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Parallel Overexpression of Seven Kallikrein Genes in Ovarian Cancer[1,2]

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ABSTRACT

Recent evidence suggests that many members of the human kallikrein (KLK) gene family are differentially regulated in ovarian cancer and have potential as diagnostic and/or prognostic markers. We used the serial analysis of gene expression and expressed sequence tag databases of the Cancer Genome Anatomy Project to perform *in silico* analyses of the expression pattern of the 15 human KLK genes in normal and cancerous ovarian tissues and cell lines. We found that seven KLK genes (*KLK5, KLK6, KLK7, KLK8, KLK10, KLK11,* and *KLK14*) are up-regulated in ovarian cancer. Probing 2 normal and 10 ovarian cancer serial analysis of gene expression libraries with gene-specific tags for each KLK indicated that whereas no expression was detected in any normal libraries (with the exception of *KLK10* and *KLK11*), these KLKs were found to be expressed with moderate densities (103–408 tags per million) in 40–60% of the ovarian cancer libraries analyzed. These data were verified by screening the expressed sequence tag databases, where 78 of 79 mRNA clones isolated for these genes were from ovarian cancer libraries. X-profiler comparison of the pools of normal and cancerous ovaries identified a significant difference in expression levels for six of the seven KLKs. We experimentally verified the overexpression of six KLK proteins in cancer *versus* normal or benign tissues with highly sensitive and specific immunofluorometric assays. A statistically significant stepwise increase in protein levels was found among normal, benign, and cancerous ovarian tissues. The expression of five KLKs showed a strong degree of correlation at the protein level, suggesting the existence of a common mechanism or pathway that controls the expression of this group of adjacent genes during ovarian cancer progression.

INTRODUCTION

The CGAP[4] is an international effort implemented by the National Cancer Institute to create a catalogue of the genes associated with cancer and to develop technological tools to support the analysis of the molecular profiles of cancer cells and their normal counterparts (1). Gene expression data contained in the electronic databases of the CGAP can be used to identify potentially informative marker genes expressed in cancer and to compare cDNA frequencies of genes of interest in normal *versus* cancer states. The project applies two main approaches, the EST method and the SAGE approach (2). Various analytical tools have also been developed for data analysis including BLAST, virtual Northern analysis, X-profiler, and digital differential display. These databases have been used successfully in recent years, and results obtained from *in silico* analysis were experimentally verified in most cases.

Epithelial ovarian cancer continues to be the leading cause of death from gynecological cancers in the United States, accounting for 14,400 deaths/year (3). Because most patients are asymptomatic until the disease has metastasized, two-thirds are diagnosed in advanced stages, with survival rates of <20%. Currently, the only tumor marker that has a well-defined and validated role in the management of ovarian cancer is CA125 (4). Serum CA125 has been evaluated for screening for ovarian cancer, differentiation between benign and malignant ovarian masses, and prognosis. This marker does not have a clear place in diagnosis, prognosis, or making treatment decisions, and it lacks specificity (5). Many potential new serum markers for ovarian cancer have been evaluated, either alone or in combination with CA125, including inhibin, prostasin, OVX1, LASA, CA15.3, and CA72-4 (4). However, none of them has a clear advantage over CA125 to date. Recently, microarray technology has been used to identify novel potential serum markers for ovarian cancer (6), and bioinformatic approaches have been successfully used to identify candidate genes for further analysis (7).

KLKs are a family of 15 genes clustered together on chromosome 19 and belong to the serine protease family of enzymes (8, 9). Prostate-specific antigen (PSA, hK3), a member of this family, is the premier tumor marker for prostate cancer (10). Accumulating evidence suggests a role for KLKs in cancer diagnosis and prognosis (11–15).

In this study, we used bioinformatic tools to examine KLK gene expression in normal and cancerous ovarian tissues and cell lines. We found strong evidence that seven KLKs are up-regulated in ovarian cancer. We then experimentally verified the overexpression of six of these KLKs at the protein level in cancer tissues and found a significant correlation between their concentration levels.

MATERIALS AND METHODS

VNBs. The mRNA sequences of the 15 human KLK genes were used to identify unique sequence tags of UniGene clusters for each KLK (for GenBank accession numbers, see Ref. 8). Two restriction digestion enzymes (*Nla*III and *Sau*3A) were used as anchoring enzymes. These sequence tags were then used to determine the levels of expression of different KLKs in 2 normal and 10 ovarian cancer libraries (4 from ovarian cancer tissues and 6 from ovarian cancer cell lines). Detailed information for these libraries is available from the web site of the CGAP.[5] A list of all libraries used for analysis is submitted as supplementary information.[2] Analyses were performed by comparing the proportion of libraries of each type (cancer *versus* normal) that show expression of each tag, in addition to the average expression densities in these libraries. mRNA sequences from the Human Genome Project were used as reference sequences. If more than one tag of the same gene appears in the same library, we only included the one with the peak level of expression (maximum tpm); the other tags were excluded to avoid inaccurate estimation of expression. Expression levels are displayed as blots with different densities and corrected as tpm to facilitate comparison.

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[4] The abbreviations used are: CGAP, Cancer Genome Anatomy Project; SAGE, serial analysis of gene expression; EST, expressed sequence tag; VNB, virtual Northern blot; tpm, tags per million; KLK, kallikrein; hK, human kallikrein protein.

[5] http://www.ncbi.nlm.nih.gov/ncicgap/.

EST Analysis. The full-length mRNA sequence of each KLK was compared against the human EST databases of the National Center for Biotechnology Information. At the time of the study, these databases included libraries from 9 normal, 3 premalignant, and 17 cancerous tissues. Expression was calculated for each KLK as the number of positive libraries in each tissue type, in addition to the total number of clones detected in each type of library.

X-profiler. X-profiler analyses of KLK gene expression were performed by comparing normal and cancerous ovarian libraries available in the SAGE databases. Because expression levels of various KLKs might be different from one cell line to another and in different types of ovarian cancer, we compared a pool of normal ovarian libraries (2 normal ovarian epithelial cell lines) against 10 different epithelial ovarian cancer libraries (4 from ovarian cancer tissues and 6 from ovarian cancer cell lines). Ovarian cancer libraries represented different histological types including serous adenocarcinoma and clear cell carcinoma. The X-profiler cutoff value was set at 2-fold difference.

Ovarian Tissue Cytosols. Human tissue extracts were prepared from 10 histologically confirmed normal ovarian tissues, 10 benign ovarian tumors, and 20 cancerous ovarian tissues. Tissue extraction was as follows: frozen human tissues (0.2 g) were pulverized on dry ice to fine powders. Two ml of extraction buffer [50 mM Tris-HCl buffer (pH 8.0) containing 150 mM NaCl, 5 mM EDTA, and 1% NP40 surfactant] were added to the tissue powders, and the mixture was incubated on ice for 30 min with repeated shaking and vortex mixing every 10 min. Mixtures were centrifuged at 14,000 $\times$ *g* at 4°C for 30 min. The supernatants representing the tissue extracts were collected and stored at −80°C until use. Our procedures have been approved by the Institutional Review Board of Mount Sinai Hospital.

Immunofluorometric Assays. KLK protein levels were measured by ELISA-type time-resolved immunofluorometric assays developed in our laboratory. These sandwich-type immunoassays use monoclonal and polyclonal antibodies developed against recombinant proteins. All assays were shown to be specific, with no cross-reactivity from other KLKs (12, 16, 17). The protein level of each KLK was normalized according to the total protein content of each sample and expressed as ng KLK/mg total protein.

Statistical Analysis. Correlation analyses were performed using Spearman correlation coefficient. The analysis of differences between the variables in the three groups was performed with the Kruskal-Wallis test.

RESULTS

First, to verify the reliability and sensitivity of the bioinformatic analysis, we compared the *in silico* pattern of expression of each KLK in normal tissues to the previously published experimental data on tissue KLK expression (8). Results obtained were concordant with the published data. For example, most EST and SAGE clones for *KLK2*, *KLK3*, and *KLK4* were found in the prostate, whereas *KLK5* was highly expressed in normal breast tissue and skin (data not shown). Also, for those KLKs that show differential expression in ovarian cancer (see below), levels of expression were about the same between normal and cancer tissues for other malignancies, further verifying the accuracy of our results.

Table 1 *In silico analysis of KLK gene expression in normal and ovarian cancer tissues using the SAGE databases*

KLK	Library Type	Positivity[a]	Average density[b]
KLK5	Normal ovary	0/2	0
	Ovarian cancer	5/10	177
KLK6	Normal ovary	0/2	0
	Ovarian cancer	5/10	262
KLK7	Normal ovary	0/2	0
	Ovarian cancer	4/10	103
KLK8	Normal ovary	0/2	0
	Ovarian cancer	6/10	192
KLK10	Normal ovary	1/2	20
	Ovarian cancer	4/10	179
KLK11	Normal ovary	1/2	20
	Ovarian cancer	4/10	408
KLK14	Normal ovary	0/2	0
	Ovarian cancer	6/10	192

[a] Number of positive libraries out of the total examined.
[b] Expression densities corrected as number of tpm.

Table 2 *In silico analysis of KLK gene expression in libraries from normal, premalignant, and ovarian cancer tissues as determined by EST database*

KLK	Library Type	Positivity	No. of clones
KLK5	Normal ovary	0/9	0
	Premalignant	0/3	0
	Ovarian cancer	5/17	16
KLK6	Normal ovary	0/9	0
	Premalignant	0/3	0
	Ovarian cancer	1/17	21
KLK7	Normal ovary	1/9	1
	Premalignant	0/3	0
	Ovarian cancer	4/17	5
KLK8	Normal ovary	0/9	0
	Premalignant	0/3	0
	Ovarian cancer	4/17	5
KLK10	Normal ovary	0/9	0
	Premalignant	0/3	0
	Ovarian cancer	3/17	20
KLK11	Normal ovary	0/9	0
	Premalignant	0/3	0
	Ovarian cancer	3/17	8
KLK14	Normal ovary	0/9	0
	Premalignant	0/3	0
	Ovarian cancer	1/17	3

Of the 15 KLK genes analyzed in this study, 7 genes (*KLK5-8*, *KLK10*, *KLK11*, and *KLK14*) were found to be up-regulated in ovarian cancer tissues and cell lines, compared with normal ovary. Probing all ovarian libraries from different sources (cancerous and normal from tissues and cell lines) with *KLK5*-specific sequences revealed that *KLK5* is not detectable in normal ovary as determined by SAGE analysis (Table 1) and EST library screening (Table 2). These data are consistent with previous experimental reports showing no expression of *KLK5* in normal ovary (18). However, VNB analysis showed that *KLK5* is expressed in 5 of 10 ovarian cancer libraries with an average blot density of 177 tpm (Table 1). These data were further verified by screening the EST databases using the BLAST search engine; *KLK5* was expressed in 5 of 17 ovarian cancer EST libraries, whereas 16 *KLK5* mRNA clones were isolated, compared with no clones in libraries from normal or premalignant lesions (Table 2). X-profiler analysis showed a significant difference when comparing *KLK5* gene expression between normal and malignant pools of ovarian libraries (Table 3).

KLK6 was originally cloned as a gene that is highly expressed in ovarian cancer but not in normal ovaries (19). This finding was later confirmed by analyzing *KLK6* expression patterns in normal tissues by PCR (20) and by immunohistochemistry (21). *In silico* analysis in the current study showed that whereas *KLK6* was not detectable by VNB in normal ovary, it was detected in 5 of 10 ovarian cancer SAGE libraries with a relatively high blot density (262 tpm; Table 1). In addition, 21 ovarian cancer EST clones were detected, compared with no clones for normal or premalignant ovarian tissues and cell lines (Table 2). X-profiler analysis further indicated a significant difference of *KLK6* expression (expression factor, 0.953) when comparing normal and malignant pools of ovarian libraries (Table 3).

KLK8 was also found to be expressed at a fairly decent density (average, 192 tpm) in 6 of 10 ovarian cancer libraries but not in libraries from normal ovaries. Analysis of the EST database also showed no expression in any of the nine normal ovarian libraries screened, whereas five *KLK8* EST clones were detected in four ovarian cancer libraries (Table 2). This was further confirmed by X-profiler analysis (Table 3). A similar overexpression pattern was observed for *KLK10*, as shown in Tables 1–3.

Weak *KLK11* expression was detected by SAGE analysis in normal ovarian libraries. However, expression density was about 20-fold higher in ovarian cancer tissues and cell lines (20 *versus* 408 tpm for normal and cancer, respectively; Table 1). In addition, all ESTs

Table 3 *X-profiler analysis of KLK gene expression in normal versus cancerous ovarian tissues and cell lines*

KLK	Average gene expression		Factor of gene expression[c]
	Cancer[a]	Normal[b]	
KLK5	18	0	0.752
KLK6	36	0	0.953
KLK7	22	0	0.823
KLK8	23	0	0.838
KLK11	17	1	0.557
KLK14	23	0	0.838

[a] A pool of 10 ovarian cancer SAGE libraries.
[b] A pool of two normal ovarian SAGE libraries.
[c] A factor of 1 represents the highest statistical significance.

detected for this gene were from cancer libraries, not normal libraries (Table 2). This was also confirmed by X-profiler analysis (Table 3). Whereas no *KLK14* clones were detected in normal SAGE or EST libraries, mRNA tags were found in 6 out of 10 cancer libraries by VNB with an average density of 192 tpm, and 5 EST clones from an ovarian cancer library (Tables 1 and 2).

No other KLKs were found to be differentially expressed in ovarian cancer by these methods. For instance, *KLK3* was found to be expressed in 1 of 2 normal ovarian libraries compared with 3 out of 10 ovarian cancer libraries, with an average density of 41 tpm in normal compared with 45 tpm in cancer (data not shown).

Overexpression of KLK Proteins in Ovarian Cancer. To verify the *in silico* mRNA overexpression results and to examine whether these mRNA up-regulations reflect enzyme changes at the protein level, we compared protein expression levels of six up-regulated KLKs in normal, benign, and cancerous ovarian tissues (*KLK14* could not be examined due to lack of an ELISA assay for this KLK). As shown in Fig. 1 and Table 4, there is a stepwise increase in the expression levels of these KLKs among normal, benign, and malignant tissues. These differences were statistically significant by the Kruskal-Wallis test for hK11 ($P < 0.001$), hK5 ($P = 0.048$), hK6 ($P = 0.044$), and hK10 ($P = 0.027$) but not for hK7 and hK8 ($P > 0.05$; Fig. 1). When comparing normal and cancer tissues, the median levels of expression increased between 2-fold (hK5) to ~80-fold (hK11; Table 4).

By Spearman correlation, moderate to strong correlations were observed between the expression levels of five of these KLKs (hK5,

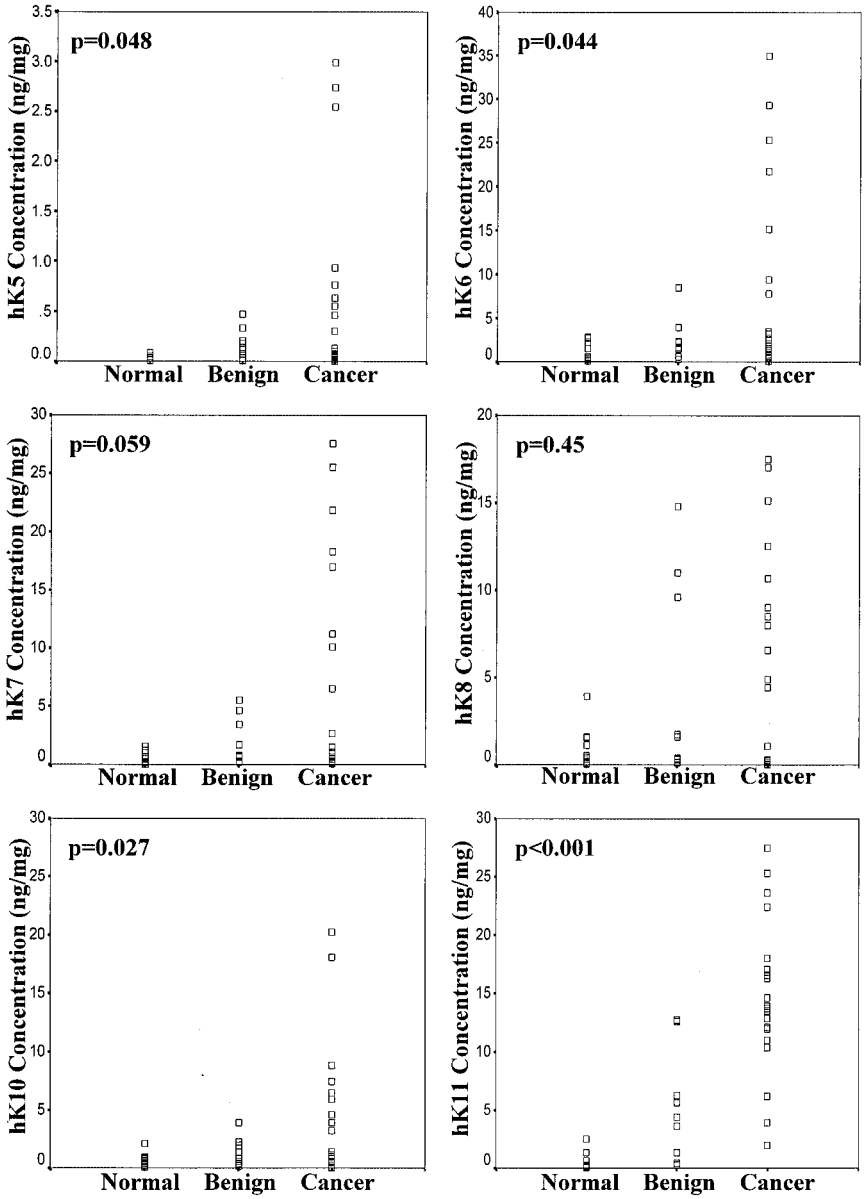

Fig. 1. KLK protein expression in normal ($N = 10$), benign ($N = 10$), and cancerous ($N = 20$) ovarian tissues. KLK levels are expressed in ng/mg total protein. *P*s were calculated by Kruskal-Wallis test. For discussion, see Results.

hK6, hK7, hK8, and hK10), and the results were highly statistically significant ($r_s = 0.45–0.74$ and $P < 0.001$; Table 5). hK11 appears to follow a different expression pattern and does not correlate well with any of the other proteins (Table 5).

DISCUSSION

Our results show that 7 of the 15 human KLKs are up-regulated in ovarian cancer. The differential expression of certain KLKs in ovarian cancer has been documented previously (11, 22, 23). *KLK4* has prognostic value in ovarian cancer patients (13, 24), and *KLK5* overexpression is an indicator of poor prognosis (25). We have also shown recently that hK6 (15), hK10 (26, 27), and hK11 (12) are useful serological diagnostic markers for ovarian cancer because their serum levels are higher than normal in >50% of ovarian cancer patients. *KLK6* was reported to be overexpressed in ovarian cancer, and it has been suggested to play a role in cancer invasion (19, 28). *KLK7* and *KLK8* were also reported to be overexpressed in ovarian cancer patients compared with normal subjects (14, 28, 29). Also, *KLK9* was shown to have prognostic value in ovarian cancer (30).

It has been reported previously that four of the most centromeric KLKs (*KLK2*, *KLK3*, *KLK4*, and *KLK15*) are differentially expressed in prostate cancer and are androgen-regulated (8, 31). Here we provide strong evidence that many members of the telomeric cluster of adjacent KLKs (*KLK5-14*) are overexpressed in ovarian cancer. This points out the possibility for the presence of a locus control region or another common regulatory mechanism for these KLKs. Because many of these genes are regulated by steroid hormones, especially estrogens (8), it will be interesting to investigate whether their up-regulation in cancer is a result of a hormonal derangement.

We used two independent databases (EST and SAGE) to generate our results. We calculated the proportion of positive libraries from each tissue type, in addition to the density of expression. We did not attempt to quantify KLK gene expression from the EST databases because many EST libraries are normalized or subtracted, which could bias the results. It is further emphasized that *in silico* analysis data should always be experimentally confirmed. Possible sources of bias include sequence errors, presence of mutations associated with certain malignancies, unequal presentation of cDNAs in different libraries, and expression of splice variants in certain malignancies.

Lercher *et al.* (32), during global analysis of >11,000 genes in 14 different tissues, selected a cutoff value for gene expression as being low (≤37 tpm) and intermediate or high (≥134 tpm). If this cutoff is applied to our data, we could conclude that whereas KLK gene expression is absent or low in normal ovarian tissues, medium to high levels of expression of 6 of the 15 KLKs are seen in ovarian cancer.

Table 4 *Descriptive statistics of protein levels of six KLKs in ovarian tissue extracts*
All values are in ng kallikrein/mg total protein.

	hK5	hK6	hK7	hK8	hK10	hK11
Normal tissues ($N = 10$)						
Mean	0.041	1.43	0.50	0.96	0.66	0.51
SE	0.006	0.33	0.16	0.37	0.18	0.25
Median	0.05	1.45	0.34	0.47	0.59	0.17
Minimum	0.01	0.05	0.00	0.03	0.08	0.03
Maximum	0.08	2.83	1.56	3.91	2.11	2.50
Benign tissues ($N = 10$)						
Mean	0.16	2.37	1.94	3.99	1.27	4.75
SE	0.04	0.74	0.60	1.76	0.37	1.49
Median	0.12	1.60	1.20	0.99	1.03	4.01
Minimum	0.01	0.29	0.22	0.09	0.12	0.25
Maximum	0.47	8.43	5.48	14.80	3.90	12.76
Cancer tissues ($N = 20$)						
Mean	0.62	9.0	7.27	5.80	4.88	14.65
SE	0.21	2.5	2.13	1.38	1.27	1.49
Median	0.11	3.15	1.26	4.63	3.53	13.81
Minimum	0.00	0.05	0.09	0.00	0.03	1.92
Maximum	2.99	35.03	27.56	17.49	20.23	27.52

Table 5 *Correlations between protein levels of six KLKs in ovarian tissue extracts*[a]

		hK6	hK7	hK8	hK10	hK11
hK5	r_s	0.53	0.74	0.55	0.61	0.11
	P	<0.001	<0.001	<0.001	<0.001	0.48
hK6	r_s		0.64	0.52	0.45	0.34
	P		<0.001	0.001	0.003	0.034
hK7	r_s			0.66	0.59	0.23
	P			<0.001	<0.001	0.14
hK8	r_s				0.390	0.17
	P				0.013	0.29
hK10	r_s					0.22
	P					0.15

[a] r_s, Spearman correlation.

Dong *et al.* (13) and Obiezu *et al.* (24) previously reported higher levels of expression of *KLK4* in ovarian cancer compared with normal ovarian tissues. In this study, no detectable expression of *KLK4* was found in any normal or cancerous tissue, as determined by SAGE or EST analysis. This might reflect the fact that *KLK4* is expressed in the ovaries at very low amounts, so that the mRNA is not detectable, except by using a highly sensitive technique such as reverse transcription-PCR.

Our current results, indicating KLK overexpression at the protein level in ovarian cancer, are in accord with previous observations of serum levels of a few of these KLKs in ovarian cancer. We have previously shown that serum levels of hK6 (15), hK10 (27), and hK11 (12) are elevated in ovarian cancer patients. Similar data have been generated for hK5, hK7, and hK8.[6]

Several hypotheses can be proposed regarding the possible involvement of KLKs in ovarian cancer. Being serine proteases, they could be implicated in tumor progression through extracellular matrix degradation. Previous studies have shown that serine protease overexpression is associated with unfavorable clinical prognosis of various forms of cancer (33). Ovarian cancer is a "hormonal" malignancy. KLKs, being under steroid hormone regulation (8), may represent downstream targets through which hormones affect the initiation or progression of ovarian cancer. The statistically significant positive correlations between expression levels five of six of these KLKs (Table 5) and the parallel overexpression, as well as data presented elsewhere (22), are supportive of an emerging idea that a number of these enzymes are part of a cascade enzymatic pathway. Direct evidence is currently lacking.

In conclusion, here we provide strong evidence suggesting that seven KLKs are up-regulated in ovarian cancer. These data were confirmed by examining several databases, in addition to protein analysis of KLKs in tissue cytosols. Further experimentation is necessary to establish the usefulness of these KLKs for diagnosis, prognosis, and treatment of ovarian cancer.

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