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ORIGINAL ARTICLE

Phenotypic subtypes predict outcomes in colorectal cancer

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ABSTRACT

Background: Colorectal cancer (CRC) is the second leading cause of cancer-related deaths globally. The Colorectal Cancer Subtyping Consortium used the transcriptome-based method to classify CRC according to four molecular subtypes, each showing different genomic alterations and prognoses: CMS1 (microsatellite instable [MSI] immune), CMS2 (canonical), CMS3 (metabolic), and CMS4 (mesenchymal). To expedite the clinical implementation of such methods, easier and preferably tumor phenotype–based methods are needed. In this study, we describe a method to divide patients into four phenotypic subgroups using immunohistochemistry. Moreover, we analyze disease-specific survival (DSS) among different phenotypic subtypes and the associations between the phenotypic subtypes and clinicopathological variables.

Methods: We categorized 480 surgically treated CRC patients into four phenotypic subtypes (immune, canonical, metabolic, and mesenchymal) using the immunohistochemically determined CD3–CD8 tumor–stroma index, proliferation index, and tumor–stroma percentage. We analyzed survival rates for the phenotypic subtypes in different clinical patient subgroups using the Kaplan–Meier method and Cox regression analysis. Associations between phenotypic subtypes and clinicopathological variables were examined using the chi-square test.

Results: Patients with immune subtype tumors exhibited the best 5-year DSS, while mesenchymal subtype tumors accompanied the worst prognosis. The prognostic value of the canonical subtype showed wide variation among different clinical subgroups. Immune subtype tumors were associated with being female, stage I disease, and a right-side colon location. Metabolic tumors, however, were associated with pT3 and pT4 tumors, and being male. Finally, a mesenchymal subtype associated with stage IV disease, a mucinous histology, and a rectal tumor location.

Conclusions: Phenotypic subtype predicts patient outcome in CRC. Associations and prognostic values for subtypes resemble the transcriptome-based consensus molecular subtypes (CMS) classification. In our study, the immune subtype stood out with its exceptionally good prognosis. Moreover, the canonical subtype showed wide variability among clinical subgroups. Further studies are needed to investigate the concordance between transcriptome-based classification systems and the phenotypic subtypes.

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Phenotypic subtype; CMS; consensus molecular subtypes; colorectal cancer; colon cancer; rectal cancer; immunohistochemistry

Background

The global incidence of colorectal cancer (CRC) is expected to rise from 1.8 million in 2018 to 2.2 million by 2030 [1]. Representing the second-leading cause of cancer-related deaths, with approximately 880,000 deaths reported in 2018, CRC causes a substantial disease burden [1,2]. As a molecularly heterogeneous disease with varying outcomes and drug responses, better methods of classifying CRC tumors are needed.

In 2015, the Colorectal Cancer Subtyping Consortium [3] proposed a consensus-based molecular subtype (CMS) classification for CRC based on six previous gene expression–based classification systems [4–9]. The consortium concluded that CRC could be divided into four molecular subtypes each showing different genomic alterations and prognoses: CMS1 (microsatellite instable [MSI] immune), CMS2 (canonical), CMS3 (metabolic), and CMS4 (mesenchymal) [3].

However, the gene expression–based analysis of clinical samples is expensive and time-consuming, hampering its use in routine clinical pathology. Since the genomic alterations and tumor developmental pathways differ between CMS subgroups, the subgroups are also phenotypically different. As such, promising methods to categorize patients among CMS subtypes according to a tumor's phenotypic characteristics determined with immunohistochemistry (IHC) have been developed [10–12]. For instance, Roseweir et al. [10] proposed a method which classifies tumors along four phenotypic subtypes using three IHC parameters: the *immunoscore*, the proliferation index, and the tumor–stroma percentage (TSP).

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Microsatellite instable (MSI)–immune CMS1 tumors are associated with immune infiltration of CD3-positive T-helper 1 (Th1) and CD8-positive cytotoxic T cells (CTLs) [3,13]. The *immunoscore*, which also has an exceptional prognostic value on its own, is determined using the intratumoral and stromal densities of CD3- and CD8-positive lymphocytes. Specifically, a high *immunoscore* is an important phenotypic characteristic present in CMS1 tumors [10,14,15]. CMS1 tumors appear to have the best prognosis of the CMS subtypes [3]. Moreover, in stage I–III CRC, MSI has also been associated with a better prognosis [16].

The chromosomal instability of *canonical* CMS2 tumors leads to changes in the WNT and MAPK pathways (also referred to as the canonical Wnt pathway), which in combination with the upregulation of β-catenin is implicated in leading to a high cancer cell proliferation in CRC carcinogenesis [17]. A high proliferation index reflects the proliferative activity of CMS2 tumors [18].

In *mesenchymal* CMS4 tumors, the expression of genes involved in the epithelial–mesenchymal transition (EMT) are upregulated and show a gene-expression profile indicative of a high stromal infiltration of the tumor cells [3]. Moreover, cancer-associated fibroblasts (CAFs) present in the tumor-associated stroma take part in extracellular matrix remodeling and inducing EMT. In addition, the amount of tumor-associated stroma mirrors the activity of CAFs and a tumor's invasive capabilities [19]. TSP is calculated from the proportion of tumor-infiltrating stroma, serving as a prominent phenotypic characteristic in CMS4 tumors [10,20]. CMS4 tumors carry the worst prognosis in the CMS classification [3].

Metabolic CMS3 tumors undergo metabolic changes to a glycolytic state in order to survive in a hostile environment [21–24]. The metabolic dysregulation of CMS3 tumors is difficult to measure using routine IHC methods. However, these tumors are thought to be concentrated in the phenotypic group with a combined low *immunoscore*, a low proliferation index, and a low TSP [10]. While all of the aforementioned markers could be readily used in clinical settings, further studies are needed to determine their ability to distinguish between different CRC subtypes.

In this study, we describe a novel method of classifying CRC into phenotypic subgroups. To do so, we assessed the CD3–CD8 tumor–stroma index [25], applying methods resembling the *immunoscore* [26], the proliferation index, and TSP on 550 colorectal cancer patients. We categorized patients along four phenotypic subtypes according to these tumor characteristics, and analyzed the differences in the disease-specific survival (DSS) among patients, comparing different phenotypic subtype groups and the associations between the phenotypic subtypes and clinicopathological variables.

Material and methods

Patients and clinical information

We retrospectively examined 550 patients who underwent surgery for the removal of a primary tumor for CRC in the Department of Surgery at Helsinki University Hospital (Finland) between 1998 and 2005. The elective CRC patients included in our analysis were consecutively selected. Essential clinical data were collected from patients' medical records. Patients with a previous cancer or an autoimmune condition were excluded from the analysis.

Staging was performed at the time of surgery, and metachronous metastasis detected during surveillance was interpreted as disease recurrence. Tumors were divided by location as right-sided colon tumors (located proximal to the splenic flexure), left-sided colon tumors, and rectal tumors. We excluded tumors located in the splenic flexure from the analyses of tumor location.

Cause of death and survival data were provided by the Finnish Digital and Population Data Services Agency and Statistics Finland. The Surgical Ethics Committee of Helsinki University Hospital approved the study protocol (Dnro HUS 226/E6/06, extension TMK02 §66 17.4.2013). Permission to examine the archived tissue samples without requiring individual patient consent was obtained from the National Supervisory Authority of Health and Welfare (Valvira Dnro 10041/06.01.03.01/2012).

Sample preparation

Samples were prepared in the Department of Pathology at Helsinki University Hospital. All analyses were performed using samples taken from the primary tumor. After retrieving samples from the archives, an experienced pathologist (JH) marked representative areas of the tumor from hematoxylin- and eosin-stained slides. A semiautomatic tumor microarray (TMA) instrument was used to punch 1-mm-diameter cylinders from the selected areas. The cylinders were subsequently embedded into new TMA paraffin blocks that were used for the immunohistochemical (IHC) staining. Each block contained a maximum of 56 cylinders. After construction, the TMA paraffin blocks were cut into 4-μm-thick sections using a microtome to enable IHC staining.

CD3 and CD8 immunostaining

We used an automatic Roche Ventana BenchMark ULTRA (F. Hoffman-La-Roche AG). Pretreatment (deparaffinization, rehydration, and antigen retrieval) was performed by incubating the slides for 64 min in a Ventana Cell Conditioning (CC1) solution at 95 °C. Then, slides were incubated for 40 min with primary antibodies (ready-to-use rabbit monoclonal CD3 antibody [Ventana, clone 2GV6] or mouse monoclonal CD8 antibody [Novocastra, clone 4B11; diluted to 1:50]) at 37 °C. Visualization of primary antibodies was carried out using a Ventana Ultraview DAB detection kit and counterstaining with Meyers's hematoxylin.

Determination of the proliferation index and cytokeratin immunostaining

Pretreatment was carried out in a pretreatment module (Agilent Technologies Inc., Dako), where slides were treated in an EnVision Flex target retrieval solution (Dako, DM828) at

98 °C for 15 min. Next, slides were placed in an Autostainer 480 (Lab Vision Corp), where they were incubated with the primary antibodies: mouse monoclonal antihuman Ki-67 antigen antibodies (Dako, clone MIB-1, diluted to 1:100) for 1 h at room temperature or mouse monoclonal antihuman epidermal cytokeratin Pan Keratin antibodies (Thermo Scientific, clone AE1/AE3, diluted 1:200) overnight at +5 °C. Then, slides were incubated for 30 min with HRP labeled EnVision Flex/HRP secondary antibodies (Dako, SM802). Finally, visualization was carried out through 10-min incubation with EnVision Flex DAB chromogen (Dako, DM827) and slides were counterstained with Meyer's hematoxylin.

Evaluation of immunohistochemistry

Scoring was performed without knowledge about patients' clinical data and outcomes. All samples were scored first by an investigator (JK) using a light microscope and, then, reviewed by an experienced pathologist (JH). If disagreements emerged regarding a sample score, they were discussed and consensus was achieved.

The CD3 and CD8 densities were scored separately from the tumor epithelium (i.e. intratumoral areas) and the surrounding stroma. The intratumoral densities of CD3 and CD8 ($CD3^T$ and $CD8^T$) were scored on a 0–3 scale: score 0 (no positive cells); score 1 (scattered individual positive cells); score 2 (small clusters of positive cells); and score 3 (abundant organized staining). The stromal CD3 and CD8 ($CD3^S$ and $CD8^S$) were scored on a 0–4 scale: score 0 (no positive cells); score 1 (sparsely scattered individual cells); score 2 (scattered small cell clusters); score 3 (medium positive cell clusters); and score 4 (abundant organized staining).

In the evaluation of the proliferation index, the percentage of MIB1-immunopositive cells among the tumor epithelium was visually evaluated with a 10% precision.

TSP was evaluated by visually approximating the percentage of the stromal tissue among the cytokeratin-positive tumor epithelium in the sample with a 10% precision.

Determining the CD3–CD8 tumor–stroma index

The intratumoral and stromal densities of CD3- and CD8-positive immune cells were dichotomized as low and high: intratumoral areas receiving an initial score of 0 in the immunostaining were considered 'low', while scores 1–3 were considered 'high', whereas stromal areas receiving an initial score of 0–3 in the immunostainings were categorized as 'low' and a score of 4 as 'high'. The dichotomized intratumoral and stromal CD3 and CD8 densities were used to calculate a 0–4-point CD3–CD8 tumor–stroma index. One CD3–CD8 tumor–stroma index point was given for every 'high'-density staining.

Determining the phenotypic subtypes

The CD3–CD8 tumor–stroma index, proliferation index, and TSP were categorized as high and low in order to enable the determination of the phenotypic subtypes. Tumors scored as 3–4 for the CD3–CD8 tumor–stroma index were considered high. Samples containing 50% or more MIB1-immunopositive cells were categorized as having a high proliferation index. Specimens with 50% or more stromal tissue were categorized as a high TSP.

Figure 1 provides a schematic representation of the determination of the phenotypic subtypes. Tumors with a high CD3–CD8 tumor–stroma index were classified as group 1 (*immune*). Samples with a low CD3–CD8 tumor–stroma index and a high TSP were classified as group 4 (*mesenchymal*). The remaining specimens with a low CD3–CD8 tumor–stroma index and a low TSP were divided into group 2 (*canonical*) and group 3 (*metabolic*) based on the proliferation index. Tumors with a high proliferation index were considered *canonical* and those with a low proliferation index were classified as *metabolic*. The phenotypic subtype was successfully determined in 480 patients (87.2%).

Statistical analysis

For all statistical analyses, we used IBM SPSS Statistics version 27 for Mac (IBM SPSS Statistics, version 27; SPSS, Inc., an IBM Company, Chicago, IL, USA). The Pearson's chi-square test was applied for the evaluation of associations between the phenotypic subtypes and clinicopathological variables. The Kaplan–Meier method was used to construct survival curves, which were compared using the log-rank method. Hazard ratios (HRs) were assessed using the Cox proportional hazards model. DSS was calculated from the date of surgery until the date of death due to CRC. In the multivariable model, we included age, gender, stage, grade, tumor location, and phenotypic classification. Stage and grade were

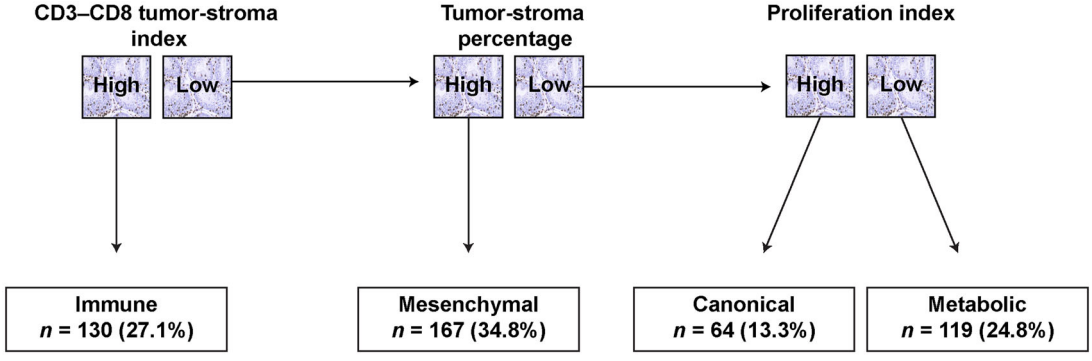

Figure 1. Flowchart illustrating the determination of the phenotypic subtypes according to the CD3–CD8 tumor–stroma index, proliferation index, and tumor–stroma percentage.

Table 1. Associations between the phenotypic subtype and clinicopathological variables among 480 colorectal cancer patients.

Clinicopathological variable	Phenotypic subtype				p Value[a]
	Immune *n* (%)	Canonical *n* (%)	Metabolic *n* (%)	Mesenchymal *n* (%)	
Age					
<69	59 (25.8%)	28 (12.2%)	57 (24.9%)	85 (37.1%)	0.713
≥69	71 (28.3%)	36 (14.3%)	62 (24.7%)	82 (32.7%)	
Gender					
Female	74 (32.2%)	27 (11.7%)	45 (19.6%)	84 (36.5%)	**0.016**
Male	56 (22.4%)	37 (14.8%)	74 (29.6%)	83 (33.2%)	
Stage (I–IV)					
I	42 (43.4%)	16 (16.2%)	18 (18.2%)	23 (23.2%)	**<0.001**
II	40 (28.8%)	19 (13.7%)	34 (24.5%)	46 (33.1%)	
III	38 (22.1%)	22 (12.8%)	50 (29.1%)	62 (36.0%)	
IV	10 (14.5%)	7 (10.1%)	17 (24.6%)	35 (50.7%)	
pT					
1–2	49 (40.2%)	24 (19.7%)	22 (18.0%)	27 (22.1%)	**<0.001**
3–4	80 (22.9%)	37 (10.6%)	96 (27.4%)	137 (39.1%)	
pN					
Negative	84 (33.3%)	36 (14.3%)	55 (21.8%)	77 (30.6%)	**0.003**
Positive	43 (19.6%)	26 (11.9%)	63 (28.8%)	87 (39.7%)	
pM					
Nonmetastasized	119 (29.2%)	55 (13.5%)	101 (24.8%)	132 (32.4%)	**0.018**
Metastasized	10 (14.5%)	7 (10.1%)	18 (26.1%)	34 (49.3%)	
Grade (WHO)					
1	12 (30.8%)	7 (17.9%)	9 (23.1%)	11 (28.2%)	0.817
2	92 (26.7%)	47 (13.6%)	83 (24.1%)	123 (35.7%)	
3	9 (34.6%)	3 (11.5%)	8 (30.8%)	6 (23.1%)	
4	9 (34.6%)	3 (11.5%)	8 (30.8%)	6 (23.1%)	
Tumor location					
Right colon	61 (44.9%)	19 (14.0%)	24 (17.6%)	32 (23.5%)	**<0.001**
Left colon	27 (22.9%)	15 (12.7%)	39 (33.1%)	37 (31.4%)	
Rectum	42 (18.7%)	30 (13.3%)	56 (24.9%)	97 (43.1%)	

[a]Pearson chi-square test.
The significant p values ($p<0.05$) appear in bold.

processed as categorical covariates. The multivariable model fulfilled the assumption of constant hazards, and we detected no significant interaction terms between the variables. The two-tailed threshold for statistical significance was set to $p < 0.05$.

Results

Patients

The median age among patients in the cohort was 69.2 years (interquartile range [IQR] 59.2–77.4 years) at the time of diagnosis, and 52.6% of patients were male. By the end of the follow-up period, 379 patients (69.0%) had died due to CRC and the median follow-up time was 6.44 years (IQR 2.00–14.9). The sixth edition of the AJCC colon and rectum cancer staging was used to determine the TNM stage [27]. Table S1 summarizes the patient characteristics.

The CD3–CD8 tumor–stroma index was successfully determined in 499 patients (90.7%), the proliferation index in 507 patients (92.2%), and TSP in 480 patients (87.2%). The scoring failed in some cases due to missing tumor tissue or deficient TMA spots. Representative images of the IHC CD3, CD8, cytokeratin, and Ki-67 stainings appear in Figure S1.

Associations between phenotypic subtypes and clinicopathological variables

Table 1 summarizes the associations between the phenotypic subtypes and clinicopathological variables. The *immune* subtype was associated with female gender ($p = 0.017$), stage I disease ($p < 0.001$), pT1–2 status ($p < 0.001$), negative lymph nodes (pN, $p = 0.002$), nonmetastasized disease ($p = 0.009$), and a location in the right colon ($p < 0.001$, Table 1). The *metabolic* subtype was associated with locally advanced (pT3–4) tumors ($p < 0.001$) and male gender ($p = 0.017$, Table 1). The *mesenchymal* subtype was associated with stage IV disease ($p < 0.001$), locally advanced (pT3–4) tumors ($p < 0.001$), positive lymph nodes (pN, $p = 0.002$), metastasized disease ($p = 0.017$), and a rectal tumor location ($p < 0.001$, Table 1).

Survival analysis

The 5-year DSS among patients with the *immune* subtype was 85.2% (95% confidence interval [CI] 78.9–91.5%), 71.7% (95% CI 59.9–83.5%) among those with the *canonical* subtype, 69.2% (95% CI 60.2–78.0%) among the *metabolic* subtype, and 57.1% (95% CI 49.0–65.1%) among the *mesenchymal* subtype (log-rank test: $p < 0.001$, Figure 2(A)). Among stage I–III patients, the 5-year DSS for *immune* subtype tumors was 89.4% (95% CI 83.7–95.1%), 78.1% (95% CI 66.5–89.7%) among *canonical* subtype tumors, 77.5% (95% CI 69.1–85.9%) among *metabolic* subtype tumors, and 70.1% (95% CI 61.9–78.3%) among *mesenchymal* subtype tumors (log-rank test: $p = 0.003$, Figure 2(B)).

Subgroup analysis

Table 2 summarizes the hazard ratios (HRs) for the phenotypic subtypes among the different groups. Exhibiting the

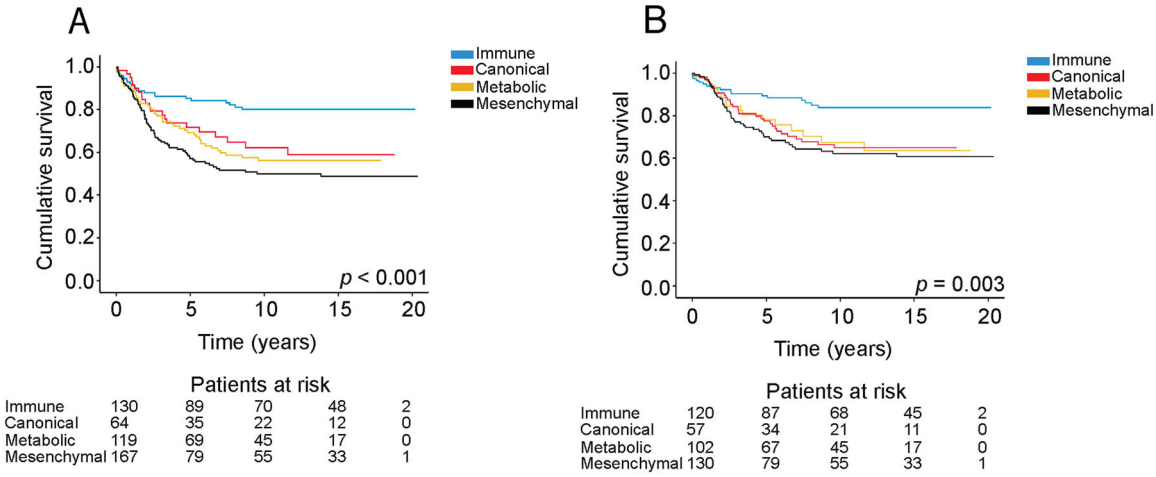

Figure 2. Disease-specific survival of (A) stage I–IV and (B) stage I–III CRC patients according to phenotypic subtype. Survival curves were drawn according to the Kaplan–Meier method, with the *p* value based on the log-rank test.

Table 2. Subgroup analysis of phenotypic subtypes. Immune subtype served as the reference value in the Cox proportional hazards model.

	Phenotypic subtype							
Clinicopathological Variable	Immune HR (95% CI)	Number of Patients	Canonical HR (95% CI)	Number of Patients	Metabolic HR (95% CI)	Number of Patients	Mesenchymal HR (95% CI)	Number of Patients
Age								
<69	1.00	59	4.82 (1.92–10.9)	28	**3.43 (1.53–7.72)**	57	**3.95 (1.83–8.50)**	85
≥69	1.00	71	0.95 (0.39–2.33)	36	1.92 (1.00–3.69)	62	**2.75 (1.51–4.99)**	82
Gender								
Female	1.00	74	1.63 (0.64–4.14)	27	**2.68 (1.29–5.56)**	45	**3.43 (1.80–6.56)**	84
Male	1.00	56	**2.32 (1.05–5.11)**	37	**2.13 (1.06–4.30)**	74	**2.74 (1.39–5.40)**	83
Stage (I–IV)								
I	1.00	42	0.80 (0.08–7.70)	16	0.70 (0.07–6.70)	18	1.17 (0.19–6.99)	23
II	1.00	40	1.38 (0.25–7.54)	19	2.15 (0.63–7.35)	34	**3.47 (1.14–10.6)**	46
III	1.00	38	**2.89 (1.26–6.59)**	22	2.08 (0.99–4.38)	50	**2.23 (1.08–4.57)**	62
IV	1.00	10	1.35 (0.41–4.45)	7	2.30 (0.88–6.03)	17	1.81 (0.74–4.42)	35
pT								
1–2	1.00	49	1.92 (0.48–7.68)	24	1.01 (0.19–5.52)	22	2.81 (0.79–9.98)	27
3–4	1.00	80	**2.54 (1.32–4.89)**	37	**2.19 (1.27–3.77)**	96	**2.55 (1.53–4.25)**	137
pN								
Negative	1.00	84	1.93 (0.61–6.08)	36	2.21 (0.84–5.81)	55	**3.78 (1.61–8.84)**	77
Positive	1.00	43	**2.05 (1.01–4.14)**	26	**1.88 (1.02–3.45)**	63	**2.19 (1.23–3.90)**	87
pM								
Nonmetastasized	1.00	119	**2.35 (1.19–4.66)**	55	**2.22 (1.23–4.03)**	101	**2.71 (1.55–4.73)**	132
Metastasized	1.00	10	1.36 (0.41–4.49)	7	2.26 (0.87–5.88)	18	2.15 (0.87–5.35)	34
Grade (WHO)								
1–2	1.00	104	**2.25 (1.59–2.90)**	54	**2.10 (1.52–2.68)**	92	**3.08 (2.54–3.60)**	134
3–4	1.00	18	**2.67 (1.16–4.18)**	6	**4.85 (3.65–6.05)**	16	**3.04 (1.72–4.36)**	12
Tumor location								
Right colon	1.00	61	0.95 (0.27–3.39)	19	**3.11 (1.37–7.08)**	24	**3.08 (1.44–6.60)**	32
Left colon	1.00	27	0.59 (0.16–2.18)	15	1.51 (0.68–3.37)	39	1.91 (0.86–4.23)	37
Rectum	1.00	42	**12.6 (2.89–55.2)**	30	**6.44 (1.48–28.0)**	56	**10.4 (2.52–43.1)**	97

Abbreviations: HR: hazard ratio; CI: confidence interval.
Statistically significant ($p<0.05$) HRs appear in bold.

best prognosis in an earlier analysis, the *immune* subtype was used as the reference value in the Cox regression analysis. Compared to the *immune* subtype, in patients under 69 years old, *canonical* (HR 4.82, 95% CI 1.92–10.9, $p < 0.001$), *metabolic* (HR 3.43, 95% CI 1.52–7.72, $p = 0.003$), and *mesenchymal* subtypes (HR 3.95, 95% CI 1.83–8.50, $p < 0.001$) indicated a worse survival (Figure S2A). The *metabolic* (HR 1.92, 95% CI 1.00–3.69, $p = 0.049$) and *mesenchymal* subtypes (HR 2.87, 95% CI 1.58–5.22, $p < 0.001$) indicated a worse prognosis among patients 69 years or older (Figure S2B). Among patients with right-sided colon cancer, those with the *metabolic* (HR 3.11, 95% CI 1.37–7.08, $p = 0.007$) and *mesenchymal* subtypes (HR 3.08, 95% CI 1.44–6.60, $p = 0.004$) exhibited a worse prognosis (Figure S3A). Interestingly, the phenotypic subtype was not a significant prognostic factor among those with left-sided colon cancer (Figure S3B). Rectal cancer patients with the *immune* subtype had a substantially better DSS compared with the *canonical* (HR 12.6, 95% CI 2.89–55.2, $p < 0.001$), *metabolic* (HR 6.44, 95% CI 1.48–28.0, $p = 0.013$), and *mesenchymal* subtypes (HR 10.4, 95% CI 2.52–43.1, $p = 0.001$, Figure S3C). The *immune* subtype also emerged as a favorable prognostic factor in stage III disease compared with patients with *canonical* (HR 2.89, 95% CI 1.26–6.59, $p = 0.012$) and *mesenchymal* subtypes (HR 2.23, 95% CI 1.08–4.57, $p = 0.029$, Figure S4C). Phenotypic subtype was not a significant prognostic factor in patients with stage I, stage II,

Table 3. Multivariable Cox regression analysis for disease-specific survival among colorectal cancer patients.

Multivariable analysis		
Clinicopathological Variable	HR (95% CI)	*p* Value
Age		
<69	1.00	**0.042**
≥69	1.41 (1.01–1.95)	
Gender		
Female	1.00	0.426
Male	1.15 (0.84–1.60)	
Stage (I–IV)		
I	1.00	**<0.001**
II	2.08 (0.85–5.13)	
III	7.41 (3.20–17.2)	
IV	25.2 (10.5–60.3)	
Grade (WHO)		
1	1.00	0.675
2	0.92 (0.49–1.74)	
3	1.16 (0.44–3.07)	
4	1.29 (0.56–2.94)	
Tumor location		
Right colon	1.00	0.180
Left colon	1.21 (0.77–1.91)	
Rectum	0.84 (0.54–1.32)	
Phenotypic subtype		
Immune	1.00	**0.010**
Canonical	2.10 (1.14–3.88)	
Metabolic	2.06 (1.21–3.51)	
Mesenchymal	2.40 (1.44–4.02)	

Abbreviations: HR: hazard ratio; CI: confidence interval.
Significant *p* values ($p<0.05$) appear in bold.

or stage IV disease (Figures S4A, S4B, and S4D). In addition, the *immune* subtype indicated a better prognosis in patients with pT3 and pT4 tumors, negative lymph nodes, positive lymph nodes, nonmetastasized disease, and grade 1–2 and grade 3–4 tumors (Table 2).

Multivariable analysis

In the multivariable analysis, an older (≥69 years) age (HR 1.41, 95% CI 1.01–1.95, $p = 0.042$, Table 3), and stage III (HR 7.41, 95% CI 3.20–17.2) or stage IV (HR 25.2, 95% CI 10.5–60.3) disease ($p < 0.001$, Table 3) at the time diagnosis emerged as independent unfavorable prognostic factors. Having *immune, canonical* (HR 2.10, 95% CI 1.14–3.88), *metabolic* (HR 2.06, 95% CI 1.21–3.51), or *mesenchymal* (HR 2.40, 95% CI 1.44–4.02) subtype tumors carried a significant independent prognostic value ($p = 0.010$, Table 3).

Discussion

This study describes a novel method of categorizing CRC according to phenotypic subgroups, a method as yet not validated against the CMS classification, but which might prove more practical to implement in a clinical setting. We found that immunohistochemically determined phenotypic CRC subtypes predict outcomes among 480 CRC patients in a manner resembling the transcriptome-based CMS classification [3,10,28]. Here, patients falling into the *immune* subtype exhibited the best prognosis and patients with the *mesenchymal* subtype exhibited the worst prognosis. Prognosis among patients with the *canonical* and *metabolic* subtypes varied across different subgroups.

In line with the CMS classification [3], our *immune* subtype (resembling CMS1) associated with right-sided tumors and being female. Specifically, the *mesenchymal* subtype we identified was associated with advanced disease, resembling the original CMS4 subgroup. In comparison, Guinney et al. [3] also reported associations between CMS1 and a high histological grade, and between CMS2 and left-sided colon tumors. Our findings related to survival rates and associations demonstrate a good prognosis and a right-side location of the *immune* tumor subtype, along with a worse prognosis among *mesenchymal* subtype tumors. These findings correspond with earlier CMS results suggesting that the phenotypic subtypes at least partly identify similar patients.

Other studies have described a CMS mini-classifier tool based on the immunohistochemistry of four gene product proteins (CDX2, FRMD6, HTR2B, and ZEB1) combined with the MSI determination [11,12]. Such studies reported similar associations to ours between a right-sided tumor location and the CMS1 subgroup [11]. However, in their study, the *mesenchymal* subtype was not significantly associated with advanced disease, contrary to our findings and indications from the original CMS classification [3,11]. Thus, the CMS mini-classifier does not differentiate between CMS2 and CMS3 tumors; but, if combined with β-catenin determination, it can be used to allocate patients to all subgroups [11]. Moreover, using only MSI to determine CMS1 patients does not recognize hypermutated tumors with microsatellite stability (MSS), which are also associated with a CMS1-like immune reaction [29].

In the subgroup analysis, the exceptionally good prognosis among *immune* subtype patients under the age of 69 stood out in our findings. Generally, among young patients, CRC tends to associate with advanced disease and high recurrence rates [30]. Since stage IV patients underwent surgery of the primary tumor with curative intent, we argue that it is also important to assess the prognostic value of the phenotypic subgroups among patients with stage IV disease. Moreover, including stage IV patients did not change the primary findings reported here. Even though the subgroups are relatively small and the analysis must be interpreted carefully, our results indicate that the *immune* subtype appears to be a strong protective factor among younger patients. Moreover, rectal cancer patients with an *immune* subtype tumor had an exceptionally good prognosis in our series. None of the *immune* subtype rectal cancer patients who were under 69 ($n = 20$), male ($n = 18$), or who had stage III–IV disease ($n = 11$) died from CRC during follow-up. In line with CMS1 tumors, the prevalence of *immune* subtype tumors unfortunately appears to decrease in the left colon and rectum compared with tumors in the right colon [31].

The prognosis for CRC patients with a high proliferation index varies between studies [32–34]. One explanation could be that, although highly proliferative tumors are often more aggressive, they are also metabolically more active and might respond better to adjuvant chemotherapy. Interestingly, in our study, the prognosis among patients

with *canonical* tumors who also had a high proliferation index varied substantially in different clinical subgroups. For instance, in patients aged 69 or older and in patients with colon cancer, prognosis among individuals with *canonical* tumors closely resembled the favorable prognosis accompanying *immune* tumors. However, in rectal cancer patients and among patients younger than 69, those with *canonical* tumors exhibited a worst prognosis. Notably, Roseweir et al. [28] used a method similar to ours to classify 1030 stage I–III CRC patients treated with a curative intent according to phenotypic subgroups. As in our study, prognosis among *canonical* patients with stage I or II disease resembled the favorable prognosis found among *immune* tumor patients; however, the prognosis among stage III *canonical* tumor patients was remarkably worse and resembled the *mesenchymal* tumor subgroup. Thus, it appears that clinicopathological factors greatly influence prognosis among *canonical* tumor patients. In young patients with rectal tumors and advanced disease in particular, a *canonical* subtype apparently associates with a poor prognosis.

The strengths of our study include the well-characterized cohort with reliable data on survival obtained from Statistics Finland and the Finnish Digital and Population Data Services Agency. In addition, the long follow-up time allowed us to precisely determine patient outcomes. By contrast, the limitations to this study include the single-center setting and the incomplete data on comorbidities.

According to our results the phenotypic subgroups seem to associate with other clinical parameters and predict outcomes in a fashion resembling genetically determined CMS subgroups. Determining CMS subgroups with a few IHC markers would expedite establishing outcome predictions and a more accurate adjuvant therapeutic direction. Interestingly, Roseweir et al. [28] have already shown that using a method similar to ours to identify phenotypically determined *immune* tumor patients yields a better response to FOLFOX adjuvant chemotherapy compared with CAPOX therapy.

The IHC methods used in our study are available at a low cost compared with genomic assessments, and are readily translatable to clinical practice. Interpreting the immunohistochemistry is straightforward and can be easily taught to pathologists as well as other clinicians. However, agreement between the phenotypic subtypes and CMS has not yet been thoroughly investigated. In addition, assessing TSP from small areas of tumor tissue, such as TMA spots, differs from assessing the proportion of the tumor-associated stroma from whole-tissue sections. Thus, assessing TSP from whole-tissue sections instead of TMA might provide different results and needs to be addressed in the future. A study in which the phenotypic subtypes and CMS are both assessed and compared, preferably in a large cohort, is needed in order to explore how well the phenotypic properties correspond to the actual molecular genetic profile of the tumor and the associated cells.

To conclude, it seems that the phenotypic CRC subtypes predict outcome in a manner resembling the genetically determined CMS classification. The phenotypic characteristics of tumors can be reliably determined using a semiquantitative IHC method. More specifically, the *immune* subtype stood out in this study with an exceptionally good prognosis. In addition, *mesenchymal* subtype patients exhibited the worst prognosis among the entire cohort as well as in most subgroups. Prognosis among *canonical* subtype patients greatly varied among different subgroups. Although all markers could be readily used in clinical settings, additional studies are still needed to validate the concordance of transcriptome-based and phenotypic assessments.

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Data availability statement

The data are available upon request from the corresponding author.

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