「Ras/Raf誘導性細胞老化における重要因子のゲノム探索」ポスター	藤本舞、穴井元暢、藤田隆教、山本尚吾、山中遼太、油谷浩幸、金田篤志	第37回日本分子生物学会	平成26年11月25日～11月27日	国内
「大腸前がん病変の遺伝子変異およびエピジェネティック異常の解析」　ポスター	酒井英嗣、梅沢翔太郎、内山詩織、大久保秀則、日暮琢磨、遠藤宏樹、松坂恵介、船田さやか、高根希世子、金田篤	第73回日本癌学会学術総会	平成26年9月25日	国内
"KRAS G12D and G12V Specific Alkylating Agent (KR12) inhibits growth of colon cancer with those KRA mutations in vitro as well as in vivo." 口演	Hiroki Nagase, Kiriko Hiraoka, Takahiro Inoue, Takayoshi Watanabe, Ken-Ichi	American Association for Cancer Research ANUAL MEETING 2014	平成26年4月5日-9日	国外
「Precision Medicineを目指したオンコジェニックドライバーを標的とした治療」口演	永瀬浩喜、平岡桐子、井上隆博、渡部隆義、越川信子、尾崎俊文	第23回癌病態治療研究会	平成26年6/12-13	国内
「これからのがん予防とがん治療　―個人個人に合わせたがんの予防と治療の考え方―」口演	永瀬浩喜	市民公開講座	平成26年6月8日	国内
「KRASコドン12変異を標的とした分子標的アルキル化剤」口演	永瀬浩喜、平岡桐子、井上貴博、越川信子、渡部隆義	第18回　日本癌分子標的治療学会	平成26年6月26日	国内
「がんドライバーミューテーション特異的アルキル化剤の開発」口演	永瀬浩喜、平岡桐子、井上貴博、越川信子、渡部隆義	第２8回　モロシヌス研究会	平成26年6月28日	国内
"Antitumor efficacy of a novel alkylating agent targeting KRAS codon 12 mutations in murine xenograft models of human colorectal cancer"ポスター	井上貴博、平岡桐子、養田裕行、丸喜明、鈴木有生、杉本博一、篠原憲一、渡部隆義、高取敦志、越川信子、板東俊和、杉山弘、尾崎俊文、永瀬浩喜	平成26年度 がん若手研究者ワークショップ	平成26年9月3日～9月6日	国内

「変異型KRASを標的とした塩基配列特異的アルキル化剤によるヒト大腸癌細胞移植マウスの抗腫瘍効果」口演	井上貴博、平岡桐子、養田裕行、丸喜明、杉本博一、篠原憲一、渡部隆義、高取敦志、越川信子、板東俊和、杉山弘、尾崎俊文、永瀬 浩喜	第73回日本癌学会学術総会	平成26年9月25日～27日	国内
「変異型 KRAS 遺伝子を標的とした新規アルキル化剤による大腸がん抗腫瘍効果の検討」ポスター	平岡桐子、井上貴博、養田裕行、杉本博一、篠原憲一、渡部隆義、越川信子、高取敦志、板東俊和、杉山弘、尾崎俊文、永瀬浩喜	第73回日本癌学会学術総会	平成26年9月25日～27日	国内
「ZEB1/E-cadherin 転写制御を標的とした新規ピロールイミダゾールポリアミドの開発」ポスター	養田裕行、高取敦志、渡部隆義、篠原憲一、井上貴博、平岡桐子、越川信子、尾崎俊文、永瀬浩喜	第73回日本癌学会学術総会	平成26年9月25日～27日	国内
「RUNX2 によるヒト膵臓がん細胞のゲムシタビン耐性獲得機構の解析」ポスター	杉本博一、中村瑞代、下里修、永瀬浩喜、尾崎俊文	第73回日本癌学会学術総	平成26年9月25日～27日	国内
「MMP-9を標的とした脂肪族/芳香族アミノ酸ペアを有するPIポリアミドによる遺伝子抑制」ポスター	渡部隆義、越川信子、尾崎俊文、板東俊和、杉山弘、永瀬浩喜	第73回日本癌学会学術総会	平成26年9月25日～27日	国内
「RUNX2 によるTAp73 の抑制を介したDNA 損傷応答機構の制御」ポスター	尾崎俊文、杉本博一、中村瑞代、下里修、永瀬浩喜	第73回日本癌学会学術総会	平成26年9月25日～27日	国内
"A novel anti-cancer agent of DNA-alkylating Pyrrole-Imidazole polyamide conjugate targeting KRAS Codon 12 Mutant DNA" 口演	Hiroki Nagase, Kiriko Hiraoka, Takahiro Inoue, Nobuko Koshikawa, Takayoshi Watanabe	52st JSCO 2014	平成26年8月28-30日	国内

「Pyrrole-Imidazole Polyamide Drug Conjugate (PDC)」 「ＤＮＡ結合化合物によるゲノム・エピゲノムの変更 -新たな抗がん治療薬開発へのアプローチ-」口演	Hiroki Nagase	東京医科歯科大学　大学院講義セミナー	平成26年10/09	国内
"Targeting specific DNA sequences by N-methylpyrrole and N-methylimidazole polyamides provides insights for the development of novel diagnostic and therapeutic drugs."ポスター	Atsushi Takatori, Hiroyuki Yoda, Kiriko Hiraoka, Kenichi Shinohara, Takayoshi Watanabe, Nobuko Koshikawa, and Hiroki Nagase.	The 28th International Mammalian Genome Conference	平成26年October 26-29th	国外
"Allele-specific knock-down of KRAS mutations in cancers by using a novel alkylating Pyrrole-Imidazole Polyamide (KR12)."口演	Atsushi Takatori, AKiriko Hiraoka, Takahiro Inoue, Takayoshi Watanabe, Ken-Ichi Shinohara, Nobuko Koshikawa, Ozaki Toshinori. and Hiroki Nagase	The 28th International Mammalian Genome Conference	平成26年October 26-29th	国外
「ゲノム認識エピジェネティック変更化合物の開発」口演	永瀬浩喜、渡部隆義、養田裕行、越川信子、高取敦志	文部科学省科学研究費補助金・新学術領域研究「生殖細胞のエピゲノムダイナミクスとその制御」第２回公開シンポジウム	平成26年10月	国内
「千葉県がんセンターにおけるClinical Genomics の視点に基づく治療法開発の取り組み」口演	永瀬　浩喜	千葉がんシンポジウム臨床研究総合センターシンポジウム	平成26年12/13	国内
「RASコドン12変異を標的とした分子標的アルキル化剤」口演	永瀬　浩喜	革新的がん医療シーズ育成領域　革新的シーズ育成分野7　「転写機能をターゲットとした創薬」会議	平成26年12/23	国内
「ドライバー遺伝子変異を標的としたアルキル化剤による治療法の開発」口演	永瀬 浩喜	第37回近畿小児血液・がん研究会	平成27年2/14	国内
"PI polyamide-drug-conjugates(PDC) towards clinical application"口演	永瀬　浩喜	「武装PIポリアミド（PIポリアミド薬物複合体）の臨床応用に向けた取り組み」京都大学　学術講演会	平成27年2/13	国内

″Alkylating agents targeting oncogenic driver mutations″口演	永瀬浩喜	私立大学戦略的研究基盤形成支援事業「ゲノム化学に基づく先進医療開発研究拠点」平成26年度報告会	平成27年2/28	国内
「千葉県がんセンターキャンサーバイオバンク　ー Clinical Genomicsへの活用ー」口演	横井左奈、鶴岡成一、滝口伸浩、伊丹真紀子、巽康年、大平美紀、山口武人、永瀬浩喜	クリニカルバイオバンク　ネットワーキング会合	平成27年3/8	国内

２．学会誌・雑誌等における論文掲載

掲載した論文（発表題目）	発表者氏名	発表した場所（学会誌・雑誌等名）	発表した時期	国内・外の別
Methylation epigenotypes and genetic features in colorectal laterally spreading tumors	Sakai E, Ohata K, Chiba H, Matsuhashi N, Doi N, Fukushima J, Endo H, Takahashi H, Tsuji S, Yagi K, Matsusaka K, Aburatani H, Nakajima A,	Int J Cancer	平成26年10月	国外
Aberrant promoter methylation of PPP1R3C and EFHD1 in plasma of colorectal cancer patients.	Takane K, Midorikawa Y, Yagi K, Sakai A, Aburatani H, Takayama T,	Cancer Med	平成26年10月	国外
DNA methylation accumulation and its predetermination of future cancer phenotypes.	Kaneda A, Matsusaka K, Sakai E, Funata S	J Biochem	平成26年8月	国外
Quantitative DNA methylation analysis for epigenotyping of	Kaneda A, Yagi K	Methods Mol Biol	平成27年	国外
Nr4a3, a possibile oncogenic factor for neuroblastoma associated with CpGi methylation within the third exon.	Uekusa S, Kawashima H, Sugito K, Yoshizawa S, Shinojima Y, Igarashi J, Ghosh S, Wang X, Fjiwara K, Ikeda T, Koshinaga T, Soma M and Nagase H	Int J Oncol	平成２６年５月	国外

A novel gene regulator, pyrrole-imidazole polyamide targeting ABCA1 gene increases cholesterol efflux from macrophages and plasma HDL concentration.	Tsunemi A, Ueno T, Fukuda N, Watanabe T, Tahira K, Haketa A, Hatanaka Y, Tanaka S, Matsumoto T, Matsumoto Y, Nagase H, Soma M.	J Mol Med	平成２６年５月	国外
Inhibition of human osteosarcoma cell migration and invasion by a gene silencer, Pyrrole-Imidazole polyamide, targeting to the human MMP9 NFkB binding site.	Kojima T, Wang X, Fujiwara K, Osakaa S, Yoshida Y, Osaka E, Taniguchi M, Ueno T, Fukuda N, Soma M, Tokuhashi Y and Nagase H.	Biol Pharm Bull	平成２６年９月	国外
Identification of Frequent Differentially Methylated Region in Sporadic Bladder Cancers.	Hasegawa R, Fujiwara K, Obinata D, Kawashima H, Shinojima Y, Igarashi J, Wang X, Ghosh S, Nagase H, Takahashi S.	Urol Int.	平成２６年９月	国外
Pyrrole-imidazole polyamide targeted to break fusion sites in TMPRSS2 and ERG gene fusion represses prostate tumor growth.	Obinata D, Ito A, Fujiwara K, Takayama K, Ashikari D, Murata Y, Yamaguchi K, Urano T, Fujimura T, Fukuda N, Soma M, Watanabe T, Nagase H, Inoue S, Takahashi S.	Cancer Sci	平成２６年１０月	国外
Genome-wide screening of aberrant DNA methylation which associated with gene expression in mouse skin cancers.	Fujiwara K, Ghosh S, Liang P, Morien E, Soma M, Nagase H.	Mol Carcinog	平成２７年３月	国外

Intracellular fragment of NLRR3 (NLRR3-ICD) stimulates ATRA-dependent neuroblastoma differentiation.	Akter J, Takatori A, Islam S, Nakazawa A, Ozaki T, Nagase H, Nakagawara A.	Biochem Biophys Res Commun	平成２６年１０月	国外
Runt-related transcription factor 2 attenuates the transcriptional activity as well as DNA damage-mediated induction of pro-apoptotic TAp73 to regulate chemo-sensitivity.	Ozaki T, Sugimoto H, Nakamura M, Hiraoka K, Yoda H, Sang M, Fujiwara K, Nagase H	FEBS J	平成２７年１月	国外
Identification of a novel E-box binding PI polyamide inhibiting MYC-driven cell-proliferation.	Mishra R, Watanabe T, Kimura M, Koshikawa N, Ikeda M, Uekusa S, Kawashima H, Wang X, Igarashi J, Choudhury D, Grandori C, Kemp C, Ohira M, Verma N, Kobayashi Y, Takeuchi J, Koshinaga T, Nemoto N, Fukuda N, Soma M, Kusafuka T, Fujiwara K, Nagase H.	Cancer Sci	平成２７年１月	国外
Inhibition of KRAS codon 12 mutants using a novel DNA-alkylating pyrrole-imidazole polyamide conjugate.	Hiraoka K, Inoue T, Taylor RD, Watanabe T, Koshikawa N, Hiroyuki Yoda H, Shinohara K, Takatori A, Sugimoto H, Maru Y, Denda T, Fujiwara K, Balmain A, Ozaki T, Bando T, Sugiyama H, Nagase H.	Nat Commun	平成２７年(in press)	国外

（注１）発表者氏名は、連名による発表の場合には、筆頭者を先頭にして全員を記載すること。
（注２）本様式はexcel形式にて作成し、甲が求める場合は別途電子データを納入すること。

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Methylation epigenotypes and genetic features in colorectal laterally spreading tumors

Eiji Sakai[1,2], Ken Ohata[3], Hideyuki Chiba[3], Nobuyuki Matsuhashi[3], Noriteru Doi[4], Junichi Fukushima[4], Hiroki Endo[1], Hirokazu Takahashi[1], Shingo Tsuji[5], Koichi Yagi[5], Keisuke Matsusaka[2], Hiroyuki Aburatani[5], Atsushi Nakajima[1] and Atsushi Kaneda[2,5,6]

[1] Department of Gastroenterology, Yokohama City University School of Medicine, Yokohama, Japan
[2] Department of Molecular Oncology, Graduate School of Medicine, Chiba University, Chiba, Japan
[3] Department of Gastroenterology, Kanto Medical Center, NTT East, Tokyo, Japan
[4] Department of Diagnostic Pathology, Kanto Medical Center, NTT East, Tokyo, Japan
[5] Genome Science Division, Research Center for Advanced Science and Technology, The University of Tokyo, Tokyo, Japan
[6] CREST, Japan Science and Technology Agency, Saitama, Japan

Aberrant DNA methylation plays an important role in genesis of colorectal cancer (CRC). Previously, we identified Group 1 and Group 2 methylation markers through genome-wide DNA methylation analysis, and classified CRC and protruded adenoma into three distinct clusters: high-, intermediate- and low-methylation epigenotypes. High-methylation epigenotype strongly correlated with *BRAF* mutations and these aberrations were involved in the serrated pathway, whereas intermediate-methylation epigenotype strongly correlated with *KRAS* mutations. Here, we investigated laterally spreading tumors (LSTs), which are flat, early CRC lesions, through quantitative methylation analysis of six Group 1 and 14 Group 2 methylation markers using pyrosequencing. Gene mutations in *BRAF, KRAS* and *PIK3CA*, and immunostaining of TP53 and CTNNB1 as well as other clinicopathological factors were also evaluated. By hierarchical clustering using methylation information, LSTs were classified into two subtypes; intermediate-methylation epigenotype correlating with *KRAS* mutations ($p = 9 \times 10^{-4}$) and a granular morphology (LST-G) ($p = 1 \times 10^{-7}$), and low-methylation epigenotype correlating with CTNNB1 activation ($p = 0.002$) and a nongranular morphology (LST-NG) ($p = 1 \times 10^{-7}$). Group 1 marker methylation and *BRAF* mutations were barely detected, suggesting that high-methylation epigenotype was unlikely to be involved in LST development. *TP53* mutations correlated significantly with malignant transformation, regardless of epigenotype or morphology type. Together, this may suggest that two molecular pathways, intermediate methylation associated with *KRAS* mutations and LST-G morphology, and low methylation associated with CTNNB1 activation and LST-NG morphology, might be involved in LST development, and that involvement of *TP53* mutations could be important in both subtypes in the development from adenoma to cancer.

Cancer Genetics

Colorectal cancer (CRC) arises through accumulation of multiple genetic and epigenetic alterations.[1–3] Somatic mutations in *KRAS, APC* and *TP53* are well-known genetic alterations, which were demonstrated in the model of adenoma-carcinoma sequence.[4] Recent exome sequencing studies revealed the involvement of somatic mutations of other genes, *e.g., SMAD4, PIK3CA, TCF7L2, ARID1A* and TET family genes, or amplification of *ERBB2* and *IGF2*.[5,6] In addition, aberrant DNA methylation of gene promoters has been reported to be a major epigenetic mechanism for silencing tumor-suppressor genes involved in colorectal carcinogenesis.[2,7]

We previously performed DNA methylation analysis of CRC on genome-wide scale and identified Group 1 and Group 2 methylation markers to classify CRC into three distinct subsets,[8,9] which has also been reported by other groups.[10–12] High-methylation epigenotype [or CpG island methylator phenotype (CIMP)[13]] showed methylation of both Group 1 and Group 2 markers, while intermediate-methylation epigenotype showed methylation of Group 2 but not Group 1 markers, and low-methylation epigenotype showed methylation of neither Group 1 nor Group 2 markers. High- and intermediate-methylation epigenotypes strongly correlated with *BRAF* and *KRAS* mutations, respectively, and low-methylation epigenotype correlated with the absence of these oncogene mutations, suggesting the existence of at least three distinct pathways in genesis of CRC.[9]

Key words: laterally spreading tumor (LST), colorectal cancer, DNA methylation, TP53, KRAS
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Correspondence to: Atsushi Kaneda, MD, PhD, Department of Molecular Oncology, Graduate School of Medicine, Chiba University, Inohana 1-8-1, Chuo-ku, Chiba 260-8670, Japan, Tel.: +81-43-226-2039, Fax: +81-43-226-2039, E-mail: kaneda@chiba-u.jp

What's new?
Although most colorectal cancers develop from adenomatous polyps, some arise via an alternative pathway involving non-protruding lateral extensions along the luminal wall. These laterally spreading tumors (LSTs) are molecularly not well characterized. Here the authors investigated epigenetic and genetic alterations in LSTs and correlated these with morphological appearances. While colorectal cancers showed high, intermediate, and low DNA methylation phenotypes, LSTs only showed intermediate and low methylation phenotypes, associated with unique genetic mutations and morphological phenotypes. These studies lay the groundwork for the molecular characterization of these untypical early colorectal cancer lesions.

Through the analysis of protruded adenoma, it was shown that the methylation epigenotype is mostly formed already at adenoma stage.[14] As serrated adenomas showed high methylation and *BRAF* mutations, high-methylation/CIMP(+) CRCs with microsatellite instability have been considered to develop through the serrated pathway.[14,15] Conventional non-serrated adenomas have been classified into intermediate-methylation epigenotype associated with *KRAS* mutations and low-methylation epigenotype associated with less frequent *KRAS* mutations.[14]

The majority of sporadic CRCs are thought to develop from adenomatous polyps through the adenoma-carcinoma sequence. It is hypothesized, however, that some CRC cases develop through alternative pathways such as the serrated pathway and the *de novo* pathway.[16,17] These nonpolypoid colorectal neoplasms that do not adopt a macroscopic protruding appearance have been documented not only in Japan[18–22] but also in Western countries.[23,24] They are characterized by lateral extensions along the luminal wall with a low vertical axis, and those with a diameter >10 mm are called laterally spreading tumors (LSTs).[22] Molecular alterations in LSTs have not been fully investigated, but the incidence of *KRAS* mutations and *TP53* abnormalities was less common, for example, in nonpolypoid lesions than in similar-sized polypoid lesions.[25–28] Epigenetic alterations of LST have not been well clarified, and the relationship between genetic and epigenetic alterations, neither.

To clarify the involvement of epigenetic alterations in LSTs, here we analyzed methylation of Group 1 and Group 2 markers quantitatively, and epigenotyped LSTs using unsupervised hierarchical clustering. We also evaluated genetic alterations in *KRAS, BRAF* and *PIK3CA* and the expression status, using immunostaining, of TP53 and CTNNB1, which are all major aberrations frequently identified in colorectal carcinogenesis.[5,6] We also elucidated the correlation among methylation epigenotypes, genetic alterations and tumor pathologies in LSTs. Although high-methylation epigenotype and *BRAF* mutations were barely involved in LST development, two distinct epigenotypes were found in LSTs: intermediate-methylation epigenotype that correlated with *KRAS* mutations and was significantly associated with a granular morphology (LST-G) and low-methylation epigenotype that correlated with CTNNB1 activation and was significantly associated with a nongranular morphology (LST-NG). Involvement of TP53 mutation, suggested to be important in enabling the development from adenoma to cancer of both LST subtypes, was also implicated.

Material and Methods

Clinical samples

A total of 125 colorectal LST samples were obtained from patients who underwent endoscopic submucosal dissection at the Yokohama City University Hospital and Kanto Medical Center, NTT East, with written informed consents. Tumors were resected endoscopically with complete en bloc excision, fixed in 10% formalin and then embedded in paraffin. Subsequently, 10-μm-thick formalin-fixed, paraffin-embedded tissue specimens underwent laser capture microdissection (Carl Zeiss, Jena, Germany) to obtain tumor cells. DNA was extracted using QIAamp DNA FFPE Tissue Kit (Qiagen, Hilden, Germany). More than 500 ng of DNA was obtained from each of 108 LST (51 LST-G and 57 LST-NG) samples, which were used in the subsequent experiments. Two experienced gastroenterologists determined the macroscopic appearance of LSTs: LST-G was composed of superficially spreading granulonodular lesions and LST-NG was composed of nonpolypoid lesions slightly elevated or sometimes depressed (Fig. 1). The height was less than half the diameter of the lesion and the thickness was less than twofold the adjacent normal mucosa. Patients with familial adenomatous polyposis, hereditary nonpolyposis colorectal carcinoma and colitis-associated carcinoma were excluded from the study, as were patients with villous and serrated neoplasms. Ethics Committees in Yokohama City University, The University of Tokyo, Chiba University and Kanto Medical Center, NTT East approved this study.

Histological evaluation

The specimens were cut at 4 μm thick and stained with hematoxylin and eosin. Two experienced pathologists performed histopathological and immunohistochemical examinations. According to the Vienna classification,[29] colorectal neoplasms were diagnosed into one of four categories: low-grade dysplasia, high-grade dysplasia, carcinoma *in situ* (intraepithelial noninvasive carcinoma) and invasive carcinoma (invading beyond the lamina propria). Two experienced pathologists determined the tumor cell content. In this study, carcinoma *in situ* and invasive carcinoma were

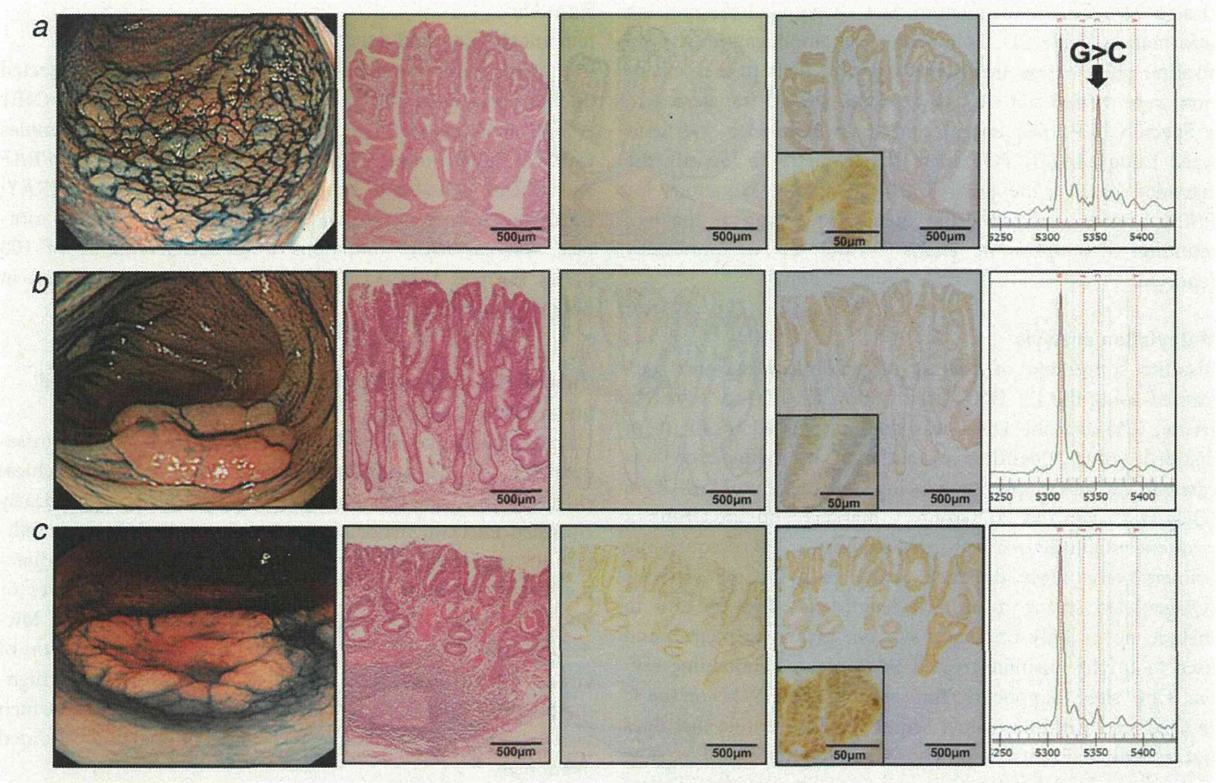

Figure 1. Macroscopic appearance of LST samples and molecular alterations. Endoscopic images after administration of 0.1% indigo carmine solution (most left), HE staining (second left), immunostaining of *TP53* and CTNNB1 (middle and second right) and MassARRAY result at KRAS_35 (most right) are representatively shown. (*a*) An LST-G adenoma, showing granular morphology, *TP53* staining(−), CTNNB1 activation(−) (negative nuclear expression, weak cytoplasmic expression and positive membrane expression) and *KRAS* mutation(+), was composed of low exophytic polypoid-like adenomatous tubules with low-grade dysplasia. (*b*) An LST-NG adenoma, showing nongranular morphology, no central depression, *TP53* staining(−), CTNNB1 activation(−) (negative nuclear expression, weak cytoplasmic expression and negative membrane expression) and *KRAS* mutation(−), was composed of low smooth nonpolypoid adenomatous tubules with high-grade dysplasia. (*c*) An LST-NG with *TP53* staining(+), CTNNB1 activation(+) (positive nuclear expression, weak cytoplasmic expression and negative membrane expression) and *KRAS* mutation(−) showed central depression, corresponding to the cancer lesion with submucosal invasion.

considered as cancer, and low- and high-grade dysplasia as adenoma. When LST samples contained both adenoma and carcinoma components, they were classified into the cancer group and only carcinoma components were microdissected.

Immunohistochemistry

Immunohistochemistry (IHC) for TP53 was conducted using DO-7 anti-mouse monoclonal antibody (Santa Cruz Biotechnology, Dallas, TX) as previously described,[9] and samples with nuclear staining were considered to be TP53-IHC(+) and thus designated as *TP53* mutation(+). IHC for CTNNB1 was performed using anti-mouse monoclonal antibody (BD Transduction Laboratories) as previously described.[30] CTNNB1 accumulates in the cytoplasm and nucleus regardless of the activating mechanism of the WNT signaling pathway, *e.g.*, *APC* mutation,[31] and therefore, we assessed expression of CTNNB1 using immunostaining. The CTNNB1 activation score, as previously described,[30] was calculated by adding the nuclear score (+2 = positive expression; +1 = weak expression; 0 = no expression), cytoplasmic score (+2 = positive expression; +1 = weak expression; 0 = no expression) and membrane score (0 = positive membrane expression; +1 = negative membrane expression), and if the sum was ≥+3 then the sample was considered to be CTNNB1 activation(+). For comparison, 38 protruded adenoma samples, 28 intramucosal CRC samples and 60 advanced CRC samples obtained from Yokohama City University Hospital were also analyzed for CTNNB1 expression using IHC and CTNNB1 activation scores.

Mutation analysis

BRAF(1799) and *KRAS*(34, 35, 37 and 38) mutations were analyzed using genotyping assay on the MassARRAY platform as we have previously described.[14] Briefly, polymerase chain reaction (PCR) amplification primers and a post-PCR extension primer were designed using the MassARRAY Assay

Design 3.0 software (Sequenom, San Diego, CA) (Supporting Information Table S1). Following PCR amplification, shrimp alkaline phosphatase treatment and post-PCR primer extension were carried out. Reaction products were transferred to a SpectroCHIP (Sequenom) and mass difference was analyzed using MALDI-TOF mass spectrometry to identify the extended base at the possible mutation site. Mutations for *PIK3CA* were analyzed using direct sequencing at the two mutation hot spots in exons 9 and 20 as previously reported.[32]

Methylation analysis

Bisulfite conversion of 500 ng of genomic DNA was performed using the EZ DNA Methylation Kit (Zymo Research, Irvine, CA) and the DNA was then suspended in 40 μL of distilled water. Quantitative validation of methylation was carried out using pyrosequencing on the PyroMark Q96 (Qiagen).[33] For the 20 Group 1 markers and 25 Group 2 markers established in our previous study,[9] pyrosequencing primers were newly designed, using Pyro Q-CpG software (Qiagen), to ensure a product length of <100 bp and to include no or only one CpG site per primer sequence, and used to amplify bisulfite-treated DNA regions containing several CpG sites (Supporting Information Table S2). For the C in CpG sites within a primer sequence, a nucleotide that does not anneal to C or U was chosen, *e.g.*, adenosine (A). The biotinylated PCR product was bound to Streptavidin Sepharose High Performance (Amersham Biosciences, Uppsala, Sweden), washed and denatured using a 0.2 mol/L NaOH solution. After addition of 0.3 μmol/L sequencing primer to the single-stranded PCR product, pyrosequencing was carried out according to the manufacturer's instructions. Analysis was performed using methylation control samples (0, 25, 50, 75 and 100%),[9,33] and it was confirmed to be highly quantitative for six Group 1 markers and 14 Group 2 markers (Supporting Information Fig. S1).

Statistical analysis

Correlations between LST morphologies and clinicopathological factors were analyzed using the Fisher's exact test, except for age and tumor size, which were analyzed using the Student's *t*-test. Correlations between LST morphologies and/or methylation epigenotype and tumor locations were analyzed using the Kruskal–Wallis test. The differences in methylation rates for each marker were analyzed using the Student's *t*-test. The differences in mutation rates between adenoma and cancer groups were analyzed using the Fisher's exact test. Unsupervised two-way hierarchical clustering was carried out based on the Euclidean distance and the complete linkage-clustering algorithm using Cluster 3.0 software. The heat map was drawn using Java Tree View software. To reveal factors associated with malignant transformation and submucosal invasion of LSTs, univariate and multivariate logistic regression analyses were performed.

Results

Immunostaining and mutation analysis

We performed immunostaining on TP53 and detected nuclear staining in 40 of 108 LST samples (Fig. 1). CTNNB1 activation status was also assessed using IHC and 31 samples were considered to be CTNNB1 activation(+) (Fig. 1). *BRAF* and *KRAS* mutations were analyzed using MassARRAY; *BRAF* mutation was rare and was detected only in two samples, whereas *KRAS* mutations were detected in 51 of 108 (47%) samples (Fig. 1). *PIK3CA* mutation was detected in only one sample.

Quantitative DNA methylation analysis and unsupervised hierarchical clustering

Using quantitative methylation data obtained from pyrosequencing, we performed two-way unsupervised hierarchical clustering on 108 LST samples (Fig. 2). LSTs were clearly classified into two major clusters: a cluster with higher methylation of Group 2 markers, considered as intermediate-methylation epigenotype, and another cluster with lower or no methylation of Group 2 markers, considered as low-methylation epigenotype. No cluster with high methylation of Group 1 markers was detected, suggesting that high-methylation epigenotype was not involved in LSTs, which was similar to findings for conventional nonserrated adenomas.[14]

Genetic alterations and other clinicopathological factors were compared between intermediate- and low-methylation epigenotypes (Fig. 2). Intermediate-methylation LST samples showed a significantly higher frequency of *KRAS* mutations than low-methylation LST samples ($p = 9 \times 10^{-4}$), indicating that intermediate-methylation epigenotype correlated with *KRAS* mutations in LSTs as well as in CRCs and protruded adenomas.[9,14] Conversely, low-methylation LST samples showed a significantly higher frequency of CTNNB1 activation than intermediate-methylation LST samples ($p = 0.002$). Occurrences of *KRAS* mutations and CTNNB1 activation were mutually exclusive ($p = 0.006$, Fisher's exact test). *BRAF* mutations were rare, but also occurred in a mutually exclusive manner to *KRAS* mutations and CTNNB1 activation. There was no correlation between methylation epigenotype and tumor location.

Most interestingly, intermediate-methylation epigenotype showed a strong correlation with LST-G, and low-methylation epigenotype showed a strong correlation with LST-NG ($p = 1 \times 10^{-7}$), suggesting that the two distinct epigenetic characteristics mostly reflect two different macroscopic morphologies of these lesions (Fig. 2).

Correlation of macroscopic morphology with methylation and other factors

When LST-G and LST-NG cases were compared, there were no significant differences in sex and age between 51 LST-G and 57 LST-NG cases (Table 1). Tumor size of LST-G

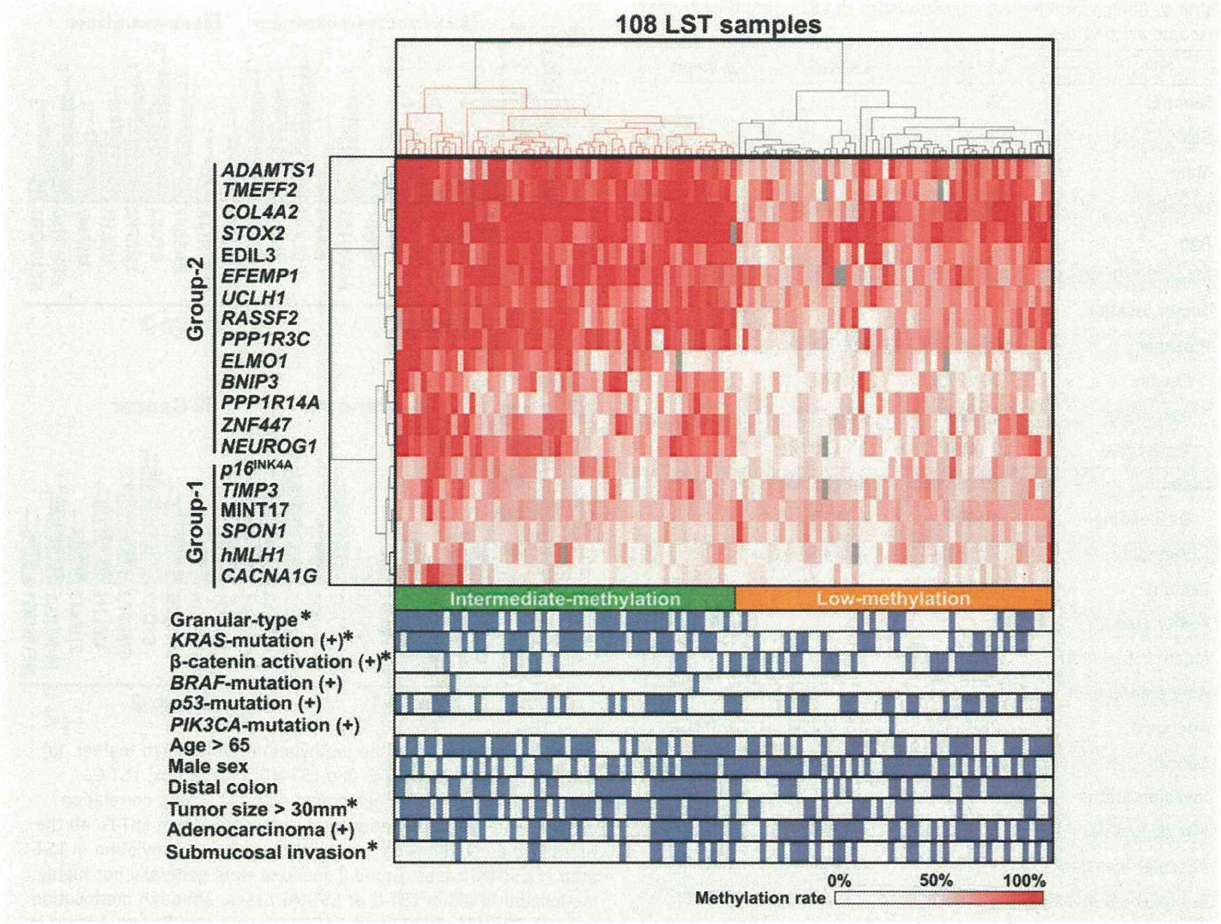

Figure 2. Epigenotyping of 108 LST samples. Using quantitative methylation data from Group 1 and Group 2 markers, LST samples were analyzed using unsupervised two-way hierarchical clustering (top) and compared with clinicopathological factors (bottom). LSTs were clearly classified into two major clusters. The left cluster of 56 cases showed higher methylation of Group 2 markers and correlated with *KRAS* mutations ($p = 9 \times 10^{-4}$), corresponding to intermediate-methylation epigenotype. The right cluster of 52 cases showed less or no methylation of Group 2 markers and correlated with CTNNB1 activation ($p = 0.002$), corresponding to low-methylation epigenotype. Intermediate-methylation LSTs highly associated with granular morphology (LST-G), and low-methylation LSTs highly associated with nongranular morphology (LST-NG) ($p = 1 \times 10^{-7}$). There was no significant difference in *TP53* mutation frequency between intermediate- and low-methylation epigenotypes ($p = 0.8$). *$p < 0.05$, Fisher's exact test.

samples was significantly larger than that of LST-NG samples (41.0 ± 19.9 *vs.* 27.6 ± 9.9, $p = 2 \times 10^{-5}$). There was no correlation between LST morphology and tumor location. Compared with LST-G cases, however, carcinoma components and submucosal invasion were detected in LST-NG cases more frequently (45 *vs.* 68%, $p = 0.02$ and 16 *vs.* 40%, $p = 0.006$, respectively).

The methylation rate of each marker was compared between LST-G and LST-NG cases (Fig. 3*a*). In agreement with the strong correlation between intermediate-methylation and LST-G cases, all the Group 2 markers showed substantially higher methylation in LST-G than in LST-NG cases. Group 1 markers were not highly methylated in either LST-G or LST-NG cases, although methylation rates of *CDKN2A*, *TIMP3* and *CACNA1G* were significantly higher in LST-G than in LST-NG cases. No significant difference was detected between LST-G and LST-NG cases with regard to patient age (67.1 ± 11.7 *vs.* 66.7 ± 10.4, $p = 0.9$) or tumor cell content ($75.4 \pm 6.2\%$ *vs.* $75.9 \pm 8.7\%$, $p = 0.8$), suggesting that the differences in methylation levels were not due to age-related changes or tumor purity differences, but reflected distinct epigenetic features of these two tumor groups.

Mutation status was compared between LST-G and LST-NG samples (Table 1). The frequency of *KRAS* mutations in LST-G samples was substantially and significantly higher than in LST-NG samples ($p = 1 \times 10^{-4}$). Conversely, the frequency of CTNNB1 activation in LST-G samples was substantially lower than in LST-NG samples ($p = 2 \times 10^{-8}$). There was no significant difference in the frequency of *TP53* mutations between LST-G and LST-NG cases ($p = 0.2$).

Table 1. Clinicopathological characteristics of LSTs according to macroscopic morphology

	LST-G	LST-NG	*p*-Value
Number	51	57	
Sex			
Male	24 (47%)	37 (65%)	0.08
Female	27 (53%)	20 (35%)	
Age			
Mean ± SD (years)	67.1 ± 11.7	66.7 ± 10.4	0.9
Tumor location			0.9
Proximal	24 (47%)	29 (51%)	
Cecum	14 (27%)	2 (4%)	
Ascending	8 (16%)	12 (21%)	
Transverse	2 (4%)	15 (26%)	
Distal	12 (24%)	14 (25%)	
Descending	3 (6%)	6 (11%)	
Sigmoid	9 (18%)	8 (14%)	
Rectum	15 (29%)	14 (25%)	
Tumor size			
Mean ± SD (mm)	41.0 ± 19.9	27.6 ± 9.9	2×10^{-5}*
Adenoma/cancer			
Adenoma	28 (55%)	18 (32%)	0.02*
Cancer	23 (45%)	39 (68%)	
Invasion status			
Lymph invasion	1 (2%)	4 (7%)	0.4
Vascular invasion	1 (2%)	5 (9%)	0.2
Submucosal invasion	8 (16%)	23 (40%)	0.006*
Mutation			
KRAS	34 (67%)	17 (30%)	1×10^{-4}*
BRAF	2 (4%)	0 (0%)	0.22
TP53	15 (29%)	25 (44%)	0.16
PIK3CA	0 (0%)	1 (2%)	1
CTNNB1 activation	2 (4%)	29 (51%)	2×10^{-8}*

Tumor locations were classified into three groups: proximal (cecum, ascending and transverse colon), distal (descending and sigmoid colon) and rectum. Tumor size was recorded as the maximum diameters of the extirpated specimen. *p*-Values were analyzed using the Fisher's exact test, the Student's *t*-test for age and tumor size or the Kruskal–Wallis test for tumor location.
*p-Value < 0.05.

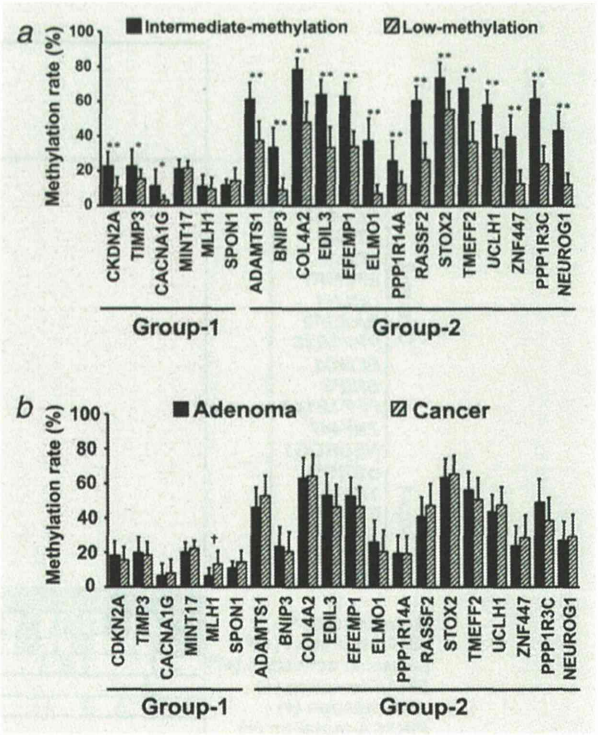

Figure 3. Comparison of the methylation rate of each marker. (*a*) Comparison between LST-G and LST-NG. Closed box, LST-G. Hatched box, LST-NG. In agreement with the strong correlation between intermediate-methylation epigenotype and LST-G, all the Group 2 markers showed substantially greater methylation in LST-G than in LST-NG cases. Group 1 markers were generally not highly methylated in either LST-G or LST-NG cases, although methylation rates of *CDKN2A*, *TIMP3* and *CACNA1G* were significantly higher in LST-G than in LST-NG cases. *$p < 0.05$, **$p < 0.001$, Student's *t*-test. (*b*) Comparison between adenoma and cancer. Closed box, adenoma. Hatched box, cancer. There was no significant difference of Group 2 marker methylation between adenoma and cancer. Group 1 markers were not highly methylated in either adenoma or cancer, although the methylation rate of *MLH1* was significantly higher in cancer than in adenoma ($^{\dagger}p = 0.004$).

Comparison between adenoma and cancer

Epigenetic alterations were compared between adenoma and cancer groups (Fig. 3*b*). Regarding methylation rates, there was no significant difference in Group 2 marker methylation between adenoma and cancer. Group 1 markers were not highly methylated in either adenomas or cancers, although the methylation rate of *MLH1* was significantly higher in cancers than in adenomas. Overall, these data are in good agreement with our previous report that methylation accumulation was mostly completed by the adenoma stage in protruded adenomas and CRCs.[9,14]

As for gene mutation (Fig. 4), there were no significant differences in the frequency of *KRAS* mutation between adenoma and cancer groups, either in LST-G (57 *vs.* 78%, $p = 0.1$, Fisher's exact test) or in LST-NG (22 *vs.* 33%, $p = 0.5$). Also, in the frequency of CTNNB1 activation, no significant differences were detected between adenoma and cancer groups, either in LST-G (0 *vs.* 9%, $p = 0.2$) or in LST-NG (39 *vs.* 56%, $p = 0.3$). However, the frequency of *TP53* mutation was substantially and significantly higher in cancer group, both in LST-G (14 *vs.* 48%, $p = 0.01$) and in LST-NG (6 *vs.* 62%, $p = 5 \times 10^{-5}$). *BRAF* and *PIK3CA* mutations were detected in only two LST-G samples and one LST-NG sample, respectively, which gave no significant difference between adenoma and cancer.

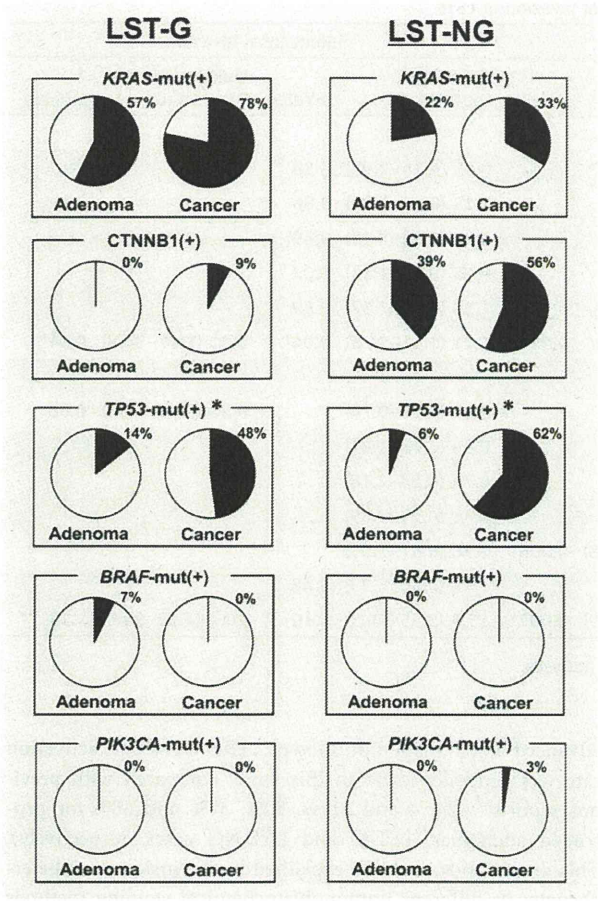

Figure 4. Comparison of mutation rates between adenoma and cancer. The frequency of *TP53* mutation was substantially and significantly higher in cancer group, both in LST-G (14 *vs.* 48%, $p = 0.01$, Fischer's exact test) and in LST-NG (6 *vs.* 62%, $p = 5 \times 10^{-5}$). The frequencies of *KRAS* mutations and CTNNB1 activation were not significantly different between adenoma and cancer groups, either in LST-G and LST-NG. The frequencies of *BRAF* and *PIK3CA* mutations in LSTs were very low, both in adenoma and cancer. *p-Value < 0.05.

Univariate and multivariate logistic regression analyses

To gain insight into the contribution of each clinicopathological factor to malignant transformation and submucosal invasion of LSTs, univariate and multivariate logistic regression analyses were performed with the following parameters: age (>65 years old), sex, tumor size (>30 mm), large nodule, central depression, *KRAS* mutation, *TP53* mutation and CTNNB1 activation (Table 2).

TP53 mutation was identified as an independent predictor of malignant transformation in both LST-G [odds ratio (OR) 6.84; 95% confidence interval (CI) 1.59–29.4, $p = 0.01$) and LST-NG cases (OR 24.6; 95% CI 2.51–240.8, $p = 0.006$). Having *KRAS* mutation and CTNNB1 activation were not predictive of malignant transformation. These data suggest that methylation accumulation and *KRAS* mutation might be necessary to form intermediate-methylation LST, mostly LST-G, but these factors are required for adenoma formation, and *TP53* mutation might contribute to cancer development. As for low-methylation LST, mostly LST-NG, CTNNB1 activation might be involved in adenoma formation, and again *TP53* mutation might contribute to cancer development.

In LST-G, a large nodule was identified as an independent predictor for malignant transformation (OR 4.89; 95% CI 1.30–18.4, $p = 0.02$) and for submucosal invasion (OR 6.21; 95% CI 1.11–34.8, $p = 0.04$). In LST-NG, central depression was identified as a predictor for malignant transformation (OR 35.0; 95% CI 3.68–332.9, $p = 0.002$) and submucosal invasion (OR 20.7; 95% CI 4.58–94.0, $P < 0.0001$).

Discussion

LSTs are a morphologically unique type of colorectal tumor characterized by laterally extended growth with minimal vertical growth. Our quantitative methylation analysis using Group 1 and Group 2 markers successfully clustered LSTs into two epigenotypes: intermediate and low methylation. Intermediate-methylation epigenotype correlated significantly with having a *KRAS* mutation and mostly reflected LST-G morphology. Low-methylation LSTs mostly reflected LST-NG morphology with significant association with CTNNB1 activation. In both epigenotypes, no increase in methylation levels was detected when comparing adenoma and cancer. Instead, *TP53* mutations were strongly associated with cancer development.

Existence of three methylation epigenotypes in CRC[9–12] had suggested that distinct molecular pathways were involved in genesis of CRC. High-methylation/CIMP(+) CRC is considered to develop through the serrated pathway, and precursor lesions of high-methylation CRC have different macroscopic morphology, *i.e.*, serrated adenoma.[14,15] Intermediate- and low-methylation epigenotypes had not shown macroscopically different features so far, including our previous analysis of conventional protruded adenomas.[14] Our study of LSTs, other types of early lesions of CRC, strikingly showed a strong association of the two different epigenotypes with macroscopically different features. Distribution of oncogene mutations and CTNNB1 activation was mutually exclusive. These might suggest that distinct molecular pathways are implicated in CRC. LST-G cases with intermediate methylation and *KRAS* mutations are suggested to lead to intermediate-methylation CRC, and LST-NG cases with low methylation are suggested to lead to low-methylation CRC, with an association with CTNNB1 activation.

There have been a couple of reports that investigated aberrant methylation in LSTs. Hiraoka *et al.* reported a significant correlation between CIMP-high and LST-G,[34] whereas Hashimoto *et al.* reported no significant correlation between morphological appearance and aberrant methylation.[35] This discrepancy might be explained by the following reasons. First, they determined the methylation status using

Table 2. Factors associated with malignant transformation and submucosal invasion of LSTs

	Malignant transformation				Submucosal invasion			
	Univariate OR (95% CI)	*p*-Value	Multivariate OR (95% CI)	*p*-Value	Univariate OR (95% CI)	*p*-Value	Multivariate OR (95% CI)	*p*-Value
LST-G								
Age > 65 years	1.63 (0.52–5.06)	0.40			0.65 (0.14–2.97)	0.58		
Male sex	0.56 (0.18–1.71)	0.31			1.15 (0.25–5.21)	0.86		
Tumor size > 30 mm	1.08 (0.33–3.56)	0.90			0.72 (0.15–3.48)	0.69		
KRAS mutation	2.70 (0.78–9.35)	0.12			0.23 (0.05–1.13)	0.07		
TP53 mutation	5.50 (1.44–21.0)	0.01*	6.84 (1.59–29.4)	0.01*	1.55 (0.32–7.52)	0.59		
Large nodule	3.90 (1.19–12.8)	0.02*	4.89 (1.30–18.4)	0.02*	6.21 (1.11–34.8)	0.04*	6.21 (1.11–34.8)	0.04*
LST-NG								
Age > 65 years	1.05 (0.34–3.22)	0.93			0.24 (0.08–0.74)		0.26 (0.06–1.08)	0.06
Male sex	0.26 (0.06–1.04)	0.06			0.54 (0.18–1.64)			
Tumor size > 30 mm	0.56 (0.18–1.76)	0.32			0.71 (0.23–2.18)			
KRAS mutation	1.75 (0.48–6.39)	0.40			0.74 (0.23–2.39)			
TP53 mutation	27.2 (3.27–226.1)	0.002*	24.6 (2.51–240.8)	0.006*	2.38 (0.81–7.05)			
CTNNB1 activation	2.03 (0.65–6.36)	0.22			1.46 (0.50–4.24)	0.48		
Central depression	38.3 (4.55–321.4)	8×10^{-4}*	35.0 (3.68–332.9)	0.002*	15.7 (5.55–44.3)	$<10^{-4}$*	20.7 (4.58–94.0)	$<10^{-4}$*

p-Values were calculated using univariate and multivariate logistic regression analyses.
*p-Value < 0.05.

methylation-specific PCR, which could have an advantage in sensitivity to detect methylated DNA molecules, but was not quantitative and therefore not suitable for classification using methylation information.[36] Second, they analyzed several CIMP-related markers (≅Group 1 markers), which might not detect intermediate-methylation epigenotype accurately.[9] Quantitative methylation analysis using Group 1 and Group 2 markers is considered to be necessary, and here we classified LSTs into intermediate- and low-methylation epigenotypes using these two types of markers.

Recently, methylation rates and *BRAF* mutations in CRC were reported to increase gradually along colorectal subsites from the rectum to the ascending colon.[37,38] However, *BRAF* mutations were hardly observed in either LST-G or LST-NG cases, and there was no correlation between methylation rates and tumor locations in this study. High-methylation epigenotype was not detected either. It is suggested that although high-methylation epigenotype/CIMP with a *BRAF* mutation is a feature of the serrated pathway,[36] it is not frequently involved in LSTs.

CTNNB1 is a central player in the WNT pathway and is considered to be involved in the development of intramucosal and invasive cancer. CTNNB1 activation status has been reported to vary widely in conventional adenoma/carcinoma.[39–42] To confirm the reliability of the CTNNB1 activation score used in this study, we performed additional IHC on 38 protruded adenomas, 28 protruded intramucosal CRCs and 60 advanced CRC samples. Consistent with previous reports,[41,42] CTNNB1 activation increased from 24% for adenoma to 46% for protruded intramucosal CRC and 63% for advanced CRC (data not shown). The CTNNB1 activation rate was generally lower in this study compared with previous reports[43] (24, 4 and 51 *vs.* 50%, 37% and 68% for protruded adenomas, LST-G and LST-NG cases, respectively). This discrepancy could be explained by intrinsic tumor heterogeneity or different immunohistochemical staining methods including antibodies and decision criteria. Importantly, CTNNB1 activation in LST-G samples was significantly lower than in protruded adenomas and in LST-NG samples, which was at a similar level in protruded intramucosal CRCs and advanced CRCs. It has been suggested that activation of WNT signaling preferentially occurs in LST-NG formation, which involves low methylation and less frequent *KRAS* mutations, and might, therefore, need other molecular aberrations.

KRAS mutations have been observed in 35–58% of sporadic CRCs[4,6,44,45] and in a similar fraction of LSTs: 46% in whole LST cases including 42% in adenomas and 48% in cancers with no significant increase in the *KRAS* mutation frequency. The methylation rate was not increased from adenoma to cancer either, suggesting that methylation epigenotype and its correlation with *KRAS* mutations may be already completed at the adenoma stage. This is in good agreement with our previous analysis of protruded adenomas and CRCs, showing that no more methylation had accumulated from adenoma to advanced cancer, suggesting that additional molecular events other than methylation and *KRAS* mutation should be necessary for cancer to develop.

The frequency of *TP53* mutations showed a substantial and significant increase from adenoma to cancer in both

intermediate- and low-methylation LSTs or in both LST-G and LST-NG cases. It has been reported that loss of *TP53* function was a late event in colorectal carcinogenesis, and heralded the transition from preinvasive to invasive disease.[4] Abnormalities have been found in 4–26% of adenomas, 50% of adenomas with invasive foci and 50–75% of CRCs.[46] Our multivariate logistic regression analysis of LSTs showed similar results, in that a *TP53* mutation is an independent risk factor for malignant transformation of both LST-G and LST-NG cases, but not submucosal invasion. This suggests that loss of *TP53* function may also be important for LSTs to develop from adenoma to carcinomas in both low- and intermediate-methylation LSTs. Although *PIK3CA* has been reported to be mutated in 10–30% of advanced CRCs, its mutation is rare in protruded adenomas and/or early-stage CRCs.[47] We confirmed that this tendency was also observed in LSTs: no mutation in LST adenomas and only one mutation in LST cancer.

For factors other than *TP53*, a large nodule in LST-G cases and central depression in LST-NG cases were significant risk factors for submucosal invasion (Table 2), and the same factors have also been previously reported.[48] Importantly, although the presence of an advanced pathology in conventional adenomas gradually increased with their size,[49] tumor size was not associated with an advanced pathology in LSTs. LSTs were rather difficult to detect when using optical or virtual colonoscopy because of their flat shape.[50] Early and careful detection and subsequent endoscopic resection of these lesions, especially LSTs with central depression, were suggested to be necessary to reduce CRC mortality. Considering the clinical application of methylation markers for early detection of LSTs, we demonstrated that epigenotype formation was completed by the adenoma stage not only in protruded adenomas[14] but also in LSTs. Therefore, it is suggested that detection of aberrant methylation in plasma cell-free DNA derived from tumor cells may be helpful for early detection of these flat-shaped malignancies. The major purpose of this study was to epigenotype LSTs, and the classifier marker genes used in this study were rarely methylated in LST-NG cases. However, there was another type of methylation marker genes, *e.g., CDO1* and *PENK*, which were not classifiers but were aberrantly methylated in all three CRC epigenotypes.[9] Although further studies are needed, methylation analyses of these commonly methylated genes in tumor tissues and plasma cell-free DNA may lead to the clinical application of these methylation markers for the early detection of LSTs.

In summary, LSTs were classified into two distinct groups: intermediate-methylation LSTs with *KRAS* mutations showing granular morphology and relatively large size, and low-methylation LSTs showing nongranular morphology and relatively small size, associated with CTNNB1 activation. Although methylation accumulation and its correlation to *KRAS* mutation are completed at the adenoma stage, having a *TP53* abnormality may be important in malignant transformation of LSTs, regardless of their methylation epigenotype.

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References

1. Grady WM, Carethers JM. Genomic and epigenetic instability in colorectal cancer pathogenesis. *Gastroenterology* 2008;135:1079–99.
2. Jones PA, Baylin SB. The epigenomics of cancer. *Cell* 2007;128:683–92.
3. Kaneda A, Feinberg AP. Loss of imprinting of IGF2: a common epigenetic modifier of intestinal tumor risk. *Cancer Res* 2005;65: 11236–40.
4. Vogelstein B, Fearon ER, Hamilton SR, et al. Genetic alterations during colorectal-tumor development. *N Engl J Med* 1988;319: 525–32.
5. Seshagiri S, Stawiski EW, Durinck S, et al. Recurrent R-spondin fusions in colon cancer. *Nature* 2012;488:660–4.
6. Cancer Genome Atlas Network. Comprehensive molecular characterization of human colon and rectal cancer. *Nature* 2012;487:330–7.
7. Feinberg AP, Ohlsson R, Henikoff S. The epigenetic progenitor origin of human cancer. *Nat Rev Genet* 2006;7:21–33.
8. Kaneda A, Yagi K. Two groups of DNA methylation markers to classify colorectal cancer into three epigenotypes. *Cancer Sci* 2010;102:18–24.
9. Yagi K, Akagi K, Hayashi H, et al. Three DNA methylation epigenotypes in human colorectal cancer. *Clin Cancer Res* 2010;16:21–33.
10. Ogino S, Kawasaki T, Kirkner GJ, et al. CpG island methylator phenotype-low (CIMP-low) in colorectal cancer: possible associations with male sex and KRAS mutations. *J Mol Diagn* 2006;8: 582–8.
11. Shen L, Toyota M, Kondo Y, et al. Integrated genetic and epigenetic analysis identifies three different subclasses of colon cancer. *Proc Natl Acad Sci USA* 2007;104:18654–9.
12. Hinoue T, Weisenberger DJ, Lange CP, et al. Genome-scale analysis of aberrant DNA methylation in colorectal cancer. *Genome Res* 2012;22: 271–82.
13. Toyota M, Ahuja N, Ohe-Toyota M, et al. CpG island methylator phenotype in colorectal cancer. *Proc Natl Acad Sci USA* 1999;96:8681–6.
14. Yagi K, Takahashi H, Akagi K, et al. Intermediate methylation epigenotype and its correlation to KRAS mutation in conventional colorectal adenoma. *Am J Pathol* 2012;180:616–25.
15. Kambara T, Simms LA, Whitehall VL, et al. BRAF mutation is associated with DNA methylation in serrated polyps and cancers of the colorectum. *Gut* 2004;53:1137–44.
16. Weisenberger DJ, Siegmund KD, Campan M, et al. CpG island methylator phenotype underlies sporadic microsatellite instability and is tightly associated with BRAF mutation in colorectal cancer. *Nat Genet* 2006;38:787–93.
17. Iwase T, Kushima R, Mukaisho K, et al. Overexpression of CD10 and reduced MUC2 expression correlate with the development and progression of colorectal neoplasms. *Pathol Res Pract* 2005; 201:83–91.
18. Muto T, Kamiya J, Sawada T, et al. Small "flat adenoma" of the large bowel with special reference to its clinicopathologic features. *Dis Colon Rectum* 1985;28:847–51.
19. Minamoto T, Sawaguchi K, Ohta T, et al. Superficial-type adenomas and adenocarcinomas of the colon and rectum: a comparative morphological study. *Gastroenterology* 1994;106: 1436–43.
20. Tada S, Iida M, Matsumoto T, et al. Small flat cancer of the rectum: clinicopathologic and endoscopic features. *Gastrointest Endosc* 1995;42:109–13.

21. Watari J, Saitoh Y, Obara T, et al. Natural history of colorectal nonpolypoid adenomas: a prospective colonoscopic study and relation with cell kinetics and K-ras mutations. *Am J Gastroenterol* 2002;97:2109–15.
22. Kudo S, Kashida H, Nakajima T, et al. Endoscopic diagnosis and treatment of early colorectal cancer. *World J Surg* 1997;21:694–701.
23. Rembacken BJ, Fujii T, Cairns A, et al. Flat and depressed colonic neoplasms: a prospective study of 1000 colonoscopies in the UK. *Lancet* 2000; 355:1211–14.
24. Soetikno R, Friedland S, Kaltenbach T, et al. Nonpolypoid (flat and depressed) colorectal neoplasms. *Gastroenterology* 2006;130:566–76.
25. Minamoto T, Sawaguchi K, Mai M, et al. Infrequent K-ras activation in superficial-type (flat) colorectal adenomas and adenocarcinomas. *Cancer Res* 1994;54:2841–4.
26. Morita T, Tomita N, Ohue M, et al. Molecular analysis of diminutive, flat, depressed colorectal lesions: are they precursors of polypoid adenoma or early stage carcinoma? *Gastrointest Endosc* 2002;56:663–71.
27. Saitoh Y, Waxman I, West AB, et al. Prevalence and distinctive biologic features of flat colorectal adenomas in a North American population. *Gastroenterology* 2001;120:1657–65.
28. Yashiro M, Carethers JM, Laghi L, et al. Genetic pathways in the evolution of morphologically distinct colorectal neoplasms. *Cancer Res* 2001;61: 2676–83.
29. Schlemper RJ, Riddell RH, Kato Y, et al. The Vienna classification of gastrointestinal epithelial neoplasia. *Gut* 2000;47:251–5.
30. Kawasaki T, Nosho K, Ohnishi M, et al. Correlation of beta-catenin localization with cyclooxygenase-2 expression and CpG island methylator phenotype (CIMP) in colorectal cancer. *Neoplasia* 2007;9:569–77.
31. Morin PJ, Sparks AB, Korinek V, et al. Activation of beta-catenin-Tcf signaling in colon cancer by mutations in beta-catenin or APC. *Science* 1997; 275:1787–90.
32. Iida S, Kato S, Ishiguro M, et al. PIK3CA mutation and methylation influences the outcome of colorectal cancer. *Oncol Lett* 2012;3: 565–70.
33. Matsusaka K, Kaneda A, Nagae G, et al. Classification of Epstein-Barr virus-positive gastric cancers by definition of DNA methylation epigenotypes. *Cancer Res* 2011;71:7187–97.
34. Hiraoka S, Kato J, Tatsukawa M, et al. Laterally spreading type of colorectal adenoma exhibits a unique methylation phenotype and K-ras mutations. *Gastroenterology* 2006;131:379–89.
35. Hashimoto K, Shimizu Y, Suehiro Y, et al. Hypermethylation status of APC inversely correlates with the presence of submucosal invasion in laterally spreading colorectal tumors. *Mol Carcinog* 2008;47:1–8.
36. Ogino S, Cantor M, Kawasaki T, et al. CpG island methylator phenotype (CIMP) of colorectal cancer is best characterised by quantitative DNA methylation analysis and prospective cohort studies. *Gut* 2006;55:1000–6.
37. Yamauchi M, Morikawa T, Kuchiba A, et al. Assessment of colorectal cancer molecular features along bowel subsites challenges the conception of distinct dichotomy of proximal versus distal colorectum. *Gut* 2012;61:847–54.
38. Rosty C, Young JP, Walsh MD, et al. PIK3CA activating mutation in colorectal carcinoma: associations with molecular features and survival. *PLoS One* 2013;8:e65479.
39. Clevers H. Wnt breakers in colon cancer. *Cancer Cell* 2004;5:5–6.
40. Wong NA, Pignatelli M. Beta-catenin—a linchpin in colorectal carcinogenesis? *Am J Pathol* 2002; 160:389–401.
41. Kobayashi M, Honma T, Matsuda Y, et al. Nuclear translocation of beta-catenin in colorectal cancer. *Br J Cancer* 2000;82:1689–93.
42. Wong SC, Lo ES, Lee KC, et al. Prognostic and diagnostic significance of beta-catenin nuclear immunostaining in colorectal cancer. *Clin Cancer Res* 2004;10:1401–8.
43. Sugimoto T, Ohta M, Ikenoue T, et al. Macroscopic morphologic subtypes of laterally spreading colorectal tumors showing distinct molecular alterations. *Int J Cancer* 2010;127:1562–9.
44. Schubbert S, Shannon K, Bollag G. Hyperactive Ras in developmental disorders and cancer. *Nat Rev Cancer* 2007;7:295–308.
45. Normanno N, Tejpar S, Morgillo F, et al. Implications for KRAS status and EGFR-targeted therapies in metastatic CRC. *Nat Rev Clin Oncol* 2009;6:519–27.
46. Worthley DL, Whitehall VL, Spring KJ, et al. Colorectal carcinogenesis: road maps to cancer. *World J Gastroenterol* 2007;13:3784–91.
47. Whitehall VL, Rickman C, Bond CE, et al. Oncogenic PIK3CA mutations in colorectal cancers and polyps. *Int J Cancer* 2012;131: 813–20.
48. Rotondano G, Bianco MA, Buffoli F, et al. The Cooperative Italian FLIN Study Group: prevalence and clinico-pathological features of colorectal laterally spreading tumors. *Endoscopy* 2011; 43:856–61.
49. Winawer SJ, Zauber AG, Fletcher RH, et al.; US Multi-Society Task Force on Colorectal Cancer; American Cancer Society. Guidelines for colonoscopy surveillance after polypectomy: a consensus update by the US Multi-Society Task Force on Colorectal Cancer and the American Cancer Society. *Gastroenterology* 2006;130:1872–85.
50. Goto H, Oda Y, Murakami Y, et al. Proportion of de novo cancers among colorectal cancers in Japan. *Gastroenterology* 2006;131:40–6.

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ORIGINAL RESEARCH

Aberrant promoter methylation of *PPP1R3C* and *EFHD1* in plasma of colorectal cancer patients

Kiyoko Takane[1,2], Yutaka Midorikawa[1,3], Koichi Yagi[4], Ayako Sakai[3,5], Hiroyuki Aburatani[3], Tadatoshi Takayama[1] & Atsushi Kaneda[2,3,6]

[1]Department of Digestive Surgery and Pathology, Nihon University School of Medicine, Tokyo, Japan
[2]Department of Molecular Oncology, Graduate school of Medicine, Chiba University, Chiba, Japan
[3]Genome Science Division, Research Center for Advanced Science and Technology, The University of Tokyo, Tokyo, Japan
[4]Department of Gastrointestinal Surgery, Graduate School of Medicine, The University of Tokyo, Tokyo, Japan
[5]Sysmex Corporation, Kobe, Japan
[6]CREST, Japan Science and Technology Agency, Saitama, Japan

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Correspondence
Atsushi Kaneda, Department of Molecular Oncology, Graduate school of Medicine, Chiba University, Inohana 1-8-1, Chuo-ku, Chiba 260-8670, Japan.
Tel: +81-43-226-2039; Fax: +81-43-226-2039; E-mail: kaneda@chiba-u.jp
Tadatoshi Takayama, Department of Digestive Surgery and Pathology, Nihon University School of Medicine, Oyaguchikamicho 30-1, Itabashi-ku, Tokyo 173-0032, Japan.
Tel: +81-3-3972-8111; Fax: +81-3-3957-8299; E-mail: takayama.tadatoshi@nihon-u.ac.jp

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Abstract

Aberrant DNA methylation is a common epigenetic alteration involved in colorectal cancer (CRC). In our previous study, we performed methylated DNA immunoprecipitation-on-chip analysis combined with gene re-expression analysis by 5-aza-2′-deoxycytidine treatment, to identify methylation genes in CRC genome widely. Among these genes, 12 genes showed aberrant hypermethylation frequently in >75% of 149 CRC samples but did not in normal samples. In this study, we aim to find out any of these methylation genes to be utilized for CRC detection using plasma DNA samples. Primers for methylation-specific PCR and pyrosequencing were designed for seven of the 12 genes. Among them, *PPP1R3C* and *EFHD1* were rarely hypermethylated in peripheral blood cells, but frequently hypermethylated in 24 CRC tissue samples and their corresponding plasma samples. In plasma samples, *PPP1R3C* was methylated in 81% (97/120) of CRC patients, but only in 19% (18/96) of noncancer patients ($P = 6 \times 10^{-20}$, Fisher's exact test). In combined analysis with *EFHD1*, both genes were methylated in 53% (64/120) of CRC patients, but only in 4% (4/96) of noncancer patients ($P = 2 \times 10^{-16}$), giving high specificity of 96%. At least one of the two genes was methylated in 90% (108/120) of CRC patients, and 36% (35/96) of control patients, giving high sensitivity of 90%. Compared with low sensitivity of carcinoembryonic antigen (17% at stage I, 40% at stage II) and CA19-9 (0% at stage I, 13% at stage II) for early-stage CRCs, sensitivity of aberrant methylation was significantly higher: *PPP1R3C* methylation at 92% (11/12) for stage I and 77% (23/30) for stage II, and methylation of at least one gene at 100% (12/12) for stage I and 87% (26/30) for stage II. *PPP1R3C* methylation or its combined use of *EFHD1* methylation was highly positive in CRC plasma samples, and they might be useful in detection of CRC, especially for early-stage CRCs.

Introduction

For cancer treatment, early detection of disease leads to favorable outcomes for patients, and it is important to develop screening tests with high sensitivity and specificity, especially for early-stage cancer [1]. In colorectal cancer (CRC) screening, stool blood tests and measurement of tumor markers in serum, such as

carcinoembryonic antigen (CEA) and carbohydrate antigen (CA19-9), are conventional methods that have been used. The fecal occult blood test, however, has a low specificity, ranging from 0.3% to 0.5% [2]. CEA and CA19-9 are not frequently positive in CRC at early stages (I and II), and their sensitivities are <50% [3]. These methods are not satisfactory for early cancer detection, and a new, noninvasive technique to detect early-stage malignancies with higher sensitivity than these protein markers would be useful as a first screening test, before the need of invasive examinations, for example, barium enemas and colonoscopies [4, 5].

Cell-free DNA derived from solid tumor cells circulates in the blood stream; therefore, detection of tumor DNA in plasma/serum could be an attractive method for cancer screening [6]. For example, detection of mutated *RAS* gene fragments [7] and microsatellite aberrations [8] in plasma/serum of cancer patients have been demonstrated. But these methods can detect only a fraction of cancer cases with specific genomic aberrations such as *RAS* mutations, and the development of screening methods to detect the majority of cancer cases are urgently needed. Aberrant DNA methylation of promoter CpG islands is a common epigenetic alteration to inactivate tumor suppressor genes in CRC and in other cancers [9, 10]. Detection of genetic mutations is rather difficult to apply to cancer screening because it is necessary to examine many possible mutation sites per gene. When DNA methylation is analyzed, only one promoter region per gene needs to be examined.

In detection of aberrantly methylated DNA in plasma samples, Lofton-Day et al. identified three blood-based molecular biomarkers including *TMEFF2*, *NGFR*, and *SEPT9* that were useful for CRC screening [11]. Thereafter, the concentration of *SEPT9* methylated DNA could be measured with higher sensitivity and specificity and detected in a majority of CRCs at all stages and colorectal locations [12].

A subgroup of CRC shows aberrant CpG island methylation at a significantly higher frequency, which is called CpG island methylator phenotype (CIMP) [13, 14]. We [15] and other groups [16–18] performed comprehensive methylation analysis of CRC samples and reported three distinct DNA methylation epigenotypes of CRC: high-, intermediate-, and low-methylation epigenotypes. In the analysis, we performed methylated DNA immunoprecipitation-on-chip analysis of CRC cell lines combined with microarray analysis of gene re-expressions by 5-aza-2′-deoxycytidine treatment, and established methylation genes to epigenotype CRC [15]. These epigenotyping genes included two major groups of genes: Group-1 genes specifically methylated in high-methylation/CIMP(+) CRCs and Group-2 genes methylated in both high- and intermediate-methylation CRCs. These genes therefore classify CRC into three epigenotypes: high-methylation/CIMP(+) CRCs with methylation of Group-1 and Group-2 genes, intermediate-methylation CRCs with methylation of Group-2 genes, and low-methylation CRCs without methylation of either group of genes. Besides these genes, another type of genes was found to be hypermethylated in all or most CRC cases regardless of epigenotype [15].

In this study, we aim to find out whether any of these commonly hypermethylated genes could be utilized for CRC detection using plasma DNA samples. For candidate genes showing aberrant methylation in >75% of CRC samples but in none of normal samples in the previous analysis, we first checked methylation status of peripheral blood cells. Genes rarely methylated in peripheral blood cells underwent subsequent methylation analysis using plasma DNA samples of CRC and noncancer patients. Methylation was analyzed using methylation-specific PCR [19] in conjunction with pyrosequencing [20], which was used for the validation of the methylation-specific amplification. It was found that *PPP1R3C* methylation alone or in combination with *EFHD1* methylation showed high sensitivity and specificity, and these genes could be used to detect CRC, especially at early stage.

Material and Methods

Clinical samples

Peripheral blood was collected from 96 patients undergoing surgical operations for benign diseases including inguinal hernia, appendicitis, and gallbladder stones (noncancer group), and from 120 patients undergoing surgical operations for CRC (CRC group). Corresponding primary CRC tissue samples were also collected from 24 CRC patients. All samples were collected with written informed consent and the surgery was done in the Department of Digestive Surgery, Graduate School of Medicine, Nihon University. Tissue samples were immediately frozen in liquid nitrogen and stored at −80°C. Frozen materials were microscopically examined for the determination of cancer cell content by pathologists, and it was confirmed that all 24 samples contained at least 40% cancer cells. DNA was extracted using QIAamp DNA Mini Kit (Qiagen, Valencia, CA) according to the manufacture's protocol. Peripheral blood was put in an ethylenediaminetetraacetic acid vacutainer coated tube and centrifuged at 1200*g* at room temperature for 15 min. From 3 mL of the supernatant plasma, cell-free genomic DNA was extracted using QIAamp Circulating Nucleic Acid Kit (Qiagen). The Ethics Committees of Nihon University, Chiba University, and The University of Tokyo certified this study.

Characteristics of the study population

The 120 CRC patients were 67.7 ± 11.4 years old (mean ± standard error), ranging 30–88, and included 71 males and 49 females, whereas the 96 noncancer patients were 63.0 ± 13.6 years old, ranging 24–87 ($P = 1$, t-test vs. CRC patients), and included 67 males and 29 females ($P = 0.1$, Fisher's exact test vs. CRC patients). Twenty (17%) CRC patients underwent neoadjuvant chemotherapy. As for tumor location, 41 (34%) were at proximal colon (10 in cecum, 15 in ascending colon, 16 in transverse colon), 37 (31%) at distal colon (4 in descending colon, 33 in sigmoid colon), and 42 (35%) at rectum. For AJCC (American Joint Committee on Cancer) stages, 12 (10%) were at stage I, 30 (25%) at stage II, 12 (10%) at stage III, and 66 (55%) at stage IV.

Bisulfite treatment of genomic DNA

By bisulfite treatment, unmethylated cytosine is converted to uracil—that is, recognized as thymine (T) after PCR, but methylated cytosine is not converted—that is, recognized as cytosine (C) after PCR. Unmethylated DNA and methylated DNA are therefore distinguishable by detecting the difference of T and C in the sequence after bisulfite treatment. Bisulfite conversion of 500 ng of genomic DNA from each tissue sample was performed using Zymo EZ DNA Methylation Kit (Zymo Research, Irvine, CA), and the DNA was eluted in 30 μL of 10 mEq Tris buffer. For plasma samples, genomic DNA isolated from 3 mL of plasma was treated with bisulfite in the same manner. To check the quality of bisulfite-converted DNA sample as PCR template, 5.3 kb upstream region of *MYOD* (chr11:17,735,751-17,735,847) [21] was amplified by PCR and the PCR product was visualized using ethidium bromide after agarose gel electrophoresis. Primers for *MYOD* were 5′-TGATTAATTT AGATTGGGTT TAGAGAAGGA -3′ (forward) and 5′-CTCCCTCTAT CCCCTAACAA ACTT-3′ (reverse). PCR product length was 97 bp and annealing temperature was 62°C. This region contains no CpG site, and should therefore be amplified regardless of methylation status.

Methylation control samples (0% and 100%) were prepared as previously described [15]. Briefly, human peripheral lymphocyte DNA was amplified using GenomiPhi v2 DNA amplification kit (GE Healthcare Life-Science, Uppsala, Sweden). The amplified DNA was not methylated at all in any CpG sites, and was used as unmethylated (0%) control. The amplified DNA was methylated by *Sss*I methylase and used as fully methylated (100%) control. These control samples were also treated with bisulfite using Zymo EZ DNA Methylation Kit.

Methylation-specific PCR

Methylation status was determined by methylation-specific PCR [19]. To design primers, Pyro Q-CpG software (Qiagen) was used to obtain the genomic DNA sequence after bisulfite conversion, by converting C at non-CpG sites to T and retaining C at CpG sites as C. Forward and reverse primers were designed to contain multiple C's, especially at the 3′ end of primer. When annealing temperature is high enough, the primers would anneal to methylated allele only, and unmethylated allele containing T at CpG sites should not be recognized and amplified.

Methylation genes in CRC were selected from genes identified in our previous study [15], in which bisulfite sequencing primers were designed in the 5′ region of each gene. The PCR products were 200–400 bp, and were analyzed in the methylation assay using MALDI-TOF-MS (matrix-assisted laser desorption ionization–time-of-flight–mass spectrometry) [22]. In this study, primers for methylation-specific PCR were designed within these regions, with PCR products being ≤100 bp, because these analyzed regions were located in 5′ CpG islands of genes and confirmed to be aberrantly methylated in CRC. PCR was performed using 5 μL of bisulfite-modified DNA as a template, and FastTaq polymerase (Roche, Basel, Switzerland). The annealing temperature for the PCR was determined to amplify 100% methylation control sample only, and not to amplify 0% methylation control sample. Among 12 candidate genes, *COL4A2*, *TSPYL5*, *TMEFF2*, *RASSF2*, *SPG20*, *EDIL3*, *CIDEB*, *ADAMTS1*, *EFHD1*, *STOX2*, *PPP1R3C*, and *UCHL1*, such primers could be designed for seven genes, *COL4A2*, *TSPYL5*, *EDIL3*, *ADAMTS1*, *EFHD1*, *STOX2*, and *PPP1R3C*. Primer sequences for these genes and the number of analyzed CpG sites are shown in Table 1.

Pyrosequencing analysis

To confirm that methylation-specific PCR specifically amplified the methylated allele, the methylation status of the PCR product was quantitatively sequenced using pyrosequencing as previously described [23]. Briefly, the biotinylated PCR product was bound to streptavidin Sepharose beads HP (GE Healthcare Life Sciences), washed and denatured using a 0.2 mol/L NaOH solution. After addition of 0.3 μmol/L sequencing primer to the purified, single-stranded PCR product, pyrosequencing was carried on PyroMark Q24 MD System (Qiagen) with Pyro Q-CpG software (Qiagen) according to the manufacturer's instructions. Primer sequences and conditions, and the number of analyzed CpG sites are shown in Table 1. Methylation control samples (0% and 100%) were analyzed in every assay to check that no PCR