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Original article

Genomic alterations in signet ring and mucinous patterned colorectal carcinoma

Hyunchul Kim[a,1], Bo-Hyung Kim[b,1], Donghwan Lee[c], Eun Shin[a,d,*]

[a] Department of Pathology, Hallym University Dongtan Sacred Heart Hospital, Hwaseong, Gyeonggi, South Korea
[b] Department of Clinical Pharmacology and Therapeutics, Kyung Hee University College of Medicine and Hospital, Seoul, South Korea
[c] Department of Statistics, Ewha Womans University, Seoul, South Korea
[d] Department of Pathology, Seoul National University Bundang Hospital, Seongnam, Gyeonggi, South Korea

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ABSTRACT

Background: The genetic alterations (GAs) in two specific histological subtypes of colorectal cancer (CRC), signet ring cell colorectal carcinoma (SRC) and mucinous colorectal carcinoma (MC), are not well known. In the present study, we employed next-generation sequencing to perform genetic profiling of SRC and MC, and compared the spectrum of GAs with the alterations found in conventional type colorectal cancer (CON).

Materials and methods: We selected 46 CRCs comprising 17 SRCs and mucinous carcinoma with signet ring cell component (SRCCs), 17 MCs, and 12 CONs with microsatellite stability or microsatellite instability-low. Deep sequencing was performed using a targeted cancer panel composed of 171 cancer-related genes. SMAD4 protein expression was evaluated by immunohistochemical staining.

Results: We detected 108 mutations in 18 different genes. Overall, 2.34 GAs were detected per tumor (range, 0–14). The overall frequency of GA and alteration in targetable genes was less prevalent in SRC/SRCC compared to the frequency of alteration in MC/CON ($p = 0.040$ and $p < 0.001$, respectively). The GA profile of SRC/SRCC included *TP53* (8/17, 47.1%), *SMAD4* (5/17, 29.4%), *KRAS* (4/17.23.5%), *APC* (4/17.23.5%), *PIK3CA*, *ATM*, *BRAF*, and *PIK3R1* (1/17, 5.9%, each). *KRAS* mutation was significantly less prevalent in SRC/SRCC compared to the number of *KRAS* mutations in MC (12/17, 70.6%) and CON (9/12, 75.0%) ($p = 0.015$ and 0.01, respectively). Compared to the 152 non-hypermutated CONs from TCGA database, *SMAD4* alteration was predominant in SRC/SRCC ($p = 0.045$) with aberrant loss of SMAD4 expression (13/17, 76.5%) compared to the *SMAD4* alterations in CON (5/15, 33.3%) ($p = 0.031$). Accordingly, *KRAS* (12/17, 70.6%), *APC* (6/17, 35.3%), *SMAD4*, *TP53* (4/17, 23.5%, each), *PIK3CA* (3/17, 17.6%), *AKT1*, *ATM*, *BRAF*, *EGFR*, and *EZH2* (1/17, 5.9%, each) were altered in MC. *APC* and *TP53* mutations were less frequent in MC compared to those in TCGA-CON ($p < 0.001$ and 0.003, respectively) whereas *KRAS* mutation was prevalent ($p = 0.041$).

Conclusion: Alterations of known cancer associated genes and targetable genes in SRC/SRCC are infrequent. The profile of GAs in SRC/SRCC and MC differs from the GA profile of CON. Specifically, *SMAD4* mutation and loss of SMAD4 expression is frequently found in SRC/SRCC. The genetic profiles revealed in the present study may aid in developing precision medicine for CRC treatment based on histological subtype.

1. Introduction

Signet ring cell colorectal carcinoma (SRC) is a type of adenocarcinoma with prominent intracellular mucus secretion. The tumor is histologically distinguished by cells with abundant intracytoplasmic mucin, which compels the nuclei aside. The cells may be arranged independently or in loose clusters and may spread diffusely throughout the bowel wall [1]. More than 96% of signet ring cell type cancers have been diagnosed in the stomach; however, signet ring cell carcinoma is a rare subtype of colorectal malignancy reported in 0.1%–2.4% of patients [2–7]. SRC shows aggressive behavior such as peritoneal seeding and has been associated with poor prognosis [2–9]. Thus, signet ring cell carcinoma is a rare, and highly malignant type of colorectal carcinoma (CRC). Additionally, mucinous carcinoma is characterized by abundant extracellular mucin production by tumor cells, which are distinguished to have 50% or more of the mucinous component. MCs

* Corresponding author at: Department of Pathology, Hallym University Dongtan Sacred Heart Hospital, Hwaseong, Gyeonggi, South Korea.
E-mail address: sea4197@gmail.com (E. Shin).
[1] These authors contributed equally to this work.

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account for 8%–10% of colon carcinomas and 33% of rectal cancers [10]. In principle, mucinous carcinoma is reported to show aggressive behavior and has been associated with poor prognosis in patients [11–13], and microsatellite instability (MSI) influences the overall survival of patients with MC. Patients with mucinous type CRC and high level of MSI (MSI-H) are reported to have better prognosis than patients with non-mucinous type CRC and MSI-H; however, patients with mucinous CRC and microsatellite stable (MSS) show worse prognosis compared to patients with non-mucinous CRC and MSS. Signet ring and mucinous patterns are more prevalent in MSI-H-related carcinomas compared to those in MSS-related carcinomas [14–17]. However, other genetic alterations (GAs) in signet ring cell carcinoma or mucinous carcinoma are not yet well known.

GA may determine the phenotype of cancer and may thus be responsible for the biological and clinical heterogeneity corresponding to phenotype. Moreover, new therapies are often designed to target specific GA. In the present study, we aimed to identify the GA profile of signet ring cell carcinoma and mucinous carcinoma, the two distinct histological subtypes of CRC. As the MSI state of CRC profoundly alters the genetic profile and its clinical features, we specifically focused on MSS or low-level microsatellite instability (MSI-L) tumors. We performed next-generation sequencing to study the genetic profile of SRC and MC, and compared the spectrum of GA in SRC and mucinous carcinoma to the genetic profile of a conventional type CRC (CON).

2. Materials and methods

2.1. Samples

We queried the pathology database to identify all cases of CRC that were reported as signet ring cell or mucinous type between 2003 and 2012 at Seoul National University Bundang Hospital. From a total of 220 cases, including 51 SRC and 169 mucinous carcinoma cases, 31 MSI-H CRC and 152 cases with unknown MSI state were excluded and 37 cases were eventually submitted for slide review. All tissue slides of the selected cases were confirmed by a pathologist for their histological subtype. The signet ring cells and mucinous portion of each case were documented in terms of percentage. If the CRC tissue sample had 50% or more of signet ring cells, it was confirmed to be a SRC whereas that with 50% or more of extracellular mucin was considered as mucinous carcinoma; therefore, 12 cases were classified as SRC, and 25 cases as mucinous carcinoma. Signet ring cell component was macro-dissected for DNA extraction from 12 SRCs. Some mucinous carcinomas were found to have significant signet ring cells and we dissected the signet ring cell area from these 5 mucinous carcinomas with signet ring cell component (SRCC), having 20% to 50% of signet ring cells. Further, The 20 remaining mucinous carcinomas were defined as pure mucinous colorectal carcinoma (MC) and the mucinous component was chosen for DNA extraction. Twelve CON with MSS or MSI-L were also selected as controls. Representative histological images are shown in Supplementary Fig. 1. This study was approved by the Institutional Review Board for Research Using Human Subjects at the Seoul National University Bundang Hospital (Institutional Review Board No. B-1606-351-301).

2.2. Assessment of microsatellite instability

Among the 220 CRC cases reported as signet ring cell or mucinous type by pathology, MSI testing was performed on 68 cases for clinical purposes. The MSI state was evaluated by fragmentation assay analysis using an automated DNA sequencer (ABI 3731 Genetic Analyzer; Applied Biosystems, Foster City, CA, USA) by analyzing 2 mononucleotide repeats BAT-25 and BAT-26, and 3 dinucleotide repeats D5S346, D17S250, and D2S123, as recommended by the National Cancer Institute. According to a standardized definition, MSI-H was defined as at least 2 of the 5 markers showing MSI, and MSI-L was defined as 1 of the 5 markers showing MSI. Tumors without MSI were classified as MSS [18].

2.3. Genomic DNA extraction

The blocks with the highest percentage of tumor cells showing specific characteristics such as signet ring cell component for SRC or SRCC and mucinous component for MC were selected on reviewing the hematoxylin and eosin-stained slides and the representative areas were macroscopically circled on the slides. Four slices of 200 μm thickness were sectioned from each of the formalin-fixed paraffin-embedded tissue (FFPE) blocks and mounted on glass slides. The sections were dissected using a scalpel by correlating with the marks on the hematoxylin and eosin-stained slides, and collected in a micro-tube. DNA was extracted using the QIAamp DNA FFPE Tissue Kit (Qiagen, Valencia, CA, USA) according to the manufacturer's instructions. The quality and quantity of FFPE extracted DNA were assessed using agarose gel electrophoresis (1%) and Qubit dsDNA HS Assay Kit with Qubit 2.0 fluorometer (Life Technologies, Carlsbad, CA, USA) following the manufacturer's instructions.

2.4. Deep sequencing using a targeted cancer panel

FFPE DNA was sheared into fragments with a mean peak size of approximately 180 bp to 200 bp using Adaptive Focused Acoustics (Covaris, Woburn, MA, USA). A library was constructed using the SureSelectXT protocol (Agilent Technologies, Santa Clara, CA, USA) with Axen Cancer Panel 2 (Macrogen, Seoul, Korea) comprising 171 cancer-related genes. The complete list of genes that were analyzed is shown in Supplementary Fig. 2. The quality of DNA was evaluated using the 2100 Bioanalyzer (Agilent Technologies, San Francisco, CA, USA). The libraries were then quantified using a Qubit dsDNA HS Assay Kit and Qubit 2.0 fluorometer (Life Technologies, Carlsbad, CA, USA). The libraries were paired-end sequenced (2×150 bp) on a NextSeq 500 system (Illumina, San Diego, CA, USA) with high-throughput using sequencing by synthesis technology to a depth coverage of approximately $2000\times$.

2.5. Variant detection

The sequence reads were trimmed using Cutadapt before mapping on the reference genome (GRCh37/hg19). The trimmed reads were aligned using Burrows-Wheeler transform (BWA)-MEM and poorly mapped reads were removed using SAMtools (v1.3.1). Duplicate reads were identified and recalibration was performed using Picard MarkDuplicates and GATK BaseRecalibrator, respectively. Somatic mutations such as single nucleotide variants and small insertions and deletions were identified using MuTect2. To discard false positive variants, the oxoG artifacts [19], low variant allele frequency ($< 2\%$), and low depth ($< 100\times$) were excluded from the data. These limits of detection were determined based on in-house validation data using dilutions of HD701 and HD753 reference samples of Horizon with NA12878. All variants of two samples were detected at the level of 2% allele frequency and a $100\times$ depth of coverage. Sensitivity and specificity were tested using 75 positive variants and 174 negative variants from 78 positive clinical samples. These were validated at the level of 100% sensitivity and specificity. Based on these results, the Axen cancer panel was certified as a comprehensive cancer panel service (5-1 and 5-2) by Korea Ministry of Food and Drug Safety. In addition, cytosine deamination artifacts were also excluded where C:G > T:A variants were detected on CpG dinucleotides and their frequency was less than 5%. Finally, germline variants were removed if the minor allele frequency was $\geq$ 5% in ExAC_EAS or if the minor allele frequency was $\geq$5% in the Macrogen Korean Population Database. All the remaining variants were annotated using SnpEff and SnpSift (v4.2i) with dbNSFP (v2.9.3), and clinical significance was analyzed using ClinVar database.

Table 1
Clinicopathological features of 49 patients with colorectal carcinoma.

Case	Sex	Age	Location	pT	pN	Stage	SRC component (%)	Mucinous component (%)	MSI state	K-ras mutation
SRC01	F	28	Descending	4b	2b	3c	100	> 50	MSS	Not done
SRC02	M	71	Ascending	3	2b	3c	80	> 50	MSS	Not done
SRC03	M	33	Transverse	4a	2b	3c	100	> 50	MSI-L	Not done
SRC04	F	78	Sigmoid	4a	2b	3c	100	0	MSS	Not done
SRC05	M	20	Transverse	4a	1a	3b	95	> 50	MSS	Negative
SRC06	M	78	Cecum	3	2b	3c	95	20	MSS	Not done
SRC07	M	60	Rectum	3	2b	3c	60	10	MSS	Negative
SRC08	M	54	Descending	4a	2b	3c	90	> 50	MSI-L	Not done
SRC09	M	21	Rectosigmoid	4a	2b	3c	70	> 50	MSS	Negative
SRC10	M	54	Descending	3	2b	3c	80	> 50	MSS	Not done
SRC11	M	45	Rectosigmoid	3	2b	3c	80	> 50	MSI-L	Not done
SRC12	M	72	Rectum	3	2b	3c	100	> 50	MSS	Not done
SRCC01	M	43	Rectosigmoid	3	2a	3b	30	> 50	MSS	Not done
SRCC02	M	24	Transverse	3	2b	3c	30	> 50	MSS	Negative
SRCC03	M	74	Hepatic flexure	4a	2b	3c	20	> 50	MSS	Negative
SRCC04	F	78	Descending	3	1b	3b	20	> 50	MSS	Not done
SRCC05	M	70	Rectum	4b	0	2c	30	> 50	MSS	Not done
MC03	F	39	Sigmoid	3	0	2a	0	> 50	MSS	Not done
MC04	M	76	Sigmoid	4a	0	2b	0	> 50	MSS	Not done
MC05	F	58	Ascending	3	2b	3c	0	> 50	MSS	Not done
MC06	F	45	Rectum	3	1a	3b	0	> 50	MSS	Not done
MC08	M	60	Rectum	4b	0	2c	0	> 50	MSS	Not done
MC09	F	71	Transverse	4a	2b	3c	0	> 50	MSS	Not done
MC10	M	63	Rectum	4a	2a	3c	0	> 50	MSS	Not done
MC11	F	69	Rectum	3	1a	3b	5	> 50	MSS	Not done
MC12	F	53	Descending	3	0	2a	0	> 50	MSS	Not done
MC13	M	56	Sigmoid	4b	1a	3c	3	> 50	MSS	Not done
MC14	M	63	Rectum	3	2b	3c	0	> 50	MSS	Not done
MC15	F	37	Sigmoid	3	1b	3b	0	> 50	MSS	Not done
MC16	F	73	Sigmoid	2	0	1	5	> 50	MSS	Not done
MC17	F	71	Ascending	4b	0	2c	0	> 50	MSS	Not done
MC18	M	84	Rectosigmoid	3	1b	3b	0	> 50	MSS	Not done
MC19	M	49	Rectosigmoid	4a	1a	3b	0	> 50	MSS	Not done
MC20	M	84	Rectum	3	0	2a	0	> 50	MSS	Not done
CON01	F	56	Sigmoid	4b	0	2c	0	0	MSS	Not done
CON02	F	44	Transverse	4b	2a	3c	0	0	MSS	Not done
CON03	M	63	Sigmoid	4a	0	2b	0	0	MSS	Negative
CON04	F	47	Rectum	4b	1b	3c	0	0	MSS	Not done
CON05	M	78	Rectum	4a	2b	3c	0	0	MSS	Not done
CON06	F	77	Sigmoid	3	2b	3c	0	0	MSI-L	Not done
CON07	M	82	Descending	4a	1b	3b	0	0	MSS	Not done
CON08	M	69	Rectum	3	2a	3b	0	0	MSS	Positive
CON09	F	75	Rectosigmoid	3	2b	3c	0	0	MSS	Not done
CON10	M	78	Ascending	3	0	2a	0	0	MSS	Not done
CONl11	M	74	Sigmoid	3	2a	3b	0	0	MSS	Not done
CON12	M	77	Rectosigmoid	3	2b	3c	0	0	MSS	Not done

Abbrevation: F, female; M, male; SRC, signet ring cell; MSI, microsatellite instability; MSS, microsatellite stable; MSI-L, microsatellite, low; MSI-H, microsatellite, high.

Variant calling was performed only in regions targeted by the cancer panel. Fusion genes were identified using an in-house script that discriminates plausible gene fusion events from structural variations as predicted by LUMPY. Copy number variations were identified by an in-house script calculating the depth for each targeted region by dividing the read depth per exon to the estimated normal reads per exon using an in-house reference. The biases of each normalized values were reduced by singular value decomposition.

2.6. Immunohistochemistry

Immunohistochemical staining was performed using a BenchMark XT automated immunostaining system (Ventana Medical System, Tucson, AZ, USA) for SMAD4 staining. After reviewing the slides, suitable paraffin tissue blocks were selected. For the SRC/SRCC group, blocks with the signet ring cell component were chosen whereas for the MC group, blocks with a mucinous component were chosen. Four micrometer thick sections were cut from each of the paraffin tissue blocks, and were mounted on positively charged slides. Subsequent to epitope retrieval at 100 °C for 24 min in cell conditioning buffer (pH 8.4) in the autostainer, endogenous peroxidase activity was blocked by immersing the slides in a 3% hydrogen peroxidase solution for 4 min at 37 °C. The samples were incubated with SMAD4 primary antibody (Catalog No. ab40759; clone Ep618Y; rabbit monoclonal; Abcam; 1:600 dilution) at 37 °C, for 16 min and then incubated with a mixture of horseradish peroxidase-labeled antibodies composed of goat anti-rabbit antibody and goat anti-mouse antibody included in the UltraView Universal DAB kit (cat. no. 760-500; Ventana Medical Systems, Inc.) for 8 min at 37 °C. Following treatment with 0.04% hydrogen peroxide in a phosphate buffer solution and DAB chromogen containing 0.2% 3,3′-diaminobenzidine tetrahydrochloride at 37 °C for 8 min, samples were treated with copper sulfate (5 μg/l) at 36 °C for 4 min (all agents used in these processes were included in the UltraView Universal DAB kit; cat. no. 760-500; Ventana Medical Systems, Inc.). Slides were counterstained with 0.5% modified Mayer's hematoxylin at 37 °C for 8 min and then post-counterstained with Bluing reagent at 37 °C for 4 min. Normal mucosa, stromal cells, and lymphocytes were used as internal controls. SMAD4 was stained in the nucleus and cytoplasm, simultaneously. As per the previously described scoring system [20,21], when more than 5% of tumor cells were positively stained in the nucleus, the case was

considered positive.

2.7. Statistical analysis

We compared the frequency of GA between the histological subtypes and with The Cancer Genome Atlas (TCGA)-CRC database. The GAs between the groups were compared using Man-Whitney U test, Kruskal-Wallis test or Fisher's exact test using R (http://www.r-project.org/) tool and the SPSS (v21.0) software package (SPSS, Chicago, IL. USA). $p < 0.05$ was considered statistically significant.

3. Results

3.1. Patient characteristics

Among 49 cases including 12 SRCs, 5 SRCCs, 20 MCs, and 12 CON cases, 3 MC cases failed library construction; therefore, 46 cases were finally submitted for deep sequencing. From the 12 SRCs, 9 cases showed more than 50% of mucinous component and 3 cases showed MSI-L. Six cases of SRC were located on the left side, 4 cases on the right side of the colon, and 2 cases were on the rectum. The patients' median age was 54 years (range, 20–78 years). The signet ring cell component of SRCC was observed to be 20%–30%. All SRCC cases showed MSS. Two SRCCs were located on the left side, 2 on the right side of the colon, and 1 was on the rectum. All MCs showed MSS. From the total number of MC cases, 3 MCs had a minor signet ring cell carcinoma component (5% or less). Eight cases were located on the left side, 3 cases on the right side, and 6 cases were on the rectum. The median age of patients with MC was 65.5 years (range, 37–84 years). From the 12 CONs, 2 cases were located on the right side, 3 cases were on the rectum, and 7 cases were on the left side. The median age of CON cases was 74.5 years (range 44–82 years). All CONs were MSS except for 1 case, which was diagnosed as MSI-L. The detailed clinicopathological features of 49 patients included in the study cohort are illustrated in Table 1.

3.2. Genomic alterations in colorectal carcinoma with signet ring cell and/ mucinous component

Massive parallel sequencing revealed 46 CRCs with 108 mutations in 18 different genes. Overall, 2.34 GAs per tumor were detected (range, 0–14). The mean ratio of GAs in SRC, SRCC, and MC showed no statistical difference (SRC *vs*. SRCC; $p = 0.383$, SRC *vs*. MC; $p = 0.067$, SRCC *vs*. MC; $p = 0.544$ by Man-Whitney U test). However, the mean ratio of GAs in SRCC/SRC was significantly lower than the mean ratio of GAs in CON/MC (mean GA: SRCC/SRC = 1.63, CON/MC = 2.79; $p = 0.040$) (Supplementary Fig. 3A). On excluding MC13, an outlier with remarkably high frequency of GA (N = 14), the mean frequency of GA was 2.09 and a decreasing order was observed with CON (mean frequency = 2.6), MC (mean frequency = 2.25), and SRCC/SRC (mean frequency = 1.59) ($p = 0.107$).

The most common alteration in SRC/SRCC was point mutation in Tumor protein 53 gene (*TP53*) (6 missense mutation and 2 nonsense mutation, 8/17, 47.1%) and the second common alteration was missense mutation in SMAD family member 4 (*SMAD4*) (5/17, 29.4%). These two genes showed higher frequency of alterations in SRC/SRCC compared to their GA frequency in MC and CON. Mutation in the KRAS proto-oncogene, GTPase (*KRAS*) (all missense mutation), and Adenomatous polyposis coli (*APC*) (all nonsense mutation), each was found in 4 cases of SRC/SRCC (for each gene: 4/17, 23.5%). Missense mutation of phosphatidylinositol-4,5-bisphosphate 3-kinase catalytic subunit alpha (*PIK3CA*), ATM serine/threonine kinase (*ATM*), B-Raf proto-oncogene, serine/threonine kinase (*BRAF*), and PIK3 regulatory subunit 1 (*PIK3R1*) was found in 1 SRC/SRCC case each (for each gene: 1/17, 5.9%). *KRAS* mutation in SRC/SRCC (4/17, 23.5%) was less prevalent compared to its mutation in MC (12/17, 70.6%) and CON (9/12, 75.0%). The differences between histological groups were found to be statistically significant ($p = 0.015$ and 0.01, respectively).

Missense mutation in the *KRAS* gene was revealed to be the most common GA in MCs (12/17, 70.6%). We detected *APC* mutation in 6 MC cases (6/17, 35.3%). Missense mutation in *SMAD4* and *TP53* was observed in 4 MC cases (for each gene: 4/17, 23.5%), whereas missense mutation of *PIK3CA* was found in 3 MC cases (3/17, 17.6%). Missense mutation of *AKT1*, *ATM*, *BRAF, EGFR,* and *EZH2* was found in 1 case each (for each gene: 1/17, 5.9%) and nonsense mutation of *MSH2*, *MSH6*, and *NF1* was detected in 1 case each (for each gene: 1/17, 5.9%). *BRCA2, PTEN*, and *PTK3R1* each were found to be mutated in 1 case (for each gene: 1/17, 5.9%) and were all revealed to be frameshift mutations.

Like MC, mutations in *KRAS* (9/12, 75.0%) and *APC* (7/12, 58.3%) were the most frequent and the second common mutation observed in CON. Five CON cases showed alteration in *TP53* (5/12, 41.7%) including 2 nonsense mutations, 1 splice site mutation, and 2 missense mutations. Missense mutation in *PIK3A* and F-Box And WD repeat domain containing 7 (*FBXW7*) was independently observed in 3 CON cases (for each gene: 3/12, 25%). *FBXW7* was only found to be mutated in CON cases whereas none of the SRC/SRCC or MC showed *FBXW7* mutation, thereby showing marginal intergroup differences between the groups (SRC/SRCC *vs*. CON, $p = 0.06$; MC *vs*. CON, $p = 0.067$). *SMAD4* and cyclin dependent kinase inhibitor 2A (*CDKN2A*) were observed to be individually mutated in 1 CON case (for each gene: missense mutation, 1/12, 8.3%).

When comparing GAs between SRC, SRCC, and MC, *KRAS* mutation was less in SRC (3/12, 25%) and in SRCC (1/5, 20%) than in MC (12/17, 70.6%). The difference was statistically significant between SRC and MC ($p = 0.025$), whereas a statistical difference was not found between SRCC and MC ($p = 0.116$). The frequencies of other genes showed no statistical difference between SRC, SRCC, and MC. We also subclassified SRC/SRCC based on mucin contents. Three cases were classified as low mucin content (0–20%) and 14 cases as high mucin content (> 50%); however, we found no statistical difference in GA according to the mucin contents (data not shown).

An oncoprint showing genes mutated in each group is illustrated in Fig. 1 and the comparison of their GA frequency is shown in Fig. 2. Detailed lists of the observed GAs in each group are illustrated in Supplementary Tables 1–3.

3.3. Potentially targetable alterations in colorectal carcinoma with signet ring cell and/ mucinous component

The Axen cancer panel T2 included 21 genes (Supplementary Fig. 2B) that are potentially targetable. Thus, their altered product can be targeted either using agents that are already approved by the US Food and Drug Administration for other indications or are currently undergoing clinical trials. Potentially targetable GAs were identified in 30 cases (30/47, 63.8%) of the total samples. *KRAS* was evaluated as the most prevalently altered gene among the other potentially targetable genes investigated in this study. Point mutation in *PIK3CA, EGFR,* and *BRAF*, and deletion in phosphatase and tensin homolog (*PTEN*) were also observed in the study. The mean frequencies of alterations in targetable genes of CON and MC were 1 and 1.1 per case, and 0.35 per SRC/SRCC case, respectively. The statistical difference between groups was found to be significant by Kruskal-Wallis test ($p = 0.006$). The difference in the ratio of CON/MC (mean = 1) and SRCC/SRC (mean = 0.35), was found to be statistically significant ($p < 0.001$) (Supplementary Fig. 3B). An oncoprint showing genes mutated in each group is shown in Supplementary Fig. 4.

3.4. Comparison of genetic alterations with TCGA colorectal cancer database

We compared the GAs in SRC/SRCC and MC with the GA profile

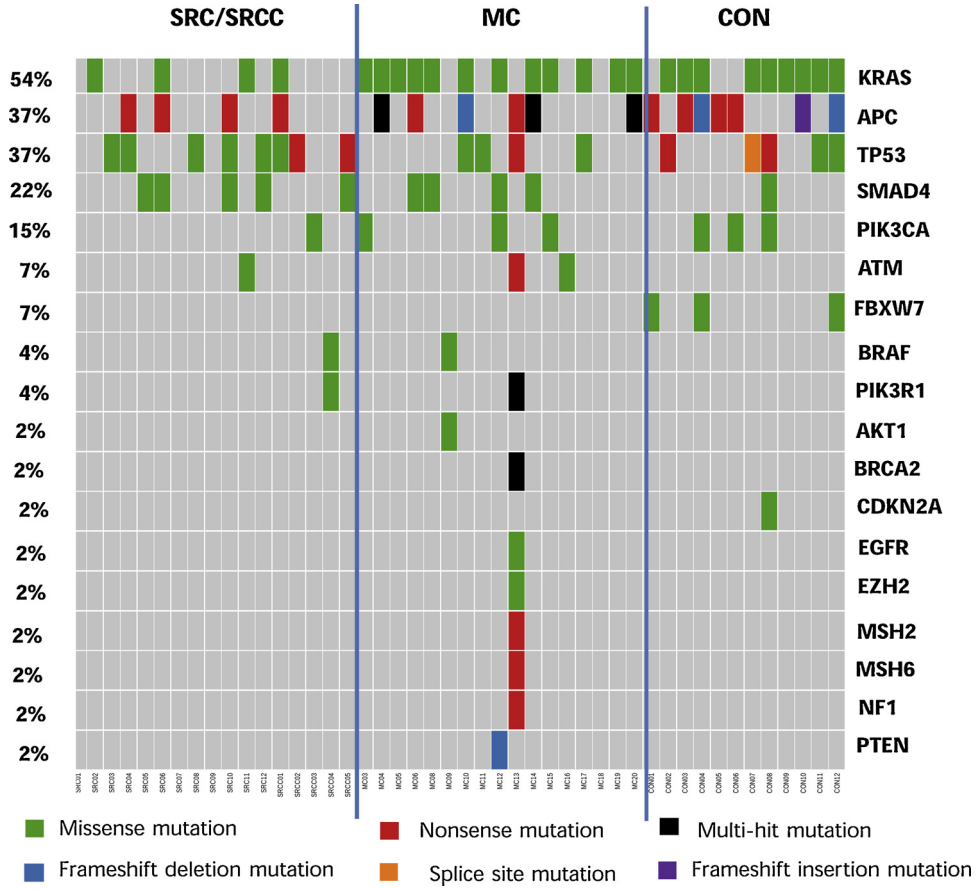

Fig. 1. Oncoprint showing mutated genes. Each column denotes an individual tumor and each row represents a gene. Colors indicate the type of genetic alteration as indicated in the legend below the oncoprint.

reported in TCGA-CRC. As our CRC cohort consisted of MSS or MSI-L cases, we sorted out the non-hypermutated cases with the conventional histological subtypes from the available CRC cases in the TCGA dataset. Consequently, 152 non-hypermutated TCGA-CRC cases with conventional type colorectal carcinoma (TCGA-CON) were used for comparative analysis. Alteration in *SMAD4* was found to be more prevalent in SRC/SRCC (5/17,29.4%) compared to the *SMAD4* alterations in TCGA-CON (18/152, 11.8%) ($p = 0.045$). *APC* mutation was found to be less common in SRC/SRCC (4/17 23.5%) compared to that in TCGA-CON (118/152, 77.6%) ($p < 0.001$) (Fig. 3A). The frequency of *APC* mutation was less in MC (6/17, 35.3%) compared to its mutation frequency in TCGA-CON (118/152, 77.6%) ($p = 0.001$) and overall, *TP53* mutation was found to be less common (MC; 4/17, 23.5% *vs.* TCGA-CON; 96/152, 63.2%) ($p = 0.003$). However, *KRAS* mutation was highly prevalent in MC (12/17, 70.6%) compared to the corresponding mutations in TCGA-CON (66/152, 43.4%) ($p = 0.041$) (Fig. 3B).

3.5. Immunohistochemical expression of SMAD4 in colorectal carcinoma based on histological subtype

Mutation in *SMAD4* was the second most common alteration in

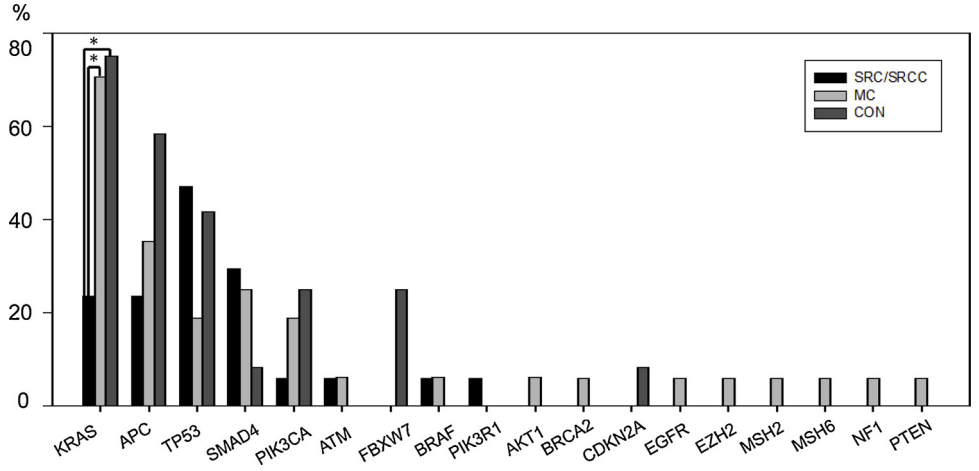

Fig. 2. Comparative analysis of the frequencies of genetic alterations based on the histological subtype of colorectal cancer (*$p < 0.05$).

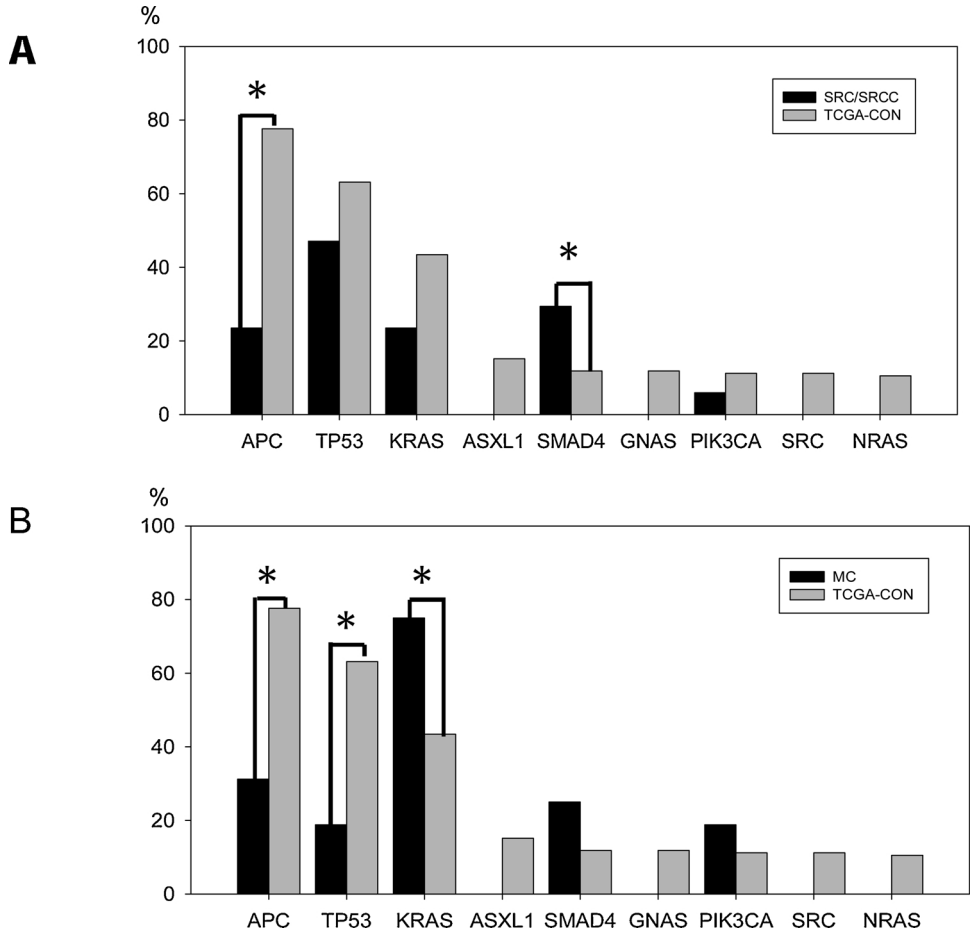

Fig. 3. (A) Comparative analysis of the frequencies of genetic alterations in SRC/SRCC and TCGA-CON. (A) Comparative analysis of the frequencies of genetic alterations in MC and TCGA-CON (*$p < 0.05$).

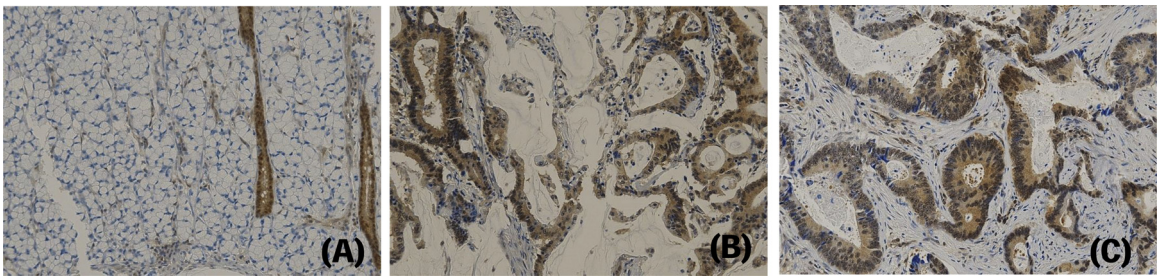

Fig. 4. Representative immunohistochemical images of SMAD4 staining. (A) Negative staining in SRC, (B) Positive staining in MC, and (C) Positive staining in CON (Original magnification ×200).

SRC/SRCC and was found to be frequent in SRC/SRCC compared to TCGA-CON. Thus, we investigated whether the protein expression level of SMAD4 in SRC/SRCC also differed from its protein expression in CRC of other histological subtypes. We performed immunohistochemical staining for SMAD4 in 50 CRC cases including 17 SRC/SRCCs, 18 MCs, and 15 CONs. Representative immunohistochemical images for SMAD4 staining are shown in Fig. 4. SMAD4 expression was found to be 23.5% (4/17), 55.6% (8/18), and 66.7% (10/15) in SRC/SRCC, MC, and CON, respectively. A statistically significant difference was found between the expression levels of SMAD4 in SRC/SRCC and CON ($p = 0.031$) (Supplementary Fig. 5). SMAD4 expression and *SMAD4* gene mutation showed no statistical association ($p = 0.172$). Relationships between histologic subtypes and clinicopathologic parameters are presented in Supplementary Table 4.

4. Discussion

With the development of high-throughput screening and systematic analysis, varied efforts for the stratification of CRC based on genetic and molecular characteristics have been accomplished. Tumor mutation burden/MSI state is an important and primary criterion in such classifications. The MSI-H group has been known to be associated with specific histological subtypes such as signet ring cell or mucinous histology [8,14,22,23]. Approximately one-third of CRCs with signet ring cell type have MSI-H [8,22], whereas the overall frequency of MSI-H observed in CRC is known to be only 15%. Other molecular alterations related to the MSI state including *BRAF* mutation and MutL homolog 1 (*MLH1*) loss of function have also been reported to have signet ring cell or mucinous histology [23]. For example, Yalcin et al. recently reported that BRAF mutation must be closely associated with the presence of

signet ring cells [24]. However, GAs of the remaining two-thirds of signet ring cell type CRC that do not belong to the MSI-H group are largely unknown; the same is true for the mucinous type CRC. Recently, although consensus molecular subtypes classify CRC with MSS or non-hypermutated state [25], GA based on histological subtype is not included in this classification. In the present study, we aimed to define GA events in specific histological subtypes, *i.e.*, signet ring cell or mucinous type CRC with MSS or MSI-L, and to compare their spectrum of GAs with the alterations detected in CON cases from our hospital. We also analyzed them using the TCGA data.

Using a deep targeted sequencing method and cancer gene panel, we found that the overall frequency of alteration in known cancer-related genes of the SRC/SRCC group was significantly less compared to the alterations of the same genes in the MC or CON group. Recently, the Asian Cancer Research Group has described 4 molecular subtypes of gastric cancer using gene expression data [26]. Among these, MSS/epithelial to mesenchymal transition group is included as a gastric cancer of diffuse type with the worst prognosis. Besides distinct clinical features such as tendency for early recurrence and highest recurrence frequency, the MSS/epithelial to mesenchymal transition subtype showed the least number of somatic mutation events compared to other groups, which is similar to the data of the SRC/SRCC group in our study.

Thus, the common known somatic mutations involved in carcinogenesis are likely to be less frequent in signet ring cell type cancer of the lower and upper gastrointestinal tract with MSS or MSI-L state, and it can be assumed that cancers of this unique histological subtype in an anatomically different location might have common genetic characteristics. We found that alteration in targetable genes was less prevalent in SRC/SRCC. This might lead to difficulties in treating such an aggressive cancer with known target agents.

From the investigated GAs, the frequency of *KRAS* mutation in SRC/SRCC was significantly less compared to its mutation in MC or CON and similarly, *APC* mutation, another key GA in CRC, was also less prevalent in SRC/SRCC compared to its mutation frequency in TCGA-CON. Some previous studies have also reported that *KRAS* mutation in SRC is less common compared to its mutation frequency in CON [5,22,27]. Recently Nam et al. performed whole exome sequencing of 5 colorectal SRCs with MSS. They reported that most mutations were commonly found in SRC and TCGA colorectal adenocarcinoma, whereas APC nonsense mutation was observed in only one SRC case similar to the present study [28]. These data imply that carcinogenesis of SRC/SRCC might be different from that of CON.

SMAD4 codes for the common intracellular mediator of the TGF-β superfamily. The TGF-β pathway is involved in the regulation of cell proliferation, differentiation, apoptosis, and migration [1 20 29 40]. Dysregulation of TGF-β signaling promotes epithelial to mesenchymal transition, which is considered a key player in promoting tumor invasion [29–31]. *In vitro* experiments have proved that TGF-β signaling regulates epithelial differentiation and inhibits intestinal epithelial proliferation in normal colonic mucosa [32,33]. Many reports have shown that mutation or silencing of *SMAD4* is involved in gastrointestinal tumorigenesis [34,35]. In the present study, while the overall frequency of GA in SRC/SRCC was found to be less, alterations in *P53* and *SMAD4* were the two most common GAs and somatic mutation of *SMAD4* in SRC/SRCC was more prevalent compared to its mutation frequency in CON. We also observed frequent loss of SMAD4 protein expression in SRC/SRCC upon immunohistochemical staining. Frequencies of immunohistochemical loss of SMAD4 have been reported as 13%–63% in colorectal cancer in previous literature [36] and according to TCGA data, the low mRNA rate for colorectal adenocarcinoma and for mucinous colorectal carcinoma is 15.38% and 18.18%, respectively. In the present study, the SMAD4 loss rate was 76.5%, which was higher compared to the previously reported expression loss or low mRNA rate of SMAD4. In CRC, *SMAD4* mutation has been linked to aggressive tumor behavior [36] and mucinous histology [37], and reduced SMAD4 expression has also been reported to be associated with poor prognosis of CRC [21,36,38]. Results from previous studies and a relatively high frequency of *SMAD4* mutation and SMAD4 expression loss in the SRC/SRCC group of the present study suggests that *SMAD4* mutation or expression loss of SMAD4 might be associated with aggressive tumor biology and may be the basis for this histological subtype of CRC. Although there has been no targeted therapy against loss of *SMAD4*, a few studies have shown the link between *SMAD4* loss and response to specific chemotherapeutic drugs such as topoisomerase inhibitors [39]. Further evaluation is required for the potential use of *SMAD4* loss or genetic mutation in determining the treatment options for SRC/SRCC.

SMAD4 mutation and SMAD4 expression loss did not show a statistical relationship in the present study. Although association between somatic mutations and their residing genes is not fully understood, mutation type is assumed to have the strong influence on transcriptional level. For example, expression of tumor suppressor genes is especially influenced by truncation mutation. Alazzouzi et al. showed that missense mutations in *SMAD4*, a tumor suppressor gene did not result in loss of SMAD4 protein levels, and a truncating mutation resulted in only moderately low protein levels [38]. The SMAD4 mutations observed in our study were all missense mutations, which might result in discordance between the somatic mutation of *SMAD4* and SMAD4 protein loss.

Song et al. reported that *APC* inactivation was observed less frequently in mucinous colorectal carcinoma compared to its frequency in non-mucinous carcinoma irrespective of the MSI status [40]. Ogino et al. showed that *TP53* inactivation was less prevalent in the mucinous group compared to its inactivation frequency in the non-mucinous group [23]. On the contrary, some have also reported that *TP53* inactivation in mucinous colorectal carcinoma is influenced by tumor location or MSI state [40,41]. In the present study, GA in *APC* and *TP53* were less prevalent in MC group compared to their GA frequency in CON. On comparing with the TCGA-CON, a statistically significant difference was observed between the two groups. These results imply that the alteration state of two major genes, *APC* and *TP53* in MC is different from the alteration pattern in non-mucinous carcinoma.

As the leading cause of hypermutation in CRC is dysfunction in the mismatch repair system, patients with CRC showing mismatch repair deficiency are expected to develop a hypermutated phenotype. Consequently, hypermutated CRC is considered relevant to MSI-H CRC and *vice versa* [25]. To date, the threshold for hypermutated tumors in CRC is defined as 12 per Mb of the mutation rate using whole-exome sequencing [42]. One MC case of the present study was an outlier with a high frequency of GA with 14 somatic mutations. Only 473,610 bp of the exons were used for targeted deep sequencing in our study, and so, only a limited number of targeted exon sequencing was performed rather than whole exome sequencing. Thus, it was difficult to confirm if this case is a hypermutated tumor. Repeated MSI testing for this case was performed in another laboratory using a fragmentation assay analysis with an ABI Prism 3130 Genetic analyzer (Applied Biosystems, Foster City, CA, USA), revealing an MSS state. Although nonsense mutations of *hMSH2* and *hMSH6* were observed by the NGS method, mismatch repair proteins including hMLH1, hMSH2, hMSH6, and PMS2 were not altered as determined by immunohistochemistry staining. Alteration of *POLE* was also not observed.

The major limitation of the present study is the limited number of cases in each group, suggesting the need for validation with a larger cohort to confirm the genetic characteristics of SRC/SRCC and MC observed by our targeted deep sequencing analyses. We tried to compare the GAs between SRC, SRCC, and MC and to analyze the genetic profiles of CRC with signet ring cell component according to mucin content; however, there was barely any statistically significant intergroup difference. These results might probably arise from the small sample size in each group. A larger sample size will facilitate a more exact understanding of the genetic characteristics and pathogenesis of CRC according to the content of signet ring cells or mucin.

5. Conclusion

Known cancer-associated genes and targetable genes are infrequently altered in SRC/SRCC. The profiles of GA in SRC/SRCC and MC were different from the GA profile of CON, obtained from the TCGA database (TCGA-CON). Specifically, mutation of *SMAD4* and loss of SMAD4 expression were prevalent in SRC/SRCC.

Ethics approval and consent to participate

This study has been performed in accordance with the declaration of Helsinki and the research protocol has been approved by the Seoul National University Bundang Hospital Ethic committee (IRB File No. B-1606-351-301). For this type of study, formal consent is not required. This article does not contain any studies with human participants or animals performed by any of the authors.

Declaration of Competing Interest

The authors declare that they have no conflict of interest.

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Appendix A. Supplementary data

Supplementary material related to this article can be found, in the online version, at doi:https://doi.org/10.1016/j.prp.2019.152566.

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