ORIGINAL RESEARCH

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Systemic inflammation is associated with circulating cell death released keratin 18 fragments in colorectal cancer

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ABSTRACT

Systemic inflammation is a stage-independent marker of poor prognosis in colorectal cancer (CRC), activated in a complex, multifactorial process. It has been proposed that one of the main factors driving systemic inflammation may be tumor necrosis. Keratin 18 (KRT18) fragments are released from dead cells and their serum levels are markers for apoptotic and necrotic cell death. In CRC, high KRT18 levels associate with advanced disease, but their relationship with tumor necrosis and systemic inflammation is unknown. In this study, serum total soluble KRT18 (tKRT18) and apoptosis-related, caspase-cleaved fragment (aKRT18) levels were measured preoperatively from 328 CRC patients, and their difference was calculated to assess necrosis related KRT18 (nKRT18) levels. The relationships of these markers with tumor necrosis, clinicopathologic features, systemic inflammation markers (C-reactive protein, albumin, and 13 cytokines), and survival were analyzed. High serum tKRT18, aKRT18, and nKRT18 levels showed association with a higher extent of tumor necrosis, distant metastasis, and increased levels of several markers of systemic inflammation, including CXCL8. High serum tKRT18 (multivariable HR 1.94, 95% CI 1.28–2.95, p = .002) and nKRT18 (multivariable HR 1.87, 95% CI 1.24–2.82, p = .003) levels were associated with poor overall survival independent of potential confounding factors. Our results show that tumor necrosis in CRC contributes to serum levels of KRT18 fragments, and both necrosis and KRT18 levels associate with systemic inflammation. Moreover, we show that serum tKRT18 and nKRT18 levels have independent prognostic value in CRC. Our observations confirm the link between cell death and systemic inflammation.

Introduction

Colorectal cancer (CRC) is one of the most common causes of cancer deaths in the Western world,¹ and TNM staging is the major prognostic parameter in CRC.² Additional histopathologic features such as high tumor grade and lymphatic invasion can be used to further identify high-risk patients, and especially in stage II disease, to optimize treatment. Moreover, an increased area of necrosis, an uncontrolled process of cell death, and systemic inflammation have been reported to represent stage-independent markers of poor prognosis in CRC.³⁻⁷

Systemic inflammation is characterized by elevated levels of serum C-reactive protein (CRP) and several proinflammatory cytokines such as interleukin 1 receptor antagonist (IL1RN), IL6, and C-X-C motif chemokine ligand 8 (CXCL8, also known as IL8).^{3,8} Clinically important is that systemic inflammation contributes to the development of cachexia, a wasting syndrome linked to involuntary loss of body weight.9 The activation of systemic inflammation in cancer is a complex, multifaceted process, which is currently not well understood.

Necrotic cell death may stimulate systemic inflammation, and high extent of tumor necrosis has been associated with increased

serum IL6 and CRP levels in CRC.¹⁰ Keratins such as keratin 18 (KRT18) are intracellular structural proteins, which are released from dead cells and can be used as serum biomarkers of cell death. KRT18 is widely expressed by a variety of single-layered epithelial cells, including gastrointestinal tract epithelium, hepatocytes, and CRC cells.^{11,12} During apoptotic cell death, caspase-cleaved KRT18 fragments (aKRT18) are released, whereas full-length KRT18 (nKRT18) is released during necrosis.^{13,14} Different forms of KRT18 are measured by specific enzyme-linked immunosorbent assays (ELISAs) using antibodies M30 and M65. The M30 antibody detects aKRT18, whereas the M65 antibody binds both aKRT18 and nKRT18, thus detecting total KRT18 (tKRT18).15

Serum tKRT18 and aKRT18 levels have been shown to be elevated in various malignancies including colorectal, esophageal, head and neck, lung, and gastric cancer.¹⁶⁻¹⁹ In CRC patients, increased serum tKRT18 and aKRT18 levels have been associated with advanced stage.²⁰ Moreover, high tKRT18 or aKRT18 levels have been shown to be associated with poor prognosis in nonsmall-cell lung cancer, gastric cancer, pancreatic cancer, and CRC.^{18,20-23} However, the studies evaluating CRC patients have been limited by small sample sizes and the lack of comparison of

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the prognostic performance of serum KRT18 levels to that of other relevant serum biomarkers.^{20,22}

Therefore, the main objective of this study was to evaluate the prognostic significance of preoperative circulating KRT18 levels in a well-characterized cohort of 328 CRC patients. Based on two earlier studies, as well as findings on other cancers, we hypothesized that higher KRT18 levels would be associated with adverse outcome. As secondary analyses, we assessed the relationships between the circulating KRT18 levels and the extent of tumor necrosis, systemic inflammatory markers, and other clinicopathologic features. We hypothesized that necrotic cell death released KRT18 fragments would be associated with the extent of tumor necrosis, reflecting the release of KRT18 fragments from the dying tumor cells, and with markers of systemic inflammation, reflecting the activation of systemic inflammation by necrotic cell death and the release of intracytoplasmic fragments into the circulation.

Materials and methods

Patients and controls

The study was based on 328 newly diagnosed CRC patients operated in the Oulu University Hospital between 2006 and 2014, who had signed an informed consent to participate and were eligible for the study.^{8,24} The patients with earlier or simultaneously diagnosed other malignant diseases were excluded. Clinical data were collected from the clinical records and a questionnaire. The follow-up data were acquired from the clinical records and Statistics Finland,^{25,26} and study endpoints were cancer-specific survival (CSS; time from operation to cancer death) and overall survival (OS; time from operation to death from any cause).²⁴ The study was accepted by the Ethics Committee of the Oulu University Hospital (42/2005, 122/2009) and performed according to the principles of the Declaration of Helsinki. The study was designed in accordance with the REMARK guidelines.²⁷ Control serum samples were acquired from healthy cataract surgery patients (Oulu University Hospital; n = 50, age ≥ 65 y).

Histopathologic analysis

The tumors were staged according to TNM8 classification and graded according to the WHO2010 criteria.² Klintrup-Mäkinen Score of peritumoral inflammatory infiltrate was evaluated using hematoxylin and eosin (HE) stained sections.²⁸ The percentage of tumor tissue showing coagulative necrosis (characteristic necrosis appearance with increased eosinophilia and nuclear shrinkage, fragmentation, and disappearance in the HE-stained sections) was evaluated as described earlier.⁷ Tumor necrosis index, an approximation of the total amount of necrosis in the tumor tissue necrosis in the HE slides by the maximum tumor diameter.

Immunohistochemistry and immune cell counting

Tissue microarrays with 1-4 (median 3) cores of 3.0 mm diameter including both the invasive margin (IM) and the

center of the tumor (CT) were constructed for immunohistochemical analysis.^{29,30} Immunohistochemistry was conducted on 3.5 µm sections cut from the tissue microarray paraffin blocks for six immune cell markers (CD3, CD8, CD68, FoxP3, mast cell tryptase, neutrophil elastase).²⁹ For immune cell counting, images were captured from the CT and the IM and the cell densities were counted using a computer-assisted cell counting method³¹ utilizing software.32 ImageJ, а freeware image analysis Intraepithelial (CT-IEL) immune cells (CD3, CD8) were counted manually from the captured images due to the inadequacy of the automatic cell counting to segregate the intraepithelial cells from those in the stroma.²⁹ Mismatch repair (MMR) enzyme screening status for MLH1, MSH2, MSH6, and PMS2 was examined with immunohistochemistry, as described earlier.^{24,33,34} BRAF V600E-specific VE1 immunohistochemistry was conducted.^{24,35} The fraction of proliferating cancer cells (MKI67 score) was evaluated with MKI67 immunohistochemistry.⁷

Analysis of serum samples

Preoperative serum samples were collected in tubes without clot activator. The samples were centrifuged and stored at -70°C until the analysis. Blood leukocyte counts, serum CRP levels, and serum albumin levels were measured in the laboratory of Oulu University Hospital, and modified Glasgow Prognostic Score (mGPS) was calculated from CRP and albumin values as previously described.³⁶ Serum levels of 27 cytokines were measured by Bio-Plex Pro Human pre-manufactured 27-Plex Cytokine Panel (Bio-Rad, Hercules, CA, USA) from patients operated between April 2006 and January 2010.8 Out of the 27 cytokines, 13 cytokines (IL1RN, IL4, IL6, IL7, CXCL8, IL9, IL12, IFNG, CXCL10, CCL2, CCL4, CCL11, and PDGF) with three (1.5%) or fewer values outside the assay working range were included in this study. Serum levels of aKRT18 were measured by M30Apoptosense® enzyme-linked immunosorbent assay (ELISA), and tKRT18 by M65* ELISA (Peviva AB, Bromma, Sweden). All the measurements were done in duplicate according to manufacturer's instructions blinded to clinicopathologic data. Units were defined against a synthetic peptide standard (1 U/L = 1.24 pM). As the M65 ELISA measures both caspase-cleaved (apoptosis) and non-caspase cleaved KRT18 (necrosis), the level of KRT18 released during necrosis (nKRT18) was calculated as M65-M30.

Statistical analyses

The statistical analyses were conducted using IBM SPSS Statistics for Windows version 25.0 (IBM Corporation, Armonk, NY, USA) and R version 3.5.1 (R Foundation for Statistical Computing, Vienna, Austria), using the packages tidyverse (v.1.2.1), survival (v.2.42–6), survminer (v.0.4.3), and plotROC (v.2.2.1). The statistical significances of the associations between categorical and continuous variables were analyzed by Mann–Whitney test (comparing two classes) or Kruskal–Wallis test

Table 1. Serum tKRT18, aKRT18, and nKRT18 levels in relation to clinical and pathological characteristics of CRCs.

Group	tKRT18, U/L, median (IQR)	P value	aKRT18, U/L, median (IQR)	P value	nKRT18, U/L, median (IQR)	P value
Age < 65 (n = 120) ≥ 65 (n = 208)	476.5 (355.4–690.6) 483.7 (374.8–695.9)	0.828	191.8 (146.8–268.7) 177.6 (140.4–249.4)	0.195	289.2 (165.7–467.8) 302.6 (191.3–474.8)	0.448
Sex Male (n = 177) Female (n = 151)	463.2 (337.1–679.6) 516.3 (384.2–734.7)	0.081	177.3 (145.6–257.3) 191.6 (140.8–247.2)	0.799	277.8 (169.6–436.1) 304.7 (206.3–512.1)	0.085
BMI <25 (n = 112) 25–30 (n = 134) >30 (n = 74)	462.2 (342.5–672.8) 447.0 (329.5–631.4) 608.7 (462.3–793.7)	5.2E-5	177.5 (138.5–239.3) 180.4 (141.8–258.1) 207.1 (157.5–271.5)	0.081	285.6 (192.4–429.8) 257.8 (146.9–416.1) 384.3 (275.3–556.7)	6.1E-5
Coronary artery disease No (n = 267) Yes (n = 61)	485.9 (360.7–691.6) 453.2 (385.1–707.8)	0.979	190.1 (146.1–249.5) 161.3 (134.4–262.6)	0.190	298.9 (177.4–470.1) 298.7 (211.3–494.2)	0.512
Asthma No (n = 292) Yes (n = 36)	480.7 (374.8–691.9) 503.4 (336.2–705.5)	0.629	191.4 (143.1–257.6) 156.7 (130.8–208.2)	0.042	298.8 (191.2–467.8) 313.1 (181.7–496.8)	0.812
Diabetes No (n = 271) Yes (n = 57)	466.5 (363.4–670.0) 569.0 (398.2–732.9)	0.103	182.9 (142.2–248.8) 192.3 144.8–277.4)	0.289	285.3 (183.2–457.0) 343.1 (209.7–510.3)	0.137
Use of cholesterol lowering r No $(n = 223)$ Yes $(n = 105)$	nedication 491.5 (365.4–692.4) 464.0 (366.1–691.8)	0.545	191.6 (146.1–253.9) 170.2 (137.3–251.8)	0.156	303.1 (178.9–471.0) 285.7 (196.9–441.5)	0.916
Use of blood pressure loweri No (n = 149) Yes (n = 179)	ng medication 463.2 (347.5–624.0) 491.8 (376.6–756.3)	0.091	191.3 (143.1–245.4) 184.9 (141.9–264.6)	0.846	279.1 (152.9–422.2) 308.0 (208.6–512.1)	0.023
Aspirin use No (n = 248) Yes (n = 80)	487.2 (364.1–690.6) 464.8 (374.0–704.5)	0.664	191.4 (145.6–253.7) 165.9 (137.0–252.9)	0.238	302.4 (183.6–470.7) 284.9 (196.0–479.0)	0.895
Preoperative radiotherapy or No $(n = 260)$ Yes $(n = 68)$	chemoradiotherapy 503.6 (376.2–725.3) 432.5 (321.7–586.9)	0.048	188.1 (141.9–266.0) 182.8 (150.3–225.8)	0.609	306.5 (191.3–482.9) 259.5 (166.6–360.8)	0.056
Tumor location Proximal colon (n = 107) Distal colon (n = 70) Rectum (n = 151)	491.8 (379.4–709.9) 519.1 (387.6–738.3) 463.2 (339.3–665.7)	0.176	193.7 (138.1–270.6) 186.2 (152.5–271.1) 185.5 (146.4–243.1)	0.780	303.5 (189.5–500.2) 336.1 (217.5–460.3) 277.2 (170.2–443.4)	0.174
WHO grade Grade 1 (n = 69) Grade 2 (n = 218) Grade 3 (n = 40)	453.2 (337.1–623.5) 503.4 (374.8–692.0) 490.1 (362.9–791.6)	0.257	161.9 (137.7–223.8) 191.6 (142.8–257.1) 205.3 (144.8–277.5)	0.062	266.3 (185.1–420.9) 306.1 (194.4–458.0) 313.0 (159.7–595.6)	0.444
TNM stage Stage I (n = 68) Stage II (n = 110) Stage III (n = 109) Stage IV (n = 41)	488.7 (336.8–662.1) 466.1 (362.2–634.1) 461.1 (379.2–630.8) 775.0 (416.1–2090.5)	0.002	162.0 (139.6–218.0) 177.6 (142.3–258.8) 182.9 (141.2–233.8) 269.4 (211.1–515.2)	8.8E-5	282.0 (173.9–482.6) 286.2 (188.1–396.6) 285.3 (174.2–410.2) 471.0 (217.5–1053.7)	0.008
Depth of invasion T1 (n = 13) T2 (n = 75) T3 (n = 219) T4 (n = 21)	613.4 (322.8–728.1) 456.5 (315.8–629.8) 478.7 (374.7–691.9) 567.9 (410.5–886.3)	0.082	166.0 (141.1–206.3) 166.8 (137.9–225.6) 192.8 (142.3–270.5) 191.6 (151.0–285.1)	0.121	430.6 (185.9–510.3) 265.2 (160.3–414.3) 298.7 (179.1–452.5) 394.3 (264.7–588.5)	0.066
Nodal metastases N0 (n = 184) N1 (n = 92) N2 (n = 52)	471.2 (342.5–637.2) 454.0 (365.7–694.7) 634.0 (424.4–883.7)	0.003	170.9 (141.9–241.6) 184.7 (136.9–254.4) 219.5 (172.2–294.0)	0.009	285.5 (185.9–421.3) 289.0 (174.6–439.5) 390.3 (233.9–656.6)	0.015
Distant metastases M0 (n = 287) M1 (n = 41)	466.5 (363.4–636.1) 775.0 (416.1–2090.5)	9.5E-5	175.9 (141.9–239.9) 269.4 (211.1–515.2)	8.0E-6	285.7 (179.1–416.9) 471.0 (217.5–1053.7)	0.001
Lymphatic invasion No (n = 178) Yes (n = 147)	477.9 (360.1–662.0) 488.6 (378.9–745.9)	0.477	175.3 (141.6–244.1) 194.8 (146.4–270.6)	0.134	286.2 (195.7–458.0) 304.6 (164.2–471.0)	0.800
Blood vessel invasion No (n = 272) Yes (n = 53)	478.3 (363.5–664.5) 540.3 (365.9–939.7)	0.156	178.9 (141.9–345.8) 219.0 (153.0–303.9)	0.028	292.4 (189.9–443.0) 345.8 (174.5–709.4)	0.170
Klintrup-Mäkinen score 0 (n = 166) 1 (n = 162)	483.1 (353.8–501.6) 481.0 (376.6–633.9)	0.473	204.2 (154.2–259.0) 167.0 (140.2–242.4)	0.009	294.8 (177.6–501.6) 300.3 (207.5–427.5)	0.701
BRAF VE1 immunohistochem Negative (n = 297) Positive (n = 30)	istry 479.2 (363.6–691.8) 548.7 (378.7–744.1)	0.302	185.8 (142.7–248.8) 192.6 (131.8–281.0)	0.747	298.7 (187.9–459.0) 335.0 (207.6–503.2)	0.326

Table 1. (Continued).

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Group	tKRT18, U/L, median (IQR)	P value	aKRT18, U/L, median (IQR)	P value	nKRT18, U/L, median (IQR)	P value		
MMR enzyme status								
MMR-deficient (n = 35)	466.5 (377.5–631.9)	0.831	142.3 (123.6–264.6)	0.072	292.8 (189.5–471.0)	0.981		
MMR-proficient ($n = 293$)	483.5 (363.5-703.1)		190.7 (147.7–251.3)		298.9 (188.1-471.0)			
Modified Glasgow Prognostic Score								
0 (n = 258)	464.8 (356.1–625.4)	8.5E-5	185.2 (142.1–240.9)	0.021	284.9 (173.6–406.3)	3.5E-5		
1 (n = 63)	660.7 (356.1–625.4)		220.7 (152.6–344.6)		423.6 (248.3–720.6)			
2 (n = 6)	742.9 (376.0–5428.0)		134.3 (94.8–1633.8)		645.6 (238.6–3723.7)			

Abbreviations: IQR: interquartile range, MMR: mismatch repair. P values are for Mann–Whitney or Kruskal–Wallis test.

(comparing three or more classes). Correlations between two continuous variables were determined using Pearson correlation coefficients (r). Cytoscape 3.7.2 was used to create a correlation network with the Prefuse force directed algorithm weighted by the statistical significances of the associations between individual variables. Multiple linear regression analysis of the correlation of serum tKRT18, aKRT18, and nKRT18 levels with selected clinicopathological factors was conducted. Receiver operating characteristics (ROC) analysis was used to determine optimal cutoff values, with the shortest distance to the coordinate (0,1), in discriminating survivors from nonsurvivors (CSS). Univariate survival was performed according to the Kaplan–Meier method. Backward conditional stepwise Cox regression was used for multivariate survival modeling. Considering multiple hypothesis testing, a two-tailed p < .01 was considered statistically significant.

Results

Serum tKRT18, aKRT18 and nKRT18 levels in patients and controls

The characteristics of CRC patients and healthy controls are presented in Supplementary Table 1. In CRC patients, the median tKRT18, aKRT18, and nKRT18 levels (U/L) were 483.4, 185.9, and 298.9, and a strong correlation existed between circulating tKRT18 and aKRT18 levels (Pearson r = 0.865, p < .001). The median serum tKRT18,

Table 2. Correlations between serum tKRT18, aKRT18, and nKRT18 level, the markers of systemic inflammation, cytokines, Ki-67, and tumor necrosis.

	tKRT18			aKRT18			nKRT18					
	Unadjusted		Adjusted		Unadjusted		Adjusted		Unadjusted		Adjusted	
	Pearson r	р	Beta	р	Pearson r	р	Beta	р	Pearson r	р	Beta	р
Systemic inflammatory market	rs											
Serum C-reactive protein	0.285	1.6E-7	0.190	0.001	0.244	8.0E-6	0.126	0.024	0.219	7.6E-5	0.154	0.010
Serum albumin	-0.138	0.013	-0.094	0.079	-0.048	0.385	0.006	0.910	-0.148	0.008	-0.118	0.033
Blood NLR	0.116	0.037	0.119	0.034	0.113	0.043	0.094	0.084	0.073	0.191	0.080	0.173
Blood leukocytes	0.170	0.002	0.099	0.086	0.101	0.068	0.012	0.834	0.181	0.001	0.141	0.020
Blood neutrophils	0.181	0.001	0.103	0.069	0.146	0.009	0.056	0.314	0.155	0.006	0.101	0.088
Blood lymphocytes	0.034	0.547	-0.056	0.386	0.006	0.921	-0.076	0.231	0.059	0.296	0.001	0.993
Blood monocytes	0.224	4.9E-5	0.158	0.005	0.133	0.017	0.047	0.389	0.228	3.9E-5	0.191	0.001
Blood eosinophils	0.030	0.593	0.012	0.818	-0.023	0.682	-0.048	0.360	0.041	0.470	0.028	0.614
Blood basophils	0.047	0.402	0.031	0.583	-0.037	0.510	-0.045	0.408	0.025	0.660	0.003	0.952
Cytokines												
IL1RN	0.262	0.001	0.140	0.090	0.234	0.005	0.102	0.214	0.263	0.001	0.185	0.032
IL4	0.111	0.182	0.027	0.731	0.108	0.195	0.027	0.733	0.112	0.181	0.048	0.567
IL6	0.385	1.8E-6	0.284	3.9E-4	0.380	2.4E-6	0.275	0.001	0.317	1.2E-4	0.243	0.004
IL7	0.124	0.135	0.028	0.731	0.117	0.161	0.008	0.925	0.175	0.036	0.109	0.195
CXCL8	0.570	6.1E-14	0.522	4.3E-9	0.496	2.0E-10	0.397	1.2E-5	0.504	1.3E-10	0.498	1.1E-7
IL9	0.190	0.022	0.112	0.160	0.305	1.9E-4	0.233	0.003	0.121	0.149	0.051	0.541
IL12	0.147	0.077	0.094	0.240	0.045	0.592	-0.016	0.844	0.185	0.026	0.156	0.059
IFNG	0.129	0.121	0.059	0.459	0.108	0.193	0.043	0.587	0.136	0.104	0.085	0.304
CXCL10	0.313	1.2E-4	0.275	3.6E-4	0.324	6.8E-5	0.280	2.5E-4	0.260	0.002	0.231	0.004
CCL2	0.187	0.024	0.106	0.196	0.203	0.014	0.113	0.164	0.109	0.195	0.036	0.675
CCL4	0.033	0.688	0.011	0.891	0.044	0.599	0.021	0.785	0.047	0.573	0.029	0.722
CCL11	-0.091	0.276	-0.078	0.318	-0.090	0.282	-0.076	0.331	-0.124	0.139	-0.115	0.161
PDGF	0.044	0.597	-0.045	0.571	-0.002	0.983	-0.102	0.198	0.069	0.414	-0.001	0.992
Tumor properties												
MKI67 index	-0.224	0.007	-0.159	0.048	-0.232	0.005	-0.159	0.045	-0.168	0.047	-0.116	0.170
Tumor necrosis index	0.264	3.0E-6	0.206	0.001	0.224	7.2E-5	0.103	0.091	0.195	0.001	0.165	0.012
Tumor necrosis percentage	0.205	2.2E-4	0.162	0.006	0.203	2.4E-4	0.113	0.052	0.146	0.009	0.129	0.039
Maximum tumor diameter	0.217	0.012	0.153	0.072	0.046	0.600	-0.045	0.597	0.171	0.050	0.134	0.135

The correlations were adjusted for tumor stage variables (T1-2 vs. T3-4; N0 vs. N1-2; M0 vs. M1), preoperative radiotherapy/chemoradiotherapy and body mass index by multiple linear regression. Abbreviations: NLR: neutrophil to lymphocyte ratio; IL: interleukin; IFN: interferon; CCL: Chemokine (C-C motif) ligand; CXCL: Chemokine (C-X-C motif) ligand; PDGF: Platelet-derived growth factor.

aKRT18 and nKRT18 levels were significantly higher in CRC patients ≥ 65 y compared to healthy controls (tKRT18: 483.7 vs. 195.1 U/L, p < .001; aKRT18: 177.6 vs. 112.2 U/L, p < .001; nKRT18 302.6 vs. 76.8 U/L, p < .001; Supplementary Table 1, Supplementary Figure 1).

Serum tKRT18, aKRT18, and nKRT18 levels in relation to basic clinicopathologic parameters

Table 1 shows the relationships between serum levels of KRT18 molecules and clinicopathologic variables. Obese CRC patients (BMI>30) had increased tKRT18 and nKRT18 levels (p < .001). Higher tumor stages were associated with elevated tKRT18 (p = .002), aKRT18 (p < .001) and nKRT18 (p = .008) levels. Higher Klintrup-Mäkinen Scores indicating pronounced peritumoral inflammatory infiltrate were associated with decreased aKRT18 levels (p = .009), and activated systemic inflammation measured by mGPS associated with increased tKRT18 (p < .001) and nKRT18 levels (p < .001). There were no statistically significant associations between serum KRT18 levels and preoperative radiotherapy or chemoradiotherapy.

Correlation of serum aKRT18, tKRT18 and nKRT18 levels and tumor necrosis index, systemic inflammation markers, MKI67 and tumor-infiltrating immune cells

We hypothesized that serum KRT18 levels would reflect tumor necrosis and be associated with systemic inflammation. The correlations between serum tKRT18, aKRT18 and nKRT18 levels and tumor necrosis, the markers of systemic inflammation, cytokines, and proliferation marker MKI67 are presented in Table 2 and Figure 1. In support our hypothesis, tKRT18 (beta = 0.206, p = .001) and nKRT18 levels (beta = 0.165, p = .012, borderline statistical significance considering multiple hypothesis testing) were associated with tumor necrosis index. Moreover, tKRT18 levels associated with areal percentage of tumor necrosis (beta = 0.162, p = .006), whereas maximum tumor diameter showed no significant association with serum KRT18 levels. There were also positive correlations between serum KRT18 levels and several systemic inflammatory markers; tKRT18 showed positive multivariable-adjusted correlations with CRP (beta = 0.190, p = .001), blood monocyte count (beta = 0.158, p = .005), IL6 (beta = 0.385, p < .001), CXCL8 (beta = 0.522, p < .001), and CXCL10 (beta = 0.275, p < .001), while aKRT18 levels were associated with serum



Figure 1. Correlation network of the interrelationships between serum keratin 18 levels, tumor necrosis index, blood immune cells, cytokines, CRP, and albumin. Individual variables are presented by nodes and their associations are presented by edges. Only the correlations with p < .01 are shown and the edge length illustrates the significance of the association. The correlations between tumor necrosis index and keratin 18 levels with other variables are presented by green (positive correlation) and red (negative correlation) edges, whereas the interrelationships between other variables are presented by gray edges.

IL6 (beta = 0.275, p = .001), CXCL8 (beta = 0.397, p < .001), IL-9 (beta = 0.233, p = .003), and CXCL10 (beta = 0.280, p < .001). nKRT18 levels were associated with blood monocyte count (beta = 0.191, p = .001), IL6 (beta = 0.243, p = .004), CXCL8 (beta = 0.498, p < .001), and CXCL10 (beta = 0.231, p = .004). Serum KRT18 levels did not show statistically significant associations with tumor-infiltrating immune cells (Supplementary Table 2).

Multivariate analysis

Multiple linear regression was used to further investigate the relative significance of potential determinants of serum KRT18 levels. Based on the univariate analyses, the factors included in the initial models were invasion through muscularis propria, nodal metastasis, distant metastasis, systemic inflammation (mGPS), BMI, Klintrup-Mäkinen Score of peritumoral inflammatory infiltrate, and tumor necrosis. Of these factors, the most prominent indicator of serum tKRT18 (beta = 0.250, p < .001) and aKRT18 (beta = 0.363, p < .001) levels was distant metastasis and the main predictor of serum nKRT18 level was mGPS (beta = 0.180, p = .002) (Table 3). However, when serum cytokines were considered for the inclusion in the models,

Table 3. Multiple linear regression model of tKRT18, aKRT18, and nKRT18 levels in colorectal cancer patients.

	Independent	Beta	p value
tKRT18	Model 1		
	Distant metastasis	0.317	2.1E-8
	Model 2		
	Distant metastasis	0.262	4.0E-6
	mGPS	0.209	2.2E-4
	Model 3		
	Distant metastasis	0.290	4.1E-7
	mGPS	0.189	0.001
	BMI	0.155	0.005
	Model 4		
	Distant metastasis	0.250	1.7E-5
	mGPS	0.160	0.004
	BMI	0.168	0.002
aKRT18	Tumor necrosis index Model 1	0.161	0.005
	Distant metastasis	0.376	1.9E-11
	Model 2		
	Distant metastasis	0.400	9.7E-13
	BMI	0.164	0.002
	Model 3		
	Distant metastasis	0.363	3.5E-10
	BMI	0.171	0.001
	Tumor necrosis index	0.124	0.026
nKRT18	Model 1		
	mGPS	0.255	9.0E-6
	Model 2		
	mGPS	0.215	2.4E-4
	Distant metastasis	0.151	0.010
	Model 3		
	mGPS	0.201	0.001
	Distant metastasis	0.172	0.004
	BIVII	0.112	0.050
	MODEL 4	0 100	0.000
	Distant material	0.180	0.002
		0.143	0.018
	DIVII Turre en recerceie in deur	0.123	0.031
	Tumor necrosis index	0.118	0.048

Abbreviations: mGPS: modified Glasgow Prognostic Score. BMI body mass index. The independents considered for the inclusion in the models were invasion through muscularis propria, nodal metastasis, distant metastasis, mGPS, BMI, Klintrup-Mäkinen Score, and tumor necrosis index. CXCL8 was the major determinant of all KRT18 fragments, showing strong associations with tKRT18 (beta = 0.580, p < .001), aKRT18 (beta = 0.503, p < .001) and nKRT18 (beta = 0.520, p < .001) levels (Supplementary Table 3).

Survival analyses

The primary aim of this study was to evaluate the prognostic significance of serum tKRT18, aKRT18, and nKRT18 levels. ROC analysis showed that serum tKRT18, aKRT18 and nKRT18 levels were capable of discriminating survivors from non-survivors (tKRT18: AUC = 0.656, 95% CI = 0.581-0.730, *p* < .001, aKRT18: AUC = 0.666, 95% CI = 0.591–0.741, *p* < .001, nKRT18: AUC = 0.626, 95% CI = 0.546-0.705, p = .001) (Supplementary Figure 2). Kaplan-Meier plots indicated that increased serum tKRT18 (>680 U/L), aKRT18 (>186 U/L) and nKRT18 (>420 U/L) were associated with worse CSS and OS (p < .001 for all) (Figure 2). In multivariate Cox proportional hazard regression models, adjusting for tumor stage, systemic inflammation, and other clinicopathologic features, tKRT18 and nKRT18 were independent predictors of poor OS (tKRT18: HR 1.94, 95% CI 1.28–2.95, p = .002, nKRT18: HR 1.87, 95% CI 1.24–2.82, p = .003), whereas aKRT18 was not significantly associated with CSS or OS (Table 4).

Discussion

In this study, we found that all KRT18 fragments associated with advanced disease stage and systemic inflammation, and tKRT18 associated with the extent of tumor necrosis. In multivariate linear regression models, serum CXCL8 level was the main determinant of all three KRT18 forms. Serum levels of tKRT18 and nKRT18 predicted poor OS independent of disease stage, systemic inflammatory markers, and other clinicopathologic features. These results suggest that cell death and systemic inflammation are strongly connected in CRC patients, and the alterations in serum KRT18 levels in CRC reflect subsequent disease course.

During the past few decades, it has become evident that tumors can induce systemic changes long before gross metastatic disease appears,^{37,38} and many of these changes are involved in systemic inflammation. However, the factors underlying systemic inflammation in cancer have not been well characterized. Tissue necrosis may rapidly provoke a systemic inflammatory response required for the removal of dead tissues. The potential significance of tumor necrosis in CRC associated systemic inflammation has been highlighted by Richards *et al.*³⁹ and Guthrie *et al.*,¹⁰ who reported that increasing amount of tumor necrosis in CRC is associated with higher mGPS and serum IL6 levels. Our present study adds to this by showing a strong link between serum cell-death-related KRT18 fragments and an assemblage of systemic inflammatory markers.

Tumor necrosis was associated with all three forms of serum KRT18 in univariate analyses, and it showed a statistically significant multivariable-adjusted association with tKRT18 (p = .001) and a tendency toward multivariable-adjusted association with nKRT18 levels (p = .012, borderline statistical significance considering multiple hypothesis testing). These



Figure 2. Kaplan–Meier survival curves for 120-month cancer-specific survival (CSS) according to serum levels of (a) tKRT18, (b) aKRT18 and (c) nKRT18, and for overall survival (OS) according to (d) tKRT18, (e) aKRT18 and (f) nKRT18.

findings support the hypothesis that tumor necrosis contributes to serum KRT18 levels in CRC. However, the associations between serum KRT18 levels and tumor necrosis were weaker than those between serum KRT18 levels and systemic inflammation. This may be related to the inadequacy of tumor necrosis percentage or tumor necrosis index, based on the evaluation of the primary tumor specimens, to estimate the necrotic tumor mass present in the body. In addition, KRT18 released from other epithelial tissues may be involved in activating systemic inflammation, as KTR18/KRT8 constitutes the predominant keratin pair of nonmalignant simple epithelial cells such as gastrointestinal tract epithelium,⁴⁰ in addition to being widely expressed by adenocarcinomas.

In particular, the liver is a potential source of the KRT18 fragments. KRT8/KRT18 represents the characteristic and only keratin pair of normal hepatocytes.⁴⁰ Indeed, an increase in KRT18 fragment levels is an established marker of liver injury.⁴¹ Potential mechanism leading to liver injury in CRC may be related to changes in the extra-tumoral intestinal mucosa or tumor secreted or induced mediators, or metastasis. When the intestinal epithelial barrier is disrupted, and intestinal permeability is increased, bacteria or bacterial components

can enter the liver via the portal circulation. Animal CRC models have shown a strong association between circulating IL6 levels and intestinal permeability.^{42,43} In the liver, gutderived endotoxin, lipopolysaccharide (LPS) of gram-negative bacteria, induces Kupffer cell activation and liver injury that is reflected to circulating aKRT18 and tKRT18 and nKRT18 levels.⁴⁴ Thus, part of serum KRT18 in CRC patients may originate from the liver, representing a consequence rather than cause of systemic inflammation. Moreover, liver is the most common site of metastasis in CRC patients, and metastatic spread may result in cell damage in the metastatic site. Accordingly, in patients with metastatic CRC, alkaline phosphatase, and aspartate transaminase levels, markers of liver injury have been shown to correlate with tKRT18 and aKRT18.²⁰ All in all, multiple tissues may contribute to circulating KRT18 levels, and the potential sources besides necrotic tumor cells in CRC remain hypothetical.

Our study analyzed serum levels of 13 cytokines and identified that CXCL8 was the most important predictor of serum levels of all KRT18 forms. In the visualization of the systemic inflammatory marker network (Figure 1), CXCL8, along with IL6, clustered at the center having a strong correlation with

Table 4. Cox regression model for the independent prognostic significance of serum tKRT18, aKRT18, and nKRT18 level.

	CSS			OS			
	HR	95%CI	p value	HR	95%CI	p value	
tKRT18							
Age (<65 vs. ≥65)	1.83	1.09-3.08	0.023	2.09	1.35-3.22	0.001	
Nodal metastases (N0 vs. N1-N2)	3.41	1.67-6.95	0.001	2.63	1.70-4.07	1.5E-5	
Distant metastases (M0 vs. M1)	5.85	3.33-10.30	9.0E-10	4.15	2.54-6.78	1.3E-8	
Lymphatic invasion (No vs. Yes)	2.11	1.07-4.13	0.030				
Serum tKRT18 (≤680 U/L vs. >680 U/L)	1.89	1.12-3.21	0.018	1.94	1.28-2.95	0.002	
aKRT18							
Age (<65 vs. ≥65)	1.85	1.10-3.12	0.020	2.07	1.34-3.19	0.001	
Nodal metastases (N0 vs. N1-N2)	3.59	1.78-7.27	3.7E-4	2.69	1.74-4.15	8.0E-6	
Distant metastases (M0 vs. M1)	6.43	3.75-11.01	1.3E-11	4.10	2.49-6.76	3.1E-8	
mGPS (0 vs. 1–2)				1.60	1.04-2.46	0.034	
Serum aKRT18 (≤186 U/L vs. >186 U/L)	1.74	1.02-2.96	0.043	1.37	0.91-2.07	0.132	
nKRT18							
Age (<65 vs. ≥65)	1.82	1.08-3.06	0.025	2.07	1.35-3.20	0.001	
Nodal metastases (N0 vs. N1-N2)	3.41	1.68-6.95	0.001	2.64	1.70-4.08	1.3E-5	
Distant metastases (M0 vs. M1)	5.99	3.42-10.47	3.6E-10	4.31	2.65-6.99	3.6E-9	
Lymphatic invasion (No vs. Yes)	2.14	1.09-4.19	0.027				
Serum nKRT18 (≤420 U/L vs. >420 U/L)	1.86	1.11-3.14	0.019	1.87	1.24-2.82	0.003	

The initial model included age, tumor location, tumor invasion, nodal metastases, distant metastases, preoperative radiotherapy or chemoradiotherapy, lymphatic invasion, mGPS, coronary artery disease, asthma, diabetes, use of cholesterol-lowering medication, use of blood pressure-lowering medication, aspirin use and serum KRT18. The final model is the model following backward elimination of nonsignificant covariates. Abbreviations: CSS: cancer-specific survival; OS: overall survival; CI: confidence interval; HR: hazard ratio; mGPS: modified Glasgow Prognostic Score. CSS: n = 328; median follow-up time 66.5 months (IQR 37.6–86.7); 71 (21.6%) events; 8 (2.4%) cases excluded from the analysis because of missing values. OS: n = 328; median follow-up time 66.5 months (IQR 37.6–86.7); 108 (32.9%) events; 8 (2.4%) cases excluded from the analysis because of missing values.

a high number of other systemic inflammatory markers. CXCL8 is a pro-inflammatory chemokine, which recruits neutrophils to the site of inflammation.⁴⁵ In addition, neutrophils synthesize and release CXCL8 in response to injury-released intracellular damage-associated molecular patterns (DAMPs).⁴⁶ These findings suggest that neutrophils and CXCL8 may represent a potential mechanistic link between cell death and systemic inflammation. Interestingly, serum CXCL8 levels are not statistically significantly associated with patient survival in multivariable models,⁴⁷ whereas this study indicates that serum tKRT18 and nKRT18 predict poor OS independent of disease stage, systemic inflammatory markers, and other clinicopathologic features. This supports the multifactorial background underlying the prognostic significance of serum KRT18 levels in CRC.

Improved prognostic parameters are needed to classify CRC into more homogenous, therapeutically relevant groups. The results of this study support the relevance of tKRT18 and nKRT18 as additional prognostic markers in CRC. This finding confirms the results of two previous studies.^{20,22} Relative to these studies, the major advantages of our study are a larger patient cohort and extensive evaluation of additional circulating biomarkers for comparison. Some of these markers, including mGPS, have been shown to be associated with patient outcome in a large group of independent studies.⁴⁸ In the present study, the serum tKRT18 and nKRT18 had stronger prognostic value than mGPS. However, our study was still limited by the lack of an independent validation cohort and by a relatively small number of patients in more specific patient subgroups, such as stage II patients. Therefore, further studies are required to evaluate, whether serum KRT18 levels could help to identify high-risk patients in these subgroups, in combination with other systemic inflammation-based markers such as mGPS, and whether these measurements could be used to guide the treatment of the patients.

In conclusion, elevated serum KRT18 levels in CRC are associated with adverse clinical outcome and systemic inflammation, especially higher serum CXCL8 levels. These results support a link between cell death, systemic inflammatory response, and patient prognosis in CRC.

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Disclosure of potential conflict of interest

The authors declare that there is no conflict of interest.

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