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Kallikrein-related peptidase 7 (KLK7) is a proliferative factor that is aberrantly expressed in human colon cancer

Abstract: Emerging evidence indicates that serine proteases of the tissue kallikrein-related peptidases family (*KLK*) are implicated in tumorigenesis. We recently reported the ectopic expression of KLK4 and KLK14 in colonic cancers and their signaling to control cell proliferation. Human tissue kallikrein-related peptidase 7 (KLK7) is often dysregulated in many cancers; however, its role in colon tumorigenesis has not yet been established. In the present study, we analyzed expression of KLK7 in 15 colon cancer cell lines and in 38 human colonic tumors. In many human colon cancer cells, KLK7 mRNA was observed, which leads to KLK7 protein expression and secretion. Furthermore, KLK7 was detected in human colon adenocarcinomas, but it was absent in normal epithelia. KLK7 overexpression in HT29 colon cancer cells upon stable transfection with a KLK7 expression plasmid resulted in increased cell proliferation. Moreover, subcutaneous inoculation of transfected cells into nude mice led to increased tumor growth that was associated with increased tumor cell proliferation as reflected by a positive Ki-67 staining. Our results demonstrate the aberrant expression of KLK7 in colon cancer cells and tissues and its involvement in cell proliferation *in vitro* and *in vivo*. Thus, KLK7 may represent a potential therapeutic target for human colon tumorigenesis.

Keywords: cancer; colon; *in vivo*; kallikrein; proliferation.

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Introduction

Colorectal cancer (CRC), like other cancers, manifests with accumulated defects in pathways regulating cell proliferation, differentiation, and apoptosis. The defects include alterations that lead to novel or increased function of oncogenes or loss of function of tumor suppressor genes (Wong et al., 2007; Fearon, 2011). The essential role of proteolytic enzymes such as matrix metalloproteinases (MMPs) and various serine proteases in colon cancer progression and metastasis is also well known. Proteases have long been associated with colon cancer progression because of their ability to degrade extracellular matrices, and facilitate invasion and metastasis (Mook et al., 2004). However, recent studies have shown that these enzymes can target a diversity of substrates and promote some steps of tumor development (Emami and Diamandis, 2007; Lopez-Otin and Matrisian, 2007). Besides their contribution to cancer progression by degrading extracellular matrix proteins, it is now clear that a subclass of proteases including some serine proteases like KLKs serve as signaling molecules controlling cell functions through specific membrane receptors, the protease-activated receptors (PARs). The PARs family consists of four G-protein-coupled receptors, namely PAR1, PAR2, PAR3, and PAR4 (Dery et al., 1998; Macfarlane et al., 2001; Adams et al., 2011), which are abundantly expressed in colon cancer (Darmoul et al., 2001, 2003, 2004b; Gratio et al., 2009). We have previously demonstrated that some serine proteases such as thrombin and trypsin behave as very robust growth factors through PAR1 and PAR2 and induce cell proliferation and motility in human colon cancer cells (Darmoul et al., 2003, 2004a,b; Gratio et al., 2009, 2010, 2011). Very recently, our group and others have shown that some members of the tumor-associated serine protease family

of kallikrein-related peptidases (KLK) signal through PARs to control cell behavior as well (Oikonomopoulou et al., 2006b; Ramachandran and Hollenberg, 2008; Ramsay et al., 2008; Gratio et al., 2009, 2011; Oikonomopoulou et al., 2010; Chung et al., 2012).

The KLK superfamily consists of 15 (KLK1-KLK15) trypsin- or chymotrypsin-like serine proteases (Borgono et al., 2004; Emami and Diamandis, 2007). KLK enzymes are secreted into the extracellular space of a wide range of tissues. Although primarily known for their clinical applicability as cancer biomarkers, recent evidence implicates KLKs in many cancer-related processes (Borgono and Diamandis, 2004; Obiezu and Diamandis, 2005; Emami and Diamandis, 2007; Gratio et al., 2009, 2011; Oikonomopoulou et al., 2010; Chung et al., 2012). Primarily, they have been shown to promote or inhibit neoplastic progression, acting individually and/or in cascades with other KLKs and proteases (Sotiropoulou et al., 2009).

Recently, we have structurally and biochemically characterized a series of KLK proteases including KLK7 (Debela et al., 2006). KLK7 displays a chymotrypsin-like serine protease activity with a preferred cleavage C-terminal to aliphatic and aromatic residues.

Originally, KLK7 expression was found in the skin and the major biological functions of the enzyme were thus connected to skin physiopathology and related epithelial tissues, such as in keratinization, stratum corneum formation, and skin desquamation (Eissa and Diamandis, 2008; Ovaere et al., 2009). KLK7 expression has been examined either individually or in panels as diagnostic or prognostic factors in different types of cancers (Talieri et al., 2009a,b; Avgeris et al., 2012; Kontos and Scorilas, 2012; Kontos et al., 2012). KLK7 expression was found to be elevated in breast carcinoma (Li et al., 2009), prostate adenocarcinoma (Jamaspishvili et al., 2011), ovarian adenocarcinoma (Kyriakopoulou et al., 2003; Prezas et al., 2006; Dong et al., 2010; Dorn et al., 2014), and lung carcinoma (Planque et al., 2005). In colon cancer, elevated KLK7 levels were found to be associated with unfavorable prognostic value in patients with colon cancer (Talieri et al., 2009b; Kontos et al., 2012).

Our recent studies have demonstrated that KLK4 and KLK14 are expressed in colonic tumors and can both signal selectively through different PARs to stimulate colon cancer cell growth. Herein, we report that KLK7 is ectopically expressed in many colon cancer cells *in vitro* and in resected tumors from patients with colon cancers. Moreover, HT29 colon cancer cells overexpressing KLK7 showed increased cell proliferation *in vitro* and greater tumor growth *in vivo* in mouse xenograft colon cancer model.

These data, presented at the International Symposium on Kallkreins and Kallikrein-Related Peptidases (Toronto, Ontario, Canada, September 28 to October 1, 2013), point to an important role for KLK7 in colon cancer tumorigenesis.

Results

Human colon cancer cell lines in culture express KLK7

KLK7 mRNA transcript levels were investigated in 15 human colon cancer cell lines by Reverse transcription-polymerase chain reaction (RT-PCR) analysis. As shown in Figure 1, 6 of 15 analyzed human colon cancer cells express KLK7 mRNA. A strong KLK7 mRNA signal (corresponding to the predicted size of 569 bp) was detected in SW48, T84, SW620, and HCT8 cells (Figure 1A). Weak

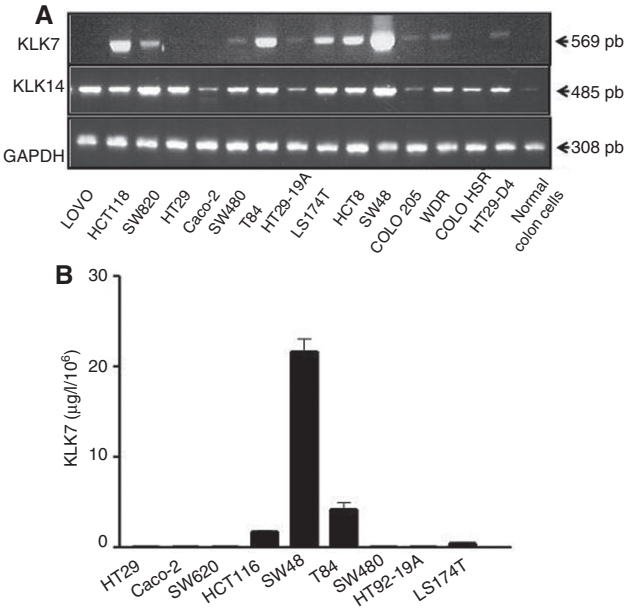

Figure 1 Expression of KLK7 in human colon cancer cell lines. (A) Four micrograms of total RNA were reverse-transcribed and PCR-amplified with KLK7, KLK14, or GAPDH primers. A single PCR-amplified product of the predicted size (569 bp) for KLK7 was visualized after electrophoresis on a 2% agarose gel (upper). Note that KLK7 is present in SW620, a cell line from a lymph node of a primary adenocarcinoma from which SW480 was derived. Contrary to KLK7, KLK14 is expressed in most of colon cancer cell lines (lower). (B) Cell culture supernatants were collected from colon cancer cells in culture, and KLK7 expression was determined by ELISA (as previously described by Shaw and Diamandis, 2007). Protein values represent the mean concentration of KLK7 expressed by 10^6 cells.

expression of KLK7 was detectable in LS174T and HCT116 cells. KLK7 was barely detectable or absent in SW480, LoVo, WIDR, Colo-HSR, Caco-2, Colo205, and HT29 cells and in both HT29-D4 and HT29-19A cell lines, which represent two differentiated clones derived from the HT29 parental cell line (Augeron and Laboisse, 1984; Fantini et al., 1986) (Figure 1A). Under our experimental conditions, KLK7 mRNA is absent in epithelial cells isolated from normal human colon (Figure 1A). This expression profile is quite different from that of KLK14, another member of the KLK family, which is expressed in almost all colonic cancer cells analyzed but not in normal colonic cells (see Figure 1A).

KLK7 protein expression was investigated by measuring KLK7 secretion into the conditioned media from human colon cancer cells *in vitro*. The antigen levels of KLK7 in various cell line supernatants were quantified using an immunoassay (see Materials and methods). As shown in Figure 1, KLK7 protein is secreted by few human colon cancer cell lines (Figure 1B). The amount of KLK7 in these supernatants was corrected for the total cell number. The highest KLK7 levels (23 μg/l; ~3 nM) were observed in the conditioned media from SW48 cells followed by HCT116, T84, and LS174T cells. KLK7 immunoreactivity in the other cell lines (HT29, Caco-2, SW620, SW480, HT29-19A, LoVo) was very low or below the detection limit of the current protocol. These data are in good agreement with the KLK mRNA expression analysis, and they suggest that a series of colon cancer cell lines express and secrete KLK7 that could potentially act in autocrine manner to control cell behavior.

KLK7 is expressed in colon cancer tumors *in vivo*

Because the ectopic expression of KLK7 in colon cancer cells *in vitro* was evident, we investigated the KLK7 expression in human colonic adenocarcinomas versus normal colonic epithelium by immunohistochemistry. Thirty-seven of 38 tumors tested were immunoreactive for KLK7. Table 1 shows the immunoreactivity scores of KLK7 for each tumor in relation with tumor stage, histological type, and differentiation grade. No significant association between the different clinicopathological variables, and the KLK7 staining scores were found (Supplementary Table 1). As depicted in Figure 2, immunoreactivity is detected in the low-grade dysplastic (Figure 2A) colonic mucosa contiguous to the cancerous lesion (Figure 2B), being seen mainly in the apical side of the epithelium. KLK7 expression is clearly seen in the mild dysplastic colonic mucosa (Figure 2C), and expression increases in intensity as the dysplasia progresses to cancer (Figure 2D and E). In contrast, in normal human colonic mucosa from control subjects without colonic cancer, no staining for KLK7 was observed in epithelial cells (Figure 2F).

The intensity of labeling varies among the different patient's adenocarcinomas analyzed (Figure 2). Staining was localized in the cytoplasmic compartment and appeared strong in the apical part (Figure 2B and D) and also in the basal part (Figure 2C) of the cells. Interestingly, KLK7 expression in the 'intravascular tumor emboli' was very strong (Figure 2E). No specific staining was seen in negative control sections where the primary antibody was omitted (not shown). These observations show that human colonic adenocarcinomas aberrantly express higher levels of KLK7 than normal colonic mucosa from healthy cases and suggest that KLK7 expression may represent a potential marker for colonic carcinogenesis.

Effects of KLK7 overexpression in transfected HT29 cells on proliferation *in vitro*

To mimic KLK7 overexpression seen in colonic tumors, we stably transfected HT29 colon cancer cells in which endogenous KLK7 expression was weak/negative (see Figure 1 with a KLK7 expression vector; see Materials and methods). Expression and secretion of KLK7 was examined by enzyme-linked immunoassay (ELISA) in conditioned medium from KLK7-transfected cells and control cells (Figure 3, inset). KLK-transfected cells exhibit KLK7 antigen levels similar to those found constitutively expressed by some other cell lines (~18 μg/l) (see Figure 1B), whereas no immunoreactive KLK7 was detected in the conditioned medium of control cells.

KLK7 is known to contribute to cancer progression in several tumor types (Avgeris et al., 2012; Kontos and Scorilas, 2012). This prompted us to evaluate the effect of KLK7 on cell growth. As shown in Figure 3, overexpression of KLK7 in HT29 cells significantly increased the cell number (2.5-fold) as compared with control cells (Figure 3). Overall, these data suggest that overexpression of KLK7 can stimulate colon cancer cell proliferation *in vitro*.

Effect of KLK7 on the tumor growth in a mouse xenograft colon cancer model

The observed increased proliferation of HT29-KLK7-transfected cells *in vitro* prompted us to investigate whether overexpression KLK7 in human HT29 colon carcinoma

Table 1 Estimation of KLK7 expression in colon carcinoma epithelial cells.

Cases	Tumor site	Histological type	Tumor stage	Differentiation grade	Scores
1	Right colon	Adenocarcinoma	$TisN_0M_0$	1	200
2	Sigmoid	Adenocarcinoma	$T_1N_0M_x$	1	160
3	Right colon	Adenocarcinoma with villus adenoma	$T_1N_0M_x$	1	200
4	N/A	Adenocarcinoma	$T_2N_0M_0$	2	140
5	N/A	Adenocarcinoma	$T_2N_0M_0$	1	360
6	N/A	Adenocarcinoma	$T_2N_0M_0$	1	160
7	N/A	Adenocarcinoma	$T_2N_0M_0$	2	140
8	N/A	Adenocarcinoma	$T_2N_0M_0$	1	140
9	N/A	Mucinous adenocarcinoma	$T_2N_0M_0$	2	40
10	N/A	Adenocarcinoma	$T_2N_0M_0$	2	25
11	N/A	Adenocarcinoma	$T_2N_0M_0$	2	360
12	N/A	Adenocarcinoma	$T_2N_0M_0$	2	400
13	Cecum	Adenocarcinoma	$T_2N_0M_x$	2	60
14	Cecum	Adenocarcinoma	$T_2N_0M_x$	1	70
15	N/A	Adenocarcinoma	$T_2N_0M_1$	2	160
16	N/A	Mucinous adenocarcinoma	$T_2N_1M_0$	2	180
17	N/A	Adenocarcinoma	$T_2N_1M_1$	3	270
18	Cecum	Mucinous adenocarcinoma	$T_3N_0M_0$	2	100
19	N/A	Mucinous adenocarcinoma	$T_3N_0M_0$	2	210
20	N/A	Adenocarcinoma	$T_3N_0M_0$	2	360
21	N/A	Adenocarcinoma with villus adenoma	$T_3N_0M_0$	2	100
22	Cecum	Mucinous adenocarcinoma	$T_3N_0M_x$	1	15
23	Rectum	Adenocarcinoma	$T_3N_0M_x$	1	0
24	Right colon	Adenocarcinoma	$T_3N_0M_x$	1	5
25	Right colon	Adenocarcinoma	$T_3N_1M_x$	2	180
26	Right colon	Adenocarcinoma	$T_3N_1M_x$	3	300
27	Left colon	Adenocarcinoma	$T_3N_1M_x$	2	200
28	Left colon	Mucinous adenocarcinoma	$T_3N_1M_x$	1	300
29	Right colon	Adenocarcinoma	$T_3N_1M_x$	1	200
30	Right colon	Adenocarcinoma	$T_3N_1M_x$	1	10
31	Right colon	Adenocarcinoma	$T_3N_2M_x$	2	240
32	Right colon	Adenocarcinoma	$T_3N_2M_x$	2	90
33	Sigmoid	Adenocarcinoma	$T_3N_2M_x$	2	180
34	N/A	Adenocarcinoma	$T_3N_4M_1$	2	360
35	Right colon	Adenocarcinoma	$T_4N_0M_x$	2	180
36	Right colon	Adenocarcinoma	$T_4N_1M_x$	2	160
37	Sigmoid	Adenocarcinoma	$T_4N_2M_x$	1	95
38	N/A	Adenocarcinoma	$T_4N_2M_1$	2	240

N/A, not applicable.

Staining intensity was often variable from place to place and scored as 0, negative; 1, weak; 2, moderate; 3, strong; 4, intense. Differentiation grade is scored as 1, well; 2, moderately; 3, poorly differentiated. Results of scores were obtained by multiplying the percentage of positive cells by the intensity (maximum=400).

cells can also influence tumor growth *in vivo*. Therefore, HT29-KLK7 cells and HT29-vector control cells were inoculated subcutaneously into the flank of nude mice, resulting in the development of tumors at the site of inoculation. Tumors were detectable at day 5 but developed slowly until day 14 (Figure 4). Thereafter, tumors increased rapidly until the end of the experiment (day 24). In mice inoculated with HT29-KLK7 cells, a substantial increase in tumor volume (474±34.27 mm^3) was detected compared with the control mice (256±42.5 mm^3) (Figure 4). Moreover, mice inoculated with the HT29-vector control cells revealed a mean tumor mass of 0.44±0.076 g (n=6). In contrast, mice injected with the HT29-KLK7 cells showed increase in mean tumor mass of 0.65±0.037 g (n=8) compared with the control tumors (Figure 5A). In addition, in HT29-KLK7-derived tumors, we found increased cell proliferation as revealed by Ki-67 staining, compared with controls (Figure 5B). Autopsy of mice did not reveal any obvious metastatic sites of tumor development.

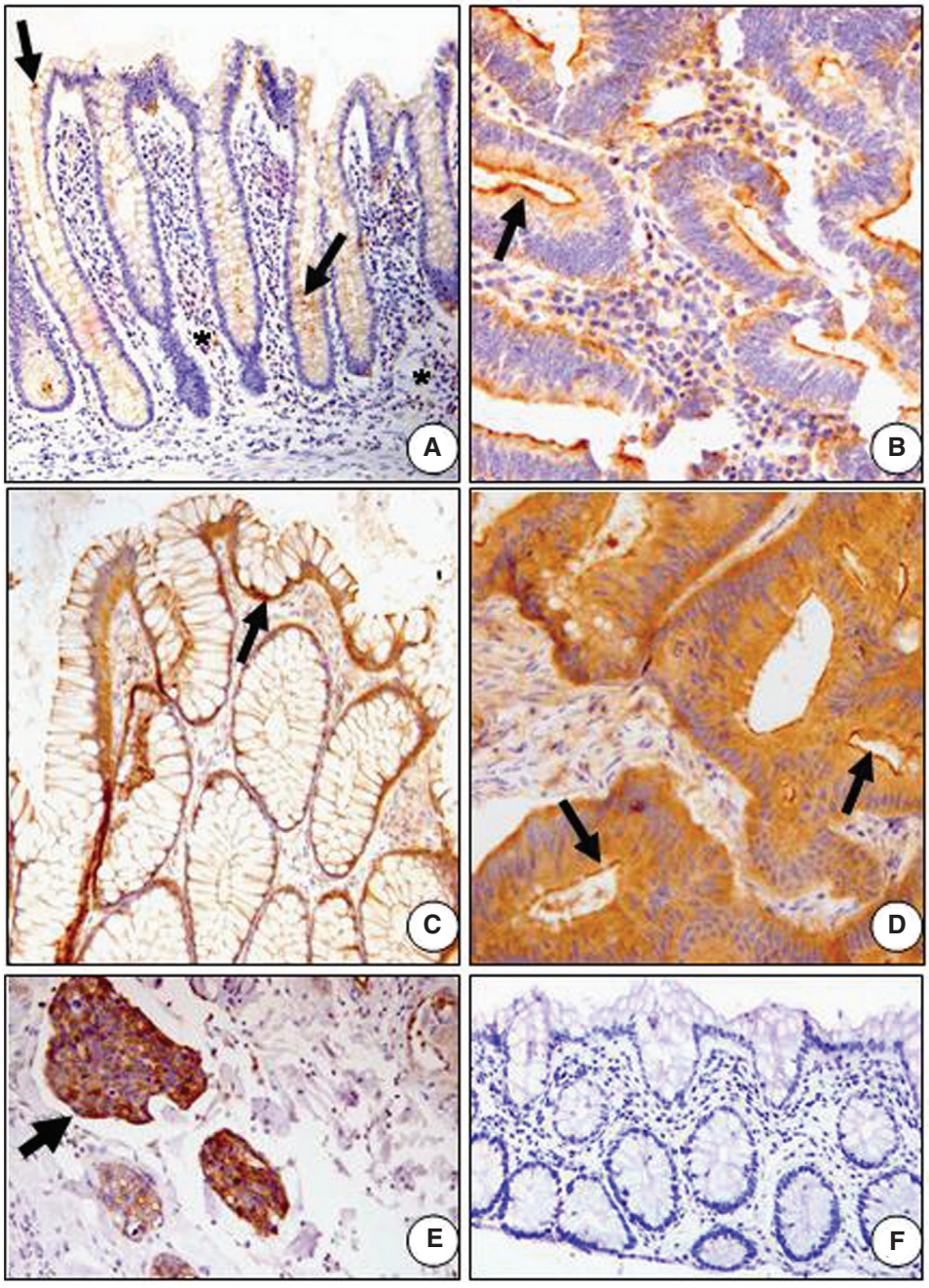

Figure 2 Representative immunostaining for KLK7 in paraffin sections of normal colonic mucosa and colonic tissues from patients with adenocarcinomas.
(A) Discrete KLK7 immunoreactivity in the epithelium (arrows) of the low-grade dysplastic colonic mucosa contiguous to a left colon adenocarcinoma (original magnification ×100). Asterisks (*) show few stromal cell staining. (B) Same patient as in (A). KLK7 immunoreactivity appeared to be distinguishable into a predominantly apical/cytoplasmic pattern (arrows) (original magnification ×200). (C) In the dysplastic colonic mucosa contiguous to the cancerous lesion, clear immunoreactivity is present in the epithelium with a predominantly basal/cytoplasmic pattern (arrows) (original magnification ×100). (D) Same patient as in (C). KLK7 high immunoreactivity is uniformly present in the cytoplasmic compartment of the cancerous epithelial cells (arrows) (original magnification ×200). (E) Third patient. KLK7 expression is seen in an intravascular tumor embolus (thick arrow) (original magnification ×100). (F) KLK7 immunoreactivity is absent in normal sigmoid glandular colonic mucosa from control healthy subjects (original magnification ×100).

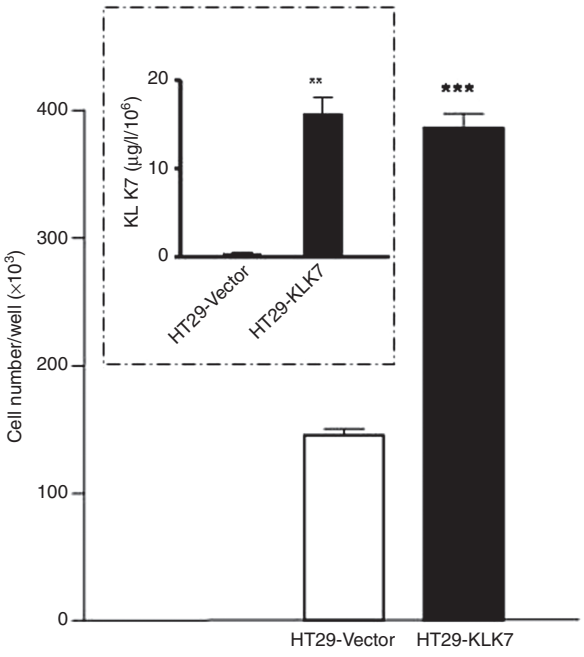

Figure 3 Effect of KLK7 overexpression in HT29 cell proliferation. Transfected HT29-KLK7 and HT29-vector cells were seeded in 48 cluster wells in DMEM medium and 10% of FCS. After 96 h, cells from triplicate wells were counted for each condition. Data are given as mean±SE of three different experiments. ***$p<0.001$, HT29-KLK7 cells versus control cells (vector-transfected). The inset shows representative KLK7 antigen levels in transfected HT29-KLK7 and HT29-vector control. Conditioned medium was harvested from HT29-vector and HT29-KLK7-transfected cells, and the KLK7 concentration was assessed by ELISA. Data are means±SE of three different experiments. **$p<0.01$, HT29-KLK7 cells versus control cells (vector-transfected).

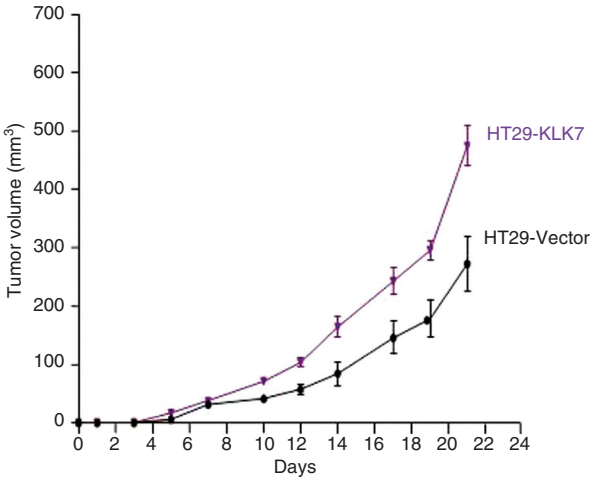

Figure 4 Effect of KLK7 overexpression on tumor volume in a mouse xenograft colon cancer model.
HT29-vector or HT29-KLK7 cells (• or ▲) were inoculated in the flank of nude mice at day 0 (six or eight groups). The development of tumors was followed by caliper measurement. Data are mean±SE of six tumors in the control group and eight tumors derived from HT29-KLK7 cells. Note that tumors with KLK7 overexpression showed an increased tumor growth rate as compared with the control vector-transfected cells.

Discussion

Our main finding is that there is a marked upregulation of KLK7 in colon cancer-derived glandular epithelial cells, relative to non-cancer-derived tissues, and that HT29 colon cancer-derived cells that overexpress KLK7 exhibit a dramatic increase in cell proliferation *in vitro* and expansion of tumor growth *in vivo* in mouse xenograft models. Thus, KLK7 can be put forward as a likely aberrantly expressed colon cancer-produced proteinase with tumorigenic functions. KLK7-triggered proliferation can now be added to KLK4 and KLK14 (Gratio et al., 2010, 2011; Chung et al., 2012) as potential key elements that play a role in the colon tumorigenic process (i.e., proliferation).

Although KLK7 has been found to be a useful biomarker in many types of cancers (Avgeris et al., 2012; Kontos and Scorilas, 2012; Devetzi et al., 2013; Dorn et al., 2014), including colon cancer (Talieri et al., 2009a,b; Kontos et al., 2012), to our knowledge, this is the first report demonstrating a proliferative role for KLK7 that is overexpressed in colon cancer.

Our study shows that KLK7 is expressed in nearly all colonic tumors (97%) tested *in vivo* as well as in several colon cancer cell lines (40%) in culture. In good agreement, those colon cancer cell lines that express KLK7 mRNA also produce and secrete the respective protein (as demonstrated by ELISA measurements in cell culture supernatants). Interestingly, expression of KLK7 does not correlate with the differentiation state of colon cancer cell lines. Indeed, KLK7 expression was absent in undifferentiated cells such as HT29 and polarized enterocyte-like cells such as CL19A and Caco-2 (Darmoul et al., 2001). Our analysis indicated high KLK7 mRNA expression in cell lines derived from metastatic sites or prone to metastasis: (a) in SW620, a lymph node-derived cell line developed from the primary adenocarcinoma of the same patient from which SW480 cell line (showing lower KLK7 expression) was established; (b) in T84, a cell line obtained from a lung metastasis of colon carcinoma; and (c) in HCT116, a metastatic colon cancer cell line. These detection patterns suggest that KLK7 may be considered as a potential progression marker for colon cancer. In agreement with this observation, clinical data indicate that upregulation of KLK7 expression in tumor tissues is correlated with poor prognosis of patients with colon cancer (Talieri et al., 2009a,b; Kontos et al., 2012). Overexpression of KLK7 expression might be regulated by

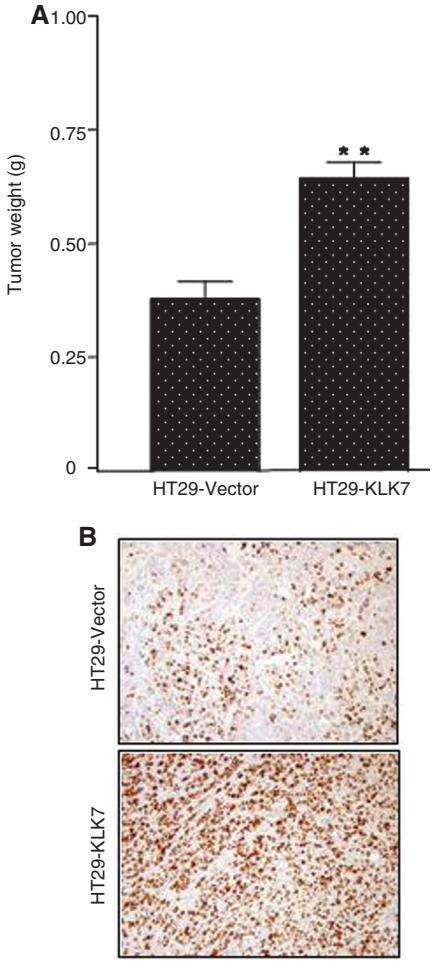

Figure 5 KLK7 overexpression effect on tumor growth *in vivo*. (A) After 24 days, tumors were harvested and weighed. Tumors derived from HT-29 KLK7-transfected cells weighed significantly more than tumors in the control groups. Data are depicted as mean±SE. $^{**}p<0.01$ HT29-KLK7 cells versus control cells (vector-transfected). (B) Representative images of immunohistochemical detection of Ki-67 in xenografts derived from mice bearing KLK7-transfected HT29 cells and control tumors. Note that staining of Ki-67 was detected in the nucleus, with a more intense staining in HT29-KLK7 tumors as compared with tumors from HT29-vector (control cells) (original magnification ×100).

tumor-microenvironment-derived soluble factors. Indeed, it has been suggested that the activity of kallikrein-related peptidases can be modulated by the tumor microenvironment (Prezas et al., 2006).

Although several studies indicate that, in many cancers, expression of KLKs is under multiple regulatory mechanisms such as hormonal and epigenetic mechanisms (Pampalakis et al., 2006; Lawrence et al., 2010), the mechanism whereby the *KLK7* gene is switched on in colon cancer is unknown. Interestingly, although KLK7 mRNA expression is found in 11 (73%) of 15 colon cancer cell lines, KLK14 mRNA is present in all colon cancer cell lines analyzed (Figure 1) (Gratio et al., 2011). In addition, although KLK7 and KLK14 are co-expressed in most of the colon cancer cells, KLK7 and KLK14 are differentially regulated in colonic cancers. Indeed, it has been reported that some KLKs, including KLK7, use alternative promoters to generate organ- and disease-specific transcripts (Dong et al., 2008). Another mechanism that regulates KLK7 mRNA expression could be a cross-talk with some oncogenes often mutated in cancer. In colon cancer, expression of KLK6, another member of the kallikrein family has been shown to be modulated by the proto-oncogene (KRAS), which is frequently mutated in colonic cancers (Henkhaus et al., 2008). Further studies on KLK7 regulation in colon cancer cells are thus warranted to evaluate the possible role of oncogenes or other signaling mechanisms that may control KLK7 expression.

In resected tumors, we have demonstrated by immunohistochemistry that KLK7 is present in cancer mucosal tissues but is expressed at a very low level in the adjacent 'normal-appearing' mucosa, from patients with colon cancer, removed from sites located far from the neoplastic tissue (Figure 2). Normal colonic mucosa from control healthy cases does not stain at all with the KLK7 antibody. This is in line with the absence of KLK7 mRNA by RT-PCR in isolated epithelial cells from healthy human colons. Interestingly, expression of KLK7 is not associated with the type of tumor type, tumor stage, or with the degree of tumor differentiation. The data regarding KLK7 expression in normal colon and colonic tumors have been controversial. The first study using *in silico* analysis did not report differences in KLK7 expression between colonic tumors and their paired normal colonic mucosa (Yousef et al., 2004). In concordance with our study, Talieri et al. (2009b), using a RT-PCR semiquantitative method, found that the *KLK7* gene is upregulated in colon cancer tissues. In another study, using ELISA to uncover the expression pattern of a panel of kallikrein-related peptidases (KLK5–8, KLK10, KLK11, KLK13–15), KLK7 has been found to be significantly overexpressed in colon cancer samples compared with their normal paired mucosa. Furthermore, elevated KLK7 levels were found to be associated with unfavorable survival in patients with colon cancer (Talieri et al., 2009a,b; Kontos et al., 2012). Absence of KLK7 expression in normal colonic tissues, found in the present study, is in agreement with a previous report by Shaw and Diamandis (2007), who also reported a lack of KLK7 expression in normal colonic tissues extracts, as measured by ELISA and RT-PCR. The discrepancy between the previously mentioned published data and our new findings are most likely due to specimen selection (normal tissue or paired colonic mucosa) and/or differences in the applied KLK7 detection methods (e.g.,

in silico, qRT-PCR and ELISA, immunohistochemistry) or type of antibody used. Because different KLK7 mRNA variants exist (Dong et al., 2003), it may also be possible that the discrepancy is due to the detection of different KLK mRNA variants, using different primers and different antibodies that recognize different epitopes. Our cell-targeted approach showing a marked upregulation of KLK7 only in the epithelial cells of adenocarcinoma tissue but not in glandular epithelial cells in normal mucosal tissues leads to a more precise evaluation of the sites of KLK7 upregulation and might explain failure to detect significant cancer-associated changes of KLK7 levels in others studies.

A number of studies have linked KLKs with unfavorable prognosis of many types of cancers including colon cancer (Emami and Diamandis, 2008; Kontos et al., 2012; Sotiropoulou and Pampalakis, 2012). We previously showed that KLK4 and KLK14 induce a signaling pathway that leads to colon cancer cell proliferation (Gratio et al., 2010, 2011). Herein, we show also that KLK7-expressing HT29 cells proliferate faster *in vitro* and develop larger tumors in nude mice. Thus, KLK7 may be a promising new biomarker in colonic tumors, but it may also be considered as a crucial contributor to the development of human colon cancer. Whether endogenously released KLK7 contributes to colon cancer development *in vivo* in human is not known but certainly warrants further investigation. In our current work, although the enzyme concentration produced by colonic tumors is sufficient to induce cell proliferation, we could not determine whether all of the KLK7 produced in the cell media or in the tumors remains catalytically active or is rapidly inhibited by tumor-produced serine proteinase inhibitors (Oikonomopoulou et al., 2010). For this purpose, as new tools on studying chymotrypsin-like proteases like KLK7 become available in the near future, it is important that studies evaluating the levels of different forms of the KLK7 enzyme (i.e., pro-form, active form, degradation fragments, partially or fully inhibited form) and their individual functions come into sharper focus.

That said, the mechanism by which released KLK7 contributes to colon cancer development *in vivo* is not known. We and others recently showed that some KLKs are potent regulators of PARs (Oikonomopoulou et al., 2006a; Ramsay et al., 2008; Gratio et al., 2010, 2011; Chung et al., 2012). Because KLK7 displays a chymotrypsin-like serine protease activity with a preferred cleavage C-terminal to aliphatic and aromatic residues, it appears at first that KLK7 unlikely induces colon cancer cell proliferation *via* PARs in the way a trypsin-like protease such as trypsin, thrombin, KLK4, or KLK14 would do (Oikonomopoulou et al., 2006a; Ramsay et al., 2008; Gratio et al., 2010, 2011; Chung et al., 2012). In line with this, Stefansson et al. (2008) found that neither KLK7 nor KLK8 induced PAR2 signaling. However, it has been reported by Debela et al. (2008) as well as Yoon et al. (2007) that KLK7 can also cleave post arginine despite its chymotryptic preference. Induction of a differential receptor signaling by cleavage at a chymotryptic site by KLK7 cannot be excluded either. Whether KLK7 signaling through other aberrantly expressed PARs in colon cancer can directly influence KLK7-induced tumor development is presently under investigation.

Several data indicate that KLKs act individually or/and in complex networks or 'KLKs cascades' that may involve cross talk with other KLKs, serine proteases, or metalloproteases (Sotiropoulou et al., 2009; Beaufort et al., 2010). Overexpression of KLK7 in colonic cancer might be involved in cancer progression by modulating other enzymatic pathways important in cancer metastasis. Recently, in pancreatic carcinoma, KLK7 has been shown to cleave pro-MMP9-generating active MMP9 (Ramani et al., 2011), an important member of the MMP family, whose overexpression is related to poor prognosis in colon cancer patients with colon cancer (Mook et al., 2004). KLK7 also induces shedding of cell adhesion proteins such as E-cadherin (Johnson et al., 2007), desmoglein 2 (Ramani et al., 2008), fibronectin (Ramani and Haun, 2008b) and urokinase-type plasminogen activator receptor (Ramani and Haun, 2008a) to promote cell proliferation and invasion in pancreatic cancer. These KLK7 targets and their subsequent functions in cancer are under investigation.

Our observations indicate that KLK7 has the potential not only to act as a prognostic marker for colon cancer but it can now also be considered as a multi-functional regulator of colon tumorigenesis, together with KLK4 and KLK14. This makes KLK7 also an attractive therapeutic target that should be investigated in the future for its clinical value in colon cancer patients' therapeutics.

Materials and methods

Cell culture

The human colon cancer cell lines HT29, SW480, HCT116, Caco-2, HCT-8, LoVo, LS-174T, SW620, and T84 were obtained from American Type Culture Collection (ATCC, Rockville, MD, USA). The Cl.19A cell line was a gift from Dr. C. Laboisse (Université de Nantes, Nantes, France), and the HT29-D4 cell line was a gift from Dr J. Marvaldi (Université d'Aix-Marseille, Marseille, France). They were routinely cultured in 25-cm^2 plastic flasks (Costar, Cambridge, MA, USA) as recommended by ATCC. Cells were maintained at 37°C in humidified atmosphere of 5% CO_2/air in DMEM containing 4.5 g/l glucose (Life

Technologies SAS, Saint Aubin, France), supplemented with 10% fetal calf serum (FCS) with the exception of Caco-2 cells, which were maintained in 20% FCS and 1% nonessential amino acids. T84 cells were maintained in DMEM-Ham's F-12 (DMEM-F12; 1:1) supplemented with 10% FCS (Life Technologies SAS, Saint Aubin, France).

Human tissues were processed according to French guidelines for research on human tissues (Agence Nationale d'Accréditation et d'Evaluation en Santé, 2001). Fresh normal human colons with no digestive disease were obtained in the past from France-Transplant according to French bioethics law (French Bioethics Advisory Committee, 2004) and Kessler (2008).

Colonic epithelial cells were isolated by manual shaking in a dispersing solution containing ethylenediaminetetraacetic acid (EDTA, 2.5 mM) as previously described (Darmoul et al., 1994). Cells were used for total RNA isolation.

Proliferation assay

Determination of cellular proliferation was accomplished by direct cell count; 20 000 cells/well were seeded in 48 cluster wells (Costar) in DMEM medium and 10% of FCS. After 24, 48, 72, or 96 h of culture, cells were detached from triplicate wells by trypsin/EDTA and counted in a hemacytometer. Cell death was evaluated with trypan blue. No significant cell death was observed in pRc/RSV and pRc/RSV-KLK7-transfected cells.

Reverse-transcription polymerase chain reaction

Four micrograms of total RNA were reverse transcribed using oligo (dT) primer. Amplifications were conducted using the resulting cDNAs. Twenty-five percent of the cDNA mixture was amplified using the human KLK7 sense primer 5′-GCC CAG GGT GAC AAG ATT ATT-3′ and antisense primer 5′-GTA CCT CTG CAC ACC AAC GG-3′ (detecting 569 bp) that anneal to exons 3 and 6, respectively. The primers thus anneal to the coding region of three KLK7 mRNA variants (Dong et al., 2008). KLK14 sense primer 5′-CAC TGC GGC CGC CCG ATC-3′ and antisense primer 5′-GGC AGG GCG CAG CGC TCC-3′ (detecting 485 bp) that anneal to exons 4 and 7, respectively were used as described elsewhere (Gratio et al., 2011). GAPDH cDNA amplification was used as an internal control with sense primer 5′-TCG GAG TCA ACG GAT TTG GTC GTA-3′ and antisense primer 5′-AGC CTT CTC CAT GGT GGT GAA GA-3′ (detecting 308 bp). Each of the 30 cycles of amplification have three-step program (denaturation, annealing and extension: 94°C–62°C for KLK7 or 59°C for KLK14-72°C, each for 40 s). The KLK7 and KLK14 PCR products were identified by electrophoresis in 2% agarose gel followed by SyberSafe staining.

Transfection and selection

The coding sequence of KLK7 cDNA (comprising 774 bp and encoding for the KLK7 full-length protein with 253 amino acids) was inserted into the mammalian expression vector pRc/RSV (Invitrogen, Carlsbad, CA, USA) to construct the recombinant plasmid pRc/RSV-KLK7 as described (Prezas et al., 2006). pRc/RSV and pRc/RSV-KLK7 were transfected into HT29 cells and using X-tremeGENE 9 Reagent (Roche Applied Science, Mannheim, Germany) according to the manufacturer's instructions. At 48 h after transfection, selective medium containing G418 antibiotic (Invitrogen) was added to the transfected cell cultures, at a final G418 concentration of 2 mg/ml. Two weeks after transfection, G418-resistant colonies were expanded. The neo-resistant *KLK7*-transfected HT29 were denoted as HT29-KLK7. For KLK7-negative controls, HT29 cells were also transfected with empty pRc/RSV vector and were selected using G418; the neo-resistant cells were designated as HT29-vector. The levels of *KLK7*-secreted protein were determined by ELISA as previously described (Shaw and Diamandis, 2007).

Animals

Five- to 6-week-old athymic (*nu*/*nu*) mice were purchased from Harlan (Gannat, France Gannat). Details of the mouse study were submitted to and approved by the Debré-Bichat Ethics Committee on Animal Experimentation (http://www.bichat.inserm.fr/comite_ethique.htm), protocol number 2012-15/773-0105 in accordance with the French law on animal protection.

Tumorigenicity assay in nude mice

Exponentially growing HT29-KLK7 or HT29-vector cells were harvested, washed with PBS, and resuspended at a concentration of 10^7/ml. Cells (10^6/100 µl) were then inoculated subcutaneously into the flank of mice. All nude mice developed tumors following injection of 10^6 cancer cells. Tumor development was followed by caliper measurements in three dimensions (length, width, and height), and the ellipsoid volume of the tumor was calculated using the modified ellipsoid formula [(length×width×height)/2] and reported in cubic millimeters. The body weight of mice was similar after 24 days in both groups.

Tissue specimen preparation and immunohistochemistry

Immunohistochemistry was performed on archival formalin-fixed paraffin-embedded tissue, samples from three cases with normal colonic mucosa, and 21 cases of colonic adenocarcinomas (Pathology Department, Bichat-Claude Bernard Hospital, Paris), of which 15 adenocarcinomas were located in the right colon and six in the left colon. Seventeen samples of tissue microarray (along with their matched normal tissue controls) (CliniSciences, Montrouge, France) were also analyzed. Tissues were used in accordance with the requirement of the Human Research Committee of the Bichat-Claude Bernard Hospital and according to the Declaration of Helsinki, which is adopted by French Bioethical Law (French Bioethics Advisory Committee, 2004). Tumors were staged according to the Seventh International Union Against Cancer (UICC) Edition. Immunostaining was performed using a Menarini Bond Max automat (Rungis, France). Sections were first incubated with a rabbit polyclonal antibody raised against human recombinant KLK7 (0.7 µg/ml) (GeneTex, Irvine, CA, USA). Thereafter, sections were incubated with biotinylated secondary antibody diluted 1:200, followed by

a polymer detection kit (Menarini). The peroxidase activity was revealed using diaminobenzidine, and nuclei were counterstained with Mayer's hemalum. In control experiments, the primary antibody was replaced with the antibody diluent. In separate experiments, we have demonstrated that the anti-KLK7 antibody stains the human mammary gland and the skin that constitutively express KLK7, whereas no staining could be observed in normal ovary tissue (Yousef et al., 2000) (data not shown).

KLK7 immunostaining was assessed by three independent observers using a semiquantitative methodology. First, the percentage of immunostained epithelial cancer cells was evaluated, and second, the staining intensity was scored on a scale where 0 represents no staining; 1, weak; 2, moderate; 3, strong; 4, intense staining. We calculated an immunostaining score for each tumor by multiplying the percentage of immunostained cells by the staining intensity class (maximal score 400).

Statistical analysis

The results are expressed as means±SE for a series of experiments and for immunohistochemistry analysis. Differences between data were tested by Student's *t*-tests for unpaired data or with nonparametric test when needed. For animal analysis, differences between the animal groups were evaluated by Newman-Keuls multiple comparison test. A p value of <0.05 was considered statistically significant.

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