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RESEARCH ARTICLE

Evaluation and prognostic significance of human tissue kallikrein-related peptidase 10 (KLK10) in colorectal cancer

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Abstract The prognosis of patients with colorectal cancer (CRC) is assessed through conventional clinicopathological parameters, which are not always accurate. Members of the human kallikrein-related peptidases gene family represent potential cancer biomarkers. The aim of this study was to investigate the expression of human tissue kallikrein-related peptidase 10 (KLK10) by immunohistochemistry in CRC, to correlate this expression with various histopathological and clinical variables, and to evaluate its significance as a predictor of disease outcome. KLK10 expression was evaluated by immunohistochemistry and a combined expression score was calculated for each case based on intensity and percentage of positivity. A statistically significant positive association was observed between KLK10 and tumor stage and liver metastases ($p=0.015$ and $p=0.035$, respectively). Paradoxically, a negative association was observed between KLK10 and tumor grade ($p=0.009$). Kaplan–Meier survival curves and univariate analysis showed that both KLK10 expression and stage had statistically significant correlations with disease-free survival (DFS) ($p=0.030$ and $p<0.001$, respectively) and overall survival ($p=0.010$ and $p=0.001$, respectively). Cox multivariate analysis showed that both KLK10 expression and stage were independent predictors of unfavorable DFS ($p=0.057$ and $p=0.001$, respectively) and overall survival ($p=0.009$ and $p=0.001$, respectively). In conclusion, KLK10 immunostaining is an independent prognostic marker in patients with CRC.

Keywords KLK · Kallikreins · KLK10 · Colorectal cancer · Prognosis

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Non-standard abbreviations

CRC	Colorectal cancer
PS	Proportion score
IS	Intensity score
TS	Total score
IHC	Immunohistochemistry

Introduction

Serine proteases are a family of enzymes that utilize a uniquely activated serine residue to catalytically hydrolyze peptide bonds [1, 2]. Kallikreins are a subgroup of serine proteases that co-localize to chromosomal region 19q13.4 and are expressed in a wide range of tissues [3]. Many members of the human kallikrein-related peptidases family of enzymes have been shown to be dysregulated in different malignancies and to have the potential to be used as cancer diagnostic/prognostic markers [4–7]. Many kallikreins including KLK6, 8, and 10 were shown to be dysregulated in colon cancer compared to normal colon [8].

According to recent cancer statistics, colorectal cancer (CRC) is the third most common malignancy and the second leading cause of cancer-related deaths in the United States [9]. The lifetime risk for CRC is 5–6% and is influenced by the heterogeneous etiology of the disease, involving genetic and environmental factors [9, 10]. For many decades, the Dukes' classification and TNM staging system have been the gold standards for predicting outcome and implementing therapeutic strategies in the management of CRC patients [11]. However, recent studies have shown the presence of heterogeneity of the behavior among patients even those with the same stage. Stepping into a new era of "personalized medicine", the introduction of new genetic molecular markers is urgently needed to substratify patients into smaller subgroups with subsequent individualization of management plans according to disease severity as determined by biological rather than anatomical parameters [12]. This will have a significant impact on patient management by reserving intensive treatments only for those with an aggressive disease and in the meantime, avoiding the cost and side effects for patients who are not suitable candidates for a specific treatment [13].

A recent study analyzed the expression of a panel of KLKs in CRC and concluded that the multiparametric combination of a group of KLKs can increase the accuracy of prediction of patients' survival beyond the traditional clinical information [14]. KLK10 is a member of the human kallikrein-related family of peptidases. Initial data showed that KLK10 is dysregulated in many cancers including breast, ovarian, and testicular cancers [15–17]. Interestingly, the pattern of KLK10 dysregulation was found to be cancer specific. The gene was downregulated in breast cancer, whereas it showed upregulation in ovarian cancer [18].

Multiple mechanisms were shown to account for KLK10 dysregulation in cancer including hypermethylation [19], and the frequent inactivation and loss of KLK10 expression was a critical step towards carcinogenesis [20]. Furthermore, it was suggested that KLK10 expression and its methylation status could be used as a molecular marker for breast cancer. Similar uses were proposed for acute lymphoblastic leukemia [20]. Recent data suggests an additional functional role for KLK10 as a downstream target for multiple miRNAs [21].

A recent study examined the examination of KLK10 mRNA expression and its association with CRC progression was studied using semi-quantitative PCR. The results suggest that KLK10 gene expression can be used as a marker of unfavorable prognosis for CRC [22].

The aim of this study was to investigate the expression KLK10 by immunohistochemistry in CRC to correlate the expression with various histopathological and clinical outcomes and to evaluate its potential use as a biomarker.

Materials and methods

Patients

Included in the study were 62 patients, 32 males and 30 females, median age 71 years (range 42–91), who underwent subtotal-colectomy for colorectal carcinoma (CRC) (median tumor size 5.0 cm, range 0.9–14 cm) in Enangelismos Hospital, Athens, Greece. Diagnosis was confirmed by a pathologist for each slide used in the study. Tumors were graded according to the WHO grading system and staged according to the modified Astler-Coller system (MAC). Two out of 62 (3%) CRCs were well differentiated, 42/62 (68%) moderately differentiated, and 18/62 (29%) poorly differentiated. Six out of 62 (10%) CRCs were stage A, 23/62 (37%) stage B, 24/62 (39%) stage C, and 9/62 (14%) stage D. Regional lymph nodes metastasis was observed in 34/62 (55%) of cases. Liver metastasis was reported in 9/59 (15%) patients; clinical data for three patients was unavailable. The median follow-up period, available for 56 patients was 62 months (range 1–68). Relapse was observed in 21/56 (38%) patients and death in 20/56 (36%) patients. The median disease-free survival (DFS) period was 45 months (range 1–62) and the median overall survival (OS) was 51 months (range 1–64) (Tables 1 and 2).

Immunohistochemical staining

The immunohistochemical staining was performed on 4 μm thick paraffin sections of tissues fixed in buffered formalin, according to a streptavidin-biotin-peroxidase protocol using the DAKO LSAB+Kit. A KLK10-specific rabbit polyclonal antibody was raised in-house against KLK10. This antibody was tested and showed no cross-reactivity with other members of the kallikrein-related family of peptidases. Staining procedures included deparaffinization in warm xylene for 5 min with two changes of xylene at room temperature, followed by rehydration by transfer through graded alcohols. Endogenous peroxidase activity was blocked with

Table 1 Distribution of numerical clinicopathological variables of the study

Variable	Mean±SE	Range	Quartiles		
			25 (Median)	50	75
Patient age (years; n=62)	68.8±1.3	42–91	63.0	71.0	77.0
Tumor size (cm; n=62)	5.17±0.28	0.90–14.00	3.50	5.00	6.12
DFS (months; n=56)	35.3±3.1	1.0–62.0	12.0	45.0	55.7
OS (months; n=56)	39.1±2.8	1.0–64.0	22.2	51.0	57.0

0.5% H_20_2 in methanol for 10 min. The sections were pretreated with 10 mmol/L citrate buffer (pH 6.1) in a microwave for 5 min and incubated overnight at 4°C with the KLK10 primary rabbit polyclonal antibody (1:500) in 3% BSA. After two washes of the sections in 50 mM Tris buffer (pH 7.6), the biotinylated Link (DAKO Corporation USA) was applied for 15 min and a streptavidin–peroxidase conjugate followed for another 15 min. The enzymatic reaction was developed in a freshly prepared solution of 3,3 -diaminobenzidine tetrahydrochloride using DAKO Liquid DAB substrate-chromogen solution for 10 min (brown color). The sections were then counterstained with haemalum, dehydrated, cleared in xylene, and mounted. In selected tissues, the primary antibody was replaced by a non-immune rabbit serum (1:500) in 3% BSA in order to assess non-specific binding.

Table 2 Associations between *KLK10* status and other clinicopathological variables

Variable	Total	No. of patients (%)		p value
		KLK10 negative	*KLK10* positive	
Sex				
Males	32	18 (56.3)	14 (43.8)	
Females	30	20 (66.7)	10 (33.3)	0.44[a]
Nodal status				
Negative	29	19 (65.5)	10 (34.5)	0.61[a]
Positive	33	19 (57.6)	14 (42.4)	
Grade				
I	2	0 (0.0)	2 (100.0)	
II	43	23 (53.5)	20 (46.5)	0.009[b]
III	17	15 (88.2)	2 (11.8)	
Stage				
A	6	1 (16.7)	5 (83.3)	
B	23	17 (73.9)	6 (26.1)	0.015[b]
C	24	17 (70.8)	7 (29.2)	
D	9	3 (33.3)	6 (66.7)	
Liver metastasis				
No	53	35 (66.0)	18 (34.0)	
Yes	8	2 (25.0)	6 (75.0)	0.035[a]
X	1			

[a] Fisher's exact test

[b] Chi-Square test

Evaluation of the immunohistochemical staining

A combination of a proportion score (PS) and an intensity score (IS) was used to assess KLK10 immunostaining: PS (proportion of positive tumor cells on the studied section): 0, none, 1, <1%, 2, 1–10%, 3, 11–30%, 4, 31–75%, 5, >75%. IS (intensity of staining by tumor cells): 0, none, 1, weak, 2, moderate, 3, strong. A total score (TS) with a range between 0 and 8 was obtained by the addition of PS and IS. The TS was simplified to a final score with a range between 0 and 4, by putting TS 1 and 2, TS 3 and 4, TS 5 and 6, and TS 7 and 8 together, respectively.

Statistical analysis

The X-tile algorithm was used to generate an optimal cutpoint for *KLK10*, as it is a gene with no established cut points regarding its expression in colorectal cancer. Having corrected for the use of minimum p value statistics, the X-tile software yielded an optimal cutoff of final score staining (≥2+), equal to the 60th percentile, with a calculated Monte Carlo p value of <0.05. Associations between dichotomous clinicopathological parameters and *KLK10* expression status were evaluated by the χ^2 test or the Fisher's exact test, where appropriate. Cox proportional hazard regression model was developed to evaluate the association between the prognostic markers and disease-free (DFS) or overall survival (OS) of patients. Survival analyses were also performed by constructing Kaplan–Meier DFS and OS curves. Differences between curves were evaluated by the log–rank test.

Results

In the normal colonic epithelium, absorptive cells showed subnuclear cytoplasmic staining with a focal patchy pattern. Goblet cells showed a similar staining pattern, but most mucin droplets remained unstained (Fig. 1a). In CRC, only 24/62 cases (39%) showed positive staining (using a cut off value for a total score greater than 2). The immunostaining

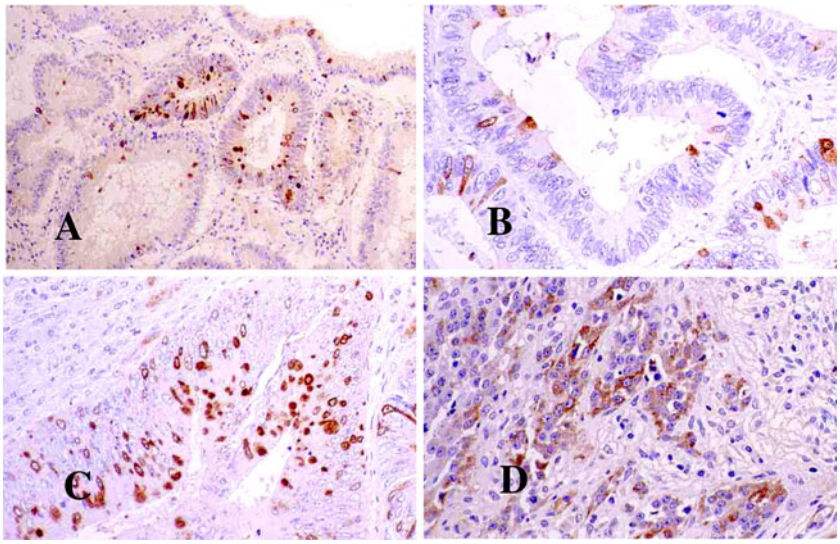

Fig. 1 Cytoplasmic immunohistochemical expression of KLK10 with a patchy distribution in **a** Normal colon mucosa (magnification 100×), **b** well-differentiated colorectal carcinoma (CRC) (magnification 200×), **c** moderately differentiated CRC (magnification 200×), **d** poorly differentiated CR (magnification 200×)

in CRCs was cytoplasmic, mostly with a patchy distribution (Fig. 1b–d). There was no significant staining in mesenchymal tissues, except mild to moderate expression in nerves and endothelium.

The distributions of the numerical clinical pathological variables of the subjects involved in this study are summarized in Table 1. As shown in Table 2, there is a statistically significant difference in KLK10 expression among the different tumor stages (p=0.015). There is also a positive correlation between KLK10 expression and liver metastases (p=0.035) (Table 2). A trend towards higher expression was seen in node-positive patients, although this did not reach statistical significance. Paradoxically, a statistically significant negative association was observed between KLK10 expression and tumor grade (p=0.009) (Table 2).

Cox univariate analysis (Table 3) showed that both stage and KLK10 expression are associated with shorter disease-free survival (HR, 2.97, p<0.001, and HR, 2.62, p=0.030, respectively). Both were also associated with significantly decreased overall survival (HR, 2.9, p=0.001 and HR, 3.12, p=0.010, respectively). In multivariate analysis, only stage retained its value as an independent predictor of unfavorable disease-free survival (HR, 2.76, p=0.001) and both KLK10 expression and stage were independent indicators of poor overall survival (HR, 3.63, p=0.009 and HR, 2.88, p=0.001, respectively) (Table 3).

Table 3 Associations between *KLK10* and disease-free and overall survival

Variable	Disease-free survival			Overall survival		
	HR[a]	95% CI[b]	p value	HR[a]	95% CI[b]	p value
Univariate analysis (n=56)						
KLK10						
Negative	1.00			1.00		
Positive	2.62	1.09–6.23	0.030	3.12	1.31–7.47	0.010
Stage (ordinal)	2.97	1.61–5.50	<0.001	2.90	1.58–5.34	0.001
Grade (ordinal)	0.78	0.33–1.85	0.58	0.81	0.33–1.95	0.63
Multivariate analysis[c] (n=54)						
KLK10						
Negative	1.00			1.00		
Positive	2.54	0.97–6.64	0.057	3.63	1.37–9.63	0.009
Stage (ordinal)	2.76	1.53–4.99	0.001	2.88	1.54–5.37	0.001
Grade (ordinal)	0.29	0.29–2.72	0.85	1.14	0.36–3.57	0.82

[a] Hazard ratio (HR) estimated from Cox proportional hazard regression model

[b] Confidence interval of the estimated HR

[c] Multivariate models were adjusted for patient stage and tumor grade

As shown in Fig. 2, patients who are KLK10 negative had a significantly longer disease-free survival compared to those who are KLK10 positive (p=0.021). Also, KLK10 positive patients had statistically significant shorter overall survival compared to those who are KLK10 negative (p= 0.007) (Fig. 3).

Discussion

Our results show that KLK10 over-expression correlates with poor prognosis in colorectal cancer, being associated with liver metastases, decreased disease-free, and overall survival. Our results are in agreement with a recently published report that analyzed KLK10 expression in colorectal cancer at the mRNA level [22]. Another report showed that KLK10 mRNA expression level significantly correlated with lymphatic invasion and clinical stage of colorectal cancer [23]. A third report showed that KLK10 expression is up-regulated in colon cancer with higher expression closely correlating with advanced disease stage, which predicts a poorer prognosis [24]. An earlier in silico analysis showed that three kallikrein genes, KLK6, 8, and 10, are overexpressed in colon cancer compared to normal colon [8]. Our findings show a negative correlation between KLK10 expression and tumor grade, indicating that these two parameters are affected by independent mechanisms. One explanation is the presence of great heterogeneity within the same histological grade. It will be interesting to conduct a study of KLK10 expression within each grade. This might be also helpful in substratifying members of each grade according to KLK10 expression.

Reports showed the dysregulation of KLK10 in other cancers as well. For instance, kallikreins 5, 7, 8, and 10 were shown to be abundantly expressed in human oral squamous cell carcinoma with an implication in malignant

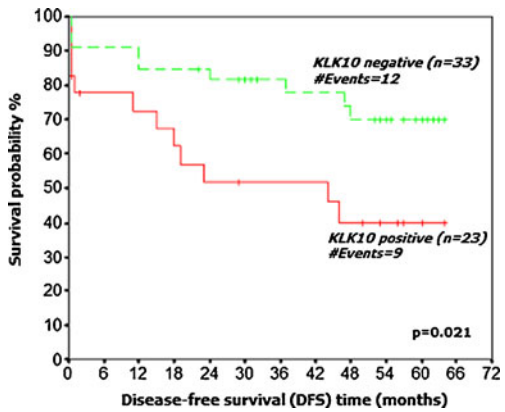

Fig. 2 Kaplan–Meier survival curve showing that KLK10-positive patients have significantly lower disease-free survival compared to those who are KLK10-negative (p=0.021)

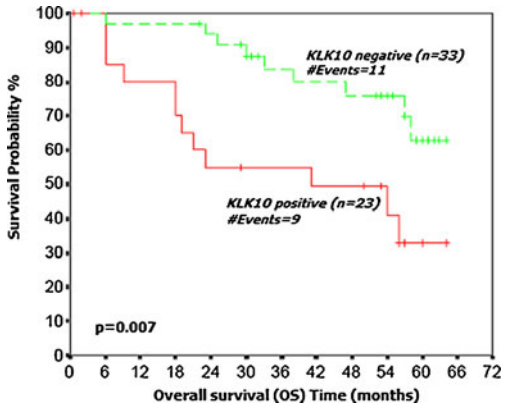

Fig. 3 Kaplan–Meier survival curve comparing overall survival between KLK10-positive and -negative colorectal cancer patients. Patients who are KLK10 negative have a significantly longer overall survival compared to those who are KLK10 positive (p<0.007)

progression [25]. Also, studies reported dysregulation of KLK10 and KLK6 in pancreatic ductal adenocarcinoma [26], kidney cancer [27], and ovarian cancer [18].

Evidence is evolving about the involvement of KLK10 in colorectal cancer pathogenesis. There are multiple mechanisms of involvement of KLK10 and other kallikreins in carcinogenesis. Kallikreins, as serine proteases, have been shown to degrade extracellular matrix proteins including collagen, fibrinogen, laminin, and fibronectin [28]. Also, it has been recently shown that KLK10 can be a direct target of miRNAs with a subsequent effect on cellular proliferation [21]. It appears, however, that the mechanisms by which kallikreins are involved in cancer vary depending on the specific kallikrein in question and the type of cancer. For example, in contrast to its upregulation in colorectal and ovarian cancer, earlier data suggested that KLK10 acted as a tumor suppressor in breast cancer, with its function controlled via epigenetic mechanisms [19]. Also, a recent report revealed that a hyperactive TGFβ-TGFβR-Smad2 signaling axis is needed to maintain epigenetic silencing of critical EMT genes, including KLK10, for breast cancer progression [29].

Interestingly, other kallikreins were also found to be dysregulated in colorectal cancer, such as KLK6 [30]. The co-dysregulation of multiple kallikreins in colorectal cancer supports the hypothesis of the presence of a kallikrein cascade that is involved in cell proliferation and differentiation [31, 32]. As such, kallikrein-related peptidases represent attractive therapeutic targets for different malignancies.

Immunohistochemical analysis (IHC) has a number of advantages over mRNA or total protein analysis, including the ability to identify the specific cells that express the biomarker of interest (e.g., epithelial versus stromal cells) and the accurate sublocalization of expression at the cellular level (cytoplasmic versus membranous versus nuclear) [33].

Immunohistochemistry also allows for semi-quantification to be performed on formalin-fixed paraffin embedded tissues. The fact that IHC can be readily performed on formalin-fixed clinical specimens carries the advantage of quick implementation of new biomarkers either alone or in combination with the existing markers in prognostic models. These results, once validated on a larger independent set, can be translated into a clinical test.

In conclusion, our results show that KLK10 expression, assessed by immunohistochemistry, is an independent indicator of poor prognosis. Assessment of KLK10 can be incorporated to a multi-molecular prognostic model to achieve better and more accurate assessment of disease prognosis.

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Conflicts of interest None.

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