

Investigation of clinicopathological characters and gene expression features in colorectal signet-ring cell carcinoma utilizing CMS classification

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Abstract. Signet ring cell carcinoma (SRCC) is a rare pathological type of colorectal cancer, of which the clinicopathological features and genetic background have not yet been fully investigated. Previous research has focused on the optimization of colorectal cancer treatment utilizing consensus molecular subtyping (CMS). However, it is not known what type of CMS would be designated to SRCC treatment. In the current study, of 1,350 patients diagnosed with colorectal cancer who underwent surgery, 14 were diagnosed with SRCC. The case-control cohort that fit the clinical background of the SRCC case was constructed. Statistical comparison between the SRCC group and the case-control cohort was performed among clinicopathological variables. SRCC and well to moderately adenocarcinoma case mRNA were submitted to microarray analysis and CMS analysis. Compared with the case-control cohort, the SRCC group was located more in the right-sided colon, the lymphatic invasion was more severe and the peritoneal dissemination was more frequent. The cancer-specific survival and the progression-free survival were significantly worse in the SRCC group compared with the case-control cohort. Microarray and CMS analysis identified that one SRCC case was significantly well assigned in the CMS 4 group and the other case was assigned in the CMS 1 group. Gene set analysis revealed the upregulation of EMT

related genes and the downregulation of fatty acid, glycolysis, differentiation, MYC, HNF4A, DNA repair genes. In conclusion, the clinical characteristics of SRCC are severe but there is a possibility of the presence of different phenotypes according to CMS analysis.

Introduction

Signet ring cell carcinoma (SRCC) is a rare histological type in colorectal cancer (CRC) and it dominates only 1% of them (1). The prognosis of SRCC patients is extremely poor and it is difficult to improve it in spite of the multimodal treatment including surgery (2). Thus, to fight against this type of deadly disease, several analyses were attempted to find early clinicopathological signs and genetic features that specifically reflect the nature of SRCC in the colorectal cancer field (1-6).

The commonly speculated clinical factors of colorectal SRCC are relatively younger patients, female-dominated, advanced disease stage, right-sided, and treatment-resistant (7-14). Additionally, CpG island methylator phenotype-high (CIMP-high), microsatellite instability-high (MSI-high), and BRAF mutation were commonly reported as genetic features of SRCC (15-18). According to these analyses, the specific character of colorectal SRCC was gradually being unveiled. However, there still be a long way to go to establish a more efficient treatment strategy for SRCC because it is not fully characterized yet.

Consensus Molecular Subtype (CMS) analysis was proposed by Guinney *et al* (19). They tried to characterize colorectal cancer into 4 subtypes by utilizing gene expression profile data set being aggregated from different gene expression analysis platforms. Several clinical trials were performed and the benefit of CMS analysis was reported (20-22). The morbidity of SRCC in colorectal cancer is so rare that it is not clearly known about where this histological type of cancer is designated in CMS analysis.

The purpose of this study is to re-confirm the clinicopathological specificity of SRCC in colorectal cancer and try to elucidate the SRCC's gene expression profile and the type

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Abbreviations: MYC, myelocytomatosis; SRCC, signet ring cell carcinoma

Key words: colorectal cancer, signet ring cell carcinoma, consensus molecular subtyping analysis

of classification in CMS analysis. Of course, there would be some limitations while utilizing the CMS analysis because the classification is not widely clinical used yet and still needs to be fine-tuned because 20% of the case does not fall within the four subtypes, however through this study, some clues of biological insight can be dug out about this extremely rare histological type of colon cancer.

Materials and methods

Study patients and diagnosis. A total of 1,350 patients who had been diagnosed with colorectal cancer and had undergone complete resection of the tumor from 1997 to 2011 in our department were enrolled in this study. Of these patients, 14 were pathologically diagnosed with SRCC, and the remaining 1,336 were diagnosed with well to moderately differentiated adenocarcinoma (well-mode DAC) (Fig. 1). Postoperative pathological staging was determined according to the seventh edition of the UICC TNM classification of malignant tumors. None of the enrolled patients underwent any chemotherapeutic treatment prior to surgery.

RNA extraction. Total RNA was extracted from frozen tumor sections (two SRCC, two differentiated AC, and five normal tissue samples) using the RNeasy Mini kit (Qiagen) according to the manufacturer's instructions, and the samples were stored at -80°C .

RNA amplification and labeling. cDNA was amplified from 20 ng total RNA using the Ovation[®] Pico WTA System V2 (NuGEN Technologies) according to the manufacturer's instructions. The amplified cDNA yield was checked using the NanoDrop 2000 spectrophotometer. Cyanine-3-labeled cDNA was prepared from 2.0 μg cDNA using the SureTag Complete DNA Labeling Kit (Agilent Technologies) according to the manufacturer's instructions, and then purified and concentrated using Amicon Ultra-0.5 ml Centrifugal Filters (Merck Millipore). Dye incorporation and cDNA yield were checked using the NanoDrop 2000 spectrophotometer.

cDNA microarray analysis. The cyanine-3-labeled cDNA was mixed with 1x blocking agent and 1X hybridization buffer (50 μl total volume), and the solution was hybridized to SurePrint G3 Human GE 8x60K Microarray v2.0 slides for 17 h at 65°C in a rotating hybridization oven. After hybridization, the microarrays were washed with GE Wash Buffer 1 for 1 min at room temperature followed by GE Wash Buffer 2 for 1 min at 37°C and then dried immediately by brief centrifugation. The microarrays were scanned using the High-Resolution Microarray Scanner (Agilent Technologies) to determine fluorescence intensity.

CMS classification. To perform consensus molecular subtype (CMS) classification, statistical computational software R (ver3.6.1; R Core Team (2019). R: A language and environment for statistical computing. R Foundation for Statistical Computing, Vienna, Austria. URL <https://www.R-project.org/>) was utilized. The data for the CMS analysis was prepared according to the report of Guinney *et al* (19) and the actual CMS classification was done following the instruction of R-package CMScaller

(ver0.99.1) (23). Prior to the analysis, acquired cDNA microarray raw data were normalized and standardized. According to the subtypes of CMS1, CMS2, CMS3, and CMS4 classification, two of each subtype data (total 8 data) were chosen from the crc TCGA subset (colorectal TCGA gene expression data with subtype annotation) included in the CMScaller and utilized as a reference data. SurePrint G3 microarray chip used in this SRCC study mounted more than 60,000 genes thus 5,000 gene expression data that is matched with the genes utilized in CMScaller were extracted. In the SurePrint G3 chip the same genes were analyzed multiply so that those genes data were applied to the CMS analysis after calculating normalization values according to the average of each expression data by global scaling. After the data adjustment, the emat data sheet was developed and CMS caller analysis was performed.

Statistical analysis. Analyses of the clinicopathological variables and histological status of the patients were performed using JMP Pro 14.0.0 statistical software (SAS Institute, Inc.). Clinicopathological factor correlations were compared between the SRCC and case-control data set using Student's t-test and the chi-square test. Progression-free survival (PFS) and cancer-specific survival (CSS) rates were calculated using the Kaplan-Meier method and compared using the log-rank test and Wilcoxon test. Correlations between clinicopathological factors and cancer-related death were estimated using the Cox proportional hazards model. A P-value <0.05 was considered to indicate significance.

Results

Clinicopathological characteristics of patients and outline of treatment with SRCC patients. Patients who had been treated for colorectal cancer from 1997 to 2011 at the Department of Surgery of Kurume University Hospital were enrolled in this study. Of 1,350 total patients, 14 were pathologically diagnosed with signet ring cell carcinoma (SRCC). The background and clinicopathological summary variables of SRCC the enrolled patients are summarized in Table I. There were 12 of 14 cases were male patients and the percentage of female patients was 14.2% in SRCC. The median value of SRCC patients' age was 62.57 ± 10.68 . There were only two cases of T1-T2 in tumor depth (14.2%) on the contrary remaining 14 cases were all T4a or T4b. Nine cases showed N2 or more lymph node metastasis and 5 cases were N0. There were 4 out of 5 cases presented peritoneal dissemination in distant metastasis. As a result of that pathological stage was as follows: Stage I 2 cases (14.2%), stage II 1 case (7.1%), stage III 6 cases (42.9%) and stage IV 4 cases (28.6%).

The locations of the primary tumors were right-sided in 8 (57.1%) cases and left-sided in 6 cases (42.9%). All of the SRCC patients had taken a primary tumor resection and D3 lymphadenectomy. Adjuvant chemotherapy or chemotherapy plus radiotherapy was performed in 8 out of 14 cases. There were 6 out of 8 cases who underwent adjuvant therapy experienced tumor recurrence and metastasis. Lung metastasis was verified in one case and the remaining 5 cases were all peritoneal dissemination recurrence.

Background matched case-control study. A case-controlled cohort that matched with the SRCC cohort in the same

Table I. Clinicopathological summary of fourteen cases of signet ring cell carcinoma.

Patient no.	Sex	Age	Tumor depth	Lymph node metastasis	Distant metastasis (location)	Pathological stage	Tumor location right/left (location)	Surgical procedure	Adjuvant therapy	Recurrence	Familial history
1	M	55	T3	N0	M0	IIA	R(A)	RHC+D3	no	no	no
2	M	69	T4a	N2b	M0	IIIC	L(Rb)	APR+D3	no	lung	no
3	M	53	T4a	N2a	M0	IIIC	R(A)	RHC+D3	CDDP+5FU	no	no
4	M	65	T1	N0	M0	I	R(C)	ICR+D3	no	no	yes
5	M	52	T4a	N2a	M0	IIIC	L(Rb)	APR+D3	UFT	local	yes
6	M	66	T4a	N2a	M1a (liver)	IVA	R(A)	RHC+D3	no	no	no
7	M	76	T4b	N2	M0	IIIC	L(Rb)	APR+D3	UFT+radiation	local	yes
8	F	55	T4a	N0	M1b (peritoneum)	IVB	R(C)	ICR+D3	IRIS->mFOLFOX-> FOLFIRI	peritoneum	no
9	M	58	T4a	N0	M1b (peritoneum)	IVB	R(A)	ICR+D3	FOLFIRI+Bv	no	yes
10	F	82	T4a	N2a	M0	IIIC	R(A)	RHC+D3	S-1	no	no
11	M	80	T4b	N2	M0	IIIC	L(Rb)	APR+D3	no	local	no
12	M	58	T1	N0	M0	I	L(Rb)	LAR+D3	no	no	no
13	M	49	T4a	N3	M1b (peritoneum)	IVB	R(C)	RHC+D3	FOLFOX	peritoneum	no
14	M	58	T4a	N3	M1b (peritoneum)	IVB	L(Rb)	LAR+D3	FOLFOX	no	yes

M, male; F, female; T1, tumor invades submucosa; T2, tumor invades muscularis propria; T3, Tumor invades through the muscularis propria into pericolorectal tissues; T4, tumor invades the visceral peritoneum or invades or adheres to adjacent organ or structure; Location C, caecum; A, ascending colon; Rb, lower rectum; Surgical Procedure RHC, right hemi-colectomy; APR, Abdominoperineal resection of rectum; ICR, Ileocaecal resection; LAR, low anterior resection; D3, lymphadenectomy dissection lesion 3; Adjuvant Therapy CDDP, cisplatin; 5FU, fluorouracil; UFT, tegafur; IRIS, Camptothecin and S-1(Tegafur/Gimeracil/Oteracil); FOLFOX, leucovorin calcium, fluorouracil and oxaliplatin; FOLFIRI, leucovorin calcium, fluorouracil and Irinotecan hydrochloride, S-1.

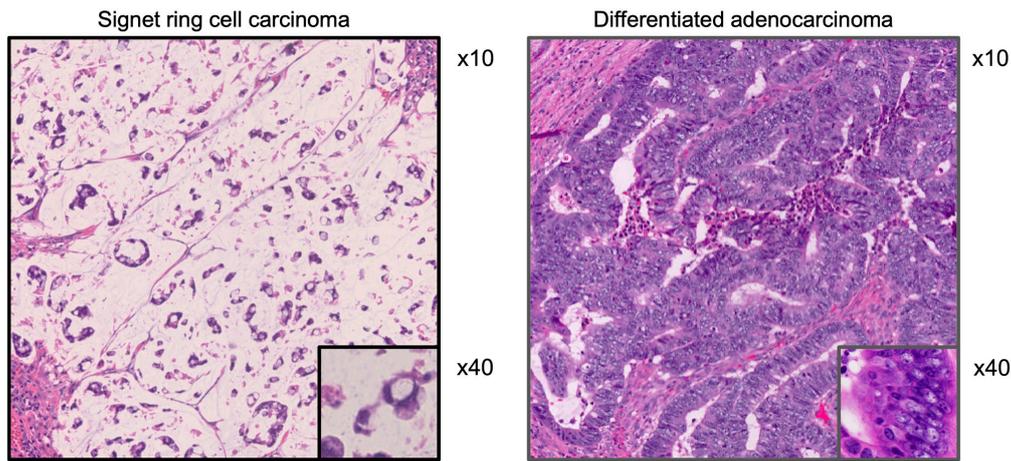


Figure 1. The representative microscopic view of signet ring cell carcinoma in colorectal cancer. Tumor tissue was stained with Hematoxylin and Eosin. The right is a low magnified view and the left is a highly magnified view.

treatment period in well and moderately differentiated adenocarcinoma cases was constructed to re-confirm the clinicopathological and prognostic features of SRCC. The cancer-specific survival (CSS) and the progression-free survival (PFS) were compared between the SRCC group and the case-control cohort group. As shown in Fig. 2 the prognosis of SRCC in CSS and in PFS was significantly worse than that of the case-control group.

Clinicopathological variables were compared between the SRCC group and the case-control group. There was no significant difference in age, gender, tumor depth, lymph node metastasis, distant metastasis, and pathological stage between the two groups (Table II). The primary location of the tumor is more frequently right-sided in the SRCC group than that in the case-control group. There was a more frequent tendency of lymphatic invasion in the SRCC group than that of in a case-control group. On the other hand, there was no significant difference in vascular invasion between the two groups. There was no significant difference between liver and lung metastasis in both groups but peritoneal dissemination more frequently occurred in the SRCC group. There was no significant difference in the status of familial history between the two groups Table II.

Microarray analysis of altered genes in SRCC. Microarray analysis was performed on mRNA samples extracted from frozen tumor tissues of SRCC and well-differentiated adenocarcinoma. Of the 58,717 differentially expressed genes extracted from the analysis, 1,445 showed significant differences between the SRCC and well-differentiated adenocarcinoma cases. The top 50 most significantly altered genes are shown in Tables III and IV.

In those tables, inflammation-related genes such as Tumor Necrosis Factor (TNF), heat shock protein (HSP), interferon-gamma (IF- γ), and interleukin-17 (IL-17) were shown as a commonly up-regulated genes in the SRCC group. Conversely, such as cadherin 1 (CDH1) and cadherin EGF Lag seven-pass G-type receptor (CELSR1) both genes related to epithelial cells were identified as a commonly down-regulated gene in SRCC.

CMS analysis of SRCC cases. According to the subtypes of CMS1, CMS2, CMS3, and CMS4 classification, two each data

were chosen from the CRC TCGA subset (colorectal TCGA gene expression data with subtype annotation) included in the CMScaller and utilized as a reference data. SRCC microarray data were processed with the reference data.

One of the SRCC data was assigned into the CMS1 however it was not statistically significant and the other data were assigned into CMS4 with a statistically significant correlation (Fig. 3). Both two cases of well-differentiated adenocarcinoma were assigned to CMS2 (Table V).

Gene Set Analysis packaged in CMS caller was performed to investigate the comprehensively altered genes in the CMS4 assigned SRCC case, then the up-regulation of epithelial Mesenchymal transition (EMT) related genes and the down-regulation of fatty acid, glycolysis, differentiation, MYC, and DNA repair-related genes were identified as mainly changed gene expression elements in SRCC (Table VI).

Discussion

Through this study, we re-confirmed that the morbidity of SRCC in colorectal cancer is quite low and the prognosis of it was significantly worse compared with the conventional histological type as previously reported. Comparison of clinicopathological factors also verified that the primary location of the tumor was right-sided and the peritoneal dissemination was frequently observed in SRCC. Microarray and following CMS analyses revealed that there was a specific gene expression pattern in colorectal SRCC.

There were only 14 SRCC cases (1.04%) out of 1,336 colorectal cancer cases who had been treated in our department from 1997 to 2011. There were several papers about the prevalence of SRCC in colorectal cancer (24-28) and each paper reported it around 1-2%. Our result re-confirmed that the prevalence of SRCC in colorectal cancer is quite rare. In this study, the primary location of SRCC was significantly more than on the right side compared with the case-control cohort group. The same fact was reported by Kim *et al* and they stated that the tumor histologically containing SRCC element tended to be on the right side of the colon (29). So it can be mentioned that there must be a common clinicopathological nature in SRCC characterizing to be in specific locations.

Table II. Comparison of clinicopathological characteristics of signet ring cell carcinoma group with well to moderately differentiated adenocarcinoma group in the case-control cohort.

Clinicopathological variables	Signet ring cell group (n=14)	Well to moderately differentiated group (n=42)	P-values
Age mean ± SD	62.57±10.68	62.57±10.42	1.0
Sex (%)			0.22
M	12 (85.71)	29 (69.05)	
F	2 (14.29)	13 (30.95)	
Tumor depth (%)			0.29
T1-T2	2 (14.29)	12 (28.57)	
T3-T4	12 (85.07)	30 (71.43)	0.29
Lymph node metastasis (%)			0.53
N ⁻	5 (35.71)	19 (45.24)/	
N ⁺	9 (64.29)	23 (54.76)	
Distant metastasis (%)			0.39
M ⁻	9 (64.29)	32 (76.19)	
M ⁺	5 (35.71)	10 (23.81)	
TNM stage (%)			0.11
0-II	3 (21.43)	19 (45.25)	
II-IV	11 (78.57)	23 (54.76)	
Tumor location (%)			0.0063 ^a
Right	8 (57.14)	8 (19.05)	
Left	6 (42.86)	34 (80.95)	
Lymphatic invasion (%)			0.053
Ly ⁻	2 (14.29)	18 (42.58)	
Ly ⁺	12 (85.71)	24 (57.18)	
Vascular invasion (%)			0.22
V ⁻	2 (14.29)	13 (30.95)	
V ⁺	12 (85.71)	29 (69.05)	
Liver Distant Metastasis (%)			0.29
-	13 (92.86)	34 (80.95)	
+	1 (7.14)	8 (19.05)	
Lung Distant Metastasis (%)			0.56
-	14 (100)	41 (97.62)	
+	0 (0)	1 (2.38)	
Peritoneum (%)			0.0358 ^a
-	10 (71.43)	39 (92.86)	
+	4 (28.53)	3 (7.14)	
Familial history of lynch syndrome related cancer (%)			0.92
Yes	6 (42.86)	17 (41.46)	
No	8 (57.16)	24 (58.58)	

SRCC, signet ring cell carcinoma; DAC, differentiated adenocarcinoma; M, male; F, female. ^aP<0.05.

There was no significant difference among lymph node metastasis between the SRCC group and case-control cohort group however, the lymphatic invasion was relatively higher and peritoneal dissemination was significantly higher in the SRCC group. The invasive character of SRCC is also clearly

shown in accordance with a previous report on the pathological characteristics of SRCC (1,17,30).

The prognosis between the SRCC group and case-control cohort group was compared and both CSS and PFS were significantly worse in the SRCC group than the case-control cohort

Table III. Top 50 upregulated genes in SRCC.

Gene symbol	Description
DMGDH	Dimethylglycine dehydrogenase
CART	Cocaine- and amphetamine-regulated transcript protein
ASB5	Ankyrin repeat and SOCS box-containing 5
SEPT4	Septin 4
SNTG2	Syntrophin, gamma 2
RYR2	Ryanodine receptor 2
CACNA2D1	Calcium channel, voltage-dependent, alpha 2/delta subunit 1
LRRTM1	Leucine rich repeat transmembrane neuronal 1
TNFSF18	Tumor necrosis factor (ligand) super family, member 18
CNGA3	Cyclic nucleotide gated channel alpha 3
EFHD1	EF-hand domain family, member D1
ARHGEF4	Rho guanine nucleotide exchange factor (GEF) 4
HSPB3	Heat shock 27 kDa protein 3
PEG3	Paternally expressed 3
INFG	Interferon, gamma
SMPX	Small muscle protein, X-linked
NPTX1	Neuronal pentraxin I
KCNJ8	Potassium inwardly-rectifying channel, subfamily J, member 8
TMOD2	Tropomodulin 2 (neuronal)
FAM49A	Family with sequence similarity 49, member A
DPP6	Dipeptidyl-peptidase 6
IL17B	Interleukin 17B
PRG-3	Proteoglycan 3
CDH19	Cadherin 19, type 2
MPDZ	Multiple PDZ domain protein
GPM6A	Glycoprotein M6A
KRTAP9-4	Keratin associated protein 9-4
MN1	Meningioma (disrupted in balanced translocation)1
ARHGAP6	Rho GTPase activating protein 6
NPC1L1	NPC1 (Niemann-Pick disease, type C1, gene)-like 1
BNC2	Basonuclin 2
ROR1	Receptor tyrosine kinase-like orphan receptor 1
PRDM8	PR domain containing 8
PCDHB5	Protocadherin beta 5
ZNF221	Zinc finger protein 221
CDH9	Cadherin 9, type 2 (T1-cadherin)
GRID2	Glutamate receptor, ionotropic, delta 2
GRIK4	Glutamate receptor, ionotropic, kainate 4
SLC4A3	Solute carrier family 4, anion exchanger, member 3
CD38	CD38 molecule

Table III. Continued.

Gene symbol	Description
LRRC21	Leucine rich repeat containing 21
KIAA1102	LIM and calponin homology domain 1
SERPINB11	Serpin peptidase inhibitor, clade B (ovalbumin), member 11
LAMP5	Lysosome associated membrane protein
GPIHBP1	Glycosylphosphatidylinositol anchored high density lipoprotein binding
GIT1	G protein-coupled receptor kinase interactor 1
LRCH2	Leucine-rich repeats and calponin homology (CH) domain containing 2
DNAJB5	DnaJ (Hsp40) homolog, subfamily B, member 5
STK33	Serine/threonine kinase 33
ZNF659	Zinc finger protein 659
SRCC, signet ring cell carcinoma.	

group. The reference cohort group contains relatively old cases however a whole of cases was case-controlled and stage IVB cases were assigned in both groups. Now, multimodal treatment was introduced in colorectal cancer therapeutic strategy and as shown in Table I FOLFOX or FOLFIRI were performed even in the SRCC group so that both two groups do not have any difference in clinical background. This result is implying that SRCC has chemotherapy resistance and it leads to a worse prognosis.

In the microarray analysis, the top 50 up- and down-regulated genes were selected (Tables III and IV). As shown in the tables, numerous genes that have not been reported in SRCC studies were identified. Notably, NPC1L1 and GPIHBP1 are involved in transporting cholesterol and absorbing lipoprotein respectively. NPC1L1 is an intracellular cholesterol transporter gene and the gene was reported to be connected with colitis-associated tumorigenesis (31). GPIHBP1 was reported to be associated with lipoprotein nutrient utilization in glioma (32). As shown in Table V, fatty acid and glycolysis associated genes were down-regulated in SRCC thus, up-regulation of these genes may imply that both energy transporting molecules are necessary for the survival of SRCC and would be a key biological target for the treatment of SRCC.

One of the SRCC cases was assigned into CMS4 and the other case was assigned into CMS1 (Fig. 3; Tables V and VI) in the CMS analysis. In the CMS analysis, colorectal cancer was classified into CMS1 to CMS4 (19), and, in brief, CMS1 is MSI immune type, CMS2 is canonical type, CMS3 is a metabolic type and CMS4 is mesenchymal type. In this study, SRCC case 1 was classified into CMS4 so case 1 was revealed to have a genetic background mainly related to epithelial-mesenchymal transition (EMT).

There was a report about a bio-physiological change in colorectal cancer cell lines after the cell lines acquired chemo agent resistance (33). And after that, a couple of clinical docu-

Table IV. Top 50 downregulated genes in SRCC.

Gene symbol	Description
FAM3D	Family with sequence similarity 3, member D
CDCA7	Cell division cycle associated 7
AREG	Amphiregulin (schwannoma-derived growth factor)
DSG2	Desmoglein 2
CEACAM5	Carcinoembryonic antigen-related cell adhesion molecule 5
TPRT	Decaprenyl Diphosphate Synthase Subunit 1
B3GNT3	UDP-GlcNAc:betaGal beta-1,3-N Acetylglucosaminyltransferase 3
PHLDA2	Pleckstrin homology-like domain, family A, member 2
ESRRA	Estrogen-related receptor alpha
GCSH	Glycine cleavage system protein H (aminomethyl carrier)
SLC22A18	Solute carrier family 22 (organic cation transporter), member 18
TFRC	Transferrin receptor (p90, CD71)
C20ORF42	Chromosome 20 open reading frame 42
TPP2	Tripeptidyl peptidase II
FLJ20272	Tetratricopeptide Repeat Domain 27
APPBP1	Amyloid beta precursor protein binding protein 1
ST14	Suppression of tumorigenicity 14 (colon carcinoma)
SMPD3	Sphingomyelin phosphodiesterase 3, neutral membrane (neutral sphingomyelinase II)
SMP3	Phosphatidylinositol glycan Anchor biosynthesis class Z
TM2D1	TM2 domain containing 1
MRPL45	Mitochondrial ribosomal protein L45
PRKDC	Protein kinase, DNA-activated, catalytic polypeptide
ABCC3	ATP-binding cassette, sub-family C (CFTR/MRP), member 3
FBP1	Fructose-1,6-bisphosphatase 1
MGC3265	Prenylcysteine Oxidase 1 Like
ZNF217	Zinc finger protein 217
OCIAD2	OCIA domain containing 2
FLJ22662	Phospholipase B Domain Containing 1
RFC3	Replication factor C (activator 1) 3, 38 kDa
SORL1	Sortilin-related receptor, L(DLR class) A repeats-containing
CDH1	Cadherin 1, type 1, E-cadherin (epithelial)
RARS	Arginyl-tRNA synthetase
LEFTY1	Left-right determination factor 1
EED	Embryonic ectoderm development
SCCPDH	Saccharopine dehydrogenase (putative)
C20ORF24	Chromosome 20 open reading frame 24
TMEM45B	Transmembrane protein 45B
SDC1	Syndecan 1
MTRF1	Mitochondrial translational release factor 1
ATP1B1	ATPase, NA ⁺ /K ⁺ transporting, beta 1 polypeptide
EPB41L1	Erythrocyte membrane protein band 4,1-like 1
TBL2	Transducin (beta)-like 2
MSI2	Musashi homolog 2 (<i>Drosophila</i>)
FANK1	Fibronectin type III and ankyrin repeat domains 1
LOC283537	Not annotated
NOC3L	Nucleolar complex associated 3 homolog (S, cerevisiae)
ALDH18A1	Aldehyde dehydrogenase 18 family, member A1
GLB1	Galactosidase, beta 1
CELSR1	Cadherin, EGF LAG seven-pass G-type receptor 1(flamingo homolog, <i>Drosophila</i>)
MGC17299	Transmembrane protein 125

Table V. Statistical analysis of CMS classification of signet ring cell carcinoma and well differentiated adenocarcinoma cases.

Case	Prediction	d.CMS1	d.CMS2	d.CMS3	d.CMS4	P-value	FDR
SRCC case 1	CMS4	0.6885880805	0.7941236723	0.7319281894	0.5330759305	0.001 ^a	0.001
SRCC case 2	NA	0.6179980022	0.7074984863	0.6460235957	0.713021697	0.569	0.569
Well case 1	CMS2	0.7670768658	0.6185472389	0.7328083907	0.7341745467	0.001 ^a	0.001
Well case 2	CMS2	0.7690166154	0.6682347108	0.7220604844	0.8455248645	0.001 ^a	0.001

SRCC, signet ring cell carcinoma; Well, well-differentiated adenocarcinoma; CMS, consensus molecular subtype; FDR, false discovery ratio; NA, not applicable. ^aP<0.05.

ments presented the association of chemoresistance and EMT related gene alteration (34-38). EMT is a gene alteration that can explain the both phenomenon of invasive and chemoresistant characteristics of SRCC so that the result is very compatible.

Another SRCC case was assigned into CMS1 but it was not statistically significant. CMS1 is an MSI immune type and it has a character of MSI-high, CIMP high, hypermutation, BRAF mutation, and worse survival after relapse. In a study of the genetic background of colorectal cancer, RAS mutations and especially BRAF mutations were found to be particularly common in SRCC (39,40). Furthermore, to take into account the result of the SRCC was significantly frequent in the right side that it reminded us that the SRCC case 2 bore CMS1 specific gene alteration on it.

The element of SRCC is considered to be the one of a poor prognostic factor however, our study revealed that SRCC cases contain a mixture of MSI-high and EMT enriched cases on it. As a result, consideration of the primary therapeutic strategy could be possible utilizing CMS analysis according to this study. One limitation of this study is the lack of information regarding the MSI and CIMP statuses of the study patients. Because MSI and CIMP affect gene expression, this is critical information. However, in this study, we could not acquire both of the information from these SRCC cases. If the information was available, it would ensure a result of this study furthermore.

Considering these limitations, further analyses involving additional SRCC cases are needed utilizing CMS analysis to identify the gene expression alterations that accurately reflect the biological features of SRCC.

Through this study, the clinicopathological severity of SRCC could be recognized. Microarray and subsequent integrative computational analyses were useful tools to comprehend the gene expression signature and to infer specific groups of genes hidden in the large information data.

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Table VI. Gene set analysis of SRCC case 1, utilizing CMS caller.

Gene set name	N Genes	Direction	P-values	FDR
EMT	199	Up	0.079	0.122
TGF- β	60	Up	0.380	0.443
LGR5 stem-cells	62	Down	0.622	0.663
CDX2	35	Down	0.124	0.173
Fatty acids	157	Down	<0.001 ^a	<0.001
Glycolysis	200	Down	<0.001 ^a	<0.001
Differentiation	612	Down	0.001 ^a	0.017
Cell cycle	200	Down	0.000	0.000
WNT	13	Down	0.245	0.312
MYC	58	Down	<0.001 ^a	<0.001
MSS	80	Down	0.000	0.000
HNF4 α	58	Down	<0.001 ^a	<0.001
DNA repair	148	Down	<0.001 ^a	<0.001
MSI	29	Down	0.991	0.991

N Genes, number of genes; FDR, false discovery ratio; EMT, epithelial-mesenchymal transition; TGF- β , transforming growth factor- β ; LGR5, leucine-rich repeat containing G protein-coupled receptor 5; CDX2, caudal type homeobox 2; WNT, Wnt signaling pathway; MYC, Myc signaling pathway; MSS, microsatellite stable; HNF4 α , hepatocyte nuclear factor 4 α ; MSI, microsatellite instable. Up, upregulated; Down, downregulated. ^aP<0.05.

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Availability of data and materials

The datasets analyzed during the current study are available in the Array Express repository, on the web page <https://www.ebi.ac.uk/arrayexpress/> ArrayExpress accession E-MTAB-9149.

Authors' contributions

KT conceived of the study and methodology, curated the data and wrote and drafted the original manuscript. TS conceived of the study, curated the data, analyzed the data and wrote,

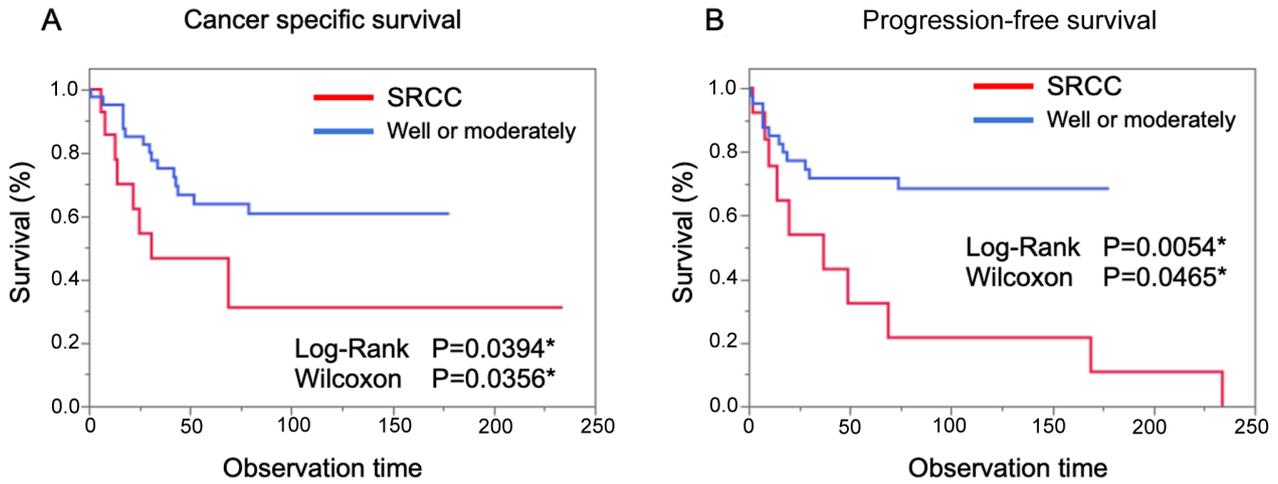


Figure 2. Survival comparisons between the case-control cohort of well or moderately differentiated adenocarcinoma cases and SRCC cases. (A) Cancer-specific survival. (B) Progression-free survival. Both survival curves were compared using the log-rank test. SRCC, signet ring cell carcinoma. *P<0.05.

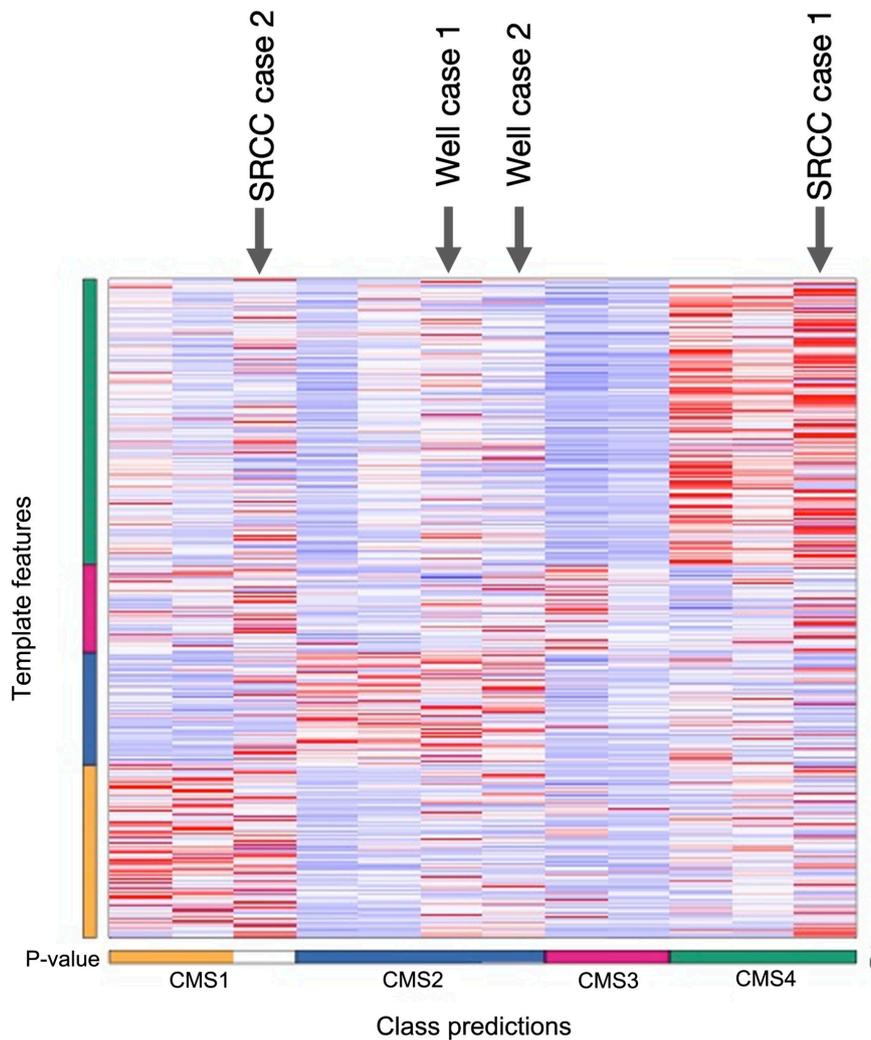


Figure 3. The color plot figure of twelve cases in CMS analysis in R utilizing the CMS caller package. The arrows are assigned SRCC and Well-differentiated adenocarcinoma samples. The remaining 9 samples data (contained in the CMS caller) were simultaneously used. Colour bars, each gene; red bar, high expression; blue bar, low expression. CMS, consensus molecular subtyping; SRCC, signet ring cell carcinoma.

reviewed and edited the manuscript. KI ran the software, was involved in data validation, performed formal analysis and

visualized the data. AK performed the IHC experiment. SN, SS, KY, MK, TY, KF, KK, TO, TY and TM were involved

in data curation. FF and JA reviewed, edited and drafted the manuscript, suggested important key points and interpreted the data in the current study. YA conceptualized the study, supervised the study and was involved in project administration. All authors read and approved the final manuscript.

Ethics approval and consent to participate

The current study was conducted in accordance with the provision of the Declaration of Helsinki and was approved by the Institutional Review Board of Kurume University Hospital (approval no. 245).

Patient consent for publication

The current study acquired written informed consent for publication from all of the patients enrolled in this study.

Competing interests

The authors declare that they have no competing interests.

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