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Aberrant methylation of *CDKN2A*, *RASSF1A* and *WIF1* in sporadic adenocarcinomatous colorectal cancer: Associations with clinicopathological features

Linh Dieu Vuong[1], Hung Viet Nguyen[2], Van-Long Truong[3], Quang Ngoc Nguyen[1,*]

[1]Pathology and Molecular Biology Center, National Cancer Hospital K, 30 Cau Buou Street, Thanh Tri, Hanoi, Vietnam
[2]Medical Image Technology Laboratory, Department of Computer Engineering, College of Engineering, Inje University, Gimhae 50834, South Korea
[3]Department of Smart Food and Drug, College of BNIT, Inje University, Gimhae 50834, South Korea

[*]**Corresponding author**
Quang Ngoc Nguyen, PhD
Pathology and Molecular Biology Center, National Cancer Hospital K, 30 Cau Buou Street, Thanh Tri, Hanoi, Vietnam.
e-mail: quangk8s@gmail.com

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ABSTRACT

Accumulating evidence support that aberrant methylation of various cancer-related genes plays an important role in the initiation and progression of colorectal cancer (CRC). This study aims to validate the accuracy of methylation specific polymerase chain reaction (MSP) to assess frequency and distribution of *GSTP1*, *CDKN2A*, *RASSF1A*, and *WIF1* methylation and analyse their correlation with clinicopathological variables in sporadic adenocarcinomatous CRC. Of the 248 CRC tissues, methylation was identified in 7.7% for *GSTP1*, 22.2% for *CDKN2A*, 33.1% for *RASSF1A*, and 54.4% for *WIF1*. Hypermethylation of *CDKN2A*, *RASSF1A*, and *WIF1* was significantly associated with adenocarcinoma ($p< 0.001$), mucinous adenocarcinoma ($p< 0.001$), and signet-ring cell adenocarcinoma subtypes ($p = 0.017$), respectively. Both *CDKN2A* and *WIF1* methylations were more common in stage II ($p = 0.012$ for *CDKN2A* and $p = 0.010$ for *WIF1*) and absence of lymph node metastasis ($p = 0.011$ for *CDKN2A* and $p = 0.012$ for *WIF1*) but were less common in stage III ($p = 0.016$ for *CDKN2A* and $p = 0.010$ for *WIF1*). *RASSF1A* methylation was associated with moderate differentiation ($p = 0.038$). These findings suggest that methylation of *CDKN2A*, *RASSF1A*, and *WIF1* may significantly contribute to CRC pathogenesis and may be considered as valuable biomarkers for accessing the development and progression of particular subtypes of colorectal cancer.

INTRODUCTION

Colorectal cancer (CRC) is a common malignant cancer as well as a leading cause of cancer mortality worldwide, and still has poor prognosis. Although the exact pathologic mechanism has not been understood fully, it is widely accepted that CRC development is resulted from the accumulation of multiple genetic and epigenetic alterations [1]. DNA promoter methylation, one of the main mechanisms of epigenetic modifications, is to be associated with development and progression of human cancers [1, 2]. Aberrant DNA promoter methylation, which is characterized by covalent addition of a methyl group to the 5′ position on Cytosine residues of CpG islands, often occurs in the earliest precursor lesion (aberrant crypt foci), and in the early stage of colorectal carcinogenesis [3, 4]. Promoter CpG island DNA hypermethylation of cancer-related genes leads to transcriptional gene silencing and importantly contributes to colorectal tumorigenesis [5].

Cyclin dependent kinase inhibitor 2A (*CDKN2A*), Ras association domain family 1 isoform A (*RASSF1A*), and Wnt inhibitory factor 1 (*WIF1*) genes function as important tumor suppressors, and their activation results in cell cycle arrest, senescence, and apoptosis [2, 6, 7]. Glutathione S-transferase pi 1(*GSTP1*) is proposed to act as a "caretaker" gene that detoxifies reactive electrophilic intermediates/carcinogenic

compounds [8]. *GSTP1*, *CDKN2A*, *RASSF1A*, and *WIF1* promoter methylations are frequent epigenetic events in various human cancers, including CRC, and crucial mechanisms leading to cell overgrowth, uncontrolled cell proliferation, tumor development and progression [6, 8, 9].

Although *GSTP1*, *CDKN2A*, *RASSF1A*, and *WIF1* inactivation by aberrant DNA methylation has been widely studied in CRC, associations between *GSTP1*, *CDKN2A*, *RASSF1A*, or *WIF1* and clinicopathological features of CRC remain controversial. Therefore, the present study was conducted to elucidate the frequency of *GSTP1*, *CDKN2A*, *RASSF1A*, and *WIF1* methylation and the correlation of each with clinicopathological data.

MATERIALS AND METHODS

Patients and tissue specimens

A total of 248 tumors of sporadic adenocarcinomatous CRC were collected for analysis in the present study. Clinical data of the patients were collected from the hospital records. Written consent was obtained from all patients was approved by the Ethnic Committee of National Cancer Hospital K (Circular No.04/2008/TT-BYT). Diagnostic pathology were evaluated by more than two pathologists based on the World Health Organization (WHO) classification (WHO, 2019) guidelines.

DNA extraction and bisulfite modification

Genomic DNA was extracted from 5 sections of 10 µm thickness of macro-dissected colorectal tumor tissues using QIAamp DNA FFPE Tissue Kit (Qiagen, Valencia, CA, USA) (containing at least 30% tumor cells). To evaluate the quality of DNA specimens, Polymerase Chain Reaction (PCR) for single-copy gene *β-globin* was carried out. DNA samples were then introduced to sodium bisulfite conversion using EpiTect Bisulfite Kit (Qiagen, Valencia, CA, USA).

Methylation specific polymerase chain reaction (MSP)

For each sample, methylation status of *GSTP1*, *CDKN2A*, *RASSF1A*, and *WIF1* were evaluated by using methylation specific polymerase chain (MSP). Sodium bisulfite-treated DNA samples were used as templates for PCR with specific primers, which were designed to be specific to either the methylated or unmethylated sequence of each gene. The reluctant PCR products were separated on 10% Poly-acrylamide gel. Each MSP was performed at least twice. Primer sequences for each gene are listed in the Table 1.

Table 1. Primer sequences.

Genes	Primers	Primer sequences (5′-3′)
β-globin NC_000011.10	Globin F	CAACTTCATCCACGTTCACC
	Globin R	GAAGAGCCAAGGACAGGTAC
GSTP1 NC_000011.10	GSTP1 Me-F1	TCGGTTAGTTGCGCGGCGATTTC
	GSTP1 Me-F2	TTCGGGGTGTAGCGGTCGTC
	GSTP1 Me-R	CGACGAAACTCCAACGAAAAC
	GSTP1 Un-F1	AGTTGTGTGGTGATTTTGGGG
	GSTP1 Un-F2	GATGTTTGGGGTGTAGTGGTTGT
	GSTP1 Un-R	CCAACAAAAACCTCACAACCT
CDKN2A/p16 NC_000009.12	P16 Me-F1	TTATTAGAGGGTGGGGCGGATCGC
	P16 Me-R1	CCACCTAAATCGACCTCCGACCG
	P16 Me-F2	TTATTAGAGGGTGGGGCGGATCGC
	P16 Me-R2	GACCCCGAACCGCGACCGTAA
	P16 Un-F1	TTATTAGAGGGTGGGGTGGATTGT
	P16 Un-R1	CCACCTAAATCAACCTCCAACCA
	P16 Un-F2	TTATTAGAGGGTGGGGTGGATTGT
	P16 Un-R2	CAACCCCAAACCACAACCATAA
RASSF1A NC_000003.12	RASSF1A Me-F	GGTTTTGCGAGAGCGCGTTTA
	RASSF1A Me-R	ACGCTAACAAACGCGAACCGA
	RASSF1A Un-F1	GGGGTTTTGTGAGAGTGTGTTTAG
	RASSF1A Un-F2	GAGAGTGTGTTTAGTTTTGTTT
	RASSF1A Un-R	TAAACACTAACAAACACAAACCAAAC
WIF1 NC_000012.12	WIF1 Me-F	CGTTTTATTGGGCGTATCGT
	WIF1 Me-R	ACTAACGCGAACGAAATACGA
	WIF1 Un-F	GGGTGTTTTATTGGGTGTATTGT
	WIF1 Un-R	AAAAAAACTAACACAAACAAAATACAAAC

Me: Methylation; Un: Unmethylation; F: Forward; R: Reverse

Statistical analysis

Statistical analysis was performed using SPSS software (IBM Corporation, New York, NY, USA). Fisher's exact test or χ^2 test was used to determine the association of variables properly. A p-value less than 0.05 (typically ≤ 0.05) is statistically significant.

RESULTS

Patient characteristics

Table 2 summarizes clinicopathological characteristics of 248 patients. The median age at diagnosis was 60 years (range, 26-90 years). Histological analysis revealed 75.4% adenocarcinomas, 21.8% mucinous adenocarcinomas, and 2.8% signet ring cell

adenocarcinomas. Most tumors were moderately differentiated (64.5%), and there were only 4.8% of tumors being well differentiated and 6.0% poorly differentiated (excepting for 61 cases without tumor differentiation evaluation). The differentiation criteria used in this study according to the WHO's classification [10]. The majority of patients (91.9%) had local disease at initial diagnosis (Table 2).

Table 2. Clinicopathological characteristics of the patients with CRC.

	Characteristics	N	%
		248	
Age	Age		
	< 60	113	45.6
	> 60	135	54.4
Gender			
	Male	140	56.5
	Female	108	43.5
Location			
	Colon	134	54.0
	Rectum	114	46.0
Histological subtype			
	Adenocarcinoma	187	75.4
	Mucinous adenocarcinoma	54	21.8
	Signet-ring cell adenocarcinoma	7	2.8
Differentiation			
	Well	12	4.8
	Moderate	160	64.5
	Poor	15	6.0
	Unknown	61	
Stage			
	I	4	1.6
	II	112	45.2
	III	112	45.2
	IV	20	8.1
Lymph node metastasis			
	Yes	132	53.2
	No	116	46.8
Distant metastasis			
	Yes	20	8.1
	No	228	91.9
Tumor size			
	< 5 cm	118	47.6
	>= 5 cm	98	39.5
	Unknown	32	

Associations between *GSTP1, CDKN2A, RASSF1A,* or *WIF1* and clinicopathological features of CRC

Aberrant promoter methylation of *GSTP1*, *CDKN2A*, *RASSF1A*, and *WIF1* was detected in 19 (7.7%), 55 (22.2%), 82 (33.1%), and 135 (54.4%) in a total of 248 colorectal tumors, respectively (Figure 1). *GSTP1* methylation tended to be associated with male patients (p = 0.053) and moderate tumor differentiation (p = 0.082), yet *GSTP1* hypermethylation did not significantly correlate with any clinicopathological feature. *CDKN2A* methylation was more common in adenocarcinoma (p< 0.001) but less common in mucinous adenocarcinoma (p< 0.001); in contrast, *RASSF1A* methylation was more frequent in mucinous adenocarcinoma (p< 0.001) but less frequent in adenocarcinoma (p = 0.002). Aberrant promoter methylation of *WIF1* occurred frequently in signet-ring cell adenocarcinoma (p =0.017) but rarely in mucinous adenocarcinoma (p = 0.009). A statistically significant correlation between methylation and pathologic stage was observed, where both *CDKN2A* and *WIF1* methylations were more common in stage II (p = 0.012 for *CDKN2A* and p = 0.010 for *WIF1*) and less common in stage III (p = 0.016 for *CDKN2A* and p = 0.011 for *WIF1*). Moreover, *CDKN2A* and *WIF1* methylations were associated with the absence of lymph node metastasis (p = 0.014 and p = 0.012, respectively). *RASSF1A* hypermethylation

significantly correlated with moderate tumor differentiation (p = 0.038) and had tendencies to be less common in stage III (p = 0.056) and in lymph node metastasis (p = 0.072) (Table 3).

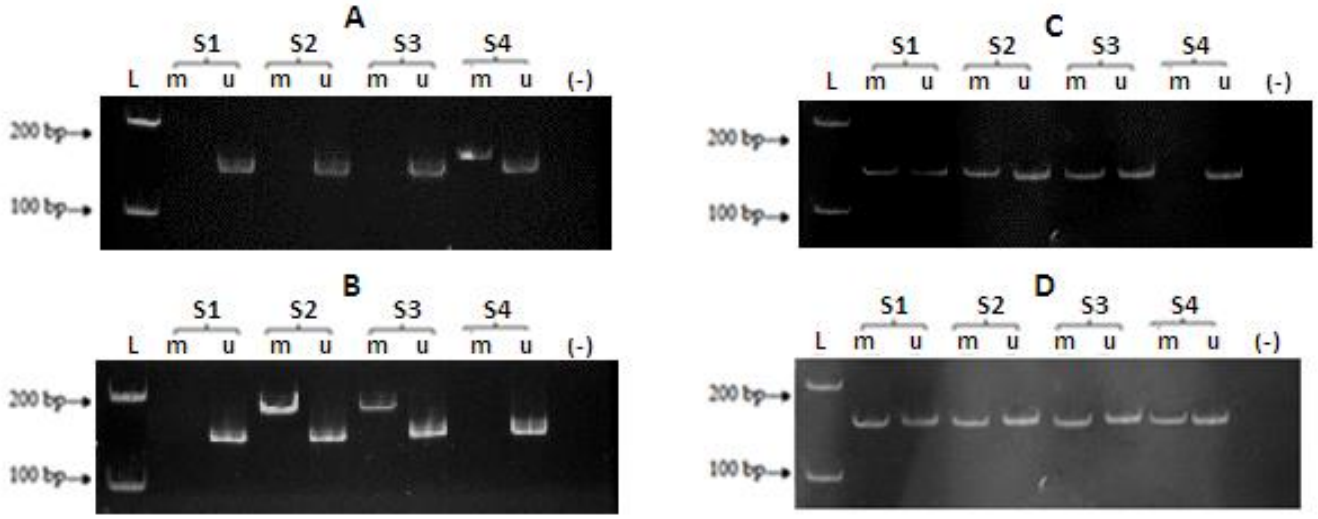

Figure 1. Representative analysis of MSP products amplified from bisulfite treated DNA with the primer sets of GSTP1 (A), RASSF1A (B), WIF1 (C) and CDKN2A (D). L: 100bp DNA ladder. (-): Negative control without DNA templates. S: colorectal cancer samples.

Table 3. *GSTP1, CDKN2A, RASSF1A*, and *WIF1* methylations and correlations with clinicopathological features.

			GSTP1 methylation			*CDKN2A* methylation			*RASSF1A* methylation			*WIF1* methylation		
			Yes	*%*	*p-value*	*Yes*	*%*	*p-value*	*Yes*	*%*	*p-value*	*Yes*	*%*	*p-value*
N		248	19	7.7		55	22.2		82	33.1		135	54.4	
Age	Age				*0.869*			*0.120*			*0.922*			*0.248*
	< 60	113	9	8.0		20	17.7		37	32.7		57	50.4	
	> 60	135	10	7.4		35	25.9		45	33.3		78	57.8	
Gender					*0.053*			*0.528*			*0.200*			*0.957*
	Male	140	15	10.7		29	20.7		51	36.4		76	54.3	
	Female	108	4	3.7		26	24.1		31	28.7		59	54.6	
Location					*0.725*			*0.931*			*0.851*			*0.787*
	Colon	134	11	8.2		30	22.4		45	33.6		74	55.2	
	Rectum	114	8	7.0		25	21.9		37	32.5		61	53.5	
Histological subtypes														
	Adenocarcinoma	187	17	9.1	*0.173*	52	27.8	*<0.001*	52	27.8	*0.002*	107	57.2	*0.123*
	Mucinous adenocarcinoma	54	2	3.7	*0.383*	3	5.6	*<0.001*	28	51.9	*0.001*	21	38.9	*0.009*
	Signet-ring cell adenocarcinoma	7	0	0.0	*1.000*	0	0.0	*0.354*	2	28.6	*1.000*	7	100.0	*0.017*
Differentiation														
	Well	12	3	25.0	*0.082*	2	16.7	*0.515*	2	16.7	*0.515*	6	50.0	*0.601*
	Moderate	160	14	8.8	*0.717*	47	29.4	*0.244*	49	30.6	*0.038*	95	59.4	*0.147*
	Poor	15	0	0.0	*0.368*	3	20.0	*0.565*	1	6.7	*0.072*	6	40.0	*0.160*
	Unknown	61												
Stages														
	I	4	0	0.0	*1.000*	1	25.0	*1.000*	2	50.0	*0.601*	2	50.0	*1.000*
	II	112	10	8.9	*0.465*	33	29.5	*0.012*	43	38.4	*0.106*	71	63.4	*0.010*
	III	112	8	7.1	*0.736*	17	15.2	*0.016*	30	26.8	*0.056*	51	45.5	*0.011*
	IV	20	1	5.0	*1.000*	4	20.0	*1.000*	7	35.0	*0.848*	11	55.0	*0.958*
Lymph node metastasis					*0.594*			*0.011*			*0.072*			*0.012*
	Yes	132	9	6.8		21	15.9		37	28.0		62	47.0	
	No	116	10	8.6		34	29.3		45	38.8		73	62.9	
Distant metastasis					*1.000*			*1.000*			*0.848*			*0.958*
	Yes	20	1	5.0		4	20.0		7	35.0		11	55.0	
	No	228	18	7.9		51	22.4		75	32.9		124	54.4	
Tumor size					*0.884*			*0.491*			*0.234*			*0.943*
	< 5 cm	118	9	7.6		30	25.4		44	37.3		68	57.6	
	>= 5 cm	98	8	8.2		21	21.4		29	29.6		56	57.1	
	Unknown	32												

DISCUSSION

Identifying molecular abnormalities has been not only essential to understand the pathogenesis of the disease better, but also valuable in diagnosis, prognosis, selection of optimal therapeutic regimens, and discovery of risk factors associated with a particular subtype [1]. Multiple studies on methylation of tumor suppressor genes, such as *CDKN2A*, *RASSF1A*, and *WIF1*, have been reported in CRC; however, their results are inconsistent. In fact, epigenetic patterns are

modulated by both endogenous and exogenous factors, including aging, ethnicity, gender, dietary habits, lifestyles, environmental factors, and medications [11, 12]. This study showed the frequency and relationship of *GSTP1*, *CDKN2A*, *RASSF1A*, and *WIF1* methylation with clinicopathological features specific to the Vietnamese CRC population.

In the present study, *GSTP1*, *CDKN2A*, *RASSF1A*, and *WIF1* promoter methylation was found in 7.7%, 22.2%, 33.1%, and 54.5% of CRC tumors, respectively. This shows that *WIF1* is a commonly methylated gene, while *GSTP1* methylation seems to be a rare event in Vietnamese CRC patients. Through extensively screening reports into CRC, the rate of *CDKN2A* (22.2%) and *RASSF1A* methylation (33.1%) was approximately the same average frequency as reported in meta-analyses [6, 7]. Whereas, our methylation frequency of 54.5% for *WIF1* is slightly lower than that in an earlier literature, which indicated frequency as high as 80.6% [13]. Generally, the frequency of *WIF1* methylation has been found to be relatively high in CRC [14], suggesting that *WIF1* hypermethylation is a frequent event in CRC. Considering the differences in genetic and environmental factors related to CRC, it is possible that prevalence of epigenetic alterations varies among studied population. Recent evidence has shown that DNA methylation is incompatible in distinct races and ethnicities [15].

Our study revealed that *CDKN2A* methylation frequently occurred in adenocarcinoma but rarely in mucinous adenocarcinoma, whereas *RASSF1A* methylation was more common in mucinous adenocarcinoma but less common in adenocarcinoma. Frequency of *WIF1* hypermethylation was positively associated with singlet-ring cell adenocarcinoma and inversely associated with mucinous adenocarcinoma. Although correlation between *CDKN2A*, *RASSF1A*, or *WIF1* methylation and histologic subtypes remains unknown, our previous study also showed a significant association between *RASSF1A* hypermethylation and mucinous adenocarcinoma in CRC [16]. These observations clearly indicated that *CDKN2A*, *RASSF1A*, and *WIF1* methylation targets different histologic subtypes of CRC. However, this study is limited by the small sample size in histologic subtypes. Thus, further studies with a larger sample size are essential to confirm this hypothesis.

Our analysis showed that *CDKN2A* hypermethylation was significantly associated with several clinicopathological characteristics toward a good prognosis. *CDKN2A* promoter methylation was found frequently in cases with early-stage and absence of lymph node metastasis. These results suggest that *CDKN2A* methylation plays a crucial role in the initiation of CRC. In contrast, several reports showed that *CDKN2A* promoter hypermethylation frequently occurred in more malignant CRC phenotype, which was associated with advanced stage and lymph node metastasis [6]. This discrepancy may be attributed to sample size, sample selection, and method used.

Similar to *CDKN2A* methylation, aberrant methylation of *WIF1* was significantly associated with tumor stage, in which *WIF1* hypermethylation frequently occurred in stage II but rarely in stage III. In addition, *WIF1* promoter methylation was found commonly in cases without lymph node metastasis. These results are consistent with a previous study, which showed a relatively high frequency of *WIF1* methylation (up to 74%) in patients with stage I and II sporadic CRC compared with 2% in healthy individuals [14]. The increased level of *WIF1* methylation and the down-regulation of WIF1 expression have been observed in colorectal adenoma tissues [17, 18]. Based on these observations, aberrant promoter methylation of *WIF1* may be related to tumor initiation.

Methylation status of *RASSF1A* was obviously correlated with moderate differentiation, which is consistent with a previous report [15]. Correlation between *RASSF1A* methylation and pathologic stage varies across various studies; some reports observed a higher level of *RASSF1A* methylation in early-stage of CRC while others reported more frequent *RASSF1A* methylation on later-stage [19, 20]. Although not significant, *RASSF1A* hypermethylation was found rarely in stage III CRC and lymph node metastasis in the present study.

In conclusion, this study reports presence of *GSTP1*, *CDKN2A*, *RASSF1A*, and *WIF1* methylation in the Vietnamese CRC population, and their correlations with clinicopathological characteristics. These observations suggest that aberrant methylation of *CDKN2A*, *RASSF1A*, and *WIF1* may be related to tumor initiation but not to tumor progression. *CDKN2A*, *RASSF1A*, and *WIF1* methylations are considered as valuable diagnostic and prognostic markers in accessing the development and progression of particular subtypes of colorectal cancer.

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AUTHOR CONTRIBUTIONS

L.D.V. and Q.N.N.: Conception and Design of the experiments. H.V.N.: Methodology and Data analysis, V-.L.T.: Data curation and Writing – original draft, L.D.V: Writing – review and editing. Q.N.N.: Supervision. All authors reviewed the manuscript.

CONFLICTS OF INTEREST

There is no conflict of interest among the authors.

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