Association of Wilms' tumor 1 gene single-nucleotide polymorphism rs16754 with colorectal cancer

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Abstract. Colorectal cancer (CRC) is one of the leading causes of cancer-related mortality worldwide. Our recent study demonstrated that the expression of Wilms' tumor 1 gene (*WT1*) is associated with surgical outcome in CRC patients. The present study aimed to investigate the genetic association of the single-nucleotide polymorphism rs16754 in the *WT1* gene with the occurrence of CRC, using an age-matched case-control study design. In addition, the correlation between genotype and *WT1* expression was investigated. Genomic DNA samples from 104 CRC cases, aged 15-65 years, and 208 healthy controls, were genotyped for rs16754 using the TaqMan genotyping method. The genotype distribution conformed to the Hardy-Weinberg equilibrium (P=0.80). The overall minor allele frequency (MAF) of rs16754 (allele A) was 0.33. The MAF among CRC cases was significantly higher compared with that in controls (0.39 vs. 0.31, respectively; P=0.03). The AA genotype was significantly associated with the disease (odds ratio = 2.51, 95% confidence interval: 1.24-5.07, P=0.01). Cases with the AA genotype exhibited a significantly poorer 3-year overall survival (60%), compared with those with the GG or GA genotypes (80%) (log-rank test, P<0.01). Reverse transcription quantitative polymerase chain reaction analysis demonstrated that the expression of *WT1* in tumor tissues was higher compared with that in normal tissue; however, there were no significant differences in its expression among different genotypes. Therefore, rs16754 was found to be associated with the occurrence and prognosis of CRC in our subjects.

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Introduction

Colorectal cancer (CRC) is among the leading causes of cancer-related mortality. In the United States, it was estimated that 142,570 individuals were diagnosed with CRC in 2010, of whom 51,370 succumbed to the disease (1). In Thailand, CRC is the third most common type of cancer in men and the fifth in women. Furthermore, CRC statistics from 4 regional registries in the country, which have been collecting data for >20 years, clearly demonstrated that the incidence rates of CRC are continuously increasing (2). Between 2001 and 2003, the estimated incidence rates of CRC in Thailand were 11.3/100,000 men and 7.9/100,000 women (2), while in 2008, the respective incidence rates were 14.7/100,000 and 11.8/100,000 (3). In Southern Thailand, where this study was conducted, the statistics between 2004 and 2007 reported CRC incidences of 12.6/100,000 for men and 9.2/100,000 for women (4).

Although surgery is the mainstay of treatment for early-stage CRC, there is an increasing use of multidisciplinary treatment. As adjuvant treatments are considered based on individual patient risk, the identification of prognostic factors is crucial for risk stratification. Well-known factors affecting outcome in CRC patients are tumor invasion, nodal status, metastatic status and carcinoembryonic antigen level (5,6).

Various histological parameters and biological markers have been investigated for possible associations with CRC. Biological markers, such as the DNA mismatch repair genes DCC, NM23-H1 and K-Ras have been verified as being able to predict disease relapse (7-10). The application of those molecular markers at the clinical level remains limited, however, due to limitations in their reproducibility and independency from other major prognosticators. Over several years, our research team has been focusing on the prognostic role of Wilms' tumor 1 gene (*WT1*) expression in CRC and have found that high WT1 expression is correlated with poor survival, regardless of tumor stage (11). These findings prompted us to investigate the genetic epidemiology of *WT1* in the Southern Thailand population and its association with the occurrence, severity and prognosis of CRC.

WT1 is among the biological markers that have been proven to act as oncogenes in various human cancers (12). *WT1*, located on chromosome 11p13, encodes a zinc finger transcription

factor that plays an important role in cell growth and differentiation. In humans, WT1 is mainly expressed in the embryonic genitourinary system. Pathologically high WT1 expression has been reported in acute leukemic cells (13), lung cancer (14), breast cancer (15) and CRC (11,16). Immunotherapy against the WT1 antigen as an adjuvant biological treatment for cancer is currently actively investigated globally (12,17).

The aim of this study was to investigate the single-nucleotide polymorphism (SNP) rs16754 of *WT1* in CRC patients. This variant was previously extensively investigated in childhood hematological malignancies (18-20), but has never been investigated in CRC. This study was undertaken to determine whether rs16754 is associated with the occurrence of CRC, as well as its correlation with pathological parameters and clinical outcomes following surgery, particularly survival probability.

Subjects and methods

Patients and controls. Patients aged 15-65 years with histologically proven colorectal adenocarcinoma, who underwent definitive surgery at Songklanagarind Hospital (Hat Yai, Thailand) between January, 2006 and June, 2013, were asked to participate in this study. A cut-off of 65 years was selected to reduce interference caused by other carcinogenic factors associated with ageing. CRC patients with familial adenomatous polyposis coli syndrome were also excluded. Blood samples were collected under informed consent. Subjects who had no history of cancer were recruited from community-based volunteers residing in the Songkhla province to be used as controls. Age-matched controls were selected on an individual basis without prior knowledge of the *WT1* rs16754 genotypes, with an age difference of ≤2 years between each case and the matched controls. In order to increase the power of the study, the number of controls was twice that of the CRC cases.

The current clinical practice guideline for CRC patients, including preoperative investigation and postoperative follow-up in our institution, was published in our previous study (21). Briefly, adjuvant chemotherapy was considered for stage III colon cancer patients, and adjuvant chemoradiation was considered for stage II and III rectal cancer cases. Patients who received neoadjuvant treatment were not included in this study. All the patients were evaluated for ≥1 year following surgery, or until death. Follow-up visits were scheduled at 1-month intervals during the first year following surgery, every 3 months during the second year, and every 6 months thereafter. Tumor staging was performed according to the sixth edition of the TNM staging system of the American Joint Committee on Cancer, version 5 (22). Our request for access to pathological samples and clinical records was approved by the Institutional Research Ethics Committee of Prince of Songkhla University (Songkhla, Thailand).

Extraction of DNA and genotyping of WT1 rs16754. Genomic DNA was isolated from peripheral blood leukocytes using the QIAamp DNA Mini kit (Qiagen, Hilden, Germany), following the manufacturer's protocol. Genotype determination of the SNP rs16754, a synonymous SNP within exon 7, was performed using the TaqMan SNP genotyping system (Applied Biosystems, Foster City, CA, USA) (Table I). The details of this method were previously reported (23). The assay mixes,

Table I. Primers and TaqMan probe used for Wilms' tumor 1 gene rs16754 genotyping.

Primers	Sequence
Forward	GCC TCC CTT CCT CTT ACT CTC T
Reverse	GATG CCG ACC GTA CAA GAG T
Reporter-1 (T)	VIC-CAC ACG TCG CAC ATC-NFQ
Reporter-1 (G)	FAM-CAC GCC GCA CAT C-NFQ

NFQ; non-fluorescence quencher; FAM, carboxyfluorescein.

including unlabeled polymerase chain reaction (PCR) primers, carboxyfluorescein (FAM) and VIC dye-labeled TaqMan minor groove binder probes of the Assays-by-Design system, were designed by Applied Biosystems. The reaction system contained 50 ng genomic DNA, 5μl 2X TaqMan™ Genotyping Master Mix, 0.25μl 40X Assay Mix, adjusted with Milli-Q H_2O to reach a total volume of 10μl. The reaction conditions included an initial step at 95˚C for 10 min, followed by 40 cycles at 95˚C for 15 sec, and at 60˚C for 60 sec in a 96-well plate including negative (no DNA) and positive controls to ensure genotyping accuracy using Prism® 7500 Fast Real-Time PCR and GeneAmp® PCR system 7500 (both from Applied Biosystems). The genotyping results were analyzed using the Applied Biosystems 7500 software, version 2.0.5, and randomly selected to be confirmed by direct sequencing. Quality control was set at a call rate of >95% and an accuracy rate of >99%.

Study of WT1 expression at the mRNA and protein level. Our study of *WT1* mRNA expression among the rs16754 genotypes used reverse transcription quantitative PCR (RT-qPCR). RNA was extracted from 66 frozen tumor tissue samples (31 GG, 20 GA and 15 AA) and 59 normal tissue samples (23 GG, 20 AG and 6 AA) with known rs16754 genotypes. RT-qPCR was performed for the *WT1* gene with *GAPDH* as an internal control using the TaqMan probe technique. The primers and probes used in our study were previously described (24). A relative expression of the *WT1* gene was calculated as $\log_{10}$ (copy number of *WT1*/copy number of *GAPDH*).

The study of WT1 protein expression and localization on tumor tissue used a WT1 immunohistochemical staining method, as described in our previous publication (11). Briefly, formalin-fixed, paraffin-embedded tissue samples from CRC patients were stained overnight at 4˚C with a mouse monoclonal anti-WT1 antibody (cat. no. clone 6FH2; Dako, Carpinteria, CA, USA), which was used as the primary antibody at a dilution of 1:100. The WT1 protein was detected by the EnVision™+ system (Dako) according to the manufacturer's instructions. Finally, the tissue sections were incubated with 3,3'-diaminobenzidine (Sigma, St. Louis, MO, USA) until a brown color developed, and then counterstained with Harris' modified hematoxylin. In negative controls, the primary antibodies were omitted. For evaluation of WT1 expression, slides of cancer specimens were analyzed by a pathologist specialised in gastroenterology (S.K.), who was blinded to the clinical information and genotyping results. To grade the tissue expression of WT1, we used the Allred scoring system, which is the standard system used to

Table II. Allele and genotype distribution of Wilms' tumor 1 gene single-nucleotide polymorphism rs16754 in the CRC and age-matched control groups.

| Variables | Subject group | | P-value |
	CRC cases	Controls	
Total no.	104	208	
Mean age (range), years	51.0 (16-63)	51.0 (17-63)	0.92
Allelotype (alleles, %)			0.03
G	126 (60.6)	289 (69.5)	
A	82 (39.4)	127 (30.5)	
Genotype (cases, %)			0.03
GG	41 (39.4)	98 (47.1)	
GA	44 (42.3)	93 (44.7)	
AA	19 (18.3)	17 (8.2)	
Minor allele genotype (cases, %)[a]			<0.01
GG/GA	85 (81.7)	191 (91.8)	
AA	19 (8.3)	17 (8.2)	

[a]Analyzed under a recessive model. CRC, colorectal cancer.

Table III. Clinical and pathological parameters of colorectal cancer patients and their correlation with the Wilms' tumor 1 gene (*WT1*) rs16754 genotype.

| Parameters | *WT1* rs16754 genotype | | P-value |
	GG/GA (%)	AA (%)	
All (n=104)	85 (81.7)	19 (18.3)	
Gender			0.20
Female	36 (87.8)	5 (12.2)	
Male	49 (77.9)	14 (22.2)	
Tumor location			0.07
Left colon/rectum	76 (84.4)	14 (15.6)	
Right colon	9 (64.3)	5 (35.7)	
Tumor size, cm (range)	5.6 (2.5-16.5)	5.5 (2.5-12)	0.82
Age, years			0.52
<40	14 (87.5)	2 (12.5)	
>40	71 (80.7)	17 (19.3)	
T-stage			0.90
1-2	8 (80.0)	2 (20.0)	
3-4	76 (81.7)	17 (18.3)	
N stage			0.51
N0	30 (79.0)	8 (21.0)	
N1-2	53 (84.1)	10 (15.9)	
M stage			0.97
M0	66 (81.5)	15 (18.5)	
M1	18 (81.8)	4 (18.2)	
AJCC stage			0.60
0-2	30 (79.0)	8 (21.0)	
3-4	54 (83.1)	11 (16.9)	
CEA, ng/dl			0.31
<5	49 (86.0)	8 (14.0)	
>5	36 (78.3)	10 (21.7)	
Differentiation			0.96
High	45 (81.8)	10 (18.2)	
Moderate/poor	35 (81.4)	8 (18.6)	
Lymphovascular/ perineural invasion			0.18
No	58 (85.3)	10 (14.4)	
Yes	23 (74.2)	8 (25.8)	
Lymph node ratio			0.07
<0.35	62 (86.1)	10 (13.9)	
>0.35	19 (70.4)	8 (29.6)	
Survival analysis[a],%			<0.01
3-year PFS	67.00	42.90	
3-year OS	80.60	59.20	

[a]Log-rank test. CEA, carcinoembryonic antigen; PFS, progression-free survival; OS, overall survival; AJCC, American Joint Committee on Cancer.

evaluate staining intensity and staining pattern. The numerical value for overall intensity (intensity score) is based on a 4-point system: 0, 1, 2 and 3 (for none, light, medium, or dark staining, respectively), while the numerical value for percentage of stained cells (proportion score) is determined by a geometric rather than linear division; no cells stained = 0; ≤1/100 cells stained = 1; ≤1/10 cells stained = 2; ≤1/3 cells stained = 3, ≤2/3 cells stained = 4; and all cells stained = 5. The sum of the two values yields the total Allred's score, which ranges between 0 and 8.

Statistical analysis. Conformation to the Hardy-Weinberg equilibrium for rs16754 was tested by its formula. A statistical analysis of the association between the genotype frequency of each SNP and the occurrence of disease was performed using the Chi-square test. Odds ratios were calculated using univariate logistic regression analysis. A survival analysis was performed using the log-rank test. Evidence of disease progression, new metastases, second primary disease and recurrence were defined as 'progression' in the progression-free survival (PFS) analysis, while cancer-related deaths were considered as failures in the overall survival (OS) analysis. Unless otherwise stated, P<0.05 was considered to indicate a statistically significant difference. All the statistical analyses used the statistical package Stata Release 13 (StataCorp LP, College Station, TX, USA).

Results

Clinicopathological characteristics. A total of 104 CRC cases, 41 women and 63 men, with a mean age of 51 years (range, 16-63 years) were included in this study. A total of 208 controls were used (67 men and 141 women), with a mean age of 51 years (range, 17-63 years), all of whom resided in the same geographic area as the patients (Table II).
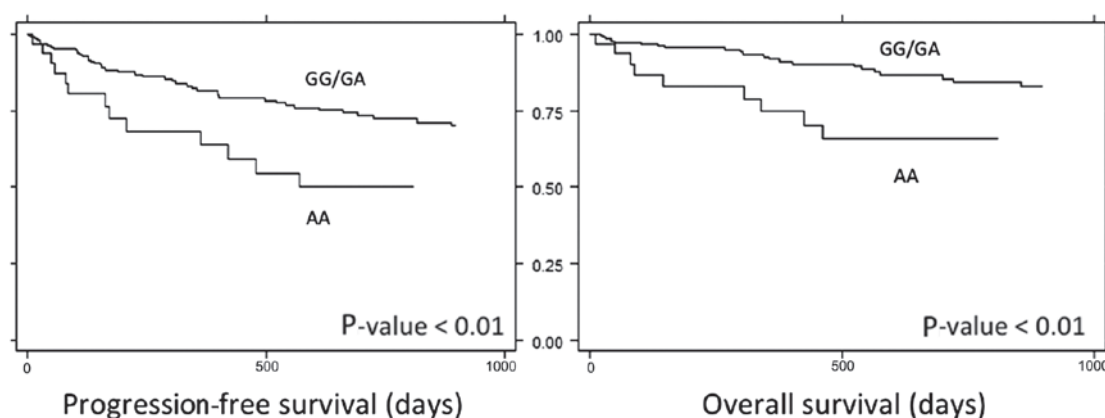
Figure 1. Kaplan-Meier curves showing the differences in progression-free and overall survival between colorectal cancer cases with different genotype groups of Wilms' tumor 1 gene rs16754 polymorphism.
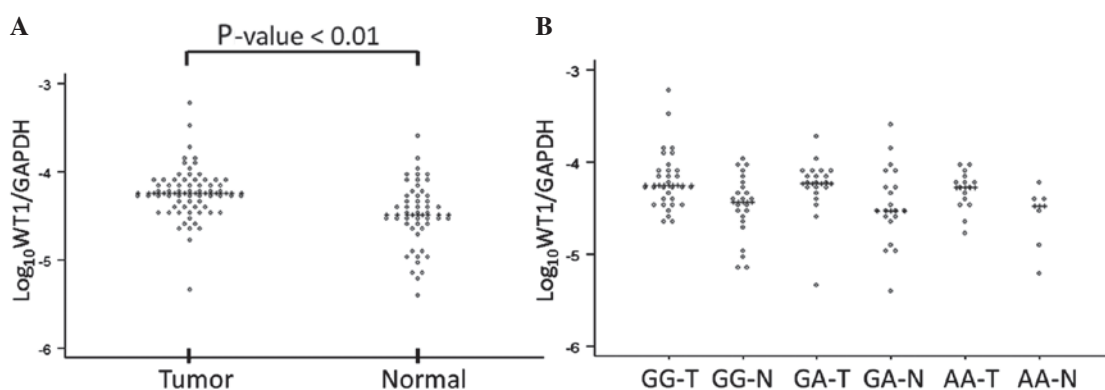

Figure 2. Relative expression of Wilms' tumor 1 gene (*WT1*) mRNA as revealed by quantitative polymerase chain reaction analysis. (A) Comparison between tumor and normal tissue and (B) normal tissue comparison of different genotype groups.

Genotyping by the TaqMan-SNP genotyping assay yielded a call rate of >99% and a reproducibility (accuracy) rate of 100%. The genotype distribution conformed to the Hardy-Weinberg equilibrium (P=0.80). The minor allele frequency (MAF) of the rs16754 (allele A) was 0.33. The MAF among CRC cases (0.39) was significantly higher compared with that in controls (0.31) (P=0.03). The AA genotype was significantly associated with CRC (odds ratio = 2.51, 95% confidence interval: 1.24-5.07, P=0.01).

The *WT1* genotype groups were not significantly associated with any clinicopathological parameters in the CRC cases (Table III). The mean follow-up period was 34 months (range, 6-90 months). When the genotypes were analyzed against survival, it was found that the AA genotype was associated with poorer PFS and OS (Fig. 1). The 3-year PFS in the AA genotype group was 43%, compared with 67% in other genotypes (P<0.01). Furthermore, the 3-year OS in the AA genotype group was 60%, compared to 81% in other genotypes (P<0.01).

On RT-qPCR, the relative expression of *WT1* in the tumor tissue was significantly higher compared with that in the adjacent normal colonic mucosa (P<0.01). However, there were no significant differences in the relative expressions among the three genotype groups (Fig. 2). On immunohistochemical staining in the 101 cases for which histological slides were obtained, the majority of CRC tumors (94 cases, 93%)

exhibited positive staining results. Apart from one case in the AA group, which displayed nuclear staining, all immunoreactivity was localized in the cytoplasm. The average Allred scores in the AA (6.4) and GA (6.7) genotype groups were higher compared with those in the GG group (5.9); however, the differences did not reach statistically significant levels (P=0.09).

Discussion

The oncologic role of *WT1* has been validated in various types of cancer, including CRC (13-16). Our previous study demonstrated a correlation between high expression of the WT1 protein and poor outcome in CRC (11). In the present study, we focused on a genetic variant of *WT1* and its association with this disease. The rs16754 SNP, located in exon 7 of *WT1*, has been found to exhibit a significant correlation between its major allele and relatively favorable clinical outcomes in hematological malignancies (18-20). According to the 1000 Genomes Browsers (browser.1000genomes.org) (25), A is the major allele of rs16754 in Western populations, while G is the major allele in East Asian populations (26,27). The MAF of 0.31 in our study was comparable with previous reports from other Asian populations in the 1000 Genomes database and in reverse correlation to the MAFs reported in European populations (18).

The CRC patients included were limited to those aged <65 years, in order to exclude potential factors associated with ageing that may affect disease development. In addition, there was a certain difficulty in recruiting older age-matched controls in our community. Our analysis demonstrated that the MAF of the rs16754 in CRC cases significantly deviated from that in the age-matched controls. The subsequent genotype analysis demonstrated a significant association between the AA genotype and CRC prognosis. As the WT1 protein expression has been identified as an unfavorable prognostic marker in CRC (11), it is possible that this variant exhibits a correlation with expression levels. Our finding of WT1 overexpression in CRC tissue was consistent with previous reports (11,16). Although differences in *WT1* expression in blood leukocytes among rs16754 genotypes have been reported in a previous study (18), a recent study on pediatric leukemia failed to detect any significant association between expression and genotype (28). Consistent with the latter study, our findings suggest that the expression level of WT1 in colonic tissue is independent of the rs16754 genotype. As the rs16754 is a synonymous SNP, the association may not indicate a direct causal association between the gene and disease development. However, the SNP may be in a linkage relationship with other disease-causal variants of *WT1*.

In conclusion, our study identified a genetic association of the rs16754 of *WT1* in a group of Thai CRC patients aged <65 years. The study also demonstrated a significant correlation between risk genotype (AA) and poorer outcome following multimodal treatment. The data suggest that this variant may be a candidate disease marker in CRC.

Acknowledgements

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