Histol Histopathol (2020) 35: 887-892

http://www.hh.um.es

Histology and Histopathology
From Cell Biology to Tissue Engineering

Tetraspanin CD63 independently predicts poor prognosis in colorectal cancer

Tuomas Kaprio[1,2], Jaana Hagström[2,3], Leif C. Andersson[3] and Caj Haglund[1,2]

[1]Department of Surgery, Helsinki University Central Hospital, [2]Research Programs Unit, Translational Cancer Biology, University of Helsinki and [3]Department of Pathology, Haartman Institute, University of Helsinki and HUSLAB, Helsinki, Finland

Summary. CD63, a member of the tetraspanin family, is expressed in endosomes and enriched in exosomes. Tetraspanins participate in a variety of physiological processes, including cellular differentiation, cell-cell fusion, and cell migration. CD63 reportedly carries both protumorigenic and tumor suppressor properties, and appears to be upregulated in breast cancer, astrocytoma, and melanoma. Yet, the effect of CD63 on cancer prognosis remains unclear, and no previous reports examined it in colorectal cancer (CRC). Identifying novel biomarkers will allow us to better differentiate patients with an increased risk of recurrence and who might benefit from adjuvant therapy. We applied immunohistochemistry with antibodies to human CD63 on 620 consecutive CRC patients treated at the Helsinki University Hospital. We evaluated the associations between CD63 expression and clinicopathological parameters and patient prognosis. We found that CD63 expression associated with an advanced stage, poor differentiation, and mucinous histology. We found no association between CD63 expression and age, sex or tumor location. CD63 expression predicted an unfavorable prognosis in CRC (p=0.00001, log-rank test) and in a subgroup of patients with metastasized CRC (p=0.011). Cox's multivariate analysis identified CD63 as an independent factor predicting an unfavorable prognosis in CRC and in the subgroup with metastasized disease.

We show for the first time that CD63 immunohistochemistry expression represents an independent marker of an unfavorable prognosis in CRC and associates with unfavorable clinicopathological parameters. Our results support the hypothesis that a higher tissue expression of CD63 in CRC, indicating an epithelial-to-mesenchymal transition (EMT)-associated secretory phenotype, associated with an adverse outcome.

Key words: Colorectal cancer, Prognosis, Tetraspanin, CD63

Offprint requests to: Tuomas Kaprio, Department of Surgery, Helsinki University Central Hospital, PO Box 440, 00029 HUS, Helsinki, Finland. e-mail: tuomas.kaprio@helsinki.fi

DOI: 10.14670/HH-18-209

Introduction

With more than 1 million new cases and half a million deaths annually, colorectal cancer (CRC) is the world's third most common cancer (Siegel et al., 2012). As with other solid tumors, metastasis represents the major cause of death from CRC (Hanahan and Weinberg, 2011). Early detection, radical surgical, and adjuvant therapy are important to clinical outcome. The stage of disease at diagnosis is the most crucial factor today for predicting patient outcome; roughly 40% of patients present with localized disease and another 40% present with regional disease (Siegel et al., 2012). Adjuvant therapy, resulting in a 10% absolute disease-specific survival benefit (O'Connor et al., 2011), is routine practice for stage III CRC patients. In stage II patients, the advantage of adjuvant therapy, however, remains unclear, whereby four out of five surgically treated stage II patients survive without chemotherapy. Currently, we cannot identify patients likely to benefit from adjuvant therapy, despite knowing multiple high-risk factors. These high-risk factors consist of T4-stage,

low differentiation grade, vascular invasion, tumor obstruction, bowel perforation, and inadequate lymph node resection. Thus, identifying new biomarkers would improve treatment decision-making.

Cellular plasticity and controlling these steps are important in the epithelial-to-mesenchymal transition (EMT) and mesenchymal-to-epithelial transition (MET), essential processes for tumor metastasis. Tetraspanins, characterized by their four transmembrane domains delimiting three intracellular and two extracellular regions, participate in a variety of physiological processes, including cellular differentiation, cell-cell fusion, and cell migration (Naour et al., 2006). Some members of the tetraspanin family reportedly inhibit metastasis, while others carry prometastatic properties. One member of the tetraspanin family, CD63, appears to be upregulated in breast cancer, astrocytoma, and melanoma (Logozzi et al., 2009; Rorive et al., 2010; Ridnour et al., 2012) Expressed on the endosomes and enriched in exoxomes, CD63 was originally reported to possess tumor suppressor properties (Radford et al., 1995; Kwon et al., 2007). More recently, however, CD63 was shown to participate in protumorigenic cell signaling, including interacting with extracellular signal-regulating kinases (Jung et al., 2012), phosphoinositide 3-kinases (Toricelli et al., 2013), β-catenin (Seubert et al., 2015), and the tissue inhibitor metalloproteinase-4 (TIMP-4) (Rorive et al., 2010). Alongside tetraspanins CD9 and CD81, CD63 serves as an exosome indicator and elevated levels of circulating exosomes in plasma were reported in CRC patients (Silva et al., 2012).

CD63 may carry pro-metastatic properties. Thus, its increased expression would indicate a poor patient prognosis. An earlier study indicated that the elevated co-expression of TIMP4/CD63 in tissue associated with worse outcomes in glioblastomas (Rorive et al., 2010). Here, we study the prognostic role of CD63 in colorectal cancer and its association with common clinicopathological parameters.

Materials and methods

Patients

The study population consists of 620 consecutive CRC patients who underwent surgery between 1990 and 2001 in the Department of Surgery at the Helsinki University Hospital (HUH). The Finnish Population Register Center provided the follow-up data needed to compute the survival statistics, and Statistics Finland provided cause-of-death information. The mean age at diagnosis was 66.2 years, with a median follow-up of 7.1 years (range, 0-20.8). The 5-year disease-specific survival (DSS) rate was 61.3% (95% confidence interval [CI] 57.2-65.4%).

This study complies with the Declaration of Helsinki, and the Surgical Ethics Committee of Helsinki University Hospital approved the study protocol (Dnro HUS 226/E6/06, extension TMK02 §66, 17 April 2013). In addition, the National Supervisory Authority of Welfare and Health granted us permission to use tissue samples without individual informed consent for this retrospective study (Valvira Dnro 10041/06.01.03.01/ 2012).

Tissue microarray

From the HUH Department of Pathology archives, we obtained formalin-fixed and paraffin-embedded tumor samples. An experienced pathologist marked representative areas of the tumor samples on hematoxylin- and eosin-stained tumor slides and 3 1.0-mm-diameter punches from each sample were mounted on recipient paraffin blocks with a semiautomatic tissue microarray instrument (Beecher Instruments, Silver Spring, MD, USA).

Immunohistochemistry

The tumor tissue microarray (TMA) blocks were freshly cut into 4-μm sections, fixed on slides, and dried at 37°C for 12 to 24 hrs. Then, continuing with deparaffinization in xylene and rehydration through a gradually decreasing concentration of ethanol-to-distilled water, TMA slides were treated in a PreTreatment module (Lab Vision Corp., Fremont, CA, USA) in an antibody-specific buffer for 20 min at 98°C for antigen retrieval. Staining of sections was performed in an Autostainer 480 (Lab Vision) using the Dako REAL EnVision Detection system (Peroxidase/DAB+, Rabbit/Mouse [Dako, Glostrup, Denmark]). Tissue samples were incubated with the CD63 primary antibody (SAB4700215, Sigma-Aldrich, Merck Life Science, Darmstad, Germany) at a dilution of 1:100 for 1 hr at room temperature. We validated the specificity of the antibody to CD63 by western blotting as reported (Kaprio et al., 2019)

Scoring of samples

The CD63 cytoplasmic expression in tumor cells was scored as negative, low, moderate or high according to the intensity. For statistical analysis, samples were grouped into negative and positive (low, moderate, and high). Stainings were scored independently by TK and JH, who were blinded to the clinical data and outcome. Differences between scores were discussed until consensus was reached. Fig. 1 provides representative images for each expression score.

Statistical analyses

We used the exact Pearson chi-square test or the exact linear-by-linear association test for ordered parameters to evaluate the association between CD63 expression and clinicopathological parameters. Disease-specific survival (DSS) was calculated from the date of

operation until the date of death from CRC or until the end of the follow-up period. The log-rank test compared survival rates using the Kaplan-Meier method. The Cox regression proportional hazard model, adjusted for sex, age, Dukes classification, and differentiation, served uni- and multivariable survival analyses. Testing of the Cox model assumption of the constant hazard ratios over time involved including each time-dependent covariate separately for each testable variable. Interaction terms were considered, although we identified none. All tests were two-sided, and we considered $p<0.05$ statistically significant. All statistical analyses were carried out using SPSS, version 24.0 (IBM SPSS Statistics, version 24.0 for Mac, SPSS, Inc., Chicago, IL, USA).

Results

Immunohistochemistry

The CD63 expression in tumor cells was primarily cytoplasmic and often granular. We found no nuclear positivity. Expression was homogenous and we saw no strengthening towards the invasive front. Of the 620 tumors represented in TMA, CD63 staining could be evaluated in 582 tumors. As such, 115 (19.8%) were scored as negative, 274 (47.1%) as exhibiting low expression, 154 (26.4%) as moderate, and 39 (6.7%) as high expression. The distribution of expression did not differ between older and newer tissue samples when the study population was split into two. In normal colonic mucosa we saw CD63 positivity in goblet and enteroendocrine cells.

Table 1. Association between CD63 expression and clinicopathological parameters.

	CD63 [n(%)]		
	Negative n=115	Positive n=467	p-value*
Age. years			
<65	56(48.7)	210(45.0)	0.531
≥65	59(51.3)	257(55.0)	
Sex			
Male	69(60.0)	368(57.4)	0.673
Female	46(40.0)	199(42.6)	
Dukes			
A	23(20.0)	57(12.2)	0.038
B	41(35.7)	162(34.7)	
C	30(26.1)	139(29.8)	
D	21(18.3)	109(23.3)	
Grade (WHO)			
1	7(6.1)	17(3.7)	0.044
2	85(74.6)	318(68.5)	
3	19(16.7)	109(23.5)	
4	3(2.6)	20(4.3)	
Missing			
Location			
Colon	57(49.6)	226(48.4)	0.836
Rectum	58(50.4)	241(51.6)	
Side			
Right	28(24.3)	119(25.5)	0.813
Left	87(75.7)	348(74.5)	
Histology			
Non-mucinous	113(98.3)	428(91.8)	0.021
Mucinous	2(1.7)	38(8.2)	

*Using the exact Pearson chi-square test or the exact linear-by-linear association test for ordered parameters.

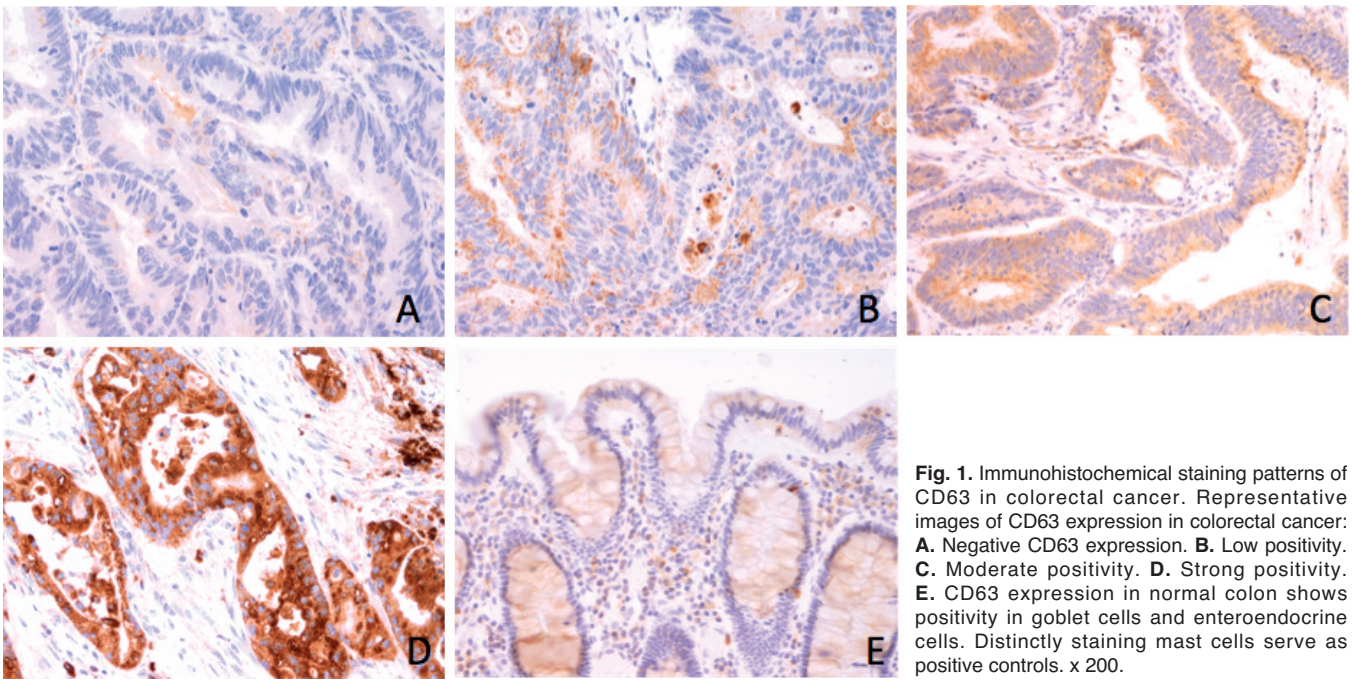

Fig. 1. Immunohistochemical staining patterns of CD63 in colorectal cancer. Representative images of CD63 expression in colorectal cancer: **A.** Negative CD63 expression. **B.** Low positivity. **C.** Moderate positivity. **D.** Strong positivity. **E.** CD63 expression in normal colon shows positivity in goblet cells and enteroendocrine cells. Distinctly staining mast cells serve as positive controls. x 200.

CD63 and clinicopathological parameters

CD63 positivity associated with an advanced stage (p=0.038), poor differentiation (p=0.044), and mucinous histology (p=0.021). We found no association between CD63 positivity with age, sex, or tumor location (colon vs. rectum or right vs. left; Table 1).

Survival analysis

Positive CD63 expression emerged as a sign of an unfavorable prognosis (p<0.00001, log-rank test); 5-year DSS for patients with a positive CD63 tumor expression was 56.7% (95% CI 52.0-61.4%) compared to 76.3% (95% CI 68.1-84.5%) among those with a negative expression. When we stratified CRC according to stage, CD63 positive expression represented a sign of an unfavorable prognosis for local disease (Dukes A-B; p=0.029) and metastasized disease (Dukes D; p=0.011.). Yet, in lymph-node positive CRC (Dukes C), we found no statistically significant relationship (p=0.076). In local disease, 5-year DSS for patients with a positive CD63 tumor expression reached 81.1% (95% CI 75.6-86.5%) compared to 90.0% (95% CI 82.4-97.6%) for

Table 2a. Cox uni- and multivariable analysis of the relative risk of death from colorectal cancer within 5 years based on CD63 expression.

CD63 expression	HR (95% CI)	P-value	N (events)	HR (95% CI)	P-value	N (events)
	Univariable			Multivariable		
Negative	1.00		115(24)	1.00		115(24)
Positive	2.11(1.68-2.54)	0.01	467 (208)	2.12 (1.69-2.55)	0.001	466(207)

CI, confidence interval; HR, Hazard ratio. Multivariable analysis included adjustment for sex, Dukes class, differentiation grade (G1-2 vs. G3-4), and histology (non-mucinous vs. mucinous).

Table 2b. Cox uni-and multivariable analysis of the relative risk of death from metastasized colorectal cancer within five years based on CD63 expression.

CD63 expression	HR (95% CI)	P-value	N (events)	HR (95% CI)	P-value	N (events)
	Univariable			Multivariable		
Negative	1.00		21(13)	1.00		21(13)
Positive	2.25(1.66-2.84)	0.007	109(93)	2.17 (2.57-2.77)	0.011	109(93)

Multivariable analysis included adjustment for sex, differentiation grade (G1-2 vs. G3-4), and histology (non-mucinous vs. mucinous).

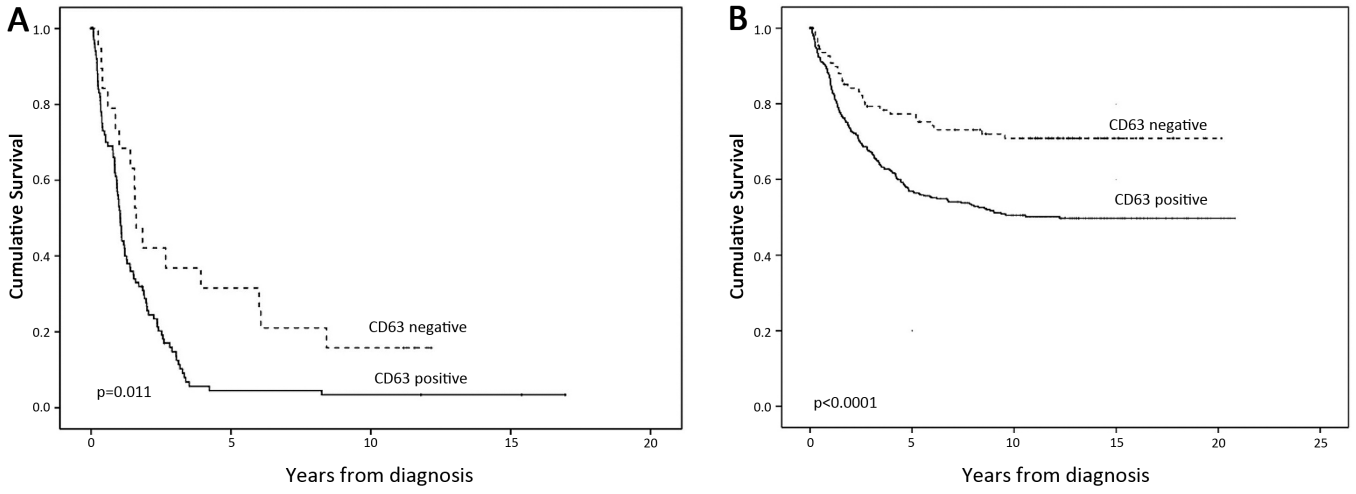

Fig. 2. CD63 expression associated with a poor prognosis. Disease-specific survival analysis based on the Kaplan-Meier method for CD63 in colorectal cancer **(A)** and Duckes D colorectal cancer **(B)** using the log-rank test.

CD63 negativity; in metastasized disease, 5-year DSS was 3.4% (95% CI 0-7.1%) compared to 26.3% (95% CI 6.5-46.1%), respectively (Fig. 2).

These results were confirmed by a 5-year univariable Cox regression analysis for CRC and for metastasized CRC, but not for the subgroup with local disease. The Cox regression multivariable analysis adjusted for age, sex, stage, and differentiation revealed that a positive CD63 expression independently predicted an unfavorable prognosis for CRC and for metastasized CRC (Tables 2a,b).

Discussion

Here, we show for the first time that CD63 expression represents an independent marker of an unfavorable outcome in colorectal cancer and in a subgroup of patients with metastasized disease. CD63 expression correlates with an advanced stage, poor differentiation, and mucinous histology.

Furthermore, CD63 expression was higher in more advanced tumors and among those with a poorer differentiation, supporting the prometastatic potential of CD63. These findings are in accordance with the literature describing CD63 as activating B-catenin, a known activator of EMT (Seubert et al., 2015). EMT is essential for tumor dissemination and the colonization of distant organs by a tumor cell (Thiery, 2002). Interestingly, the loss of CD63 expression induces the transition from the mesenchymal-to-epithelial phenotype, a process important in the formation of metastasis when tumor cells return to a more epithelial phenotype at the distant site reached (Zöller, 2008).

To our knowledge, we show for the first time that the tissue expression of CD63 serves as an independent marker of a poor prognosis in colorectal cancer. The direction of CD63 expression was the same in all the subgroups, but was above statistical significance in lymph-node positive disease. This difference seems to be more of a statistical issue, than of true biological difference. Earlier reports on prognosis in other cancers have been mixed. Increased CD63 expression predicted a poorer prognosis in glioblastomas, but required combining with TIMP4 expression to have a prognostic effect (Rorive et al., 2010). A similar effect on prognosis was also reported in gastrointestinal stromal tumours (Lewitowicz et al., 2016). However, a lack of CD63 expression correlated with a poor prognosis in stage I to II lung adenocarcinoma, but failed to independently act as a prognostic factor (Kwon et al., 2007). A recent report suggested CD63 expression to be a sign of better prognosis in pancreatic ductal adenocarcinoma (Khushman et al., 2018).

In addition to constituting a component of the exosomes/tumor-derived extracellular vesicles released, CD63 is also functionally involved in processes regulating intracellular vesicle transport (Berditchevski and Odintsova, 2007). This agrees with the higher CD63 expression in tumors with mucinous morphology, where CD63 may be implicated in the transport and secretion of mucin. We recently found evidence for an intriguing interplay between CD63 and the expression of ornithine decarboxylase antizyme inhibitor 2 (AZIN2), which also acts as a regulator of the intracellular vesicle traffic. The overexpression of AZIN2 cDNA in colon cancer cells induced the robustly enhanced release of CD63 carrying exosomes to the culture medium (Kaprio et al., 2019). Inducing EMT in colon cancer cells, however, induced the upregulated expression of endogenous AZIN2. Taken together, these findings suggest that the expression of CD63 and AZIN2 constitutes signatures of an EMT-associated secretory phenotype appearing to indicate an adverse outcome in cancer.

The ultimate molecular mechanism(s) linking an increased tumor cell expression of CD63 to a poorer prognosis in colorectal cancer remains unknown. A recent study (Nishida-Aoki et al., 2017) reported interesting findings potentially shedding some light on this phenomenon. In that study, they investigated mice xenografted with a highly metastatic clone of MDA-MB 231 breast cancer cells and treated them with antibodies to human CD63. The anti-CD63 antibodies significantly decreased the metastatic spread to the lungs and lymph nodes, but had no effect on the growth of the primary xenograft tumors. They showed that the effect was apparently mediated by the phagocytic clearance of tumor-derived CD63-positive exosomes opsonized by CD63 antibodies. This study further emphasized the role of tumor cell-derived exosomes in the formation of metastatic niches.

CD63 represents an essential cofactor for the expression of the adhesion molecule P-selectin, which mediates the heterotypic aggregation of activated platelets to cancer cells and the adhesion of cancer cells to the stimulated endothelial cells (Doyle et al., 2011). The P-selectin-mediated binding of platelets to circulating tumor cells leads to the formation of micro-emboli facilitating metastatic growth. This was demonstrated by the observation that human colon cancer cells xenografted in P-selectin knockout mice failed to display any metastatic spread in vivo (Kim et al., 1998).

Given that the present study is based on retrospective material, we have no information on the in vivo levels of tumor-derived exosomes in individual patients. However, our findings support the hypothesis that the increased expression of CD63 in colon cancer tissue serves as a signature of an EMT-associated secretory phenotype associated with adverse outcomes.

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Accepted February 19, 2020