The Relationship between Wilms' Tumor 1 (WT1) and Paired Box 8 (Pax-8) Protein Expressions in Papillary and Anaplastic Thyroid Carcinomas

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ABSTRACT. Background : We elucidated the expression of WT1 and its correlation with Pax-8 and TTF-1 as a differentiation promoter in thyroid cancer. **Methods** : We investigated the expression of WT1, Pax-8, and TTF-1 immunohistochemically in 60 primary tumors (30 well-differentiated and 15 poorly differentiated papillary carcinomas and 15 anaplastic thyroid carcinomas). **Results** : Positive staining of WT1 was found in the cytoplasm and nucleus of the cancer cells. Each positive rate of cytoplasmic staining of WT1 was 100% in the 45 papillary carcinomas and 93.3% in the 15 anaplastic carcinomas. The immunohistochemical scores for WT1 in well differentiated, poorly differentiated papillary carcinomas and anaplastic carcinomas were 5.90 ± 1.7, 6.53 ± 1.7, and 3.60 ± 2.1, respectively. The scores for the well and poorly differentiated papillary carcinomas were significantly higher than those for the anaplastic carcinomas. The expression level of WT1 showed a significant correlation with both Pax-8 and TTF-1. **Conclusions** : This is the first report to demonstrate a positive correlation of the expression of WT1 with Pax-8 and TTF-1 in clinical cases. In thyroid cancer, the decrease in expression of WT1 with anaplastic transformation is assumed to be closely related to Pax-8 and TTF-1.

Key words ① **thyroid carcinoma** ② **WT1** ③ **Pax-8** ④ **differentiation** ⑤ **TTF-1**

The Wilms' tumor 1 (WT1) gene was identified as a candidate gene among children harboring Wilms' tumor in 1990[1]. WT1 is a transcriptional repressor with zinc finger domains[2,3]. Although it was demonstrated to be a suppressor gene at initially[3], overexpression of its wild type has been reported in several tumors subsequently. Expression of WT1 has been demonstrated in leukemia[4], malignant mesothelioma[5], bone and soft tissue sarcoma[6], and in breast[7], ovarian[8], lung[9], thyroid[10], and colorectal cancers[11]. Most reports have emphasized the overexprssion of WT1 in cancer cells compared to the expression in normal tissues. Although mutated homozygous WT1 occurrs in Wilms' tumor in the kidney, WT1 plays an opposite and transactivate role in several cancers without mutations. Little is known as yet

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about these reversal mechanisms, but some investigators have noticed isoforms of WT1. WT1 has four isoforms that are formed by alternative RNA splicing[12]. In these isoforms, the presence or absence of three amino acids (lysine, threonine, and serine ; KTS) between the third and fourth zinc fingers in WT1 seems to be important, because, the insertion or omission of KTS affects DNA-binding affinity and the specificity of WT1[13)~15)]. Some reports have noted reversal effects on targets and cell proliferation depending on the presence of KTS[16), 17)].

Many target genes of WT1 have been identified, including insulin-like growth factor-2 (IGF-2)[18)], platelet-derived growth factor (PDGF) A-chain[19)], IGF1 receptor[20)], epidermal growth factor receptor (EGFR)[21)], colony-stimulating factor-1 (CSF-1)[22)], bcl-2[23)], c-myc[23)], E-cadherin[24)], cyclin E[25)], and mevalonate[26)]. Therefore, the candidate target genes of WT1 are representative and important for cell proliferation.

Although the regulatory factors for expression of WT1 are not as yet well known, paired-box (Pax)-2 and -8[27), 28)], hypoxia-inducible factor 1[29)], IGF-1[30)] and Spl[31)] have been included. In these factors, Pax is essential for all organs, and especially during development. Pax-8 is expressed in the developing and adult thyroid, the developing secretory system and, at lower levels, in the adult kidney[32)]. Therefore, the thyroid is the only adult organ in which Pax-8 is fully and persistently expressed. In the normal thyroid, Pax-8 is essential and critical for the expression of differentiation markers such as thyroglobulin (Tg), thyroid peroxidase (TPO), and thyrotropin receptor (TSH-R) in cooperation with thyroid transcription factor-1 (TTF-1)[33)]. Expression of Pax-8 and TTF-1 has also been observed in differentiated thyroid carcinoma[34)]. With the dedifferentiation of thyroid cancer, these differentiation markers and transcriptional factors would decrease and cancer cells would be acquired to change into an unfavorable phenotype[34)].

In this study, we detected the expression level of WT1 in papillary thyroid carcinomas including poorly differentiated ones and in anaplastic thyroid carcinomas using an immunohistochemical method. We investigated the relationship between clinical behavior and WT1 expression to determine whether WT1 expression in thyroid carcinomas is related to differentiation. We also elucidated the expression levels of Pax-8 and TTF-1 in such carcinomas and analyze the relationships among them and WT1.

MATERIALS AND METHODS

Materials

The subjects of this study were 45 papillary thyroid carcinoma patients (15 of well differentiated type without distant metastasis, 15 of well differentiated type with distant metastases, and 15 of poorly differentiated type), and 15 anaplastic thyroid carcinoma patients who were treated in our hospital. The thyroid function of all the patients was euthyroid before primary operations. All of the patients with papillary carcinoma had received thyroxin to suppress serum TSH during follow-up after the initial surgical treatment. Determination of the dose of thyroxin to maintain the serum TSH level under the lower limit for each patient was done by measuring the level every six months during follow-up. Distant recurrence of disease was defined by echography, chest X-ray films, and computed tomography and/or thallium or radioiodine scintigraphy during follow-up.

For this study, we used embedded paraffin sections of the primary tumor of each patient, which were resected at each initial surgical procedure, or percutaneous fine needle biopsy. Informed consent was obtained

from all enrolled patients.

Immunohistochemical procedure

The sections embedded in paraffin were cut into sections 4μm in thickness, and fixed on aminopropyltrietoxysilane-coated glass slides (Matsunami, Osaka, Japan). After typical deparaffinization, all the slides were autoclaved for 20 min at 121 ℃ in a 10mM citrate buffer (pH 6.0) for antigen retrieval. Then, the slides were incubated for two hours at room temperature with each of the following primary antibodies ; WT1 antibody, a mouse monoclonal antibody (sc-7385, Santa Cruz, CA, USA), at a concentration of 10 ug/ml, Pax-8 antibody, a goat polyclonal antibody (sc-16279, Santa Cruz, CA, USA), at a concentration of 10 ug/ml, and TTF-1 antibody, a goat polyclonal antibody (sc-8761, Santa Cruz, CA, USA), at a concentration of 10 ug/ml. After incubation, the slides were washed in PBS for 15 min, and a secondary antibody was applied using the LSAB plus kit (DAKO Corp., Carpentaria, CA, USA) according to the manufacturer's protocol. After washing, the colors were developed using new fuchsine and 5-bromo-4-chloro-3-indoxyl phosphate, nitro blue tetrazolium chloride, and indonitrotetrazolium (BCIP/NBT/INT) (DAKO Corp., Carpentaria, CA, USA). Then the slides were counterstained with hematoxylin (WAKO, Osaka, Japan), washed in distilled water for 5 min and mounted.

We used normal mouse serum instead of primary antibody in negative control studies, which were performed for all slides (data not shown).

Analysis of immunohistochemical detection

Immunostaining was evaluated by two repeated stainings of the same specimen. We blinded ourselves to the characteristics of the patients, the extent of the tumor and prognostic scores. The cells of a tissue section were evaluated as positive when they showed a distinctly specific stain when compared with the cells of negative control sections. Then, two investigators independently decided the level of intensity of positive cells of each slide. Only in the case of discordance among investigators, did we discuss and decide on the intensity together.

For these immunohistochemical analyses, except for that of WT1, we evaluated both the concentration and positive rate of stained nucleus in each section using the Allred scoring system[35]. In brief, for evaluation of the concentration of staining, the stained cells in the largest population of the section were divided into four degrees (from zero to three). For evaluation of the positive rate of the stained nucleus, the major intensity of the nucleus was divided into six degrees, zero, one (population of the stained nucleus in less than 1/100 of the entire nucleus of a cell), two (1/100-1/10), three (1/10-1/3), four (1/3-2/3), and five (>2/3). Multiplication of both scores resulted in a final quotation ranging from 0-8, which was presented as the mean ± S.D.

The expression of WT1 was evaluated with regard to both the concentration and distribution of stained cells, as previously described[36]. For evaluation of the concentration of staining, the stained cells in the largest population of the section were divided into four degrees (from zero to three). For evaluation of the distribution of stained cells, the major intensity of cells was divided into four degrees, zero, one (observation of stained cells in less then 30% of the entire population of cells.), two (<60%), and three (≧60%). Multiplication of both scores resulted in a final quotation ranging from 0-9, which was presented as the mean ± S.D.

Table 1. Background of patients

	PTC (without rec)	PTC (with rec)	poorly PTC	ATC
	n=15	n=15	n=15	n=15
Age	54 (31-72)	57 (11-73)	57 (20-82)	71 (43-83)
Gender (male: female)	3:12	5:10	5:10	6:09
Tumor size (cm)	3 (2.3-8.5)	3 (0.8-7)	2.75 (1.8-6.5)	5.5 (2.5-11)
EX				
0	6	7	6	-
1	1	0	2	-
2	8	8	7	6
Recurrent site				
Lung	-	13	1	4
Lymph node	-	7	3	6
Bone	-	6	0	2
Pleura	-	1	0	2
Brain	-	1	0	0
DFI (m)	-	42 (2-135)	23.5 (5-94)	-
OS (m)	109 (51-202)	86 (38-214)	28 (5-94)	8 (2-35)

PTC; papillary thyroid carcinoma, ATC; anaplastic thyroid carcinoma
EX: extrathyroidal infiltration
DFI; disease free interval, OS; overall survival

Statistical analysis

For statistical analysis, the F test and Spearman's rank correlation test were used, and $p<0.05$ was considered significant.

RESULTS

Table 1 shows the characteristics of patients enrolled in this study. The median ages of the well differentiated papillary carcinoma patients without recurrence or with recurrence, the poorly differentiated papillary carcinoma patients, and the anaplastic carcinoma patients were 54, 57, 57, and 71 years old, respectively. Forty-seven of the patients were women (68.3%) and 13 were men (31.7%). Among the papillary carcinoma patients, there were 23 patients with EX2 (infiltration to adjacent organs except muscles). Among the well-differentiated papillary carcinoma patients with recurrent tumors, metastatic sites appeared in the lung (86.7%), lymph nodes (46.7%), bone (40%), pleura (6.7%), and brain (6.7%). The disease-free interval and overall survival of patients with recurrent well differentiated papillary carcinomas and poorly differentiated papillary carcinomas were 42 and 86 months, and 23.5 and 28 months, respectively. The overall survival of patients with anaplastic carcinomas was eight months.

Immunohistochemical study

Positive staining of WT1 was found in the cytoplasm and nucleus of the cancer cells (Fig. 1A and 1C).

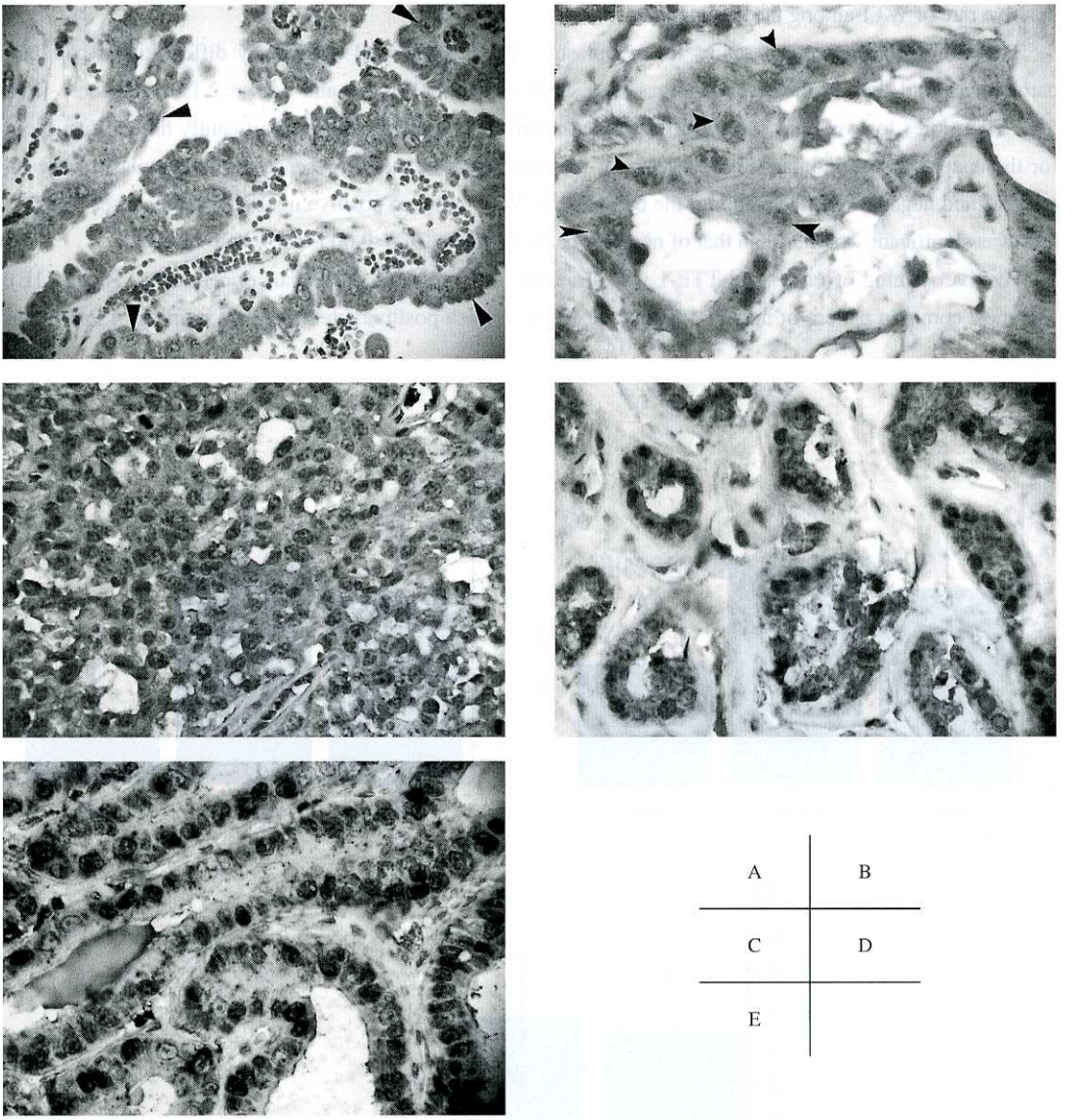

Fig. 1. Immunohistochemical results

Magnification of each figure is ×200 (A and C) and ×400 (B, D and E).

The positive staining for WT1 was developed using fuchsine, and for Pax-8 and TTF-1 by NBT/BCIP/INT. The slide for WT1 was counterstained with hematoxylin.

In all papillary thyroid carcinoma, positive staining of WT1 appears in the cytoplasm (A). Speckled nuclear staining can be seen in the same specimen. Representative nucleic staining is showed in B (Arrow head). WT1 staining in anaplastic carcinoma is shown in C. The brown staining of Pax-8 is mostly in the nucleus and partly in the cytoplasm in papillary carcinoma (D). Positive brown staining of TTF-1 appears in the nucleus (E).

Each positive rate of cytoplasmic staining of WT1 was 100% in the 45 papillary carcinomas, and 93.3% in the 15 anaplastic carcinomas, respectively. And the positive rate of nucleic staining was 37.8% in the 45 papillary carcinomas and 20% in the 15 anaplastic carcinomas. The most common pattern of nucleic staining was speckled (Fig. 1B). Diffuse staining of the nucleus was rare. There was no significant difference in the

positive rate of WT1 among the carcinomas.

The immunohistochemical scores for cytoplasmic WT1 in the well and poorly differentiated papillary carcinomas, and the anaplastic carcinomas were 5.90 ± 1.7, 6.53 ± 1.7, and 3.60 ± 2.1, respectively (Fig. 2A). The scores for well and poorly differentiated papillary carcinomas were significantly higher than those for the anaplastic carcinomas (Fig. 2A). As for the correlation of the WT1 score of the cytoplasm between positive and negative nucleic staining, the cytoplasmic WT1 score of positive nucleic staining cases (6.55 ± 2.0) was significantly higher than that of negative cases (4.95 ± 1.9, $p<0.005$ by Fischer's test).

Positive staining of Pax-8 and TTF-1 was observed in the nucleus of the cancer cells (Fig. 1D and 1E). The most common pattern of nucleic staining was diffuse. Each positive rate of nucleic staining of Pax-8 was

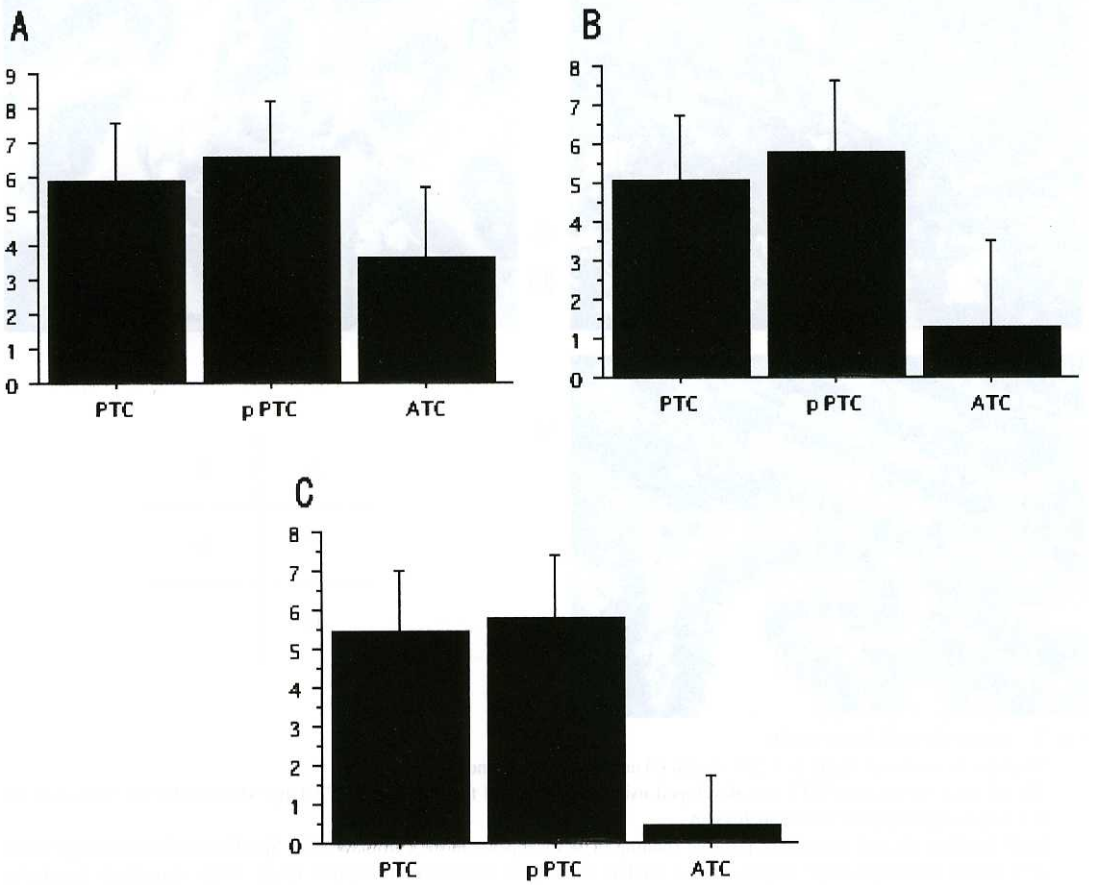

Fig. 2. The immunohistochemical scores of WT1 (A), Pax-8 (B), and TTF1 (C) among each histological type
The immunohistochemical scores for WT1 in well differentiated, and poorly differentiated papillary carcinomas, and anaplastic carcinomas were 5.90 ± 1.7, 6.53 ± 1.7, and 3.60 ± 2.1, respectively. The scores for well and poorly differentiated papillary carcinomas were significantly higher than those for anaplastic carcinomas ($p<0.001$, analyzed by Fischer's test, Fig. 2A). The immunohistochemical scores for Pax-8 in well and poorly differentiated papillary carcinomas, and anaplastic carcinomas were 5.07 ± 1.6, 5.8 ± 1.8, and 1.27 ± 2.3, respectively (Fig. 2B). The scores of Pax-8 in anaplastic carcinomas were significantly lower than those in papillary carcinomas (Fig. 2B). The immunohistochemical scores for TTF-1 in well and poorly differentiated papillary carcinomas, and anaplastic carcinomas were 5.47 ± 1.5, 5.8 ± 1.6, and 0.47 ± 1.2, respectively (Fig. 2C). The scores of TTF-1 in each histological type showed similar significance in Pax-8.

100% in the 45 papillary carcinomas, and 13.3% in the 15 anaplastic carcinomas. Each positive rate of nucleic staining of TTF-1 was 100% in the 45 papillary carcinomas, and 6.7% in the 15 anaplastic carcinomas. The positive rates for Pax-8 and TTF-1 in the anaplastic carcinomas were significant lower than those in the papillary carcinomas. The immunohistochemical scores for Pax-8 in the well and the poorly differentiated papillary carcinomas, and the anaplastic carcinomas were 5.07 ± 1.6, 5.8 ± 1.8, and 1.27 ± 2.3, respectively (Fig. 2B). The Pax8 score in the anaplastic carcinomas was significantly lower than that in papillary carcinomas (Fig. 2B). The immunohistochemical scores for TTF-1 in the well and the poorly differentiated papillary carcinomas, and the anaplastic carcinomas were 5.47 ± 1.5, 5.8 ± 1.6, and 0.47 ± 1.2, respectively (Fig. 2C). The TTF-1 score in the anaplastic carcinomas was significantly lower than that in papillary carcinomas (Fig. 2C).

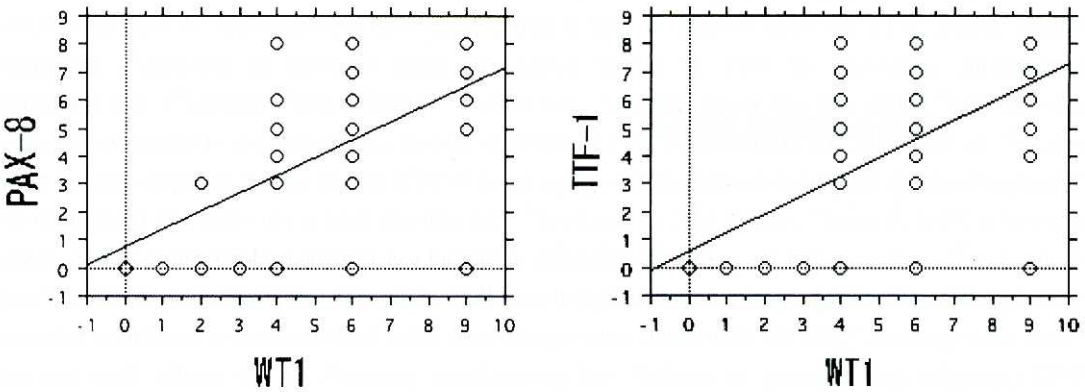

Fig. 3. The correlation of immunohistochemical scores among WT1 and Pax-8 and TTF-1 in thyroid cancers
The expression level of WT1 showed a significant correlation with both Pax-8 (Fig. 3A, $p<0.0001$) and TTF-1 (Fig. 3B, $p<0.0001$). The correlation coefficients were 0.529 and 0.531.

Table 2. Correlation between WT1 expression and clinical data in papillary carcinoma patients

		WT1 score	p value
Age		-	p=0.645
Gender	male (n=13)	6±1	p=0.507
	female (n=32)	6±2	
Tumor Size		-	p=0.398
EX	0 or 1 (n=23)	6±2	p=0.938
	2 (n=22)	6±2	
Presence of Recurrence			p=0.209
	yes (n=18)	6±1	
	no (n=27)	6±2	

WT1 score: mean ± S.D.

Correlation among the expression levels of WT1, Pax-8, and TTF-1 (Fig. 3)

The expression level of WT1 in thyroid cancer showed a significant correlation with both Pax-8 and TTF-1 (Fig. 3A and 3B). The correlation coefficients were 0.529 and 0.531, respectively.

Relationship between clinical data and the expression level of WT1 (Table 2)

Table 2 shows the relationship between clinical data and the expression level of WT1 in only patents with papillary thyroid carcinomas. With regards to age, gender, tumor size, EX, presence of recurrence, and overall survival, there was no significant relationship with the expression of WT1.

DISCUSSION

The role of WT1 in the initiation and/or progression of cancer is still unknown except in hereditary Wilms' tumor. WT1 has been thought to play a critical role with overexpression of wild-type WT1. Furthermore, expression of WT1 in cancer tissues has been observed in leukemia[4], malignant mesothelioma[5], bone and soft tissue sarcoma[6], and breast[7], ovarian[8], lung[9], thyroid[10], and colorectal cancer[11]. In this study, the expression of WT1 in papillary thyroid carcinomas was observed in all cases. There has been only two reports concerning the expression of WT1 in thyroid cancer. Its expression has been reported in 20 of 21 cases[10], and in none of ten cases[37]. The antibody used in our study and these previous studies was the same one, but the positivity of staining in thyroid cancer varied. Moreover, there have been some reports demonstrating a positive relationship between WT1 expression and prognosis in leukemia[38] and breast cancer patients[39]. On the other hand, some reports have failed to demonstrate a correlation between WT1 expression and prognosis in ovarian[8] and mesothelioma patients[40]. In our results, there was no significant relationship between the expression of WT1, the clinical parameters and prognosis. No difference was observed in the expression levels of WT1, Pax-8, and TTF-1 between well and poorly differentiated thyroid carcinomas. Therefore, there may be no relationship between WT1 and clinical behavior.

In our findings, although the localization of WT1 was mainly cytoplasmic, in about one third, it was also in the nucleus. Reports have shown the main localization of WT1 to be the nucleus in breast cancers[7], ovarian tumors[8], malignant mesotheliomas[37, 40], and desmoplastic small round cell tumors[41]. On the other hand, Oji et al. reported its expression only in the cytoplasm of thyroid[10], and colorectal cancers[11]. As with our findings, both staining of the cytoplasm and the nucleus has been reported in a small population of breast cancer[7] and mesothelioma patients[9]. The difference in the localization of WT1 in each organ has been demonstrated as a model of shuttling of multifunctional pre-mRNA binding proteins between the nucleus and the cytoplasm[42)~44)].

The presence or absence of three amino acids (lysine, threonine, and serine ; KTS) between the third and fourth zinc fingers has been thought to be important in isoforms of WT1, because, the insertion or omission of KTS affects DNA-binding affinity and the specificity of WT1[13)~15)]. These differences of alternative splicing are more important in the biological behavior of WT1. Englert et al. reported that WT1 (+KTS) or WT1 mutants exhibited a speckle pattern within the nucleus[45]. In our findings, nuclear staining of WT1 showed a speckled pattern. Therefore, overexpression of WT1 in thyroid cancer might be depended on WT1 (+KTS) or WT1 mutants.

Although the regulatory factors for expression of WT1 are not as yet well known, paired-box (Pax)-2 and

-8[27), 28)] was demonstrated as the regulatory factor. Pax-8 is also essential for the maintenance of differentiation of thyroid follicular cells. Even in adults, Pax-8 is still expressed in normal thyroid and kidney. In the thyroid, in particular, Pax-8 plays an important role as a promoter activator of differentiation markers such as Tg, TPO, and TSH-R. Like Pax-8, TTF-1 is also essential for the differentiation of thyroid cells[33)]. In adults, TTF-1 expression is still expressed in thyroid, lung[33)]. Thus, the thyroid retains genes that act mainly in development. In this study, we evaluated the relationship between the expression levels of Pax-8 and WT1, and found a significant correlation between them. There was also a significant correlation between expression levels of TTF-1 and WT1. Eccles *et al* investigated the expression levels of WT1, Pax-2, and Pax-8 in Wilms' tumor and fetal kidney by in situ hybridization[46)], but they failed to demonstrate a significant correlation among them. To the best of our knowledge, this is the first report to demonstrate a positive correlation of the expressions of WT1, Pax-8, and TTF-1 in clinical cases.

In our findings, the highest expression of WT1 was seen in papillary carcinomas. Anaplastic carcinomas showed lower expression. In thyroid cancer, the expression of WT1 seemed to decrease with dedifferentiation in the same manner as the expression levels of Pax-8 and TTF-1. In the thyroid, which expresses Pax-8 even in adults, Pax-8 is assumed to play an important role in the regulation of WT1.

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