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The potential role of follicular helper T cells and helper T cells type 1 in Warthin tumour

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Abstract:	Warthin tumour (WT) is a benign tumour of the salivary gland that proliferates in both glandular epithelial and lymphoid tissue components, and rarely exhibits cystic changes. T follicular helper cells (Tfh) are involved in the formation and maintenance of germinal centres, the differentiation of B cells into plasma cells, and the maintenance of helper T cell type 2 (Th2)-dominant humoral immune responses. T-bet induces differentiation into helper T cell type 1 (Th1) by suppressing differentiation into Tfh and enhances cellular immune responses. The objective of this study was to enhance our understanding of the immune responses and relationship between Tfh and Th1 cells in patients with WTs. In this study, we classified WTs (n=64) into solid-type (n=25) and cyst-type (n=39). We also performed immunostaining of the Tfh markers CXCR5 and CD40L, and the Th1 marker T-bet for statistical analysis. The cyst-type exhibited significant atrophy of the germinal centre area (P =0.0019), significantly fewer Tfh-positive lymphocytes in germinal centres (P <0.0001), and significantly more T-bet-positive lymphocytes in the epithelium (P =0.0017). We observed that Tfh were involved in the formation and maintenance of lymphoid follicles in WTs. In the cyst-type, Th2-dominant humoral immune responses were suppressed, and Th1-dominant cellular immune responses may have caused damage to tumour tissue.
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Response to Reviewers:	Reviewer #1: This is an important and interesting research article that Tfh and Th1 cellular immune responses are important of forming Warthin tumor. Very exciting research works have been done. We believe that this article provides new insights into the understanding of the WT immune response. Thank you for your valuable comments.

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Des. 15th, 2020

Dear Dr. and Prof. Thomas Kirchner, Editor-in-Chief of Pathology Research and Practice

Editor-in-Chief of Pathology Research and Practice

,

Please find our enclosed Original Contribution, "**The potential role of follicular helper T cells and helper T cells type 1 in Warthin tumour**", which we would like to submit for consideration for publication in the ***Pathology Research and Practice***.

We do believe to write a new and valuable Original Contribution with a substantial merit for all Readers. This article has not been published elsewhere and is not under consideration by another journal. All Authors have approved the submission of the manuscript. Prior to this submission, a native English-speaking scientific writer checked our manuscript thoroughly for grammatical and scientific content. We hope that you will consider the present manuscript for publication in the ***Research and Practice***. We are looking forward to hearing from you at your earliest convenience. Thank you so much.

Respectfully yours,

Yoshiaki Kobayashi, MD,
Department of Head and Neck Surgery, Kanazawa Medical University, 1-1 Daigaku, Uchinada, Ishikawa 920-0293, Japan. Tel: 81-76-286-3511; Fax: 81-76-218-8440 and E-mail: kobadon@kanazawa-med.ac.jp

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Original Article

The potential role of follicular helper T cells and helper T cells type 1 in Warthin tumour

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Short running head: **Roles of Tfh and Th1 in Warthin tumour**

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Abstract

Warthin tumour (WT) is a benign tumour of the salivary gland that proliferates in both glandular epithelial and lymphoid tissue components, and rarely exhibits cystic changes. T

follicular helper cells (Tfh) are involved in the formation and maintenance of germinal centres, the differentiation of B cells into plasma cells, and the maintenance of helper T cell type 2 (Th2)-dominant humoral immune responses. T-bet induces differentiation into helper T cell type 1 (Th1) by suppressing differentiation into Tfh and enhances cellular immune responses. The objective of this study was to enhance our understanding of the immune responses and relationship between Tfh and Th1 cells in patients with WTs. In this study, we classified WTs (n=64) into solid-type (n=25) and cyst-type (n=39). We also performed immunostaining of the Tfh markers CXCR5 and CD40L, and the Th1 marker T-bet for statistical analysis. The cyst-type exhibited significant atrophy of the germinal centre area (P=0.0019), significantly fewer Tfh-positive lymphocytes in germinal centres (P<0.0001), and significantly more T-bet-positive lymphocytes in the epithelium (P=0.0017). We observed that Tfh were involved in the formation and maintenance of lymphoid follicles in WTs. In the cyst-type, Th2-dominant humoral immune responses were suppressed, and Th1-dominant cellular immune responses may have caused damage to tumour tissue.

Keywords: Warthin tumour; T Follicular helper cell; Helper T cell type 1; CXCR5; T-bet

1. Introduction

First reported by Warthin in 1929[1)], Warthin tumour (WT), also known as papillary cystadenoma lymphomatosum; is the second most common benign tumour of the salivary glands after pleomorphic adenoma and comprises 2-15% of all epithelial parotid gland tumours[2)].

While the histogenesis of WTs is still debated, the most favoured hypothesis is that they are derived from aberrant ductal epithelial cells in lymph nodes located inside and outside the

parotid gland. Other factors reported to be involved in tumorigenesis are Epstein-Barr virus infection, smoking, autoimmune disease, radiation exposure, and chronic inflammation[3-5].

Clinically, WTs present as slow-growing, soft tumours that are not painful on palpation; however, they can be painful when the tumour growth advances rapidly and uncontrollably. The treatment for WTs is surgical resection, their recurrence rate is ≤2%[6], and malignant transformation of the tumours is extremely rare[7].

Radiologically, WTs present as well-demarcated round masses with slightly heterogeneous contrast enhancement internally in computed tomography. In magnetic resonance imaging, they present as well-demarcated round masses but are heterogeneous internally. T1-weighted images show a mix of isointense and hypointense regions compared to the surrounding parotid gland tissue. T2-weighted images show hyperintensity, but also regions of hypointensity with different signal intensities[8]. WTs can form cysts of various shapes and localizations that exhibit a variety of image findings from simple thin-walled cysts to lobulated multilocular cysts. Internally, the cysts often exhibit hypointensity in T1-weighted images and hyperintensity in T2-weighted images, but when the protein concentration of the contents are high, T1-weighted images show hyperintensity and T2-weighted images show hypointensity[8].

Macroscopically, WTs present as encapsulated, well-demarcated masses. The cut surface is brown, with a mixture of solid and cystic areas. The cysts contain a turbid brown fluid like muddy water[9]. Histopathologically, WTs exhibit tubular and papillary proliferation of highly columnar cells with oxyphilic and granular cytoplasm, at times accompanied by squamous metaplasia and goblet cell metaplasia. Lymphoid follicles exhibiting hyperplastic changes proliferate in the tumour stroma, and plasma cells, Langerhans cells, and mast cells are found around the lymphoid follicles. WTs with cystic changes exhibit notable acute to chronic inflammatory cell infiltration, lymphoid tissue replaced by fibrous connective tissue, and less

conspicuous lymphoid follicle formation[10]. The cysts contain secretions from oxyphil and goblet cells, necrotic substances, cholesterin needle crystals, lymphocytes, neutrophils, macrophages, and other substances[10].

B cell lymphoma 6 (*BCL6*) and *T-bet* are master genes that induce differentiation of naïve T cells into T follicular helper cells (Tfh) and helper T cell type 1 (Th1). When BCL6 is expressed in the nuclei of Tfh, C-X-C motif chemokine receptor type 5 (CXCR5), inducible T-cell co-stimulator, CD40 ligand (CD40L), and programmed cell death protein-1 express on the surface of Tfh cell membranes, which promotes the formation and maintenance of germinal centres in lymphoid follicles and the differentiation of B cells[11]. Interleukin-21 produced by Tfh induces the differentiation of B cells into plasma cells and is involved in helper T cell type 2 (Th2)-dominant humoral immune responses[12]. T-bet is a transcription factor that inhibits differentiation into Tfh and promotes Th1-dominant cellular immune responses[13]. Therefore, it is believed the differentiation of Tfh and Th1 is controlled by the coexistence and balance of T-bet and Bcl6.

In 2019, Kurose et al.[14] demonstrated that in idiopathic multicentric Castleman disease, the area of the germinal centres of lymphoid follicles and the Tfh count are significantly lower than in reactive lymphadenopathy and suggested that controlling Th2-dominant immune responses could provoke Th1-dominant immune responses. This indicates that Tfh may be involved in the formation and maintenance of lymphoid follicles in WTs, and also that Th1-dominant immune responses may be occurring in cyst-type WTs with atrophied lymphoid follicles.

In this study, we classified WTs into solid-type and cyst-type, and performed immunostaining of the Tfh-specific markers CXCR5 and CD40L, and the Th1-specific marker T-bet to examine differences in the immune responses between the two.

2. Materials and methods

2.1. Patients and tissue specimens

We examined WT patients (n=64) who underwent surgery at the Department of Head and Neck Surgery at Kanazawa Medical University Hospital from 2000 to 2018. All patients were given information and provided written consent. The study was approved by the Kanazawa Medical University ethics committee. We obtained medical information on all patients that included their age at diagnosis, sex, tumour location, tumour diameter, imaging findings, presence or absence of recurrence, and presence or absence of postoperative complications from Kanazawa Medical University Hospital's electronic medical records system. All radiographic images were evaluated by three head and neck surgeons (YK, MK, and HT). When the maximum diameter of the hyperintensity region in T2-weighted images exceeded 50% of the maximum diameter of WT, it was defined as cystic-type WT.

All tissue sections were fixed with 10% neutral buffered formalin, embedded in paraffin, and stained with haematoxylin-eosin (H&E). To evaluate the total number of CXCR5-positive and CD40L-positive Tfh, and the number of CXCR5-positive and CD40L-positive Tfh per 0.01 mm^2 in germinal centres of WT, five high-magnification fields of view with the highest cell densities were selected, and the cells were counted using an optical microscope (Leica, Dmd108). Cells with stained cell membranes were assessed as positive for CXCR5 and CD40L. To evaluate the number of T-bet positive lymphocytes infiltrating the WT epithelium, five high-magnification fields of view with the highest cell densities were selected, and the cells were counted using under an optical microscope (Leica, Dmd108). Cells with stained nuclei were assessed as positive for T-bet. All histopathological sections were evaluated by pathologists (NK and AS) and a head and neck surgeon (YK). If there was some disagreement among the specialists, a final decision was made based on the opinion of a broad-certified

pathologist (SY).

2.2. Immunohistochemistry

The same automatic staining device was used for all immunostaining (BenchMark GX, Ventana Medical System, Tucson, AZ, USA). 3,3'-diaminobenzidine solution was used to visualize the antigen-antibody reactions. The Tfh-specific markers anti-human CXCR5 monoclonal antibodies (clone 51505, 1:100 dilution; R&D systems, Minneapolis, USA) and anti-human CD40L monoclonal antibodies (clone 66502-1-Ig, 1:500 dilution; Proteintech, Rosemont, USA) were used to carry out this study. The Th1-specific marker anti-human T-bet monoclonal antibodies (clone ab91109, 1:500 dilution; abcam) were also used. The antibodies were kept warm at 37 ºC for 30 min.

2.3. Statistical analysis

All statistical analyses were carried out with the PRISM software program ver. 6 (Graph Pad Software, La Jolla, CA, USA). The t-test was used to compare histopathological data between solid-type and cyst-type WTs. Results with $P<0.05$ were considered statistically significant.

3. Results

3.1. Patient characteristics

The 64 WTs were classified as solid-type (n=25) or cyst-type (n=39) based on radiographic images. The male-female ratios of patients with the solid- and cyst-type WTs were 22:3 and 26:13, respectively; the mean ages of patients were 63.8±6.7 years (range: 48-76 years) and 60.9±10.0 years (range: 44-83 years), respectively, which is not a significant difference. The mean diameters of the solid- and cyst-type WTs were 4.1±1.4 cm and 3.9±1.8 cm, respectively,

which is not a significant difference. The operation times of the solid- and cyst-type WTs were 149.6±49.7 min and 201.6±85.8 min, respectively, being significantly longer for cyst-type WTs (P=0.0066). For postoperative complications, the most common was transient facial palsy, at four cases (16%) in the solid group and eight cases (25%) in the cyst group. Frey syndrome (n=1, 3.1%) and hematoma (n=1, 3.1%) were also seen in the cyst group. Recurrence occurred in one case in each group; in the solid and cyst groups, it was 6 years and 2 years 9 months postoperatively, respectively (Table 1).

3.2. Radiological features of WTs

All 64 WTs were located on the inferior pole of the parotid gland. The solid-type WT exhibited hypointensity with distinct boundaries that formed tumorous lesions with smooth margins in T1-weighted images, and heterogeneous hyperintensity in T2-weighted images (Fig. 1A). Similar to the solid-type, the cyst-type WT exhibited heterogeneous hyperintensity in T2-weighted images and hypointensity in T1-weighted images, but in some areas, the boundary between the tumour and surrounding tissue was indistinct (Fig. 1B).

3.3. Detailed histopathological and immunohistochemical features of WTs

Histologically, solid-type WTs (n=25) exhibited equal proliferation of both glandular epithelial components and the lymphoid tissue components, though glandular epithelial components were conspicuous in some cases and lymphoid tissue components in others. Glandular epithelial components were accompanied by tubular and papillary arrangements (Fig. 2A). The cytoplasm was highly columnar and contained acidophilic cytoplasmic granules. The lymphoid tissue components consisted of small T lymphocytes without atypia and lymphoid follicles exhibiting hyperplastic changes (Fig. 2B). Almost no lymphocyte infiltration was

observed in the glandular epithelial cells (Fig. 2C). Mucus and acidophilic secretions were accumulated inside the glandular cavity, in some cases along with bleeding (n=13, 52%), purulent inflammation (n=1, 4%), collections of foam cells (n=3, 13%), and deposition of cholesterin needle crystals (n=12, 48%). Partial fibrosis (n=9, 36%), granulation tissue proliferation (n=4, 16%), epithelioid granuloma formation (n=1, 4%), and squamous metaplasia (n=1, 4%) were observed. Fibrosis was observed between the parotid and adipose tissue around the tumour capsule (n=8, 32%).

Histologically, the cyst-type WT (n=39) had proliferation of both glandular epithelial and lymphoid tissue components, with the glandular epithelial components exhibiting marked cystic dilation (Fig. 2D). The lymphoid tissue components consisted of small T lymphocytes without atypia and lymphoid follicles exhibiting atrophic to hyperplastic changes (Fig. 2E). A large amount of lymphocyte infiltration was observed in the glandular epithelial cells (Fig. 2F). The cysts contained large amounts of mucus and acidophilic secretions, along with bleeding (n=22, 56.4%), purulent inflammation (n=13, 33.3%), collections of foam cells (n=15, 38.4%), and deposition of cholesterin needle crystals (n=15, 38.4%). Partial fibrosis (n=21, 53.8%), granulation tissue proliferation (n=12, 30.7%), epithelioid granuloma formation (n=1, 2.5%), and squamous metaplasia (n=4, 10.2%) were observed. Fibrosis was observed between the parotid and adipose tissue around the tumour capsule (n=19, 48.7%).

3.4. The sizes of areas of germinal centres

Cyst-type WTs had significantly smaller germinal centre area than solid-type WTs (0.04±0.01 vs. 0.03±0.01 mm^2, P=0.0019).

3.5. Numbers of Tfh in the germinal centres

Cyst-type WTs had significantly fewer CXCR5-positive Tfh in the germinal centres than solid-type WTs (59±36.1 vs. 20.7±16.2, $P<0.0001$) (Fig. 3A-3B, Table 2). Cyst-type WTs had significantly fewer CD40L-positive Tfh in the germinal centres than solid-type WTs (131.2±94.3 vs. 33.0±23.6, $P<0.0001$) (Fig. 3C-3D, Table 3).

3.6. Number of Tfh per 0.01 mm^2 in the germinal centres

Per 0.01 mm^2, the solid-type WT had significantly fewer CXCR5-positive Tfh in the germinal centres than the cyst-type WT (120.4±43.3 vs. 87.1±17.6, $P=0.0095$) (Table 2). Per 0.01 mm^2, the solid-type WT had significantly fewer CDL40-positive Tfh in the germinal centres than the cyst-type WT (291±58.2 vs. 173.4±40.2, $P<0.001$) (Table 3).

3.7 The numbers of Th1 in the epithelial cells

The cyst-type WT had significantly more T-bet positive cells in epithelial cells than the solid-type WT (10.9±11.9 vs. 21.2±10.3, $P=0.00179$) (Fig. 3E-3F, Table 4).

4. Discussion

To the best of our knowledge, there are no studies discussing the relationship of Tfh and Th1 in WT. The present study provides new findings on some of the immune responses seen in patients with WTs.

In solid-type WTs, Bcl6 expression and suppressed T-bet expression is thought to have promoted the differentiation of naïve T cells to Tfh. The expression of CXCR5 and CD40L on the surface of Tfh cell membranes encouraged the formation and maintenance of germinal centres in lymphoid follicles, and the differentiation of B cells, which is believed to have caused a Th2-dominant humoral immune response. In cyst-type WTs, in contrast, T-bet expression and

suppressed Bcl6 expression is thought to have suppressed the differentiation of Naïve T cells to Tfh, making it impossible for germinal centres to form or be maintained, resulting in germinal centre atrophy. In addition, this provoked a Th1-dominant cellular immune response, which is believed to have caused epithelial cell injury (Fig. 4).

C-C motif ligand 28 (CCL28) is expressed in epithelial cells in the mammary glands, salivary glands, small intestine, and large intestine, and is known as a chemotactic factor of CD4/CD8 T cells. Ogawa et al.[15] showed there to be a marked increase in CCL28 expression in intestinal epithelial cells in patients with ulcerative colitis or Crohn's disease. This may explain why cyst-type WTs with conspicuous lymphocyte infiltration in the WT epithelium had higher *CCL28* mRNA expression and tissue damage to the tumour than solid-type WTs.

Cysts arise from a wide range of factors, including tumours, hereditary diseases, prenatal developmental abnormalities, chronic inflammation, fluid retention, parasites, trauma, and blood vessel failure. If the cause of cystic changes in WTs is extensive infarction associated with circulatory disturbance[16], and if secretions from oxyphils or goblet cells, or degenerated or necrotic epithelial cells accumulate in tumour tissue, it can create chronic inflammation that damages tumour tissue.

In this study, we found that Th2-dominant humoral immune responses were suppressed and Th1-dominant cell-mediated immune responses were provoked in cyst-type WTs compared to solid-type WTs, which may have resulted in damage to tumour tissue. However, because cyst-type WTs exhibited more varied inflammatory responses than solid-type WTs, it is debatable whether Th1-dominant immune responses alone can explain everything about cyst-type WTs. Aga et al.[17] reported a large number of IgG4-positive plasma cells in the lymphoid follicles of WT and observed a marked Th2-dominant humoral immune response in WT cases. In addition, Yigit et al.[18] examined the content of cysts in primary ovarian mucinous

cystadenoma and mucinous cystic adenocarcinoma and found high concentrations of both Th1 cytokines such as IL-12 and interferon-gamma (IFN-γ) and Th2 cytokines such as IL-4 and IL-5. This indicates that cyst formation may be caused by the interaction between Th1-dominant cellular immune responses and Th2-dominant humoral immune responses. We believe it is necessary to examine the liquid contents of WT cysts and to measure cytokine levels.

In a study of complications in 108 parotid gland WT cases that underwent resection, Chulam et al.[19] observed facial neuropathy (n=51, 47.2%) and Frey syndrome (n=19, 17.6%). In our comparison of solid-type and cyst-type WTs, the operations were significantly longer and postoperative complications were more common in the cyst-type WTs. In the cyst-type WT, there was notable fibrosis and inflammatory cell infiltration, including from lymphocytes, histiocytes, and neutrophils, which may have created strong adhesion to the para-parotid tissue and adipose tissue, making dissection difficult. When operating on cyst-type WTs, great care should be taken when detaching the tumour and potential postoperative complications should be carefully considered.

Conclusion

Tfh were found to be involved in the formation and maintenance of lymphoid follicles in WT. In cyst-type WTs, Th2-dominant humoral immune responses were suppressed and Th1-dominant cellular immune responses may have caused damage to tumour tissue. In the future, more WT cases need to be examined to obtain deeper insights into the immune responses associated with WTs.

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Kanazawa Medical University for their technical guidance in the pathological and immunohistochemical analyses.

CONFLICTS OF INTEREST

The authors declare no conflicts of interest in association with the present study.

AUTHOR CONTRIBUTION

NK, XG, AS and SY participated in the conception of the study and the writing of the manuscript. YK, MK and HT performed clinical imaging and/or the pathological/ immunohistochemical analyses of the lesion specimens. All of the authors have read and approved the final manuscript.

References

1) Warthin AS. Papillary cystadenoma lymphomatosum. rare teratoid of the parotid region**.** J Cancer Res. 1929; 13:116-125.

2) Nishikawa H, Kirkham N, Hogbin BM. Synchronous extra-parotid Warthin's tumour. J Laryngol Otol. 1989; 103:792-793.

3) Gallo O, Bocciolini C. Warthin's tumour associated with autoimmune diseases and tobacco use. Acta Otolaryngol. 1997; 117:623-627.

4) Cennamo A, Falsetto A, Gallo G, Lanna M, Calleri G, Di Giacomo D. Warthin's tumour in the

parotid gland (an inflammatory or a neoplastic disease?). Chir Ital. 2000; 52:361-367.

5) Kristensen S, Tveterås K, Friedmann I, Thomsen P. Nasopharyngeal Warthin's tumour: a metaplastic lesion. J Laryngol Otol. 1989; 103:616-619.

6) Eveson JW, Cawson RA. Salivary gland tumours. A review of 2410 cases with particular reference to histological types, site, age and sex distribution. J Pathol. 1985; 146:51-58.

7) Smółka W, Markowski J, Piotrowska-Seweryn A, Paleń P, Dobrosz Z, Dziubdziela W. Mucoepidermoid carcinoma in Warthin tumour of the parotid gland. Arch Med Sci. 2015; 19:691-695.

8) Ikeda M , Motoori K, Hanazawa T, Nagai Y, Yamamoto S, Ueda T, Funatsu H, Ito H. Warthin tumour of the parotid gland: diagnostic value of MR imaging with histopathologic correlation. AJNR Am J Neuroradiol. 2004; 25:1256-1262.

9) Gary LE, Paul LA. AFIP Atlas of Tumor Pathology: Tumors of the Salvary Glands. Washington, DC, USA: ARP Press; 2008. p.85-100.

10) Morinaga S. Atlas of Salivary Gland Tumors. Tokyo, Japan: Kanahara-shuppan Press; 2005. p.64-69.

11) Gensous N, Charrier M, Duluc D, Contin-Bordes C, Truchetet ME, Lazaro E, Duffau P, Blanco P, Richez C. T Follicular helper cells in autoimmune disorders. Front Immunol. 2018;

17:1637.

12) Bryant VL, Ma CS, Avery DT, Li Y, Good KL, Corcoran LM, Rene WM, Stuart GT. Cytokine- mediated regulation of human B cell differentiation into Ig-secreting cells: predominant role of IL-21 produced by CXCR5+ T follicular helper cells. J Immunol. 2007; 179:8180–8190.

13) Nakayamada S, Kanno Y, Takahashi H, Jankovic D, Lu KT, Johnson TA, Sun H, Vahedi G, Hakim O, Handon R, Schwartzberg PL, Hager GL, O'Shea JJ. Early Th1 cell differentiation is marked by a Tfh cell-like transition. Immunity. 2011; 35, 919-931.

14) Kurose N, Guo X, Shioya A, Mizutani K, Kumagai M, Fujimoto S, Kawabata H, Masaki Y, Takai K, Aoki S, Nakamura S, Yamada S. The potential role of follicular helper T cells in idiopathic multicentric Castleman disease with and without TAFRO syndrome. Pathol Res Pract. 2019; 215, 152563.

15) Ogawa H, Iimura M, Eckmann L, Kagnoff MF. Regulated production of the chemokine CCL28 in human colon epithelium. Am J Physiol Gastrointest Liver Physiol. 2004; 287: G1062-9.

16) Yorita K, Nakagawa H, Miyazaki K, Fukuda J, Ito S, Kosai M. Infarcted Warthin tumour with mucoepidermoid carcinoma-like metaplasia: a case report and review of the literature. J Med Case Rep. 2019; 13, 12.

17) Aga M, Kondo S, Yamada K, Wakisaka N, Yagi-Nakanishi S, Tsuji A, Endo K, Murono S, Ito M, Muramatsu M, Kawano M, Yoshizaki T. Immunoglobulin class switching to IgG4 in Warthin tumour and analysis of serum IgG4 levels and IgG4-positive plasma cells in the tumour. Hum Pathol. 2014; 45, 793-801.

18) Yigit R, Massuger LFAG, Zusterzeel PLM, Pots J, Figdor CG, Torensma R. Cytokine profiles in cyst fluids from ovarian tumors reflect immunosuppressive state of the tumour. Int J Gynecol Cancer. 2011; 21, 1241-1247.

19) Chulam TC, Francisco ALN, Filho JG, Alves CAP, Kowalski LP. Warthin's tumour of the parotid gland: our experience. Acta Otorhinolaryngol Ital. 2013; 33, 393-397.

Figure Legends

Figure 1. Radiological features of solid-type and cyst-type of WTs.

A: Solid-type WT MRI image (T2-weighted image). A well-demarcated, solid mass with partial heterogeneous hyperintensity (arrow).

B: Cyst-type WT MRI image (T2-weighted image). A well-demarcated, solid mass with cystic cavities with indistinct boundaries internally (arrow).

Figure 2. Histological features of solid-type and cyst-type of WTs.

A: Low magnification of a solid-type WT. The epithelial cells exhibit tubular and papillary proliferation with conspicuous lymphoid follicle formation in the stroma (HE, x4).

B: High magnification of a solid-type WT. The germinal centres of the lymphoid follicles show hyperplastic changes (HE, x20).

C: High magnification of a solid-type WT. No lymphocyte infiltration is seen in the epithelial cells (HE, x40).

D: Low magnification of a cyst-type WT. The epithelial cells exhibit cystic dilation but without notable lymphoid follicle formation (HE, x4).

E: High magnification of a cyst-type WT. The germinal centres of the lymphoid follicles are atrophied (HE, x20).

F: High magnification of a cyst-type WT. Conspicuous lymphocyte infiltration in the epithelial cells (HE, x40).

Figure 3. Immunohistochemical features of solid-type and cyst-type WTs at high magnification (x20).

A: Solid-type WT. Numerous CXCR5-positive Tfh are observed inside germinal centres exhibiting hyperplastic changes (CXCR5).

B: Cyst-type WT. A few CXCR5-positive Tfh are seen inside atrophied germinal centres (CXCR5).

C: Solid-type WT. Numerous CD40L-positive Tfh are seen inside germinal centres exhibiting hyperplastic changes (CD40L).

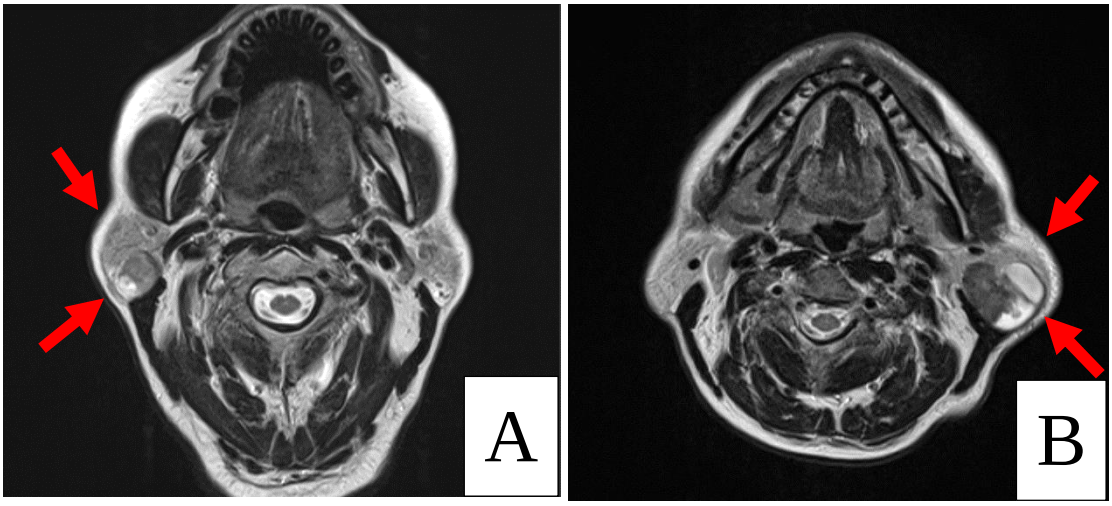
D: Cyst-type WT. A few CD40L-positive Tfh are seen inside atrophied germinal centres (CD40L).

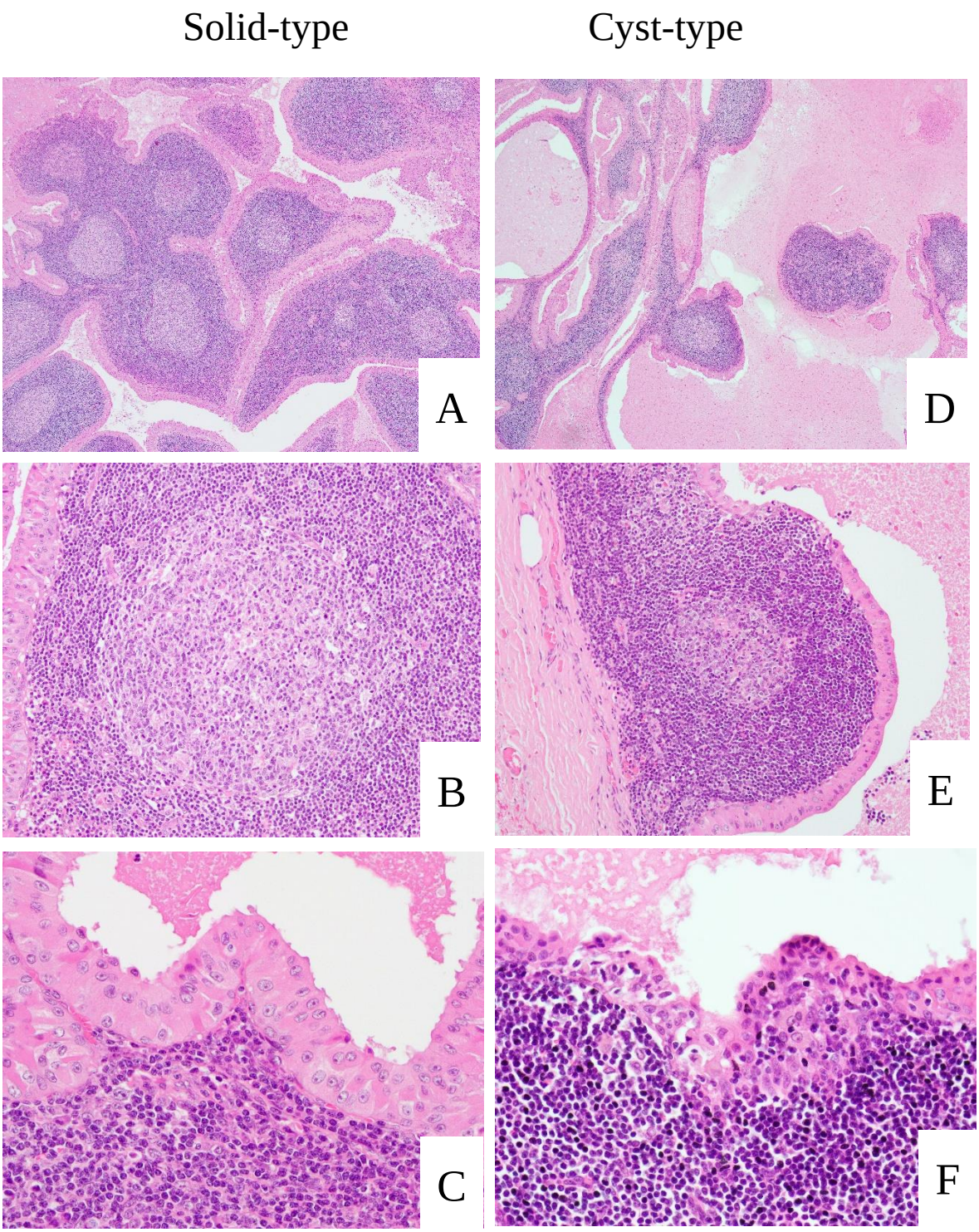
E: Solid-type WT. No T-bet-positive lymphocytes are seen in the epithelial cells (T-bet).

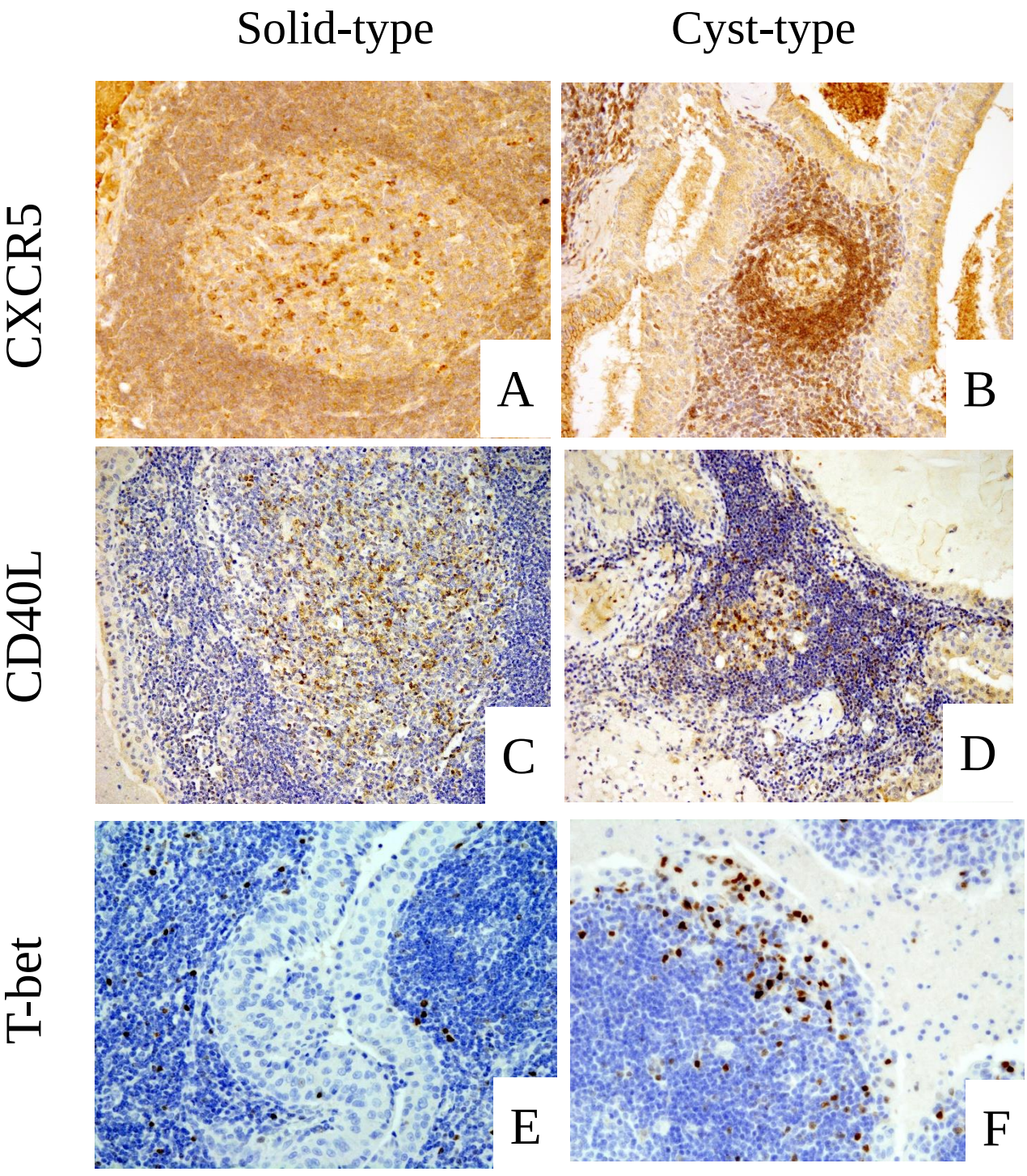
F: Cyst-type. Numerous T-bet-positive lymphocytes are seen in the epithelial cells (T-bet).

Figure 4. A schematic model of the effects of solid-type and cyst-type WTs on immune responses.

In the solid-type WT, Bcl6 expression was accompanied by differentiation into Tfh and suppression of differentiation into Th1. The lymphoid follicle germinal centres exhibit hyperplastic changes, which cause the differentiation of B cells into plasma cells and activates humoral immunity. CXCR5 and CD40L expression is observed on the surface of Tfh cell membranes. No infiltration of Th1 lymphocytes is observed in epithelial cells. In the cyst-type WT, T-bet expression is accompanied by differentiation into Th1 and suppression of differentiation into Tfh. The lymphoid follicle germinal centres exhibit atrophic changes, which suppress the differentiation of B cells into plasma cells and activates cellular immunity. There is conspicuous infiltration of Th1 lymphocytes in epithelial cells.







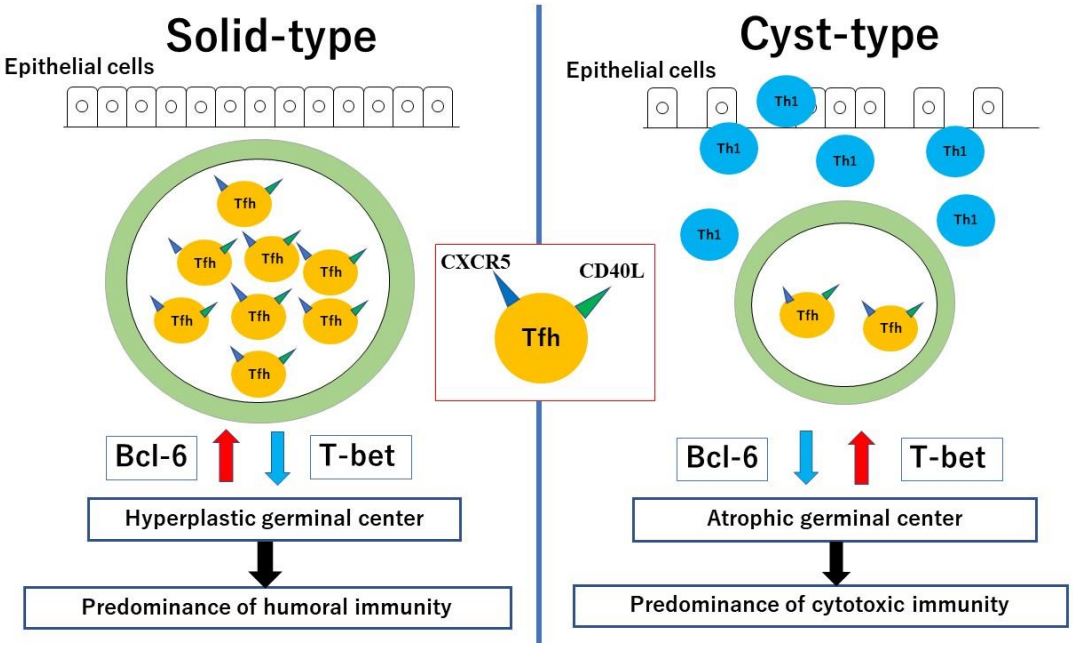


Fig.4

Table 1. Clinical patient data

	Solid-type (n = 25)
M : F	22 : 3
Age（years）	63.8±6.7
Tumor size (cm)	4.1±1.4
Operation time (minute)	149.6±49.7
Post-operative complication	4 (16.0%)
Transient facial nerve paralysis	4 (16.0%)
Frey syndrome	0 (0.0%)
Hematoma	0 (0.0%)
Recurrence	1 (4.0%)

Abbreviations: n, number; M, Male; F, Female; *,$p<0.05$;

Cyst-type (n = 39)
26 : 13
60.9±10.0
3.9±1.8
201.6±85.8*
8 (25.0%)
7 (21.9%)
1 (3.1%)
1 (3.1%)
1 (4.0%)

Table 2. Histopathological findings of $CXCR5^{+}$-Tfh

	Solid-type (n = 27)
Number of Tfh in GC	59.0 ± 36.1
Number of Tfh in GC per 0.01 mm^2	120.4 ± 43.3

Abbreviations: $CXCR5^{+}$, C-X-C chemokine receptor type 5-positive; Tfh, T
n, number; GC, germinal center; *, $p<0.05$; ****, $p<0.0001$;

Cyst-type (n = 31)
20.7 ± 16.2****
87.1 ± 17.6*

follicular helper;

Table 3. Histopathological findings of $CD40L^+$-Tfh

	Solid-type (n = 25)
Number of Tfh in GC	131.2 ± 94.3
Number of Tfh in GC per 0.01 mm^2	291.0 ± 58.2

Abbreviations: $CD40L^+$, CD40 ligand-positive; Tfh, T follicular helper; n, number; GC, germinal center; *, $p<0.05$; ****, $p<0.0001$;

Cyst-type (n = 34)
33.0 ± 23.6****
173.4 ± 40.2*

Table 4. Histopathological findings of T-bet^{+}-lymphocyte

	Solid-type (n = 22)
Number of T-bet^{+}-lymphocyte in epithelial c	10.9 ± 11.9

Abbreviations: n, number; *, $p<0.05$;

Cyst-type (n = 30)
21.2 ± 10.3*

CONFLICTS OF INTEREST

The authors declare no conflicts of interest in association with the present study.

AUTHOR STATEMENT

NK, XG, AS and SY participated in the conception of the study and the writing of the manuscript. YK, MK and HT performed clinical imaging and/or the pathological/ immunohistochemical analyses of the lesion specimens. All of the authors have read and approved the final manuscript.