RESEARCH NOTE

A study of the frequency of methylation of gene promoter regions in colorectal cancer in the Taiwanese population

CHANG-CHIEH WU[1], JEN-CHUN KUAN[2], CHIH-HSIUNG HSU[3], TZU-AN CHEN[4], CHIEN-AN SUN[3,5], TSAN YANG[6], SHINN-LONG LIN[7] and YU-CHING CHOU[3]*

[1]*Division of Colorectal Surgery, Department of Surgery, and* [7]*Department of Anesthesiology, Tri-Service General Hospital, National Defense Medical Center, No.325, Sec.2, Chenggong Rd., Neihu District, Taipei, Taiwan (Republic of China)*
[2]*Graduate Institute of Life Sciences, and* [3]*School of Public Health, National Defense Medical Center, No.161 Sec. 6, Minquan E. Rd., Neihu Dist., Taipei, Taiwan (Republic of China)*
[4]*Department of Surgery, Landseed Hospital, Taiwan No.77, Kwang-Tai Rd., Pingjen City, Tao-Yuan, Taiwan (Republic of China)*
[5]*Department of Public Health, College of Medicine, Fu-Jen Catholic University, No. 510, Jhong-Jheng Rd., Sin-Jhuang, New Taipei, Taiwan (Republic of China)*
[6]*Department of Health Business Administration, Meiho University, No.23, Pingguang Rd., Neipu, Pingtung, Taiwan (Republic of China)*

[Wu C.-C., Kuan J.-C., Hsu C.-H., Chen T.-A., Sun C.-A., Yang T., Lin S.-L. and Chou Y.-C. 2013 A study of the frequency of methylation of gene promoter regions in colorectal cancer in the Taiwanese population. *J. Genet.* **92**, 109–113]

Introduction

In 2008, colorectal cancer (CRC) was the third most commonly diagnosed cancer worldwide in males, and the second most common in females, with over 1.2 million new cancer cases and 608,700 deaths estimated to have occurred during the year (Jemal *et al.* 2011). The age-specific prevalence of CRC is higher in men than in women, and later-birth cohorts exhibit a much stronger cohort effect than earlier-birth cohorts (Brenner *et al.* 2010).

Fortunately, CRC is often preventable by early detection and removal of adenomatous polyps, and survival prospects are significantly better when CRC is diagnosed while still localized (Levin *et al.* 2008). Recent progress in our understanding of the biology of colorectal cancer has led to possible new approaches to screening (Davies *et al.* 2005). Accordingly, genetic alterations have been associated with specific steps in this polyp–adenocarcinoma sequence and may drive the histological progression of colon cancer (Yiu *et al.* 2005).

DNA methylation is the most well-studied epigenetic change (Feng *et al.* 2008), and provides a new generation of cancer biomarkers. Epigenetic modifications, particularly DNA methylation in selected gene promoters, are common molecular-level alterations in human tumours (Kim *et al.* 2010). Recent study has concluded that many gene promoter methylations result in transcriptional silencing, and so might be exploited as biomarkers for the early detection of CRC (Mitomi *et al.* 2010). Previous research shows that DNA methylation occurs frequently in the promoter regions of tumour suppressor genes, and it plays an important role in tumour development (Kneip *et al.* 2011). Multiple studies have reported association of CRC with gene mutations (Vilkin *et al.* 2009).

Transcriptional silencing of tumour-suppressor genes by hypermethylation of promoter-region CpG islands is an important pathway results in tumourigenesis development (Esteller 2007). The tumour progression of CRC is caused by a loss of function in cell-cycle regulation and mismatch repair (MMR), which is related to DNA hypermethylation of the transcription start site (Esteller 2011). Many studies have identified DNA-methylation-related genes associated with carcinogenesis pathways (Silver *et al.* 2012). For example, *O*-6-methylguanine DNA methyltransferase (*MGMT*) is a tumour-suppressor gene involved in DNA repair, and promoter hypermethylation and low expression by transcriptional silencing are associated with many diseases, including CRC (Shima *et al.* 2011). The *CDKN2A* gene (encode cyclin-dependent kinase inhibitor 2A) is considered as a tumour-suppressor gene involved in cell-cycle control, and methylation in promoter regions of this gene is a frequent event in CRC patients (Veganzones-de-Castro *et al.* 2012). The

*For correspondence. E-mail: trishow@mail.ndmctsgh.edu.tw.

Keywords. colorectal cancer; DNA methylation; Taiwanese population.

mismatch repair gene *MLH1* plays an important role in deletion of mononucleotide repeats, and loss of the repair system is one of the main mechanisms of accumulating functional changes oncogenic effects (Iacopetta *et al.* 2010).

Previous studies used methylation-specific polymerase chain reaction (MSPCR) to analyse DNA methylation of colorectal samples and associated tissues from a cohort of patients with colorectal carcinomas (Azuara *et al.* 2010; Pancione *et al.* 2010; Yi *et al.* 2011). However, there has been some criticism of the design of the case–control studies, a lack of case numbers, to represent the general population in Taiwan.

The aims of this study were to determine the DNA methylation status of three tumour-related genes (*MGMT*, *CDKN2A* and *MLH1*) using MSPCR to analyse tumour tissue samples from 132 CRC patients (264 samples) selected from the Tumor Bank, Tri-Service General Hospital, Taiwan and compare it with that of matched normal colorectal tissue samples, >10 cm away from the tumour site, of the same patients. We strongly believe such a panel of genes would be suggested to establish a gene promoter methylation patterns for detecting malignant colorectal disease in tissue from patients with early-stage colorectal cancer.

Materials and methods

Collection of subjects

All patients enrolled with informed consent in this study were purposively sampled under Tri-Service General Hospital Institution Review Board (IRB) approval (TSGHIRB approval number: 098-05-292). All patients were clinically diagnosed with CRC, and had to be able to tolerate surgical resection. Based on these criteria, pairs of specimens were obtained from 132 CRC patients (264 samples) enrolled in the study, and meeting these criteria. Sample collection was made at surgery clinics; tumour tissue and normal tissue were resected at the same time; the matched normal tissue was taken from an incision more than 10 cm away from the carcinoma site. Specimens were immediately stored in liquid nitrogen. The resection procedure was reviewed by the Department of Colorectal Surgery, Tri-Service General Hospital.

DNA isolation and sodium bisulphite treatment

Genomic DNA was extracted from tissue samples using a QIAamp DNA Mini Kit (Qiagen GmbH, Hilden, Germany) according to the manufacturer's protocol. The resulting DNA was sodium bisulphite modified using an EZ DNA Methylation Kit (Zymo Research, Orange, USA). Methylated DNA control for MSPCR assays was generated using *Sss*I methylase (Zymo Research, Orange, USA).

Methylation-specific PCR

Bisulphite-treated DNA was subjected to MSPCR using primer pairs designed to amplify the target genes specifically. The reaction solution (15 μL) contained HotStart Taq Premix (7.5 μL) (RBC Bioscience, Taipei, Taiwan), bisulphite-treated DNA (0.6 μL), and 0.6 μL aliquots of primer; PCR conditions included denaturation at 95°C for 10 min followed by 35 cycles at 95°C for 30 s, annealing temperature at 62°C, 60°C, and 53°C for 35 s, 72°C for 30 s, and final extension at 72°C for 4 min (table 1). PCR products were mixed with DNA dye (Bioman, Taipei, Taiwan), subjected to horizontal gel electrophoresis on 1.5% agarose gel for 45 min, stained with ethidium bromide for 10 min, and the results analysed by visualization under UV transillumination.

Statistical methods

The analysis involved i) descriptive statistics including the frequency of methylation of three genes (*CDKN2A*, *MLH1* and *MGMT*) in tumour and matched normal tissues and ii) inferential statistics, including the chi-square test, used to assess the significance of the differences between tumour and matched normal tissues to evaluate association of DNA

Table 1. Primer sequences, annealing temperature and product size for methylation specific PCR of three genes related to colorectal cancer.

Gene		Forward primer (5'→3')	Annealing temperature (°C)	Product size (bp)
CDKN2A	M	F: TTATTAGAGGGTGGGGCGGATCGC R: GACCCCGAACCGCGACCGTAA	62	150
	U	F: TTATTAGAGGGTGGGGTGGATTGT R: CAACCCCAAACCACAACCATAA	62	151
MLH1	M	F: ACGTAGACGTTTTATTAGGGTCGC R: CCTCATCGTAACTACCCGCG	60	118
	U	F: TTTTGATGTAGATGTTTTATTAGGGTTGT R: ACCACCTCATCATAACTACCCACA	60	124
MGMT	M	F: TTTCGACGTTCGTAGGTTTTCGC R: GCACTCTTCCGAAAACGAAACG	53	81
	U	F: TTTGTGTTTTGATGTTTGTAGGTTTTTGT R: AACTCCACACTCTTCCAAAAACAAAACA	53	93

methylation and CRC. We defined panels of genes sensitivity as the proportion of tumour tissue samples in which gene methylation was detected in one or more of the genes present in the panel. Gene panel specificity was defined as the proportion of matched normal tissue samples in which gene methylation was not detected in any of the genes in the panel. All analyses were conducted using SPSS v. 20.0.

Table 2. Clinical characteristics of 132 CRC patients.

Variable	Number of patients (per cent of total in parentheses)
Sex	
Male	64 (51.5)
Female	68 (48.5)
Surgical age	
<59	51 (38.6)
60–69	19 (14.4)
70–79	34 (25.8)
>80	28 (21.2)
Average surgical age (yr)	68.9 ± 13.7
Histologic stage	
I	14 (11.1)
II	48 (38.1)
III	40 (31.7)
IV	24 (19.0)
Recurrence or metastasis	
Yes	40 (30.3)
No	92 (69.7)
Deceased during follow-up	
Yes	32 (24.2)
No	100 (75.8)

Table 3. Methylation status in promoter region of three CRC-related genes in tumour tissue and matched normal tissue.

Gene	No. and percentage of samples methylated		
	Tumour tissue, n (%)	Matched normal tissue, n (%)	*P**
MGMT	58 (44.9)	9 (6.8)	<0.001
CDKN2A	54 (40.9)	22 (16.7)	<0.001
MLH1	21 (15.9)	4 (3.0)	<0.001

* Chi-square test

Results

Among the 132 CRC patients (264 samples) enrolled in this study, 51.5% were male and 48.5% female; the average of surgical age was 68.9. Of the cohort, 38.1% patients were classified at histological stage II, 30.3% patients experienced recurrence or metastasis after surgical excision, and 24.2% subjects died during the follow-up of this study. We collected pairs of tumour and matched normal tissue samples from each of the subjects (table 2).

DNA methylation of *MGMT*, *CDKN2A* and *MLH1* were more frequent in tumour than in matched normal tissues; in addition, the methylation patterns were not only occurred in each of the genes but also pooling the three genes as panels. Although the three genes were methylated in both tumour and matched normal tissue samples, the percentage of methylation was significantly higher in tumour tissue than it was in normal tissue (*MGMT*, 44.7% vs 6.8%; *CDKN2A*, 41.7% vs 16.7%; *MLH1*, 15.9% vs 3.0%; $P < 0.001$) (table 3).

We used panels of genes to assess sensitivity and specificity of the methylation data in distinguishing tumour and normal tissue from CRC patients. One single gene *MLH1* showed that methylation had the greatest specificity (97.0%), but it identified only 15.9% of CRC tissue samples. The panel of *MLH1* and *MGMT* genes showed higher sensitivity (52.3%), with 90.8% specificity. The panel of all three genes, *MLH1*, *MGMT*, and *CDKN2A*, showed 65.9% sensitivity and 75.8% specificity from the same CRC patients which was compared by their tumour and matched normal tissues (table 4).

Discussion

There is potential need for a screening strategy that uses biomarkers to place cancer patients in appropriate surveillance programmes for early detection of cancer. This study suggests use of a panel of genes whose methylation status

Table 4. Sensitivity and specificity of DNA methylation of three genes related to colorectal cancer evaluated in panels of one, two, or all three genes.

Panel	No. and percentage of samples methylated	
	Tumour tissue, n (%)	Matched normal tissue, n (%)
MLH1	21 (15.9)	4 (3.0)
MLH1 + *MGMT*	69 (52.3)	12 (9.1)
MLH1 + *MGMT* + *CDKN2A*	87 (65.9)	32 (24.2)

See Methods for definitions of gene panel sensitivity, and gene panel specificity.

in normal and tumour tissue from colorectal cancer patients provides useful information. DNA methylation at gene promoter is generally associated with epigenetic silencing of gene function (Kangaspeska *et al.* 2008); thus, the role of methylation might be an important feature for a significant proportion of genes (Metivier *et al.* 2008). Substantial efforts have been made to determine the cause and role of aberrant DNA methylation in colorectal cancer. In our study, we observed a trend of increasing numbers in the panels of genes and extent of colorectal cancer tissue growth an observation also noted by Kim *et al.* (2010).

We observed methylation of DNA in the promoter region of *MGMT*, *CDKN2A*, and *MLH1* genes in both normal and tumour colorectal tissue of CRC patients. The frequency of methylation in tumour tissue was 44.7% in *MGMT*, 41.7% in *CDKN2A* and 15.9% in *MLH1* in agreement with other studies on the sensitivity of correct detection of cancerous colorectal tissue (Huang *et al.* 2009; Vilkin *et al.* 2009). DNA methylation is associated with CRC and plays an important role in functional gene silencing in transformation of a normal cell to a tumour cell (Hitchins *et al.* 2011). The overall sensitivity and specificity of the DNA methylation status provided by the three genes combined were 65.9% and 75.8%, respectively.

The faecal occult blood test (FOBT) is commonly used in clinical screening for CRC. The technique is facile and efficient for medical personnel to use; however, a review study reported meta-analysis findings that FOBT may not provide adequate sensitivity to serve as a screening option for CRC (sensitivity, 36%) (Rosman and Korsten 2010). Therefore we are conducting further research with a larger cohort to establish a more sensitive and specific panel of genes as a biomarker for CRC.

The detection of DNA methylation has been used in identifying tumour tissues by other research group (Hinoue *et al.* 2012); however, our study focusses on the DNA methylation of tumour suppressor gene as a predicted biomarker with more sensitive and specific, especially for comparison of matched normal tissue from the same individual which was obtained >10 cm away from the tumour site. Additionally, there were a very few DNA aberrant methylation associated with earlier colorectal cancer studies in Taiwan. It was a larger sample size in our study that could be more representative of DNA methylation study relative to others (Chen *et al.* 2009; Huang *et al.* 2009).

Our findings show a correlation between colorectal cancer and methylation of promoter DNA of *MGMT*, *CDKN2A*, and *MLH1* genes. There are two main limitations of this study. The first is that DNA methylation, which identifies novel biomarkers of CRC among a panel of genes, lacks assessable and directly available information (DNA from blood or faeces) for use as a widely used screening tool. Second, we could not obtain information on methylation status over the course of tumour progression for each individual. Therefore, this study shows an association between the DNA methylation of tumour tissue and adjacent normal tissue. However, these three target genes may provide useful information in ongoing research as novel biomarkers in CRC. We also did not conduct quantitative analysis of DNA methylation. We suggest that researchers measure methylation of gene promoter regions by a pyro-sequence approach in future.

In the present study, we confirm a correlation between colorectal cancer and DNA methylation. For future work, we suggest recruiting high-risk individuals, for whom colorectal carcinogenesis has not yet occurred, as a cohort study base to investigate any causal-relationship between DNA methylation and cancer cell development.

Acknowledgements

The authors thank the participants in the study for their specimen donations. The study was supported by funding from the National Science Council, Republic of China (NSC 98-2314-B016-001, NSC 99-2314-B016-020, NSC 100-2314-B016-025, NSC 101-2314-B-016-029).

References

Azuara D., Rodriguez-Moranta F., de Oca J., Soriano-Izquierdo A., Mora J., Guardiola J. *et al.* 2010 Novel methylation panel for the early detection of colorectal tumors in stool DNA. *Clin. Colorectal Cancer* **9**, 168–176.

Brenner H., Altenhofen L. and Hoffmeister M. 2010 Sex, age, and birth cohort effects in colorectal neoplasms: a cohort analysis. *Ann. Int. Med.* **152**, 697–703.

Chen S. P., Chiu S. C., Wu C. C., Lin S. Z., Kang J. C., Chen Y. L. *et al.* 2009 The association of methylation in the promoter of APC and MGMT and the prognosis of Taiwanese CRC patients. *Genet. Test Mol. Biomarkers* **13**, 67–71.

Davies R. J., Miller R. and Coleman N. 2005 Colorectal cancer screening: prospects for molecular stool analysis. *Nat. Rev. Cancer* **5**, 199–209.

Esteller M. 2007 Cancer epigenomics: DNA methylomes and histone-modification maps. *Nat. Rev. Genet.* **8**, 286–298.

Esteller M. 2011 Epigenetic changes in cancer. *F1000 Biol. Rep.* **3**, 9.

Feng Q., Hawes S. E., Stern J. E., Wiens L., Lu H., Dong Z. M. *et al.* 2008 DNA methylation in tumor and matched normal tissues from non-small cell lung cancer patients. *Cancer Epidemiol. Biomarkers Prev.* **17**, 645–654.

Hinoue T., Weisenberger D. J., Lange C. P., Shen H., Byun H. M., Van Den Berg D. *et al.* 2012 Genome-scale analysis of aberrant DNA methylation in colorectal cancer. *Genome Res.* **22**, 271–282.

Hitchins M. P., Rapkins R. W., Kwok C. T., Srivastava S., Wong J. J., Khachigian L. M. *et al.* 2011 Dominantly inherited constitutional epigenetic silencing of MLH1 in a cancer-affected family is linked to a single nucleotide variant within the 5′UTP. *Cancer Cell* **20**, 200–213.

Huang C. C., Chien W. P., Wong R. H., Cheng Y. W., Chen M. C. and Lee H. 2009 NAT2 fast acetylator genotype and MGMT promoter methylation may contribute to gender difference in K-RAS mutation occurrence in Taiwanese colorectal cancer. *Environ. Mol. Mutagen.* **50**, 127–133.

Iacopetta B., Grieu F. and Amanuel B. 2010 Microsatellite instability in colorectal cancer. *Asia Pac. J. Clin. Oncol.* **6**, 260–269.

Jemal A., Bray F., Center M. M., Ferlay J., Ward E. and Forman D. 2011 Global cancer statistics. *CA Cancer J. Clin.* **61**, 69–90.

Kangaspeska S., Stride B., Metivier R., Polycarpou-Schwarz M., Ibberson D., Carmouche R. P. *et al.* 2008 Transient cyclical methylation of promoter DNA. *Nature* **452**, 112–115.

Kim M. S., Lee J. and Sidransky D. 2010 DNA methylation markers in colorectal cancer. *Cancer Metastasis Rev.* **29**, 181–206.

Kneip C., Schmidt B., Seegebarth A., Weickmann S., Fleischhacker M., Liebenberg V. *et al.* 2011 SHOX2 DNA methylation is a biomarker for the diagnosis of lung cancer in plasma. *J. Thorac. Oncol.* **6**, 1632–1638.

Levin B., Lieberman D. A., McFarland B., Andrews K. S., Brooks D., Bond J. *et al.* 2008 Screening and surveillance for the early detection of colorectal cancer and adenomatous polyps, 2008: a joint guideline from the American Cancer Society, the US Multi-Society Task Force on Colorectal Cancer, and the American College of Radiology. *Gastroenterology* **134**, 1570–1595.

Metivier R., Gallais R., Tiffoche C., Le Peron C., Jurkowska R. Z., Carmouche R. P. *et al.* 2008 Cyclical DNA methylation of a transcriptionally active promoter. *Nature* **452**, 45–50.

Mitomi H., Fukui N., Tanaka N., Kanazawa H., Saito T., Matsuoka T. *et al.* 2010 Aberrant p16((INK4a)) methylation is a frequent event in colorectal cancers: prognostic value and relation to mRNA expression and immunoreactivity. *J. Cancer Res. Clin. Oncol.* **136**, 323–331.

Pancione M., Sabatino L., Fucci A., Carafa V., Nebbioso A., Forte N. *et al.* 2010 Epigenetic silencing of peroxisome proliferator-activated receptor gamma is a biomarker for colorectal cancer progression and adverse patients' outcome. *PLoS One* **5**, e14229.

Rosman A. S. and Korsten M. A. 2010 Effect of verification bias on the sensitivity of fecal occult blood testing: a meta-analysis. *J. Gen. Intern. Med.* **25**, 1211–1221.

Shima K., Morikawa T., Baba Y., Nosho K., Suzuki M., Yamauchi M. *et al.* 2011 MGMT promoter methylation, loss of expression and prognosis in 855 colorectal cancers. *Cancer Causes Control* **22**, 301–309.

Silver A., Sengupta N., Propper D., Wilson P., Hagemann T., Patel A. *et al.* 2012 A distinct DNA methylation profile associated with microsatellite and chromosomal stable sporadic colorectal cancers. *Int. J. Cancer* **130**, 1082–1092.

Veganzones-de-Castro S., Rafael-Fernandez S., Vidaurreta-Lazaro M., de-la-Orden V., Mediero-Valeros B., Fernandez C. *et al.* 2012 p16 gene methylation in colorectal cancer patients with long-term follow-up. *Rev. Esp. Enferm. Dig.* **104**, 111–117.

Vilkin A., Niv Y., Nagasaka T., Morgenstern S., Levi Z., Fireman Z. *et al.* 2009 Microsatellite instability, MLH1 promoter methylation, and BRAF mutation analysis in sporadic colorectal cancers of different ethnic groups in Israel. *Cancer* **115**, 760–769.

Yi J. M., Dhir M., Van Neste L., Downing S. R., Jeschke J., Glockner S. C. *et al.* 2011 Genomic and epigenomic integration identifies a prognostic signature in colon cancer. *Clin. Cancer Res.* **17**, 1535–1545.

Yiu R., Qiu H., Lee S. H. and Garcia-Aguilar J. 2005 Mechanisms of microsatellite instability in colorectal cancer patients in different age groups. *Dis. Colon Rectum* **48**, 2061–2069.

Received 14 September 2012, in final revised form 27 December 2012; accepted 28 December 2012
Published on the Web: 2 April 2013