Parallel underexpression of kallikrein 5 and kallikrein 7 mRNA in breast malignancies

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Kallikrein (KLK) 5 and KLK7 were reported to be overexpressed in several cancers, but underexpressed in prostate and breast cancers. However, the expression levels of KLK5 and KLK7 in benign breast tissues and metastases, and the relationship between KLK5 and KLK7, have not been reported. In addition, the value of KLK5 and KLK7 in the diagnosis and prognosis prediction of breast cancer patients is far from clear. To further determine their role and clinical significance in breast cancer and to explore the relationship between KLK5 and KLK7, the mRNA levels of KLK5 and KLK7 in normal breast tissues, benign breast tissues, primary tumors, and lymph node metastases were detected by real-time reverse transcription–polymerase chain reaction and microarray. The relationship between KLK5 and KLK7 expression and clinicopathological parameters, and the correlation between the mRNA levels of KLK5 and KLK7 as well as the 5′-uncoding regions of KLK5 and KLK7 were analyzed. The mRNA levels of KLK5 and KLK7 were both downregulated in breast cancers relative to normal and benign tissues, and downregulated in metastases compared to primary cancers. Underexpression of KLK5 and KLK7 was correlated with postmenopausal status and positive estrogen receptor status. The mRNA levels of KLK5 and KLK7 were positively correlated in breast malignancies. Moreover, four homologous sequences and 10 transcription factors as potential regulators were found on the control regions of both KLK5 and KLK7. Thus, KLK5 and KLK7 were underexpressed in parallel, potentially with the same regulation pathways, in breast malignancies, which might contribute to the carcinogenesis and development of breast cancer. They are potential biomarkers for breast cancer. (*Cancer Sci* 2009; 100: 601–607)

The human kallikrein (KLK) gene family, located at the chromosomal locus 19q13.3-q13.4, encodes a subgroup of 15 secreted serine proteases with known or predicted trypsin-like or chymotrypsin-like activity.[1] Emerging data indicate that KLKs are implicated in various human malignancies. The classical KLK gene *KLK3* (also known as prostate-specific antigen) has proven to be a perfect biomarker for prostate cancer and has been used in clinical auxiliary diagnosis and prognosis prediction of prostate cancer.[2,3] Other KLK family members are overexpressed in many kinds of malignancies compared with their normal tissues, including ovarian, cervical, colorectal, gastric, lung, urinary bladder, pancreatic, and prostate cancer (KLK4, KLK11, KLK14, KLK15), but KLKs are underexpressed in breast and prostate cancers (KLK2, KLK3, KLK5, KLK6, KLK10, KLK13), renal cancer, and urinary bladder cancer.[4] KLK5 (also known as stratum corneum trypsin-like enzyme) and KLK7 (also known as stratum corneum chymotrypitc enzyme), which were originally identified from a keratinocyte cDNA library,[5–7] catalyze the degradation of intercellular cohesive structures in the outermost layer of the skin and contribute to the cell-shedding process of the skin surface,[6] which is similar to the shedding of tumor cells into the surrounding microenvironment.[8]

Both KLK5 and KLK7 were reported to be overexpressed in several cancers, including ovarian,[9–14] lung,[15] testicular,[16,17] urinal bladder,[18] and cervical cancers,[19,20] but underexpressed in prostate[21] and breast cancers.[22] In our previous study, KLK5 and KLK7 were also found to be significantly downregulated in breast cancers compared with their paired normal breast tissues. In addition, these two enzymes showed coexpression in skin tissue[23] and ovarian cancer[11] and KLK5 has been shown to activate KLK7 *in vitro*, indicating that KLK5 may be the physiological activator of KLK7.[23] Although both KLK5 and KLK7 were shown to be underexpressed in breast cancer, the expression levels of KLK5 and KLK7 in benign breast tissues and metastases and the correlation between KLK5 and KLK7 in breast tissues have not been reported. Furthermore, the role of KLK5 and KLK7 in cancer development is still not well defined. Their overexpression in ovarian cancer tissues is associated with unfavorable prognosis of ovarian cancer patients.[24–26] In breast cancer, however, the value of KLK5 and KLK7 in predicting prognosis is far from clear. Yousef's group has demonstrated that KLK5 overexpression is a significant predictor of reduced disease-free survival (DFS) and overall survival (OS) in breast cancer patients,[27] and Talieri and colleagues also found that breast cancer patients with KLK7-positive tumors have relatively shorter DFS and OS than patients with KLK7-negative tumors.[28] However, research by Holzscheiter *et al.* concluded to the contrary that high KLK7 mRNA status indicates a good prognosis in breast cancer.[29] To further determine their role in breast cancer development, evaluate their clinical significance in breast cancer, and explore the correlation between KLK5 and KLK7 in breast cancer, we detected their mRNA expression levels in normal breast tissues, benign breast tissues, primary breast cancers, and lymph node metastases by real-time reverse transcription polymerase chain reaction (RT-PCR) and microarray. We then correlated the expression levels with the clinical parameters of the patients, including age, clinical stage, pathological tumor size, axillary lymph node status, nuclear grade, estrogen receptor (ER) status, progestogen receptor (PR) status, and DFS.

Materials and Methods

Clinical samples. All normal breast tissues, benign breast tumors, and invasive breast cancers were collected from patients undergoing complete dissection of breast and axillary lymph nodes followed by adjuvant systematic therapy (consisting of chemotherapy combined with hormonal therapy when with positive ER status for breast cancer patients or local tumorectomy for patients with benign breast disease) at Tianjin Cancer Hospital, China, between January 2002 and June 2003. Thirty normal tissues, 108 primary tumors, 30 benign breast fibroadenomas, and 30 lymph node metastasis samples were collected for real-time RT-PCR. Of the 108 breast cancer cases: primary cancers and paired normal breast tissues were collected in 20 cases; primary cancers and paired lymph node metastases were obtained in

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Table 1. Correlation between the mRNA levels of kallikrein (KLK) 5 and KLK7 and clinicopathological factors

Clinicopathological Factors		KLK5			KLK7		
		–/+	++	*P*-value	–/+	++	*P*-value
Breast tissue	Normal	5	25	0.000†	1	29	0.000†
	Benign	3	27		0	30	
	Cancer	78	30		51	57	
	Metastases	23	7		22	8	
Age (years)	<50	33	16	0.303	17	32	0.017
	≥50	45	14		34	25	
Menopausal status	Pre/peri-	34	19	0.066	17	36	0.002
	Post-	44	11		34	21	
Tumor size (cm)	≤2 cm	36	14	0.962	28	22	0.090
	>2 cm	42	16		23	35	
Clinical stage	I + II	59	23	0.911	40	42	0.565
	III + IV	19	7		11	15	
Lymph node status	–	35	17	0.272	22	30	0.324
	+	43	13		29	27	
Lymph node-positive number	0	35	17	0.513	22	30	0.516
	1–3	18	4		13	9	
	4–9	10	5		6	9	
	≥10	15	4		10	9	
Nuclear grade	I	9	3	0.396	5	7	0.148
	II	55	18		38	35	
	III	14	9		8	15	
Estrogen receptor	+	54	13	0.020	42	25	0.000
	–	22	15		8	29	
	Missing	2	2		1	3	
Progestogen receptor	+	36	11	0.463	25	22	0.343
	–	40	17		25	32	
	Missing	2	2		1	3	
Her2	–	45	16	0.673	31	30	0.289
	+	23	10		13	20	
	Missing	10	4		7	7	
Relapse or distant metastasis	Negative	49	14	0.327	31	32	0.587
	Positive	12	7		8	11	
	<3 years	17	9		12	14	

†The mRNA expression levels of KLK5 and KLK7 in malignant breast samples (including primary breast cancers and lymph node metastases) were lower than the mRNA levels in non-malignant breast tissues (including normal and benign breast tissues).

another 20 cases; matched normal breast, primary cancers, and lymph node metastases were collected in 10 cases; and only primary cancer samples were obtained in the other 58 cases. The detailed clinical characteristics of these patients, including clinical stage, pathological tumor size, lymph node status, nuclear grade, ER status, PR status, human epidermal growth factor type2 (Her2) status, and the existence of relapse or distant metastases, are shown in Table 1. ER and PR expression were determined by immunohistochemical staining (positive when more than 15% of the nuclei showed staining). Her2 was defined as positive when more than 10% of the membrane showed staining by immunohistochemical assay. Eighty-nine cases were followed up for more than 3 years (from 36 to 65 months, median 51 months), including 21 cases that developed local relapse (nine cases) or distant metastasis (six cases to the lung including three cases with simultaneous bone metastasis, two cases to the liver, two cases to the brain, one case to the other breast, and one case to the solar plexus). Forty-nine cases with a tumor of diameter less than 5 cm, consisting of 29 node-negative cases and 20 node-positive cases, were selected for microarray analysis. In these 49 cases, primary cancers and paired normal breast tissues were collected in nine lymph node-negative cases; only primary cancer samples were obtained in the other 40 cases. In addition, RNA was extracted and pooled equally from normal breast tissue taken from 32 patients with benign or malignant breast disease as control RNA. Thirty-six cases were used in both real-time RT-PCR and microarray analysis. Tissue samples were snap-frozen in liquid nitrogen and stored at –80°C. All samples were examined by hematoxylin–eosin staining on formalin-fixed paraffin-embedded sections, and only samples with 75% or more epithelial cells were selected for real-time RT-PCR. The use of these tissues was approved by the Institutional Reviewing Board and the Research Committee.

RNA extraction and first-strand cDNA preparation. RNA was extracted with TRIZOL reagent (Invitrogen, Gaithersburg, MD, USA) according to the manufacturer's instructions. RNA quality was assessed by formaldehyde agarose gel electrophoresis and was quantified spectrophotometrically. RT for first-strand cDNA was carried out using the First-strand cDNA System (Invitrogen). In brief, 5 μg of total RNA was denatured for 5 min at 65°C and snap-cooled on ice in the presence of 0.5 μg Oligo(dT) and 10 mmol dNTP mix, followed by incubation at 42°C for 50 min with First-Strand Buffer, 0.2 μmol dithiothreitol, 40 U RNaseOUT ribonuclease inhibitor (Invitrogen), and 200 U SuperScript II in a reaction system with a total volume of 20 μL. Reactions were stopped by incubation at 70°C for 15 min.

Real-time PCR. Real-time PCR analysis was carried out using the Platinum Quantitative PCR SuperMix-UDG System (Invitrogen) according to the manufacturer's instructions. We quantified transcripts of the housekeeping gene glyceraldehyde-3-phosphate dehydrogenase (GAPDH) as a control, as described in reference 30.[30] Primers and Taqman probes of KLK5 and KLK7 were designed on the common sequences of all known splicing variants. The primers and Taqman probes of KLK5 were 5′-GCAAGACCCCCCTGGATGTG-3′, 5′-TCCCAGAGGGCACG GTGTTA-3′, and 5′(FAM)-GTTGGCGAGAACATGCTCTGTGA CCC-(TAMRA)-3′. The primers and Taqman probes of KLK7 were 5′-AGGCGTCCTGGTCAATGAG-3′, 5′-GGGTGGCGGAA TGACTT-3′, and 5′(FAM)-CCACTGCAAGATGAATGAGTACA CCG-(TAMRA)-3′. Assays were carried out using the ABI 7500 TaqMan system (Applied Biosystems, Foster City, CA, USA). PCR was carried out after incubation at 50°C for 2 min and predenaturing at 95°C for 3 min, followed by 40 cycles at 95°C for 30 s and 65°C for 1 min. Quantification of target-gene expression in the samples was accomplished by measuring the fractional cycle number at which the amount of expression reached a fixed threshold (C_T). The relative quantification was given by the C_T values, determined by triplicate reactions for test and reference samples for each target and for *GAPDH*. Triplicate C_T values were averaged and the *GAPDH* C_T subtracted to obtain ΔC_T. Relative expression levels of each target gene were determined as $2^{-\Delta C_T}$.

Microarray. The human long oligonucleotide microarray was constructed by CapitalBio Corporation (Beijing, China). The microarray consists of 5′-amino-modified 70-mer probes representing 21 329 well-characterized human genes purchased from the Operon Company (http://www.operon.com). DNA labeled with fluorescent dye (Cy5 and Cy3-dCTP) was produced by Eberwine's linear RNA amplification method[31] and subsequent enzymatic reaction as previously described.[32] All samples were labeled with Cy5, and the control, pooled from 32 normal breast tissues, was labeled with Cy3. The labeled DNA was purified, resuspended in elution buffer, and quantified. Labeled control and test samples were adjusted quantitatively based on the efficiency of Cy-dye incorporation and mixed with 30 μL hybridization solution (3× standard sodium citrate (SSC), 0.2% sodium dodecylsulfate, 25% formamide, and 5× Denhart's solution). DNA in the hybridization solution was denatured at 95°C for 3 min prior to loading on a microarray. The array was hybridized at 42°C overnight and washed consecutively with two washing solutions: 0.2% sodium dodecylsulfate and 2× SSC at 42°C for 5 min, followed

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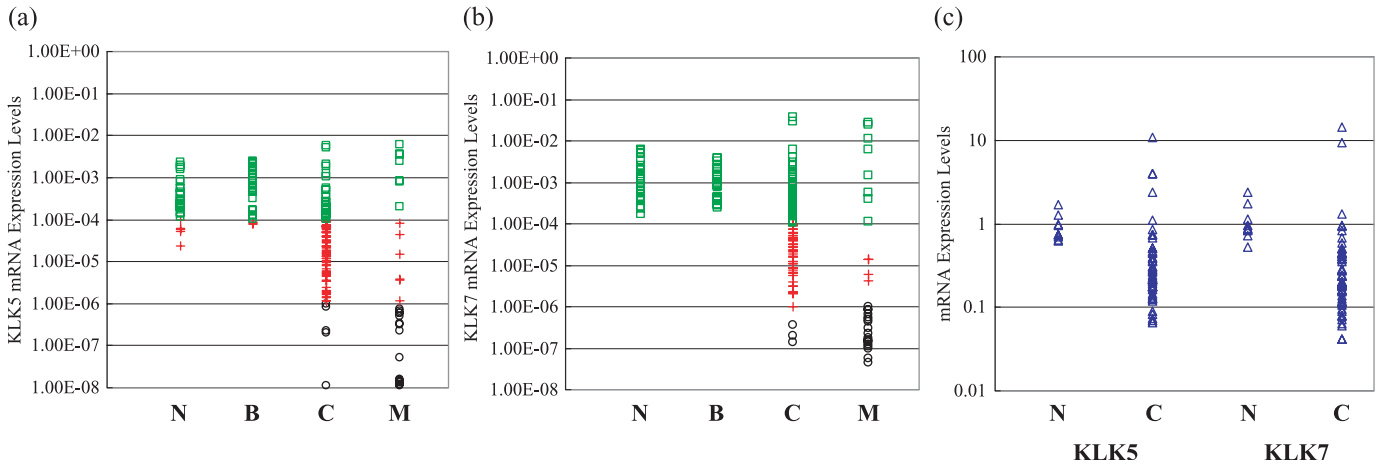

Fig. 1. Distribution of (a) kallikrein (KLK) 5 and (b) KLK7 in real-time polymerase chain reaction and (c) microarray analysis in normal breast (N), benign breast tissues (B), primary breast cancers (C), and lymph node metastases (M). Black circles indicate negative (–) KLK5 or KLK7. Red crosses indicate weakly positive (+) KLK5 or KLK7; green squares represent strong positive (++) KLK5 or KLK7. Blue triangles indicate the ratios of KLK5/KLK7 mRNA levels in breast samples relative to the normal breast control.

by 0.2% SSC for 5 min at room temperature. Arrays were scanned with a ScanArray Express Scanner (Packard Bioscience, Kanata, OT, USA), and the images obtained were analyzed with GenePix Pro 4.0 (Axon Instruments, Foster City, CA, USA).

Statistical analysis. Because the distribution of KLK5 and KLK7 mRNA expression in breast tissues did not accord with the normal distribution, the relationship between KLK5 and KLK7 and various clincopathological variables was analyzed by χ^2-test or Fisher's exact test, as appropriate. The cut-off value for distinguishing malignancies from non-malignant tissues and the threshold for classifying primary breast cancers and metastases were determined by receiver operating characteristic (ROC) curve and area under the curve. The correlation coefficients between KLK5 and KLK7 and the correlation between KLK5 and KLK7 and other members of the KLK family or transcription factors were calculated with non-parametric Spearman correlation analysis. Eighty-nine cases with more than 3 years follow up were used for survival analysis. Survival analysis was carried out according to the methods of Kaplan and Meier. All calculations were carried out using SPSS for Windows statistical software package (SPSS, Chicago, IL, USA). Furthermore, Cluster 3.0 and Treeview software (Stanford University) were used to carry out average linkage clustering for classifying these samples according to the expression levels of KLK5 and KLK7.

5′-Non-coding region analysis. To analyze the homology between the 5′-control region of KLK5 and KLK7, the upstream sequences from –2000 to their transcriptional start sites were compared using the Blast program (http://www.ncbi.nlm.nih.gov). Then the potential transcription factor binding sites on their 5′-control regions were searched in the transcription factor binding sites database (http://www.cbrc.jp/research/db/TFSEARCH.html).

Results

Expression levels of KLK5 and KLK7 mRNA in breast tissues. The mRNA levels of KLK5 and KLK7 measured by real-time RT-PCR analyses ranged from 0 to 6.11E–03 and from 4.47E–08 to 3.63E–02, respectively, in all tissues including normal breast, benign breast tissues, primary breast cancers, and lymph node metastases. The distribution of KLK5 and KLK7 was centralized in normal breast (from 2.39E–05 to 2.34E–03, and from 3.42E–05 to 6.22E–03) and benign breast tissue (from 7.82E–05 to 2.52E–03, and from 2.43E–04 to 3.94E–03) (Fig. 1). However, their distribution was more decentralized in primary breast cancers (from 0 to 5.85E–03, and from 1.43E–07 to 3.63E–02) and lymph node metastases (from 0 to 6.11E–03, and from 4.47E–08 to 2.84E–02) (Fig. 1). The distribution of KLK5 and KLK7 mRNA expression in breast tissues did not accord with the normal distribution. Based on the results of the ROC analysis, the mRNA value (1.0E–04), which could distinguish malignancies from normal breast tissues and benign breast samples, and the threshold (1.0E–06), which could classify primary cancers and metastases, were used to group all samples into three groups: –, negative (less than 1.0E–06); +, weakly positive (from 1.0E–06 to 1.0E–04); and ++, strongly positive (more than 1.0E–04).

Table 2. mRNA expression levels of kallikrein (KLK) 5 and KLK7 in breast cancers and lymph node metastases

Samples	KLK5			KLK7		
	–	+/++	*P*-value	–	+/++	*P*-value
Cancers	5	103	0.000	3	105	0.000
Metastases	17	13		18	12	

mRNA expression differences of KLK5 and KLK7 between malignant and non-malignant breast tissues. In the 30 cases with paired primary breast cancers and lymph node metastasis for real-time PCR, KLK5 mRNA levels were downregulated more than 1.5 fold in cancers in 25 of 30 cases (from 1.69 to 11 390.16 fold), upregulated in one case (7.93 fold), and equal in the other four cases. KLK7 mRNA was downregulated in cancers in 24 cases (from 1.63 to 43 447.94 fold), upregulated in three cases (from 4.37 to 26.37 fold), and equal in the other three cases. No significant difference was found in the KLK5 and KLK7 mRNA levels between benign breast samples and normal breast tissues (Table 1). KLK5 was downregulated more than 1.5 fold in lymph node metastases in 17 of 30 cases compared with their paired primary cancers, but upregulated in 8 of 30 cases. Equal levels were seen in the rest of the five cases. KLK7 was downregulated in 23 of 30 cases compared with their paired primary cancers, but upregulated in the other seven cases. KLK5 and KLK7 were negative (–) in 56.67% and 40.00% of lymph node metastases, and positive (+/++) in 43.33% and 60.00% of metastases, respectively. But they were negative in only 4.63% (5/108, KLK5) and 2.78% (3/108, KLK7) of primary cancers (Table 2). This difference was statistically significant (P = 0.000). The mRNA expression levels of KLK5 and KLK7 in malignant breast samples (including 108 primary breast cancers and 30 lymph node metastases) were lower than the

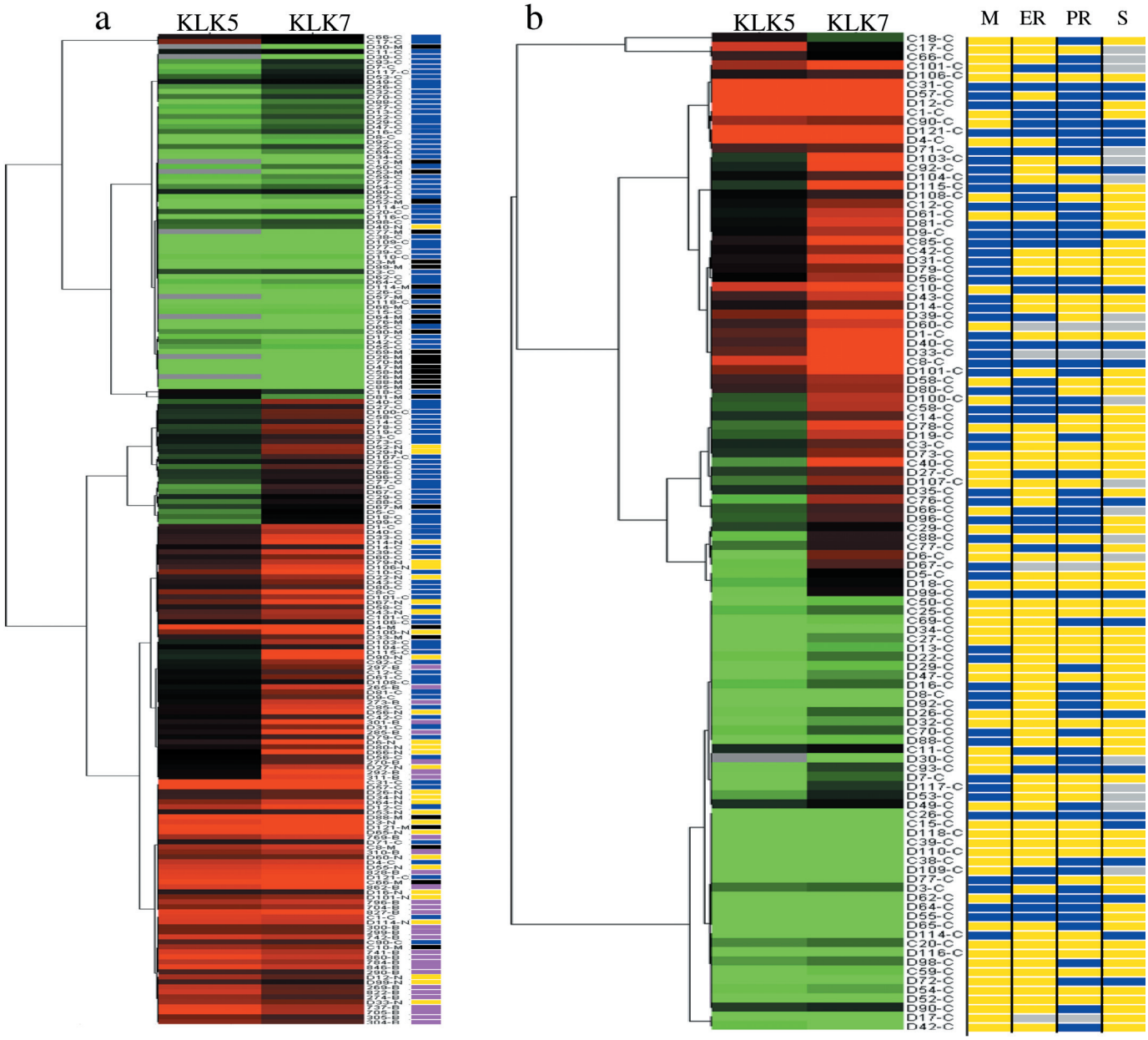

Fig. 2. Cluster results combining kallikrein (KLK) 5 with KLK7. Each row represents one breast sample. In the cluster color bar, red means strongly positive (++) KLK5/KLK7 in this sample; green represents negative or weakly positive (–/+) KLK5/KLK7; and gray means no detectable KLK5/KLK7 in the sample. (a) Four colors, yellow (normal breast), pink (benign breast tissues), blue (primary breast cancer), and black (lymph node metastases) represent different kinds of breast tissue. KLK5 and KLK7 were parallelly underexpressed in breast malignances. (b) Blue bars represent premenopausal status (M), negative estrogen receptor (ER)/progestogen receptor (PR), and relapse/distant metastases (S), respectively; yellow bars represent postmenopausal (M), positive ER/PR, and disease-free survival (S), respectively; and gray bars represent missing data.

mRNA levels in non-malignant breast tissues (including 30 normal and 30 benign breast tissues). KLK5 and KLK7 were negative or weakly positive (–/+) in 73.19% (101/138) and 52.90% (73/138) of malignant tissues, but in only 13.33% (8/60) and 1.67% (1/60) of non-malignant breast tissues ($P = 0.000$; Table 1; Fig. 1). The difference between malignant and non-malignant breast tissues combining KLK5 with KLK7 is shown in Figure 2(a). In accordance with the results of real-time PCR, both KLK5 and KLK7 were found to be downregulated in breast cancer samples compared with normal breast tissues in the microarray analysis (Fig. 1c).

Correlation between the mRNA levels of KLK5 and KLK7 and clinicopathological factors. The mRNA expression levels of KLK7, but not KLK5, were lower in patients older than 50 years than in younger patients (Table 1). Both KLK5 and KLK7 were significantly lower in postmenopausal patients than in premenopausal and perimenopausal patients ($P = 0.066$ and 0.002, respectively), and underexpressed in the ER-negative group compared with the ER-positive group ($P = 0.020$ and 0.000, respectively) (Table 1; Fig. 2). No significant differences were found between the mRNA levels of KLK5 and KLK7 and other clinicopathological parameters, including tumor size, clinical stage, nuclear grade, PR status, Her2 status, and the clinical outcome of breast cancer patients (Table 1).

Results of the 5′-non-coding sequence analysis. Ten transcription factors were found as potential regulators, binding to the 5′-control regions (the upstream sequences from –2000 to their transcriptional start sites) of both KLK5 and KLK7, including

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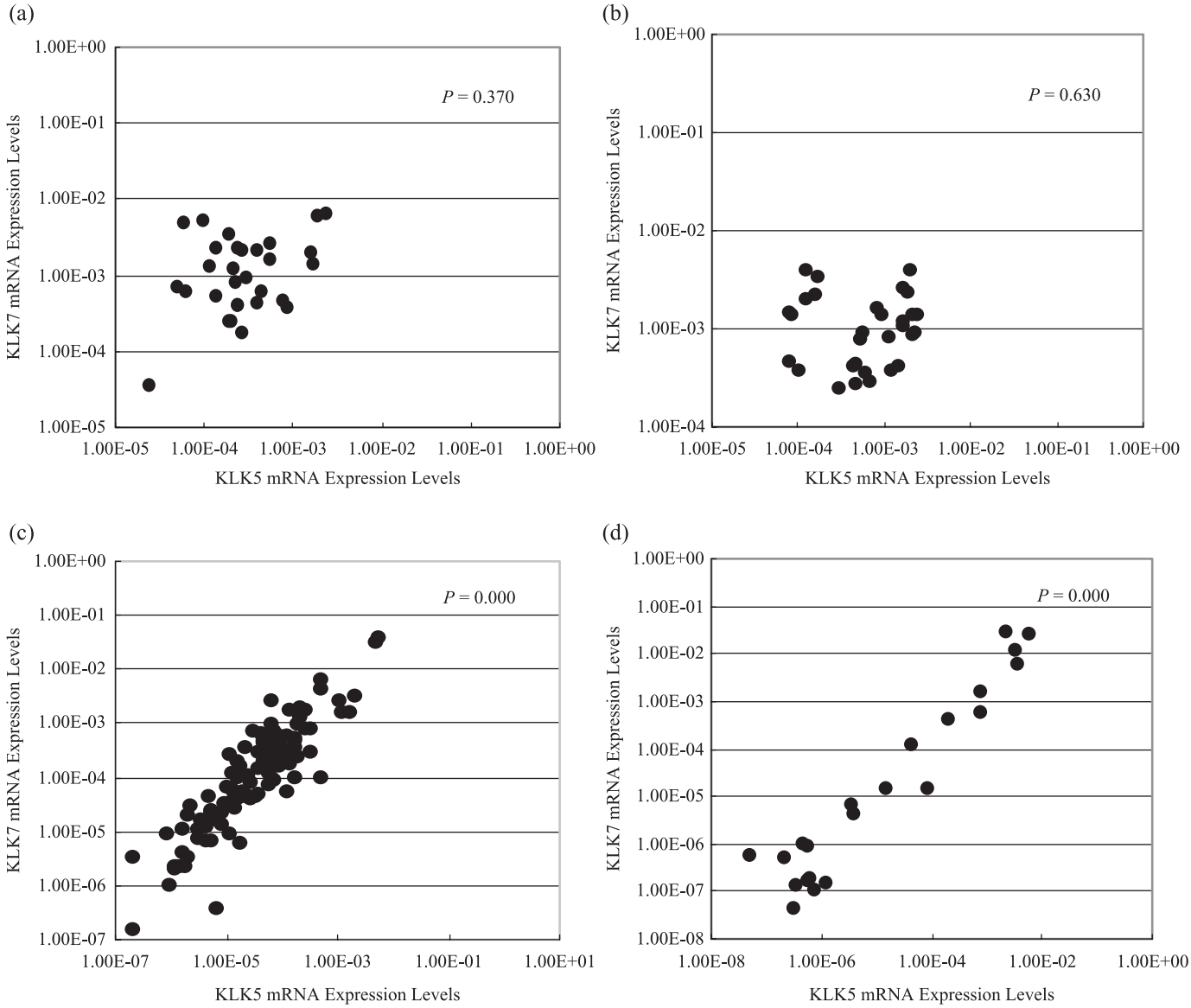

Fig. 3. The mRNA levels of kallikrein (KLK) 5 and KLK7 were positively correlated in (c) breast cancers and (d) lymph node metastases. No correlation was found in (a) normal breast and (b) benign breast tissues.

runt-factor acute myeloid leukemia 1 protein (AML1/RUNX1), GATA-binding factor 2 (GATA2), GATA-binding factor 3 (GATA3), heat shock factor 2 (HSF2), myeloid zinc finger 1 (MZF1), P300, pre-B-cell leukemia transcription factor 1 (PBX1), stimulating protein 1 (SP1), sterol regulatory element-binding protein 1 (SREBP1), and sex-determining region Y gene product (SRY). Four homologous sequences with more than 80% similarity between the 5′-control regions of KLK5 and KLK7 were found (Table 3). SREBP1 was found to bind to the same homologous region of KLK5 and KLK7. Because the mRNA levels of KLK5 and KLK7 were correlated with ER status, we searched the estrogen response element (ERE, 5′-GGTCAnnnTGACC-3′) on the 5′-control region of KLK5 and KLK7. Although there were no ERE found, two ERE half-site motifs (ERE1/2, TGACC) were identified on the upstream sequence of KLK5 (–262 and –1254), and four ERE1/2 motifs were found on the control region of KLK7 (–492 and –1669 with GGTCA, –784 and –1647 with TGACC).

Correlation between the mRNA expression levels of KLK5 and KLK7. The mRNA levels of KLK5 and KLK7 were positively correlated in breast cancers ($r = 0.869$, $P = 0.000$; Fig. 3c) and lymph node metastases ($r = 0.871$, $P = 0.000$; Fig. 3d). But no correlation was found in normal breast tissue ($r = 0.170$, $P = 0.370$; Fig. 3a) or benign breast tissue ($r = 0.092$, $P = 0.630$; Fig. 3b).

Table 3. Homologous sequences between the 5′-control regions of kallikrein (KLK) 5 and KLK7

No.	Genes	Location	Length (bp)	Homology (%)
1	KLK5	–873 to –939	103	85
	KLK7	–202 to –404		
2	KLK5	–1000 to –1096	97	85
	KLK7	–1630 to –1726		
3	KLK5	–988 to –1106	119	80
	KLK7	–441 to –557		
4	KLK5	–831 to –949	128	80
	KLK7	–1473 to –1591		

Correlation between the mRNA levels of KLK5 and KLK7 and other members of the KLK family or potential transcription factors. All

members of the KLK family and 10 potential transcription factors were present on the microarray except KLK9 and MZF1. Both KLK5 and KLK7 mRNA were correlated with the expression levels of KLK6, KLK8, KLK10, KLK12, GATA3, HSF2, PBX1 and SREBP1 in the microarray analysis (Table 4).

Table 4. Correlation between the mRNA levels of kallikrein (KLK) 5 and KLK7 and other members of the KLK family or potential transcription factors

Genes	KLK5		KLK7	
	R	*P*-value	*R*	*P*-value
KLK1	–0.112	0.407	–0.175	0.192
KLK2	–0.096	0.555	0.187	0.247
KLK3	–0.002	0.987	0.040	0.766
KLK4	0.760	0.597	–0.002	0.992
KLK5	1.000	0.000	0.851	0.000
KLK6	0.893	0.000	0.870	0.000
KLK7	0.851	0.000	1.000	0.000
KLK8	0.743	0.000	0.752	0.000
KLK10	0.442	0.001	0.411	0.001
KLK11	0.194	0.148	0.231	0.084
KLK12	–0.272	0.039	–0.266	0.044
KLK13	–0.192	0.292	–0.362	0.042
KLK14	0.109	0.418	0.124	0.357
KLK15	–0.126	0.355	–0.114	0.403
RUNX1	–0.064	0.631	0.003	0.982
GATA2	–0.262	0.047	–0.213	0.109
GATA3	–0.443	0.000	–0.384	0.003
HSF2	0.281	0.033	0.220	0.098
P300	0.071	0.589	0.083	0.536
PBX1	–0.240	0.069	–0.272	0.039
SP1	–0.101	0.492	–0.060	0.687
SREBP1	–0.326	0.012	–0.288	0.029
SRY	0.039	0.857	–0.201	0.346

Discussion

In the present study, we detected the mRNA expression levels of KLK5 and KLK7 in 30 normal breast tissues, 30 benign breast tissues, 108 primary breast cancers, and 30 lymph node metastases by real-time RT-PCR and correlated the expression levels with the clinical parameters of the patients. As a result, KLK5 and KLK7 were found to be downregulated in breast cancers relative to normal and benign breast tissues, and downregulated in lymph node metastases compared to primary breast cancers. Underexpression of KLK5 and KLK7 was correlated with postmenopausal status and positive ER status. No significant differences were found between the mRNA levels of KLK5 and KLK7 and other clinicopathological parameters or the clinical outcome of breast cancer patients. Furthermore, the mRNA levels of KLK5 and KLK7 were positively correlated in breast cancers and lymph node metastases, but no correlation was found in normal breast tissue or benign breast tissue. Underexpression of KLK5 and KLK7 in breast cancers compared with normal breast tissues was confirmed by the results of microarray.

Although it had been shown that KLK5 and KLK7 are underexpressed in breast cancers relative to normal breast tissue,[29] their expression levels in benign breast tissue and metastases and their role in the development of cancer were not well defined. The results of Talieri *et al.* indicated that KLK7 mRNA expression is positively associated with clinical stage and negatively related to PR status, and its overexpression might be a marker of unfavorable prognosis for breast cancer patients.[28] Yousef's group has demonstrated that high KLK5 expression levels are found more frequently in premenopausal and perimenopausal, node-positive, and ER-negative breast cancer patients and its overexpression is a significant predictor of reduced DFS and OS in breast cancer patients.[27] However, Holzscheiter and colleagues showed that KLK7 expression is significantly higher in ER-negative tumors than in ER-positive tumors, and high KLK7 mRNA expression reveals a better prognosis for breast cancer patients.[29] Consistent with the results reported by Holzscheiter and Yousef, our results showed that high KLK5 and KLK7 expression were found more frequently in premenopausal and ER-negative patients. However, in contrast to their studies, no significant difference was found between the mRNA levels of KLK5 and KLK7 and the clinical outcome of breast cancer patients. This discrepancy may be related to the different assay and statistical methods used. In the previous study carried out by Talieri's group, KLK7 was not detected in 54.3% of breast cancer samples based on a regular RT-PCR assay. But in the present study, KLK5 and KLK7 mRNA expression levels were detected by a real-time PCR method with higher sensitivity. In another study reported by Holzscheiter's group, the forward primer targeted the splice junction between exons 2 and 3 of *KLK7*, so only full-length KLK7 mRNA transcripts were amplified selectively. However, in the present study, not only full-length KLK7 transcripts but also the splicing variant lacking exon 2 were amplified because the KLK7 PCR product spanned the splice junction between exons 3 and 4. Moreover, Holzscheiter carried out *t*-test or ANOVA following a log-transformation. But in the present study, the distribution of KLK5 and KLK7 mRNA expression did not accord with the normal distribution, even after being log-transformed, so the relationship between KLK5 and KLK7 and various clincopathological variables was analysed by the χ^2-test or Fisher's exact test as appropriate. The difference in Chinese and Western populations might also contribute to the discrepancy observed.

Breast tissue consists of different kinds of cells: luminal epithelial cells, myoepithelial cells, fibroblasts, myofibroblasts, endothelial cells, and leukocytes. The results of real-time PCR are influenced by the relative ratio of different cell populations. So in the present study, only samples with 75% or more epithelial cells were selected for real-time RT-PCR and microarray. Moreover, the mRNA expression levels in a large sample could reflect the average level in the whole tissue specimen.

Coexpression of KLK5 and KLK7 has also been observed in skin tissue[23] and ovarian cancer.[11] But the possible mechanism for this has not been elucidated. Four homologous sequences between the 5′-control regions of KLK5 and KLK7 and 10 transcription factors potentially binding to their 5′-control regions were identified in the present study. Furthermore, both KLK5 and KLK7 mRNA were found to correlate with the expression levels of KLK6, KLK8, KLK10, KLK12, GATA3, HSF2, PBX1, and SREBP1. A possible explanation for their parallel underexpression in breast malignancies could be that KLK5 and KLK7 are regulated by the same transcription factors. Moreover, KLK5 and KLK7 are neighbors of KLK6 and KLK8, so the copy number of this locus might be another reason for their parallel underexpression.

It has been reported that KLK5 and KLK7 could be upregulated by estrogens and progestins.[33,34] But no hormone response elements were found in the 5′-transcription regulation regions of KLK5 and KLK7. By examining the transcription factor database, SREBP1 was found to be a transcription factor that might bind to the same homologous region of KLK5 and KLK7. It has been reported that SREPB1 as a potential coactivator of estrodial (E2)-ER-dependent effects might be able to bind directly to the ERE1/2 motifs, enhancing ER binding.[35] This might explain how KLK5 and KLK7 can be both upregulated by steroid hormones[34] and differentially expressed in different age groups and patients

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with different menopausal status, as well as in different ER groups, but no ERE were found on their 5′-non-coding regions.

In conclusion, we detected the mRNA levels of KLK5 and KLK7 in normal breast tissue, benign breast tissue, primary cancers, and lymph node metastases so that the role of KLK5 and KLK7 in every stage of breast carcinogenesis and the development of breast cancer could be observed. KLK5 and KLK7 were underexpressed in parallel in breast malignancies and downregulated in lymph node metastases, indicating that their downregulation may contribute to the carcinogenesis and development of breast cancer. They are potential biomarkers for breast cancer.

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References

1 Gan L, Lee I, Smith R *et al*. Sequencing and expression analysis of the serine protease gene cluster located in chromosome 19q13 region. *Gene* 2000; **257**: 119–30.
2 Stege R, Grande M, Carlstrom K, Tribukait B, Pousette A. Prognostic significance of tissue prostate-specific antigen in endocrine-treated prostate carcinomas. *Clin Cancer Res* 2000; **6**: 160–5.
3 Diamandis EP. Prostate-specific antigen: a cancer fighter and a valuable messenger? *Clin Chem* 2000; **46**: 896–900.
4 Paliouras M, Borgono C, Diamandis EP. Human tissue kallikreins: the cancer biomarker family. *Cancer Lett* 2007; **249**: 61–79.
5 Egelrud T. Purification and preliminary characterization of stratum corneum chymotryptic enzyme: a proteinase that may be involved in desquamation. *J Invest Dermatol* 1993; **101**: 200–4.
6 Hansson L, Stromqvist M, Backman A, Wallbrandt P, Carlstein A, Egelrud T. Cloning, expression, and characterization of stratum corneum chymotryptic enzyme. A skin-specific human serine proteinase. *J Biol Chem* 1994; **269**: 19 420–6.
7 Brattsand M, Egelrud T. Purification, molecular cloning, and expression of a human stratum corneum trypsin-like serine protease with possible function in desquamation. *J Biol Chem* 1999; **274**: 30 033–40.
8 Michael IP, Sotiropoulou G, Pampalakis G *et al*. Biochemical and enzymatic characterization of human kallikrein 5 (hK5), a novel serine protease potentially involved in cancer progression. *J Biol Chem* 2005; **280**: 14 628–35.
9 Dong Y, Kaushal A, Brattsand M, Nicklin J, Clements JA. Differential splicing of KLK5 and KLK7 in epithelial ovarian cancer produces novel variants with potential as cancer biomarkers. *Clin Cancer Res* 2003; **9**: 1710–20.
10 Prezas P, Arlt MJ, Viktorov P *et al*. Overexpression of the human tissue kallikrein genes KLK4, 5, 6, and 7 increases the malignant phenotype of ovarian cancer cells. *Biol Chem* 2006; **387**: 807–11.
11 Yousef GM, Polymeris ME, Yacoub GM *et al*. Parallel overexpression of seven kallikrein genes in ovarian cancer. *Cancer Res* 2003; **63**: 2223–7.
12 Shan SJ, Scorilas A, Katsaros D, Rigault de la Longrais I, Massobrio M, Diamandis EP. Unfavorable prognostic value of human kallikrein 7 quantified by ELISA in ovarian cancer cytosols. *Clin Chem* 2006; **52**: 1879–86.
13 Yousef GM, Polymeris ME, Grass L *et al*. Human kallikrein 5: a potential novel serum biomarker for breast and ovarian cancer. *Cancer Res* 2003; **63**: 3958–65.
14 Tanimoto H, Underwood LJ, Shigemasa K *et al*. The stratum corneum chymotryptic enzyme that mediates shedding and desquamation of skin cells is highly overexpressed in ovarian tumor cells. *Cancer* 1999; **86**: 2074–82.
15 Planque C, de Monte M, Guyetant S *et al*. KLK5 and KLK7, two members of the human tissue kallikrein family, are differentially expressed in lung cancer. *Biochem Biophys Res Commun* 2005; **329**: 1260–6.
16 Luo LY, Yousef G, Diamandis EP. Human tissue kallikreins and testicular cancer. *APMIS* 2003; **111**: 225–32.
17 Yousef GM, Obiezu CV, Jung K, Stephan C, Scorilas A, Diamandis EP. Differential expression of kallikrein gene 5 in cancerous and normal testicular tissues. *Urology* 2002; **60**: 714–18.
18 Shinoda Y, Kozaki K, Imoto I *et al*. Association of KLK5 overexpression with invasiveness of urinary bladder carcinoma cells. *Cancer Sci* 2007; **98**: 1078–86.
19 Santin AD, Cane S, Bellone S *et al*. The serine protease stratum corneum chymotryptic enzyme (kallikrein 7) is highly overexpressed in squamous cervical cancer cells. *Gynecol Oncol* 2004; **94**: 283–8.
20 Tian X, Shigemasa K, Hirata E *et al*. Expression of human kallikrein 7 (hK7/SCCE) and its inhibitor antileukoprotease (ALP/SLPI) in uterine endocervical glands and in cervical adenocarcinomas. *Oncol Report* 2004; **12**: 1001–6.
21 Yousef GM, Scorilas A, Chang A *et al*. Down-regulation of the human kallikrein gene 5 (KLK5) in prostate cancer tissues. *Prostate* 2002; **51**: 126–32.
22 Yousef GM, Yacoub GM, Polymeris ME, Popalis C, Soosaipillai A, Diamandis EP. Kallikrein gene downregulation in breast cancer. *Br J Cancer* 2004; **90**: 167–72.
23 Ekholm IE, Brattsand M, Egelrud T. Stratum corneum tryptic enzyme in normal epidermis: a missing link in the desquamation process? *J Invest Dermatol* 2000; **114**: 56–63.
24 Diamandis EP, Borgono CA, Scorilas A *et al*. Immunofluorometric quantification of human kallikrein 5 expression in ovarian cancer cytosols and its association with unfavorable patient prognosis. *Tumour Biol* 2003; **24**: 299–309.
25 Kim H, Scorilas A, Katsaros D *et al*. Human kallikrein gene 5 (KLK5) expression is an indicator of poor prognosis in ovarian cancer. *Br J Cancer* 2001; **84**: 643–50.
26 Kyriakopoulou LG, Yousef GM, Scorilas A *et al*. Prognostic value of quantitatively assessed KLK7 expression in ovarian cancer. *Clin Biochem* 2003; **36**: 135–43.
27 Yousef GM, Scorilas A, Kyriakopoulou LG *et al*. Human kallikrein gene 5 (KLK5) expression by quantitative PCR. an independent indicator of poor prognosis in breast cancer. *Clin Chem* 2002; **48**: 1241–50.
28 Talieri M, Diamandis EP, Gourgiotis D, Mathioudaki K, Scorilas A. Expression analysis of the human kallikrein 7 (KLK7) in breast tumors: a new potential biomarker for prognosis of breast carcinoma. *Thromb Haemost* 2004; **91**: 180–6.
29 Holzscheiter L, Biermann JC, Kotzsch M *et al*. Quantitative reverse transcription-PCR assay for detection of mRNA encoding full-length human tissue kallikrein 7: prognostic relevance of KLK7 mRNA expression in breast cancer. *Clin Chem* 2006; **52**: 1070–9.
30 Li X, Cao X, Zhang W, Feng Y. Expression level of insulin-like growth factor binding protein 5 mRNA is a prognostic factor for breast cancer. *Cancer Sci* 2007; **98**: 1592–6.
31 Guo Y, Guo H, Zhang L *et al*. Genomic analysis of anti-hepatitis B virus (HBV) activity by small interfering RNA and lamivudine in stable HBV-producing cells. *J Virol* 2005; **79**: 14 392–403.
32 Feng Y, Sun B, Li X *et al*. Differentially expressed genes between primary cancer and paired lymph node metastases predict clinical outcome of node-positive breast cancer patients. *Breast Cancer Res Treat* 2007; **103**: 319–29.
33 Yousef GM, Scorilas A, Magklara A, Soosaipillai A, Diamandis EP. The KLK7 (PRSS6) gene, encoding for the stratum corneum chymotryptic enzyme is a new member of the human kallikrein gene family – genomic characterization, mapping, tissue expression and hormonal regulation. *Gene* 2000; **254**: 119–28.
34 Yousef GM, Diamandis EP. The new kallikrein-like gene, KLK-L2. Molecular characterization, mapping, tissue expression, and hormonal regulation. *J Biol Chem* 1999; **274**: 37 511–16.
35 Lopez D, Sanchez MD, Shea-Eaton W, McLean MP. Estrogen activates the high-density lipoprotein receptor gene via binding to estrogen response elements and interaction with sterol regulatory element binding protein-1A. *Endocrinology* 2002; **143**: 2155–68.