The relationship between helper T cell subtype expression and clinicopathologic manifestation of papillary thyroid carcinoma with lymphocytic thyroiditis

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The relationship between helper T cell subtype expression and clinicopathologic manifestation of papillary thyroid carcinoma with lymphocytic thyroiditis

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ABSTRACT

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In South Korea, the incidence of thyroid cancer is one of highly occurring cancer. Out of thyroid cancers, papillary thyroid carcinoma (PTC) is the highest. PTCs were frequently accompanied by lymphocytic thyroiditis (LT). Some articles reported Hashimoto's thyroiditis (HT; extreme form of LT) increased occurrence of PTC. In contrast, others reported LT had an anti-tumor activity, which leaded to a good prognosis.

Concerning two conflicting results, this study was performed to unravel how LT was operated in cancer immunity.

Two subtypes of helper T cell (Th) cytokines were used; Th1 (IFN-γ, TNF-α, IL-2) and Th2 (IL-4, IL-10, IL-1β). The cytokine levels were measured by quantitative RT-PCR method using non-tumor tissue. Because most PTC cases represented microcarcinomas, p27 immunohistochemical marker was used as a surrogate marker for lymph nodal metastasis.

This study revealed that most PTCs have mixed Th1 (IFN-γ, TNF-α, IL-2) and Th2 (IL-4, IL-10, & IL-1β) immunity. Cytokines were always expressed more in the cases with LT than those without LT. When statistically analysis was confined to the PTC cases with LT, the cases with lower levels of cytokines had a tendency to be related to low grade expression of p27. Considering under-expression of p27 as being closely associated with lymph nodal metastasis, this result implicated that lower levels of cytokines were

frequently found in the cases with nodal metastasis. In other words, high expression of cytokines might play a role in inhibiting lymph nodal metastasis. The cases with extrathyroidal tumor extension had a tendency to show the higher levels of cytokine expression than those with intrathyroidal tumor confinement. Although this result could not determine whether it implicated an anticancer effect or not, there was a tendency to the increased level of cytokines in the cases with extrathyroidal tumor extension.

This result implicated that mixed Th1 and Th2 cytokines might have an anticancer effect, only in terms of lymph nodal metastasis.

Key words: Papillary thyroid carcinoma, Helper T cell, Cytokine, p27, Prognosis, Metastasis, Extrathyroidal extension, Lymphocytic thyroiditis, interleukin, interferon, TNF

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I. INTRODUCTION

The annual incidences of thyroid cancer are variable among countries in the world; for males, 1.2-2.6 per 100,000 and for females, 2.0-3.8 per 100,000. The groups of higher incidence are Japan, Unites states, Sweden, and France[1, 2]. In South Korea, the incidence of thyroid cancer is 0.88 per 100,000 in males and 6.68 per 100,000 in females. Out of thyroid cancers, papillary carcinoma is the highest (83.8%)[3].

Papillary thyroid carcinoma (PTC) is the most frequent among malignant thyroid tumors[3]. Hence, researches on the etiology, prognosis, and pathophysiology of PTC have been investigated extensively. Recent studies have focused on molecular pathology (e.g. E-cadherin, c-Met, and EGF-R) and genetic alteration (e.g. RET/PTC, TRK, BRAF, and p53)[4]. Most PTC cases go for indolent courses, but seldom behave aggressively in a way of frequent recurrences or metastases[5]. Shibru et al maintained that patients over age 45 with the expression of COX-2 and VEGF-C had more aggressive behavior of PTC[6]. Besides above, many researches for prognostic factors of PTC have been tried. Recently, Shibru et al found the BRAF mutations were significantly associated with aggressive behavior of thyroid cancer[7].

There have been many attempts to develop the therapeutic vaccines for cancer[8]. Contrary to many researches like these on cancer immunotherapy, researches on

the role of immunity in PTC biologic behavior have not been sufficient.

There were several reports that immunity of PTCs could influence biologic behavior. As an evidence to support this idea, there was an article about the good prognosis of PTC in association with Hashimoto's thyroiditis or concomitant lymphocytic thyroiditis[9, 10]. Traditionally, it has been reported that Th1-immunity suppresses and Th2-immunity supports for tumor growth (Th = helper T cell). However, even Th2-immunity can help the anti-tumor activity [11].

Th1 immunity induces cytotoxic CD8+ T lymphocytes (CTLs). Although CTLs can acquire anti-tumor immunity, some tumor cells escape CTL immune surveillance and survive. Hence, from a practical view, Th1 predominance in itself does not represent anti-tumor immunity. Th2 immunity provokes humoral immune reaction, e.g. antibody synthesizing immunity. A part of Th2 immunity, such as B cells and IL-10 makes a favorable situation for tumor growth. In contrast, tumor-infiltrating granulocytes-linked Th2 immunity promotes anti-tumor growth[11].

Concerning Th immunity of PTC, there were a few reports. As one of them, Mardente et al reported that a Th cytokine pattern of peripheral blood in the PTC with chronic lymphocytic thyroiditis had predominant Th2 immune reaction or mixed cell response[12]. Intrathyroidal lymphocytes in Hashimoto's thyroiditis are composed of both B cells and T cells, the majority of which are CD8+ T cells cytotoxic against thyroid follicle cells[13]. PTCs are often associated with chronic lymphocytic thyroiditis and Hashimoto's thyroiditis. Shull et al reported that diffuse lymphocytic thyroiditis was associated with papillary carcinoma without therapeutic histories[14]. Mauras and co-workers reported that three cases of thyroid cancer with Hashimoto's thyroiditis did not have recurrent disease after thyroidectomy[15].

Therefore, it is inappropriate to judge which Th subtype immunity has more superior anti-tumor activity than the other.

With above complex immune responses, analysis of Th1/Th2 interrelationship is more reasonable to grasp the essence of what real tumor immunities react in PTCs.

There were several clinical trials to induce cytotoxic immunity against thyroid cancers. Amino et al used saline homogenates of thyroid tumors and Lo Gerfo et al applied chemically altered thyroglobulins. But clinical efficacy has not been proved yet[16,17].

As aforementioned, up to now, the significance of lymphocytic infiltration in PTC has not been clear whether it contributes to inhibiting or promoting tumor growth. Some purported that lymphocytic infiltration was an anticancer immune reaction[14] and others argued that it happened de novo. With these reasons, to establish immunotherapy for thyroid cancer, disclosure of tumor immune entity should be sine qua non.

Recently with early detection of PTC, it was difficult to procure the cases of advanced tumor stage. To solve this problem, we adopted the immunohistochemical marker, p27 as a surrogate marker, to represent the possibility of lymph nodal metastasis[18].

In this research, we analyzed the pattern of Th immunity and investigated its relationship to the clinicopathologic manifestation of PTC.

II. MATERIALS AND METHODS

1. Cases and materials

After institutional board review approval (Protocol No. 08-0194), 23 patients' fresh surgical samples of thyroid papillary carcinoma with (13 cases) and without (10 cases) lymphocytic thyroiditis were enrolled with following study inclusion. The thyroid tissues were sampled separately as PTC portion and non-tumor portion in fresh state. These samples were collected in records of department of pathology in GangNam Severance Hospital with patient's agreement in the formal permission of local IRB. Non-tumor portions were used for measuring quantity of cytokine (TNF-α, IFN-γ, IL-2, IL-4, IL-10, and IL-1β) mRNA. The PTC portions were used for immunohistochemical staining for p21/kip1 (p27).

All cases included in this research were 23. The ages of 23 cases ranged from

35 from 59 years old (mean age 47).

13 cases with PTC and lymphocytic thyroiditis (LT) and 10 cases of PTC without LT were collected. The subtypes of PTC were most conventional type in 21 cases and those of only 2 cases showed follicular variant.

2. Measurement of cytokines

Non-tumor portions were used for measuring quantity of cytokine (TNF-α, IFN-γ, IL-2, IL-4, IL-10, and IL-1β) mRNA. 13 cases with PTC and lymphocytic thyroiditis (LT) were the objective group and 10 cases of PTC without LT were used as the reference group.

Quantitative RT-PCR was performed according to manual of LightCycler® 480 Real-Time PCR System[19].

A. RNA isolation

Total RNA was isolated using the High Pure RNA Tissue kit (Roche).

The acquired tissue samples (1 ~ 10 mg) were grounded by pestle instrument with adding lysis/binding buffer 400㎕. Those were sent for centrifugation at 13,000 rpm for 2 minutes. Supernatant fluid was picked up and kept in another Eppendorf tube with adding absolute ethanol 200㎕ and mixed well. This fluid was transferred into High Pure Filter tube and under centrifugation at 13,000g for 30 seconds. The filtered fluid was mixed with both DNase incubation buffer 90㎕ and DNase I working solution 10㎕ in Eppendorf tube. This mixture was transferred into filter tube and incubated at room temperature for 15 minutes. This filter tube was inserted and suspended at the entrance of Eppendorf tube. After adding 500㎕ of Wash buffer I into filter tube, it was sent for centrifugation at 8000 g for 15 seconds. Sediment fluid within Eppendorf tube was discarded. As the same way as follows; after adding 500㎕of Wash buffer II, centrifugation at 8,000 g for 15 seconds, sediment fluid within Eppendorf tube was discarded, and after adding 300㎕ of Wash buffer II, centrifugation at 13,000 g for 2 minutes, sediment fluid within Eppendorf tube was discarded.

This filter tube was transferred and inserted into the new Eppendorf tube. 90㎕ of Elution buffer was added and for centrifugation at 8000g for 1 minute. The filtered fluid within Eppendorf tube below filter tube contained extracted RNA material. Quantification of total RNA was performed by Nanodrop (ND-1000).

B. Reverse Transcription

700ng of total RNA from tissues was reverse transcribed with cDNA Synthesis kit(Roche) according to the manufacturer's recommendation.

9.4㎕ of the extracted RNA fluid was mixed with 2㎕ of random hexamer primer, resulting in total 11.4㎕. This fluid was incubated at 65℃ for 10 minutes and processed for denaturation. And then this fluid (11.4㎕) was added with 8.6㎕ of Transcriptor High Fidelity cDNA synthesis kit and incubated at 50℃ for 30 minutes, resulting in 20㎕ mixture. This mixture was temporarily kept in ice box and incubated at 85℃ for 5 minutes. This process resulted in 20 ㎕ of cDNA. Reverse transcribed cDNA was diluted 1:3 with H_2O to use in qPCR.

C. Real-time PCR

Amplification with Real ready custom panel 96(Roche) was performed according to the manufacturers recommendation.

20㎕ of cDNA fluid was mixed with 260㎕ of Probe master and 240㎕ of DW. Real-time Ready Custom Panel was designed by LightCycler 480 Real-Time PCR System (Roche Applied Science), for the target cytokine markers (TNF-α, IFN-γ, IL-2, IL-4, IL-10, and IL-1β). LightCycler 480 Real-Time System (Roche) was used to measure relative quantification: e.g. the ratio of target mRNA quantity to reference mRNA quantity.

Both target gene mRNA quantity and reference gene mRNA quantity were corrected by PCR cycle number (Cp value) by computer software. Because mRNA quantities of other target cytokines were divided by the same reference gene mRNA quantity, each value of target cytokines could be used to compare

those of other target genes, quantitatively. The used reference gene was actin. The real time qPCR was run on LC480 II (Roche). The thermal cycling conditions were followed by 45 cycles of 95℃ for 10 seconds, 60℃ for 30 seconds, and 72℃ for 1 second.

D. Interpretation of data

The reference gene value (house-keeping gene) and IL-1β value were always measured. When some cytokine values were sparse but could be measured, the values were written, such as 0.00. There were cytokine values with not-valid. This meant that despite many PCR amplification processes, reference gene was calculated, but target gene could not be detected. So we manipulated these not-valid values as zero (0) value.

Although it had been known to be meaningless to compare the rank of cytokine value in the same specimen, the expressed cytokine levels could be ranked and compared in this research via a quantitative RT-PCR method. Using the concept of relative quantification, the cytokine values were corrected for differences in quality and quantity by dividing the concentration of a target RNA by the concentration of a reference RNA in the same sample (relative ratio = concentration of target/concentration of reference)[20]. The most common way to compare expression levels of different samples is to designate one of the samples as calibrator. All other samples are compared to the calibrator. For normalization of the final results, the target/reference ratio of each sample s divided by the target/reference ratio of the calibrator sample:

Calibrator normalized ratio = (sample; concentration of target/concentration of reference)/(calibrator; concentration of target/concentration of reference)[20].

In this research, we applied the constantly expressed cytokine, IL-1β as a calibrator.

3. Immunohistochemistry

A. Procedures of immunohistochemical stain

The immunohistochemical stain for p27 protein was done in PTC tumor tissue of 23 cases.

Paraffin-embedded tissue was analyzed by immunohistochemical stain for p27 expression. Formalin-fixed paraffin-embedded sections (3μm thick) were dewaxed in xylene and rehydrated through graded alcohols to water. Endogenous peroxidase activity was blocked in 3% hydrogen peroxide. Antigen retrieval was performed in citrate buffer (pH 6.0) within a microwave pressure cooker, and endogenous biotin detection was blocked with the Avidin-Biotin blocking kit (Vector Laboratories, Inc., Burlingame, CA).

Optimum primary antibody dilutions were predetermined, and appropriate positive control samples (tissues known to be positive for the immunohistochemical marker) and negative control samples (test tissue sections without the addition of primary antibody) were used for p27 (Novocastra, Newcastle Upon Tyne, UK). Primary antibody incubation was performed at 1:200 dilutions for 1 hour. After then, slide was washed in PBS, and secondary incubation was carried out with Biotin anti-mouse/anti-rabbit IgG followed by Streptavidin-HRP (Signet Pathology System, Dedham, Massachusetts) for 30 minutes. Immunoreactivity was revealed by incubation in 3-amino-9-ethylcarbazol. Slides was counterstained in Hematoxylin and mounted with Balsam.

B. Interpretations of immunohistochemical stain

When p27 immunoreactivity was interpreted, only nuclear staining on PTC cells was regarded as positive. Its measurement was evaluated in two tiered grade system: low grade (negative staining or faint staining on high power field) and high grade (positive staining, easily detectable on lower power field) (Figure).

Based on published literature, the marker was categorized by two tiered grade: low or high[21]. Nuclear p27 was considered altered when they were less than 30% based on previously published clinically relevant levels.

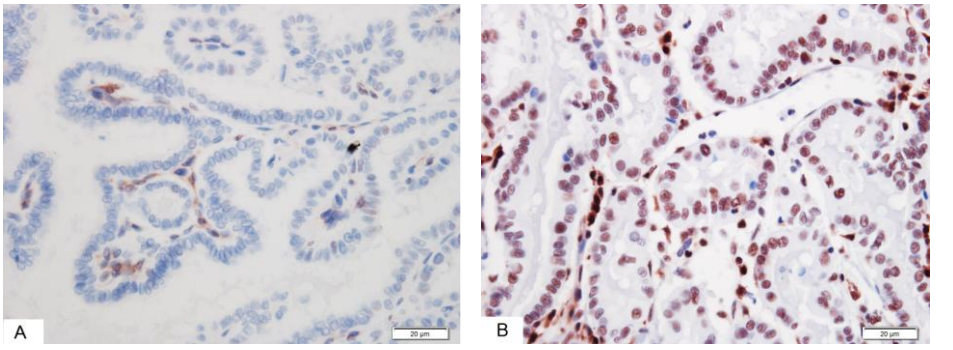

Figure 1. Immunohistochemical stain results of p27. A. Negative immunohistochemistry result of p27. The nuclei of papillary thyroid carcinoma cells were not stained for p27. This grade of nuclear staining could be classified as 'low-grade'. Under-expression of p27 was known to be associated with lymph nodal metastasis. B. Positive immunohistochemistry result of p27. The nuclei of papillary thyroid carcinoma cells were stained for p27 with brown-red color. This grade of nuclear staining could be classified as 'high-grade' (p27 immunohistochemistry, X400).

Immunoreactivity to p27 was measured. In 21 cases, the immunohistochemical stain (IHC) results were summarized in two groups; the cases with lymphocytic thyroiditis (LT) (n = 13) and the cases without LT (n = 8). Two cases were not performed, for the IHC sections for p27 were devoid of tumor tissue.

4. Statistical analysis

The Mann Whitney U test was performed to examine the relationships of cytokines (TNF-α, IFN-γ, IL-2, IL-4, IL-10, and IL-1β), p27 expression with clinical and pathologic characteristics (Table 5, 7, 8). The Fisher's Exact test was performed to examine the relationship between p27 expression and lymph nodal metastasis (Table 6). All reported *p* values were 2-sided, and significance was set at 0.05. All statistical tests were performed with SPSS, version 17.0 (SPSS, Chicago, IL).

III. RESULTS

1. Clinical manifestations and histopathologic findings

A. PTC with lymphocytic thyroiditis

The subtypes of PTCs with LT were most conventional (11 out of 13 cases, 84.61%) and those of only 2 cases were PTC, follicular variant (2 out of 13 cases, 15.38%). The tumor size ranged from 0.2 cm to 1.1 cm (average; 0.55 cm). 6 cases showed extrathyroidal tumor extension (6/13 = 46.15%). 2 cases with lymph nodal metastasis were present (2/13 = 15.38 %) (Table 1).

Table 1. Clinical manifestations and histopathologic findings of the cases with lymphocytic thyroiditis (n = 13)

Case No.	Age	Sex	Tumor size(cm)	Tumor extension	LN metastasis	Subtype
Case 1	48	M	0.5	ET	N	C
Case 2	51	M	0.4	ET	N	C
Case 3	59	F	0.7	IT	N	F
Case 4	36	M	1.1	IT	P	C
Case 5	56	F	0.2	IT	N	C
Case 6	35	F	0.7	IT	N	C
Case 7	55	F	0.3	ET	N	C
Case 8	40	F	0.5	IT	N	C
Case 9	52	F	0.4	IT	N	C
Case 10	35	F	0.9	IT	N	C
Case 11	47	F	0.6	ET	P	C
Case 14	52	F	0.5	ET	N	F
Case 16	38	F	0.9	ET	N	C

M, male; F, Female; ET, extrathyroidal extension; IT, intrathyroidal confinement; LN, lymph node; P, Positive for metastasis; N, Negative for metastasis; C, Conventional; F, Follicular variant.

Most cases of papillary thyroid carcinoma revealed the conventional subtype. Six out of thirteen cases (46.15%) showed extrathyroidal tumor extension.

B. PTC without lymphocytic thyroiditis

The subtypes of PTCs with LT were all conventional (10/10 = 100%). 3 cases with extrathyroidal tumor extension were present (3/10 = 30%). Tumor size ranged from 0.1cm to 1.5cm (average; 0.61 cm). The cases with lymph nodal metastasis were 3 cases (3/10 = 30%) (Table 2).

Table 2. Clinical manifestations and histopathologic findings of the cases without lymphocytic thyroiditis (n = 10)

Case No.	Age	Sex	Tumor size(cm)	Tumor extension	LN metastasis	Subtype
Case 12	40	F	0.2	IT	N	C
Case 13	53	F	0.1	IT	P	C
Case 15	42	F	0.9	ET	N	C
Case 17	49	F	0.2	IT	N	C
Case 18	52	F	0.5	IT	N	C
Case 19	58	M	0.5	IT	P	C
Case 20	48	F	1.2	ET	N	C
Case 22	50	F	1.5	ET	N	C
Case 23	39	F	0.7	IT	N	C
Case 25	45	F	0.2	IT	P	C

M, male; F, Female; ET, extrathyroidal extension; IT, intrathyroidal confinement; LN, lymph node; P, Positive for metastasis; N, Negative for metastasis; C, Conventional; F, Follicular variant

The tumor subtype of all cases was conventional. 3 cases (30%) showed extrathyroidal tumor extension. The cases with lymph nodal metastasis were 3 cases (30%).

2. Cytokine and immune profiles

In 23 cases, the cytokine immune profiles were summarized in two groups; the cases with lymphocytic thyroiditis (LT) (n = 13) and the cases without LT (n =

10).

The cytokines could be originated from inflammatory cells, thyroid tissue, or endothelial cells. In this research, the tissues were from the thyroid tissue near PTC.

A. PTC with lymphocytic thyroiditis

After manipulating data using the concept of calibrator with IL-1β, each sample was compared. Among 6 cytokines, TNF-α, IL-4, IFN-γ, and IL-1β were relatively well expressed (Table 3).

Based on this fact, the expressed cytokines in PTC with LT represented mixed Th1 (TNF-α, IFN-γ) and Th2 (IL-4, IL-10) immunity.

Table 3. Cytokine and immune profiles of the cases with lymphocytic thyroiditis (n = 13)

	TNF-α /IL-1β	IL-4 /IL-1β	IFN-γ /IL-1β	IL-10 /IL-1β	IL-2 /IL-1β	IL-1β /IL-1 β
Case 1	81.28	47.94	11.15	0.14	0.00	1.00
Case 2	4130.98	3173.80	1163.73	0.000.00	0.00	1.00
Case 3	3328.36	2686.57	946.27	0.00	0.00	1.00
Case 4	198.41	145.50	64.68	0.20	0.00	1.00
Case 5	276.08	258.27	93.89	0.73	0.09	1.00
Case 6	5275.74	4871.32	1875.00	0.00	0.00	1.00
Case 7	2454.55	2227.27	954.55	7.88	3.10	1.00
Case 8	108.88	91.54	40.36	1.39	0.25	1.00
Case 9	242.84	242.84	46.20	0.18	0.00	1.00
Case 10	141.74	135.65	43.39	0.20	0.10	1.00
Case 11	4.21	0.03	0.05	0.04	0	1.00
Case 14	5.81	1.54	0.39	0.11	0.09	1.00
Case 16	2.32	0	0.12	0.05	0	1.00
Median	198.41	145.5	46.2	0.16	0	1.00

To compare expression levels of different samples in quantitative RT-PCR, the concept of calibrator was used. In this study, the calibrator was IL-1β.

The value unit was the *ratio* of one cytokine to IL-1β.

TNF-α, IL-4, IFN-γ, and IL-1β cytokines were relatively well expressed. The immunity of papillary thyroid carcinoma with lymphocytic thyroiditis was mixed Th1(TNF-α, IFN-γ) and Th2 (IL-4, IL-10) pattern.

B. PTC without lymphocytic thyroiditis

After manipulating data using the concept of calibrator with IL-1β, each sample was compared. Comparing the cases with LT, the cases without LT frequently revealed non-detected cytokine level. Among 6 measured cytokines, IFN-γ, IL-10, and IL-2 level were too sparse to be measured (Table 4).

Based on this fact, the expressed cytokines in PTC without LT represented

mixed Th1 (TNF-α, IFN-γ) and Th2 (IL-4, IL-1β) immunity.

Table 4. Cytokine and immune profiles of the cases without lymphocytic thyroiditis (n = 10)

	TNF-α /IL-1β	IL-4 /IL-1β	IFN-γ /IL-1β	IL-10 /IL-1β	IL-2 /IL-1β	IL-1β /IL-1β
Case 12	5.67	0.18	0	0.13	0	1.00
Case 13	3.41	0	0.09	0	0	1.00
Case 15	28.04	16.16	0	0	0	1.00
Case 17	11.93	1.05	0	0.36	0	1.00
Case 18	43.76	0.00	0	0	0	1.00
Case 19	62.07	22.07	0	1.71	0.25	1.00
Case 20	5.06	0.08	0	0.10	0	1.00
Case 22	512.61	277.47	5.21	0	0	1.00
Case 23	8.19	2.56	1.66	0.02	0.03	1.00
Case 25	32.47	0.00	0	2.18	0	1.00
Median	19.98	0.61	0	0.06	0	1.00

To compare expression levels of different samples in quantitative RT-PCR, the concept of calibrator was used. In this study, the calibrator was IL-1β.

The value unit was the *ratio* of one cytokine to IL-1β.

The cases without lymphocytic thyroiditis frequently showed non-detected

cytokine level. The immunity of papillary thyroid carcinoma without lymphocytic thyroiditis was mixed Th1(TNF-α, IFN-γ) and Th2 (IL-4, IL-1β) pattern.

C. Prognostic parameters according to presence of lymphocytic thyroiditis

The prognostic parameters (tumor size, extrathyroidal extension, and lymph node metastasis) were not statistically related to lymphocytic thyroiditis. But the cases without LT had a tendency to lymph nodal metastasis (Table 5).

The cases with LT expressed cytokines (TNF-α, IFN-γ, IL-4, IL-10, IL-2) higher than those without LT. Among 5 cytokines, TNF-α, IFN-γ, and IL-4 were statistically significantly expressed higher in the cases with LT than those without LT ($p < 0.05$).

In terms of p27 as a surrogate marker for lymph nodal metastasis, the degree of p27 expression was not correlated with lymph nodal metastasis in this study; both low grade and high grade expression of p27 had a tendency to occur in PTC cases with LT. In contrast, nodal metastatic cases occurred slightly more in PTC cases without LT.

This discrepant result implicated p27 could not be used as an interchangeable surrogate marker for lymph nodal metastasis.

This result was against the previously known p27 result about nodal metastasis[21, 22]. Furthermore, the degree of p27 expression did not show any correlation with lymph nodal metastasis in this study ($p = 0.15$) (Table 6). Like this, as the usefulness of PTC cases without LT as a reference group was low, this study became to adopt the PTC cases with LT in analysis on the relationship between cytokine immune profile and prognostic parameters (see section D).

Besides the PTC cases in this study were most microcarcinomas, which may represent the incipient phase of tumors. The incipient phase of tumors had always a limitation to representing proper tumor staging, such as tumor size, nodal metastasis, and tumor extension.

Hence, in this study, p27 was used as a representing marker of lymph nodal metastasis in microcarcinoma (incipient tumor) cases in statistical analysis (see section D).

Table 5. Relationship between clinical parameters and the cases with lymphocytic thyroiditis

	LT (n = 13)	**Non-LT (n = 10)**	***p* value**
Tumor size(cm)	0.50(0.20-1.10)	0.50(0.10-1.50)	*0.70*
Extrathyroidal extension (n)	6 (46.15%)	3 (30%)	*0.66*
Lymph nodal metastasis (n)	2 (15.38%)	3 (30%)	*0.61*
LG p27 (n = 14)	8 (8/13 = 61.53%)	6 (6/10 =60%)	*0.65*
TNF-α	198.41(2.32-5275.74)	19.98(3.41-512.61)	*0.04*
IL-4	145.50(0.00-4871.32)	0.61(0.00-277.47)	*0.01*
IFN-γ	46.20(0.05-1875.00)	0.00(0.00-5.21)	*0.00*
IL-10	0.14(0.00-3.10)	0.06(0.00-0.25)	*0.50*
IL-2	0.00(0.00-3.10)	0.00(0.00-0.25)	*0.32*

LT, cases with lymphocytic thyroiditis; Non-LT, cases without lymphocytic thyroiditis; LG p27, low grade expression of p27 immunohistochemistry.

Statistical value is measured as 'Median'. *p* value; < *0.05*.

The value of cytokines is the ratio of each cytokine to IL-1β.

Under-expression of p27 was known to be associated with lymph node metastasis. There was no statistically significance between p27 expression and nodal metastasis according to presence of lymphocytic thyroiditis.

The levels of TNF-α, IFN-γ, and IL-4 were statistically significantly expressed higher in the cases with LT than those without LT ($p < 0.05$).

Table 6. Relationship between cytokine and nodal metastasis

	Nodal metastasis (n = 5)	**Without nodal metastasis (n = 18)**
LG p27 (n = 14)	3 (3/5 = 60%)	11 (11/18 = 61.11%)
HG p27 (n = 7)	1 (1/5 = 20%)	6 (6/18 = 33.33%)

LG p27, low grade expression of p27 immunohistochemistry; HG p27, high grade expression of p27 immunohistochemistry.

The cases with nodal metastasis (total 5); one case was not performed for p27 immunostain due to loss of cancer lesion. Under-expression of p27 was known to be associated with lymph node metastasis. There was no statistically significance between p27 expression and nodal metastasis.

D. Cytokine and immune profiles and clinical implications in the cases with lymphocytic thyroiditis

The statistical analysis in this section D was done only in the PTC cases with LT.

The amount of cytokines was lower in the cases with low grade expression of p27 than those with high grade expression of p27. But there was no statistical significance.

Under-expression of p27 was known to be associated with lymph nodal metastasis. Considering this fact, this study result implicated that the cases with lymph nodal metastasis had a tendency to have the lower levels of cytokines than those without nodal metastasis except for IL-4 (Table 7). In other words, the higher levels of cytokines might play a role in inhibiting lymph nodal metastasis.

Table 7. Relationship between p27 immunopositivity and cytokine and immune profiles in the cases with lymphocytic thyroiditis (n =13)

	LG p27 (n = 8)	HG p27 (n = 5)	*p* value
TNF-α	76.32(2.32-4130.98)	81.28(5.81-5275.74)	*0.39*
IL-4	53.85(0-3173.80)	47.94(1.54-4871.32)	*0.31*
IFN-γ	2.66(0-1163.73)	11.15(0-1875)	*0.43*
IL-10	0.07(0-7.88)	0.11(0-1.71)	*1.00*
IL-2	0(0-3.10)	0(0-0.25)	*0.75*

LG p27, low grade expression of p27 immunohistochemistry; HG p27, high grade expression of p27 immunohistochemistry.

Statistical value is measured as 'Median'. *p* value; < 0.05.

The value of cytokines; the ratio of each cytokine to IL-1β.

Under-expression of p27 was known to be associated with lymph node metastasis. Except for IL-4, the levels of TNF-α, IFN-γ, and IL-10 were lower in the cases with low grade expression of p27. This fact implicated that the increased cytokines might play a role in inhibiting lymph nodal metastasis.

The cases with extrathyroidal tumor extension had a tendency to have higher levels of cytokines (TNF-α, IFN-γ, and IL-4) than those with intrathyroidal tumor confinement (Table 8). But there was no statistical significance. Although this result could not determine whether it implicated an anticancer effect or not, there was a tendency to the increased level of cytokines in the cases with extrathyroidal tumor extension.

Taken together, although there was no statistical significance, lower levels of cytokines had a tendency to occur in the cases of nodal metastasis. This may implicate 5 cytokines (TNF-α, IFN-γ, IL-2, IL-4, & IL-10) contribute to anti-tumor activity in terms of lymph nodal metastasis.

In terms of helper T cell immunity, it could be concluded that mixed Th1 (TNF-α, IFN-γ, IL-2) and Th2 (IL-4, IL-10) immunity play a role in anti-tumor effect.

Table 8. Relationship between cytokine and tumor extension in the cases with lymphocytic thyroiditis (n = 13)

	ET (n = 6)	IT (n = 7)	*p* value
TNF-α	43.54(2.32-4130)	28.04(5.06-512.61)	*1.00*
IL-4	24.74(0-3173)	16.16(0.08-277.47)	*1.00*
IFN-γ	5.77(0.05-1163.73)	0(0-5.21)	*0.15*
IL-10	0.08(0-7.88)	0(0-0.10)	*0.23*
IL-2	0(0-0.10)	0(0-0)	*0.37*

ET, Extrathyroidal tumor extension; IT, Intrathyroidal tumor confinement

Statistical value is measured as 'Median'. *p* value; <0.05.

The value of cytokines is the ratio of each cytokine to IL-1β.

There was no statistically significant difference between the cases with ET and IT. Although this result could not determine whether it implicated an anticancer effect or not, there was a tendency to the increased level of cytokines in the cases with extrathyroidal tumor extension.

IV. DISCUSSION

This study revealed mixed Th1 (IFN-γ, TNF-α, IL-2) and Th2 (IL-4, IL-10) immunity. After focusing on the cases with lymphocytic thyroiditis (LT), statistical analysis showed the cases with lower levels of cytokines had a tendency to occur in the cases with low grade expression of p27 (surrogate marker for lymph nodal metastasis).

In view of helper T cell immunity, mixed Th1 and Th2 immunity seemed to play a role in anti-cancer activity.

There have been several studies for immune profiles of thyroiditis.

Among them, Phenekos et al reported that Hashimoto's thyroiditis (HT) and Graves' disease had two different helper T cell immunities, respectively[23]. In this report, with a preferential immunoexpression of IL-2, IFN-γ, IL-12, and IL-18, a Th1 pattern of immune response characteristic of cellular immunity

was dominant in HT. Colin et al also reported the Th1 context analyzed in peripheral lymphocytes was dominant in HT patients[24].

In contrast, Ajjan et al reported immunity of mixed Th1 and Th2 response in HT[24]. They reported that RT-PCR result showed both Th1 (IL-1α, IL-2, IL-8, IL-10, IFN-γ, TNF-α) and Th2 (IL-1β, IL-4, IL-6) immunity. This result was similar to that of this study.

In this study, PTCs with lymphocytic thyroiditis had more increased cytokine expression of TNF-α, IL-4, IFN-γ, IL10, and IL2 than those without LT. This might mean lymphocytes mainly contributed to secrete inflammatory cytokines. This assumption was consistent with the well known reports on lymphocytes as a source of TNF-α[25, 26]. In contrast to these results, some argued fibroblasts were the possible source of TNF-α[27, 28, 29]. About these two conflicting opinions, Aust et al demonstrated thyroid-derived lymphocytes were potential TNF-α producers within the thyroid, whereas unstimulated fibroblasts and thyrocytes did not produce detectable amounts of TNF- α *in vitro*[27]. Hence, the result of this study must be in accordance with that of Aust et al.

In this study, IL-4 expression had a tendency to increase in the cases with extrathyroidal tumor extension (ET) and LT, but did not show any statistical relation to prognostic parameters. This might mean IL-4 expression contributed to favor proliferative activity of PTC. Furthermore, among 5 cytokines (IFN-γ, TNF-α, IL-2, IL-4, IL-10), only IL-4 expression was higher in the cases with low grade expression of p27. This meant that IL-4 expression was increased in the cases with lymph nodal metastasis. Like this inference in this study, Vella et al demonstrated IL-4 level was increased in aggressive PTCs. They suggested that PTC cells received protection from apoptosis by IL-4 produced by activated T lymphocytes in thyroid glands[30].

In this study, IL-1β was always expressed in all cases and used as a calibrator to compare cytokine levels among cases. About the role of IL-1β in PTCs, Yip et al reported that *in vitro* test, IL-1β was anticancer factor which suppressed the proliferation and reduce the invasive potential of human papillary thyroid carcinoma cells[31]. Because IL-1β was used as a calibrator in this study, its

expression level among cases could not be compared.

Concerning the relationship between lymphocytic thyroiditis and tumor biologic behavior, most reports about this theme approved the prognostic beneficial role of CLT in PTCs.

Paulson et al[32] suggested that chronic lymphocytic thyroiditis (CLT) might have a protective role in tumor spread. As supportive evidence on this, Mitsiades et al[33] reported that Th1 cytokines, such as IFN-γ and TNF-α increased the sensitivity of both normal and neoplastic thyrocytes to FasL and TRAIL, which led to apoptosis. Furthermore, Ahn et al reported that Hashimoto's thyroiditis (HT) was definitely associated with PTC as was chronic inflammation with cancer in other locations. They also mentioned that the coexistence of HT in PTC cases introduced favorable clinical outcomes when compared with those of PTC without HT[34]. In agreement with this, Yoon et al reported that the patients of PTC with CLT had smaller tumor size, lower incidence of capsular invasion, and a significantly lower incidence of lymph nodal metastases than patients without CLT[35]. As a mechanism for these anti-tumor activities of CLT in PTCs, several articles reported that antithyroid antibodies may be able to recognize these malignant cells and destroy them in the same way as they destroy normal follicular cells, contributing to the low rate of clinical progression of these lesions[36, 37, 38, 39].

This study revealed that, in the PTC cases with LT, the cases with extrathyroidal tumor extension had the higher levels of cytokines (mixed Th1 and Th2) than those with intrathyroidal confinement. Concerning this result, there can be two different interpretations. One is that cytokines may promote the tumor invasion, and the other is that cytokines may be increased to protect tumor invasion with anti-tumor immunity.

In this study, among 6 cytokines, TNF-α was expressed higher than 5 remaining cytokines. Like two above contradictory interpretations, the role of TNF-α in tumor immunity has been known to reveal a dual contradictory facet. Pang et al reported that TNF-α has an anti-proliferative action in a human papillary thyroid carcinoma cell line[40]. In contrast to this effect of TNF-α, Ohta

et al reported that IFN-γ, IL-1β, and TGF-β1 inhibited proliferation of PTC cell lines, but TNF-α promoted proliferation of PTC cell lines[41]. In agreement with this pro-proliferative action of TNF-α, Pang et al reported occurrence of tumor resistance to anti-proliferative activity of TNF-α *in vitro* test[42]. But until now, there has been no clinical report on this phenomenon.

Like this, the role of cytokines in tumor immunity must be complex. Hence further studies should be necessary for the role of cytokines in tumor immunity in various aspects.

This study showed that p27 expression did not show any relation to lymph nodal metastasis. This may be attributed to too early stage of tumor, e.g. microcarcinoma, for the incipient tumor phase could not represent proper tumor stage.

The p27 protein was first identified as an inhibitor of cyclin E/CDK2 complexes during TGF-beta induced G1 arrest43. Phosphorylation, as such, was the mechanism primarily used for regulating p27 activity. The p27 protein possessed multiple tyrosine, serine, or threonine phosphorylation sites. The inhibitory actions of p27 on cyclin/CDK complexes were weakened by phosphorylation, directed by some signal transduction pathways[44].

The current model generally accepted is that p27 suppresses tumorigenesis by inhibiting cyclin/CDK activity in the nucleus, but exerts other functions in the cytoplasm that are potentially oncogenic[45].

There were several clinical studies about relationship between expression of p27 and lymph nodal metastasis. Khoo et al[46] maintained that underexpression of p27 was associated with lymph nodal metastasis. Similarly, Karlidag et al [18] reported that p27 expression in the non-metastasizing PTC was lower than normal thyroid tissue and higher than metastasizing PTC.

As aforementioned, our research did not show statistically significant relationship between p27 expression and nodal metastasis. As a plausible explanation for this, the cases in our study were mainly those of microcarcinoma in PTCs. So there may be a chance that even PTC cases with

low expression of p27 were too incipient to reveal lymph nodal metastasis. With this limitation of evaluating the relation between cytokine levels and nodal metastasis, well known p27 marker was used as a surrogate marker in representing the possibility of nodal metastasis in the early phase PTCs.

As aforementioned, after focusing on the cases with lymphocytic thyroiditis, the cases with low grade expression of p27 had a tendency to have lower levels of cytokines than those with high grade expression of p27. This might implicate the cases with lower levels of mixed Th1 and Th2 cytokines had a higher probability of having lymph nodal metastasis.

In this study, two cases were follicular variant of PTC. They revealed high grade expression of p27. Resnick et al. reported that the follicular variant of PTC had a higher p27 staining frequency ($p = 0.05$) than did classical papillary carcinoma. It could be inferred that follicular variant PTC may have a relative low risk for lymph nodal metastasis.

Taken together, considering lower expression of cytokines and its relation to lymph nodal metastasis in the PTC cases with LT of this study, mixed Th1 and Th2 immune cytokines may have a tendency to anti-cancer effect.

To set up the criterion of cytokine value to predict tumor prognosis, further study for determining the absolute cytokine value and immunohistochemistry of cytokine will be necessary.

V. CONCLUSION

This study revealed that most PTCs have mixed Th1 (IFN-γ, TNF-α, & IL-2) and Th2 (IL-4, IL-10, & IL-1β) immunity.

All cytokines were exclusively more expressed in the cases with lymphocytic thyroiditis(LT) than those without LT. This means these cytokines in PTC cases originate mainly from lymphocytes rather than fibroblasts or thyroid cells.

Lower levels of cytokines in PTC with LT had a tendency to be related to the low grade expression of p27. Considering p27 as a surrogate marker for lymph nodal metastasis, this result implicated that lower levels of cytokines were correlated to lymph nodal metastasis.

The cases with extrathyroidal tumor extension had a tendency to show the higher levels of cytokine expression than those with intrathyroidal tumor confinement. Although this result could not determine whether it implicated an anticancer effect or not, there was a tendency to the increased level of cytokines in the cases with extrathyroidal tumor extension.

This result implicated that mixed Th1 and Th2 cytokines had an anti-cancer effect, only in terms of lymph nodal metastasis.

To set up the criterion of cytokine value to predict tumor prognosis, further study for determining the absolute cytokine value and immunohistochemistry of cytokine should be necessary.

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ABSTRACT (IN KOREAN)

만성 림프구성 갑상선염을 동반한 유두상 갑상샘 암의 임상병리학적 양상과 보조 T 세포 아형 발현간의 상관 관계

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양 석 우

한국에서는 갑상샘암은 발생 빈도가 높은 암종 중 하나다. 갑상샘암종 중 유두상암은 가장 흔하며, 림프구성 갑상샘염을 동반한 경우가 많다. 일부 논문에서는 하시모토 갑상샘염이 유두상암의 발생을 증가시킨다고 보고 하였다. 그러나 다른 보고에서는 림프구성 갑상샘염은 항종양 작용을 하여 좋은 예후에 기여한다고 보고 하였다.

이 대립된 두 이론에 대하여 이 연구에서는 림프구성 갑상샘염이 종양 면역에 어떤 작용을 하는지에 대한 연구를 하였다.

보조 T 세포 면역의 두 종류에 해당하는 시토카인을 측정하였다; Th1 (TNF-α, IFN-γ, IL-2)와 Th2 (IL-4, IL-10, IL-1β). 시토카인 수치는 정량적 RT-PCR을 사용하였고, 조직은 종양이 없는 림프구성 갑상샘염이 포함된 조직에서 시행하였다.

이 연구 결과에서는 대부분의 유두상암은 혼합형 보조 T 세포 면역 반응을 보였다; Th1 (TNF-α, IFN-γ, IL-2)와 Th2 (IL-4, IL-10, IL-1β). 시토카인은 항상 림프구성 갑상샘염이 동반된 경우에서 높게 측정되었다. 통계학적 분석을 림프구성 갑상샘염이 동반된 경우에 국한하여 분석한 결과 시토카인 발현이 낮은 경우는, 통계학적 유의성은 없었으나, p27의 발현이 낮은 경향을 보였다. p27을 림프절 전이의 대리표지자로 분석하면, 이 연구 결과는 시토카인 발현이 낮은 경우는 림프절 전이 가능성이 높은 유두상 갑상샘암에서 발생한 경향이 많았다. 즉 높은 시토카인 발현은 림프절 전이를 억제하는 역할에 기여한다고 추론할 수 있다. 종양의 갑상샘외 침범이 있는 증례에서는 시토카인 발현이 높은 경향성이 있었다. 이 결과는

종양의 갑상샘외 침범에 대한 항종양 작용 여부를 판단할 수 없지만, 종양의 갑상샘외 침범 시 염증 반응 증가의 경향성이 높음을 의미한다.

이 연구 결과는 유두상 갑상샘암에서 림프절 전이 측면에서 Th1과 Th2 혼합형 보조 T 세포 면역의 시토카인은 항종양 작용을 하는 경향을 의미한다.

핵심 되는 말: 유두상 갑상샘암, 보조 T 세포, 시토카인, p27, 예후, 전이, 종양의 갑상샘외 침범, 림프구성 갑상샘염, 인터루킨, 인터페론, TNF