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• BRIEF REPORT •

*p*16 gene methylation in colorectal cancers associated with Duke's staging

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Abstract

AIM **To explore the association of methylation of the CpG island in the promotor of the P16 tumor suppressor gene with the clinicopath ological characteristics of the colorectal cancers.**

METHODS **Methylation-specific PCR (MSP) was used to detect P16 methylation of 62 sporadic colorectal cancer specimens.**

RESULTS **P16 methylation was detected in 42% of the tumors. Dukes' staging was associated with P16 methylation status. p16 methylation occurred more frequently in Dukes' C and D patients (75.9%) than in Dukes' A and B patients (12.1%).**

CONCLUSION **P16 methylation plays a role in the carcinogenes is of a subset of colorectal cancer, and it might be linked to poor prognosis.**

Subject headings colorectal neoplasma/pathology; genes, P16; polymerase chain reaction/METHODS; CpG region; methylation; MSP

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INTRODUCTION

The alterations of p16 gene, i.e. homozygous deletion, point mutation and promoter methylation, and their consequence, P16 gene inactivation, were frequently observed in many human tumors. In colorectal cancers (CRC), since the first two mechanisms have never been demonstrated[1], the role of P16 gene methylation in carcinogenesis has attracted considerable attention recently[2-4]. Whether P16 methylation is associated with clinicopat hological characteristics has not been clear[4,5]. In the present study, we detected the occurrence of p16 methylation in primary CRC using a recently developed methylation specific PCR method (MSP) and explored the relationship between methylated p16 and Dukes' staging and other clinicopathological characteristics of the patients.

MATERIALS AND METHODS

Tumor specimens

Fresh specimens (n = 62) of surgically resected CRC were collected from Rui Jin Hospital and were immediately frozen at -80℃ after rinsed with phosphate buffered saline.

DNA isolation

Tumor DNA was isolated and purified with Trizol reagent (GIBCO Life Technologies) according to the manufacturer's instruction.

Bisulfite conversion of DNA samples

Bisulfite conversion of DNA samples for methylation detection was carried out based on the principle that treatment of DNA with bisulfite would result in the conversion of unmethylated cytosine residues into uracil, while methylated cytosine residues remain unchanged. Thus, the DNA sequences of methylated and unmethylated genomic regions following bisulfite conversion would be different and distinguishable by sequence specific PCR primers. CpGenome DNA modification kit (Intergen, New York, NY) was used. One microgram of DNA was treated with sodium bisulfite and the bisulfite-converted DNA was resuspended in a total volume of 30 μL of TE.

Methylation-specific PCR (MSP)

MSP was performed using CpG p16 WIZ amplification kit (Intergen). With a complete chemical modification reaction, U primers amplified only unmethylated DNA, and M primers amplified only methylated DNA in the region of P16 gene promoter. W primers amplified unmodified DNA no matter they were methylated or not. Each chemically modified experimental DNA sample was amplified with primers U, M and W respectively. The PCR mixture contained 1×PCR buffer (500 mmol·L^{-1} KCl, 100 mmol·L^{-1} Tris-HCl, 10 g·L^{-1} Triton-100, 15 mmol·L^{-1} $MgCl_2$), dNTPs (each at 0.25 mmol·L^{-1}), U or M primers 1.0 μL, Ampli Taq Gold polymerase (Perkin-Elmer, Foster city, CA) 1 U, bisulfite-modified DNA 0.1 μg in a final volume of 30 μL. Reactions were started at 95℃ for 12 min. Amplification was carried out in a thermal cycler (Hybaid, Teddington, England) for 35 cycles (45 s at 95℃, 45 s at 60℃, and 60 s at 72℃), followed by a final 10 min extension at 72℃. U, M and W controls provided by the kit served as validation of the reagents and PCR conditions. PCR products were analyzed by agarose gel electrophoresis and ethidium bromide staining.

The expected PCR products visualized on the gel should be an 154 bp nucleotide for unmethylated P16 and 145 bp nucleotide for methylated p16. If the sample contains unmethylated DNA, Uprimer will produce an 154 bp products (band U), and if it contains methylated DNA, M primer will produce 145 bp products (band M). The appearance of band W from the sample indicated an incomplete bisulfite conversion, and was considered a sign of unqualified chemical modification of the sample.

Clinicopathological data

Clinicopathological data were collected after MSP for p16 methylation was accomp lished. The clinicopathological data

included age, gender of the patients, anatomical location[2] and Dukes stage of CRC.

Statistical analysis

The χ^2 test was performed to analyze the relationship between p16 methylation status and each of the clinicopathologic parameters.

RESULTS

MSP analysis of p16 methylation

Of 62 CRC specimens, 26 displayed bands M, 154 bp products, signifying that 42% of the tumors had detectable p16 methylation. Band U, 154 bp product, was visible in all specimens with varied intensity, indicating the existence of unmethylated DNA in these specimens. No band W appeared in all specimens (Figure 1).

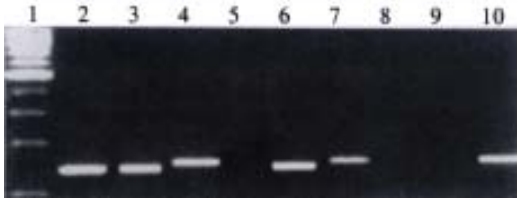

Figure 1 Electrophoresis of MSP products of p16 CpG region. 1. 100 bp marker, 2. W control (142 bp), 3. M control (145 bp), 4. U control (154 bp), 5,6,7. The same sample (from patient No.9) reactive with W, M,U primers respec tively, 6. 145 bp product, 7. 154 bp product, indicating p16 was methylated in this sample, 8,9,10. Same sample (from patient No.34) reactive with W, M, U primers respectively, 10. 154 bp product, indicating that p16 was unmethylated in this sample.

Clinicopathological correlations with p16 methylation

No correlation was found between p16 methylation and some of clinical factors, including age, gender, tumor location, etc. However, P16 methylation was signif icantly associated with Dukes' staging. Dukes' C, D patients were more likely to contain methylated p16 compared with Dukes' A, B patients (Table 1).

Table 1 Clinicopathological correlations with p16 methylation

	n	Methylated	Unmethylated
Male	35	18 (51.4)	17 (48.6)
Female	27	8 (29.6)	19 (70.4)
Age (yrs)			
<50	15	7 (46.7)	8 (53.3)
>50	47	19 (40.4)	28 (59.6)
Tumor location[2]			
Proximal	21	12 (57.1)	9 (42.9)
Distal	41	14 (34.1)	27 (65.9)
Dukes' staging			
A, B	33	4 (12.1)	29 (87.9)
C, D	29	22 (75.9)	7 (24.1)[b]

[b]$P<0.01$, *vs* A, B.

DISCUSSION

DNA methylation involves addition of a methyl group to the carbon 5 position of the cytosine ring. This reaction is catalyzed by DNA methyltransferase in the context of the sequence 5'-CG-3', which is also referred to as a CpG dinucleotide[6,7]. The potential contribution of DNA methylation to oncogenesis is mediated by one or more of mechanisms that include DNA hypomethylation, hyper methylation of tumor suppressor gene and chromosomal instability in cancers[8-13]. The methylation of gene, particularly the methylation of CpG-rich promoters, could block transcriptional activation[6]. P16 tumor suppressor gene plays a monitor role in the passage of cells through the G1 to S phase of the cell cycle by binding to cyclin-dependent kinase 4 and inhibiting its effect on cyclin D1[14-16]. Methylation of cytosine residues at CpG sites in p16 gene promotor, resulting in a silenced p16 expression, has been reported in many cell lines, including CRC, and some primary carcinomas in varied origins, such as colon, brain, breast, bladder, ovary, lung, and myeloma and so on[17-30].

In the previous studies on p16 methylation, most researchers used Southern analysis and digestion of methylation-sensitive enzymes to detect methylation[15,31]. We used MSP, in the present study, for analysis of the methylat ion status of p16. This method provided significant advantages over previous ones used for assaying methylation. MSP is much more sensitive than Southern analysis, facilitating the detection of low numbers of methylated alleles and the study of DNA from small samples. MSP allows examination of all CpG sites, not just those within sequences recognized by methylation sensitive restriction enzymes. In summary, MSP is a simple, sensitive and specific method for determining the methylation status of CpG rich region[32]. This might account for that the occurrence of p16 methylation detected by MSP (20%-50%)[4,5,33] was usually higher than those by Southern analysis and other METHODS (10%-20%)[15,31]. In our study, methylated p16 in primary CRC was found in a frequency as high as 42%, probably revealing a number more precise than those detected not by MSP.

Though p16 methylation is common in CRC cell lines[4,34,35] and has been suggested to involve also in the primary CRC[2,4,5,33,36-38], the association of p16 methylation with the clinicopathological characteristics of primary CRC has rarely been investigated and concluded with conflicts[2,5,37-39]. The role of p16 methylation in the development and progress of the primary CRC therefore awaits to be elucidated. Wiencke *et al* found that women were much more likely than men to have p16-methylated tumors and that proximal tumors were more likely to contain methylated p16. They reported that p16 methylation was also associated with poorly differentiated tumors[2]. Liang *et al* reported that the presence of p16 methylation predicted shorter survivals in colorectal cancer patients[5]. Zhang et al found that p16 methylation in CRC was not associated with clinicopathologic data[37,38]. In our study, no correlation was found between p16 methylation and various clinicopathologic factors including age, gender or tumor location. However, p16 methylation was significantly associated with Dukes' staging. Dukes C, D patients were more likely (75.9%) to contain methylated p16 as compared with Dukes' A, B patients (12.1%). Dukes' staging is a clinical classification for colorectal cancers based on the tumor size, local extent, metastatic status, i.e. lymph node involvement. Hence, Dukes' staging has been considered a most important prognostic determinant. In the present study, p16 methylation was more likely to occur in Dukes' C, D patients. This result suggested that p16 methylation might link to a more malignant outcome of CRC.

How does the existence of methylated p16 promoter affect the expression pattern of p16 protein in primary tumors Controversy has emerged over recent years on the

relationship of p16 promoter methylation and its gene expression in whole tumors. Although most of authors hold the traditional viewpoint that detectable p16 methylation necessarily link to the inactivation of p16 protein, or transcriptional silencing of p16 gene[3,10,19,31,40], coexistence of p16 methylation and p16 expression in one specimen has been frequently described[2,41-44]. It was noticeable that, in our study, band U, i.e. PCR products amplified by primer U, was visible in each specim en. Similar results were also reported by other investigators[2]. This might first be attributed to the contamination of non-neoplastic cells that naturally harbored unmethylated DNA. However, Guan *et al* demonstrated that band U appeared even in the samples acquired by microdissection which got rid of the non-neoplastic cells[4] . This indicated the possibility that tumor cells in one sample were virtually the mixture of those contained methylated and unmethylated DNA. In fact, the cell line in which p16 methylation was detected could still express p16 protein, due to a partial methylation[45]. In summary, coexistence of p16 methylation and p16 expression in one sample might reflect the cell heterogeneity, in which, as we speculated, a part of cells contained methylated p16 and loss of p16 expression whereas another part of cells kept expressing or even overexpressing p16 protein. We found that, in a previous immunohistochemical study on 71 archival specimens of CRC, p16 expression-positive tumor cells were seen in roughly 50% specimens, and these p16 expressive specimens were coincident with those with a poorer differentiation grade[46]. Other investigators have also proposed that activation but not inactivation of p16 gene was associated with primary CRC[47]. We will continue our effects to explore the correspondent relation of p16 methylation and p16 expression in the identical CRC specimen, so as to determine how the seemly paradoxical events might unite in one tumor. The elucidation of the relationship between p16 expression and p16 gene methylation in primary tumors will certainly help better understand the role of methylation of tumor suppressor genes in carcinogenesis.

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