Pathology – Research and Practice 208 (2012) 104–108

Contents lists available at SciVerse ScienceDirect

Pathology – Research and Practice

journal homepage: www.elsevier.de/prp

Original article

Evaluation and prognostic significance of human tissue kallikrein-related peptidase 6 (KLK6) in colorectal cancer

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ARTICLE INFO

Article history:
Received 7 July 2011
Received in revised form
29 September 2011
Accepted 27 December 2011

Keywords:
KLK
Kallikreins
KLK6
Colorectal cancer
Prognosis

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 6 (KLK6) 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. KLK6 expression was evaluated by immunohistochemistry and an expression score was calculated for each case. In CRC, KLK6 expression was decreased compared to normal colonic mucosa. A statistically significant, positive association was observed between KLK6 and tumor stage ($p = 0.036$), lymph node metastases ($p = 0.030$), and liver metastases ($p = 0.025$). Univariate analysis showed that KLK6 expression and stage had statistically significant correlation with disease-free survival ($p = 0.045$ and $p < 0.001$, respectively) and overall survival ($p = 0.027$ and $p < 0.001$, respectively). Cox multivariate analysis showed that KLK6 expression was an independent predictor of unfavorable overall survival ($p = 0.041$). Kaplan–Meier survival curves showed that KLK6-positive patients have statistically significant lower disease-free and overall survival. In conclusion, KLK6 immunostaining is an independent prognostic marker in patients with CRC.

Introduction

Serine proteases (SPs) are a family of enzymes that utilize a uniquely activated serine residue to catalytically hydrolyze peptide bonds [20,29]. Kallikreins are a subgroup of SPs that co-localize to chromosomal region 19q13.4 and are predominantly expressed in endocrine-related organs such as the prostate, breast, ovary, testis, and uterus [31]. 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 of being used as cancer diagnostic/prognostic markers [2,7,13,22].

The human kallikrein-related peptidase-6 (KLK6) is a member of the human kallikrein gene family, and is expressed in a wide array of normal tissues [25,30]. Using immunohistochemical analysis, Petraki et al. showed that KLK6 has generally cytoplasmic staining and is expressed in breast, prostate, kidney, endometrium, appendix, salivary gland, bile ducts, and gallbladder [19]. A splice variant of the human kallikrein-6 gene was also recently identified [15].

Early in silico analyses of KLK6 showed upregulation of the gene in cancers of the female genital and gastrointestinal tract [24]. KLK6 was also recently shown to be upregulated in colon cancer [9]. At the mRNA level, elevated KLK6 expression significantly correlates with aggressive tumor behavior and poor outcome [14]. There are multiple potential mechanisms that can underline the apparent expression of human kallikrein-6 gene in malignancy [17]. KLK6 was shown to act as a mediator of

Abbreviations: CRC, Colorectal cancer; PS, Proportion score; IS, Intensity score; TS, Total score; IHC, Immunohistochemistry.

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doi:10.1016/j.prp.2011.12.010

Table 1
Distribution of numerical clinicopathological variables of the study.

Variable	Mean ± SE	Range	Quartiles (median)		
			25	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

K-RAS-dependent migration of colon cancer cells [8]. The aim of this study was to investigate the expression of human tissue kallikrein-related peptidase 6 by immunohistochemistry in colorectal cancer, 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) at Enangelismos Hospital, Athens, Greece. Tumors were graded according to the WHO grading system and staged according to the modified Astler-Coller system (MAC). Two out of sixty-two (3%) CRCs were well differentiated, 42/62 (68%) moderately differentiated, and 18/62 (29%) poorly differentiated. Six out of sixty-two (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 (LNM) was observed in 34/62 (55%) of cases. Liver metastasis (LM) was reported in 9/59 (15%) patients; clinical data for three patients was unavailable. There was one lymph node-positive case where the exact stage was not specified. 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).

Table 2
Associations between *KLK6* status and other clinicopathological variables.

Variable	Total	No. of patients (%)		*p* value
		KLK6 negative	*KLK6* positive	
Sex				
Males	32	20 (62.5)	12 (37.5)	
Females	30	21 (70.0)	9 (30.0)	0.58[a]
Nodal status				
Negative	28	23 (82.1)	5 (17.9)	0.030[a]
Positive	34	18 (52.9)	16 (47.1)	
Grade				
I	2	2 (100.0)	0 (0.0)	
II	42	28 (66.7)	14 (33.3)	0.54[b]
III	18	11 (61.1)	7 (38.9)	
Stage				
A	6	5 (83.3)	1 (16.7)	
B	23	19 (82.6)	4 (17.4)	0.036[b]
C	24	14 (58.3)	10 (41.7)	
D	9	3 (33.3)	6 (66.7)	
Liver metastasis				
No	50	37 (74.0)	13 (26.0)	
Yes	9	3 (33.3)	6 (66.7)	0.025[a]
x	3			

[a] Fisher's exact test.
[b] Chi-square test.

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 KLK6-specific rabbit polyclonal antibody was raised in-house against KLK6 [11]. 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 0.5% H_2O_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 KLK6 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 hemalum, 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.

Evaluation of the immunohistochemical staining

Negative controls (no primary antibody) were included for every case. A combination of a proportion score (PS) and an intensity score (IS) was used to assess KLK6 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 cut-point for *KLK6*, as it is a gene with no established cutpoints regarding its expression in colorectal cancer. Having corrected the use of minimum *p*-value statistics, the X-tile software yielded an optimal cutoff of final score staining (>2), equal to the 65th percentile, with a calculated Monte Carlo p value < 0.05. Associations between dichotomous clinicopathological parameters and *KLK6* expression status were evaluated by the χ^2 test or Fisher's exact test, where appropriate. Cox proportional hazard regression models were 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.

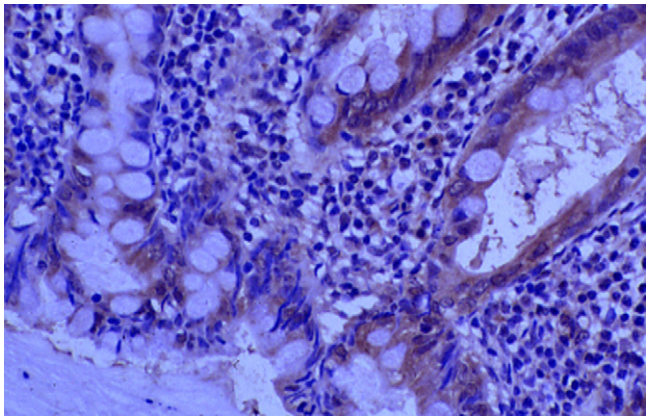

Fig. 1. Immunohistochemical expression of KLK6 in normal colonic epithelium. Magnifications 100×.

Results

In the normal colonic epithelium, absorptive cells of normal colonic mucosa showed subnuclear cytoplasmic staining. Goblet cells showed supranuclear cytoplasmic staining. Most mucin droplets remained unstained (Fig. 1). The distributions of the numerical clinical pathological variables of the subjects involved in this study are summarized in Table 1. KLK6 expression was downregulated in CRC compared to the adjacent normal colonic mucosa. In CRC, only 21/62 cases (37%) showed positive staining (using a cutoff value for total score more than 2) (Fig. 2). The immunostaining in CRCs was cytoplasmic. There was no significant staining in mesenchymal tissues, except for mild to moderate expression in nerves and endothelium.

In CRC, KLK6 staining was associated with a poor prognosis. There was a significant association between KLK6 and tumor stage, with a statistically significant higher percentage of positive cases associated with higher stages ($p = 0.036$). Also, lymph node-positive patients had a higher frequency of KLK6 positivity (17.9% in lymph node-negative versus 47.1% in lymph node-positive patients, $p = 0.30$). Moreover, patients with liver metastasis showed statistically significant more KLK6-positive cases compared to those who are negative for liver metastasis (66.7% versus 26.0%, respectively $p = 0.025$) (Table 2). There was no correlation between KLK6 and sex or age of the patient. Taken together, these results indicate higher KLK6 expression in more aggressive disease.

As shown in Table 3, the univariate analysis showed that patients who are KLK6-positive have a statistically significant worse survival (hazard ratio 1.34, 95% CI = 1.01–1.79, $p = 0.045$ for DFS and hazard ratio 1.40, 95% CI = 1.04–1.88, $p = 0.027$ for OS). In multivariate analysis, and after taking the other confounders into account, KLK6 positivity still held its significant association with poor OS (hazard ratio 1.31, 95% CI = 1.02–2.01, $p = 0.041$). Stage but not grade of tumor was also a strong predictor of poor outcome in both the univariate analysis (hazard ratio 3.05, $p = <0.001$) and the multivariate analysis (hazard ratio 2.92, $p = 0.001$). Grade was not associated with a significant, poor outcome (Table 3).

Kaplan–Meier survival curves showed that KLK6-positive patients had a significantly lower disease-free survival compared to those who are KLK6-negative ($p = 0.045$) (Fig. 3), and a significantly lower overall survival ($p = <0.001$) (Fig. 4).

Discussion

The association between kallikreins and cancer is strong and growing. KLK6 has been proposed as a biomarker for ovarian cancer, and several other kallikreins are emerging as potential new cancer biomarkers [27,28,33]. The mechanisms by which kallikreins are involved in cancer are beginning to be unraveled. KLK6, in particular, has been shown to degrade extracellular matrix proteins, including collagen, fibrinogen, laminin, and fibronectin [1,12]. The proteolysis of extracellular matrix likely removes structural inhibitors to tumor cell growth. In addition, KLK6 may have anti-apoptotic activity, thus promoting tumor cell growth [9]. Recently, a novel role of KLK6 was demonstrated in cancer by inhibiting epithelial–mesenchymal transition [16].

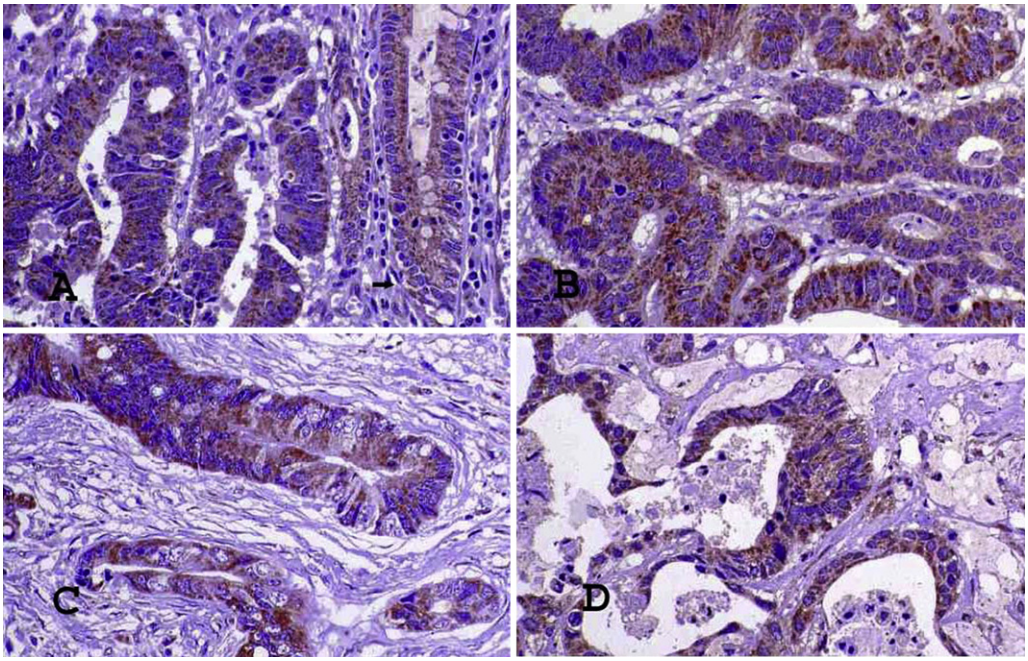

Fig. 2. Diffuse cytoplasmic immunohistochemical expression of KLK6 in colorectal adenocarcinoma (CRC). (A and C) Well-differentiated CRC (arrow in (A) staining in normal epithelium), (B) moderately differentiated CRC, (D) mucinous CRC. All magnifications 200×.

Table 3
Associations between *KLK6* 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 ($n = 56$)						
KLK6						
Negative	1.00			1.00		
Positive	1.34	1.01–1.79	0.045	1.40	1.04–1.88	0.027
Stage (ordinal)	3.05	1.63–5.72	<0.001	3.12	1.66–5.85	<0.001
Grade (ordinal)	0.92	0.39–2.12	0.83	0.95	0.41–2.25	0.91
Multivariate analysis[c] ($n = 54$)						
KLK6						
Negative	1.00			1.00		
Positive	1.31	0.94–1.81	0.11	1.42	1.02–2.01	0.041
Stage (ordinal)	2.92	1.57–5.41	0.001	2.93	1.58–5.42	0.001
Grade (ordinal)	0.62	0.23–1.68	0.35	0.56	0.21–1.59	0.28

[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.

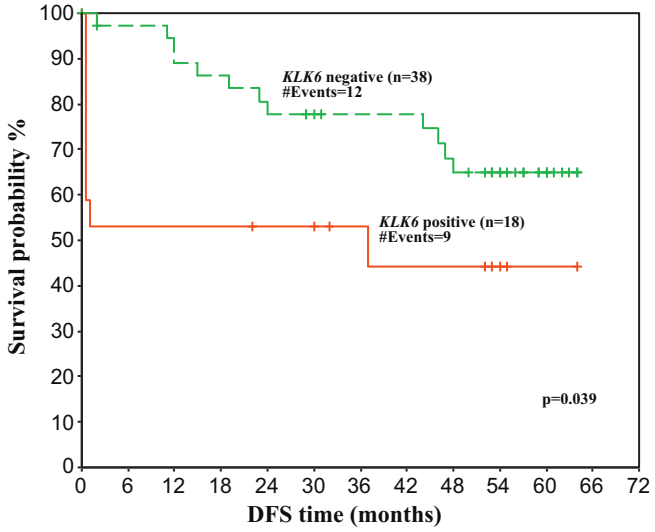

Fig. 3. Kaplan–Meier survival curve showing that KLK6-positive patients by immunohistochemistry have significantly lower disease free survival compared to those who are KLK6-negative ($p = 0.045$).

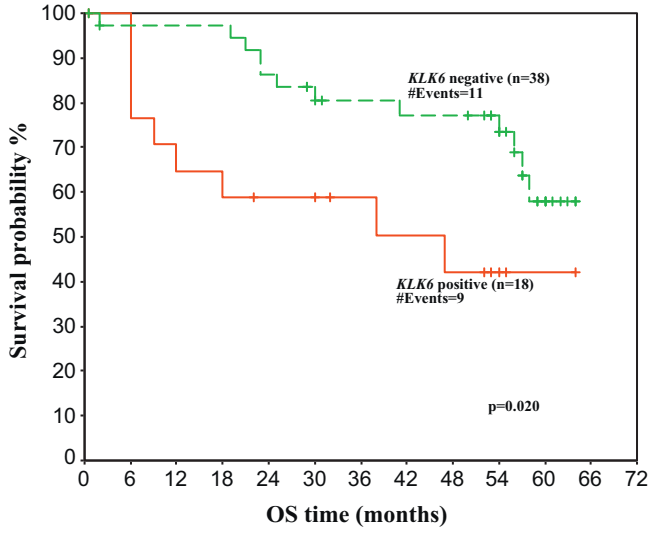

Fig. 4. Kaplan–Meier survival curve showing that CRC patients who are KLK6-negative have a significantly higher overall survival compared to those who are KLK6-positive by immunohistochemistry ($p < 0.001$).

In colorectal cancer, activating K-RAS mutations are seen in approximately 50% of cases. The upregulation of KLK6 mRNA in human colon cancer cells transfected with mutant K-RAS enhances colon cancer cell migration through laminin and Matrigel [8]. This upregulation is partly mediated through the PI3K and p42/44 MAPK pathways [8]. In keeping with previous studies, we showed that the overexpression of KLK6 in CRC is a marker of poor prognosis. Patients with higher levels of KLK6 presented at a higher stage, had more lymph node metastases, higher rates of liver metastases, and shorter overall survival. Our findings complement and confirm previous reports indicating a prognostic role for KLK6 [9].

Immunohistochemical analysis 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). 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. Interestingly, antibodies are now commercially available for KLK6 and other kallikreins, and once the results of this study are validated, it can be easily optimized as a clinical test. It should be noted, however, that the results of this study should be validated on larger scale studies, preferably on independent sets, before being accepted for clinical application.

Interestingly, our results show downregulation of KLK6 in cancer compared to normal tissues. This is in contrast to a recent in silico analysis that showed upregulation in gastrointestinal tumors but significant downregulation in breast and brain tumors [24]. This contradiction might be explained by post-transcriptional modifications, as recently hypothesized [23]. Our results, however, are in agreement with recent evidence suggesting that KLK6 is a putative tumor suppressor [16], and data showing its downregulation in salivary gland tumors compared to normal [3]. It is also possible that KLK6 might perform different functions at different stages of tumor initiation and progression.

In malignant tissues, glandular epithelia constitute the main kallikrein immunoexpression sites, and staining patterns suggest that these proteases are secreted [18,31]. The secretion of these proteases into serum makes them attractive tumor markers. Recently, a study assessed the over-expression of KLK6 in serum and CRC via RT-PCR and IHC [10].

This study identified that KLK6 was up-regulated in CRC but not in premalignant dysplastic lesions, such as tubular- or

tubulovillous adenomas, or in non-tumorous colonic tissue. As is the case with other KLKs, the identification of elevated KLK6 secretion in the serum of patients with CRC may prove to be a useful screening or diagnostic tool for CRC [4–6,21,26,32].

In conclusion, our study showed that KLK6 IHC in patients with CRC is a simple method for contributing to disease outcome prediction.

Acknowledgements

This work was supported by grants from the Canadian Cancer Society (CCS grant # 20185), the Ministry of Research and Innovation of the Government of Ontario, the Kidney Foundation of Canada, and Prostate Cancer Canada (grant # 2010-555).

References

[1] M.J. Bernett, S.I. Blaber, I.A. Scarisbrick, P. Dhanarajan, S.M. Thompson, M. Blaber, Crystal structure and biochemical characterization of human kallikrein 6 reveals that a trypsin-like kallikrein is expressed in the central nervous system, J. Biol. Chem. 277 (2002) 24562–24570.
[2] J.A. Clements, N.M. Willemsen, S.A. Myers, Y. Dong, The tissue kallikrein family of serine proteases: functional roles in human disease and potential as clinical biomarkers, Crit. Rev. Clin. Lab. Sci. 41 (2004) 265–312.
[3] M.R. Darling, L. Jackson-Boeters, T.D. Daley, E.P. Diamandis, Human kallikrein 6 expression in salivary gland tumors, J. Histochem. Cytochem. 54 (2006) 337–342.
[4] E.P. Diamandis, A. Scorilas, S. Fracchioli, M. Van Gramberen, H. De Bruijn, A. Henrik, A. Soosaipillai, L. Grass, G.M. Yousef, U.H. Stenman, M. Massobrio, A.G. Van Der Zee, I. Vergote, D. Katsaros, Human kallikrein 6 (hK6): a new potential serum biomarker for diagnosis and prognosis of ovarian carcinoma, J. Clin. Oncol. 21 (2003) 1035–1043.
[5] E.P. Diamandis, G.M. Yousef, Human tissue kallikreins: a family of new cancer biomarkers, Clin. Chem. 48 (2002) 1198–1205.
[6] E.P. Diamandis, G.M. Yousef, A.R. Soosaipillai, P. Bunting, Human kallikrein 6 (zyme/protease M/neurosin): a new serum biomarker of ovarian carcinoma, Clin. Biochem. 33 (2000) 579–583.
[7] N. Emami, E.P. Diamandis, Utility of kallikrein-related peptidases (KLKs) as cancer biomarkers, Clin. Chem. 54 (2008) 1600–1607.
[8] R.S. Henkhaus, E.W. Gerner, N.A. Ignatenko, Kallikrein 6 is a mediator of K-RAS-dependent migration of colon carcinoma cells, Biol. Chem. 389 (2008) 757–764.
[9] J.T. Kim, E.Y. Song, K.S. Chung, M.A. Kang, J.W. Kim, S.J. Kim, Y.I. Yeom, J.H. Kim, K.H. Kim, H.G. Lee, Up-regulation and clinical significance of serine protease kallikrein 6 in colon cancer, Cancer (2010).
[10] J.T. Kim, E.Y. Song, K.S. Chung, M.A. Kang, J.W. Kim, S.J. Kim, Y.I. Yeom, J.H. Kim, K.H. Kim, H.G. Lee, Up-regulation and clinical significance of serine protease kallikrein 6 in colon cancer, Cancer 117 (2011) 2608–2619.
[11] X.L. Liu, D.E. Wazer, K. Watanabe, V. Band, Identification of a novel serine protease-like gene, the expression of which is down-regulated during breast cancer progression, Cancer Res. 56 (1996) 3371–3379.
[12] A. Magklara, A.A. Mellati, G.A. Wasney, S.P. Little, G. Sotiropoulou, G.W. Becker, E.P. Diamandis, Characterization of the enzymatic activity of human kallikrein 6: autoactivation, substrate specificity, and regulation by inhibitors, Biochem. Biophys. Res. Commun. 307 (2003) 948–955.
[13] K. Mavridis, A. Scorilas, Prognostic value and biological role of the kallikrein-related peptidases in human malignancies, Future Oncol. 6 (2010) 269–285.
[14] K. Ogawa, T. Utsunomiya, K. Mimori, F. Tanaka, H. Inoue, H. Nagahara, S. Murayama, M. Mori, Clinical significance of human kallikrein gene 6 messenger RNA expression in colorectal cancer, Clin. Cancer Res. 11 (2005) 2889–2893.
[15] G. Pampalakis, L. Kurlender, E.P. Diamandis, G. Sotiropoulou, Cloning and characterization of novel isoforms of the human kallikrein 6 gene, Biochem. Biophys. Res. Commun. 320 (2004) 54–61.
[16] G. Pampalakis, E. Prosnikli, T. Agalioti, A. Vlahou, V. Zoumpourlis, G. Sotiropoulou, A tumor-protective role for human kallikrein-related peptidase 6 in breast cancer mediated by inhibition of epithelial-to-mesenchymal transition, Cancer Res. 69 (2009) 3779–3787.
[17] G. Pampalakis, G. Sotiropoulou, Multiple mechanisms underlie the aberrant expression of the human kallikrein 6 gene in breast cancer, Biol. Chem. 387 (2006) 773–782.
[18] C.D. Petraki, V.N. Karavana, K.I. Revelos, L.Y. Luo, E.P. Diamandis, Immunohistochemical localization of human kallikreins 6 and 10 in pancreatic islets, Histochem. J. 34 (2002) 313–322.
[19] C.D. Petraki, P.A. Papanastasiou, V.N. Karavana, E.P. Diamandis, Cellular distribution of human tissue kallikreins: immunohistochemical localization, Biol. Chem. 387 (2006) 653–663.
[20] R.M. Schultz, M.N. Liebman, Structure–function relationship in protein families, in: T.M. Devlin (Ed.), Textbook of Biochemistry with Clinical Correlations, Wiley-liss Inc., New York, 1997, pp. 1–2.
[21] C. Stephan, K. Jung, T. Nakamura, G.M. Yousef, G. Kristiansen, E.P. Diamandis, Serum human glandular kallikrein 2 (hK2) for distinguishing stage and grade of prostate cancer, Int. J. Urol. 13 (2006) 238–243.
[22] O.L. Tan, A.K. Whitbread, J.A. Clements, Y. Dong, Kallikrein-related peptidase (KLK) family mRNA variants and protein isoforms in hormone-related cancers: do they have a function? Biol. Chem. 387 (2006) 697–705.
[23] N.M. White, A. Bui, S. Mejia-Guerrero, J. Chao, A. Soosaipillai, Y. Youssef, M. Mankaruos, R.J. Honey, R. Stewart, K.T. Pace, L. Sugar, E.P. Diamandis, J. Dore, G.M. Yousef, Dysregulation of kallikrein-related peptidases in renal cell carcinoma: potential targets of miRNAs, Biol. Chem. 391 (2010) 411–423.
[24] G.M. Yousef, C.A. Borgono, N.M. White, J.D. Robb, I.P. Michael, K. Oikonomopoulou, S. Khan, E.P. Diamandis, In silico analysis of the human kallikrein gene 6, Tumour Biol. 25 (2004) 282–289.
[25] G.M. Yousef, E.P. Diamandis, The new human tissue kallikrein gene family: structure, function, and association to disease, Endocr. Rev. 22 (2001) 184–204.
[26] G.M. Yousef, E.P. Diamandis, Expanded human tissue kallikrein family – a novel panel of cancer biomarkers, Tumour Biol. 23 (2002) 185–192.
[27] G.M. Yousef, E.P. Diamandis, An overview of the kallikrein gene families in humans and other species: emerging candidate tumour markers, Clin. Biochem. 36 (2003) 443–452.
[28] G.M. Yousef, E.P. Diamandis, The human kallikrein gene family: new biomarkers for ovarian cancer, Cancer Treat. Res. 149 (2009) 165–187.
[29] G.M. Yousef, M.B. Elliott, A.D. Kopolovic, E. Serry, E.P. Diamandis, Sequence and evolutionary analysis of the human trypsin subfamily of serine peptidases, Biochim. Biophys. Acta 1698 (2004) 77–86.
[30] G.M. Yousef, L.Y. Luo, S.W. Scherer, G. Sotiropoulou, E.P. Diamandis, Molecular characterization of zyme/protease M/neurosin (PRSS9), a hormonally regulated kallikrein-like serine protease, Genomics 62 (1999) 251–259.
[31] G.M. Yousef, C.V. Obiezu, L.Y. Luo, A. Magklara, C.A. Borgono, T. Kishi, N. Memari, P. Michael, M. Sidiropoulos, L. Kurlender, K. Economopolou, C. Kapadia, N. Komatsu, C. Petraki, M. Elliott, A. Scorilas, D. Katsaros, M.A. Levesque, E.P. Diamandis, Human tissue kallikreins: from gene structure to function and clinical applications, Adv. Clin. Chem. 39 (2005) 11–79.
[32] G.M. Yousef, M.E. Polymeris, L. Grass, A. Soosaipillai, P.C. Chan, A. Scorilas, C. Borgono, N. Harbeck, B. Schmalfeldt, J. Dorn, M. Schmitt, E.P. Diamandis, Human kallikrein 5: a potential novel serum biomarker for breast and ovarian cancer, Cancer Res. 63 (2003) 3958–3965.
[33] G.M. Yousef, G.M. Yacoub, M.E. Polymeris, C. Popalis, A. Soosaipillai, E.P. Diamandis, Kallikrein gene downregulation in breast cancer, Br. J. Cancer 90 (2004) 167–172.