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Original article

Simple classifiers for molecular subtypes of colorectal cancer

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ABSTRACT

Background and study aim: Colorectal cancer (CRC) is a heterogeneous disease entity with a diverse biological pathogenesis. This study aims to validate the two studies published in 2013 which established a separate CRC molecular subtype classification by utilizing a rapidly accessible miniclassifier, and verify a simplified version thereof.

Patients and methods: Participants diagnosed with CRC (n = 568) were subtyped in three classifications for characteristic, and prognostic purposes. Colorectal cancer subtypes (CCS) were classified as: i) CCS1 (CDX2+, microsatellite stable (MSS)/microsatellite instability (MSI)-low), ii) CCS2 (MSI-high), and iii) CCS3 (FRMD6/ZEB1/HTR2B +, CDX2-, MSS/MSI-low]. Simplified CCS (SiCCS) subtypes were grouped as: i) CDX2 (CDX2+, MSS/MSI-low, ZEB1 $\leq$ 2), ii) MSI-H (MSI-high, CDX2/FRMD6/ZEB1/HTR2B +/-), and iii) ZEB1 (ZEB1 $\geq$ 2, CDX2-, MSS/MSI-low). New molecular classification (NMC) subtypes were defined as: i) enterocyte (E-C) (MUC2 +), ii) goblet-like (G-L) (MUC2 + and TFF3 +), iii) transit-amplifying (T-A) (CFTR +), and iv) stem-like (S-L) (ZEB1 +).

Results: In total, 53.5% (n = 304) CCS, 58.3% (n = 331) SiCCS, and 37.7% (n = 214) NMC tumours could be evaluated. CCS2 and MSI-H CRCs had the most favourable survival outcome, whereas the CCS3, ZEB1 and S-L subtypes showed the poorest prognosis. A significant overlap between CCS3, ZEB1, and S-L tumours was demonstrated.

Conclusion: There is still a need for a consensus gene expression-based subtyping classification system for CRCs, thereby allowing the categorization of most CRC tumours. This study reveals that a simple and rapidly accessible process could replace the complicated, costly and mostly inapproachable methods clinical practices that have been introduced in the majority of previous studies.

Introduction

Colorectal cancer (CRC) is a heterogeneous disease, emerging from biologically diverse pathways distinguished by various compositions of gene-based transitions within the tumour [1]. Each of these different pathways can produce a distinct subtype with specific clinicopathological tumour characteristics and patient survival, resulting in the need for different therapeutic methods. Many previous studies have contributed consistent efforts to identify a novel molecular classification of CRC in association with cellular and molecular features. Most of these studies have concentrated on and extensively investigated the following tumour markers: microsatellite instability (MSI), CpG island methylator phenotype (CIMP), and BRAF and KRAS mutations [2–9]. However, to date, there has been no consensus on internationally standardized CRC molecular classification with reliable prognostic stratification [10].

In 2013, among the several groups that have reported on CRC taxonomy, two separate studies introduced comprehensive human CRC molecular subtype classification systems by gene expression profiling [11,12]. One study by De Sousa E. Melo, et al. [11] described three main colon cancer subtypes (CCSs) by deriving a 146-gene classifier to categorize 90 patients into CCS1, CCS2 and CCS3. In addition, they provided a rapidly accessible classification tool, namely a tissue microarray-based miniclassifier using immunohistochemistry for four epithelial gene encoding proteins (FRMD6, ZEB1, HTR2B and CDX2), in combination with microsatellite status to categorize CCS1 to CCS3. Cross-validation confirmed that the tissue microarray-based miniclassifier could achieve an accurate classification when compared to the microarray-based classifier, thus resulting in significant prediction of disease outcome. The second study by Sadanandam et al. [12] introduced a

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new molecular classification (NMC) of CRCs by defining five distinct high-consensus molecular subtypes by identifying subtype associated markers using significance and prediction analyses [12]. The five subtypes were named as (i) enterocyte (E-C), defined by high expression of enterocyte-specific genes (MUC2 only) (ii) goblet-like (G-L), high mRNA expression of goblet-specific MUC2 and TFF3; (iii) transit-amplifying (T-A), a heterogeneous collection of samples with variable expression of stem cells and Wnt-target genes; (iv) stem-like (S-L), with high expression of Wnt signaling targets plus stem cell, myoepithelial and mesenchymal genes, and low expression of differentiation markers; and (v) inflammatory (I), marked by comparatively high expression of chemokines and interferon-related genes.

The aim of this work was to search for a simple classifier of the molecular classification system of the CRCs with an easy and rapid accessibility. In the present study, the CRC molecular subtyping algorithms published in the above two studies were applied to patients who had previously undergone primary resection for CRCs in the present institute (n = 568), in order to validate these previously published two high-consensus molecular classification systems. A combination of microsatellite instability and immunohistochemical expressions of the following epithelial gene-coding proteins, FRMD6, HTR2B, ZEB1, MUC2, TFF3 and CDX2 were applied, to compare patient outcomes and establish a community-based cancer subtyping for clinical practice. CDX2 is a biomarker absent in colon cancers with high level of ALCAM, a molecule expressed on colon-cancer cells with enriched tumourigenic capacity and CDX2-negative expression is an independent adverse prognostic marker of colon cancers [13]. ZEB1 plays an important role in tumour invasion and metastasis by activating urokinase plasminogen activator (uPA) and its inhibitor, plasminogen activator inhibitor-1 (PAI-1) which forms the key protease cascade of tumour invasiveness in the plasminogen activation system. ZEB1 and uPA are expressed in tumour cells at the invasive front of primary CRCs, setting ZEB1 as a potential prognostic biomarker and potential therapeutic target in CRCs [14]. Therefore, an additional classification method using only CDX2, MSI and ZEB1 was analyzed to verify whether it could represent the simplified the version of CCS.

Patients and methods

Patients and sample collection

A retrospective study for CRC classifications recruited a total of 568 CRC patients from January 2004 to December 2008. The eligibility criteria for inclusion were surgically resected CRC at the Pusan National University Hospital (PNUH) (Busan, Korea) with histological diagnosis of adenocarcinoma. All patients were chemotherapy-naive and underwent R0 resections for primary CRCs independently, before receiving any chemotherapy. Haematoxylin and eosin (H&E)-stained slides of all surgically resected tumour samples were reviewed by two pathologists. Clinicopathological variables of each patient and tumour were retrieved, including age, sex, tumour location (right or left), tumour size, histologic differentiation, lymph node (LN) status, pathologic T- and N stages, lymphovascular tumour invasion (LVI), perineural invasion (PNI), survival status, metastasis, recurrence, and response to a fluorouracil-based chemotherapy regimen (5-FU). Vital status, date of death, recurrence, and metastasis were determined through clinical records of the patients.

The study was approved by the university ethics committee, and all participating patients were informed about the study and had to provide signed, written consent before enrollment.

Tissue microarray and immunohistochemistry

A tissue microarray (TMA) composed of 568 tumour samples was constructed from standard formalin-fixed and paraffin-embedded sections that were obtained from the Department of Pathology, and the National Biobank of Korea, PNUH. All samples from the National Biobank of Korea were obtained with informed consent under institutional review board-approved protocols.

An appropriate H&E-stained section from each tumour was selected and a representative area was identified, and the congruent area in the paraffin block was collected for the TMA. A semi-automated tissue arrayer (Beecher Instruments, WI, USA) was used to construct the tissue microarrays. In each tumour, two cores with diameter of 2.0 mm were obtained using the tissue microarray instrument and inserted in a recipient block. Recipient blocks were sectioned at 3 μm, dried for 20–30 min at 60 °C, deparaffinized in xylene and rehydrated in graded alcohol as per routine practice. The sections were subsequently submerged in citrate antigen retrieval buffer, microwaved for antigen retrieval (pH 9.0), treated with 3% hydrogen peroxide in methanol to quench endogenous peroxidase activity, and then incubated with 1% bovine serum albumin overnight at 4 °C to block non-specific binding. Slides were stained manually with anti-ZEB1 (1:100, Sigma, HPA027524), anti-CFTR (1:100, Abcam, ab59394), anti-FRMD6 (1:100, Sigma, HPA001297), anti-HTR2B (1:100, Sigma, HPA012867), anti-TFF3 (1:100, R&D systems, MAB4407), and anti-MUC2 (1:100, Novocastra, NCL-MUC-2). After a secondary incubation, staining was developed using DAB + Chromogen (Dako, K5007), and slides were counterstained with hematoxylin. Only anti-CDX2 (free dilution, 0.32 mg/L, Leica, PA0535) was stained with Bond-Max (Leica) autostainer. All immunohistochemical (IHC) stainings were scored for each antibody expression in a blinded fashion by two pathologists. The two cores from each tumour were scored independently and paired at the end. If scores for the two samples were discordant, the final score for the tumour was upgraded to the higher score. ZEB1 and CDX2 were scored in the nucleus, MUC2 and TFF3 in cytoplasm whereas CTFR, FRMD6, HTR2B showed cytoplasmic and membranous expressions. Normal tissues utilized for positive control for ZEB1, CFTR, FRMD6, HTR2B and TFF3 were kidney glomeruli, epithelial cells of the lung, hepatocytes, endometrial glands and goblet cells of colon, respectively. For MUC2 and CDX2, normal colonic tissue was tested for positive control. IHC findings except CDX2 were scored from 1 to 4 according to intensity; 1 for negative, 2 for weak, 3 for moderate, and 4 for strong expression and were considered positive for tumour cells with any intensity expressions. For CDX2, all tumours in which the malignant epithelial component showed widespread nuclear expression, either in all or a majority of cancer cells, were scored as CDX2-positive. All tumours in which the malignant epithelial component either completely lacked CDX2 expression or showed faint nuclear expression in a minority of malignant epithelial cells (<10%) were scored as CDX2-negative.

The concordance between the two pathologists was analyzed by using contingency tables to calculating the Cohen's kappa Index which showed an excellent agreement (k = 0.89).

Microsatellite instability (MSI)

MSI status of the samples was determined using the MSI Analysis System, and GeneMarker version 2.6 (SoftGenetics, LLC State College, PA, USA), according to the manufacturer's instructions. Samples were considered MSI-high (MSI-H) when two or more markers were instable, and MSI-low (MSI-L) or Microsatellite Stable (MSS) were defined as one or zero out of five markers were instable, respectively. The genomic DNA from the tumour samples was isolated using the QIAamp DNA FFPE Tissue Kit (Cat. No.

56404, Qiagen, Duesseldorf, Germany) according to the manufacturer's instructions.

Molecular subtype classifications – Colorectal cancer subtype (CCS), Simplified CCS, and New Molecular classification (NMC).

Tumour subtypes in CCS classification were defined as follows: CCS1 (CDX2-positive, MSS/MSI-low, FRMD6/ZEB1/HTR2B $\leq$ 2), CCS2 (MSI-high, CDX2/FRMD6/ZEB1/HTR2B + or -), and CCS3 (FRMD6/ZEB1/HTR2B $\geq$ 2, CDX2-negative, MSS/MSI-low) (Fig. 1). In addition, a further simplified CCS (SiCCS) classification consisting of 3 groups is as follows: 1) CDX2 (CDX2-positive, MSS/MSI-low, ZEB1 $\leq$ 2), MSI-H (MSI-high, CDX2/FRMD6/ZEB1/HTR2B + or -), and 3) ZEB1 (ZEB1 $\geq$ 2, CDX2-negative, MSS/MSI-low), was investigated.

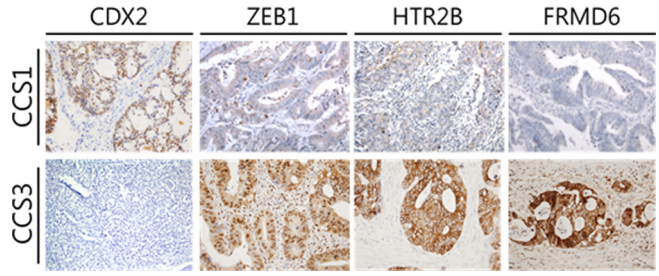

Fig. 1. Immunohistochemistry assays of patient CRC samples using subtype specific markers to assign subtypes for Colorectal Cancer Subtype (CCS) classification. Each of HTR2B, FRMD6 and ZEB1 was scored 1, 2, 3 or 4 for negative, weak, moderate or strong intensity of staining, respectively. CDX2 was scored 1 for negative, 2 for weak staining less than 10% in proportion, 3 for weak and 4 for moderate/strong stains. CCS2 was subtyped based on MSI-H status.

Tumour subtypes in NMC system were categorized as, 1) Enterocyte (E-C): MUC2 $\geq$ 2, TFF3/CFTR/ZEB1 = 1 (negative); 2) Goblet-like (G-L): MUC2 and TFF3 $\geq$ 2, CFTR/ZEB1 = 1 (negative); 3) Transit-amplifying (T-A): CFTR $\geq$ 2, MUC2/TFF3/ZEB1 = 1 (negative); and, 4) Stem-like (S-L): ZEB1 $\geq$ 2, MUC2/TFF3/CFTR = 1 (negative) (Fig. 2). The inflammatory subtype was excluded from this study as subtyping required multiple mutational molecular studies, which deviated from the focus of this study, i.e., to utilize a simple classification method.

The remaining tumours that did not fulfill the criteria to be able to be classified into the above subtypes were grouped in an "undefined" category and eliminated in the statistical analysis.

Chemotherapy with fluorouracil (5-FU) based regimen

A total of 349 (61.3%) patients were treated with fluorouracil (5-FU) based regimen as adjuvant chemotherapy, per standard protocol as a first-line treatment. Twenty-six patients (4.6%) had received chemotherapeutic agents other than the 5-FU-based regimen while the treatment status of the remaining 193 (34.0%) patients was unknown. Most of the patients received the 5-FU (capecitabine/doxifluridine) based regimen orally, according to the instructed dosage, in conjunction with other antineoplastic drugs. Eleven patients (1.9%) were infused with intravenous 5-FU-based regimen.

Tumour response was evaluated according to WHO recommendations for the evaluation of cancer treatment in solid tumours [15]. The size of the metastatic lesions was estimated from bidimensional measurements (the product of the longest diameter and the longest perpendicular diameter) using computed

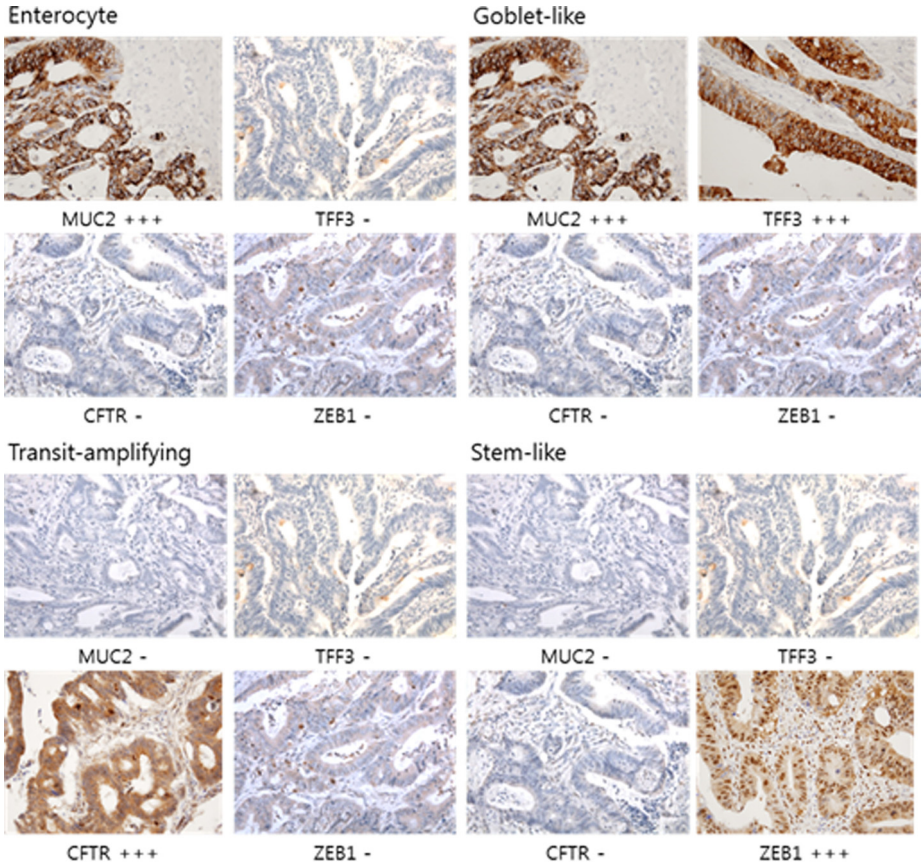

Fig. 2. Immunohistochemistry assays of patient CRC samples using subtype-specific markers MUC2, TFF3, CTFR and ZEB1, to assign subtypes for New Molecular Classification (NMC). For immunohistochemistry, each subtype-specific marker was scored 1, 2, 3 or 4 for negative, weak, moderate or strong intensity of staining, respectively.

tomography (CT) scanning. Percent change of the size was calculated by evaluating the size of the metastatic lesions before and after chemotherapy treatments, which was then used to classify patients into two groups, responders and non-responders. Patients with 50% or more decrease in the size of the metastatic lesion were classified as responders, and patients with increased size or a less than 50% decrease of the lesion were classified as non-responders [16].

Statistical analysis

To evaluate clinicopathological features of CRCs in each of CCS, simplified CCS and NMC classifications, Student's T-test and one-way ANOVA test were used. Fisher's exact test was carried out for response to 5-FU-based chemotherapy in each classification system and Chi-squared test for analysis of relationship between the CCS and NMC systems. The Kaplan-Meier method was used to calculate disease-free survival (DFS) and recurrence-free survival (RFS) from the treatment start date to the date of death, or the date that the surviving patients were last seen. All statistical analyses were performed using SPSS for windows version 21.0 (IBM, North Castle, NY, USA), and significance was set at $p < .05$.

Results

Overall patient summary

A total of 568 patients were included in this study. Patient median age at diagnosis was 64 years (range 17–91 years) and the median tumour size was 5.0 cm (range 1.5–12.5 cm). Among them, 335 (58.9%) were male and 233 (41.0%) were female patients. The tumour was located on the right side of the colon in 134 (23.6%) cases and 434 (76.4%) had tumours on the left side of the colon. MSI-H was present in 44 (7.7%) patients, while MSI-low and MSS were present in 22 (3.9%) and 502 (88.4%) patients respectively. Data on recurrence and metastasis were available in 408 and 400 patients respectively, in which recurrence was present in 25 patients (4.4%) and metastasis in 120 (21.1%) patients. The median duration of follow-up for 568 patients was 91.6 months (range 0.8–137.2 months), and 19.3 months (range 0.8–85.3 months) for the 25 patients with local recurrence.

CCS and SiCCS classifications and tumour characteristics

The number of evaluable tumours was 304 and 331 for the CCS and SiCCS classifications, respectively. Among CCS, 11.8% (n = 36) were classified as CCS1, 14.8% (n = 45) as CCS2, and 77.34% (n = 223) as CCS3. CCS1 and CCS3 were more associated with well/moderately differentiated adenocarcinomas whereas CCS2 showed a significant correlation with poorly differentiated and mucinous adenocarcinoma ($p < .0001$). CCS2 was located more commonly on the right side of the colon (60%) and CCS1 and CCS3 on the left side ($p < .0001$). T stage at diagnosis had a significant impact on CCS2 subtype as it was predominantly present in stage 3 tumours (80%, $p = .002$). The CCS3 subtype was associated with the highest rate of metastatic regional lymph nodes ($p = .002$) and advanced N stage ($p = .028$). In contrast, CCS2 showed a significant correlation with negative LN metastasis ($p = .02$), along with N0 stage ($p = .028$) (Table 1). In SiCCS, the CDX2 group was the predominant group, consisting 50.8% of the total. All the clinicopathological variables showed identical significant results with the CCS, except the tumour size, which correlated with the MSI-H group and the largest tumours (Table 2). MSI status was not evaluated for statistical significances in CCS and SiCCS to avoid any biases.

NMC subtypes and tumour characteristics

A total of 214 tumours were included in the NMC classification which comprised of 33 (15.4%), 18 (8.4%), 57 (26.6%) and 106 (49.5%) for each subtype, E-C, G-L, T-A and S-L, respectively. Significant correlations were observed between the invasion depth, LVI and PNI with the NMC subtypes. Early CRC most frequently appeared in the G-L subtype whereas S-L subtype was present in T3 stage, and the E-C subtype had mainly T4 stage tumours. The G-L subtype had the lowest association with lymphovascular tumour emboli and perineural invasions, while the E-C and S-L subtypes showed a significant correlation with positive LVI and PNI, respectively (Table 3). There was no significance with tumours located on two sides of the colon, however, when analyzed in 6 divisions (cecum, ascending, transverse, descending, sigmoid colon and rectum), the S-L subtype showed a predilection for the sigmoid colon, E-C for rectum and T-A for ascending and transverse colon ($p = .004$, data not shown in the table).

DFS and RFS in CCS/SiCCS and NMC

Kaplan-Meier curves were used to illustrate DFS across subtypes. In CCS, significant disease-free probability was observed ($p = .003$), where CCS3 had the highest mortality rate (n = 85) with average survival duration of 92.58 (85.8–99.3) months. CCS1 had 10 cases of deaths, with an average survival period of 105.1 (90.6–119.5) months. The best DFS was observed in the CCS2 subtype (n = 5) with average survival time of 124.8 (114.3–135.2) months (Fig. 3a). This result meant that MSI-H subtype is associated with the most favorable outcome among the CCS classified CRCs. This was concordant with the results of the 331 cases in SiCCS, in which the ZEB1 group showed the worst prognosis, and best survival was observed in MSI-H subtype ($p < .0001$) (Fig. 3b).

Among NMC, DFS demonstrated the best prognosis in the G-L subtype (n = 1) with survival duration of 116.3 (102.6–129.9) months, while the worst DFS was observed in the S-L subtype (n = 39). However, DFS for the NMC subtypes was not statistically significant ($p = .094$) (Fig. 3c).

Recurrence-free probabilities in CCS and NMC provided insignificant data. In the CCS classification, only 19 cases had recurrent CRCs and among them, where 1, 2 and 16 cases were designated as CCS1, CCS2, and CCS3, respectively. Kaplan-Meier recurrence survival curves showed the lowest RFS rate in CCS3 with an average recurrence-free duration of 122.15 months, while CCS1 had a relatively favorable RFS ($p = .206$) (Fig. 4a). In SiCCS, 14 instances of recurrence were observed (CDX2: 3, MSI-H: 2, and ZEB1: 9). In contrast to CCS, the RFS in SiCCS was significant, showing that MSI-H had a slightly higher RFS rate than that of CDX2, while ZEB1 was associated with the highest recurrence rate ($p = .048$) (Fig. 4b). In the NMC, a total of 14 cases of recurrence were identified (E-C: 3, G-L: 0, T-A: 6, and S-L: 5). The T-A subtype had the lowest RFS rate, whereas the G-L subtype had the highest recurrence-free probability, as there was no recurrence ($p = .564$) (Fig. 4c).

Chemotherapy response to 5-FU based regimen

Response to chemotherapy (5-FU-based regimen) in each classification system was analyzed using Fisher's exact test. Only 33, 22, and 16 cases were included in the CCS, SiCCS, and NMC classifications, respectively (Table not shown). The CCS2 and MSI-H groups had a relatively higher response rate to the 5-FU-based regimen (n = 1, 50% response in each), however, these were not statistically significant ($p = .282$, & $p = .399$ respectively). Among NMC, T-A was the only subtype with responders to the 5-FU-based regimen (n = 3, 60% response, $p = .079$). However the very low quantity of

Table 1
Relationship between colorectal cancer subtype (CCS) classification and clinicopathological characteristics in 304 patients with colorectal cancer.

Variables	Total (%)	CCS (relative % in parentheses)			p value
		CCS1	CCS2	CCS3	
Total Number	304	36 (11.8)	45 (14.8)	223 (73.4)	
Age (years)		63.67 ± 9.76	59.69 ± 12.07	63.99 ± 10.69	0.208
Size (cm)		3.80 ± 0.36	6.88 ± 3.66	4.91 ± 2.42	0.111
Sex					0.224
Male	178 (58.6)	24 (66.7)	30 (66.7)	124 (55.6)	
Female	126 (41.4)	12 (33.3)	15 (33.3)	99 (44.4)	
Location					**.0001**
Right Colon	**87 (28.6)**	**8 (22.2)**	**27 (60.0)**	**52 (23.3)**	
Left Colon	**217 (71.4)**	**28 (77.8)**	**18 (40.0)**	**171 (76.7)**	
Histological type					**.0001**
Well	**25 (8.2)**	**3 (8.3)**	**3 (6.7)**	**19 (8.5)**	
Moderately	**236 (77.6)**	**31 (86.1)**	**26 (57.8)**	**179 (80.3)**	
Poorly	**21 (6.9)**	**1 (2.8)**	**5 (11.1)**	**15 (6.7)**	
Mucinous	**22 (7.2)**	**1 (2.8)**	**11 (24.4)**	**10 (4.5)**	
Invasion depth (pT)					**.02**
T1	**14 (4.6)**	**1 (2.8)**	**3 (6.7)**	**10 (4.5)**	
T2	**36 (11.8)**	**5 (13.9)**	**1 (2.2)**	**30 (13.5)**	
T3	**220 (72.4)**	**26 (72.2)**	**36 (80.0)**	**158 (70.9)**	
T4	**34 (11.2)**	**4 (11.1)**	**5 (11.1)**	**25 (11.2)**	
Perineural invasion					**.038**
Negative	**187 (62.8)**	**23 (63.9)**	**36 (80.0)**	**134 (60.1)**	
Positive	**111 (37.2)**	**13 (36.1)**	**9 (20.0)**	**89 (39.9)**	
Lymphovascular emboli					.259
Negative	174 (57.8)	20 (55.5)	32 (71.1)	126 (56.5)	
Positive	127 (42.2)	16 (45.7)	14 (28.9)	97 (43.5)	
Lymph node metastasis					**.002**
Negative	**169 (55.6)**	**22 (61.1)**	**35 (77.8)**	**112 (50.2)**	
Positive	**135 (44.4)**	**14 (38.9)**	**10 (22.2)**	**111 (49.8)**	
Lymph node stage (pN)					**.028**
N0	**169 (55.6)**	**22 (61.1)**	**35 (77.8)**	**112 (50.2)**	
N1a(1)	**37 (12.2)**	**5 (13.9)**	**3 (6.7)**	**29 (13.1)**	
N1b(2–3)	**52 (17.1)**	**7 (19.4)**	**6 (13.3)**	**39 (17.6)**	
N2a(4–6)	**21 (6.9)**	**1 (2.8)**	**0 (0.0)**	**20 (9.0)**	
N2b(≥7)	**25 (8.2)**	**1 (2.8)**	**1 (2.2)**	**23 (10.4)**	
MSI					
MSS/MSI-L	253 (83.2)	36 (100.0)	0 (0.0)	217 (97.3)	
MSI-H	45 (14.8)	0 (0.0)	45 (1 0 0)	0 (0.0)	
Unknown	6 (2.0)	0 (0.0)	0 (0.0)	6 (2.6)	
Recurrence					.482
Present	19 (6.2)	1 (2.8)	2 (4.4)	16 (7.2)	
Absent	285 (93.8)	35 (97.2)	43 (95.6)	207 (92.8)	
Metastasis					.421
Present	91 (29.9)	12 (33.3)	10 (22.2)	69 (31.7)	
Absent	213 (70.1)	24 (66.7)	35 (77.8)	154 (68.3)	

Abbreviation: CCS, colorectal cancer subtype; MSI, microsatellite instability; MSS, microsatellite stable; MSI-L, microsatellite instability-low; MSI-H, microsatellite instability-high.
P values in boldface indicate statistical significance ($P \leq .05$).

data available for response to the 5-FU based chemotherapy regimen in this present study limited statistically significant results and a more extensive study on this chemotherapeutic response in metastatic CRCs is mandatory.

Relationship between CCS/SiCCS and NMC classifications

To evaluate the relationship between the CCS and NMC classifications, 102 patients classified by both CCS and NMC subtyping algorithms were analyzed with a chi-squared test. There was a significant interconnectivity between the CCS3 subtype of the CCS classification and the stem-like subtype in NMC classification as 43 out of 102 cases overlapped ($p < .0001$) (Table 4). Identical methods were applied to 140 cases that overlapped in SiCCS and NMC, which, when analyzed demonstrated that 43 tumours belonged to the ZEB1/S-L group (Table 5). The CCS3, ZEB1 and S-L subtypes represent the worst prognostic subtype in each classification system, and also display higher prevalence in the left colon (CCS3, ZEB1) and sigmoid colon (S-L). This relationship was supported by the results of a recent study by Guinney et al. [17].

Discussion

CRC is the third most common cancer globally [18], and fourth most common cause of death due to cancer in the Korean population [19]. Screening has shown to reduce colorectal cancer incidence and mortality [20]; however, there is still controversy regarding post-operative treatment strategies that contribute to lowering the mortality of CRCs. This is due to lack of a high-consensus, broad molecular classification, despite the high prevalence of CRC in relation to tumours of other organs. For example, for breast cancer, researchers have successfully utilized a concept known as "unbiased genome-wide analyses of gene-expression patterns" for the molecular classification of subtypes that have significant prognostic outcomes, and which contribute significantly to development of individualized treatment plans [21,22].

CRC has heterogeneous molecular pathogenesis, and develops through multiple genetic and epigenetic pathways. Over the past decades, identifying a molecular taxonomy for CRCs has been the focus of cancer genomics, to be able to provide a more personalized, optimal treatment strategy [23], which, however, has been

Table 2
Relationship between simplified colorectal cancer subtype (SiCCS) classification and clinicopathological characteristics in 331 patients with colorectal cancer.

Variables	Total (%)	SiCCS (relative% in parentheses)			p value
		CDX2	MSI-H	ZEB1	
Total Number	331	168 (50.8)	45 (13.6)	118 (35.6)	
Age (years)		63.76 ± 11.2	59.69 ± 12.1	63.65 ± 11.4	.301
Size (cm)		**4.45 ± 1.5**	**6.88 ± 3.7**	**4.82 ± 1.8**	**.043**
Sex					.357
Male	191 (57.7)	97 (57.7)	30 (66.7)	64 (54.2)	
Female	140 (42.3)	71 (42.3)	15 (33.3)	54 (45.8)	
Location					**<.0001**
Right Colon	**89 (26.9)**	**31 (18.5)**	**27 (60.0)**	**31 (26.3)**	
Left Colon	**242 (73.1)**	**137 (81.5)**	**18 (40.0)**	**87 (73.7)**	
Histological type					**<.0001**
Well	**31 (9.4)**	**20 (11.9)**	**3 (6.7)**	**8 (6.8)**	
Moderately	**254 (76.7)**	**141 (83.9)**	**26 (57.8)**	**87 (73.7)**	
Poorly	**21 (6.3)**	**2 (1.2)**	**5 (11.1)**	**14 (11.9)**	
Mucinous	**25 (7.6)**	**5 (3.0)**	**11 (24.4)**	**9 (7.6)**	
Invasion depth					**.011**
T1	**13 (3.9)**	**9 (5.4)**	**3 (6.7)**	**1 (0.8)**	
T2	**41 (12.4)**	**29 (17.3)**	**1 (2.2)**	**11 (9.3)**	
T3	**241 (72.8)**	**117 (69.6)**	**36 (80.0)**	**88 (74.6)**	
T4	**36 (10.9)**	**13 (7.7)**	**5 (11.1)**	**18 (15.3)**	
Perineural invasion					**.002**
Negative	**206 (62.2)**	**109 (649)**	**36 (80.0)**	**61 (51.7)**	
Positive	**124 (37.5**	**58 (34.5)**	**9 (20.0)**	**57 (48.3)**	
Lymphovascular emboli					.087
Negative	201 (60.7)	107 (63.7)	31 (68.9)	63 (53.4)	
Positive	128 (38.7)	59 (35.1)	14 (31.1)	55 (46.6)	
Lymph node metastasis					**.002**
Negative	**186 (56.2)**	**95 (56.5)**	**35 (77.8)**	**56 (47.5)**	
Positive	**145 (43.8)**	**73 (43.5)**	**10 (22.2)**	**62 (52.5)**	
Lymph node stage					**.004**
N0	**185 (55.9)**	**95 (56.5)**	**35 (77.8)**	**56 (47.5)**	
N1a(1)	**45 (13.6)**	**28 (16.7)**	**3 (6.7)**	**14 (11.9)**	
N1b(2–3)	**46 (13.9)**	**22 (13.1)**	**6 (13.3)**	**18 (15.3)**	
N2a(4–6)	**27 (8.2)**	**14 (8.3)**	**0 (0.0)**	**13 (11.0)**	
N2b(≥7)	**27 (8.2)**	**9 (5.4)**	**1 (2.2)**	**17 (14.4)**	
MSI					
MSS/MSI-L	282 (85.2)	166 (98.8)	0 (0.0)	116 (98.3)	
MSI-H	45 (13.6)	0 (0.0)	45 (100)	0 (0.0)	
Unknown	4 (1.2)	2 (1.2)	0 (0.0)	2 (1.7)	
Recurrence					.370
Present	14 (4.2)	3 (1.8)	2 (4.4)	9 (7.6)	
Absent	224 (67.7)	80 (47.6)	43 (95.6)	101 (85.6)	
Metastasis					.401
Present	74 (22.4)	27 (16.1)	10 (22.2)	37 (31.4)	
Absent	168 (50.8)	57 (33.9)	35 (77.8)	76 (64.4)	

Abbreviation: SiCCS, simplified colorectal cancer subtype; MSI, microsatellite instability; MSS, microsatellite stable; MSI-L, microsatellite instability-low; MSI-H, microsatellite instability-high.
P values in boldface indicate statistical significance ($P \leq .05$).

a major clinical challenge. Previously, CRCs have been described mainly with the following distinct molecular phenotypes, 1) chromosomal instability (CIN), associated with APC, KRAS and TP53 mutations [24]; 2) microsatellite instability (MSI), caused by deficiency of genetic mismatch repair, associated with AXIN1, BRAF and BAX mutations [25–27], and commonly known to be a second mutational pathway for colorectal carcinogenesis; and 3) CpG island methylator phenotype (CIMP), which shows increased hypermethylation of gene promoter regions [28–30]. Many recent studies have reported on similar classifications of CRC using comparable strategies, based on customized bioinformatic analysis on alternative data sets, and interpretation from different perspectives [23,31–33], the subtypes of which have both shown clear relationships, and an overlap with more previously applied classification systems [34]. Some recent studies for molecular classification of CRCs have proposed that best prognostic group was associated with MSI-H/BRAF-mut/p53-negative cases with Maspin cytoplasmic predominance and high CD3 score whereas worst was observed in MSS/BRAF-mut/p53 >50% with Maspin nuclear predominance and low CD3 score [35]. Another study investigated for the EGFR signaling pathway of CRC by investigating mutations of seven genes (*KRAS-BRAF-PIK3CA-PIK3R1-AKT1-MAP2K1-PTEN*), IHC of six proteins (EGFR-p110α-p85α-PTEN-phosphoAKT-phos phoMEK1), *PTEN* deletion, and MSI and integrated these results according to five previously defined groups by Jass et al. [1,36].

These large-scaled studies on gene expression signatures have made steady progress for evolution of a novel gene expression-based molecular subtyping system as a reliable source of disease stratification. Despite these efforts, there is no internationally accepted high-consensus molecular classification of CRCs.

Due to increased activity in the field of gene expression-based molecular research, many pathways of CRC development have been elucidated. However, a major challenge exists with molecular studies on gene mutations in clinical practice, due to factors such as, time constraints, cost, and difficulty in accessibility to such testing facilities. Therefore, a simple and readily feasible method for an intrinsic molecular classification of CRC is required. Thus, each of the two studies published in 2013 introduced a method of a tissue microarray-based miniclassifier using immunohistochemistry for gene-encoding proteins of colorectal cancer subtypes [11] and

Table 3
Relationship between NMC system and clinicopathological characteristics in 214 patients with colon cancer.

Variables	Total (%)	NMC (relative% in parentheses)				p value
		E-C	G-L	T-A	S-L	
Total Number	214	33 (15.4)	18 (8.4)	57 (26.6)	106 (49.5)	
Age (years)		64.70 ± 13.9	64.78 ± 11.36	62.53 ± 10.63	62.52 ± 12.03	.216
Size (cm)		9.50 ± 4.71	5.20 ± 0.00	5.5 ± 0.87	4.27 ± 1.67	.084
Sex						.229
Male	123 (57.5)	15 (45.5)	8 (44.4)	36 (63.2)	64 (60.4)	
Female	91 (42.5)	18 (54.5)	10 (55.6)	21 (36.8)	42 (39.6)	
Location						.37
Right Colon	56 (26.2)	10 (30.3)	4 (22.2)	19 (33.3)	23 (21.7)	
Left Colon	158 (73.8)	23 (69.7)	14 (77.8)	38 (66.7)	83 (78.3)	
Histological type						43.2
Well	21 (9.8)	3 (9.1)	5 (27.8)	7 (12.3)	6 (5.7)	
Moderately	164 (76.6)	23 (69.7)	6 (33.3)	44 (77.2)	91 (85.8)	
Poorly	10 (4.7)	1 (3.0)	0 (0.0)	2 (3.5)	7 (6.6)	
Mucinous	19 (8.9)	6 (18.2)	7 (38.9)	4 (7.0)	2 (1.9)	
Invasion depth						**.025**
T1	**9 (4.2)**	**1 (3.0)**	**2 (11.1)**	**4 (7.0)**	**2 (1.9)**	
T2	**27 (12.6)**	**2 (6.1)**	**2 (11.1)**	**9 (15.8)**	**14 (13.2)**	
T3	**156 (72.9)**	**21 (63.6)**	**13 (72.2)**	**42 (73.7)**	**80 (75.5)**	
T4	**22 (10.3)**	**9 (27.3)**	**1 (5.6)**	**2 (3.5)**	**10 (9.4)**	
Perineural invasion						**.019**
Negative	**133 (62.1)**	**20 (60.6)**	**17 (94.4)**	**37 (64.9)**	**59 (55.7)**	
Positive	**81 (37.9)**	**13 (39.4)**	**1 (5.6)**	**20 (35.1)**	**47 (44.3)**	
Lymphovascular emboli						**.04**
Negative	**137 (64.0)**	**18 (54.5)**	**16 (88.9)**	**32 (56.1)**	**71 (67.0)**	
Positive	**77 (36.0)**	**15 (45.5)**	**2 (11.1)**	**25 (43.9)**	**35 (33.0)**	
Lymph node metastasis						.106
Negative	113 (52.8)	16 (48.5)	14 (77.8)	32 (56.1)	51 (48.1)	
Positive	101 (47.2)	17 (51.5)	4 (22.2)	25 (43.9)	55 (51.9)	
Lymph node stage						.386
N0	113 (52.8)	16 (48.5)	14 (77.8)	32 (56.1)	51 (48.1)	
N1a(1)	32 (15.0)	2 (6.1)	1 (5.6)	10 (17.5)	19 (17.9)	
N1b(2–3)	30 (14.0)	8 (21.2)	1 (5.6)	7 (12.3)	14 (13.2)	
N2a(4–6)	25 (11.7)	3 (9.1)	2 (11.1)	5 (8.8)	15 (14.2)	
N2b(≥7)	14 (6.5)	4 (12.1)	0 (0.0)	3 (5.3)	7 (6.6)	
MSI						.119
MSS/MSI-L	190 (88.8)	29 (87.9)	12 (66.7)	50 (87.8)	99 (93.4)	
MSI-H	20 (9.3)	4 (12.1)	5 (27.8)	6 (10.5)	5 (4.7)	
Unknown	4 (1.9)	0 (0.0)	1 (5.6)	1 (1.7)	2 (1.9)	
Recurrence						.311
Present	14 (6.5)	3 (9.1)	0 (0.0)	6 (10.5)	5 (4.7)	
Absent	200 (93.5)	30 (90.9)	18 (100.0)	51 (89.5)	101 (95.2)	
Metastasis						.525
Present	60 (28.0)	10 (30.3)	5 (27.8)	20 (35.1)	25 (23.6)	
Absent	154 (72.0)	23 (69.7)	13 (72.2)	37 (64.9)	81 (76.4)	

Abbreviations: NMC, new molecular classification; E-C, enterocyte; G-L, goblet-like; T-A, transit-amplifying; S-L, stem-like; MSI, microsatellite instability; MSS, microsatellite stable; MSI-L, microsatellite instability-low; MSI-H, microsatellite instability-high.
P values in boldface indicate statistical significance ($P \leq .05$).

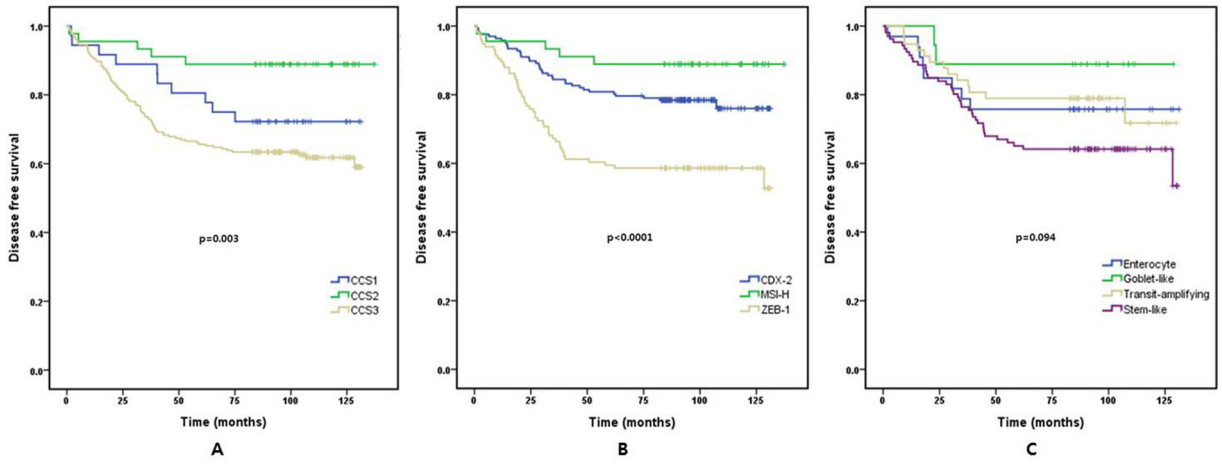

Fig. 3. Kaplan-Meier curves comparing disease free survival in colorectal cancer patients by tumour subtypes in CCS (A), CDX2/MSI/ZEB1 (B) and NMC (C). CCS3 and ZEB1 tumours have significantly worse prognosis whereas CCS2 and MSI type show better outcome. In NMC, S-L tumours have the dismal prognosis, but this is not a significant data.

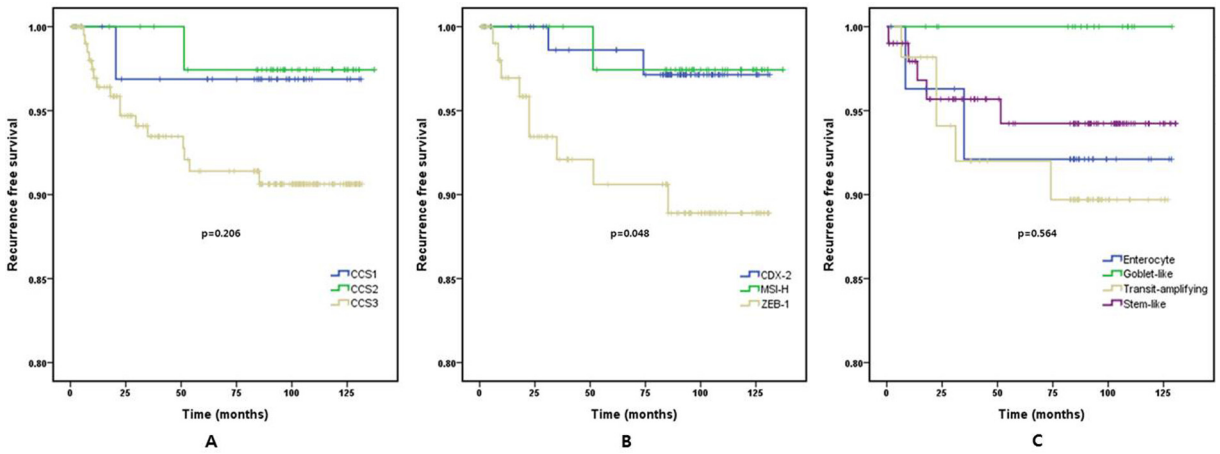

Fig. 4. Kaplan-Meier curves comparing recurrence free survival in colorectal cancer patients by tumour subtype in CCS (A), CDX2/MSI/ZEB1 (B) and NMC (C). CCS3, T-A and ZEB1 tumours showed highest recurrence rate, however, all data were not significant.

Table 4
Table showing the relationship between CCS and NMC systems.

CCS	NMC				Total	p value
	E-C	G-L	T-A	S-L		
CCS1	2	1	0	0	3	**<.0001**
	66.7%	33.3%	0.0%	0.0%	100.0%	
	10.0%	11.1%	0.0%	0.0%	2.9%	
CCS2	4	5	6	5	20	
	20.0%	25.0%	30.0%	25.0%	100.0%	
	20.0%	55.6%	24.0%	10.4%	19.6%	
CCS3	14	3	19	43	79	
	17.7%	3.8%	24.1%	54.4%	100.0%	
	70.0%	33.3%	76.0%	89.6%	77.5%	
TOTAL	20	9	25	48	102	
	19.6%	8.8%	24.5%	47.1%	100.0%	
	100.0%	100.0%	100.0%	100.0%	100.0%	

Abbreviations: CCS, colorectal cancer subtype; NMC, new molecular classification; E-C, enterocyte; G-L, goblet-like; T-A, transit-amplifying; S-L, stem-like.
[a]*P* values in boldface indicate statistical significance ($P \leq .05$).

Table 5
Table showing the relationship between SiCCS and NMC systems.

SiCCS	NMC				Total	p value
	E-C	G-L	T-A	S-L		
CDX-2	14	3	18	20	55	**.001**
	25.5%	5.5%	32.7%	36.4%	100.0%	
	53.8%	30.0%	50.0%	29.4%	39.3%	
MSI-H	4	5	6	5	20	
	20.0%	25.0%	30.0%	25.0%	100.0%	
	15.4%	50.0%	16.7%	7.4%	14.3%	
ZEB-1	8	2	12	43	65	
	12.3%	3.1%	18.5%	66.2%	100.0%	
	30.8%	20.0%	33.3%	63.2%	46.4%	
TOTAL	26	10	36	68	140	
	18.6%	7.1%	25.7%	48.6%	100.0%	
	100.0%	100.0%	100.0%	100.0%	100.0%	

Abbreviations: SiCCS, simplified colorectal cancer subtype; NMC, new molecular classification; E-C, enterocyte; G-L, goblet-like; T-A, transit-amplifying; S-L, stem-like.
[a]*P* values in boldface indicate statistical significance ($P \leq .05$).

new molecular classification system [12]. This study investigated the validity of these classification systems by examining the expression of immunohistochemical stains of common antibodies (between the two platforms) in combination with MSI status. Despite the low prevalence of MSI-H (7.7%), compared to sporadic CRCs that make up approximately 15%–20% of MSI-H [37,38], the present study showed relatively consistent results in the analysis of the CCS classification. Patients with CCS2 (MSI-H) CRCs were associated with right-sided tumours [11] and had the most favorable survival outcome, in agreement with many previous publications [24,27,39,40]. Also, CCS3 CRCs showed the worst prognosis, demonstrating significantly low DFS and RFS rates, which agreed with the findings of the study by De Sousa E. Melo et al. [11]. In addition, the simplified CCS (SiCCS), utilizing only ZEB1 positivity instead of all the three following immunohistochemistry staining, namely FRMD6/ZEB1/HTR2B, produced identical significant DFS

and RFS, compared to the original CCS. This result would suggest that ZEB1 is the most important and influential marker for CCS3, and ZEB1 could be used alone in classifying the CCS3 group. In comparison to the previous study by De Sousa E. Melo, the percentage of each subgroup was 49%, 24% and 27% in CCS1 to CCS3, respectively, which is more analogous to the results of SiCCS (50%, 14%, 36%) than CCS (12%, 15%, 73%). These differences in ratios could be associated with the results of FRMD6 and HTR2B as exclusion of these two IHC markers was the difference between CCS/SiCCS and the selection bias of the cut-off value of the antibodies is not negligible. In this study, only two tumours had serrated precursor lesions which were both classified as CCS3 but one out of them was included in ZEB1 subtype of SiCCS. Thus, due to small quantity, specific relationships between serrated lesions and CCS3/ZEB1 were unevaluable.

In NMC subtypes, several identical outcomes were observed with the results by Sadanandam et al. [12]. First, S-L tumours were mainly located in the sigmoid colon, and E-C tumours occurred most commonly in rectum. The G-L subtype was reported to be significantly associated with Duke's stage A CRCs, whereas E-C tumours were related to more advanced, stage C tumours, in concurrence with the author's results that described that early CRCs were associated with the G-L subtype, whereas T4 stage tumours were present relatively highly in the E-C subtype. Even though the DFS and RFS outcomes were not statistically significant in this study, it was similar as both results revealed that S-L tumours had the worst patient outcome with the shortest DFS, and G-L subtype showed the best prognosis. There was a major difference in the results regarding the response to the 5-FU-based chemotherapy regimen, in which the original article specified that the S-L subtype showed a beneficial response to FOLFIRI in adjuvant or metastatic setting, and the regimen in the T-A and G-L subtypes was ineffective. This was incompatible with the present result, which showed that the response to the 5-FU-based regimen in T-A subtype only, and not in any of the remaining subtypes. This result was supported by the study by Raquel et al. [41], in which the stem-like subtype had no responses to treatment with FOLFIRI, whereas patients belonging to the other subtypes responded to the treatment. Raquel et al. [41] suggested that the number of patients included in the original study by Sadanandam et al. [12] was insufficient to establish a positive correlation between classification of the tumour based on gene expression profiles and sensitivity to FOLFIRI, and therefore, the data must be confirmed by further studies to prevent erroneous correlations from leading doctors to prescribe inadequate treatments in practice [41]. Likewise, the CCS/SiCCS classifications gave contrasting results in previous studies, where CCS2/MSI-H were the subtypes with the best chemotherapeutic response to the 5-FU-based regimen in this study, which differed from the previous studies that reported that MSI-H tumours do not benefit from 5-FU-based regimens in the adjuvant setting [42,43]. However, this discrepancy could be attributed to the small sample size available for tumour recurrence, and response to the 5-FU-based chemotherapy regimen among the CCS/SiCCS and NMC subtypes, and additional, large-scaled studies are needed to examine the validity of the suggested relationships between the subtypes, treatments, and survivals outcomes.

Regarding the relationship between the CCS, SiCCS, and NMC systems, the CCS3/ZEB1 and S-L subtypes significantly overlapped between the systems (42%, and 30.7%, respectively), as expected from the results of the clinicopathological correlations in each of CCS, SiCCS, and NMC. This supports the well-established suggestion that each CRC subtype is not a completely distinct phenotype, and shares common features with other subtypes. A recently published study supports this result, in which it described four robust-consensus molecular subtypes (CMS1-4), by integrating and comparing the results of six CRC subtyping algorithms, including the CCS and NMC classifications, to resolve inconsistencies and establish the intrinsic subtypes of CRCs [17]. CMS1 comprised of the majority of MSI tumours, including the CCS2 and inflammatory subtypes, showing an association with right-sided lesions, and higher histopathological grade. CMS4 tumours showed upregulation of genes implicated in epithelial-to-mesenchymal transition, and incorporated CCS3 and stem-like subtypes, tended to be diagnosed at a more advanced stage, and were associated with worse DFS, and poor relapse-free survival. CMS2 showed high concordance with the CCS1, E-C, and T-A subtypes, which were mainly located in the left-hand side [17]. These findings therefore were vastly similar to that of the present study.

This study was conducted in order to contribution to the search for a high-consensus classification of CRC, and the authors validated the significance of the two previously introduced CRC intrinsic molecular classification systems, which utilized simple and rapidly accessible immunohistochemistry and MSI study only. Due to this simplified method, the inflammatory (I) subtype of NMC was excluded in this study as it required several molecular studies. Hence, discovering a methodologically simple way to include the inflammatory subtype in the study would modify the classification system and produce more reliable results. Also for CCS, a more simplified method using fewer immunohistochemistry stains could be expected with further detailed and thorough investigations.

In conclusion, this study suggests that the CCS and NMC subtypes, classified by several immunohistochemical stains of epithelial-encoding proteins, and the results of MSI status, agreed with previous studies in many ways, and has can be considered to be relatively reliable in its representation of distinct subtypes. Furthermore, certain subtypes from each classification system were indistinguishable from one another, due to the fact that they demonstrated a significant overlap in expression profiles, for example, the CCS3/ZEB1 and S-L subtypes. However, there were several important limitations in this study. First, only 53.5%, 58.3%, and 37.7% of the total tumour samples could be evaluated according to the CCS, SiCCS, and NMC classification protocols. This was critical as only about half of the participants were included in CCS/SiCCS and even less in NMC and left large numbers of unclassified cases, which inflicted restrictions in prognostic and predictive results. Second, due to insufficient data pool, association with serrated precursor lesions could not be determined. And third, a bias is not negligible in selecting the cut-off values of the immunohistochemical stains as it was adjusted to obtain the maximal numbers of evaluable cases. Lastly, simplification of this study induced exclusion of some of the potent markers such as K-ras, CIMP, BRAF mutation and P53, which is another impediment that could be redeemed in future large-scaled studies.

Until now, continuous studies have been ongoing for markers to set a classification of CRC for patients' clinical prognosis and predictions. However, in comparison to the results which have allowed a better clinicopathological classification of tumours, not a considerable improvement in translational medicine has been proposed. Therefore, there is still a need for establishment of a consensus gene expression-based subtyping classification system, which will hopefully unite the overlapped subtypes into one, thereby allowing the categorization of the majority of, if not all, CRC tumours.

Conflict of interest

None.

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