Differential Splicing of *KLK5* and *KLK7* in Epithelial Ovarian Cancer Produces Novel Variants with Potential as Cancer Biomarkers[1]

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ABSTRACT

Purpose: **The wild-type or variant mRNAs of several kallikrein (*KLK*) genes, such as *KLK4*, are highly expressed in ovarian carcinomas and may have potential as tumor markers. Two of these *KLK* genes (*KLK5* and *KLK7*) and their proteins (hK5 and hK7) were first identified in the skin epidermis, where hK5 may be the physiological activator of hK7. The purpose of this study was to reexamine the expression of *KLK5*/hK5 and *KLK7*/hK7 and their association and to determine whether cancer-related variant transcripts were expressed.**

Experimental Design: **The expression of *KLK5*/hK5 and *KLK7*/hK7 was analyzed in the same cohort ($n = 37$) of benign ($n = 4$) and malignant ovarian tissue ($n = 23$) samples and primary cultured cells ($n = 21$) and in 8 ovarian cancer cell lines using semiquantitative RT-PCR; Southern, Northern, and Western blot analyses; and immunohistochemistry techniques.**

Results: **We showed the concordant higher expression of both *KLK5*/hK5 and *KLK7*/hK7 in ovarian carcinomas, especially late-stage serous carcinomas, compared with normal ovaries and benign adenomas. We also found that one novel *KLK5* transcript with a short 5′-untranslated region and a novel *KLK7* transcript with a long 3′-untranslated region were highly expressed in the ovarian cancer cell lines OVCAR-3 and PEO1, respectively, but were expressed at very low levels in normal ovarian epithelial cells. Both Western blot and immunohistochemistry analyses showed that these two enzymes are secreted from ovarian carcinoma cells.**

Conclusions: **Our study demonstrated that hK5 and hK7, or more specifically, the short *KLK5* and long *KLK7* transcripts, may be useful as tumor markers for epithelial-derived serous carcinomas. However, additional clinical studies assessing serum levels of these putative biomarkers are required to confirm their usefulness in the diagnosis and/or monitoring of these tumors.**

INTRODUCTION

Ovarian carcinoma is the second most common gynecological malignancy and the leading cause of death from gynecological malignancy (1). The overall 5-year survival rate of ovarian cancer patients is less than 50% because most of these patients are diagnosed at an advanced stage of the disease, in which the primary tumor has progressed to a highly invasive and metastatic state (2). Thus, development of a reliable early diagnostic system is very important in the management and treatment of ovarian carcinomas.

The KLKs[3] are a subgroup of serine proteases that have recently been expanded to 15 members, and the *KLK* genes localized to chromosome 19q13.4 (3, 4). Many of these genes are highly expressed in ovarian cancer in comparison with normal ovary. We have previously shown high expression of *KLK4* (alternatively named prostase, *KLK-L1*, EMSP-1, and ARM1) and its mRNA variants, as well as the hK4 protein, in ovarian epithelial carcinoma (5). Others have shown that higher *KLK4* mRNA levels in ovarian cancer tissues are related to poor prognosis (6). Aberrant expression of *KLK6*/hK6 [alternatively named protease M, zyme, and neurosin (7–9)], *KLK8*/hK8 [neuropsin, ovasin, tumor-associated differentially expressed gene-14, and TADG-14 (10, 11)], *KLK9* (12), *KLK10* [normal epithelial-specific 1 gene and NES1 (13)], and *KLK11* [trypsin-like serine protease, TLSP, and hippostasin (14)] was also found in ovarian carcinomas. *KLK5* [alternatively named stratum corneum tryptic enzyme and SCTE (15)] and *KLK7* [stratum corneum chymotryptic enzyme and SCCE (16, 17)] were originally identified from a keratinocyte library, and their enzymes were purified from stratum corneum of human skin. *KLK7* catalyzes the degradation of intercellular cohesive structures in the outer-

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[3] The abbreviation used are: *KLK*, *kallikrein*; NOE, normal ovarian epithelial; BNG, benign serous adenoma; SER, serous; MUC, mucinous; END, endometrioid; CCC, clear cell carcinoma; GCT, granulosa cell tumor; nt, nucleotide; aa, amino acid(s); DIG, digoxigenin; RT-PCR, reverse transcription-PCR; UTR, untranslated region; AU, absorbance unit(s); EST, expression sequence tag; RACE, rapid amplification of cDNA ends; PI, protease inhibitor; ALP, antileukoprotease.

most layer of the skin and contributes to the cell shedding process at the skin surface (17). In addition, these two enzymes showed coexpression in skin tissue, and hK5 has now been shown to activate hK7 *in vitro*, suggesting that hK5 may be the physiological activator of hK7 (18).[4] Recently three different groups independently reported that *KLK5* (19, 20) and *KLK7*/hK7 (21) were highly expressed in ovarian cancers, respectively. However, expression of the hK5 protein in ovarian tumors has not yet been documented, and the relationship between the expression patterns of *KLK5* and *KLK7* in this tumor is not known. In addition, two *KLK7* mRNA transcripts (2 and 1.2 kb) were observed but not characterized further.

Thus, the aims of this study were to reexamine the expression of *KLK5* and *KLK7* in the same cohort of ovarian tumors compared with normal ovary. Using semiquantitative RT-PCR, immunohistochemistry, and Southern, Northern, and Western blot analyses, we demonstrated the concordant higher expression of both *KLK5* and *KLK7* and their respective proteins (hK5 and hK7) in ovarian carcinomas compared with normal ovaries and benign adenomas. In addition, we have identified novel *KLK5* and *KLK7* variant mRNA transcripts from ovarian cancer cell lines and NOE cells.

MATERIALS AND METHODS

Tumor Samples and Cell Culture. Twenty-one primary cultured ovarian epithelial cell samples [3 NOE samples, 2 SER adenomas, 8 SER carcinomas, 5 END carcinomas, and 3 CCCs], 16 ovarian tissue samples (3 normal ovaries, 3 SER carcinomas, 4 MUC tumors, and 6 GCT tumors), and 8 SER ovarian cancer cell lines were used for this study, as described previously (5). Ethics approval was obtained from the respective institutional ethics committees, and informed consent was obtained from all patients. The primary cultured cancer cells were grown as described previously (22). The ovarian cancer cell lines were grown in RPMI 1640 (Life Technologies, Inc., Gaithersburg, MD) supplemented with 10% FCS. The conditioned media, which was supplemented with 0.05% bovine albumin instead of FCS, was collected after 48 h of cell culture.

RT-PCR, Southern Blot, and DNA Sequencing Analyses. Total RNA was isolated from tumor cells or tissues using TRIzol reagent (Life Technologies, Inc.), and 2 μg of total RNA were reverse transcribed with random primers (100 ng) using Superscript II. PCR was performed with 50 ng of primers [K5Ex3S and K5Ex6AS (Fig. 1*A*) or K7Ex3S and K7Ex6AS (Fig. 1*B*)] and 1 μl of cDNA from two different primary ovarian cell lines (NOE and SER cell lines) for 25, 30, 35, and 40 cycles to determine that amplification was in the linear range (Fig. 2, *A* and *B*) and the appropriate cycle number for semiquantitative PCR. The final optimum cycling conditions chosen for further semiquantitative analysis of the samples were 94°C for 5 min followed by 35 cycles of 94°C, 64°C (*KLK5*) or 60°C (*KLK7*), and 72°C for 1 min each, and a final extension at 72°C for 7 min. PCR for β2-microglobulin, which was used as an internal control, was performed with primers 5′-TGAATTGCTATGTGTCTGGGT-3′ and 5′-CCTCCATGATGCTGCTTACAT-3′ for 35 cycles with similar PCR conditions, except for the annealing temperature (56°C). The PCR products were electrophoresed on 1.5% agarose gels.

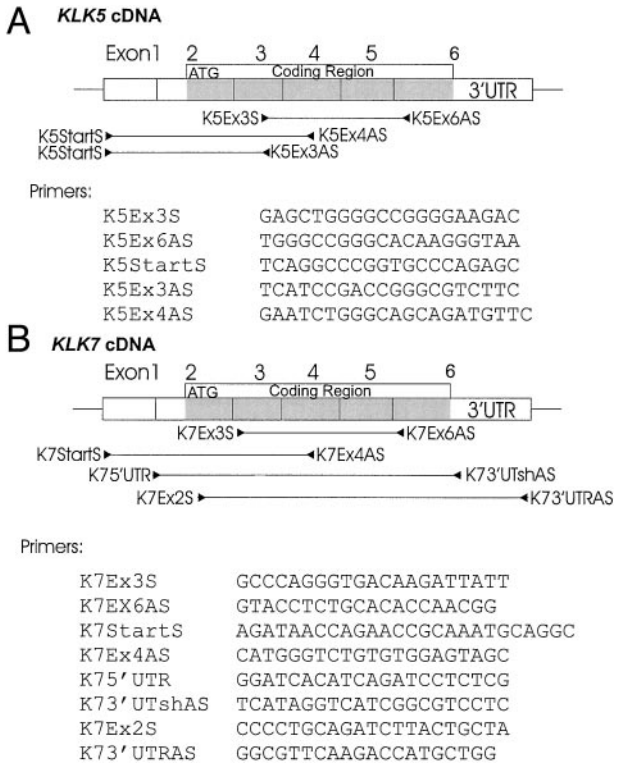

Fig. 1 Schematic diagrams of human *KLK5* (*A*) and *KLK7* (*B*) cDNA and the positions of primers used to detect *KLK5* and *KLK7* expression, respectively. The *numbered exons* indicate primarily coding exons compared with those exons with 5′-UTR and 3′-UTR sequences only. The sequences of the primers are denoted 5′ to 3′.

To examine expression of *KLK5* and *KLK7* in a larger range of samples, PCR was performed with the specific primers and optimum conditions as described above. The resulting amplicons were analyzed by Southern blot hybridization using DIG 3′-end-labeled internal *KLK5* (5′-AGTGCACTTGGGGGCTCTTGGTT-3′) or *KLK7* (5′-GCCGAGGTGCACGGTGTACTC-3′) oligonucleotide probes in Easyhyb solution (Roche) overnight at 37°C. The blots were washed and then incubated with anti-DIG antibody, and signals were detected on X-ray film after alkaline phosphatase (Roche) treatment with CDP-star as the substrate. The intensity of the bands on the Southern blots was determined by densitometry (GS-690 Imaging; Bio-Rad) using the Bio-Rad Multi-Analyst program (in AU). Representative PCR products (NOE 5, SER Ca 11, and the ovarian cancer cell line OVCAR-3 from Table 1) were also sequenced, and the DNA sequences were analyzed using the tBLASTN algorithm.

Northern Blot Analysis. Ten μg of total RNA were denatured, electrophoresed, and transferred to a Hybond-N membrane. Prehybridization was performed in UltraHyb (Clontech). The constructs for the *KLK5* and *KLK7* cRNA probes

[4] M. Brattsand and T. Egelrud. Activation of stratum corneum chymotryptic enzyme (SCCE) by stratum corneum tryptic enzyme (SCTE), manuscript in preparation.

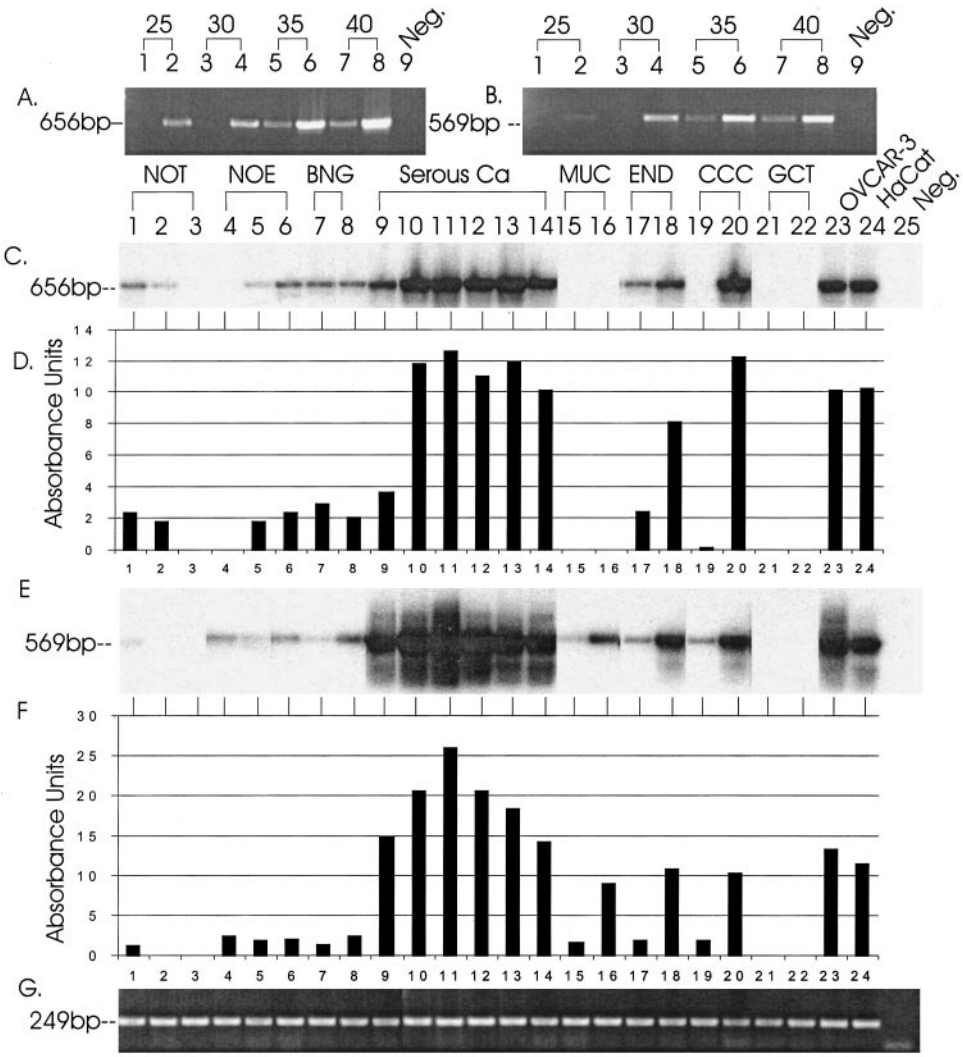

Fig. 2 *KLK5* and *KLK7* expression in normal ovaries and ovarian tumors. *A* and *B*, analysis of amplification of *KLK5* (*A*) and *KLK7* (*B*) in ovarian epithelial cells for different PCR cycles. *Lanes 1* and *2*, 25 cycles; *Lanes 3* and *4*, 30 cycles; *Lanes 5* and *6*, 35 cycles; *Lanes 7* and *8*, 40 cycles; *Lanes 1, 3, 5,* and *7*, NOE cells; *Lanes 2, 4, 6,* and *8*, SER carcinoma cells; *Lane 9*, negative control (no cDNA). *C* and *E*, Southern blot analysis of the *KLK5* and *KLK7* RT-PCR products, respectively, with DIG-labeled internal *KLK5* and *KLK7* probes. *D* and *F*, densitometric analysis of the Southern blots *C* and *E*, respectively. *G*, ethidium bromide-stained agarose gel of the RT-PCR for β2-microglobulin as an internal control. *Lanes 1–3*, normal ovarian tissues; *Lanes 4–6*, NOE cells; *Lanes 7* and *8*, primary cultured cells from SER adenomas of ovary (BNG); *Lanes 9* and *10*, primary cultured cells from stage II SER carcinomas; *Lanes 11–14*, primary cultured cells from stage III and IV SER ovarian carcinomas; *Lanes 15* and *16*, MUC carcinoma tissues; *Lanes 17* and *18*, primary cultured cells from END carcinomas; *Lanes 19* and *20*, primary cultured cells from CCCs; *Lanes 21* and *22*, GCT tissues; *Lanes 23–25*, SER ovarian carcinoma cell line OVCAR-3, keratinocyte line HaCat, and negative control respectively. From *C*−*G*, *Lanes 1–14* are the same samples as 1–14 in Table 1; *Lanes 15* and *16* are samples 30 and 29, *Lanes 17* and *18* are samples 32 and 34, *Lanes 19* and *20* are samples 37 and 38, and *Lanes 21* and *22* are samples 40 and 41 in Table 1, respectively.

were derived from the full-length EST cDNA clone (W07551; *KLK5*) and an exon 3–6 PCR product (*KLK7*; 569 bp) cloned into pGEMT (Promega, Madison, WI). After linearizing with *Xho*I, ^{32}P-labeled *KLK5* and *KLK7* cRNA probes were made using the Strip-EZ kit (Ambion, Inc., Austin, TX), and hybridization was performed overnight at 68°C. The membrane was washed and then exposed to X-ray film for visualization. A control hybridization to determine RNA loading was performed with a ^{32}P-labeled 18S oligonucleotide probe (5′-CGGCATG-TATTAGCTCTAGAATTACCACAG-3′).

Identification of a Novel *KLK5* 5′-UTR Sequence and Its Splice Variants. To determine the sequence of the different *KLK5* mRNA transcripts between the OVCAR-3 and HaCat cell lines as observed on Northern blot analysis (Fig. 3*A*), the known sequence of *KLK5* (GenBank accession number NM_012427), originally derived from HaCat cells, was subjected to homology search using the tBLASTN algorithm on the National Center for Biotechnology Information web server against the human EST database. An EST from an ovarian adenocarcinoma clone (GenBank accession number BF033594)

Table 1 Patient characteristics and expression patterns of *KLK5* and *KLK7*

Number	Histology	Stage[a]/grade[b]	Intensity[c] and summary of *KLK5* expression	Intensity and summary of *KLK7* expression
1	NOT[d]		2.3	1.2
2	NOT		1.8	0
3	NOT		0	0
4	NOE[e]		0	2.4
5	NOE[e]		1.8	1.8
6	NOE[e]		2.3 (4/6)	2 (4/6)
7	SER adenoma[e]		2.9	1.3
8	SER adenoma[e]		2 (2/2)	2.4 (2/2)
9	SER Ca[e]	IIb/2	3.6	14.8
10	SER Ca[e]	IIc/3	11.8	20.6
11	SER Ca[e]	IIIc/1	12.6	25.9
12	SER Ca[e]	IV/2–3	11	20.6
13	SER Ca[e]	IIIc/3	11.9	18.4
14	SER Ca[e]	IIIc/3	10.1	14.2
15	SER Ca[e]	IIIb/3	6.6	18.4
16	SER Ca[e]	III/1	1	10
17	SER Ca	III/3	10	12
18	SER Ca	III/2–3	9	11
19	SER Ca	III/3	9 (11/11)	4 (11/11)
20	JAM (SER)	Xenograft/3	1	0
21	CI-80-13S (SER)	IV/3	12.1	5
22	SKOV-3 (SER)	III/1	0	0
23	OVCAR-3 (SER)	III/3	10.1	12.1
24	PEO1 (SER)	III/3	11	10.3
25	PEO4 (SER)	Recurrent	10	12
26	PEO14 (SER)	III/1	9.9	10
27	OAW42 (SER)	III/NA	12 (7/8)	0 (5/8)
28	MUC adenoma		0	5.1
29	MUC Ca	I/NA	0	8.9
30	MUC Ca	I/NA	0	2.5
31	MUC Ca	II/NA	0 (0/4)	1.6 (4/4)
32	END Ca[e]	IIb/2	2.4	1.8
33	END Ca[e]	III/2–3	12.1	10.8
34	END Ca[e]	Ic/LMP	8.1	24.5
35	END Ca[e]	Ia/NA	0	6.6
36	END Ca[e]	IIIb/3	1 (4/5)	7.6 (5/5)
37	CCC[e]	Ia/2	0	1.8
38	CCC[e]	IIc/NA	12.2	10.2
39	CCC[e]	IIIb/2	8 (2/3)	18.6 (3/3)
40	GCT	I/NA	0	0
41	GCT	I/NA	0	0
42	GCT	I/NA	0	0
43	GCT	Ia/NA	0	0
44	GCT	Unstaged/NA	0	0
45	GCT	Recurrent/NA	0	0

[a] Federation of International Gynecology and Obstetrics (FIGO) stage system.
[b] Grades: 1, well; 2, moderately; 3, poorly; and 4, undifferentiated (33).
[c] Intensity of *KLK5* or *KLK7* Southern blot bands was determined by densitometric analysis (AU).
[d] NOT, normal ovarian tissues; NOE, normal ovarian epithelial cells; NA, not available; Ca, carcinoma.
[e] Primary cultured ovarian epithelial cells from NOE, serous adenoma, serous, endometrioid, and clear cell carcinomas.

revealed a different 5′-UTR sequence; primers were designed accordingly, and RT-PCR was performed. Two μg of total RNA from OVCAR-3 and NOE cells were reverse-transcribed into first-strand cDNA using the *KLK5*-specific primer (K5Ex4AS, Fig. 1*A*), as described above. PCR was performed with 1 μl of cDNA, 50 ng of *KLK5*-specific primers (K5StartS and K5Ex4AS, Fig. 1*A*), followed by a seminested PCR (K5StartS and K5Ex3AS, Fig. 1*A*) with similar PCR conditions as described above.

Identification of *KLK7* mRNA Transcripts from Ovarian Cancer. To determine the precise sequences of the two different mRNA transcripts (1.2 and 2 kb, respectively) found on Northern blot analysis (Fig. 3*B*), the published *KLK7* sequence (GenBank accession number NM_005046), originally derived from human keratinocytes, was also subjected to a homology search against the EST database using the tBLASTN algorithm. An ovarian tumor EST clone (GenBank accession number AU134435) showed further upstream 5′-UTR sequence, whereas another one had further downstream 3′-UTR sequence (GenBank accession number AA425991). Accordingly, four sets of primers spanning these different regions (Fig. 1*B*) were designed, and RT-PCR was performed with these primers and cDNA from the ovarian cancer cell line PEO1, with NOE cells as the control. After *KLK7* gene-specific reverse transcription

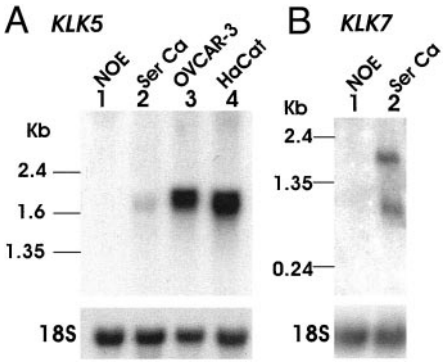

Fig. 3 Northern blot analysis of mRNA hybridized with the ^{32}P-labeled cRNA *KLK5* probe (*A*), *KLK7* probe (*B*), and 18S control from normal ovary and ovarian carcinomas. *Lane 1*, NOE cells; *Lane 2*, SER ovarian carcinoma cells (*Ser Ca*); *Lane 3*, ovarian carcinoma cell line OVCAR-3; *Lane 4*, HaCat cells.

with K7Ex4AS primer, PCR was performed with 1 μl of cDNA and 50 ng of primers (K7StartS and K7Ex4AS; Fig. 1*B*) with conditions similar to those described above, except that the annealing temperature was 58°C. To examine the 3′-UTR sequence of *KLK7*, 3′-RACE was first performed with the universal 3′-UTR primer (Promega), and PCR amplification was then performed with 1 μl of cDNA and 50 ng of *KLK7*-specific primers (K75′UTR and K73′UtshAS, Fig. 1*B*) as described above. To determine the long *KLK7* mRNA transcripts, PCR was performed with K7Ex2S and K73′UTRAS primers (Fig. 1*B*).

Western Blot Analysis. Cytoplasmic extracts (150 μg of protein) from primary cultured NOE cells, ovarian tumor cells (BNG, Ser Ca 1 and 2, OVCAR-3, and PEO1), HaCat cells, and conditioned media from ovarian cancer cell line PEO1 were electrophoresed on denaturing 10% SDS-polyacrylamide gels, and the protein was then transferred to a Protran membrane (Schleicher and Schuell, Dassel, Germany). After confirming equivalent protein loading by Ponceau S (Sigma) staining, the membrane was blocked with 5% skim milk in TBS/Tween 20 for 2 h at room temperature and then incubated with anti-hK5 pro-region antibody (Pe-C1; 0.4 μg/ml), anti-hK5 active region peptide antibody (0.2 μg/ml), and anti-hK7 antibody (anti-SCCE antibody; 0.6 μg/ml) overnight at 4°C, respectively. The production, specificity, and characterization of Pe-C1 and anti-SCCE antibodies have been described previously (17, 18, 23). Anti-hK5 active region antibody is a polyclonal rabbit antiserum raised against and affinity-purified toward a mixture of the peptides, RIRPTKDVRPINVSSHC (midregion) and CEDAY-PRQIDDTMF (COOH-terminal region). All three antibodies were affinity-purified, and preabsorption experiments and cross-reactivity experiments against hK5 (anti-hK7 antibodies) or hK7 (anti-hK5 antibodies) were performed to confirm their specificity (18). Although all these antibodies have not been tested for cross-reactivity against all 15 KLKs, which are not yet available commercially, it is unlikely that they will cross-react with any other KLKs because of the low homology (25–44%) between all of the family members (3) and the specificity of the peptides chosen. The blot was washed and then incubated with a horseradish peroxidase goat antirabbit IgG conjugate (Dako, Glostrup, Denmark; 1:2000 dilution) at room temperature. The signals were visualized on X-ray film by enhanced chemiluminescence (SuperSignal West Femto System; Pierce, Rockford, IL). After the original membrane was stripped, Western blot analysis was performed with β-tubulin antibody (1:2000 dilution; Pierce) as an internal control for equal loading.

Immunohistochemistry. Formalin-fixed paraffin blocks from four SER ovarian tumors and two normal ovaries were sectioned (4 μm), deparaffinized, and rehydrated, and then antigen retrieval was performed by microwave heat treatment in 5% urea in 0.1 M Tris buffer (pH 9.5). After H_2O_2 treatment to block endogenous peroxidase, the sections were incubated overnight with anti-hK5 pro-region antibody (3 μg/ml), anti-hK5 active region peptide antibody (1 μg/ml), or anti-hK7 antibody (3 μg/ml) at 4°C, respectively. Then the EnVision$^+$ peroxidase (antirabbit) polymer (Dako) detection system was used with 3,3′-diaminobenzidine (Sigma) as the chromogen. The sections were counterstained with Mayer's hematoxylin. Normal goat serum (10%) replaced the primary antibodies as a negative control.

RESULTS

Expression of *KLK5* and *KLK7* in Normal Ovaries and Ovarian Tumors. Twenty-one primary cultured ovarian epithelial cell samples (3 NOE samples, 2 SER adenomas, 8 SER carcinomas, 5 END carcinomas, and 3 CCCs), 16 ovarian tissue samples (3 normal ovaries, 3 SER carcinomas, 4 MUC tumors, and 6 GCT tumors), and 8 SER ovarian cancer cell lines were examined for expression of *KLK5* and *KLK7* by semiquantitative RT-PCR over 35 cycles, followed by Southern blot and densitometry analyses. The results for *KLK5* and *KLK7* expression in a representative group of samples are shown in Fig. 2, *C–F*, respectively. β2-Microglobulin, which was used as an internal control (Fig. 2*G*), showed a consistent pattern of expression in all samples, indicating the integrity of the RNA. The *KLK5* and *KLK7* PCR products from NOE cells, the primary cultured cells from a SER ovarian carcinoma, and the ovarian cancer cell line OVCAR-3 (*Lanes 6*, *10*, and *23* in Fig. 2, *C* and *E*) were sequenced, and the *KLK5* and *KLK7* sequences were identical to those reported previously (15, 17, 24, 25). The *KLK5* and *KLK7* expression results and clinical information on all tumor tissues and cell lines are summarized in Table 1.

KLK5 expression was detected in the normal ovaries (4 of 6) as well as SER epithelial ovarian tumors (benign, 2 of 2; malignant, 11 of 11; cell lines, 7 of 8), END carcinomas (4 of 5), and CCCs (2 of 3). On densitometric analysis, a higher level of *KLK5* expression (>5 AU) was observed in 9 of 11 SER carcinomas and 6 of 8 SER-derived cancer cell lines compared with the normal ovaries, benign adenomas, END carcinomas, and CCCs that are positive. However, no *KLK5* was detected in MUC tumors (0 of 4) or GCTs (0 of 6; Fig. 2, *C* and *D*; Table 1). The expression pattern for *KLK7* was similar to *KLK5* [normal ovaries, 4 of 6; BNGs, 2 of 2; SER carcinomas, 11 of 11; SER cancer cell lines, 5 of 8; MUC tumors, 4 of 4; END carcinomas, 5 of 5; and CCCs, 3 of 3 (Fig. 2, *E* and *F*; Table 1)]. Ten of 11 SER carcinomas and 5 of 8 SER cancer cell lines showed higher *KLK7* level (>5 AU) than normal ovaries, be-

nign adenomas, MUC carcinomas, END carcinomas, and CCCs, whereas no *KLK7* mRNA was found in GCTs (Fig. 2*E*; Table 1). In addition, eight SER carcinomas with high *KLK5* levels also showed high *KLK7* expression, and comparable levels of expression for both genes were observed in all samples. The present results suggest that both *KLK5* and *KLK7* are coordinately highly expressed by epithelial ovarian carcinomas, especially SER carcinomas.

Northern blot hybridization with the full-length ^{32}P-labeled *KLK5* cRNA probe showed a faint band in the SER ovarian carcinoma and an intense *KLK5* transcript of 1.6–1.7 kb in the ovarian cancer cell line OVCAR-3 and HaCat control, whereas no visible band was detected in normal ovary (Fig. 3*A*). In addition, OVCAR-3 showed a larger *KLK5* mRNA transcript than the human keratinocyte cell line (HaCat; Ref. 15). Northern blot hybridization with a ^{32}P-labeled *KLK7* probe revealed 1.2- and 2-kb transcripts, as reported previously in skin and ovarian cancer tissues, respectively (17, 21), in the SER ovarian carcinoma sample but not in the NOE cells. These results confirmed the findings of the RT-PCR and Southern blot analyses that both *KLK5* and *KLK7* are more highly expressed in SER carcinomas compared with normal tissues. Hybridization with ^{32}P-labeled 18S was used as an internal control and showed the relatively equivalent loading of RNA.

Identification of a Novel *KLK5* 5′-UTR and Its Splice Variant. Analysis of the EST database suggested that the differences in *KLK5* transcript length may be due to differences in the 5′-UTR region. To determine the 5′-UTR sequence of *KLK5* in the normal ovary and ovarian cancer cell line OVCAR-3, RT-PCR and seminested PCR were performed with *KLK5*-specific primers (K5StartS, K5Ex4AS, and K5Ex3AS, Fig. 1*A*). Two PCR products of 290 and 494 bp (Fig. 4*A*) were noted in OVCAR-3 cells, but only the 494-bp band was seen in NOE cells. Comparison of these two sequences with the published genomic sequence (GenBank accession number AF135028) revealed two novel 5′-UTR sequences derived from a new exon further upstream of the known exon 1 (exon 2 in Fig. 4*B*). One of these novel 5′-UTR sequences (314 bp; GenBank accession number AF435980) was found in both NOE and OVCAR-3, whereas the shorter new exon sequence (112 bp; GenBank accession number AF435981) was found in OVCAR-3 only (Fig. 4*B*). The known 5′-UTR exon 1-derived sequence from HaCat cells (15) is identical to the last 31-bp at the 3′-end of the *KLK5* long exon 1 sequence. The two novel *KLK5* mRNA variants have an identical coding region, as published previously (15, 24), and they would be translated into an identical protein, although their 5′-UTR nt sequences are different. Possibly this would also account for the small size difference between the OVCAR-3 and HaCat *KLK5* transcripts seen on the Northern blot (Fig. 3*A*).

Identification of *KLK7* Splicing Variant mRNA Transcripts from Ovarian Cancer. Analysis of the GenBank and EST database suggested that the differences in the length of the two *KLK7* transcripts may be due to differences in the 5′-UTR and/or 3′-UTR regions. Using RT-PCR, 3′-RACE, and sequencing analyses, two *KLK7* mRNA transcripts were identified from NOE cells and PEO1. The long *KLK7* transcript (1756 bp; GenBank accession number AF411214; Fig. 4*C*) has 6 exons and contains an additional exon (188 bp; exon 1 in Fig. 4*C*) further upstream compared with other published sequences (GenBank accession numbers AF166330 and NM_005046), but the coding exons and 3′-UTR (748 bp) are consistent with these previously published sequences. The ATG codon is located at nt 247, and a protein, hK7, of 253 aa is predicted (Fig. 4*E*), which is identical to the enzyme purified from human skin (17). In comparison to the long form, the short *KLK7* mRNA transcript (1054 bp; GenBank accession number AF411215; Fig. 4*D*) contains 5 exons with exon 2 deleted and contains only 177 bp in the 3′-UTR region. The short *KLK7* transcript generates a protein of 181 aa because a different protein translation start site (ATG at nt 332) is used and encodes a shortened protein sequence without a pre- and pro-region (Fig. 4*E*). Both the long and short *KLK7* transcripts may account for the 2 and 1.2 kb on the Northern blots when allowing 100–200 bp for a poly(A) tail. The truncated hK7 does not contain the histidine residue of the catalytic triad (Fig. 4*E*). Both short and long *KLK7* transcripts were expressed by NOE and PEO1 cells as determined by 3′-RACE and RT-PCR, respectively, although PEO1 cells predominantly expressed the longer transcript (Fig. 4, *F–H*).

Western Blot Analysis of hK5 and hK7. Eight different ovarian cancer cell lines, primary cultured benign and malignant ovarian epithelial cells were analyzed by Western blot with anti-hK5 and anti-hK7 antibodies, respectively (Fig. 5). Fig. 5, *A* and *B*, shows a representative Western blot with anti-hK5 antibodies of the primary cultured NOE cells, BNG cells, SER ovarian carcinoma cells, and ovarian cancer cell lines. The anti-hK5 pro-region antibody detected a M_r 60,000 protein in these cells (Fig. 5*A*, *Lanes 1–4*), and a faint band (M_r ≈40,000) was seen in PEO1 cells. The anti-hK5 active region peptide antibody detected M_r 100,000, M_r 60,000, and M_r 36,000 bands from HaCat cells; the M_r 100,000 and M_r 60,000 bands were also seen from the extracts of ovarian cancer cells. However, a M_r 36,000 band was detected in the conditioned media of PEO1 cells by the anti-hK5 active region antibody (Fig. 5*A*) but was not detected by the pro-region antibody (data not shown). The anti-hK7 antibody recognized proteins of M_r 60,000 and M_r 30,000 in the HaCat control and ovarian cancer cells (Fig. 5*B*) but only recognized a M_r 30,000 band from the conditioned media of PEO1. Primary cultured ovarian cancer cells and cell lines showed higher hK5 and hK7 expression than NOE and adenoma cells (Fig. 5, *A* and *B*). In addition, hK5 and hK7 were detected from the conditioned media of ovarian cancer cell line PEO1, suggesting that these enzymes can be secreted by cancer cells. Western blot analysis of β-tubulin expression showed the relatively equal loading of protein.

Expression of hK5 and hK7 in Ovarian Cancer Tissues. On immunohistochemistry, using either anti-hK5 pro-region or active form region antibody, only weak hK5 staining was observed in the surface epithelium of benign SER cystadenoma (Fig. 6, *A* and *E*), whereas hK5 was found predominantly at the apical membrane and cytoplasm of cancer cells (Fig. 6, *B*, *C*, *F*, and *G*). In addition, the staining intensity detected by the anti-active hK5 peptide antibody was stronger than that of the anti-pro-form antibody. It also appears that extracellular secretion of hK5 was detected as immunoreactive material at the apical membrane of these glandular structures (Fig. 6*G*), which is consistent with our finding on Western blot analysis (Fig. 5*A*). Using the anti-hK7 antibody, only weakly focal hK7 staining

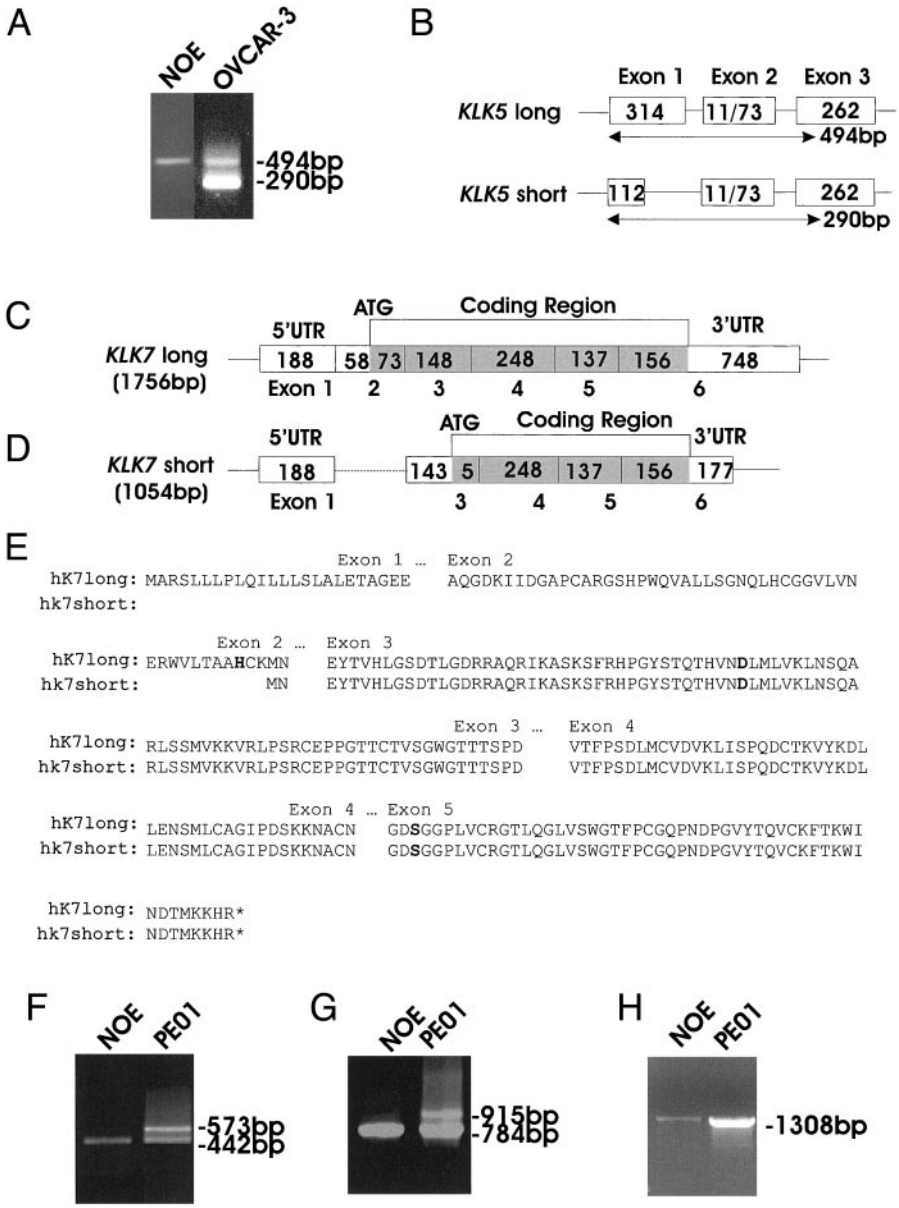

Fig. 4 Schematics and RT-PCR analysis of *KLK5* (*A* and *B*) and *KLK7* (*C–H*) transcripts in normal and malignant ovarian epithelial cells. *A*, expression of *KLK5* 5′-UTR region by RT-PCR in NOE and OVCAR-3 cells with primers K5StartS and K5Ex3AS indicating the 494- and 290-bp products. *B*, schematic diagrams showing the first three exons of *KLK5* and the different 5′-UTR regions in ovarian epithelial cells. *Boxed numbers* indicate size (in bp) of exons; *slash* indicates ATG coding start site in exon 2. The RT-PCR product sizes are indicated below. *C* and *D*, schematic representation of the long *KLK7* (*C*) and short *KLK7* (*D*) transcripts. *Boxed numbers* indicate size (in bp) of exons; *slash* indicates ATG coding start site in exon 2 or exon 3. The 5′-UTR, 3′-UTR, and coding regions are indicated. *E*, aa sequence alignment of long and short forms of hK7. The five exons of the coding region are marked, and the introns are indicated by a *dotted line*. The aa that constitute the catalytic triad, histidine (*H*), aspartic acid (*D*), and serine (*S*) are marked in *bold*. *Asterisk* indicates the end of the protein sequence. *F–H*, expression of both long and short *KLK7* transcripts by RT-PCR using different primers shown in Fig. 1*B* and visualized by ethidium bromide-stained agarose gel. *F*, K7Starts and K7Ex4AS primers showing long *KLK7* (573 bp) and short *KLK7* (exon 2 deleted; 442 bp) in NOE and PEO1 cells. *G*, K75′UTR and K73′UtshAS primers showing expression of long (915 bp) and short (784 bp) *KLK7* transcripts in NOE and PEO1 cells. *H*, expression of *KLK7* long transcript (1308 bp) in NOE and PEO1 cells by RT-PCR with K7Ex2S and K73′UTRAS primers.

was found in benign SER cystadenoma cells (Fig. 6*I*), whereas strong staining was observed in the apical membrane and cytoplasm of ovarian carcinoma cells (Fig. 6, *J* and *K*) and the cancer cells at the invasive front (Fig. 6*J*). Like hK5, extracellular secretion of hK7 from the cancer cells was also observed (Fig. 6*K*). No staining was observed in the negative control, where 10% normal goat serum replaced the primary antibodies (Fig. 6, *D, H,* and *L*).

DISCUSSION

Ovarian cancer is the leading cause of death in all gynecological malignancies. Most ovarian cancer patients are diagnosed at a late stage, and at this time cancer has already progressed and metastasized. The KLKs, like several other serine proteases, are increasingly associated with tumor progression (26). We demonstrated concordant high levels of *KLK5*/hK5 and *KLK7*/hK7 in ovarian carcinomas, especially in SER carcinomas, compared with normal ovaries and BNGs. We have identified novel 5′-UTR sequences of *KLK5* derived from a previously undescribed exon further upstream and further characterized the two *KLK7* mRNA transcripts in NOE and ovarian carcinoma cell lines. In addition, differential expression of these *KLK5* and *KLK7* variant transcripts was found between NOE and ovarian cancer cell lines. To our knowledge, this is also the first study to show higher hK5 expression in ovarian cancers than in NOE and benign SER cystadenoma and to provide evidence that hK5 and hK7 can be secreted by ovarian cancer cells.

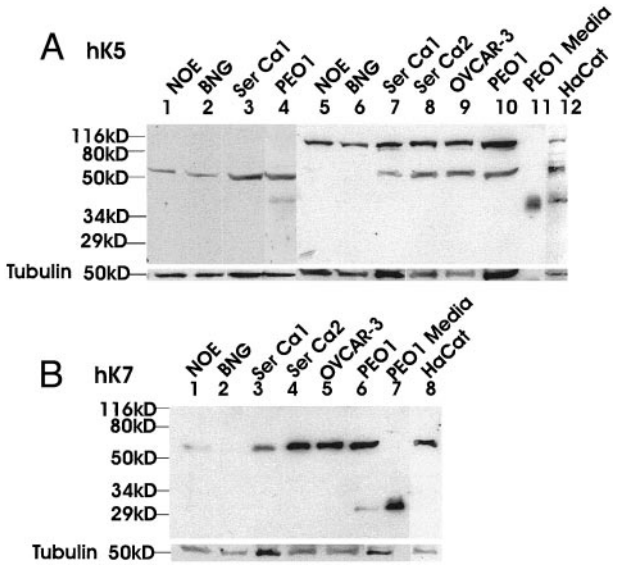

Fig. 5 Expression of hK5 and hK7 in NOE cells, ovarian cancer cell lines, and ovarian tumor cells by Western blot analysis. *A*, Western blot with anti-hK5 antibodies. *Lanes 1–4*, Western blot with anti-hK5 pro-form region antibody; *Lanes 5–12*, Western blot with anti-hK5 peptide antibody to the active form. *Lane 1*, NOE cells; *Lane 2*, BNG; *Lane 3*, SER ovarian carcinoma cells; *Lane 4*, ovarian cancer cell line PEO1; *Lane 5*, NOE cells; *Lane 6*, BNG; *Lanes 7* and *8*, SER ovarian carcinoma cells; *Lanes 9* and *10*, ovarian cancer cell lines OVCAR-3 and PEO1; *Lane 11*, PEO1 conditioned media; *Lane 12*, keratinocyte cell line HaCat. *B*, Western blot with anti-hK7 antibody. *Lane 1*, NOE cells; *Lane 2*, BNG; *Lanes 3* and *4*, SER ovarian carcinoma cells; *Lanes 5* and *6*, ovarian cancer cell lines OVCAR-3 and PEO1; *Lane 7*, PEO1 conditioned media; *Lane 8*, HaCat. Reincubation of the Western blots with a β-tubulin antibody shows the equivalent protein loading.

Consistent with previous studies by other groups (19–21), the current results showed higher mRNA levels of *KLK5* and *KLK7* in ovarian carcinomas, especially SER carcinoma, than in benign ovaries. Historically, KLKs are primarily expressed in or localized to (glandular) epithelium (27). Thus, the primary localization of *KLK5*/hK5 and *KLK7*/hK7 to epithelial cell-derived tumors is not unexpected. To date, there is not a specific biomarker for epithelial ovarian carcinomas, which account for approximately 90% of all ovarian cancers. We also found that no *KLK5* mRNA was detected in MUC tumors or GCTs, nor was *KLK7* mRNA detected in GCTs. The relative lack of expression in MUC and GCT tissues, which are likely to have a lower percentage of epithelial cells, also supports the notion that these and other *KLKs* are excellent biomarkers for epithelial ovarian carcinomas (5–14).

Taken together with the reports of high expression of *KLK4*/hK4 (5, 6), *KLK5* (19, 20), *KLK6*/hK6 (7, 8), *KLK7*/hK7 (21), *KLK8*/hK8 (10, 11), *KLK10*/hK10 (13), and *KLK11*/hK11 (14) in ovarian carcinomas, these data demonstrate that the *KLK* loci are increasingly more important in ovarian tumorigenesis, particularly in SER epithelial carcinomas. Indeed, studies from our group (5) and others (6–11, 13–14, 19–21) indicated that aberrant expression of *KLKs* is present in late-stage and high-grade ovarian cancers and predicted shorter survival time, although *KLK9* expression was found to be a favorable prognostic marker for this disease (12). In our study, the majority of epithelial carcinomas analyzed are from late-stage (III or IV) disease. Moreover, hK6 (9), hK10 (13), and hK11 (14) have been found to be higher in the sera of late-stage ovarian cancer patients, and their potential as tumor markers for the diagnosis of this cancer has been suggested. Our present results that hK5 and hK7 can be secreted by ovarian cancer cells suggests they have similar potential as serum markers for the diagnosis and monitoring of this disease.

Previous studies suggested that hK7 degrades the cohesive structures between individual corneocytes in the stratum corneum that is the genesis for cellular desquamation or shedding of skin cells (17, 28). The data from our immunohistochemical study (the observation that the invading cancer cells expressed high hK7) suggest that hK7 may have the potential to degrade the surrounding matrix as the tumor progresses. We also showed that those SER carcinoma cells with high *KLK5* levels had concordantly high *KLK7* levels and the identical localization of hK5 and hK7 in ovarian cancer tissues. In previous studies, we have also shown the coexpression of hK5 and hK7 in skin tissue and suggested that there may be a relationship between these two enzymes (15, 18). Indeed, we have now confirmed the interaction between recombinant hK5 and hK7 *in vitro* in an activation cascade.[4] Thus, it is reasonable to hypothesize a similar role of these two enzymes in ovarian cancer invasion.

This process of tumor invasion is a complex event involving the activation and inhibition of these serine proteases during tumor progression. The secreted form (Fig. 5) is at the theoretical molecular weight for these enzymes (M_r 36,000 for hK5 and M_r 30,000 for hK7). The high molecular weight protein bands of hK5 or hK7 in ovarian cancer samples may be representative of pro-forms (M_r 60,000), complexes of these enzymes with other proteins (M_r 60,000 and M_r 100,000), possibly their different respective inhibitors (18), or dimers of the active enzymes (M_r 60,000). Because these SDS-PAGE gels were run under reducing conditions, there are unlikely to be any dimers present. The observation that the antibody to the hK5 pro-form (with clear specificity for pro-hK5 and not active hK5; Ref. 18) only detects the M_r 60,000 band from ovarian samples and not the M_r 100,000 or M_r 36,000 bands that were detected by the anti-active-hK5 antibody suggests that this is either the pro-form only or a complex of the pro-form with another protein. Pro-forms of KLKs are not known to complex with other protein/inhibitors because KLK/inhibitor complexes are normally covalent complexes of the active enzyme and an inhibitor (27). The substantial apparent molecular weight difference of M_r 24,000 between the active (M_r 36,000) and pro-hK5 (M_r 60,000) forms is unlikely to be entirely due to the clipping of the pro-region only (66 aa), although this would appear to be the explanation at this time. Similarly, the M_r 60,000 hK7 band likely represents the pro-form or a complex with an unknown protein. Although intracellular PIs are largely unknown, the PIs PI6 (M_r 30,000) and PI3 (M_r 40,000) were found to bind to prostatic cytosolic active hK2 (27) and hK8 in mouse brain (29), respectively. These PIs or other unknown inhibitors (binding proteins) may be complexed with active hK5 or hK7, a point that remains to be clarified. It has been reported that ALP is the major inhibitor of hK7 and may also be involved in the process of desquamation (30). The expression of ALP and hK7 was found to be coordi-

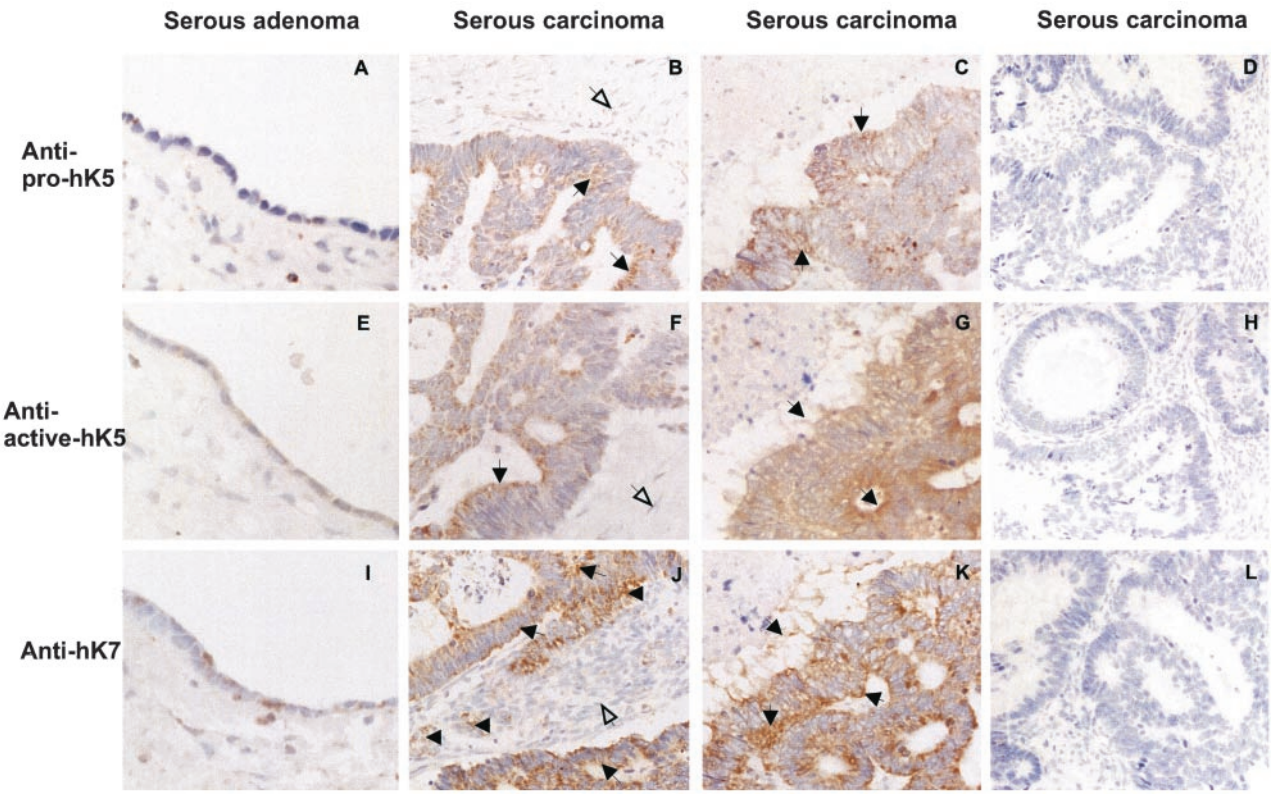

Fig. 6 Representative localization of hK5 and hK7 in ovarian tumor biopsies. *A–C*, hK5 expression as detected with the anti-hK5 pro-form antibody. *E–G*, hK5 expression as detected with anti-hK5 peptide antibody to active form. *I–K*, hK7 expression as detected with anti-recombinant hK7 antibody. Benign SER cystadenoma of ovary showing weak hK5 (*A* and *E*) and hK7 (*I*) expression in the surface epithelium. Moderately differentiated SER ovarian carcinomas showing cytoplasmic (*B, C, F,* and *G*) and apical membrane (*F* and *G*) expression of hK5 (*solid arrows*). hK7 expression in moderately differentiated SER ovarian carcinoma showing apical membrane and cytoplasmic expression (*solid arrow*), and the invading cancer cells showing cytoplasmic (*arrowhead*) expression (*J*), apical membrane (*open arrow*) and cytoplasmic (*solid arrow*) expression (*K*). No staining shown by stroma cells (*open arrows*). Negative control (*D, H,* and *L*) performed with 10% normal goat serum instead of primary antibodies on SER ovarian carcinoma sections. The *brown color* is the 3,3′-diaminobenzidine stain for the antibodies, and the Mayer hematoxylin counterstains the nucleus (*blue*). Magnification: *A, E,* and *I,* ×120; *B, C, F, G, J,* and *K,* ×60; *D* and *H,* ×80; and *L,* ×100.

nated in ovarian cancers (31), although whether the high molecular weight hK7 band in our study is hK7 complexed with ALP remains to be determined. In addition, the NOE and BNG cells showed weak hK5 immunoactivity as detected by the anti-pro-region antibody or only higher molecular weight bands (M_r 100,000) detected by the anti-active form antibody. These results suggest that all of the hK5 is bound in an inhibitor complex and that no active hK5 was present in these cells. The presence of such inhibitor(s) might modulate the shedding of tumor cells so that identification of these inhibitors and their modes of action may be useful in a new therapeutic approach.

In addition to the high expression of *KLK5*/hK5 and *KLK7*/hK7 in ovarian carcinomas, two novel *KLK5* splice variants with an additional upstream exon were found in the ovarian cancer cell line OVCAR-3, whereas only one transcript was found in NOE cells. We also identified two *KLK7* mRNA transcripts from NOE and PEO1 cells. These two *KLK7* transcripts have a similar additional upstream 188-bp exon, whereas the short form, which is predominantly expressed by NOE cells, has no exon 2 and has only 177 bp in the 3′-UTR. The *KLK5* mRNA variants with different 5′-UTR can be translated into the same hK5 protein. However, the two *KLK7* transcripts encode different proteins. Multiple mRNA variants with different 5′-UTR sequences in several genes, such as mouse estrogen receptor α gene (32), have been reported in different tissues, and it is suggested that different promoter utilization and alternative splicing generate these mRNA variants, often in a tissue-specific manner. Our present results showed different 5′-UTR sequences of *KLK5* and *KLK7* in ovarian tissues from HaCat cells and suggest that different promoters of these two *KLKs* are present in these tissues. In addition, our present results showed that there was selective use of the different *KLK5* 5′-UTR sequences in ovarian cancer cells compared with NOE cells. Thus, the use of these variant *KLK5* promoters may be important in tumorigenesis because their normal regulation may be altered in cancer cells. The function, if any, of the shorter exon 2 deleted *KLK7* variant, which would not encode a serine protease and which is the predominant form expressed by NOE cells, has yet to be determined. However, the long *KLK7* transcript would encode the full-length pre-pro- enzyme, enabling secreted active hK7 enzyme to be present in the cancer cells but not NOE cells. As reported previously, *KLK4* (5) and *KLK8* (11) variants are also

found in ovarian cancer. Thus, variant mRNA transcripts are a common feature of the human *KLK* family, particularly in tumorigenesis, and may represent a novel regulatory mechanism as well as potential biomarkers for cancer.

The differential expression of *KLK5* and *KLK7* variant transcripts between normal ovaries and ovarian cancers suggests the potential of their use diagnostically on RT-PCR analysis of tumor biopsies for the early detection of ovarian cancer. RT-PCR analysis on circulating tumor cells may also be useful for the prediction of metastatic disease and poorer prognosis of ovarian cancer patients. hK5 and hK7, or these variant proteins, if secreted, may be useful serum markers [perhaps along with other KLKs (9, 14)] for SER epithelial carcinomas for which a specific biomarker is currently not available.

In summary, the results from this study demonstrate the differential expression of *KLK5*/hK5 and *KLK7*/hK7 between normal ovaries and ovarian carcinomas, with particularly high expression in epithelial-derived SER carcinomas. The presence of different mRNA transcripts of *KLK5* and *KLK7* in NOE cells compared with ovarian cancer suggests that these variant *KLK* transcripts are important in ovarian tumorigenesis and their potential as biomarkers for cancer. The secretion of hK5 and hK7 by ovarian cancers may allow their use as potential serum markers for this tumor, perhaps in a multiplex KLK profile. The concordant high expression of both *KLK5* and *KLK7* in ovarian cancer would allow the interaction of these two enzymes in an activation cascade in this disease. Additional studies are required to determine the precise functions of hK5 and hK7 in ovarian tumorigenesis and their usefulness in the diagnosis and/or monitoring of this cancer.

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