FULL PAPER

BJC

British Journal of Cancer (2013) 109, 1004–1012 | doi: 10.1038/bjc.2013.430

Keywords: CpG island methylator phenotype; microsatellite instability; colorectal cancer; prognosis

Prognostic implication of the CpG island methylator phenotype in colorectal cancers depends on tumour location

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Background: Colorectal cancer (CRC) is usually categorised as proximal or distal CRC. Recently, many researchers have tried to determine the molecular heterogeneity of CRCs along bowel subsites. However, the differential effects of the CpG island methylator phenotype (CIMP) and microsatellite instability (MSI) on the clinical outcome according to tumour location are not well-known.

Methods: We analysed clinicopathologic and molecular characteristics, including CIMP, MSI, *KRAS* and *BRAF* mutations, in 734 CRCs according to bowel subsites. And the prognostic value of CIMP and MSI was analysed according to tumour location.

Results: We found a linear increase of female predominance, T, N category, stage, differentiation, absence of luminal necrosis, tumour -infiltrating lymphocytes, Crohn's-like lymphoid reaction, serration and mucin production from the rectum to caecum. CpG island methylator phenotype -high and MSI-high gradually increased from the rectum to caecum. CpG island methylator phenotype is a poor prognostic factor of overall survival (hazard ratio (HR): 4.13, 95% confidence interval (CI): 1.27–13.46) and disease-free survival (HR: 2.90, 95% CI: 1.04–8.08) in rectal cancers.

Conclusion: Clinicopathologic and molecular profiles of CRCs gradually change along bowel subsites, and the prognostic implication of CIMP is different according to tumour location.

Colorectal cancer (CRC) is one of the most common malignancies worldwide. In Korea, CRC is the third most commonly diagnosed malignancy and the fourth cause of cancer-related death. Over the past decade, the incidence of CRC had rapidly increased, and 25 782 patients were newly diagnosed in 2010 (Jung *et al*, 2013). Colorectal cancers are a heterogeneous disease in terms of molecular carcinogenesis. Currently, three molecular pathways are generally accepted, which are chromosomal instability (CIN), microsatellite instability (MSI) and CpG island methylator phenotype (CIMP). Chromosomal instability is characterised by alterations in the number and structure of chromosomes, and the accumulation of somatic mutations of proto-oncogenes and tumour suppressor genes (Fearon and Vogelstein, 1990; Smith *et al*, 2002). Microsatellite instability is caused by a defective mismatch repair system and is characterised by alterations in the number of repeat nucleotide(s), leading to frame-shift mutations of the corresponding genes (Boland and Goel, 2010). CpG island methylator phenotype is characterised by widespread cancer-specific hypermethylation of numerous promoter CpG island loci (>1000 genes) (Hinoue *et al*, 2012). Although the majority of cancer-specific hypermethylation events do not have functional consequences, $<10\%$ of cancer-specific hypermethylation events have a strong inverse relationship with their gene expression levels and inactivate cancer-related genes (Hinoue *et al*, 2012). The CIN

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Received 4 March 2013; revised 27 June 2013; accepted 4 July 2013; published online 30 July 2013

pathway is known to be in an exclusive relationship with the MSI pathway, which partially overlaps with the CIMP pathway (The Cancer Genome Atlas Network, 2012).

CRCs with MSI and/or CIMP have distinctive clinicopathologic features compared with CRCs with CIN. Colorectal cancers with MSI are more frequently associated with female gender, poor differentiation or mucinous histology, tumour-associated lymphoid reaction, large tumour size and favourable survival than CRCs with CIN (Popat *et al*, 2005; Boland and Goel, 2010; Roth *et al*, 2012). Colorectal cancers with CIMP show some shared features with CRCs with MSI, such as female preponderance, poor differentiation and tumour-associated lymphoid reaction, but CRCs with CIMP are more frequent in older-aged patients and tend to be associated with poor clinical outcome compared with CRCs with MSI (Ogino *et al*, 2006, 2009; Barault *et al*, 2008).

During the past decades, an accumulating series of studies have reported epidemiological, clinicopathological and molecular differences between proximal colon cancer (proximal to the splenic flexure), distal colon cancer (distal to the splenc flexure) and rectal cancer (rectosigmoid and rectum) (Bufill, 1990; Breivik *et al*, 1997; Komuro *et al*, 2005; Saltzstein and Behling, 2007; Li and Lai, 2009; Slattery *et al*, 2009; Benedix *et al*, 2010; Yamauchi *et al*, 2012b). Proximal colon cancers are frequently observed in women and in older patients, whereas rectal cancers are more likely to be found in men than in women, and in people slightly younger than people with proximal or distal colon cancers. Whereas initial symptoms of rectal cancers include rectal bleeding and difficulty with stool passage, initial clinical manifestations of proximal colon cancers and distal colon cancers are anaemia and change in bowel habits, respectively (Majumdar *et al*, 1999). On a molecular level, rectal cancers or distal colon cancers are associated with CIN and *TP53* mutation, whereas proximal colon cancers are associated with CIMP, MSI and *BRAF* mutation (Russo *et al*, 2005; Sugai *et al*, 2006; Lee *et al*, 2008; Nosho *et al*, 2008; Hinoue *et al*, 2012).

Recently, Benedix *et al* (2011) subdivided the proximal colon and distal colon into four and three subsites, respectively, and compared colon cancers from these seven subsites based on demographic factors and clinicopathological features. This study revealed that there are considerable differences in multiple parameters, including age of onset, gender, tumour grade, histological subtype, lymphatic invasion and AJCC TNM stages, within proximal colon cancers and distal colon cancers. Subsequent studies by Benedix *et al* (2012) and Yamauchi *et al* (2012b) have demonstrated differences in the molecular features, including CIMP, MSI, *KRAS* and *BRAF* mutation, in cancers from seven or nine bowel subsites. However, the prognostic implication of diverse molecular alterations along bowel subsites is not well understood.

Geographical differences in both the incidence of CRC and the ratio of colon to rectal cancers are well known, with a higher incidence of CRC and a greater ratio of colon to rectal cancers in western population compared with that of the eastern population (Sung *et al*, 2005; Jemal *et al*, 2011). Furthermore, the prevalence of CIMP, MSI and *BRAF* mutation is significantly lower in CRCs from eastern population than in CRCs from western population (Kang, 2011; Rhee *et al*, 2012), even though the exact reason of low prevalence of those molecular alterations in eastern population is still not known. Although subsite-dependent differences in clinicopathologic and molecular features have been demonstrated in CRCs from western population, little is known regarding bowel subsite-dependent differences in clinicopathologic and molecular features in CRCs from eastern population (Benedix *et al*, 2012; Yamauchi *et al*, 2012b). Previously, we reported the clinicopathologic and prognostic implications of CIMP and MSI in CRCs, and the implications in four molecular subtypes in CRCs according to a combination of CIMP and MSI in a relatively limited number of CRCs ($n=320$) (Kim *et al*, 2009). In this study, we investigated clinicopathologic and molecular characteristics, including CIMP and MSI, in an independent set of 734 CRCs according to tumour location. Moreover, we performed survival analysis to reveal the differences in prognostic implications of CIMP and MSI depending on location.

MATERIALS AND METHODS

Study subjects. Nine hundred and eighty-nine CRC patients underwent curative surgery in Seoul National University Hospital, Seoul, Republic of Korea, from January 2004 to December 2006. After exclusion of 255 CRC patients (refusal of molecular study ($n=108$), non-invasive cancers ($n=30$), familial adenomatous polyposis ($n=11$), multiple occurrences ($n=56$), neoadjuvant chemo- and/or radiotherapy ($n=34$), recurrent tumour s ($n=16$)) and formalin-fixed paraffin-embedded tissues from 734 CRC patients were selected for this study. This study was approved by the Institutional Review Board.

Clinicopathologic analysis. Clinicopathologic characteristics, including age, sex, tumour location and TNM stage, were obtained from electronic medical records. Tumour location was initially divided into the caecum, ascending colon, hepatic flexure, transverse colon, splenic flexure, descending colon, sigmoid colon, rectosigmoid and rectum, according to AJCC sixth edition. Then, tumours located proximal to the splenic flexure were designated proximal colon cancers, tumours located in from splenic flexure to rectosigmoid junction were designated distal colon cancers and cancers in the rectosigmoid junction and rectum were considered as rectal cancers. Through microscopic examination of representative sections of tumours, two pathologists (Bae and Kang) evaluated the following parameters without knowledge of the CIMP, MSI, *KRAS* and *BRAF* mutation status of the specimen: tumour differentiation (tumour grade), luminal necrosis, Crohn's-like lymphoid reaction, number of tumour-infiltrating lymphocytes, luminal serration and extraglandular mucin production (Ogino *et al*, 2006). Overall survival (OS) and disease-free survival (DFS) data were extracted from the patients' medical records or from death registry offices.

***KRAS/BRAF* mutation and MSI analysis.** Through microscopic examination of histologic slides, representative tumour portions were marked on the tissue slides, and then the marked areas were subjected to manual microdissection. The dissected tissues were collected into microtubes containing lysis buffer and proteinase K, and were incubated at 55 °C for 2 days. Following centrifugation, the supernatants were transferred into a newly labelled microtube. The samples were then placed into a 95 °C heat block for 10 min to inactivate the proteinase K. Direct sequencing of *KRAS* codons 12 and 13, and allele-specific PCR analysis for *BRAF* codon 600 were performed as previously described (Kim *et al*, 2009). Microsatellite instability status was determined by NCI 5 markers, including BAT25, BAT26, D2S123, D5S346 and D17S250. Microsatellite instability-high (MSI-H) samples were defined as two or more markers being unstable, MSI-low (MSI-L) as 1 marker being unstable, and microsatellite stable (MSS) in the absence of instability.

Analysis of CIMP. DNA samples were bisulphite-modified using the EZ DNA methylation kit (Zymo Research, Orange, CA, USA). The modified DNA samples were analysed for their methylation status of eight CIMP-specific CpG islands (*CACNA1G*, *CDKN2A*, *CRABP1*, *IGF2*, *MLH1*, *NEUROG1*, *RUNX3* and *SOCS1*) by using a methylation-specific, probe-based, real-time PCR technology (the MethyLight assay). The DNA methylation at each methylation marker was reported as a percentage of methylated reference (PMR), that is, $PMR = 100 \times [(\text{methylated reaction/ALU})_{\text{sample}} / (\text{methylated reaction/ALU})_{M.SssI\text{-reference}}]$ (Kim *et al*, 2009).

MethyLight analysis was performed in quintiplicate and of the five measured values; the median was regarded as a representative value of methylation level for each marker and each sample. Each marker was considered methylated when median PMR was greater than 4. CpG island methylator phenotype-high (CIMP-H) was defined as $\geqslant 5$ methylated promoters, CIMP-low (CIMP-L) as 1 to 4 methylated promoters, and CIMP-0 as 0 methylated promoters.

Statistical analysis. SAS software (version 9.3 for Microsoft Windows; SAS Institute, Cary, NC, USA) was used for statistical analyses, and Kaplan–Meier plots were constructed using R software. The age of each group was compared using Kruskal–Wallis test. Clinicopathologic characteristics between and among groups were compared using Pearson's χ^2-test and Fisher's exact test for categorical variables. Kruskal–Wallis test was used for age of each group and ordinal variables, such as TNM stage and tumour differentiation. Mantel–Haenszel linear-by-linear association χ^2-test was used to reveal linear tendency of ordinal and categorical variables according to tumour location. Kaplan–Meier survival curve, log-rank test and Cox-proportional hazard model were used for survival analysis. For multivariate survival analysis, all the possible prognostic variables were initially included in Cox-proportional hazard model, then statistically significant variables were selected by backward elimination. Time-dependent covariate method was used to test proportional hazard assumption. All statistical tests were two-sided, and statistical significance was defined as $P<0.05$, except survival analysis. In survival analysis, statistical significance was defined as $P<0.0125$ by Bonferroni correction (total cases, proximal colon, distal colon and rectum cases).

RESULTS

Clinicopathologic characteristics of CRCs according to CIMP and MSI status. Patient characteristics are described in Table 1 and Supplementary Table S1. A total of 734 CRC patients (median age, 62 years; range, 20–90 years) were included. The male to female ratio was 1.55: 1 (446 males and 288 females). Tumour location was the caecum in 51, ascending colon in 94, hepatic flexure in 20, transverse in 35, splenic flexure in 9, descending in 34, sigmoid in 249, rectosigmoid in 74 and rectum in 168 patients. Collectively, 200 patients had proximal colon cancer, whereas 292 and 242 patients had distal colon cancer and rectal cancer, respectively. Median duration of follow-up was 56.5 months (0.3–89.8 months). Detailed clinicopathologic and histologic characteristics according to CIMP and MSI are summarised in Supplementary Table S1 and S2. CpG island methylator phenotype-L CRCs and CIMP-H CRCs were detected in 403 patients (54.9%) and 47 patients (6.4%), respectively. Microsatellite instability-L CRCs and MSI-H CRCs were detected in 37 patients (5.0%) and 65 patients (8.9%), respectively. Whereas CIMP-H CRCs were associated with older age at diagnosis ($P=0.035$), MSI-H CRCs were associated with younger age at diagnosis ($P=0.036$). CpG island methylator phenotype-H CRCs and MSI-H CRCs were frequently observed in the proximal large intestine ($P<0.001$), but no difference between sex according to CIMP and MSI status was observed ($P=0.445$ in CIMP, $P=0.716$ in MSI). Although CIMP-H CRCs showed advanced T, N category and overall stage, MSI-H CRCs were associated with less frequent distant metastasis. *BRAF* mutation was more frequent in CIMP-H CRCs than in CIMP-0,L CRCs ($P=0.005$).

On microscopic examination, CIMP-H CRCs and MSI-H CRCs were strongly associated with increased numbers of tumour-infiltrating lymphocytes, luminal serration and mucin production ($P<0.001$ for each variable). Colorectal cancers with CIMP-H showed more frequent poor differentiation than CIMP-0,L CRCs ($P<0.001$). Absence of luminal necrosis was frequent only in MSI-H CRCs ($P<0.001$).

Clinicopathologic and molecular characteristics of CRCs according to tumour location. Clinicopathologic and histologic characteristics along the bowel subsites are summarised in Tables 1 and 2. We found a linear trend among female occurrence, advanced T category, N category, overall stage, differentiation, absence of luminal necrosis, increased number of tumour-infiltrating lymphocytes, Crohn's-like lymphoid reaction, serration, mucin production, CIMP-H and MSI-H from the rectum to the caecum, using the Mantel–Haenszel linear-by-linear association χ^2-test. Prevalence of CIMP-H showed clearest linear association along the bowel subsites, except for absence of CIMP-H in the splenic flexure.

Supplementary Figure S1 displays the bowel-subsite distribution of four molecular subtypes, which were generated by the combinatory status of CIMP and MSI. The CIMP-0,L/MSS, MSI-L subtype exhibited progressive decreases in proportion from the rectum to caecum, whereas the CIMP-H/MSS,MSI-L and CIMP-H/MSI-H subtypes displayed a tendency towards a progressive increase from the rectum to caecum.

Different prognostic implication of CIMP and MSI according to tumour location. Initially, we performed survival analysis to determine the prognostic value of CIMP-H and MSI-H in the 734 CRCs. In the univariate survival analysis, CIMP-H CRCs showed worse OS ($P=0.009$) and DFS ($P=0.008$) compared with CIMP-0,L CRCs (Supplementary Figure S2a and b). Microsatellite instability status did not correlate with clinical outcome (Supplementary Figure S2c and d). Multivariate survival analysis revealed that only TNM stage, tumour differentiation and adjuvant chemotherapy were independent prognostic factors. CpG island methylator phenotype was not an independent prognostic factor for entire CRCs ($P=0.451$ in OS and $P=0.337$ in DFS) (Supplementary Table S3). Supplementary Figure S3 shows the Kaplan–Meier survival curves of four molecular subtypes according to tumour location.

As two thirds of the 734 CRCs were located in the sigmoid colon to rectum, and the prevalence of CIMP-H and MSI-H was low in this study population, it was impossible to analyse adjusted prognostic value of CIMP and MSI according to 9 subsites (adjusted for TNM stage, tumour differentiation and adjuvant chemotherapy status). Instead, we evaluated the prognostic implication of CIMP and MSI in proximal colon cancers, distal colon cancers and rectal cancers. In 200 proximal colon cancers, the CIMP status did not correlate with clinical outcome (Figure 1). Proximal colon cancers with MSI-H showed a tendency of better prognosis in univariate analysis, but the statistical significance was marginal ($P=0.055$ in OS, $P=0.131$ in DFS) (Figure 2). In 292 distal colon cancers, CIMP-H tended to show poor prognosis, reaching no statistical significance in univariate analysis ($P=0.178$ in OS, $P=0.150$ in DFS) (Figure 1). CpG island methylator phenotype-H was associated with poor clinical outcome in rectal cancers in univariate analysis ($P<0.001$ in OS, $P<0.001$ in DFS). Multivariate survival analysis showed marginal significance for CIMP-H in rectal cancers after Bonferroni correction (hazard ratio (HR): 4.13, 95% confidence interval (CI): 1.27–13.46, $P=0.019$ in OS; HR: 2.90, 95% CI: 1.04–8.08, $P=0.042$ in DFS) (Figure 1 and Table 3). The HR of CIMP-H compared with CIMP-0,L was gradually decreased from the rectum to the proximal colon ($P<0.001$ in OS and DFS) (Table 3).

DISCUSSION

In this study, we evaluated clinicopathologic and molecular characteristics of CRCs along bowel subsites. Despite a trend

Table 1. Clinicopathological features of colorectal cancers according to subsites

Variables	Number of patients (total $n=734$)	Caecum ($n=51$, 7.0%)	Ascending ($n=94$, 12.8%)	Hepatic flexure ($n=20$, 2.7%)	Transverse ($n=35$, 4.8%)	Splenic flexure ($n=9$, 1.2%)	Descending ($n=34$, 4.6%)	Sigmoid ($n=249$, 33.9%)	Recto-sigmoid ($n=74$, 10.1%)	Rectum ($n=168$, 22.9%)	*P*-value
Age											0.073[a]
Median (range) (years)	62 (20–90)	63 (28–81)	63 (29–85)	62 (45–76)	66 (41–77)	52 (27–65)	59 (20–80)	62 (32–90)	59 (34–83)	62 (36–87)	
Sex											0.012[b]
Male	446 (60.8%)	22 (43.1%)	53 (56.4%)	13 (65.0%)	19 (54.3%)	7 (77.8%)	23 (67.3%)	156 (62.6%)	41 (55.4%)	112 (66.7%)	
Female	288 (39.2%)	29 (56.9%)	41 (43.6%)	7 (35.0%)	16 (45.7%)	2 (22.2%)	11 (32.4%)	93 (37.4%)	33 (44.6%)	56 (33.3%)	
T category											<0.001[b]
1	27 (3.7%)	0 (0.0%)	3 (3.2%)	0 (0.0%)	2 (5.7%)	1 (11.1%)	2 (5.9%)	9 (3.6%)	4 (5.4%)	6 (3.6%)	
2	109 (14.8%)	5 (9.8%)	5 (5.3%)	0 (0.0%)	5 (14.3%)	0 (0.0%)	3 (8.8%)	31 (12.5%)	7 (9.5%)	53 (31.5%)	
3	535 (72.9%)	41 (80.4%)	71 (75.5%)	18 (90.0%)	22 (62.9%)	7 (77.8%)	26 (76.5%)	188 (75.5%)	59 (79.7%)	103 (61.3%)	
4	63 (8.6%)	5 (9.8%)	15 (23.8%)	2 (10.0%)	6 (17.1%)	1 (11.1%)	3 (8.8%)	21 (8.4%)	4 (5.4%)	6 (3.6%)	
N category											0.219[b]
0	374 (50.9%)	19 (37.3%)	49 (52.1%)	10 (50.0%)	21 (60.0%)	8 (88.9%)	20 (58.8%)	125 (50.2%)	34 (46.0%)	88 (52.4%)	
1	201 (27.4%)	12 (23.5%)	26 (27.7%)	5 (25.0%)	7 (20.0%)	1 (11.1%)	10 (29.4%)	66 (26.5%)	26 (35.1%)	48 (28.6%)	
2	159 (21.6%)	20 (39.2%)	19 (20.2%)	5 (25.0%)	7 (20.0%)	0 (0.0%)	4 (11.8%)	58 (23.3%)	14 (18.9%)	32 (19.0%)	
M category											0.771[b]
0	612 (83.4%)	43 (84.3%)	79 (84.0%)	16 (80.0%)	29 (82.9%)	7 (77.8%)	32 (94.1%)	198 (79.5%)	61 (82.4%)	147 (87.5%)	
1	122 (16.6%)	8 (15.7%)	15 (16.0%)	4 (20.0%)	6 (17.1%)	2 (22.2%)	2 (5.9%)	51 (20.5%)	13 (17.6%)	21 (12.5%)	
Stage											0.058[b]
I	111 (15.1%)	4 (7.8%)	6 (6.4%)	0 (0.0%)	6 (17.1%)	1 (11.1%)	4 (11.8%)	35 (14.1%)	8 (10.8%)	47 (28.0%)	
II	238 (32.4%)	15 (29.4%)	42 (44.7%)	9 (45.0%)	14 (40.0%)	6 (66.7%)	15 (44.1%)	77 (30.9%)	24 (32.4%)	36 (24.4%)	
III	264 (36.0%)	24 (47.1%)	31 (33.0%)	7 (35.0%)	9 (25.8%)	0 (0.0%)	13 (38.2%)	86 (34.5%)	29 (39.2%)	65 (38.7%)	
IV	121 (16.5%)	8 (15.7%)	15 (15.9%)	4 (20.0%)	6 (17.1%)	2 (22.2%)	2 (5.9%)	51 (20.5%)	13 (17.6%)	20 (11.9%)	
CIMP											<0.001[b]
CIMP-0,L	687 (93.6%)	41 (80.4%)	79 (84.0%)	17 (85.0%)	31 (88.6%)	9 (100.0%)	31 (91.2%)	243 (97.6%)	73 (98.6%)	163 (97.0%)	
CIMP-H	47 (6.4%)	10 (19.6%)	15 (16.0%)	3 (15.0%)	4 (11.4%)	0 (0.0%)	3 (8.8%)	6 (2.4%)	1 (1.4%)	5 (3.0%)	
MSI											<0.001[b]
MSS,MSI-L	669 (91.1%)	39 (76.5%)	75 (79.8%)	18 (90.0%)	30 (85.7%)	5 (55.6%)	28 (82.3%)	238 (95.6%)	73 (98.6%)	163 (97.0%)	
MSI-H	65 (8.9%)	12 (23.5%)	19 (20.2%)	2 (10.0%)	5 (14.3%)	4 (44.4%)	6 (17.7%)	11 (4.4%)	1 (1.4%)	5 (3.0%)	
KRAS mutation ($n=695$)											0.811[b]
Wild type	512 (73.7%)	36 (75.0%)	68 (73.1%)	15 (75.0%)	22 (66.7%)	8 (100.0%)	25 (73.5%)	175 (75.8%)	48 (70.6%)	115 (71.9%)	
Mutant type	183 (26.3%)	12 (25.0%)	25 (26.9%)	5 (25.0%)	11 (33.3%)	0 (0.0%)	9 (26.5%)	56 (24.2%)	20 (29.4%)	45 (28.1%)	
BRAF mutation ($n=728$)											0.169[b]
Wild type	689 (94.6%)	48 (94.1%)	87 (93.5%)	16 (84.2%)	33 (94.3%)	8 (88.9%)	33 (97.1%)	232 (94.3%)	70 (95.9%)	162 (96.4%)	
Mutant type	39 (5.4%)	3 (5.9%)	6 (6.5%)	3 (15.8%)	2 (5.7%)	1 (11.1%)	1 (2.9%)	14 (5.7%)	3 (4.1%)	6 (3.6%)	

Abbreviations: CIMP = CpG island methylator phenotype; MSI = microsatellite instability; MSS = microsatellite stable.
[a]Kruskal–Wallis test.
[b]Mantel–Haenszel linear-by-linear association χ^2-test.

towards an increasing percentage of right colon cancers in the eastern population, the prevalence of CIMP-H, MSI-H and *BRAF* mutation was low compared with that of the western population (Takada *et al*, 2002; Chang *et al*, 2006; Gao *et al*, 2012; Lin *et al*, 2012). There were gradual changes of gender distribution, T category, histologic features and the prevalence of CIMP-H and MSI-H along the bowel subsites. CpG island methylator phenotype-H CRCs were more frequently associated with older age, advanced T, N category and overall stage, poor differentiation and *BRAF* mutation. Shared features between CIMP-H CRCs and MSI-H CRCs were increased numbers of tumour-infiltrating lymphocytes, luminal serration and mucin production. Although CIMP was not an independent prognostic factor in entire CRCs, rectal cancers with CIMP-H showed poor clinical outcome.

Despite morphologic continuity, the large intestine demonstrates regional differences in embryology, anatomy, physiology and biochemistry (Iacopetta, 2002). The proximal and distal large intestines, which are derived from the midgut and hindgut, respectively, show differences in the capillary network of mucosa (Skinner and O'Brien, 1996), height of mucosal crypts (Arai and Kino, 1989), expression of blood group antigens (Wolf *et al*, 1989), expression of glycoconjugates (Caldero *et al*, 1989), carbonic anhydrase level in epithelial cells (Fleming *et al*, 1995), fermentation reactions producing short-chain fatty acids (Macfarlane *et al*, 1992), metabolism of bile acids (Thomas *et al*, 2001), expression of various isoforms of cytochrome P-450 (Mercurio *et al*, 1995), activity of ornithine decarboxylase (Zehnter *et al*, 1996), expression of Na^+/H^+ exchanger isoforms and profiles of genes expressed in epithelial cells (Glebov *et al*, 2003). However, these biological features do not change abruptly at the border between the proximal large intestine and distal large intestine. Rather, most of these features change gradually along the long axis of the large intestine.

Table 2. Histological features of CRCs according to subsites

Variables	Number of patients ($n=734$)	Caecum ($n=51$, 7.0%)	Ascending ($n=94$, 12.8%)	Hepatic flexure ($n=20$, 2.7%)	Transverse ($n=35$, 4.8%)	Splenic flexure ($n=9$, 1.2%)	Descending ($n=34$, 4.6%)	Sigmoid ($n=249$, 33.9%)	Recto-sigmoid ($n=74$, 10.1%)	Rectum ($n=168$, 22.9%)	*P*-value
Gross type											0.227[a]
Fungating	481 (65.5%)	30 (58.8%)	67 (71.3%)	9 (45.0%)	19 (54.3%)	6 (66.7%)	20 (58.8%)	163 (65.5%)	48 (64.9%)	119 (70.8%)	
Infiltrative	253 (34.5%)	21 (41.2%)	27 (28.7%)	11 (55.0%)	16 (45.7%)	3 (33.3%)	14 (41.2%)	86 (34.5%)	26 (35.1%)	49 (29.2%)	
Differentiation											0.047[a]
WD	54 (7.4%)	3 (5.9%)	7 (7.5%)	0 (0.0%)	4 (11.4%)	0 (0.0%)	3 (8.8%)	17 (6.8%)	7 (9.5%)	13 (7.7%)	
MD	652 (88.8%)	43 (84.3%)	79 (84.0%)	20 (100.0%)	30 (85.7%)	9 (100.0%)	31 (91.2%)	224 (89.0%)	67 (90.5%)	149 (88.7%)	
PD	28 (3.8%)	5 (9.8%)	8 (8.5%)	0 (0.0%)	1 (2.9%)	0 (0.0%)	0 (0.0%)	8 (3.2%)	0 (0.0%)	6 (3.6%)	
Luminal necrosis											0.001[a]
Absent	74 (10.1%)	9 (17.7%)	18 (19.2%)	0 (0.0%)	7 (20.0%)	1 (11.1%)	3 (8.8%)	14 (5.6%)	4 (5.4%)	18 (10.7%)	
Present	660 (89.9%)	42 (82.3%)	76 (80.8%)	20 (100.0%)	28 (80.0%)	8 (88.9%)	31 (91.2%)	235 (94.4%)	70 (94.6%)	150 (89.3%)	
Tumour budding											0.083[a]
Absent	30 (4.1%)	1 (2.0%)	2 (2.1%)	0 (0.0%)	2 (5.7%)	1 (11.1%)	3 (8.8%)	6 (2.4%)	3 (4.1%)	12 (7.1%)	
Present	704 (95.9%)	50 (98.0%)	92 (97.9%)	20 (100.0%)	33 (94.3%)	8 (88.9%)	31 (91.2%)	243 (97.6%)	71 (95.9%)	156 (92.9%)	
Tumour-infiltrating lymphocytes											<0.001[a]
Low (<8/HPF)	554 (75.5%)	27 (52.9%)	61 (64.9%)	15 (75.0%)	28 (80.0%)	6 (66.7%)	27 (79.4%)	196 (78.7%)	62 (83.8%)	132 (78.6%)	
High (⩾8/HPF)	180 (24.5%)	24 (47.1%)	33 (35.1%)	5 (25.0%)	7 (20.0%)	3 (33.3%)	7 (20.6%)	53 (21.3%)	12 (16.2%)	36 (21.4%)	
Crohn's-like lymphoid reaction											0.017[a]
Absent	601 (81.9%)	37 (72.5%)	73 (77.7%)	13 (65.0%)	30 (85.7%)	5 (55.6%)	30 (88.2%)	209 (83.9%)	67 (90.5%)	137 (81.5%)	
Present	133 (18.1%)	14 (27.5%)	21 (22.3%)	7 (35.0%)	5 (14.3%)	4 (44.4%)	4 (11.8%)	40 (16.1%)	7 (9.5%)	31 (18.5%)	
Luminal serration											0.005[a]
Absent	692 (94.3%)	46 (90.2%)	84 (89.4%)	19 (95.0%)	32 (91.4%)	9 (100.0%)	33 (97.1%)	234 (94.0%)	73 (98.6%)	162 (96.4%)	
Present	42 (5.7%)	5 (9.8%)	10 (10.6%)	1 (5.0%)	3 (8.6%)	0 (0.0%)	1 (2.9%)	15 (6.0%)	1 (1.4%)	6 (3.6%)	
Mucin production											<0.001[a]
Absent	644 (87.7%)	41 (80.4%)	69 (73.4%)	16 (80.0%)	28 (80.0%)	7 (77.8%)	31 (91.2%)	228 (91.6%)	70 (94.6%)	154 (91.7%)	
Present	90 (12.3%)	10 (19.6%)	25 (26.6%)	4 (20.0%)	7 (20.0%)	2 (22.2%)	3 (8.8%)	21 (8.4%)	4 (5.4%)	14 (8.3%)	

Abbreviations: CRC = colorectal cancer; MD = moderately differentiated; PD = poorly differentiated; WD = well differentiated.
[a]Mantel–Haenszel linear-by-linear association χ^2-test.

About three decades ago, epidemiologic studies reported differences of age and sex distribution between proximal colon cancers and distal CRCs (Jensen, 1984). Bufill (1990) suggested that proximal colon cancers and distal CRCs have arisen from different genetic pathways. Later on, genetic and epigenetic studies revealed the preference of CIMP and MSI in proximal colon cancers and CIN in distal CRCs (Weisenberger *et al*, 2006; Ogino *et al*, 2009). Some investigators proposed trichotomous subdivision of CRCs, which considers rectal cancers distinct from distal colon cancers (Li and Lai, 2009). However, such a dichotomous or trichotomous division of the large bowel was challenged by results of a recent study, demonstrating that differential clinicopathological or molecular features between proximal and distal CRCs do not change abruptly at the splenic flexure or between the sigmoid and the rectosigmoid, but show gradual change along the large bowel (Yamauchi *et al*, 2012a, b). Yamauchi *et al* (2012b) analysed the frequencies of molecular changes in nine subsites along the large intestine by using multivariate logistic regression and linear regression analysis, and found gradual increases in the frequencies of CIMP-H, MSI-H and *BRAF* mutations from the rectum to the ascending colon. Colorectum continuum theory is supported by findings of the present study that there was a linear correlation between female predominance, advanced T category, poorer differentiation, and CIMP-H and MSI-H from the rectum to caecum. Furthermore, there were gradual variations of histologic features towards the proximal end, such as the absence of luminal necrosis, increased numbers of tumour-infiltrating lymphocytes, Crohn's-like lymphoid reaction, mucin production and luminal serration. Results of the present study and Yamauchi *et al* (2012b) study indicate that the sample size is critical to assess colorectum continuum and that a large-scale study should be designed to assess coloretum continuum.

In the Yamauchi *et al* (2012b) study, caecal cancer did not follow linear relationships between tumour location and CIMP-H, MSI-H and *BRAF* mutation frequencies. In addition, caecal cancers did not follow the linearity trend in terms of age of onset, histologic differentiation, extracellular mucin production or signet ring cell component. In contrast, our study demonstrated that caecal cancers followed linear relationships between tumour location and molecular or clinicopathological features. In the study by Benedix *et al* (2011), which analysed 29 568 cases of colon cancer, caecal cancers followed the linear trend in terms of age of onset,

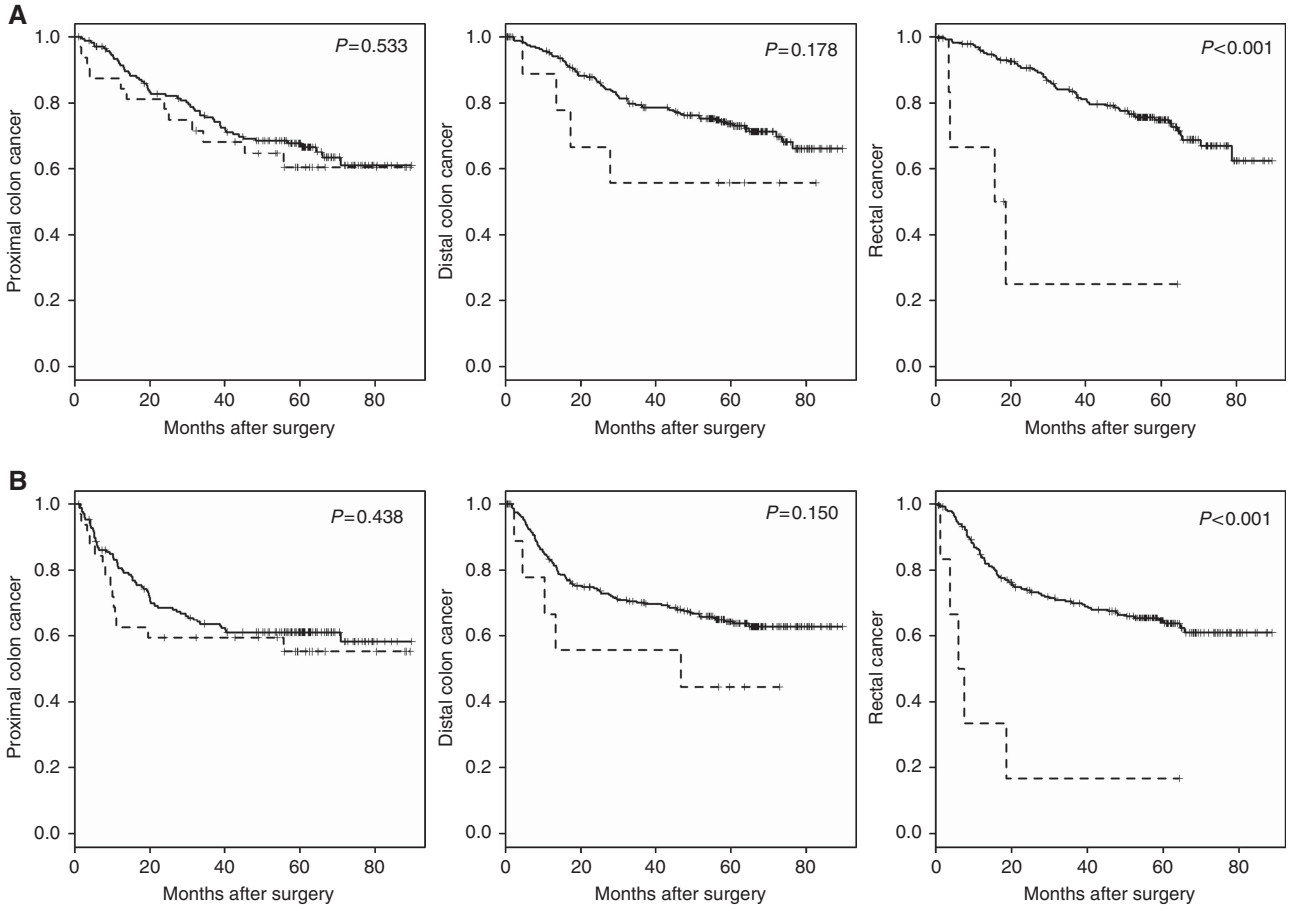

Figure 1. Kaplan–Meier survival curves by CIMP status in different tumour location. (**A**) Overall survival and (**B**) disease-free survival (linear line, CIMP-0,L; dashed line, CIMP-H).

tumour grade and histological subtype, but were distinct from other subsite cancers because of the high proportion of AJCC III/IV tumours and lymphatic invasion. The frequency of *KRAS* mutation in caecal cancers was higher than that of other CRCs in two studies (Yamauchi *et al*, 2012b; Rosty *et al*, 2013). In our study, the frequency of *KRAS* mutation in caecal cancers was not different from that in cancers of the other subsites. Discrepancies between our study and Yamauchi *et al*'s study might be related to general screening/detection and/or environmental/racial (genetic) differences. Or a difference in the proportion of caecal cancer between our current study and Yamauchi *et al*'s study (7.0% *vs* 16.8%) might contribute to the discrepancies.

Most studies have shown that CIMP-H CRCs are associated with poor clinical outcome (Shen *et al*, 2007; Ogino *et al*, 2009). However, an independent role for CIMP in clinical outcome is still controversial (Kim *et al*, 2009; Sanchez *et al*, 2009; Dahlin *et al*, 2010). When we performed survival analysis on 734 CRCs, CIMP was not an independent prognostic factor, but when survival analysis was restricted to rectal cancers, CIMP-H CRCs showed worse clinical outcome, and HR of CIMP-H compared with CIMP-0,L decreases from the rectum to the proximal colon. These findings indicate that differential clinicopathologic and molecular profiles of CRCs along the bowel subsites might influence the prognostic role of CIMP.

Traditional epidemiologic studies showed controversial results of site-specific risk of lifestyle and environmental factors in CRCs, such as body mass index, physical activity, and red meat and fibre consumption (Hjartaker *et al*, 2013; Robsahm *et al*, 2013). Inconsistent results from epidemiologic studies might originate from molecular heterogeneity of CRCs along the bowel subsites. These obstacles might be overcome by 'molecular pathological epidemiology', which enable pathway-specific epidemiologic study (Ogino *et al*, 2011, 2013). In the Iowa Women's Health Study, cigarette smoking was a risk factor for proximal colon cancers, CIMP-H CRCs and *BRAF*-mutated CRCs (Limsui *et al*, 2010). Post-menopausal hormone therapy was strongly associated with *KRAS* wild type in distal CRCs (Limburg *et al*, 2012). Hughes *et al* (2012) reported that body mass index is the risk factor for *BRAF* wild-type CRCs and MSS CRCs in two prospective cohort studies.

In our knowledge, this study is the largest molecular analysis of CRC patients in the eastern population, clearly demonstrating low frequencies of CIMP-H and MSI-H, and the first study that has focused on prognostic implication of CIMP and MSI according to tumour location. There are some limitations to our study. First, we cannot exclude the possibility of bias from retrospective and single institutional selection of study population, but another single institutional study showed similar distribution of age, sex and tumour location in 896 CRCs (Cheung *et al*, 2008). Second, molecular alterations in rectal cancers were underrepresented because of the exclusion of CRCs with preoperative chemo, and/or radiation therapy history. Third, the lower proportion of CIMP-H and MSI-H CRCs compared with that of the western population could bias the prognostic role of CIMP-H and MSI-H. Fourth, as two-third of CRCs are located in the sigmoid colon to rectum, we could not reveal a linear trend for prognostic implication of CIMP

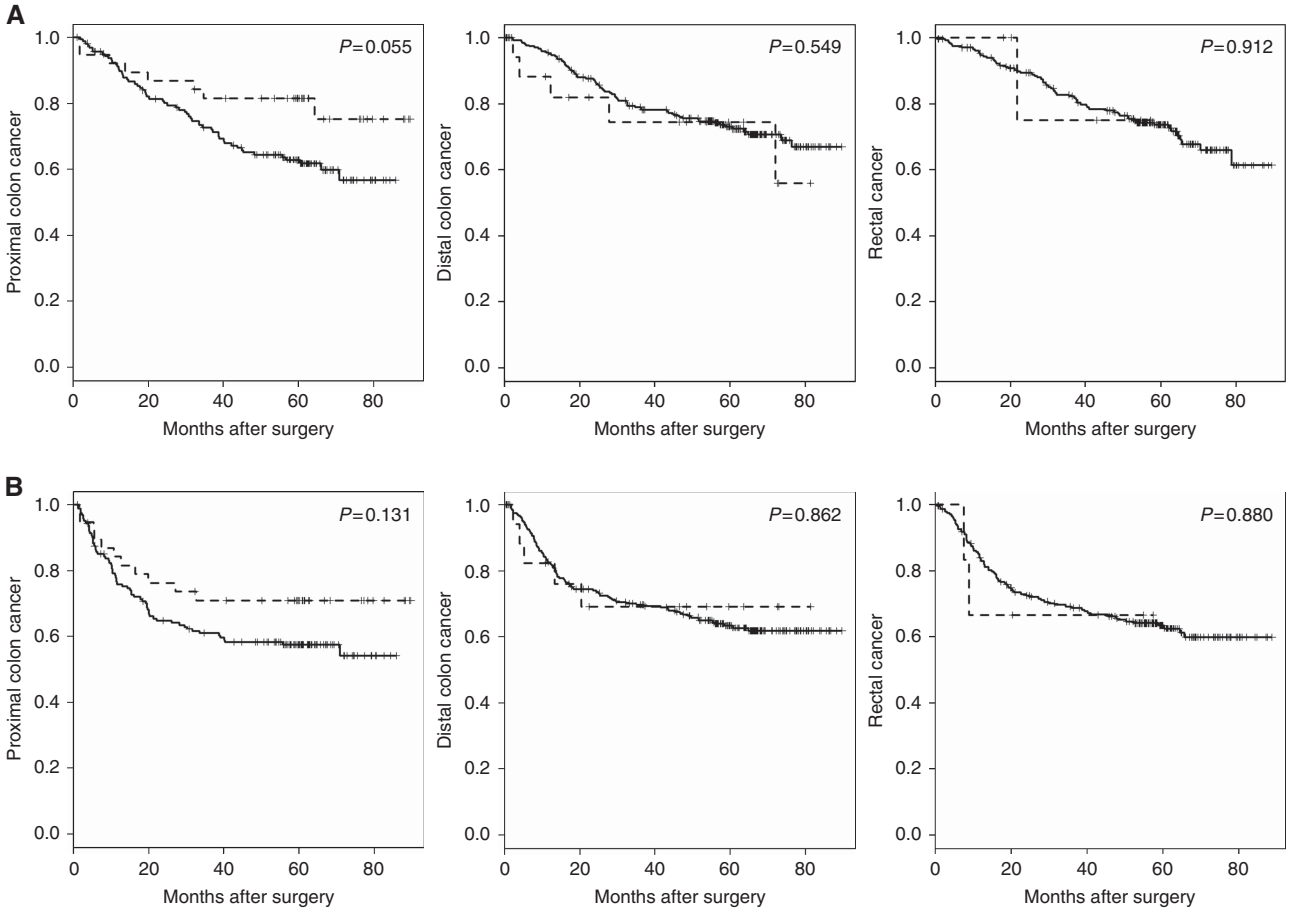

Figure 2. Kaplan–Meier survival curves by MSI status according to tumour location. (**A**) Overall survival and (**B**) disease-free survival (linear line, MSS,MSI-L; dashed line, MSI-H).

Table 3. Adjusted prognostic implication of CIMP-H and MSI-H according to tumour location of CRCs

		Overall survival		Disease-free survival	
Variables	**Tumour location**	**HR (95% CI)**	***P*-value**	**HR (95% CI)**	***P*-value**
CIMP	Proximal colon cancers	0.84 (0.42–1.69)	0.618	1.00 (0.53–1.88)	0.993
	Distal colon cancers	1.35 (0.47–3.90)	0.579	1.31 (0.51–3.36)	0.575
	Rectal cancers	4.13 (1.27–13.46)	0.019	2.90 (1.04–8.08)	0.042
	p for trend		<0.001		<0.001
MSI	Proximal colon cancers	0.80 (0.36–1.78)	0.585	1.15 (0.58–2.29)	0.688
	Distal colon cancers	1.26 (0.49–3.21)	0.633	0.96 (0.38–2.39)	0.924
	Rectal cancers	0.82 (0.11–6.01)	0.846	0.91 (0.22–3.75)	0.897
	p for trend		0.072		<0.001

Abbreviations: CI = confidence interval; CIMP-H = CpG island methylator phenotype-high; CRC = colorectal cancer; HR = hazard ratio; MSI-H = microsatellite instability-high. *TNM stage, tumour differentiation and adjuvant chemotherapy status were adjusted.

and MSI along nine subsites. Despite these limitations, our study showed the clinicopathologic and molecular heterogeneity of CRCs along the bowel subsites and revealed that the prognostic role of CIMP differs with tumour location.

In conclusion, clinicopathologic and molecular profiles of CRCs gradually change along the bowel subsites, and the prognostic implication of CIMP is affected by tumour location. Different biological behaviour of CRCs according to tumour location might be a clue to investigation of colorectal carcinogenesis and personalised cancer prevention and treatment.

ACKNOWLEDGEMENTS

This study was supported by a Mid-career Researcher Program through the National Research Foundation of Korea (NRF) funded by the Ministry of Education, Science and Technology (MEST) (2011-0015646), a grant from the National R&D Program for Cancer Control, Ministry of Health & Welcare, Republic of Korea (0720540), and a Priority Research Centers Program through NRF) funded by MEST (2009-0093820).

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