Differential expression of CD133 based on microsatellite instability status in human colorectal cancer

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Differential expression of CD133 based on microsatellite instability status in human colorectal cancer

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ABSTRACT

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The association between the types of genomic instability and cancer stem cell (CSC) has not been elucidated in colorectal cancer (CRC). Thus, this study aimed to investigate the expressions of CSC markers with respect to microsatellite instability (MSI) status in human CRC.

Immunostainings for CD133, CD44 and CD166, and K-ras mutation analysis were performed on 50 MSI-high (MSI-H), and 50 microsatellite stable (MSS) CRC tissues. In 11 MSS and MSI-H CRC cell lines, CD133 expression and DNA methylation statuses of the CD133 promoter were determined. The proportion of CD133 positive cells and the ability of colosphere formation were compared between

HCT116 cells and HCT116+Chr3 cells (hMLH1-restored HCT116 cells).

Immunohistochemistry for CSC markers revealed that high CD133 expression was more frequent in MSS cancers than in MSI-H (P<0.001, 74.0% vs. 28.0%, respectively), and related with short disease-free survival. Neither CD44 nor CD166 expression differed significantly with respect to MSI status. K-ras mutation showed no association with expressions of CD133, CD44, or CD166. CD133 expression was relatively high in the MSS cell lines compared to those in MSI-H, and showed a reverse correlation with DNA methylation of the CD133 promoter. hMLH1-restored HCT116 cells increased proportions of CD133 positive cells and colosphere forming ability, compared to those in HCT116 cells.

In conclusion, high levels of CD133 expression were observed more frequently in MSS CRC than in MSI-H, suggesting that differential expression of colon CSC markers may be linked to tumor characteristics dependent on MSI status.

Key words : Colorectal cancer, Cancer stem cell, CD133, Microsatellite instability

Differential expression of CD133 based on microsatellite instability status in human colorectal cancer

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I. INTRODUCTION

Chromosomal instability (CIN) and microsatellite instability (MSI) are two major types of genomic instability involved in colorectal carcinogenesis. Approximately 15% of all sporadic colorectal cancer (CRC)s display high frequency MSI(MSI-H), while the remaining 85% are microsatellite stable (MSS) and develop from gross chromosomal aberrations or chromosomal instability. Tumors with MSI-H tend to be more proximal in location, frequent poorly differentiated, mucinous, and markedly infiltrated by lymphocytes.[1] Moreover, MSI CRCs are associated with a better prognosis than are MSS tumors.[2]

A growing body of evidence supports the concept that human cancers

originate from an aberrant stem cell population.[3-5] According to the cancer stem cell (CSC) theory, a small defined subset of undifferentiated progenitor or cancer initiating cells generate and maintain a tumor cell population through continual self-renewal and differentiation.[6] Several studies have demonstrated the existence of colon CSCs through the isolation of a tumorigenic CRC stem cell population based on the expressions of specific cell surface markers, including CD133, CD44, and CD166.[7-9] Evidence suggests that these CSCs are involved not only in tumor formation but also in metastasis, and that tumor stem cells show relatively high resistance to chemical and irradiation therapies.[10-13] In addition, recent studies show that promoter hypermethylation of the CD133 CpG islands is associated with its absent or low expression in colon cancer cell lines,[14, 15] suggesting a possible relationship between CSC marker expression and the CpG island methylator phenotype (CIMP) in MSI-associated sporadic CRCs. However, the association between the types of genomic instability (CIN/MSI) and CSC has not been elucidated. Thus, this study aimed to investigate the expressions of CSC markers with respect to MSI status in human CRC tissues and cell lines.

II. MATERIALS AND METHODS

1. Clinical samples

Case selection was based on CRC specimens from patients who underwent curative surgical resection between 2006 and 2009 at Severance Hospital, Yonsei University, Seoul, Korea. Fifty patients with sporadic CRC showing MSI-H were recruited and an equal number of age/gender matched patients with MSS CRCs were subsequently enrolled by random selection. Patients were excluded from the study if they (1) met Amsterdam criteria II, (2) suffered from familial adenomatous polyposis(FAP) or had a family history of FAP, (3) suffered from inflammatory bowel disease, (4) had a history of surgical resection for CRC, and (5) had multiple CRCs located in both the proximal and distal colon.[16] The histological diagnosis was based on standard haematoxylin and eosin stained sections, according to the guidelines of the World Health Organization (WHO). Clinical data were retrospectively reviewed. The Institutional Review Board of Severance Hospital approved this study.

2. Microsatellite instability

Microsatellite analysis was performed using a panel of five National Cancer Institute (NCI) workshop-recommended consensus microsatellite markers (BAT25, BAT26, D2S123, D17S250, and D5S346).[17] The details of microsatellite analysis are described elsewhere.[18] Cases showing a microsatellite shift at two or more markers were classified as MSI-H. Cases showing no microsatellite instability were classified as MSS.

3. Immunohistochemistry

Immunohistochemical stainings were performed on the 4μm sections of formalin-fixed, paraffin-embedded surgical tumor samples. Sections were mounted, deparaffinized in xylene, and rehydrated in descending concentrations of ethanol. Antigen retrieval was performed using citrate buffer (10mM, pH6.0) heated in a pressure cooker for 5min. Blocking of endogenous peroxidases was accomplished by incubating sections in 3% hydrogen peroxide for 5min. Mouse anti-CD133 antibody (Miltenyi Biotec, Bergisch Gladbach, Germany, 1:100dilution), mouse anti-CD44 antibody (SantaCruz Biotechnology, SantaCruz, CA, 1:100dilution), and mouse anti-CD166 antibody (Novocastra, Newcastle, UK, 1:100dilution) were incubated with sections overnight at 4°C. Immunostaining was performed using the Envision^{+} Dual Link System and diaminobenzidine (DAB) visualization (Dako, Carpinteria, CA) for CD133, CD166 staining and Vectastain ABC kit (Vector Laboratories, Burlingame, CA) for CD44 staining according to the manufacturer′s instructions. Sections were counterstained with hematoxylin. Negative controls underwent the same protocol with the primary antibody omitted.

4. CD133, CD44, and CD166 expressions

The specimens were evaluated semi-quantitatively with no knowledge of patients′ clinical information. Only membranous immunostaining of tumor cells was evaluated in this study because the colon CSC isolation was based on the cell surface expressions of CD133, CD44, and CD166, as in previous studies.[7-9] Five medium power (magnifying power of x100) microscopic fields per tumor and staining were evaluated in the CD133 and CD44 assessments, while the entire stained area of each section was examined in CD166 assessment.

In the CD133 immunostaining evaluation, tumors were given a semi-quantitative score of <50% (low) or ≥50% (high) CD133+.[19] Gland positivity was defined as either membranous staining or staining of shed cellular debris in the tumor glands.[19] With regard to CD44 expression, tumors with less than 50% CD44-positive cells were considered as CD44-low, and those with 50% or more positive tumor cells, as CD44-high.[20] In the CD166 immunostaining evaluation, tumors that contained any cells with membranous CD166 expression were scored as CD166-positive, and those with no membranous CD166 expression, as CD166-negative.[21]

5. K-ras mutation analysis of human CRC tissues

DNA was extracted from five 10-μm-thick paraffin sections containing a

representative portion of tumor tissue, and specific mutations in K-ras exon 2 (codons 12/13) were identified as described previously.[22]

6. Mutation analysis for coding mononucleotide repeats and sequencing

Among 50 MIS-H DNA samples extracted from five 10-μm-thick paraffin sections containing tumor tissues, thirty-nine samples which have sufficient amount of genomic DNA were selected for coding mononucleotide repeats (cMNRs) analysis. In addition, the genomic DNA was also extracted from 5 MSI-H cell lines (LoVo, HCT116, RKO, DLD1, HCT15). After searching the coding area of CD133 genes containing cMNR with a length of 8 or more nucleotides, specific primers were designed to amplify the cMNR in these genes. The sequences of the primers used are as follows: forward, 5′-TCCTCATCTTCAGTGGTGGAC-3′; reverse, 5′-TTGAAGCTGTTCTGCAGGTG-3'. After purifying PCR product by DNA purification kit (MEGAquick-spin™ Total Fragment DNA Purification Kit, iNtRON Biotechnology), the DNA was subjected to sequencing (Genotech, Daejeon, Korea).

7. Cell culture

Five MSI-H cell lines (LoVo, HCT116, RKO, DLD1, HCT15) and six MSS

cell lines (SW480, HT29, Caco-2, SW620, COLO205, WiDr) were obtained from American Type Culture Collection (ATCC; Manassas, VA) and grown in Dulbecco′s Modified Eagle′s Medium (DMEM) supplemented with 10% fetal bovine serum. All media were supplemented with 2mM L-glutamine, 100U/mL penicillin, 100 mg/mL streptomycin, and 1x MEM non-essential amino acid mix (NEAA). Cells were incubated at 37℃ in a humidified atmosphere of 5%CO_2 in air. The MSI status of each colon cancer cell lines have been confirmed in previous reports,[23-25] and were consistent with those of other previously established data.[26] HCT116+Chr3 cells, which are HCT116 cells with hMLH1 restored by transfer of chromosome 3 containing a single wild-type copy of the hMLH1 gene,[27] were a generous gift from Prof. Dong Kyoung Chang (Sungkyunkwan University School of Medicine, Seoul, Korea).

8. Reverse transcription-polymerase chain reaction analysis

Semi-quantitative RT-PCR experiments were conducted to measure CD133 mRNA expressions in the 11 colon cancer cell lines. Total cellular RNA was isolated using the RNeasy Mini RNA isolation kit (Qiagen, Valencia, CA). Reverse transcription (RT) of total RNA (4μg/20μl) to cDNA was performed using oligo dT primers and the EasyScript Plus first strand cDNA synthesis kit

(ABM, Richmond, BC, Canada) according to the manufacturer′s protocol.

Primers for CD133 and β-actin were designed based on previous data.[28] The primers were transcribed with 4 μg isolated total RNA using 2X PCR Taq MasterMix (ABM, Richmond, BC, Canada) on a PCR cycler (Eppendorf, Westbury, NY) with the following reaction conditions: PCR amplification (denaturation at 94°C for 30sec, annealing at 60-61°C for 30sec, chain extension at 72°C for 30sec) for 35cycles and a final chain extension at 72°C for 10min. The amplified products were separated on 2.5% agarose gels, stained with ethidium bromide (1μg/ml), and visualized using a transilluminating UV chamber (Alpha Innotech, San Leandro, CA). β-actin expression served as an internal standard.

9. Western blot analysis

Briefly, cells were lysed and the protein concentration of lysates was determined using the BCA Protein Assay kit (Pierce, Rockford, IL). Protein samples were prepared by boiling after the addition of denaturing sample buffer. Samples containing 10μg protein were subjected to SDS-PAGE and transferred onto a Hybond-P PVDF membrane (Amersham, Freiburg,

Germany). After blocking with 5% bovine serum albumin(BSA) in 0.1% TBST (Tween20 in TBS solution) for 60minutes, membranes were probed with rabbit polyclonal antibodies to CD133 (1:1000; Cell Signaling, MA), and incubated at room temperature overnight. Bound antibodies were detected with peroxidase-labeled goat anti-rabbit IgG antibody and visualized using enhanced chemiluminescence reagents (Amersham). For a loading control, the monoclonal anti-β-actin antibody (Santa Cruz Biotechnology, Santa Cruz, CA) at a dilution of 1:2000 was used.

10. Bisulphite conversion of DNA and pyrosequencing

Genomic DNA was extracted from cell lines using the Wizard Genomic Purification Kit (Promega, Madison, WI). Sodium bisulphite modification of 500ng DNA was carried out with the EZ DNA methylation kit (Zymo Research, CA) following the manufacturer′s protocol. To test the methylation status of the CD133 promoter CpG island, PCR primer sequences for pyrosequencing were obtained from the PyroMark Assay Database (Biotage AB, Uppsala, Sweden). These primers were designed to hybridize with CpG-free regions to secure methylation-independent amplification. The sequences of the primers used are as follows: forward, 5′-GGAGGGTAGTTGTATTTTAAGTAAGG-3′; reverse, biotin-5′-

CCCCTACACTCCCTTCTTACAA-3′; and sequencing primer, 5′-TTTGAGGGGGGAAAAGA-3′. HotStart Taq polymerase was used to amplify 20μl bisulphite–treated DNA. PCR conditions were 95°C for 5min, followed by 50cycles of: 95 °C for 30s, 61 °C for 30s, 72 °C for 30s, followed by 72 °C for 5min and finally 4 °C. After PCR, the biotinylated strand was captured on streptavidin-coated beads (Amersham Bioscience, Uppsala, Sweden) and incubated with sequencing primers. All samples and blank controls for each reaction were then analyzed via pyrosequencing using the PyroMark Q24 system (Biotage). Subsequent quantification of methylation density was conducted using Pyro-Mark Q24 software. Bisulphite-treated DNA from 18Co cells, a human myofibroblast cell line, was assessed as a negative control.

11. Flow cytometric analysis

Flow cytometeric analysis of CD133 was performed in HCT116 and HCT116+Chr3 cells. The prepared cells were detached by accutase (Millipore, Billerica, MA, U.S.A), and washed with phosphate buffered saline (PBS), and then resuspended in FACS buffer (1x PBS, 1% BSA), 2 mM ethylene diamine tetraacetic acid [EDTA]). Phycoerythrin (PE)-conjugated anti-CD133 antibody (Miltenyi Biotec, Bergisch Gladbach, Germany) was added and

incubated for 10 min on ice. Samples were then washed and analyzed by using BD LSRII (BD Biosciences, Franklin Lakes, NJ, U.S.A) coupled to a computer with data analysis software (BD FACS Diva software).

12. Colosphere culture assay

The sphere-forming ability as cancer stem cell characteristics was evaluated by colosphere culture assay. HCT116 cells and HCT116+Chr3 cells were plated with 2,000 cells/well in 24-well ultra-low adhesive plates (Corning Incoporated, NY, U.S.A), and cultured in serum-free DMEM-F12 supplemented with B27 (Life Technologies, Carlsbad, CA, U.S.A), 20 ng/mL epidermal growth factor (R&D Systems, Minneapolis, MN, U.S.A), and 10 ng/mL fibroblast growth factor (R&D Systems, Minneapolis, MN). They were cultured in 5% CO_2 incubator at 37°C with a medium change every 3 days. To measure the ability of colosphere formation, the number of colospheres was counted under microscope at day 14.

13. Statistical analysis

Continuous variables were compared using Student′s *t*-test, and categorical variables were compared using a Chi-square test or Fisher′s exact test. Disease-free survival was calculated from the date of surgery to the date of

disease recurrence. Survival was evaluated using the Kaplan–Meier method. The log-rank test was used to compare the cumulative survival durations in the patient groups. Multivariate analyses were carried out using the Cox proportional-hazards model. *P* values of <0.05 were considered statistically significant.

III. RESULTS

1. Clinicopathologic characteristics of CRC patients

The mean age of patients was 60.8±8.7years. All patients were histologically diagnosed with adenocarcinoma. Patients with MSI-H and MSS tumors did not differ significantly in tumor histology or TNM stage, however, proximal and undifferentiated (poor or mucinous) tumors were more often observed in MSI-H than in MSS tumors (Table 1). These site-specific and histological differences between MSI-H and MSS tumors are consistent with previous observations,[17, 29, 30] and imply that the mismatch repair pathway has a pivotal role in proximal colon cancer carcinogenesis compared with distal CRC, and two different genetic mechanisms, MSI and CIN, contribute to carcinogenesis in the proximal and distal colon, respectively.[31, 32]

The majority of patients with stage II (82.5%) and stage III (92.9%) cancers received 5-flourouracil (5-FU)-based chemotherapy postoperatively.

Postoperative radiation therapy was also performed in selected rectal cancer patients (22.0% in stage II, 75.0% in stage III). The median follow-up duration after colorectal resection was 36.0 months (interquartile range 28.4–42.5 months).

Table 1. Clinicopathological characteristics and associations with MSI status in colorectal cancer subjects.

	MSI-H (n=50)	MSS (n=50)	*P* value
Age , years, mean±SD	60.6±9.2	61.0±8.2	0.784
Gender			1.000
Male	31(62.0%)	31(62.0%)	
Female	19(38.0%)	19(38.0%)	
Location (%)			<0.001
Proximal colon	36 (72.0%)	10 (20.0%)	
Distal colon and rectum	14 (28.0%)	40 (80.0%)	
Differentiation (%)			0.002
Well/moderate	38 (76.0%)	49 (98.0%)	
Poor/mucinous	12 (24.0%)	1 (2.0%)	
T stage			0.786
T2	4(8.0%)	8(16.0%)	
T3	44(88.0%)	37(74.0%)	
T4	2(4.0%)	5(10.0%)	
N stage			0.786
N0	37 (74.0%)	35 (70.0%)	
N1	9 (18.0%)	12 (24.0%)	
N2	4 (8.0%)	3 (6.0%)	
TNM Stage			0.504
I	3(6.0%)	6(12.0%)	
II	34(68.0%)	29(58.0%)	
III	13(26.0%)	15(30.0%)	

MSI-H, microsatellite instability-high; MSS, microsatellite stable

2. CD133, CD44 and CD166 expressions according to the MSI status of CRC tissue

Figure 1 shows representative immunostained samples for semi-quantitative scoring of each colon CSC marker. High expressions of CD133 and CD44, and positive expression of CD166 were observed in 51%, 73%, and 74% of samples, respectively. Results for each CSC marker relative to MSI status are summarized in Table 2. High CD133 expression was observed more frequently in MSS tumors than in MSI-H tumors (P <0.001, 74.0% vs. 28.0%, respectively). High CD44 expression did not differ between MSI-H and MSS tumors (P =0.499, 76.0% vs. 70.0%, respectively). CD166-positive expression was observed in 74% of both MSS and MSI-H tumors.

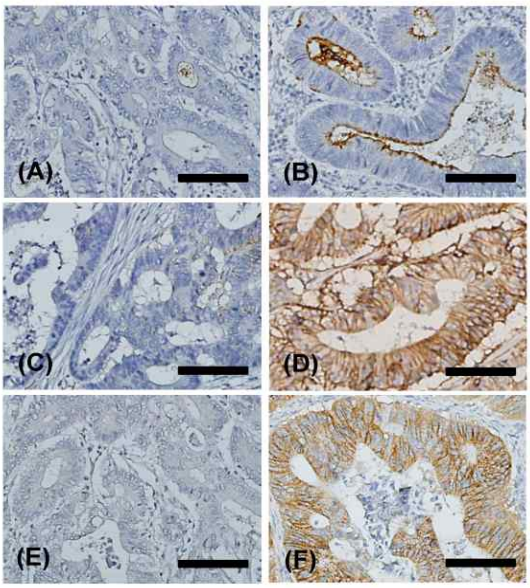

Figure 1. Semi-quantitative scoring of immunohistochemical stainings for CD133, CD44, and CD166 in human colorectal cancer tissue. Tumors that showed no staining or less than 50% positively stained glands were scored as CD133-low (A), and tumors with more than 50% positively stained glands were scored as CD133-high (B). Tumors with less than 50% of cells expressing CD44 were scored as CD44-low (C), and tumors with 50% or more, as CD44-high (D). Tumors without CD166 staining were scored as CD166-negative (E), and tumors presenting any membranous staining for CD166, as CD166-positive (F). Original magnification, ×200. All scale bar, 50μm.

Table 2. Scoring of immunohistochemical staining for cancer stem cell markers and association with MSI status in colorectal cancer tissue.

		MSI-H (n=50)	MSS (n=50)	*P* value
CD133				<0.001
	Low	36 (72.0%)	13 (26.0%)	
	High	14 (28.0%)	37 (74.0%)	
CD44				0.499
	Low	12 (24.0%)	15 (30.0%)	
	High	38 (76.0%)	35 (70.0%)	
CD166				1.000
	Negative	13 (26.0%)	13 (26.0%)	
	Positive	37 (74.0%)	37 (74.0%)	

MSI-H, microsatellite instability-high; MSS, microsatellite stable

3. Disease-free survival according to MSI status and CSC marker expression

Because of the relatively short follow-up duration, we estimated the disease-free survival rather than overall survival using Kaplan-Meier analysis. Patients with MSS tumors had shorter disease-free survival times than did those with MSI-H tumors (P =0.005) [Figure 2(A)], which is consistent with the known prognostic implications of MSI status. In regard to CSC marker expression, high CD133 expression showed a significant negative correlation with disease-free survival (P=0.044) [Figure 2(B)], whereas neither CD44 nor CD166 expression did not correlated with tumor recurrence (P =0.150 and P =0.186, respectively) [Figure 2(C) and (D)].

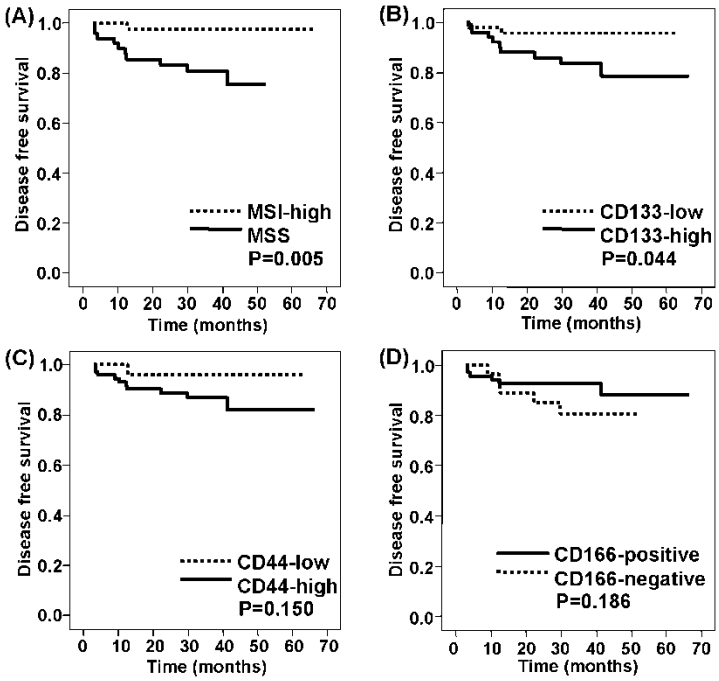

Figure 2. Kaplan-Meier survival curves comparing disease-free survival times based on the MSI status, CD133, CD44, and CD166 expression. The details of patients groups are as follows: (A) MSI-high *vs.* MSS, (B) CD133-low *vs.* high, (C) CD44-low *vs.* high, and (D) CD166-positive *vs.* negative. The groups were compared using the log-rank test. MSI, microsatellite instability; MSS, microsatellite stable

Meanwhile, when multivariate Cox regression analysis of disease free survival including both MSI status and CD133 expression under controlling potent confounder of tumor stage was performed, the statistical significance of CD133 expression was lost (hazard ratio [HR] 1.446, 95% confidence interval [CI] 0.290–7.210, *P*=0.653), however MSI was persistently significant (HR 10.274, 95% CI 1.194–88.432, *P*=0.034) (Table 3).

Table 3. Prognostic factors for recurrence in multivariate analysis (Cox proportional-hazards model).

Variable	HR	95% CI	*P* value
TNM stage (every one stage increment)	11.453	2.535-51.754	0.002
MSI status (MSS)	10.274	1.194-88.432	0.034
CD133 expression (High)	1.446	0.290-7.210	0.653

HR, hazard ratio; CI, confidence interval; MSI, microsatellite instability; MSS, microsatellite stable

4. K-ras mutation status and CSC marker expression in CRC tissues

Because Ras/ERK signaling may participate in the maintenance of CSC properties,[33-36] the association between CSC marker expression and K-ras mutation was investigated. K-ras mutation was observed in 32% of MSS

tumors and 34% of MSI-H tumors. The frequency of K-ras mutation was not associated with high or positive CD133, CD44, or CD166 expression in these human CRC tissues (Table 4). Moreover, K-ras mutation did not correlate with CSC markers expression in the subgroup analysis both MSI-H and MSS tumors respectively (Table 5).

Table 4. Cancer stem cell marker expression according to K-ras mutation status in colorectal cancer tissue.

		K-ras mutant (n=33)	K-ras wild-type (n=67)	*P* value
CD133				0.619
	Low	15 (45.5%)	34 (50.7%)	
	High	18 (54.5%)	33 (49.3%)	
CD44				0.061
	Low	5 (15.2%)	22 (32.8%)	
	High	28 (84.8%)	45 (67.2%)	
CD166				0.097
	Negative	12 (36.4%)	14 (20.9%)	
	Positive	21 (63.6%)	53 (79.1%)	

MSI-H, microsatellite instability-high; MSS, microsatellite stable

Table 5. Cancer stem cell marker expression according to K-ras mutation status in MSS and MSI-H subgroups of colorectal cancer.

	MSI-H tumor			MSS tumor		
	K-ras mutant (n=17)	K-ras wild-type (n=33)	*P* value	K-ras mutant (n=16)	K-ras wild-type (n=34)	*P* value
CD133			0.873			0.508
Low	12 (70.6%)	24 (72.7%)		3 (18.8%)	10 (29.4%)	
High	5 (29.4%)	9 (27.3%)		13 (81.3%)	24 (70.6%)	
CD44			0.510			0.099
Low	3 (17.6%)	9 (27.3%)		2 (12.5%)	13 (38.2%)	
High	14 (82.4%)	24 (72.7%)		14 (87.5%)	21 (61.8%)	
CD166			0.282			0.203
Negative	6 (35.3%)	7 (21.2%)		6 (37.5%)	7 (20.6%)	
Positive	11 (64.7%)	26 (78.8%)		10 (62.5%)	27 (79.4%)	

MSI-H, microsatellite instability-high; MSS, microsatellite stable

5. Identification of cMNR-containing CD133 genes in MSI-H CRC tissues and cell lines

Because many target genes containing cMNR sequences and frequent mutations in these regions have been identified by a mutational analysis of MSI-H tumors,[25, 37-42] moreover, the number of nucleotides in the MNR have

Table 6. List of coding mononucleotide repeat-containing CD133 genes in MSI-H CRC tissues

Patient	Sequence (A8)
Patient 1	CATTCCCACAGTGACTGC**AAAAAAAA**TAGAGGCACTTACGGCACTCT
Patient 2	CATTCCCACAGTGACTGC**AAAAAAAA**TAGAGGCACTTACGGCACTCT
Patient 3	CATTCCCACAGTGACTGC**AAAAAAAA**TAAAGGCACTTACGGCACTCT
Patient 4	CATTCCCACAGTGACTGC**AAAAAAAA**TAGAGGCACTTACGGCACTCT
Patient 5	CATTCCCACAGTGACTGC**AAAAAAAA**TAAGGCACTTACGGCACTCTT
Patient 6	CATTCCCACAGTGACTGC**AAAAAAAA**TAGAGGCACTTACGGCACTCT
Patient 7	CATTCCCACAGTGACTGC**AAAAAAAA**TAGAGGCACTTACGGCACTCT
Patient 8	CATTCCCACAGTGACTGC**AAAAAAAA**TAGAGGCACTTACGGCACTCT
Patient 9	TCTTCCCACAGTGACTGC**AAAAAAAA**TAGAGGCACTTACGGCACTCT
Patient 10	CATTCCCACAGTGACTGC**AAAAAAAA**TAGAGGCACTTACGGCACTCT
Patient 11	CATTCCCACAGTGACTGC**AAAAAAAA**TAGAGGCACTTACGGCACTCT
Patient 12	CATTCCCACAGTGACTGC**AAAAAAAA**TAGAGGCACTTACGGCACTCT
Patient 13	CATTCCCACAGTGACTGC**AAAAAAAA**TAGAGGCNNTAACGGCANTCT
Patient 14	CATTCCCACAGTGACTGC**AAAAAAAA**TAGAGGCACTTACGGCACTCT
Patient 15	CATTCCCACAGTGACTGC**AAAAAAAA**TAGAGGCACTTACGGCACTCT
Patient 16	TCTTCCCACAGTGACTGC**AAAAAAAA**TAGAGGCACTTACGGCACTCT
Patient 17	CATTCCCACAGTGACTGC**AAAAAAAA**TAGAGGCACTTACGGCACTCT
Patient 18	CATTCCCACAGTGACTGC**AAAAAAAA**TAGAGGCACTTACGGCACTCT
Patient 19	CATTCCCACAGTGACTGC**AAAAAAAA**TAGAGGCACTTACGGCACTCT
Patient 20	CATTCCCACAGTGACTGC**AAAAAAAA**TAGAGGCACTTACGGCACTCT
Patient 21	CATTCCCACAGTGACTGC**AAAAAAAA**TAGAGGCACTTACGGCACTCT
Patient 22	CATTCCCACAGTGACTGC**AAAAAAAA**TAGAGGCACTTACGGCACTCT
Patient 23	CATTCCCACAGTGACTGC**AAAAAAAA**TAGAGGCACTTACGGCACTCT
Patient 24	CATTCCCACAGTGACTGC**AAAAAAAA**TAGAGGCACTTACGGCACTCT
Patient 25	CATTCCCACAGTGACTGC**AAAAAAAA**TAGAGGCACTTACGGCACTCT
Patient 26	CATTCCCACAGTGACTGC**AAAAAAAA**TAGAGGCACTTACGGCACTCT
Patient 27	CATTCCCACAGTGACTGC**AAAAAAAA**TAGAGGCACTTACGGCNNTCT
Patient 28	CATTCCCACAGTGACTGC**AAAAAAAA**TAGAGGCACTTACGGCACTCT

Patient 29	CATTCCCACAGTGACTGC**AAAAAAAA**TAGAGGCACTTACGGCACTCT
Patient 30	CATTCCCACAGTGACTGC**AAAAAAAA**TAGAGGCACTTACGGCACTCT
Patient 31	CATTCCCACAGTGACTGC**AAAAAAAA**TAGAGGCACTTACGGCACTCT
Patient 32	CATTCCCACAGTGACTGC**AAAAAAAA**TAGAGGCACTTACGGCACTCT
Patient 33	CATTCCCACAGTGACTGC**AAAAAAAA**TAGAGGCACTTACGGCACTCT
Patient 34	CATTCCCACAGTGACTGC**AAAAAAAA**TAGAGGCACTTACGGCACTCT
Patient 35	CATTCCCACAGTGACTGC**AAAAAAAA**TAGAGGCACTTACGGCACTCT
Patient 36	CATTCCCACAGTGACTGC**AAAAAAAA**TAGAGGCACTTACGGCACTCT
Patient 37	CATTCCCACAGTGACTGC**AAAAAAAA**TAGAGGCACTTACGGCACTCT
Patient 38	CATTCCCACAGTGACTGC**AAAAAAAA**TAGAGGCACTTACGGCACTCT
Patient 39	CATTCCCACAGTGACTGC**AAAAAAAA**TAGAGGCACTTACGGCACTCT

Table 7. List of coding mononucleotide repeat-containing CD133 genes in MSI-H CRC cell lines

Cell line	Sequence (A8)
HCT116	CATTCCCACAGGTGACTGC**AAAAAAAA**TAGAGGCACTTACGGCACTC
LoVo	CATTCCCACAGGTGACTGC**AAAAAAAA**TAGAGGCACTTACGGCACTC
DLD-1	CATTCCCACAGGTGACTGC**AAAAAAAA**TAGAGGCACTTACGGCACTC
HCT15	CATTCCCACAGGTGACTGC**AAAAAAAA**TAGAGGCACTTACGGCA
RKO	CATTCCCACAGGTGACTGC**AAAAAAAA**TAGAGGCACTTACGGCACTC

been reported to be related to increased mutational frequency,[43-45] cMNR with a length of 8 or more nucleotides in CD133 gene was examined to untie the mechanism of CD133 expression in MSI-H CRC tissues and cell lines. All samples had cMNR [(A)8] in their CD133 genes, however there was no evidence of length alterations (frameshift mutations) in cMNR (Table 6 and Table 7).

6. CD133 expression in the colon cancer cell lines: RT-PCR, Western blot analysis, and DNA methylation of the CD133 promoter CpG island

To complement the findings of CRC tissues, the CD133 expression was determined in CRC cell lines according to MSI status. Using RT-PCR, we found that relative CD133 mRNA levels correlated with protein levels in Western blot analysis [Figures 3(A), (B)]. In Western blots using six MSS colon cancer cell lines, four lines (WiDr, HT-29, Caco-2, colo205) showed significant amounts of CD133 protein, a very weak band for SW620 cells, and no identifiable band for SW480[Figure 3(B)]. However, among five MSI-H cell lines, one (LoVo) showed only very weak CD133 expression, and the other four showed none [Figure 3(B)].

In bisulphite conversion of DNA and pyrosequencing analysis, the CD133 expression in colon cancer cell lines correlated inversely with promoter methylation except for two cell lines (Colo205 and LoVo) [Figure 3]. Three of six MSS CRC cell lines were unmethylated, and all five MSI cell lines were methylated [Figure 3(C)], suggesting that in MSI CRC, the CpG island of the CD133 promoter might be highly methylated.

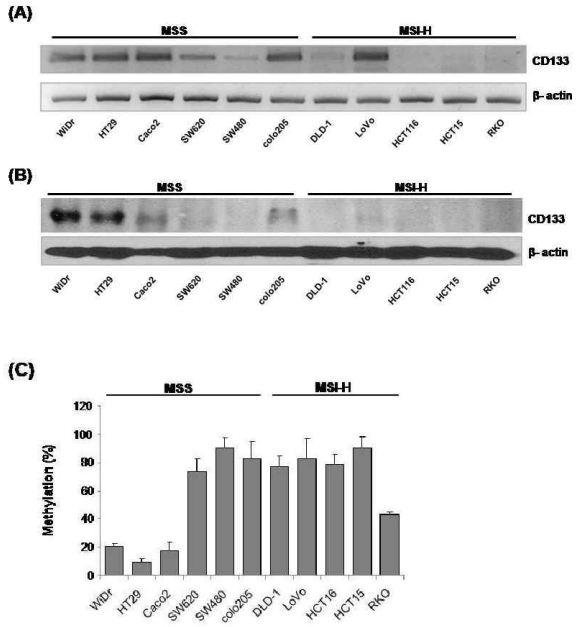

Figure 3. Analysis of CD133 expression and DNA methylation status of the CD133 promoter CpG island in six MSS and five MSI CRC cell lines. The CD133 mRNA and protein levels were determined using RT-PCR(A) and Western blot analysis(B), respectively. The methylation status of the CD133 promoter was analyzed using bisulphite conversion of DNA and pyrosequencing(C). MSI-H, microsatellite instability-high; MSS, microsatellite stable; CRC, colorectal cancer.

7. Comparison of CD133 expression and colosphere formation between HCT116 and HCT116+Chr3 cells

To investigate the direct effect of changing MSI status on CSC, we compared the proportion of CD133 positive cells and the ability of colosphere formation between HCT116 cells and HCT116+Chr3 cells. HCT116 cells

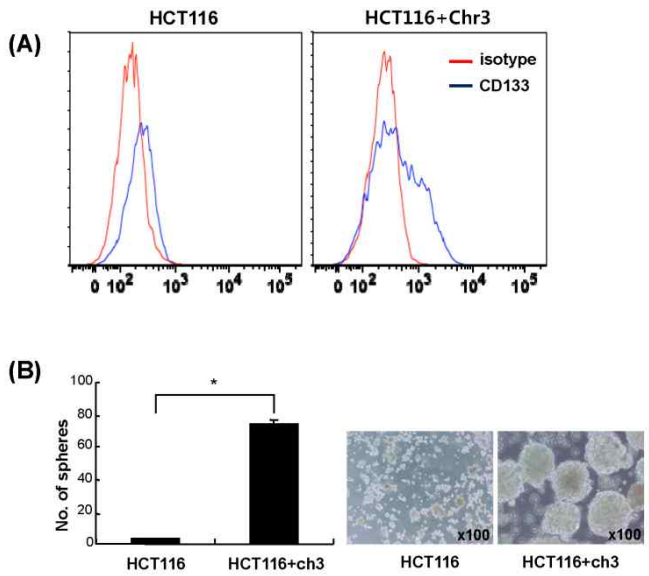

Figure 4. Comparisons of CD133 expression and colosphere formation between HCT116 and HCT116+Chr3 cells. In HCT116 cells and HCT116+Chr3 cells, the proportion of CD133 positive cells(A) and the ability of colosphere formation(B) were determined using flow cytometric analysis and colosphere culture assay, respectively. Data shown are mean ± S.E. of three independent experiments. *$P < 0.05$.

have inactivating hMLH1 mutations and exhibit MSI, and HCT+Chr3 cells show restored DNA mismatch repair activity and corrected MSI by transfer of chromosome 3 containing a wild-type copy of the hMLH1 gene into HCT116 cells.[27]

HCT116+Chr3 cells showed significantly higher proportion of CD133 positive cells (46.2%) than that in HCT116 cells (3.7%), and increased ability of colosphere formation (Figure 4), suggesting that MSS colorectal cancer contains more CD133 positive cells with CSC characteristics.

IV. DISCUSSION

Considering the prominence of two major genomic instability types, CIN and MSI, and increasing evidence for the role of CSCs in CRC development, it was sought to determine the relationship between MSI status and CSC marker expression in CRC. In this study, it was demonstrated that high CD133 expression was infrequently observed in MSI-H CRC tissues and cell lines.

The pentaspan transmembrane glycoprotein CD133 was originally described as a hematopoietic stem cell marker[46] and was subsequently shown to be expressed by a number of progenitor cells including those of the epithelium.[47] CD133 expression has been used to identify CSC in CRC,[7, 8]

however, whether CD133 is a coincidental marker for tumorigenesis or a causal element in tumorigenesis remains open to debate.

Evidence suggests that CSCs resist conventional anticancer therapies in diverse tumor types,[11] including CRC,[48] and that high CD133 expressions in cancer tissues and sera of CRC patients indicate a poor prognosis.[19, 20, 49-53] Colorectal cancer with MSI generally has a better prognosis than does CRC with MSS. We also observed poor prognoses for CRC with MSS and highly expressed CD133. These findings, together with the results of this study showing high CD133 expression in MSS CRC, support the premise that poor prognoses of patients with MSS tumors may be associated with the higher CD133 expression in MSS CRC compared to that in MSI CRC.

Pino et al. demonstrated that expressions of epithelial to mesenchymal transition (EMT) markers were significantly associated with the absence of MSI.[54] They concluded that better prognosis for MSI tumors may reflect the absence of EMT, which is related to features of advanced disease such as metastasis and resistance to chemotherapy.[55] Recent studies have shown that EMT is associated with the generation of cancer cells with stem cell-like characteristics[56] and one of the major inducer for EMT is transforming growth factor-β (TGFβ) in several type of cancers including breast cancer, hepatocellular carcinoma, and lung cancer.[35, 57, 58] In line with these observations, Pino et al, as aforementioned, demonstrated that TGF β-1 also

induce EMT in human CRC with MSS, however tumor cells with MSI were unresponsive to TGF β-1 signaling possibly due to mutant transforming growth factor-β receptor type II (TGFBR2) gene.[54] Additionally, Pirozzi et al showed that TGF β-1 upregulate several genes of stemness including CD133 in primary lung cancer cell line, although the data in the CRC have not yet been reported.[58] Moreover, CD133 may be a target gene of WNT/β-catenin signaling,[59] which may participate in EMT in colorectal tumor cells.[60, 61] Results of these studies point to a complex web of relationships linking CD133 expression, EMT, and MSI status in CRC development. In this study, it was tried to untangle this web by examining the relationship between CSC marker expression and MSI status.

The reason for low CD133 expression in MSI tumors is not known but may involve methylation in the gene promoter region. A significant proportion of sporadic MSI-associated CRCs arise through a process that involves the CIMP.[62] MSI tumors frequently show promoter methylation, a mechanism of gene silencing, at known tumor suppressor genes or functionally unknown genes.[63]

Recently, Yi et al. demonstrated that the promoter CpG island of CD133 is largely methylated in cells with absent or low expression of this marker protein, and that lack of such methylation is prominent in purely CD133+

cells.[14] Another study confirmed this observation using different colon cancer cell lines.[15] The results of this study for CD133 promoter CpG island methylation in cell lines agreed with corresponding results for these cell lines in a previous report[14] and showed an inverse correlation between CD133 expression and promotor methylation density in both MSS and MSI cell lines. Considering that methylation is more often involved in MSI-H tumors than in MSS tumors, it could be inferred that absent or low expression of CD133 in MSI-H tumors may reflect the relatively high frequency of CD133 promotor methylation in these tumors. However, CD133 promoter methylation status is very heterogeneous within individual tumors and cell lines and the dynamic regulation of CD133 expression does not correlate directly with promoter methylation status.[14, 15] Recent results showed no relationship between CD133 protein expression on cell surfaces and CD133 mRNA, and no correlation between changes in CD133 expression and promoter methylation status during differentiation of the HT29 cells.[64] Some cell lines and primary tumors do not show reverse correlation between CD133 promoter methylation and CD133 expression,[14, 15] which was also noted in our results using cell lines. Thus, post-translational modification of CD133 mRNA or protein may combine with promoter regulation in the complex regulation of CD133 expression.

In addition to promoter methylation, cMNR with a length of 8 or more nucleotides in CD133 gene was examined in this study, since many cancer-

associated genes have been found to harbor mutations at mono- or dinucleotide repeats in the coding sequences in cancers with MSI.[37, 41] However, in the present study no mutation was observed in the cMNR [(A)8] of CD133 genes among MSI-H CRC tissues and cell lines. Regarding these results, because it was explored 8 nucleotide repeated sequences alone, further assay might be needed for the other cMNRs with lower number of sequence in CD133 genes. Nevertheless, considering that the chance of mutation is higher as the number of repeated nucleotide is increasing,[43-45] the possibility for the detection of frameshift mutation in the further examination seems to be low. Taken together, although the methylation status of CD133 promoter was not confirmed by in vivo data, it appears that promoter methylation rather than cMNR mutation partly contribute to the low expression of CD133 in MSI-H CRC.

More recently, Neumann et al. demonstrated the association between CD133 expression and loss of hMLH1 expression in right side colon cancer and showed that high CD133 expression was more frequently observed in patients with positive hMLH1 expression compared with in those with negative hMLH1 (35%[33/95] versus 0%[0/19] respectively).[65] Although they included only right sided colon cancer patients and analyzed CD133 expression not based on MSI status but hMLH1 expression, considering that epigenetic inactivation of hMLH1 gene by promoter hypermethylation is generally acknowledged as a main mechanism for development of sporadic

MSI-H CRCs,[17, 63, 66] the results of Neumann et al. are in line with the findings of this study for the more frequent observation of high levels of CD133 expression in MSS CRC than in MSI-H. In contrast to the results of this study, data from another study showed a higher level of CD133 mRNA in MSI CRC than in MSS CRC.[67] However, that study presented mRNA levels as the only measure of CD133 expression and in only a small number of samples. Considering the manifest complexity of CD133 regulation, such as post-translational modulation,[64] assessment of cell surface CD133 protein levels versus mRNA levels may be the basis for this discrepancy, but further study should be performed to clarify the reason of this discrepancy.

Among the patients with sporadic CRCs, K-ras mutations occur in 30-35%, typically in a tumor arising in a pathway involving chromosomal instability.[68] Evidence suggests that Ras/ERK signaling participates in the emergence and maintenance of CSC-like properties.[34-36, 69] In this study, however, there was no association between CSC marker expression and K-ras mutation, although K-ras mutant CRC showed a tendency towards higher expression of CD44 compared to K-ras wild-type CRC (P=0.061). The subgroup analysis according to MSI status sustained this observation. Tabu et al. showed that, while Ras/ERK may contribute to CD133 protein expression, this is not observed in the Caco-2 colon cancer cell line.[70] Similarly, no association between CD133 expression and K-ras mutation status was found in CRC tissues.

Regarding the incidence of K-ras mutation in MSI-H and MSS tumors, this study showed no difference in K-ras mutation between MSI-H and MSS CRCs (34% versus 32%, *P*=0.832), which is inconsistent with previous reports showing higher K-ras mutation rate in MSS CRCs than MSI-H CRCs.[71, 72] In a previous report, Nash et al. reported that K-ras mutation was more frequent in MSS tumors than MSI-H tumors (38% versus 22%, *P*=0.002) in CRC patients (stage I~ IV).[71] This discrepancy may be related to possible bias in age/gender matched selection of MSS CRC patients, a relatively small number of patients, and partially, the fact that this study did not include stage 4 patients, because stage 4 CRCs have rare MSI-H phenotype without significant difference of K-ras mutation rate, compared to other stages.[71]

As for the prognostic analysis, both MSI status and CD133 expression were significant prognostic factors for disease free survival in univariate analysis, however the statistical significance of CD133 vanished in multivariate analysis, whereas MSI status constantly maintained statistical significance. These findings suggest that MSI status is more dominant prognostic marker than CD133 expression. Although the reason for this finding is unclear, the consecutive process, such as differential induction of EMT based on MSI status and increased CSC markers by EMT,[35, 56-58] as mentioned above, might result in high CD133 expression in MSS tumor, suggesting the MSI status might be one of crucial precedent determinants for the expression of CD133.

Meanwhile, as previously mentioned, Neumann et al suggested novel algorithm including hMLH1, CD133, and beta catenine expression for predicting the risk of distant metastasis in right sided colon cancer.[65, 73] They showed that, in hMLH1-positive cases, combined high scores of CD133 and beta-catenin were associated with a very high rate of distant metastases (94.4%), whereas the risk was intermediate for carcinomas with either low CD133 and ／ or low beta-catenin expression (P=0.0007). However, it is unclear whether CD133 expression is an independent predictor for prognosis regardless of hMLH1 expression, since they did not perform multivariate analysis for prognosis including both hMLH1 and CD133 expression. Further large scale study is warranted to elucidate this issue.

The absence of a relationship between CD44/CD166 expression and MSI status in this experiments may reflect the heterogeneity in surface marker expression among tumor stem and progenitor cells, which may change as the tumor evolves. Future study will clarify the regulatory properties and interrelationships of CSC markers, especially with respect to the genomic instability status of the CRC.

V. **CONCLUSION**

It was found that high CD133 expression was more frequently observed in MSS than in MSI-H colorectal cancer tissues and cell lines. These findings suggest that differential expressions of colon CSC markers may be related to differences in tumorigenic pathways, such as the CIN/MSS, MSI, and CIMP pathways in colorectal cancer.

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<ABSTRACT (IN KOREAN)>

인간 대장직장암에서 현미부수체 불안정성에 따른 CD133의 발현양상 분석

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박 재 준

대장직장암에서 유전적 불안정성과 암줄기세포와의 관계에 대해서는 잘 알려져 있지 않다. 본 연구에서는 인간 대장직장암에서 현미부수체 불안정성 (microsatellite instability, MSI)에 따른 암줄기세포 표지자의 발현을 조사하였다.

CD133, CD44, 와 CD166 발현을 각각 50명의 MSI-high (MSI-H)와 현미부수체 안정형 (microsatellite stable, MSS)

대장직장암 환자의 수술 조직에서 면역조직화학염색법으로 확인하였다. 또한, 이 환자들의 수술조직으로 K-ras변이 분석도 시행하였다. 총 11개의 MSI-H와 MSS 대장직장암 세포주들에서 CD133의 발현과 CD133 promoter의 DNA methylation 상태를 조사하였다. HCT116 세포주와 HCT116+Chr3 (hMLH1 유전자가 복구된 HCT116 세포주)에서 CD133 양성 세포들의 비율과 colosphere 형성 능력을 비교 분석하였다.

암줄기세포 표지자의 면역조직화학염색결과에서 CD133의 높은 발현이 MIS-H 종양에 비하여 MSS 종양에서 더 빈번하게 관찰되었으며 (P<0.001, 각각 28.0% 대 74.0%), 이는 짧은 무병생존기간과 관련이 있었다. 반면에 CD44와 CD166발현은 모두 MSI상태에 따라서 차이를 보이지 않았다. CD133발현은 MSI-H 세포주에 비하여 MSS 세포주에서 비교적 높게 나타났으며 이는 CD133 promoter의 DNA methylation과 역의 상관관계를 보였다. HCT116+Chr3 세포주는 HCT116 세포주와 비교하였을 때 CD133 양성 세포의 비율이 증가되었고 높은 colosphere 형성능력을

보였다.

결론적으로 CD133의 높은 발현이 MIS-H 종양에 비하여 MSS 종양에서 더 빈번하게 관찰되었으며 이는 대장암 암줄기세포 표지자의 발현이 MSI 상태에 따르는 종양의 특성과 관련이 있을 것임을 시사한다.

핵심되는 말 : 대장직장암, 암줄기세포, CD133, 현미부수체 불안정성