# Immunohistochemical Analysis of WT1 Antigen Expression in Various Solid Cancer Cells

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**Abstract.** *For a peptide-pulsed dendritic cell (DC) vaccine to work effectively in cancer treatment, it is significant that the target protein is expressed in cancer cells. Wilms' tumor 1 (WT1) has been identified as a molecular target for immune cell therapy of cancer. We evaluated the protein expression levels of WT1 in various solid tumors, as well as mucin 1 (MUC1) or major histocompatibility complex (MHC) class l molecules. Seven hundred and thirty-eight patients whose tissue samples were examined by immunohistochemical analysis agreed to undergo DC vaccine therapy. The positive staining of WT1 in tumor cells was observed in 25.3% of patients, with only 8.5% of them showing moderate to strong expression; moreover, WT1 tended to localize in the nucleus and cytoplasm. A positive staining of tumor cells by an anti-MHC class l monoclonal antibody was observed in 98.6% and by an anti-MUC1 monoclonal antibody in 76.8% of the patients. In relation to the application of cancer-specific immunotherapy, these findings provide useful information for determining the efficacy of MUC1- and WT1-targeted therapy.*

Dendritic cell (DC)-based vaccines pulsed with various tumor-specific antigens (TSAs) have been developed for cancer immunotherapy (1). Our previous data suggest that immunotherapy with tumor antigen-pulsed immature DCs with zoledronate leads to activation of Vγ9γδ T cells and induction of CD40L on Vγ9γδT cells (2). The activated Vγ9γδT cells secret T helper (Th)1-cytokines, such as interferon (IFN)-γ, and enhance the expansion of tumor antigen-specific $CD8^+$ cells by tumor antigen-pulsed immature DCs. We utilized zoledronate-pulsed DCs as cancer vaccines with various cancer antigens in treatment of solid tumors (3-6). For DC-based cancer vaccines, some reports have described insufficient clinical responses despite the good immunoresponses indicating delayed-type hypersensitivity (DTH) (7). Immune check-point inhibitors, such as antibodies against programmed cell death protein 1 (PD-1) and cytotoxic T-lymphocyte-associated protein 4 (CTLA4), are clinically used for patients with advanced or recurrent melanoma and non-small cell lung cancer to reverse immune suppression and activate effector T cells (8, 9). Furthermore, the efficacy of immune check-point inhibitors was reported to correlate with disorders related to TSAs, oncogenic viral proteins or DNA repair pathway mutations (10).

Tumor antigens can be categorized as TSAs, cancer/testis (CT) antigens or tumor-associated antigens (TAAs) (11, 12). TSAs are abnormal proteins that arise from non-synonymous somatic mutations in tumor cells; however, such antigens are not expressed in normal cells. CT antigens can be potential targets as cancer vaccines because their expression is normally restricted to the germ cells of the testis or ovary or certain tumor cells (11, 13). TAAs are overexpressed normal proteins, such as Wilms' tumor 1 (WT1) (14, 15) or mucin 1 (MUC1) that regulate growth-promoting functions (16). The antigenicity of TAAs was reported to depend on the levels of abnormal expressions (17) because of the lower affinity of the T cell receptor (TCR) against TAAs than of TCR against TSAs (18).

For DC vaccines loaded with various TAAs peptides, their phase I/IIa clinical trial for immunotherapy was carried out in elderly patients with acute myeloid leukemia (AML), using pulsed DCs with a modified WT1 peptide and zoledronate (19). In that trial, three human leukocyte antigen (HLA)-A2402-positive elderly patients with AML were enrolled. The induction of immunoresponses to the WT1 peptide detected as DTH was indicated in two of the three patients, with a transient decrease in the number of leukemic cells being observed in these two patients. Unfortunately, a rapid expansion of leukemic cells was observed in the patient showing no immunoresponses to the WT1-specific peptide

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after the third vaccination. Recently, we studied a DC vaccine pulsed with zoledronate and an overlapping pool of peptides derived from the full length of WT1 for patients with WT1-expressing solid tumors as both $CD8^+$ cytotoxic T lymphocytes (CTLs) and $CD4^+$ T-helper cells against WT1 can be potentially induced without HLA restriction. However, in order to be recognized by CTLs with low-affinity TCRs, it is essential that a sufficient amount of TAAs, such as WT1 or MUC1, should be presented by major histocompatibility complex (MHC) class I on tumor cells (20).

In the present study, we examined the protein expression levels of WT1 in various solid tumors, as well as MUC1 or MHC class I molecules by immunohistochemical analysis. We also analyzed the organ and histopathological profiles of WT1 protein expression in various malignancies classified on the basis of ICD10 and ICD-O-3.

## Materials and Methods

*Patients' background*. Between October 2007 and September 2014, tumor samples were obtained intraoperatively or by biopsy from 738 patients who provided their informed consent to four facilities of the Seta Clinic Group. The patients agreed to undergo DC vaccine therapy and their tissue samples were examined by immunohistochemical analysis for the expression levels of WT1, MUC1 and MHC class I molecules. The mean age of the 738 patients was 58.6 years (range=6-87) and the male-to-female ratio was 1:1.10 (352:386). In this study, 55 cancer types (ICD-10) and 24 histological types (ICD-O-3M) were included (Table I).

*Specimens*. A total of 738 specimens were examined, comprising of 72 stomach cancers, 68 colon cancers, 47 rectum cancers, 63 pancreas cancers, 120 lung cancers, 48 breast cancers and 63 ovarian cancers. The remaining tumor types are summarized in Table I. The prepared paraffin-embedded or formalin-fixed tissues were examined at Tokyo Central Pathology Laboratory Company. The histological diagnosis of each tumor was confirmed on the basis of hematoxylin and eosin staining results and the pathological reports provided by each medical facility.

*Immunohistochemistry*. A mouse monoclonal anti-human WT1 protein antibody (immunogen:human WT1 protein consisting of N-terminal amino acids 1-181, clone 6F-H2; Dako, Glostrup, Denmark), an anti-MUC-1 glycoprotein mouse monoclonal antibody (Novacastra Laboratories Ltd., Newcastle, UK) and an anti-HLA class I-A, B, C mouse monoclonal antibody (Hokudo Co., Ltd., Hokkaido, Japan) were used for the detection of WT1, MUC1 and MHC class l antigens, respectively.

*WT1 immunohistochemical staining method*. The mouse monoclonal anti-WT1 antibody (clone 6F-H2; Dako), an enzyme antibody LSAB method (labeled streptavidin-biotin) (21), and a Ventana Benchmark XT (Ventana Medical Systems, Inc., Arizona, USA) device were used for the immunohistochemical staining. Tissue sections were prepared as follows: enzyme and heat treatment for 8 and 60 min, respectively, to activate the antigen, reaction with the primary antibody for 32 min, reaction with a secondary antibody for 8 min and counterstaining with hematoxylin and eosin for 8 min.

*Evaluation method*. The relative ratio (proportion) and positive reaction strength (*i.e.*, staining intensity) were determined to analyze antigen expression. The level and distribution of expression were subjectively estimated and positive reaction strength was described as -, +, ++ and +++ (Table ll).

## Results

*Immunohistochemical findings of WT1, MUC1 and MHC class I protein expressions in tumor cells*. We studied WT1 protein expression in various solid tumors by immunohistochemical staining using monoclonal antibodies against WT1, MUC1 and MHC class I molecules. The results of the immunohistochemical staining of WT1 expressed in various solid tumors are shown in Figure 1. For malignant pleural mesothelioma, a strong expression of the WT1 protein was observed in the nucleus of tumor cells (Figure 1A). A weak expression of WT1 protein was observed only in the cytoplasm of cancer cells and in both the nucleus and cytoplasm of breast cancer and malignant pleural mesothelioma cells, respectively (Figure 1B and C). It is shown in Figure 1D that no WT1 protein expression was found in one patient with adenocarcinoma of the pancreas. Immunohistochemical staining showed MUC1 expression at the luminal and/or apical site of cancer cells (Figure 2A and B). MHC class I molecules were expressed in most of the solid tumors; however, loss or down-regulation of the expression of MHC class I molecules was observed in few tumors (Figure 2C and D).

*Analysis of WT1, MUC1 and MHC class I protein expression in various tumors classified on the basis of ICD10*. We categorized the WT1 expression patterns in tumors classified on the basis of ICD10 and ICD-O-3. Additionally, we also studied the expression patterns of MUC1, as one of the TAAs, and MHC class I molecules, as antigen presentation-associated molecules in various tumors. The expression levels of WT1, MUC1 and MHC class I in various tumors classified on the basis of ICD10 are shown in Table III. The expression of WT1 substantially differed depending on the tumor site, classified on the basis of ICD10. On the other hand, MUC1 expression was observed in most solid tumors. For malignant mesothelioma, WT1 expression was found in all tumors. Additionally, a high frequency of WT1 expression was shown (39.3%; 46/117) for the malignancies of female genital organs (ICD10; C52-C59), including cancers of the ovary (52.4%; 33/63). The frequency of WT1 expression was also relatively high in cancers of the bile duct (C23-24, 41.2%; 7/17), lung (C34, 35.0%; 42/120), breast (C50, 25.0%; 12/48) and prostate (C61, 28.6%; 4/14).

Regarding MUC1 protein expression, it was found in most solid tumors in the lip, oral cavity and pharynx (C02-13), respiratory and intrathoracic organs (C30-C38), skin

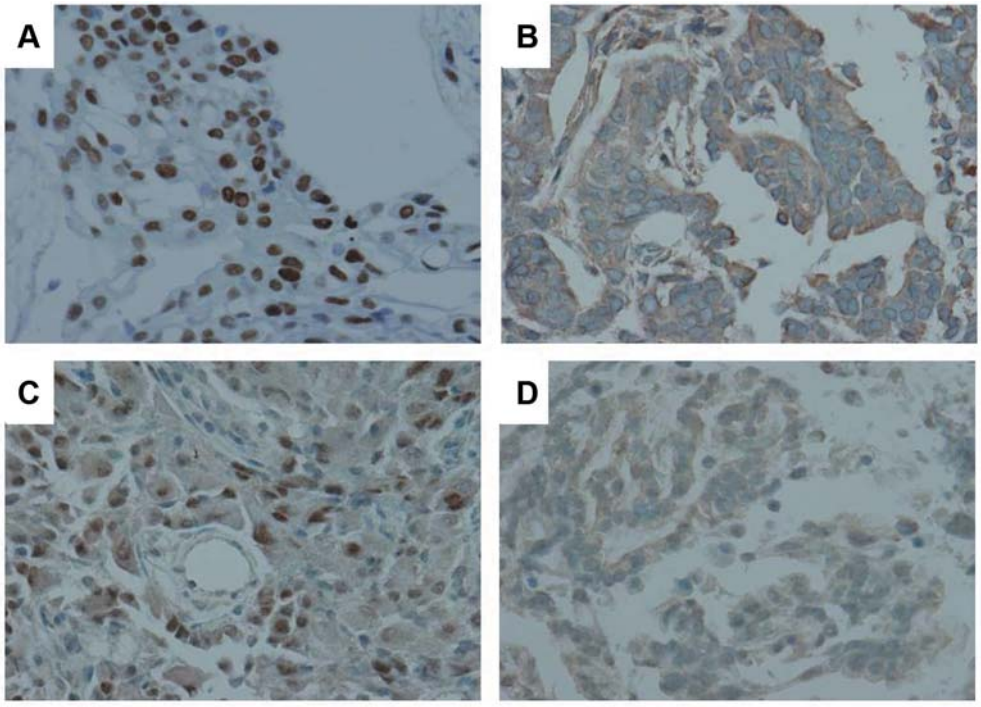


Figure 1. *Immunohistochemical staining of Wilms' tumor 1 (WT1) protein by monoclonal anti-WT1 antibody. Pleural mesothelioma showing 90% nuclear staining (A), breast cancer showing 90% cytoplasmic staining (B), pleural mesothelioma showing 80% cytoplasmic and nuclear staining (C), adenocarcinoma of the pancreas showing negative staining (D). Original magnification, ×400.*

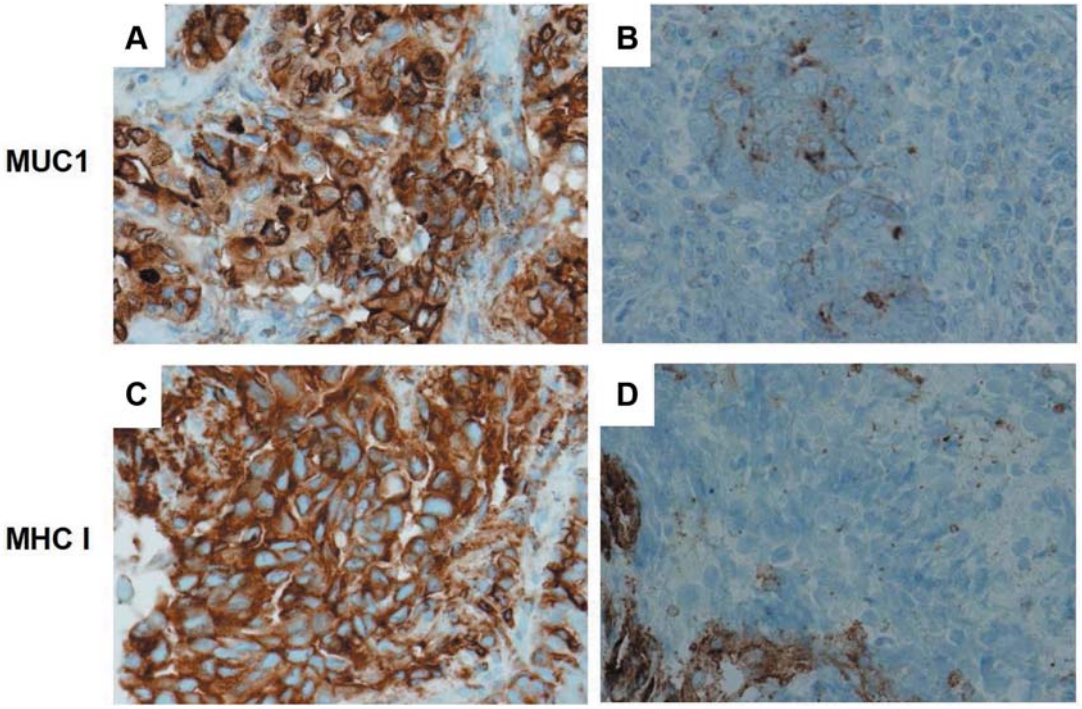


Figure 2. *Immunohistochemical staining of mucin 1 (MUC1) protein (A), (B) and major histocompatibility complex (MHC) class I molecules (C), (D). Pancreatic cancer showing cell membrane and nuclear staining (A) and negative staining (B). Adenocarcinoma of the lung showing cell membrane staining (C), small cell carcinoma of the lung showing negative staining (D). Original magnification, ×400.*

Table I. *Age and gender for each tumor type of malignancy.*

| ICD-10 | Tumor type | Number of patients | Median age (range) | | Gender (M:F) |
|---|---|---|---|---|---|
| Malignant neoplasms of lip, oral cavity and pharynx | | | | | |
| C02 | Tongue cancer | 6 | 53.5 | (23-77) | 1:5 |
| C03 | Gum cancer | 1 | 81 | - | 1:0 |
| C04 | Floor of mouth cancer | 1 | 66 | - | 1:0 |
| C06 | Mouth cancer | 1 | 63 | - | 1:0 |
| C07 | Parotid gland cancer | 1 | 67 | - | 1:0 |
| C08 | Salivary gland cancer | 1 | 58 | - | 1:0 |
| C10 | Oropharyngeal cancer | 3 | 53 | (39-70) | 2:1 |
| C11 | Nasopharyngeal cancer | 2 | 34 | (20-48) | 1:1 |
| C13 | Hypopharyngeal cancer | 2 | 67.5 | (63-72) | 2:0 |
| Malignant neoplasms of digestive organs | | | | | |
| C15 | Esopharyngeal cancer | 26 | 64 | (45-85) | 20:6 |
| C16 | Stomach cancer | 72 | 62 | (15-85) | 47:25 |
| C17 | Small intestine cancer | 11 | 60 | (37-84) | 9:2 |
| C18 | Colon cancer | 68 | 65 | (23-85) | 34:34 |
| C19 | Rectosigmoid junction cancer | 3 | 62 | (60-65) | 2:1 |
| C20 | Rectal cancer | 47 | 60 | (29-87) | 27:20 |
| C22 | Liver cancer | 22 | 65 | (25-83) | 12:10 |
| C23 | Gallbladder cancer | 5 | 55 | (40-78) | 5:0 |
| C24 | Bile duct cancer | 12 | 61.5 | (42-82) | 9:3 |
| C25 | Pancreatic cancer | 63 | 60 | (32-86) | 34:29 |
| Malignant neoplasms of respiratory and intrathoracic organs | | | | | |
| C30 | Nasal cavity and middle ear cancers | 2 | 68 | (65-71) | 0:2 |
| C31 | Accessory sinus cancer | 1 | 44 | - | 1:0 |
| C32 | Laryngeal cancer | 1 | 59 | - | 1:0 |
| C33 | Tracheal cancer | 2 | 43 | (27-59) | 2:0 |
| C34 | Lung cancer | 120 | 64 | (33-84) | 74:46 |
| C37 | Thymus cancer | 3 | 36 | (36-58) | 1:2 |
| C38 | Mediastinal cancer | 1 | 72 | - | 0:1 |
| Malignant neoplasms of bone and articular cartilage | | | | | |
| C40 | Osteosarcoma | 2 | 57.5 | (50-65) | 2:0 |
| C41 | Chondrosarcoma | 1 | 16 | - | 1:0 |
| Melanoma and other malignant neoplasms of skin | | | | | |
| C43 | Malignant melanoma | 8 | 60.5 | (22-77) | 3:5 |
| C44 | Skin cancer | 3 | 80 | (37-80) | 2:1 |
| Malignant neoplasms of mesothelial and soft tissues | | | | | |
| C45 | Malignant mesothelioma | 5 | 62 | (36-74) | 5:0 |
| C48 | Peritoneal cancer | 5 | 68 | (39-70) | 0:5 |
| C49 | Sarcoma | 4 | 30.5 | (27-50) | 3:1 |
| Malignant neoplasm of breast | | | | | |
| C50 | Breast cancer | 48 | 50 | (29-85) | - |
| Malignant neoplasms of female genital organs | | | | | |
| C52 | Vaginal cancer | 1 | 76 | - | - |
| C53 | Cervical cancer | 14 | 50 | (33-75) | - |
| C54 | Corpus uteri cancer | 26 | 61 | (38-74) | - |
| C55 | Uterine cancer | 12 | 45.5 | (31-65) | - |
| C56 | Ovarian cancer | 63 | 51 | (19-82) | - |
| C57 | Fallopian tube cancer | 1 | 60 | - | - |
| Malignant neoplasms of male genital organs | | | | | |
| C61 | Prostate cancer | 14 | 65 | (55-77) | - |
| C63 | Testicular cancer | 1 | 79 | - | - |
| Malignant neoplasms of urinary tract | | | | | |
| C64 | Kidney cancer | 9 | 56 | (12-68) | 8:1 |
| C65 | Renal pelvis cancer | 3 | 63 | (54-70) | 2:1 |
| C66 | Ureter cancer | 4 | 74 | (70-82) | 2:2 |
| C67 | Bladder cancer | 10 | 64.5 | (46-78) | 8:2 |
| C68 | Urethral cancer | 1 | 38 | - | 0:1 |

Table I. *Continued*

Table I. *Continued*

| ICD-10 | Tumor type | Number of patients | Median age (range) | | Gender (M:F) |
|---|---|---|---|---|---|
| Malignant neoplasms of eye, brain and other parts of central nervous system | | | | | |
| C70 | Malignant meningioma | 1 | 14 | - | 0:1 |
| C71 | Malignant brain tumor | 9 | 44 | (6-80) | 4:5 |
| Malignant neoplasms of thyroid and other endocrine glands | | | | | |
| C73 | Thyroid cancer | 3 | 59 | (53-73) | 2:1 |
| C74 | Adrenal gland cancer | 1 | 55 | - | 0:1 |
| C75 | Parathyroid cancer | 1 | 63 | - | 0:1 |
| Malignant neoplasms of ill-defined, secondary and unspecified sites | | | | | |
| C80 | Cancer of unknown primary site | 5 | 47 | (39-64) | 3:2 |
| Malignant neoplasms, stated or presumed to be primary, of lymphoid, hematopoietic and related tissues | | | | | |
| C84 | Peripheral T-cell lymphoma | 2 | 74.5 | (69-80) | 1:1 |
| C85 | Malignant lymphoma | 4 | 71 | (63-85) | 2:2 |
| | Total | 738 | 61 | (6-87) | 352:386 |

(C44), mesothelial tissues (C45), breast (C50), female genital organs (C52-57) and urinary tract (C64.65). For cancers of the digestive organs (C15-25), the MUC1 protein was expressed in more than 80% of the tumors in the esophagus, small intestine, bile duct or pancreas; however, the MUC1 positivity rates were 50-60% for cancers of the stomach, colon, rectum, liver and gallbladder. In this study, MHC class I expression was also investigated in various solid tumors and MHC class I molecules were found to be expressed in all cancer cells. We found nine malignant tumors with loss of MHC class I expression among 645 solid tumors, including four lung cancers, two corpus uteri cancers, one esophageal cancer, one ovarian cancer and one thyroid cancer.

*Analysis of WT1 protein expression in various tumors histologically classified on the basis of ICD-O-3*. We studied the expression patterns of WT1 in various solid tumors classified on the basis of ICD-O-3 (Table IV). For the histological types of cancer, the frequencies of WT1 expression were 44.2% (19/43) in cystic, mucinous and serous tumors, such as serous adenocarcinoma, mucinous cancer and signet ring cell carcinoma (M844-849); 62.5% (5/8) in complex mixed and stromal tumors, such as carcinosarcoma and adenosarcoma (M893-899); and 100% (5/5) in malignant mesothelioma (M905). Additionally, these three types of solid tumors demonstrated moderate to strong expression of WT1 (++ and +++) in cancer cells. A high expression positivity rate of WT1 was observed in myosarcoma (80.0%; 4/5), although a strong expression of WT1 was found only in one patient. For other histological types of cancer, the positivity rates of WT1 expression ranged from 6.3 to 50.0%; however, these tumor cells showed a weak expression of WT1. For the localization analysis of WT1 in cancer cells, the expression frequencies were 33.7, 59.4 and 4.3% in the nucleus, cytoplasm and both nucleus and cytoplasm of tumor cells, respectively (Table V). In most tumors with a strong expression of WT1, such as malignant mesothelioma, cystic, mucinous, serous tumors and complex mixed and stromal tumors, the WT1 protein localized in the nucleus of cells. However, for tumor cells with a weak (+) WT1 expression, the WT1 protein distributed uniformly in the cytoplasm of cancer cells. There were few tumors with the WT1 protein distributed in both the nucleus and cytoplasm.

Table II. *Evaluation method for immunohistochemical analysis*.

| Relative ratio (proportion) | |
|---|---|
| (–) | A positive cell is not recognized as a neoplastic cell (0%) |
| (+) | About 1/4 of the neoplastic cells are positive cells (less than 1/2) |
| (++) | About 1/2 of the neoplastic cells are positive cells (less than 3/4) |
| (+++) | About 3/4 of the neoplastic cells are positive cells with high reaction strength (intensity) (with the positive cells mostly neoplastic cells) |
| **Positive reaction strength (intensity)** | |
| (–) | Negative |
| (+) | Weak reaction: observable but judged to be relatively weak under high magnification |
| (++) | Moderate reaction: observable but judged to be slightly weak under low magnification |
| (+++) | Strong reaction: easily observable under low magnification |

Table III. *Results of immunohistochemical analysis of Wilms' tumor 1 (WT1), major histocompatibility complex (MHC) class I and mucin 1 (MUC1).*

| ICD-10 | Tumor type | WT1 | | MUC1 | | MHC-I | |
|---|---|---|---|---|---|---|---|
| | | No. of positive cases | Positive ratio (%) | No. of positive cases | Positive ratio (%) | No. of positive cases | Positive ratio (%) |
| Malignant neoplasms of lip, oral cavity, and pharynx | | | | | | | |
| C02 | Tongue cancer | 1/6 | 16.7 | 6/6 | 100.0 | 6/6 | 100.0 |
| C03 | Gum cancer | 0/1 | 0.0 | 0/1 | 0.0 | 1/1 | 100.0 |
| C04 | Floor of mouth cancer | 1/1 | 100.0 | 1/1 | 100.0 | 1/1 | 100.0 |
| C06 | Mouth cancer | 0/1 | 0.0 | 1/1 | 100.0 | 1/1 | 100.0 |
| C07 | Parotid gland cancer | 1/1 | 100.0 | 1/1 | 100.0 | 1/1 | 100.0 |
| C08 | Salivary gland cancer | 0/1 | 0.0 | 1/1 | 100.0 | 1/1 | 100.0 |
| C10 | Oropharyngeal cancer | 0/3 | 0.0 | 3/3 | 100.0 | 3/3 | 100.0 |
| C11 | Nasopharyngeal cancer | 0/2 | 0.0 | 1/2 | 50.0 | 2/2 | 100.0 |
| C13 | Hypopharyngeal cancer | 0/2 | 0.0 | 2/2 | 100.0 | 2/2 | 100.0 |
| Malignant neoplasms of digestive organs | | | | | | | |
| C15 | Esopharyngeal cancer | 5/26 | 19.2 | 20/21 | 95.2 | 20/21 | 95.2 |
| C16 | Stomach cancer | 13/72 | 18.1 | 37/63 | 58.7 | 61/61 | 100.0 |
| C17 | Small intestine cancer | 2/11 | 18.2 | 8/10 | 80.0 | 10/10 | 100.0 |
| C18 | Colon cancer | 6/68 | 8.8 | 28/56 | 50.0 | 57/57 | 100.0 |
| C19 | Rectosigmoid junction cancer | 0/3 | 0.0 | 0/2 | 0.0 | 2/2 | 100.0 |
| C20 | Rectal cancer | 5/47 | 10.6 | 24/42 | 57.1 | 41/41 | 100.0 |
| C22 | Liver cancer | 2/22 | 9.1 | 9/17 | 52.9 | 17/17 | 100.0 |
| C23 | Gallbladder cancer | 2/5 | 40.0 | 3/5 | 60.0 | 5/5 | 100.0 |
| C24 | Bile duct cancer | 5/12 | 41.7 | 8/10 | 80.0 | 10/10 | 100.0 |
| C25 | Pancreatic cancer | 19/63 | 30.2 | 56/62 | 90.3 | 60/60 | 100.0 |
| Malignant neoplasms of respiratory and intrathoracic organs | | | | | | | |
| C30 | Nasal cavity and middle ear cancers | 2/2 | 100.0 | 2/2 | 100.0 | 2/2 | 100.0 |
| C31 | Accessory sinus cancer | 0/1 | 0.0 | 1/1 | 100.0 | 1/1 | 100.0 |
| C32 | Laryngeal cancer | 0/1 | 0.0 | 0/1 | 0.0 | 1/1 | 100.0 |
| C33 | Tracheal cancer | 1/2 | 50.0 | 2/2 | 100.0 | 2/2 | 100.0 |
| C34 | Lung cancer | 42/120 | 35.0 | 97/109 | 89.0 | 101/105 | 96.2 |
| C37 | Thymus cancer | 1/3 | 33.3 | 2/3 | 66.7 | 3/3 | 100.0 |
| C38 | Mediastinal cancer | 0/1 | 0.0 | 0/0 | 0/0 | | |
| Malignant neoplasms of bone and articular cartilage | | | | | | | |
| C40 | Osteosarcoma | 0/2 | 0.0 | 1/2 | 50.0 | 2/2 | 100.0 |
| C41 | Chondrosarcoma | 0/1 | 0.0 | 0/1 | 0.0 | 1/1 | 100.0 |
| Melanoma and other malignant neoplasms of skin | | | | | | | |
| C43 | Malignant melanoma | 3/8 | 37.5 | 1/4 | 25.0 | 7/7 | 100.0 |
| C44 | Skin cancer | 0/3 | 0.0 | 3/3 | 100.0 | 2/2 | 100.0 |
| Malignant neoplasms of mesothelial and soft tissues | | | | | | | |
| C45 | Malignant mesothelioma | 5/5 | 100.0 | 3/3 | 100.0 | 3/3 | 100.0 |
| C48 | Peritoneal cancer | 4/5 | 80.0 | 4/5 | 80.0 | 5/5 | 100.0 |
| C49 | Sarcoma | 1/4 | 25.0 | 0/4 | 0.0 | 4/4 | 100.0 |
| Malignant neoplasm of breast | | | | | | | |
| C50 | Breast cancer | 12/48 | 25.0 | 45/45 | 100.0 | 43/43 | 100.0 |
| Malignant neoplasms of female genital organs | | | | | | | |
| C52 | Vaginal cancer | 1/1 | 100.0 | 0/0 | 1/1 | 100.0 | |
| C53 | Cervical cancer | 3/14 | 21.4 | 11/13 | 84.6 | 11/11 | 100.0 |
| C54 | Corpus uteri cancer | 6/26 | 23.1 | 17/22 | 77.3 | 20/22 | 90.9 |
| C55 | Uterine cancer | 2/12 | 16.7 | 9/11 | 81.8 | 10/10 | 100.0 |
| C56 | Ovarian cancer | 33/63 | 52.4 | 51/54 | 94.4 | 55/56 | 98.2 |
| C57 | Fallopian tube cancer | 1/1 | 100.0 | 1/1 | 100.0 | 1/1 | 100.0 |
| Malignant neoplasms of male genital organs | | | | | | | |
| C61 | Prostate cancer | 4/14 | 28.6 | 5/11 | 45.5 | 12/12 | 100.0 |
| C63 | Testicular cancer | 0/1 | 0.0 | 0/0 | 0/0 | | |
| Malignant neoplasms of urinary tract | | | | | | | |
| C64 | Kidney cancer | 1/9 | 11.1 | 8/9 | 88.9 | 9/9 | 100.0 |
| C65 | Renal pelvis cancer | 1/3 | 33.3 | 3/3 | 100.0 | 3/3 | 100.0 |

Table III. *Continued*

Table III. *Continued*

| ICD-10 | Tumor type | WT1 | | MUC1 | | MHC-I | |
|---|---|---|---|---|---|---|---|
| | | No. of positive cases | Positive ratio (%) | No. of positive cases | Positive ratio (%) | No. of positive cases | Positive ratio (%) |
| C66 | Ureter cancer | 0/4 | 0.0 | 4/4 | 100.0 | 4/4 | 100.0 |
| C67 | Bladder cancer | 0/10 | 0.0 | 9/9 | 100.0 | 9/9 | 100.0 |
| C68 | Urethral cancer | 0/1 | 0.0 | 1/1 | 100.0 | 1/1 | 100.0 |
| Malignant neoplasms of eye, brain and other parts of central nervous system | | | | | | | |
| C70 | Malignant meningioma | 0/1 | 0.0 | 1/1 | 100.0 | 1/1 | 100.0 |
| C71 | Malignant brain tumor | 1/9 | 11.1 | 1/5 | 20.0 | 7/7 | 100.0 |
| Malignant neoplasms of thyroid and other endocrine glands | | | | | | | |
| C73 | Thyroid cancer | 0/3 | 0.0 | 2/3 | 66.7 | 2/3 | 66.7 |
| C74 | Adrenal gland cancer | 0/1 | 0.0 | 0/0 | 0/0 | | |
| C75 | Parathyroid cancer | 0/1 | 0.0 | 0/1 | 0.0 | 1/1 | 100.0 |
| Malignant neoplasms of ill-defined, secondary and unspecified sites | | | | | | | |
| C80 | Cancer of unknown primary site | 1/5 | 20.0 | 2/5 | 40.0 | 5/5 | 100.0 |
| Malignant neoplasms, stated or presumed to be primary, of lymphoid, hematopoietic and related tissues | | | | | | | |
| C84 | Peripheral T-cell lymphoma | 0/2 | 0.0 | 1/1 | 100.0 | 2/2 | 100.0 |
| C85 | Malignant lymphoma | 0/4 | 0.0 | 1/2 | 50.0 | 4/4 | 100.0 |
| Total | 187/738 | 25.3 | 497/647 | 76.8 | 636/645 | 98.6 | |

## Discussion

We evaluated the WT1 expression in cancer tissues of 738 patients using the anti-WT1 monoclonal (6F-H2) antibody. The positive staining of WT1 in tumor cells was observed in 25.3% of patients, indicating that WT1 was not strongly expressed in many cancer cells. WT1 was expressed in malignant mesothelioma, peritoneal cancer and ovarian cancer (malignancy classification according to ICD-10). On the other hand, malignant mesothelioma, serous adenocarcinoma, mucinous cancer, signet ring cell carcinoma, carcinosarcoma and adenosarcoma (malignancy classification according to ICD-O-3) showed strong WT1 expressions. These findings may provide potentially meaningful information when considering WT1-targeted therapies, such as peptide vaccine and DC vaccine therapies. Antigenic expression in cancer tissues is one of the important factors for the aforementioned antigen-specific immunotherapies. Despite the clinical development of WT1-targeted therapies in recent years, the expression level of WT1 in cancer tissue remains controversial (22, 23).

Nakatsuka *et al*. focused on issues associated with the analysis of the expression of WT1 in the cytoplasm, as well as the nucleus of cells (23). Even though we confirmed the cytoplasmic, as well as nuclear, WT1 expression, a low WT1 positivity rate was observed. Such discrepancies may be explained by the current use of non-standardized immunohistochemical techniques for measuring WT1 expression levels in cancer cells. Therefore, to effectively implement WT1-targeted therapies, the staining method should be standardized. Similarly, the same issue-solving must be considered when using the tissue expression of programmed death-ligand 1 (PDL-1) as a biomarker of PD-1/PD-L1 pathway blockade for WT1-targeted therapies (24).

From the results of this study, WT1 was found to have the tendency to localize in the nucleus and cytoplasm. In malignancies of the female genital organ and mesothelioma, WT1 was observed to mainly localize in the nucleus and cytoplasm in cancers of the digestive organs. Interestingly, among adenocarcinomas, WT1 localization in the nucleus was observed in 94.1% of patients with ovarian cancer, while only 6.8% of patients with other cancer types showed mostly cytoplasmic WT1 localization.

WT1 was expressed (+, ++ and +++) in 25.3% of the patients; however, only 8.5% of them showed moderate to strong expression (++ and +++). It is in our practice to apply the DC vaccine therapy to patients with more than moderate (++) WT1 expression. Therefore, immunohistochemical staining is essential for DC vaccine therapy.

Since TSAs are not encoded in the normal host genome, oncogenic viral proteins and abnormal proteins arising from mutated somatic cells have recently been receiving attention as a target for immunotherapy (25). TAAs, including WT1, have versatility but not affinity compared with TSAs; thus,

Table IV. *Intensity of Wilms' tumor 1 (WT1) immunohistochemical staining.*

| ICD-O-3 | Histological type | Case number (%) | | | | | | |
|---|---|---|---|---|---|---|---|---|
| | | – | | 1+ | | 2+ – 3+ | | Total |
| M801-M804 | Large cell carcinoma<br>Undifferentiated carcinoma<br>Small cell carcinoma | 20 | (71.4) | 7 | (25.0) | 1 | (3.6) | 28 |
| M805-M808 | Squamous cell carcinoma | 64 | (75.3) | 18 | (21.2) | 3 | (3.5) | 85 |
| M809-M811 | Basal cell carcinoma | 1 | (100.0) | 0 | | 0 | | 1 |
| M812-M813 | Transitional cell carcinoma | 15 | (93.8) | 1 | (6.3) | 0 | | 16 |
| M814-M838 | Adenocarcinoma | 363 | (77.2) | 79 | (16.8) | 28 | (6.0) | 470 |
| M843 | Mucoepidermoid carcinoma | 1 | (50.0) | 1 | (50.0) | 0 | | 2 |
| M844-M849 | Serous adenocarcinoma<br>Mucinous adenocarcinoma<br>Signet ring cell carcinoma | 24 | (55.8) | 3 | (7.0) | 16 | (37.2) | 43 |
| M850-M854 | Infiltrating duct adenocarcinoma<br>Paget disease | 24 | (77.4) | 4 | (12.9) | 3 | (9.7) | 31 |
| M855 | Acinar cell carcinoma | 1 | (100.0) | 0 | | 0 | | 1 |
| M856-M857 | Adenosquamous carcinoma | 3 | (75.0) | 1 | (25.0) | 0 | | 4 |
| M858 | Malignant thymoma | 1 | (50.0) | 1 | (50.0) | 0 | | 2 |
| M872-879 | Malignant melanoma | 5 | (62.5) | 3 | (37.5) | 0 | | 8 |
| M881-M883 | Fibromyxosarcoma | 2 | (100.0) | 0 | | 0 | | 2 |
| M885-M888 | Liposarcoma | 1 | (100.0) | 0 | | 0 | | 1 |
| M889-M892 | Myosarcoma | 1 | (20.0) | 3 | (60.0) | 1 | (20.0) | 5 |
| M893-M899 | Adenosarcoma<br>Carcinosarcoma | 3 | (37.5) | 0 | | 5 | (62.5) | 8 |
| M904 | Synovial sarcoma | 2 | (100.0) | 0 | | 0 | | 2 |
| M905 | Malignant mesothelioma | 0 | (0.0) | 0 | | 5 | (100.0) | 5 |
| M906-M909 | Germ cell tumor | 1 | (100.0) | 0 | 0 | 1 | | |
| M912-M916 | Hemangiosarcoma | 0 | (0.0) | 1 | (100.0) | 0 | | 1 |
| M918-M924 | Chondrosarcoma | 2 | (100.0) | 0 | | 0 | | 2 |
| M938-M948 | Malignant glioma | 7 | (87.5) | 1 | (12.5) | 0 | | 8 |
| M949-M952 | Neuroepithelioma | 1 | (100.0) | 0 | | 0 | | 1 |
| M953 | Malignant meningioma | 1 | (100.0) | 0 | | 0 | | 1 |
| M959-M972 | Malignant lymphoma | 6 | (100.0) | 0 | | 0 | | 6 |
| Unknown | Unknown | 2 | (50.0) | 1 | (25.0) | 1 | (25.0) | 4 |
| Total | 551 | | (74.7) | 124 | (16.8) | 63 | (8.5) | 738 |

TAAs may not be capable of eliciting effective antitumor immunoresponses. Therefore, an effective extensive induction of the immunoresponses of T lymphocytes, along with DCs, CD4+ T cells and CD8+ T cells in the body, using long peptides, is a way to realize the beneficial effects of DC vaccine therapy *via* TAAs (26).

Positive staining of tumor cells by the anti-MUC1 monoclonal antibody was observed in 76.8% of samples. Many samples of cancer tumor cells showed MUC1 expression, whereas a weak expression was observed in some types of cancer of the stomach, colon, rectum, liver or gallbladder. These findings provide information useful for determining the efficacy of MUC1-targeted therapy similarly to WT1-targeted therapy. MUC1, a TAA, is expressed in various types of somatic cells; consequently, there is an undeniable possibility that lymphocytes are anergic (27) towards MUC1 and may not also be activated by a vaccine.

A positive staining of tumor cells by the anti-MHC class l monoclonal antibody was observed in 98.6% of samples. For application of specific immunotherapy, this finding indicates that the presence of MHC class l is not a limiting factor. It is the selection of the appropriate cancer antigens that is of importance. Therefore, $CD8^+$ T lymphocyte-based therapies, in relation to the cancer immunity cycle, can form the basis for immune-cell therapy. Inhibitory NK cell receptors recognize self-MHC class l molecules, that prevent NK cell activation (28), on the basis of which the 'missing-self' hypothesis has been proposed. In the practice of NK cell therapy, this finding shows the importance of confirming the presence of rare MHC class l abnormalities by prior examinations.

In conclusion, relating to the application of cancer-specific immunotherapy, these findings provide information useful for determining the efficacy of MUC1- and WT1-targeted therapy.

Table V. *Distribution of Wilms' tumor 1 (WT1) protein in cells determined by immunohistochemical staining.*

| ICD-O-3 | Histological type | Case number (%) | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|
| | | Nucleus | | Cytoplasm | | Nucleus & cytoplasm | | Unknown | | Total |
| M801-M804 | Large cell carcinoma<br>Undifferentiated carcinoma<br>Small cell carcinoma | 2 | (25.0) | 6 | (75.0) | 0 | | 0 | | 8 |
| M805-M808 | Squamous cell carcinoma | 4 | (19.0) | 16 | (76.2) | 1 | (4.8) | 0 | | 21 |
| M812-M813 | Transitional cell carcinoma | 0 | | 1 | (100.0) | 0 | | 0 | | 1 |
| M814-M838 | Adenocarcinoma | 22 | (20.6) | 79 | (73.8) | 3 | (2.8) | 3 | (2.8) | 107 |
| M843 | Mucoepidermoid carcinoma | 0 | | 1 | (100.0) | 0 | | 0 | | 1 |
| M844-M849 | Serous adenocarcinoma<br>Mucinous adenocarcinoma<br>Signet ring cell carcinoma | 18 | (94.7) | 1 | (5.3) | 0 | | 0 | | 19 |
| M850-M854 | Infiltrating duct adenocarcinoma<br>Paget disease | 2 | (28.6) | 5 | (71.4) | 0 | | 0 | | 7 |
| M856-M857 | Adenosquamous carcinoma | 0 | | 1 | (100.0) | 0 | | 0 | | 1 |
| M858 | Malignant thymoma | 0 | | 0 | | 1 | (100.0) | 0 | | 1 |
| M872-879 | Malignant melanoma | 2 | (66.7) | 0 | | 1 | (33.3) | 0 | | 3 |
| M889-M892 | Myosarcoma | 3 | (75.0) | 0 | | 1 | (25.0) | 0 | | 4 |
| M893-M899 | Adenosarcoma<br>Carcinosarcoma | 5 | (100.0) | 0 | | 0 | | 0 | | 5 |
| M905 | Malignant mesothelioma | 4 | (80.0) | 0 | | 1 | (20.0) | 0 | | 5 |
| M912-M916 | Hemangiosarcoma | 0 | | 1 | (100.0) | 0 | | 0 | | 1 |
| M938-M948 | Malignant glioma | 0 | | 0 | | 0 | | 1 | (100.0) | 1 |
| Unknown | Unknown | 1 | (50.0) | 0 | | 0 | | 1 | (50.0) | 2 |
| Total | | 63 | (33.7) | 111 | (59.4) | 8 | (4.3) | 5 | (2.7) | 187 |

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