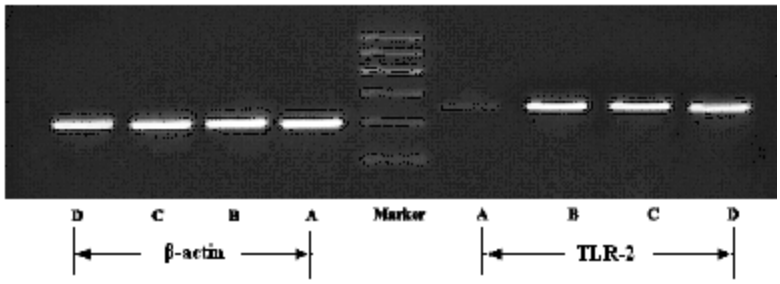

Fig. 1. The TLR-2 expression in SGI, NSG, SGBT and SGMT tissue by PCR. A: SGMT group, B: SGBT group, C: SGI group, D: NSG group, β-actin were used as control gene for normalization

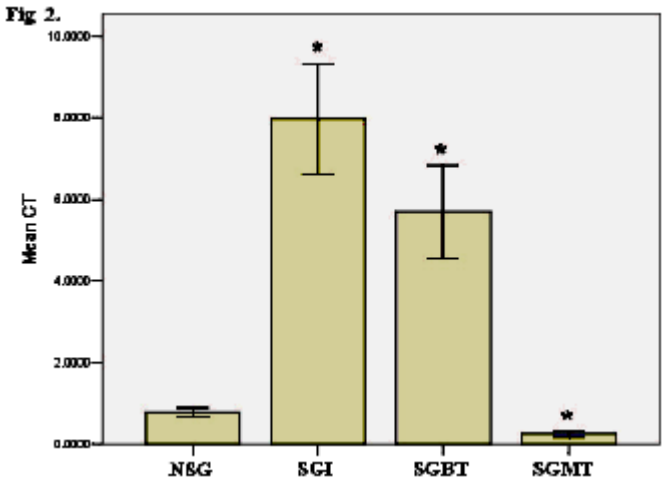

Fig. 1.The differences of TLR-2mRNA expression in SGI, NSG, SGBT and SGMT tissue were detected by RT-qPCR..(* Compared with the normal tissue, *P<0.05)

secretion is through the same transductional pathway. The ability of TLR-2 to induce also HBD-3 may amplify its therapeutic potential responsible for human inflammation microenvironment steady state disorders.(Paoletti, *et al.*, 2012; Shin, *et al.*, 2010;Scharf, *et al.*, 2010;Varoga, *et al.*, 2009.). This suggests an increased innate advertence of the organism in case of an inflammation but also in the border region of a tumor by invading body defending AMPs.

For the first time, we detected decreased expression levels of TLR-2 in SGMT when compared to NSG and, especially, when compared to the SGI. In the presence, Ribeiro FV et al, observed gene expressions of TLR-2 were higher in periodontitis when compared to periodontally healthy sites ($P < 0.05$). (Ribeiroet *et al.*, 2012.)TLR-2 is known to be constitutively expressed in epithelial tissue. Janardhanam SB et al, reperted the tissue epithelium and the salivary epithelial cells expressed reduced TLR-2 increasing proteins and transcripts in OLP (Janardhanammet *et al.*, 2011.). Lu H, *et al.* found TLR2 could activate dendritic cells (DC) and T cells by PSK, and oral administration of PSK in neu transgenic mice significantly inhibits breast cancer growth. (Lu *et al.*, 2011.) They postulated that TLR-2 seems to play an important role in control of transcription and induction of apoptosis in SGI and SGBT. Decreased expression levels of TLR-2 in SGMT could result in decreased apoptosis and therefore be the reason for tumor progression. Furthermore, Mahadevan NR *et al.* they suggested that transmissible ER stress is a mechanism through which tumor cells can control myeloid cells by directing them toward a proinflammatory phenotype, onsistent with the fact that a second signal through TLR-2 combined with exposure to tumor ER stress-conditioned medium, thus facilitating tumor progression. (Mahadevan *et al.*,

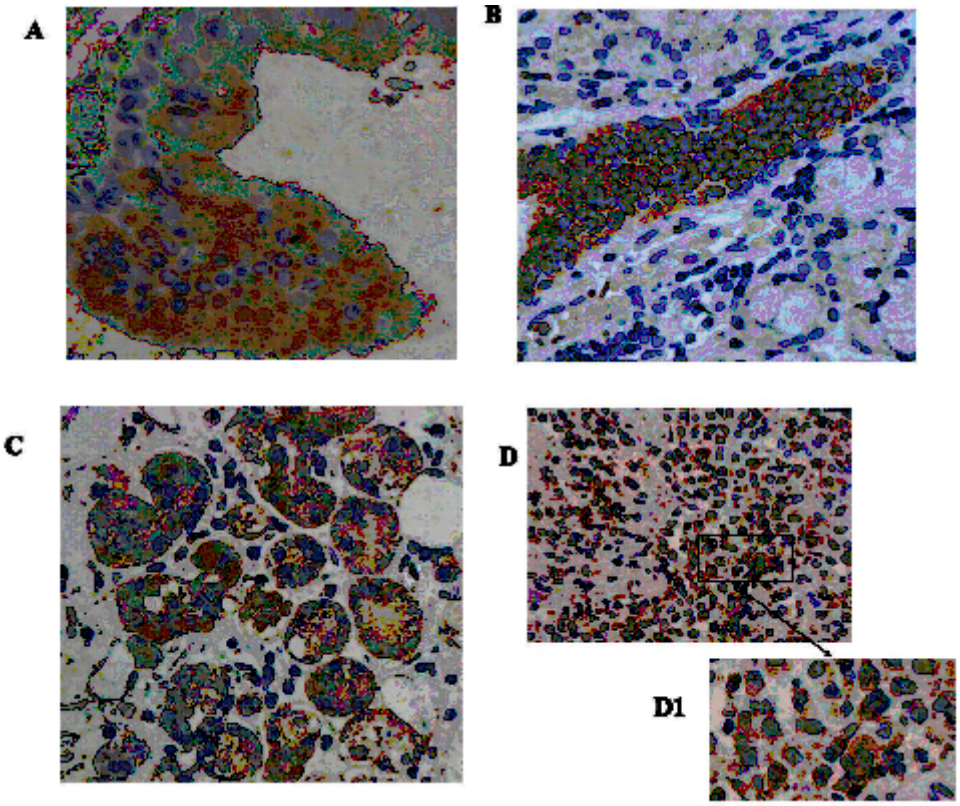

Fig. 1. The differences of TLR-2 protein expression in SGI, NSG, SGBT and SGMT tissuses were detected by immunohistochemical assay, A: SGI group, B: NSG group, C: SGBT group, D: SGMT group. Visible some shading in malignant tumor nuclear(Arrow shown D1).(×400)

2011) We found a shift of TLR-2 from the cytoplasm to the tumor nucleus in the SGMT, comparing NSG, SGI, and SGBT. The protein accumulated in the nucleus although the gene was down regulated. The reason and biological impact of the nuclear migration of the protein is not clear up to now. But hypothetically, nuclear accumulation of TLR-2 protein seems to cause down regulation of the gene promoter. In conclusion, our present study suggests that in SGMT compared to NSG, SGI and SGBT tissue reduced gene expression and changed protein localization of TLR-2 from the cytoplasm to the nucleus might play a potential role in tumor development.

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