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ORIGINAL ARTICLE

Association of CTTN polymorphisms with the risk of colorectal cancer

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Purpose: Various studies searching for biomarkers to predict tumor metastasis or prognosis in both esophageal squamous cell carcinoma (ESCC) and head and neck squamous cell carcinoma (HNSCC) are currently underway. However, few data have been reported on its association with colorectal cancer (CRC). Single nucleotide polymorphisms (SNPs) are the most common known form of human genetic variation and may contribute to an increased susceptibility to cancer including CRC. The present study aimed to investigate whether the polymorphisms in the *CTTN* gene are associated with susceptibility to CRC in the Korean population. **Methods:** A case-control study was performed to examine the relationship between the CTTN g.-9101C>T, g.-8748C>T, and g.72C>T polymorphisms and the risk of CRC. Polymerase chain reaction-restriction fragment length polymorphism analysis of g.-8748C>T, g.-9101C>T and Taqman analysis of g.72C>T were performed on blood samples from 218 patients with CRC and 533 control individuals. The g.-9101C>T, g.-8748C>T, and g.72C>T SNPs in CTTN and their haplotypes were analyzed. **Results:** The genotype and allele frequencies of g.-9101C>T, g.-8748C>T, and g.72C>T did not differ between the patient group and the control group. Further, the haplotype of CTTN g.-9101C>T, g.-8748C>T, and g.72C>T did not differ between patient group and the control group. However, the genotype and allele frequencies of CTTN g.-9101C>T were significantly increased in the lymph node positive CRC group compared to the control group. **Conclusion:** The CTTN g.-9101C>T polymorphism may influence lymph node positive CRC.

Key Words: Genetic polymorphism, Human CTTN protein, Colorectal neoplasms

INTRODUCTION

Recent developments in genetics have revealed the entire sequence of the human genome. As a result, many studies have shown associations between specific polymorphisms and disease [1]. In particular, colorectal cancer (CRC) appears to have a high frequency of diverse genetic polymorphisms that are contributing factors, including genetic tendencies and personal factors, etc., which usually converge. In addition, health screening colonoscopies are actively performed, which have shown that CRC is one of the 3 leading causes of cancer related death in men and women worldwide. Even among patients with CRC who undergo potentially curative resection, 40 to 50% ulti-

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mately relapse and die of metastatic disease.

Although tumor-nodes-metastasis (TNM) classification is useful for staging CRC in patients and selecting specific treatments for them, it is not sufficient; patients at the same stage have various outcomes, which suggests that conventional staging procedures cannot precisely predict cancer prognosis. Therefore, there is a great need to identify molecular markers of more aggressive colorectal tumor phenotypes to appropriately select patients for adjuvant systemic or targeted therapy. In the sixth edition of the American Joint Committee on Cancer (AJCC) cancer staging manual, nonanatomical factors were judiciously added to the classifications that modified stage groups. This shift away from purely anatomic information has been extended in the 7th edition of the AJCC cancer staging manual. Relevant markers are so important and are required by clinicians in order to make clear treatment decisions that they have been included in the groupings. Example markers used in staging CRC include perineural invasion, microsatellite instability, pre-operative or pre-treatment carcinoembryonic antigen, K-Ras (V-Ki-ras2, Kirsten rat sarcoma viral oncogene) gene analysis, and 18q loss of heterozygosity assay, and many other biological markers associated with the prognosis of malignant tumors have been studied [2,3].

Identification of the factors regulating carcinogenesis and the progression of CRC would contribute to reducing the occurrence of CRC, as well as improving the clinical outcome for the treatment of the disease. To better understand the underlying mechanism of carcinogenesis in CRC, genome-wide association studies (GWAS) are being performed around the world. Efforts to detect the most important single nucleotide polymorphisms (SNPs) for disease are an area of intense study. In epidemiological studies, GWAS generally examine all or most of the genes of different individuals of a particular species to identify the extent to which the genes vary from individual to individual. The variations in genotypes are then associated with specific diseases or malignancies. With regard to the biological significance of SNPs in the progression of CRC, several studies have been reported. In high-grade soft tissue sarcoma, patients with the Gly/Gly genotype of the FGFR4 SNP were found to have a better prognosis with regard to cumulative overall and metastasis free survival [4].

Cortactin is an actin-binding protein that activates the Arp2/3 complex, which regulates the actin cytoskeleton and inhibits debranching of dendritic actin networks [5]. Cortactin is overexpressed in many types of human cancers, including head and neck squamous cell carcinoma (HNSCC) and esophageal squamous cell carcinoma, colorectal, gastric, hepatocellular, breast, and ovarian cancers. Most frequently, cortactin overexpression occurs through chromosomal amplification of the 11q13 region; however, overexpression has also been reported in tumors in the absence of this amplification. Actin cytoskeleton remodeling has effects on cell migration, motility, and adhesion, as well as tumor invasion and metastasis [5,6]. In some studies, overexpression of cortactin was correlated with histological differentiation, T and N stage in gastric cancer, depth of invasion in CRC, and poor prognosis in patients with lymph node metastasis [7,8]. However, only few studies have examined the association of CTTN polymorphisms and haplotypes with CRC. For this reason, our study was aimed at investigating the association of the CTTN g.-9101C>T, g.-8748C>T, and g.72C>T polymorphisms with the risk of CRC.

METHODS

For this case-control study, blood samples were collected from 218 consecutive patients who were diagnosed with CRC at our hospital between June 2001 and August 2007. As controls, we collected blood samples from 533 healthy volunteers. The CTTN g.-9101C>T, g.-8748C>T, and g.72C>T polymorphisms were analyzed in the 2 groups retrospectively, and their association with CRC was assessed. TNM cancer staging criteria from the 7th edition of the AJCC manual were applied, and T1 and T2 were placed in the Ta group, and T3 and T4 were placed in the Tb group, respectively. Regional lymph nodes were applied to the N0 in the N(−) group, and the N1 and N2 in the N(+) group, respectively. The study protocol was approved by the Ethics Committee and written-informed consent was obtained from all participants.

Analysis of CTTN polymorphisms

Genomic DNA was extracted from the peripheral blood of individuals in the patient and control groups and the *CTTN* gene g.-9101C>T, g.-8748C>T polymorphisms were analyzed using the polymerase chain reaction-restriction fragment length polymorphism (PCR-RFLP) method, and g.72C>T was analyzed by the TaqMan method. In CRC patients, the haplotype of these CTTN SNPs was analyzed from peripheral blood collected before surgery.

PCR-RFLP

Portions of the *CTTN* gene containing either the 9101C>T or -8748C>T polymorphic sites were partially amplified using a primer set for -9101C>T and -8748C>T (CTTN-PF1; TCCCAGGTGAGTACCCATGTGGT and CTTN-PR1; TCGCGGCCAGGCGACGCCACA). An initial PCR denaturation step was performed at 95°C for 5 minutes, followed by 30 cycles of denaturation at 98°C for 10 seconds, annealing at the melting temperature of each primer pair for 15 seconds, and extension at 72°C for 30 seconds, with a final 10-minute extension at 72°C. The PCR products for -9101C>T and -8748C>T were digested with 2 U of *Eco* 52 I for 12 hours at 37°C and with 1 U of *Nar* I for 12 hours at 37°C, respectively, and then separated on a 1.5% agarose gel and visualized under UV with ethidium bromide. Restriction enzyme digestion of the PCR products for -9101C>T (630 bp) and -8748C>T (630 bp) yielded 2 fragments of 490 bp and 140 bp (Fig. 1).

TaqMan analysis

The assay reagents for the g.72C>T (rs2298397) polymorphism in the *CTTN* gene were designed by Applied Biosystems (Foster City, CA, USA). The reagents consisted of a 40× mix of unlabeled PCR primer and TaqMan MGB probes (FAM and VIC dye-labeled). A 10 μL reaction was optimized with 0.125 μL of 40× reagent, 5 μL of 2× TaqMan Genotyping Master mix (Applied Biosystems), and 2 μL containing 50 ng of genomic DNA. The PCR conditions were as follows: 1 cycle of 95°C for 15 minutes, 50 cycles at 95°C for 10 seconds and 60°C for 45 seconds. The PCR was performed in a Rotor-Gene thermal cycler RG6000 (Corbett Life Science, Mortlake, Australia). The samples were read and analyzed using Rotor-Gene 1.7.40 software (Corbett Life Science). The reference sequence for the *CTTN* gene was based on the sequence of human chromosome 11, 11q13.

Statistical analysis

We determined whether the allelic distribution of the SNPs was in Hardy-Weinberg equilibrium using the chi-square test. The allele and genotype frequencies of these SNPs were compared between the patients and controls using the chi-square test or Fisher's exact test. We also tested the frequencies of these haplotypes based on the expectation maximization algorithm using SNPAlyze software (Dynacom Co., Mobara, Japan). The association of the haplotype frequencies with CRC was evaluated by a permutation test. In all cases, a P-value of <0.05 was considered statistically significant.

RESULTS

Genotype and allele frequencies of the CTTN polymorphisms in CRC patients and the controls and the risk of CRC

The genotype frequencies of the CTTN g.-9101C>T polymorphism were 97.0% (TT), 3.0% (TC), and 0% (CC) in the patient group, and 98.6% (TT), 1.4% (TC), and 0% (CC) in the control group. The genotype frequencies of the

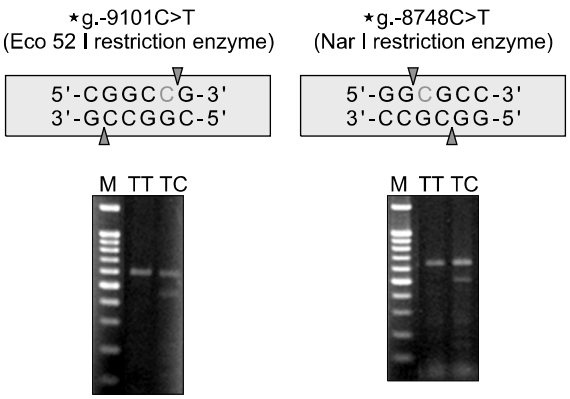

Fig. 1. CTTN genotyping restriction fragment length polymorphism result. Restriction enzyme digestion of the polymerase chain reaction products for g.-9101C>T (630 bp) and g.-8748C>T (630 bp) yielded 2 fragments of 490 bp and 140 bp.

CTTN g.-8748C>T polymorphism were 97.9% (TT), 2.0% (TC), and 0% (CC) in the patient group, and 94.2% (TT), 5.8% (TC), and 0% (CC) in the control group. The genotype frequencies of the CTTN g.72C>T polymorphism were 78.4% (CC), 21.1% (CT), and 0.46% (TT) in the patient group, and 78.1% (CC), 20.1% (CT), and 1.9% (TT) in the control group. The genotype and allele frequencies of CTTN g.-9101C>T, g.-8748C>T, and g.72C>T did not differ between the patient group and the control group (Table 1).

Genotype and allele frequencies of the CTTN polymorphisms according to the T and N stages of CRC and the controls and the risk of CRC

The genotype and allele frequencies of CTTN g.-9101C>T, g.-8748C>T, and g.72C>T did not differ between the 2 CRC groups according to T stage. However, the genotype and allele frequencies of CTTN g.-9101C>T were significantly higher in the lymph node-positive CRC group than in the control group (P = 0.036 and P = 0.039, respectively) (Tables 2, 3).

Table 1. Genotype and allele frequencies of the CTTN polymorphisms between patients with colorectal cancer and the control group and the risk of colorectal cancer

Position[a]	Genotype/ allele	Total		P-value[b]
		Control	CRC	vs. CRC
g.-9101C>T (rs12576561)	TT	430 (98.6)	97 (97.0)	0.522
	TC	6 (1.4)	3 (3.0)	
	CC	0 (0)	0 (0)	
	T	866 (99.3)	197 (98.5)	0.381
	C	6 (0.7)	3 (1.5)	
g.-8748C>T (rs11825736)	TT	404 (94.2)	91 (97.9)	0.348
	TC	25 (5.8)	2 (2.2)	
	CC	0 (0)	0 (0)	
	T	833 (97.1)	184 (98.9)	0.204
	C	25 (2.9)	2 (1.1)	
g.72C>T (rs2298397)	CC	416 (78.1)	171 (78.4)	0.331
	CT	107 (20.1)	46 (21.1)	
	TT	10 (1.9)	1 (0.5)	
	C	939 (88.1)	388 (89.0)	0.658
	T	127 (11.9)	48 (11.0)	

Values are presented as number (%).
CRC, colorectal cancer.
[a]Calculated from the translation start site. [b]P-values were determined by Fisher's exact test or chi-square test from a 2 × 3 contingency table.

Haplotype frequencies of the CTTN polymorphisms between CRC patients and the controls and the risk of CRC

The major haplotype of CTTN g.-9101 C>T, g.-8748C>T, and g. 72C>T was T-T-C, T-T-T. The haplotype did not differ between CRC patients and the controls (Table 4).

Haplotype frequencies of the CTTN polymorphisms according to T and N stages of CRC and the the controls and the risk of CRC

The haplotype frequencies of CTTN g.-9101C>T, g.-8748C>T, and g.72C>T did not differ between CRC groups and the control groups (Tables 5, 6).

DISCUSSION

There has been an explosion of information concerning the natural genetic variation in the human genome and its functional and clinical significance. This scientific progress is beginning to allow us to disentangle the complex role that genetic factors play in the causation and progression of common multi-factorial diseases [1,2]. Recently, the incidence of CRC in Korea has been increasing sharply, and it currently ranks second among digestive organ malignancies. This is thought to be related to the rapid changes in the lifestyle of Koreans over past few decades as a result of industrialization and high-calorie food and fat increased intake. Multi-factorial diseases such as cancer occur as a result of the interplay between a number of genetic and environmental factors. These genetic factors may differ from those in single gene disorders in that the effect of individual genes is small; in other words, the probability of a genetic change resulting in disease is low. Only in combination with numerous other genetic and environmental variables does a polymorphic gene predispose to disease [9-11]. Depending on the frequency of naturally occurring DNA sequences and their ability to cause disease, these variations are referred to as either polymorphisms (a frequency of >1% in the normal population) or mutations (a frequency of <1%, which usually results in disease). However, not all mutations result in disease, and some polymorphisms are functionally im-

Table 2. Genotype and allele frequencies of the CTTN polymorphisms among the Ta and Tb groups of colorectal cancer patients and the control group and the risk of colorectal cancer

Position[a]	Genotype/ allele	T[b]			P-value[c]	
		Control	Ta	Tb	vs. Ta	vs. Tb
g.-9101C>T (rs12576561)	TT	430 (98.6)	23 (100)	74 (96.1)		
	TC	6 (1.4)	0 (0)	3 (3.9)	0.852	0.300
	CC	0 (0)	0 (0)	0 (0)		
	T	866 (99.3)	46 (100)	151 (98.1)	0.266	0.140
	C	6 (0.7)	0 (0)	3 (2.0)		
g.-8748C>T (rs11825736)	TT	404 (94.2)	20 (100)	71 (97.3)		
	TC	25 (5.8)	0 (0)	2 (2.7)	0.541	0.557
	CC	0 (0)	0 (0)	0 (0)		
	T	833 (97.1)	40 (100)	144 (98.6)	0.622	0.410
	C	25 (2.9)	0 (0)	2 (1.4)		
g.72C>T (rs2298397)	CC	416 (78.1)	47 (90.4)	124 (74.7)		
	CT	107 (20.1)	5 (9.6)	41 (24.7)	0.101	0.250
	TT	10 (1.9)	0 (0)	1 (0.6)		
	C	939 (88.1)	99 (95.2)	289 (87.1)	0.033	0.631
	T	127 (11.9)	5 (4.8)	43 (13.0)		

Values are presented as number (%).
[a]Calculated from the translation start site. [b]Primary tumor stages were applied to the T1 and T2 in the Ta group and T3 and T4 in the Tb group. [c]P-values were determined by Fisher's exact test or chi-shared test from a 2 × 3 contingency table.

Table 3. Genotype and allele frequencies of the CTTN polymorphisms in the N (−) and N (+) colorectal cancer groups and the control group and the risk of colorectal cancer

Position[a]	Genotype or allele	Lymph node[b]			P-value[c]	
		Control	N (−)	N (+)	vs. N (−)	vs. N (+)
g.-9101C>T (rs12576561)	TT	430 (98.6)	56 (100)	40 (93.0)		
	TC	6 (1.4)	0 (0)	3 (7.0)	0.677	0.036
	CC	0 (0)	0 (0)	0 (0)		
	T	866 (99.3)	112 (100)	83 (96.5)	0.517	0.039
	C	6 (0.7)	0 (0)	3 (3.5)		
g.-8748C>T (rs11825736)	TT	404 (94.2)	51 (100)	39 (95.1)		
	TC	25 (5.8)	0 (0)	2 (4.9)	0.208	0.970
	CC	0 (0)	0 (0)	0 (0)		
	T	833 (97.1)	102 (100)	80 (97.6)	0.099	1.000
	C	25 (2.9)	0 (0)	2 (2.4)		
g.72C>T (rs2298397)	CC	416 (78.1)	93 (78.2)	77 (78.9)		
	CT	107 (20.1)	26 (21.9)	20 (20.4)	0.304	0.835
	TT	10 (1.9)	0 (0)	1 (1.0)		
	C	939 (88.1)	212 (89.1)	174 (88.8)	0.739	0.904
	T	127 (11.9)	26 (10.9)	22 (11.2)		

Values are presented as number (%).
[a]Calculated from the translation start site. [b]Regional lymph nodes were applied to the N0 in the N (−) group and the N1 and N2 in the N (+) group. [c]P-values were determined by Fisher's exact test or chi-square test from a 2 × 3 contingency table.

portant and have been implicated in disease pathogenesis. Polymorphisms exist in several forms, and changes involving single nucleotides are referred to as SNPs. SNPs may occur at a frequency as high as 1 in every 185 base pairs, with up to 16 million polymorphisms in the human genome [12].

Cortactin was first identified as a major substrate for Src kinase. Because it localized to cortical actin structures, it

was named cortactin. At that time, little was known about its function, except that it bound to actin filaments, had an SH3 domain, and that its C-terminus was phosphorylated by Src kinase [13]. Subsequently, the cortactin gene was found to be identical to *Ems1*, a gene that is frequently overexpressed in breast and head and neck cancers due to its presence in the 11q13 amplicon. Amplification of 11q13 has been frequently tied to poor prognosis, including higher pathological stage, lymph node and distant metastasis, and decreased survival [14,15]. Although many other genes are present within this amplicon, the consistent overexpression of cortactin in 11q13 amplified tumors along with its ubiquitous presence in cell motility structures, such as lamellipodia and invadopodia, have generated a great deal of interest in the role of cortactin in tumor invasion [13-15].

In HNSCC, 30 to 40% of tumors contain the 11q13 amplicon, and it clearly correlates with poor patient prognosis, including decreased survival [16]. In addition, a number of interesting genes are present in this amplicon, including *TPC2*, *ORAOV1/TAOS1*, *FGF4*, *CCND1*, *FGF19*, *FGF3*, *FLJ10261*, *FADD*, *PP1A1*, *TMEM1GA*, and *SHANK2* [17]. Of these, cyclinD1 and EMS1/cortactin have been considered to be the best candidates for promoting tumor aggressiveness, since unlike many of the other genes in the amplicon, they are consistently overexpressed upon amplification [18]. Between these 2 genes, cortactin has been more highly correlated with poor prognosis in HNSCC and estrogen receptor-negative breast cancers, whereas cyclin D1 has been associated with poor prognosis in estrogen receptor-positive breast cancers [15,19].

Timpson et al. [20] reported that co-overexpression of

Table 4. Haplotype frequencies of the CTTN polymorphisms between colorectal cancer patients and the controls and the risk of colorectal cancer

Haplotype			Frequency[a]		P-value[b]
g.-9101C>T	g.-8748C>T	g.72C>T	Control	CRC	vs. CRC
T	T	C	0.866	0.877	0.764
T	T	T	0.097	0.093	0.824
Others			0.029	0.012	-

CRC, colorectal cancer.
[a]Values were constructed using the expectation maximization algorithm with genotyped single nucleotide polymorphisms. [b]P-values were determined by permutation test.

Table 5. Haplotype frequencies of the CTTN polymorphisms among the Ta and Tb groups of colorectal cancer patients and the control group and the the risk of colorectal cancer

Haplotype			Frequency[a]			P-value[b]	
g.-9101C>T	g.-8748C>T	g.72C>T	Control	Ta	Tb	vs. Ta	vs. Tb
T	T	C	0.866	0.912	0.867	0.539	0.962
T	T	T	0.097	0.088	0.094	0.796	0.936
Others			0.029	0	0.016	-	-

Primary tumor stages were applied to the T1 and T2 in the Ta group and T3 and T4 in the Tb group.
[a]Values were constructed using the expectation maximization algorithm with genotyped single nucleotide polymorphisms. [b]P-values were determined by permutation test.

Table 6. Haplotype frequencies of the CTTN polymorphisms among the N (−) and N (+) groups of colorectal cancer patients and the control group and the risk of colorectal cancer

Haplotype			Frequency[a]			P-value[b]	
g.-9101C>T	g.-8748C>T	g.72C>T	Control	N (−)	N (+)	vs. N (−)	vs. N (+)
T	T	C	0.866	0.895	0.851	0.474	0.763
T	T	T	0.097	0.105	0.081	0.713	0.614
Others			0.029	0	0.027	-	-

Regional lymph nodes were applied to the N0 in the N (−) group and the N1 and N2 in the N (+) group.
[a]Values were constructed using the expectation maximization algorithm with genotyped single nucleotide polymorphisms. [b]P-values were determined by permutation test.

cyclin D1 and cortactin in HNSCC cells promotes resistance to gefitinib, an epidermal growth factor receptor antagonist drug, as defined by the IC_{50} for proliferation. Rothschild et al. [21] examined HNSCC cells with 11q13 amplification, and found that generally cortactin plays an important role in migration and invasion and also promotes resistance to gefitinib-induced inhibition of migration; however, it is unclear whether any of the other genes in the amplicon contribute to this phenotype.

A few studies have examined the function of cortactin in tumors grown in mice. In a spontaneous model of breast tumorigenesis, the expression of cortactin under the mouse mammary tumor virus promoter did not increase the number of new tumors, regardless of the presence or absence of cyclin D1 overexpression. Therefore, cortactin is unlikely to act as a tumor initiator [22]. This finding is not inconsistent with the known cortactin biology, since 11q13 amplification usually occurs as a late change in cancer, and further, it suggests that cortactin expression may be important mainly in the promotion of tumor progression. How might cortactin promote tumour progression? The role of cortactin in regulating the formation of actin-based structures intimately associated with cell motility, its localization to podosome-like structures in transformed cells, and the enhancement of cell migration upon cortactin overexpression are all consistent with a role in cancer cell invasion and metastasis [23-25].

In CRC, Hirakawa et al. [26] demonstrated that localization of cortactin at the cell periphery was significantly associated with lymph node involvement with lymph node involvement. In addition, the results indicate that the association between ZO-1 and cortactin plays an important role in regulating cell adhesion and spreading. However, research studies on CTTN polymorphism are extremely rare. Based on the current study, the relationship between CTTN polymorphism and CRC will provide important information for pathogenesis and treatment.

Furthermore, recent studies have suggested that, compared with single SNP approaches for genetic association studies, analyses based on haplotypes can significantly improve robustness and the power for detecting associations between markers and disease [1,27]. Ryk et al. [28] reported that some haplotypes of the *XRCC1* gene may contribute to genetic susceptibility for non-small cell lung cancer. Furthermore, smoking status appears to play an important role in the association of the *XRCC1* polymorphisms and haplotypes with the risk of non-small cell lung cancer. Another study showed that the IL1B-1464-C/C genotype was associated with increased prevalence of atrophic gastritis in the antrum of the stomach and that IL1RN*2 individually, or in its haplotype configuration, is linked to the presence of premalignant lesions in Caucasians [29]. For CRC, Park et al. [30] reported that the common haplotype, *TIMP2-418*G303*G*, was associated with an increased risk for CRC compared with the controls, while the *MMP2-735*C-1575*G* common haplotype was associated with decreased susceptibility for CRC compared with the controls. Although the haplotype of CTTN g.-9101C>T, g.-8748C>T, and g.72C>T did not differ between CRC and the controls, genetic polymorphisms often vary significantly among different groups. Therefore, large-scale studies are needed to clarify the associations of the single polymorphisms, gene-gene interactions, and gene-environment interactions with CRC in well-defined groups.

In conclusion, the genotype and allele frequencies of CTTN g.-9101C>T were significantly higher in lymph node-positive CRC patients compared to the controls. However, the genotype and allele frequencies of g.-8748C>T and g.72C>T did not differ between the CRC patient group and the control group. In addition, the haplotype of CTTN g.-9101C>T, g.-8748C>T, and g.72C>T did not differ between the CRC patient group and the control group. The results showed an association between a specific CTTN polymorphism and lymph node metastasis of CRC. However, further studies with large populations and the CTTN g.-9101C>T, g.-8748C>T, and g.72C>T polymorphisms are require to elucidate their role in CRC.

CONFLICTS OF INTEREST

No potential conflict of interest relevant to this article was reported.

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