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Immunohistochemical localization of human kallikreins 6, 10 and 13 in benign and malignant prostatic tissues

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Human kallikreins 6, 10 and 13 (hK6, hK10 and hK13) are expressed by many normal, mainly glandular tissues, including prostatic epithelium. Some kallikreins may function as tumor suppressors or are downregulated during cancer progression. The aim of this study was to evaluate the immunoexpression of these kallikreins in benign and malignant prostatic tissues and correlate their expression with prostate cancer (PC) prognosis. Included in the study were 25 cases of nonmalignant prostate and 179 cases of PC. Among them, 122 PC cases were immunostained for hK6, 94 for hK10 and 113 for hK13, respectively. The follow-up period for a subset of 68 patients who had undergone radical prostatectomy (RP) was 1–58 months (mean = 13.4 ± 1.7 and median = 8.0 months). A cutoff value of 0.2 μg/l of serum PSA was established as a biochemical recurrence threshold. Follow-up information was available for 26/55 RP cases stained for hK6, 14/32 cases stained for hK10 and 25/59 cases stained for hK13. Gleason score (GS) 7 carcinomas were stratified as 7a and 7b, according to the primary grade. PC with GS 2–7a were histologically categorized as low malignant (LM) and PC with GS 7b–10 as high malignant (HM). The immunohistochemical method of streptavidin–biotin–peroxidase using monoclonal and polyclonal antibodies was performed. In the benign prostate and in prostatic intraepithelial neoplasia, a cytoplasmic immunostaining of varying intensity was evident. In PC, the immunoexpression of all kallikreins was decreased: 102/122 cases (84%) were positive for hK6, 73/94 (78%) for hK10 and 97/113 (86%) for hK13, respectively. A statistically significant difference in expression was found, in comparison to nonmalignant prostates ($P = 0.029$, 0.009 and 0.045, respectively). Also, a positive correlation was observed between the immunoexpression of these three kallikreins. Concerning the histological grade, HM-PC expressed all three kallikreins with a slightly higher percentage than LM-PC: 79 *vs* 88% for hK6, 76 *vs* 79% for hK10 and 76 *vs* 92% for hK13. These differences were statistically significant only in the case of hK13 ($P = 0.024$). Serum PSA did not correlate with kallikrein immunoexpression in PC. Furthermore, there was no significant correlation between kallikrein expression and pathological stage or recurrence, in the cases of RP. All three kallikreins are expressed in the nonmalignant and malignant prostate, with cancer tissues demonstrating slightly lower expression. Expression levels did not correlate with aggressiveness and they do not seem to have value for prostate cancer prognosis.
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Introduction

Human tissue kallikreins are members of a multigene family that includes 15 genes (KLK1–KLK15 for genes, hK1–hK15 for proteins), all located on chromosome 19q13.4. KLK6, KLK10 and KLK13 are expressed in many normal, mainly glandular tissues, among them the prostatic epithelium. There are suggestions that some kallikreins may function as tumor suppressors and that they are downregulated during cancer progression.[1,2] We previously examined the immunohistochemical distribution of hK6, hK10 and hK13 in different human tissues.[3–5] Prostatic epithelium strongly expressed these three kallikreins. The aim of this study was to evaluate the immunoexpression of these kallikreins in benign and malignant prostatic tissues and to correlate their expression with the degree of histological differentiation of prostate cancer, with pathological stage and biochemical recurrence.

Materials and methods

Patients and tissue samples

Included in this study were 25 patients with benign prostatic disease (benign prostatic hyperplasia (BPH) and prostatitis) and 179 patients with prostate cancer (PC). The median serum PSA of the first group (available in 19 cases) was 2.8 (0.5–23) μg/l and of the second group (available in 108 cases), 43 (1.6–500) μg/l. The tissue material of the first group concerned transrectal ultrasound (TRUS) biopsies and of the second group, TRUS biopsies (95 cases; 53%), transurethral resection material (16 cases; 9%) and radical prostatectomy (RP) specimens (68 cases; 38%). The follow-up period for the 68 patients who had undergone RP was 1–58 months (mean = 13.4 and median = 8 months). A cutoff value of 0.2 μg/l of serum PSA on 4–6-month interval monitoring was used to establish biochemical recurrence.

Paraffin sections, 4 mm thick, of tissues fixed in buffered formalin were used for hematoxylin–eosin staining and the performance of the immunohistochemical staining for the three kallikreins. In total, 122/179 cases were stained for hK6, 94/179 for hK10 and 113/179 for hK13. In all, 62 cases were stained for both hK6 and hK10, 73 for hK6 and hK13, 60 for hK10 and hK13, and 45 for all three kallikreins. Staining for each kallikrein was performed on separate occasions and not all tissues were stained for all kallikreins. Follow-up information of biochemical recurrence for patients who had undergone RP was available for 26/55 cases stained for hK6, 14/32 cases stained for hK10 and 25/59 cases stained for hK13. In total, 16/26 (62%), 10/14 (71%) and 15/25 (60%) patients relapsed, respectively.

Histological grading and staging data

Grading was established according to the Gleason score (GS) differentiation system (range 2–10).[6] GS 7 carcinomas were stratified as 7a and 7b, according to the predominant grade (predominant grade in 7a = 3, and in 7b = 4). A 4-scale grouping was used for the final evaluation of the histological grading as follows: A (GS ⩽ 6), B (GS = 7a), C (GS = 7b) and D (GS = 8–10). For statistical analysis, a second 2-scale grouping (I and II) was also performed, combining groups A and B (low malignant (LM) PC) and C and D (high malignant (HM) PC) subgroups. Among the 122 cases stained for hK6, 57 (47%) belonged to group I and 65 (53%) to group II. Among the 94 cases stained for hK10, 33 (35%) belonged to group I and 61 (65%) to group II. Among the 113 cases stained for hK13, 42 (37%) belonged to group I and 71 (63) to group II. Extracapsular extension was observed in 33/55 (60%), 18/32 (56%) and 32/59 (54%) of RP cases stained for hK6, hK10 and hK13, respectively. Invasion of the seminal vesicles was observed in 15/55 (27%), 11/32 (34%) and 15/59 (25%) of RP cases stained for hK6, hK10 and hK13, respectively. Metastatic invasion of the iliac lymph nodes was observed in 3/55 (5.4%), 3/32 (9.4%) and 4/59 (6.8%) of RP cases stained for hK6, hK10 and hK13, respectively (Tables 1–3).

Table 1 Relationship between hK6 immunoexpression and other variables in 122 patients with prostate cancer

Variable	*Patients*	*No. of patients (%)*		*P*
		hK6 negative	*hK6 positive*	
4-scale histological grading[a]				
A	32	6 (18.75)	26 (81.25)	0.475[c]
B	25	6 (24)	19 (76)	
C	24	4 (16.7)	20 (83.3)	
D	41	4 (9.8)	37 (90.2)	
2-scale histological grading[b]				
I	57	12 (21.1)	45 (78.9)	0.193[c]
II	65	8 (12.3)	57 (87.7)	
Extracapsular extension				
–	22	8 (36.4)	14 (63.6)	0.475[c]
+	33	9 (27.3)	24 (72.7)	
Seminal vesicle invasion				
–	40	13 (32.5)	27 (67.5)	0.677[c]
+	15	4 (26.7)	11 (73.3)	
Lymph node invasion				
–	52	17 (32.7)	35 (67.3)	0.233[c]
+	3	0 (0)	3 (100)	
Biochemical recurrence				
–	10	2 (20)	8 (80)	0.868[d]
+	16	4 (25)	12 (75)	

[a]A (GS ⩽ 6), B (GS=7a), C (GS=7b), D (GS=8–10).
[b]I (GS ⩽ 7a), II (GS=7b–10).
[c]Pearson's χ^2 test.
[d]Log-rank test.

Immunohistochemistry

The streptavidin–biotin–peroxidase protocol, using the DAKO LSAB+Kit Peroxidase was performed. Specific polyclonal and monoclonal antibodies for the three kallikreins, raised by immunizing with full-length recombinant hK6, hK10 and hK13, respectively, were used (dilutions: 1 : 500 for polyclonal and 1 : 150 for monoclonal antibodies).[7–9] Staining procedures included

Table 2 Relationship between hK10 immunoexpression and other variables in 94 patients with prostate cancer

Variable	*Patients*	*No. of patients (%)*		*P*
		hK10 negative	*hK10 positive*	
4-scale histological grading[a]				
A	19	5 (26.3)	14 (73.7)	
B	14	3 (21.4)	11 (78.6)	0.973[c]
C	18	4 (22.2)	14 (77.8)	
D	43	9 (20.9)	34 (79.1)	
2-scale histological grading[b]				
I	33	8 (24.2)	25 (75.8)	0.745[c]
II	61	13 (21.3)	48 (78.7)	
Extracapsular extension				
–	14	5 (35.7)	9 (64.3)	0.400[c]
+	18	4 (22.2)	14 (77.8)	
Seminal vesicle invasion				
–	21	7 (33.3)	14 (66.7)	0.365[c]
+	11	2 (18.2)	9 (81.8)	
Lymph node invasion				
–	29	8 (27.6)	21 (72.4)	0.833[c]
+	3	1 (33.3)	2 (66.7)	
Biochemical recurrence				
–	4	0 (0)	4 (100)	0.974[d]
+	10	2 (20)	8 (80)	

See Table 1.

Table 3 Relationship between hK13 immunoexpression and other variables in 113 patients with prostate cancer

Variable	*Patients*	*No. of patients (%)*		*P*
		hK13 negative	*hK13 positive*	
4-scale histological grading[a]				
A	22	4 (18.2)	18 (81.8)	0.096[c]
B	20	6 (30)	14 (70)	
C	22	2 (9.1)	20 (90.9)	
D	49	4 (8.2)	45 (91.8)	
2-scale histological grading[b]				
I	42	10 (23.8)	32 (76.2)	0.024[c]
II	71	6 (8.5)	65 (91.5)	
Extracapsular extension				
–	27	8 (29.6)	19 (70.4)	0.103[c]
+	32	4 (12.5)	28 (87.5)	
Seminal vesicle invasion				
–	44	10 (22.7)	34 (77.3)	0.435[c]
+	15	2 (13.3)	13 (86.7)	
Lymph node invasion				
–	55	11 (20)	44 (80)	0.810[c]
+	4	1 (25)	3 (75)	
Biochemical recurrence				
–	10	2 (20)	8 (80)	0.288[d]
+	15	1 (6.7)	14 (93.3)	

See Table 1.

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 microwave for 5 min and incubated overnight at 4°C with the primary rabbit polyclonal and mouse monoclonal antibodies 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.

Statistics

Statistical analysis was carried out with the SPSS 10.0 software, using the χ^2 test, the Kruskal–Wallis test and the log-rank test for the Kaplan–Meier survival analysis. A P-value <0.05 was considered significant.

Results

The immunoexpression of the three kallikreins in the benign and the cancerous prostatic epithelium was cytoplasmic. Any staining, weak or intense, was considered as positive. In all 25 cases of normal, inflamed and hyperplastic prostate, an immunostaining of varying intensity in the prostate columnar cells, with only a slight heterogeneity in the staining pattern, was observed (Figure 1a and b). Although the basal cells remained mostly unstained, the kallikreins were often expressed in basal cell hyperplasia. Foci of high-grade prostatic

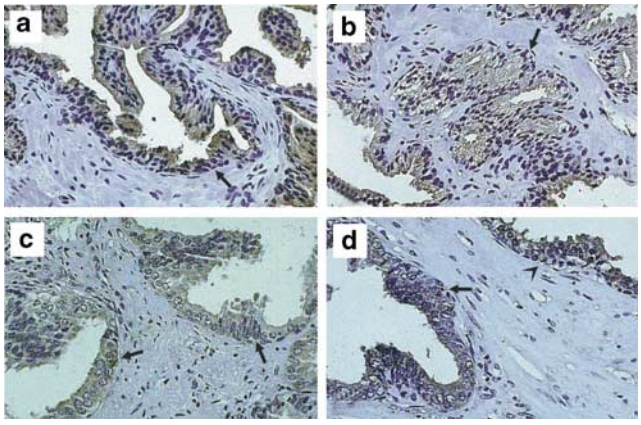

Figure 1 *Immunohistochemical expression of hK6 (a) and hK10 (b) by the columnar cells of benign prostatic epithelium (arrow). Polyclonal antibody, magnification ×200; (c) immunohistochemical expression of hK13 by high-grade PIN (arrows). Polyclonal antibody, magnification ×200; (d) immunohistochemical expression of hK6 by the columnar cells of benign prostate epithelium (arrowhead) and in high-grade PIN (arrow). Monoclonal antibody, magnification ×200.*

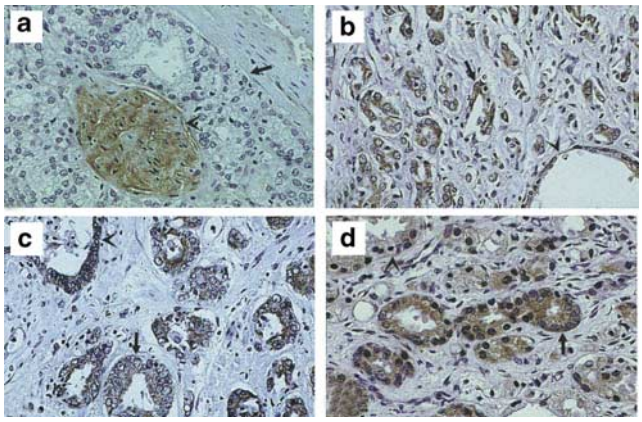

Figure 2 *(a) Immunohistochemical expression of hK10 by a nerve (arrowhead). Absence of immunoreactivity in the surrounding GS 8 prostate adenocarcinoma (arrow). Polyclonal antibody, magnification ×200; (b) immunohistochemical expression of hK6 by the columnar cells of benign prostate epithelium (arrowhead) and in the adjacent GS 6 prostate adenocarcinoma (arrow). Polyclonal antibody, magnification ×200; (c) immunohistochemical expression of hK13 by the columnar cells of benign prostate epithelium (arrowhead) and in the adjacent GS 6 prostate adenocarcinoma (arrow). Monoclonal antibody, magnification ×200; (d) Immunohistochemical expression of hK10 by the columnar cells of benign prostate epithelium (arrowhead) and in the adjacent GS 6 prostate adenocarcinoma (arrow). Monoclonal antibody, magnification ×200.*

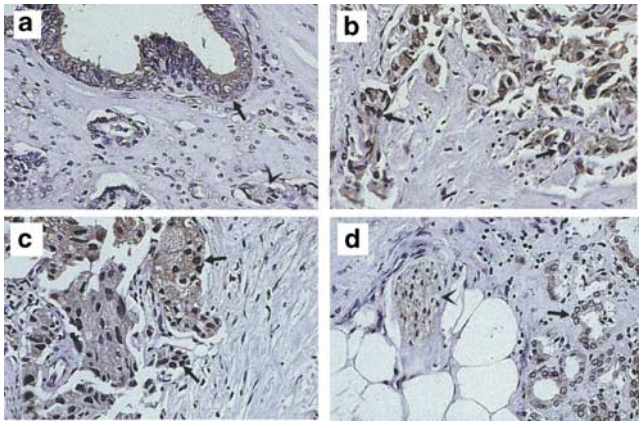

Figure 3 *(a) Immunohistochemical expression of hK10 by high-grade PIN (arrow). Absence of immunoreactivity in the adjacent GS 6 prostate adenocarcinoma (arrowhead). Polyclonal antibody, magnification ×200; (b) immunohistochemical expression of hK13 by a GS 8 prostate adenocarcinoma (arrows). Monoclonal antibody, magnification ×200; (c) immunohistochemical expression of hK13 by a GS 8 prostate adenocarcinoma (arrows). Polyclonal antibody, magnification ×200; (d) immunohistochemical expression of hK6 a GS 6 prostate adenocarcinoma (arrow) with extracapsular extension. Immunoreactivity of a nerve as well (arrowhead). Monoclonal antibody, magnification ×200.*

intraepithelial neoplasia (PIN) found in cases of PC, mostly expressed the proteins (Figure 1c and d). In the cancerous prostate, the immunoexpression of all kallikreins was generally decreased, with considerable heterogeneity in the staining pattern: 102/122 cases (84%) were positive for hK6, 73/94 (78%) were positive for hK10 and 97/113 cases (86%) were positive for hK13, respectively (Figure 2a–d). This downregulation in PC was statistically significant (Pearson χ^2 test, $P=0.029$, 0.009 and 0.045 for hK6, hK10 and hK13, respectively) (see also Figure 3a).

A positive correlation was found between hK6 and hK10, hK6 and hK13, as well hK10 and hK13 immunoexpression in the cancerous prostate (Pearson χ^2 test, $P=0.001$, 0.000, 0.000, respectively). From the 45 cases that were immunostained for hK6, hK10 and hK13, 34 (76%) showed similar expression patterns for the three kallikreins.

Concerning histological grade, HM-PC expressed all three kallikreins at a slightly higher percentage than LM-PC: 79 *vs* 88% for hK6, 76 *vs* 79% for hK10 and 76 *vs* 92% for hK13. The difference in the expression between LM-PC and HM-PC was statistically significant only for hK13 (χ^2 test, $P=0.024$) (Figure 3b and c). It is worth mentioning that by using the 4-scale grading, the difference between the groups was significant only at the 0.10 level (indicatively statistically significant—$P=0.096$). This could be explained by the observation that the percentage of the hK13 positive GS$\leqslant$6 carcinomas was slightly higher than the percentage of the hK13 positive GS 7A carcinomas (no statistically significant difference) (Tables 1–3).

In RP specimens, the extracapsular extension of prostate cancer was associated with a slightly higher percentage of kallikrein immunoexpression than the intraprostatic localization, as follows: positivity of 74 *vs* 65% for hK6, 78 *vs* 64% for hK10 and 87 *vs* 70% for hK13, respectively (Figure 3d). However, these differences were not statistically significant. A higher percentage of cancers with seminal vesicle invasion expressed the three kallikreins, in comparison with cancers with no seminal vesicle invasion: 73 *vs* 68% for hK6, 82 *vs* 67% for hK10 and 87 *vs* 77% for hK13, respectively. These differences were not statistically significant. There was also no significant difference in the expression of the three kallikreins between the cases with or without positive iliac lymph nodes (Tables 1–3).

Kaplan–Meier survival curves demonstrated that there was no statistically significant difference in the biochemical recurrence rate between patients with hK6/10/13-positive and hK6/10/13-negative tumors ($P=0.87$, 0.97, 0.29, for the three kallikreins, respectively). Finally, we found no difference in pre-operative PSA between hK6-, hK10- or hK13- positive or -negative-tumors.

Discussion

Among all prostate cancer biomarkers, prostate-specific antigen is the most valuable and is currently used for the diagnosis and monitoring of the disease.[10] Another emerging prostatic biomarker is human glandular kallikrein 2 (hK2).[11–15] More recently, a few other members of the kallikrein family, such as hK4 and hK15[16,17] have also been shown to have potential as prostatic biomarkers. Human kallikrein 11 (hK11) appears to be a promising new biomarker for prostatic and ovarian carcinoma.[18] Other members of the kallikrein family have already been shown to be highly expressed in prostatic epithelium, including human kallikreins 6, 10 and 13.[3–5] None of these newly identified kallikreins has been examined by immunohistochemistry for their prognostic value in prostatic carcinoma.

Previously, Darson *et al*[19,20] provided evidence that hK2 expression by immunohistochemistry may have value for identifying more aggressive prostate tumors. Quantitative analysis of prostatic tissue extracts did not confirm these findings.[21] Furthermore, prostate-specific antigen expression in malignant *vs* nonmalignant prostatic tissue suggests that PSA is lower in malignancy.[21,22]

In this investigation, we verified expression of the three kallikreins, hK6, hK10 and hK13 in the columnar cells of prostatic epithelium. In comparison to normal or hyperplastic prostatic tissues, the expression of these three kallikreins in malignancy appears to be decreased. These data are similar to those reported for PSA and hK2.[21,22] This immunohistochemical expression does not seem to correlate much with indicators of tumor aggressiveness, including GS, extracapsular extension, seminal vesicle invasion or lymph node status. Furthermore, we found no correlation of immunohistochemistry with biochemical recurrence or with preoperative PSA. These data suggest that these immunohistochemical markers are not useful for prostate cancer prognosis.

We have previously examined the serum concentration of human kallikreins 6 and 10 in various malignancies and found no elevations of these kallikreins in prostate cancer, despite significant elevations in ovarian cancer.[23,24] These combined data do not support a diagnostic or prognostic value of the three studied kallikreins in prostatic carcinoma. Other androgen-regulated kallikreins, such as hK4, hK11 and hK15 may have more potential for this purpose.[16–18]

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