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Clinicopathological significance and prognostic value of Wilms' tumor gene expression in colorectal cancer

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Abstract.
BACKGROUND: Colorectal cancer (CRC) is one of the most commonly diagnosed cancers, and novel effective treatments and diagnostic tools are urgently required.
OBJECTIVE: The selection of appropriate targeting tumor-associated antigens (TAAs) is critical for immunotherapy. Therefore, we analyzed TAA expression levels and investigated their relationship with clinical factors in adjacent normal mucosa (ANM) and CRC tissue.
METHODS: We obtained specimens of CRC primary tumors and matched ANM from 137 patients with CRC who underwent surgical resection. The mRNA levels of seven TAAs, Wilms' tumor gene (*WT1*), kinetochore associated-2 (*KNTC2*), cell division cycle associated-1 (*CDCA1)*, M phase phosphoprotein-1 (*MPHOSPH1*), DEP domain-containing 1 (*DEPDC1*), 34-kDa translocase of the outer mitochondrial membrane (*TOMM34*) and ring finger protein-43 (*RNF43*), were analyzed using quantitative real-time reverse transcription-polymerase chain reaction, and their relationships with clinicopathological factors and the cell cycle were analyzed.
RESULTS: The expression levels of all seven TAAs were significantly higher in CRC tissues than in ANM. Expression levels of *WT1* in CRC tissues did not correlate with the cell cycle. Furthermore, *WT1* expression in CRC tissues was significantly related to tumor progression, lymph node metastasis, distant metastasis and clinical stage.
CONCLUSIONS: WT1 is a potential marker for prognosis and tumor progression in CRC.

Keywords: Colorectal cancer, tumor-associated antigens, Wilms' tumor gene

1. Introduction

Colorectal cancer (CRC) is one of the most commonly diagnosed cancers worldwide, and accounts for an estimated 9.4% of all malignancies [1]. In Asian countries, the incidence of CRC has increased approximately 2–4-fold over the past few decades [2]. The most effective treatment for CRC is surgery, but approximately 60% of patients who undergo curative re-

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section experience local recurrence or distant metastasis [3]. Despite advances in surgical techniques and chemotherapeutic options, the survival rate of CRC has not substantially improved; around 20% of patients with CRC die from recurrence of the disease [1]. Thus, novel effective treatments and diagnostic tools are required to treat CRC. New immunotherapies that target tumor-associated antigens (TAAs) have been used to treat CRC, in addition to conventional treatments [4]. It has been demonstrated that CD8 cytotoxic T lymphocytes (CTLs) recognize epitope peptides derived from TAAs presented on MHC Class I molecules and then lyse the tumor cells. TAAs are proteins that are known to be overexpressed in and broadly distributed among malignant cells of various origins [5,6]. Many TAAs in various human malignancies were identified using cDNA expression cloning methods [7]. Immunotherapy is an active therapeutic approach designed to trigger the immune system to respond to tumor-specific antigens and attack tumor cells [8] and immunotherapy strategies include the use of peptides in TAAs. With antigenic epitope peptide vaccines derived from these TAAs, clinical trials have been initiated by multiple groups to treat cancer patients [9–11]. Several vaccines for peptides, such as those for Wilms' tumor gene (*WT1*) [4,12], 34-kDa translocase of the outer mitochondrial membrane (*TOMM34*), and ring finger protein 43 (*RNF43*) [13] have been suggested to be clinically beneficial for metastatic CRC, in particular, *TOMM34* and *RNF43*, which have been examined in phase I clinical trials [13]. These clinical trials have led to the possibility of developing new peptide-based cancer immunotherapies. However, these therapies have seen only a few recent successes and a tremendous number of failures [14]. In fact, clinical trials of TAA cell-targeted strategies are not always accepted because of the lack of reliable markers and the poor understanding of their behavior. However, combined immunotherapy and conventional chemotherapy treatments can simultaneously kill cancer cells and boost antitumor immune responses, and thus have significant advantages [15,16]. There have been reports that treatment with chemotherapy such as cyclophosphamide or gemcitabine can augment the antitumor effects of cancer immunotherapy [17], and immunotherapy has the potential to improve the effect of cancer treatments. Therefore, selection of appropriate targeting TAAs for effective immunotherapy is critical. An ideal TAA should have the characteristics of tumor-specific-antigens, which are silenced in normal tissues but overexpressed in cancer tissues. Immunotherapy is often recommended for advanced cancer patients in addition to surgery and chemotherapy, and thus it is preferable that the expression levels of TAAs are significantly higher in malignancies with tumor progression. The identification of TAAs at an early stage in tumor progression has clear implications for the continued development of cancer immunotherapy. Although TAA expression is generally associated with tumor progression and immunogenicity in various cancer types, little is known about their expression in relation to CRC progression [13]. In the present study, we analyzed TAA expression levels and investigated their relationship with clinical factors in ANM and CRC tissues using quantitative real-time reverse transcription-polymerase chain reaction (qPCR).

2. Materials and methods

2.1. Patients and samples

Tissue specimens were obtained from 137 patients (70 men, 67 women; age range, 30–91 years) who underwent surgical resection for CRC at Sagamihara National Hospital between 2010 and 2013. As control samples, resection specimens were taken from matched adjacent normal tissue (ANM) in CRC patients, and additional samples were taken from 10 patients with non-neoplastic colorectal polyps (CP). The ANM specimens were obtained from the margin of the CRC primary tumor, $\geqslant$ 10 cm away. All specimens were confirmed as CRC, ANM and CP by a pathologist. The ANM and CP specimens were further confirmed to be free of cancer cells by the pathologist. The diameters of the primary tumors (T), numbers of lymph nodes with metastases (N) and development of distant metastases (M) were classified according to the Union for International Cancer Control (UICC) TNM staging system. The study protocol was approved by the Research Ethical Committee of Sagamihara Hospital (Sagamihara, Japan). Informed consent for this study was obtained from all patients.

2.2. Preparation of tissue specimens

After surgery or biopsy, one-half of each CRC, ANM and CP specimen was immediately soaked in RNAlater RNA Stabilization Reagent (Qiagen, Hilden, Germany) and stored at -80°C until RNA extraction.

2.3. RNA extraction and cDNA synthesis

CRC, ANM and CP specimens were homogenized with a vortex-type homogenizer (Shakeman 2; Bio

Table 1
qPCR primer sequences

Targets	Forward primers (5′–3′)	Reverse primers (5′–3′)
GAPDH	GCACCGTCAAGGCTGAGAAC	ATGGTGGTGAAGACGCCAGT
HPRT	GGCAGTATAATCCAAAGATGGTCAA	GTCAAGGGCATATCCTACAACAAAC
ACTB	TGGCACCCAGCACAATGAA	CTAAGTCATAGTCCGCCTAGAAGCA
SDHA	TGGGAACAAGAGGGCATCTG	CCACCACTGCATCAAATTCATG
B2M	CTATCCAGCGTACTCCAAAGA	AAGACAAGTCTGAATGCTCCAC
UBC	TCCCTTCTCGGCGATTCTG	TCTGCATTGTCATGAGGCGAT
rRNA	CGACCATAAACGATGCCGAC	GGTGGTGCCCTTCCGTCAAT
TBP	CCATGACTCCCGGAATCCCTAT	ATAGGCTGTGGGGTCAGTCCA
WT1	AGGGTACGAGCGATAACCACAC	CTCAGATGCCGACCGTACAAGA
CDCA1	AGAAGCATGCCGTGAAACGTA	CCATCTGAAAGCTGCTTGAACTC
KNTC2	AACCAAGGACCTGGAAGCTGAAC	TTTGGAATTCTCAGCACCTTTAGGA
MPHOSPH1	AATTGGTGTAAACCTGGCCACTA	CTTGCTTCTACATTTGAGAGCTTTG
DEPDC1	GGTTCTGATTATGCCTACTGGTTGA	TGGAATCTATCCATGTTCCAGCTTA
TOMM34	AAGCCAGAGTTCTGAAGGAAGAAGG	AGCTTGAGGGCTTCTGTGCAGT
RNF43	TGGTTACATCAGCATCGGACTTG	TGGAGTCTTCGACCTGGTTCTTG
CYCLIN A	CACTCTACACAGTCACGGGACAAAG	GTCTGGTGAAGGTCCATGAGACA

Medical Science, Tokyo, Japan). Total RNA was extracted using an RNeasy Lipid Tissue Kit (Qiagen). The quality and concentration of total RNA were validated using an Agilent 2100 Bioanalyzer (Agilent, Santa Clara, CA). Freshly isolated total RNA from CRC tissues and ANM was converted to cDNA using a PrimeScript RT Reagent Kit (Takara Bio, Shiga, Japan) according to the manufacturer's instructions.

2.4. qPCR

qPCR was performed using a Bio-Rad CFX96 system (Bio-Rad, Hercules, CA) following the manufacturer's protocols. Specific primers for *WT1*, cell division cycle associated 1 (*CDCA1*), kinetochore associated 2 (*KNTC2*), M phase phosphoprotein 1 (*MPHOSPH1*), DEP domain containing 1 (*DEPDC1*), *TOMM34*, *RNF43* and cyclin A (*CCNA2*) and the housekeeping genes glyceraldehyde-3-phosphate dehydrogenase (*GAPDH*), hypoxanthine phosphoribosyltransferase (*HPRT*), β-actin (*ACTB*), succinate dehydrogenase complex, subunit A (*SDHA*), β2-microgl ohulin ($B2M$), ubiquitin C (*UBC*), 18S ribosomal RNA (*rRNA*) and TATA-box binding protein (*TBP*) were purchased from Takara Bio (Otsu, Japan) (Table 1). The amplified products using the individual primer pairs were cloned into the pGEM-T vector (Promega, Madison, WI). The same plasmid was linearized by enzymatic digestion for use as a quantification standard. The sequences were confirmed by DNA sequencing using a CEQ8000 Genetic Analysis System (Beckman Coulter, Fullerton, CA). The quality and concentration of the quantification standard were validated using an Agilent 2100 Bioanalyzer (Agilent). The PCR reaction consisted of 5 μl of SsoFast EvaGreen Supermix (Bio-Rad), 3.5 μl of RNase/DNase-free water, 0.5 μl of 5 μM primer mix, and 1 μl of cDNA in a final volume of 10 μl. The cycle number for qPCR was 50. Calculated copy numbers were normalized based on the copy numbers of the housekeeping gene *ACTB*. All experiments were performed in duplicate.

2.5. Statistical analyses

To compare the results from the qPCR assays between expression levels in CRC, ANM and CP samples, we used one-way ANOVAs with Tukey-Kramer multiple comparison post-tests. Correlations between the expression levels of TAAs and *CCNA2* used Spearman's rank coefficients. Correlations between TAA expression levels and clinical stages used Kruskal-Wallis tests and Pearson product-moment coefficients. One-way ANOVAs with Tukey-Kramer multiple comparison post tests were performed for comparisons of the clinical stages. All statistical analyses were carried out using StatView software (SAS, Cary, NC). Results of comparisons are shown as mean values. P-values of $<$ 0.05 was considered significant.

3. Results

3.1. Selection of appropriate housekeeping genes in CRC

We investigated the mRNA levels of three housekeeping genes in CRC and ANM using qPCR (Fig. 1). *GAPDH*, *HPRT* and *UBC* mRNA levels were significantly higher in CRC tissues than in ANM ($P < 0.001$), but *ACTB* and *TBP* were not significantly higher ($P = 0.62$ and $P = 0.55$, respectively).

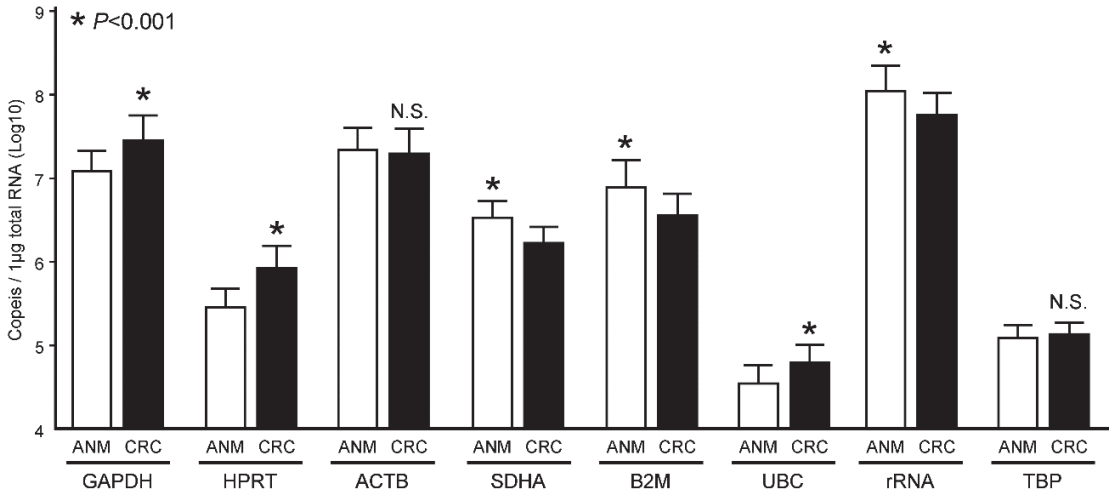

Fig. 1. Selection of appropriate housekeeping genes in CRC. mRNA expression levels of housekeeping genes were measured by qPCR in each tissue sample. Open and filled bars indicate ANM or CRC respectively; vertical error bars indicate mean ± SD. The expression levels of *ACTB* and *TBP* were almost equal in ANM and CRC tissues. Statistical analyses used Wilcoxon signed-rank tests. $^{*}P < 0.001$.

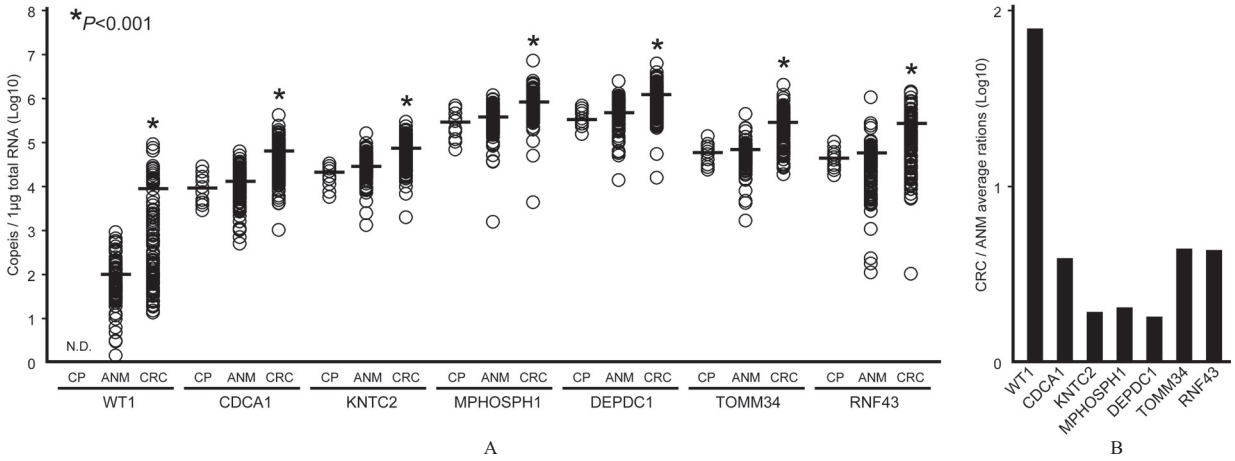

Fig. 2. Gene expression profiles of seven TAAs in ANM, CRC and CP tissues. Relative mRNA expression levels of TAAs in ANM, CRC and tissues were examined by qPCR. *ACTB* was used as an internal control. All experiments were performed in duplicate. (A) mRNA levels of all seven TAAs were significantly higher in CRC tissues than in ANM and CP samples. Statistical analyses used one-way ANOVAs with Tukey-Kramer multiple comparison post-tests. $^{*}P < 0.001$. (B) Comparisons of mRNA expression levels are shown as CRC/ANM average ratios. The mRNA expression of *WT1* was increased by approximately 82-fold, while other TAAs were increased around 2–5-fold.

3.2. *mRNA expression profiles of seven TAAs in NOM, ANM and CRC*

We next determined the mRNA levels of seven TAAs in 137 paired CRC tissues and ANM, and 10 CPs using qPCR. Expression levels of all seven TAAs were significantly increased in CRC tissues than in ANM and CP samples ($P < 0.001$; Fig. 2A). In the ANM, the mRNA expression levels of *WT1* were lower than other TAAs. The average expression level of *WT1* was approximately 82-fold higher in CRC tissue than ANM, whereas the other TAAs were only 2–5-fold higher (Fig. 2B). Moreover, in the CP samples, mRNA expression levels of WT1 were not detected.

3.3. *Correlations between TAA expression levels and CCNA2 in CRC*

We analyzed expression levels of *CCNA2* and TAAs in CRC tissues (Fig. 3), using 44 randomly selected samples from the 137 samples. *CCNA2* expression was strongly correlated with expression of *CDCA1*,

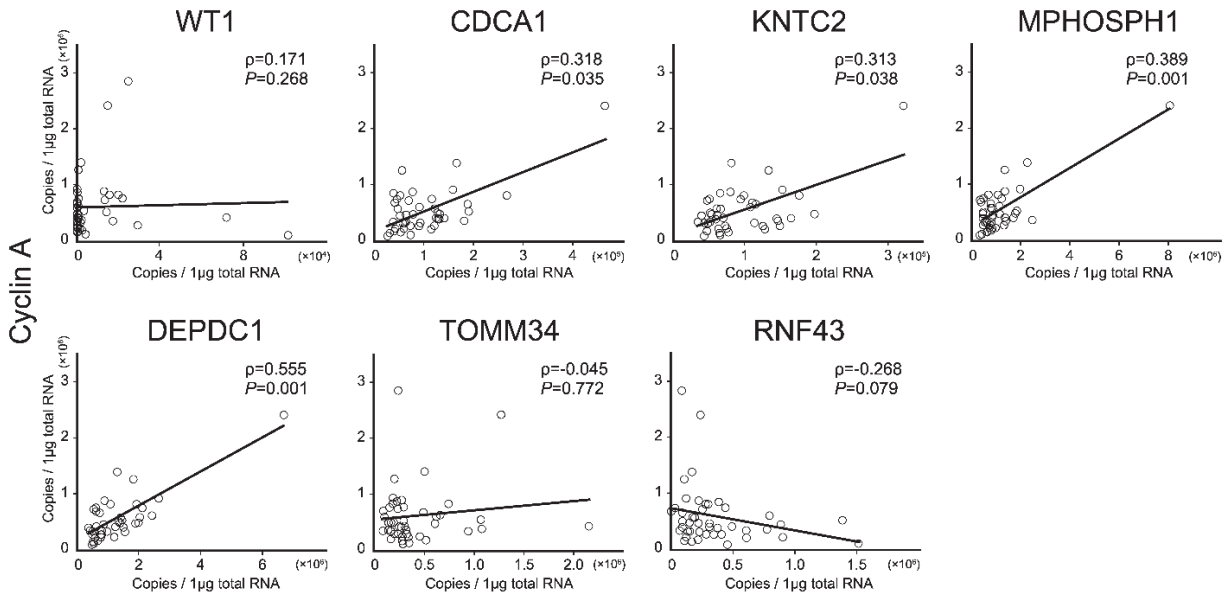

Fig. 3. Correlations between mRNA expression levels of TAAs and *CCNA2* in CRC. The mRNA expression levels of TAAs and *CCNA2* were measured by qPCR. *ACTB* was used as an internal control. Expression levels of *CCNA2* in CRC tissues significantly correlated with *CDCA1*, *KNTC2*, *MPHOSPH1*, and *DEPDC1* in CRC tissues, but not with *WT1*, *TOMM34* and *RNF43*. Statistical analyses used Spearman's rank correlation coefficient.

KNTC2, *MPHOSPH1* and *DEPDC1* in CRC tissues ($P = 0.035$, $P = 0.038$, $P = 0.001$, and $P = 0.001$, respectively), but not with the expression of *WT1*, *TOMM34* and *RNF43* ($P = 0.268$, $P = 0.772$ and $P = 0.079$, respectively).

3.4. Clinicopathological factors and expression levels of WT1, TOMM34 and RNF43 in CRC

Next, we analyzed clinicopathological factors and *WT1*, *TOMM34*, and *RNF43* levels in CRC tissues (Table 2). Specimens of advanced T stage had significantly greater *WT1* expression ($P = 0.039$), but not *TOMM34* and *RNF43*, than early-stage specimens. Similarly, specimens with lymph node metastasis and distant metastasis had significantly greater *WT1* expression than negative specimens ($P = 0.023$ and $P = 0.036$, respectively), but not *TOMM34* and *RNF43*. Moreover, fifteen of the 137 CRC patients who underwent surgery died within 5 years, and the specimens from these patients had greater WT1 expression than those from patients who remained alive, but there was no significant difference ($P = 0.259$). TOMM34 and RNF43 were also found to not be significant.

We also found significant differences between *WT1* mRNA expression levels and clinical stages in CRC tissues ($P = 0.011$; Table 2), but not *TOMM34*, and *RNF43*. Clinical stages were correlated highly with *WT1* expression levels in CRC ($r = 0.2541$; $P = 0.002$; Fig. 4). However, we found no significant associations between expression of *WT1*, *TOMM34* and *RNF43* in terms of sex, age or site of tumor (Table 2).

4. Discussion

In the present study, we analyzed the mRNA expression levels of seven TAAs in specimens from 137 CRC patients. First, we selected an appropriate housekeeping gene to use as a standard in our analysis. Among the eight known housekeeping genes, GAPDH is considered to be an inappropriate internal standard gene for cancer because of the hypoxic conditions in cancer tissues [18]; Zhong et al. reported that *GAPDH* mRNA expression levels in cell lines are increased by 21.2–75.1% under hypoxic conditions [19]. Jacques et al. also reported that the coefficients of correlation between expression levels of *TBP* in colon samples and CRC had quite a positive result in their experiment [20]. Another report suggested that *ACTB* is the most stable gene in diabetic glomeruli and primary mesangial cells [21]. Surprisingly, in our study, the expression levels of ACTB and *TBP* were almost equal in ANM and CRC tissues. Therefore, *ACTB* was se-

Table 2
Comparative analyses of clinicopathological characteristics and *WT1*, *TOMM34*, and *RNF43* status in patients with CRC

Clinicopathological characteristics		Number of patients (%)	*WT1*[c]	*TOMM34*[c]	*RNF43*[c]
All cases		137 (100.0)			
Gender[a]	Male	67 (48.9)	0.815	0.865	0.672
	Female	70 (51.1)			
Age[a]	$<$ 65 years	40 (29.2)	0.998	0.446	0.935
	$\geqslant$ 65 years	97 (70.8)			
Site of tumors[b]	Rectum	32 (23.4)	0.144	0.067	0.906
	Sigmoid colon	42 (30.7)			
	Descending colon	8 (5.8)			
	Transverse colon	11 (8.0)			
	Ascending colon	34 (24.8)			
	Cecum	10 (7.3)			
Survival[a]	Survival	122 (89.1)	0.259	0.567	0.691
	Death	15 (10.9)			
T stage[a]	pT1/T2	21 (15.3)	**0.039**	0.723	0.799
	pT3/T4	116 (84.7)			
N stage[a]	pN0	57 (41.6)	**0.023**	0.831	0.081
	pN1-N3	80 (58.4)			
M stage[a]	M0	115 (83.9)	**0.036**	0.503	0.997
	M1	22 (16.1)			
Clinical stage[b]	I	16 (11.7)	**0.011**	0.926	0.345
	II	41 (29.9)			
	III	58 (42.3)			
	IV	22 (16.1)			

[a] Mann–Whitney U test; [b] Kruskal–Wallis test; [c] P-values; significant differences are in boldface.

lected as the internal standard gene in the present study. Moreover, we analyzed *TBP* as an internal control to obtain reliable results. The calculated copy numbers were normalized based on the copy numbers of *TBP* and an analogous result was obtained using *ACTB*. We found that, although the mRNA expression levels of all seven TAAs were significantly greater in CRC tissues than in ANM, mRNA expression of *WT1* in CRC tissue was approximately 82-fold higher than in ANM.

Expression of TAAs is restricted to a limited set of tissues such as the fetal kidney, ovary, testis, spleen, and mesothelial cell lining of visceral organs [5,6]. However, in the present study, TAA expression was also detected in the ANM tissues. Although TAAs that decorate cancer cells are targets for the immune system, many are not truly cancer-specific because they can also be found on normal tissues [22]. This arises through the presence of pre-malignant cells that express TAAs in normal-appearing colorectal mucosal tissues. This concept is field cancerization, or the field effect, which was first described by Slaughter et al. in oral carcinoma [23]. The concept of field cancerization implies that tumor carcinogenesis may even occur in the ANM. A previous study showed that gene expression in ANM cells in CRC patients was not quite normal, and this may have important implications for CRC prognosis and progression [22]. Moreover, in the present study, mRNA expression levels of *WT1* were not detected in the CP samples. It is thought that *WT1* has strong tissue specificity in CRC [12]. In selecting appropriate TAAs, their expression in adjacent tissues is important. Ideal TAAs for peptide vaccines would show strongly specific expression and correlation with clinicopathological factors. In fact, the correlation of *WT1* mRNA with CRC pathological conditions strengthens the clinical significance of *WT1* in CRC.

Regarding the cell cycle, we investigated the correlations between TAAs and a cyclin family member. Cell proliferation follows an orderly progression through the cell cycle, which is governed by protein complexes composed of cyclins such as cyclins A, B1, B2, C, D1–3, and E and cyclin-dependent kinases [24]. Cyclin A achieves its maximal level during the S and G_2 phases, and is thus regarded as a regulator of the transition to mitosis; it may directly affect the entry into mitosis of cancer cells, and therefore mediate the high proliferative activity of cancer cells [25]. The cell cycle of cancer cells is faster than that of normal cells, and the expression level of cyclin A is known to be increased in cancer cells [26]. We investigated the association between TAA expression levels and cyclin A (*CCNA2*), and revealed some close relationships in CRC tissues. Identification of TAAs that are not af-

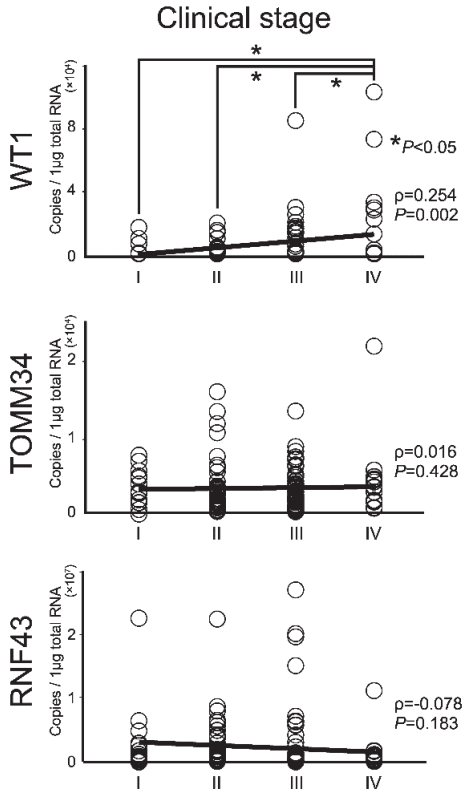

Fig. 4. Correlations among expression levels of *WT1*, *TOMM34* and *RNF43* and CRC clinical stage. mRNA expression levels of *WT1*, *TOMM34*, and *RNF43* and clinical stage were evaluated in CRC tissue. *WT1* mRNA expression correlated only with clinical stage ($r = 0.254$; $P = 0.002$). Statistical analyses used the Pearson product–moment correlation and one-way ANOVAs with Tukey–Kramer multiple comparison post-tests. $^{*}P < 0.05$.

fected by cell proliferation was an important consideration. In the present study, we found no correlations between *CCNA2* and *WT1*, *TOMM34*, and *RNF43* in CRC tissues. Among the CRC clinicopathological factors, only *WT1* showed a significant association with lymph node metastasis, distant metastasis, T-stage, or clinical stage, whereas the other TAAs did not. Therefore, *WT1* could be an appropriate TAA marker for tumor progression in CRC.

WT1 was originally isolated as a tumor-suppressor gene that is inactivated in a subset of Wilms' tumors and mutated in the germline of children with a genetic predisposition to this kidney neoplasm in childhood [27–29]. The wild-type WT1 protein was shown to be expressed in cancer cells derived from various kinds of cancers, including colon cancer [12], and overexpressed in primary leukemia [30], breast cancer [31,32], lung cancer [33], bone and soft-tissue sarcoma [34], and head and neck squamous cell carcinoma [35]. Recently, a National Cancer Institute pilot project to prioritize cancer antigens made efforts to develop both a priority-ranked list of known cancer antigens, and a list of weighted "ideal" antigen criteria/characteristics for evaluating TAAs [36]. Interestingly, WT1 headed this list, supporting translational research for further design of WT1-based cancer vaccines [36].

The mRNA expression levels of *WT1* in CRC correlated with distant metastasis, clinical stage, and lymph node metastasis. Additionally, fifteen of the 137 CRC patients who underwent surgery died within 5 years, and the specimens from these patients had greater *WT1* expression than those from patients who remained alive, but there was no significant difference. Our collection of CRC tissue specimens was examined between 2010 and 2013, therefore, we could not analyze the 5-year survival rate. We will continue the investigation of survival rates in the future. However, we did find that the expression of *WT1* in CRC tissues was significantly related to tumor progression, lymph node metastasis, distant metastasis and clinical stage, thus *WT1* expression may be correlated with survival rate. Moreover, Oji et al. [12] reported that *WT1* mRNA expression in CRC was significantly higher than that in normal-appearing colorectal mucosal tissues, with no significant correlations between *WT1* mRNA expression and clinical factors and TNM stages. Although our results were not consistent with this report, the number of samples in our study was much larger, and therefore *WT1* expression may reflect tumor progression and metastatic activity. These analyses of *WT1* will increase our understanding of TAAs in CRC pathogenesis, and could further lead to novel treatment and early diagnostic tools and offer new strategies for TAA-based immunotherapies in CRC.

5. Conclusions

WT1 is an appropriate TAA for CRC, and *WT1* mRNA expression in CRC primary tumors could be a novel independent marker for prognosis and tumor progression.

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