Human Kallikrein 4 (*KLK4*) Is Highly Expressed in Serous Ovarian Carcinomas[1]

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ABSTRACT

Previous studies indicated that a new member of the human kallikrein (*KLK*) gene family, *KLK4*, was expressed in prostate, breast, and endometrial carcinoma cell lines and may have potential as a tumor marker. The aim of this study was to examine the expression of *KLK4* in the normal ovary and ovarian tumors of different histology, stage, and differentiation and to determine its association with ovarian tumor progression. Using reverse transcription-PCR, Southern blot, and densitometry analyses, we found the level of *KLK4* expression was higher in late stage serous (SER) epithelial-derived ovarian carcinomas than in normal ovaries, mucinous epithelial tumors, and granulosa cell tumors. *KLK4* was highly expressed in all of the SER ovarian carcinoma cell lines (eight of eight), SER epithelial carcinomas (11 of 11), and two adenomas, whereas it was expressed at a lower level (or not at all) in normal ovaries (four of six), mucinous epithelial tumors (three of four), endometrioid carcinomas (four of five), clear cell carcinomas (two of three), or granulosa cell tumors (three of six). Of particular interest, *KLK4* mRNA variants were detected in SER ovarian carcinoma cell lines and primary cultured ovarian tumor cells, but they were not present in normal ovaries. *In situ* hybridization analysis showed that *KLK4* mRNA transcripts are localized to adenocarcinoma cells of ovarian tumor tissues. Similarly, immunohistochemical staining of ovarian carcinoma sections showed immunoreactivity to *KLK4* protein product (hK4) antipeptide antibodies. In addition, intracellular hK4 levels, as detected on Western blot analysis, were induced by 100 nM estrogen treatment of the estrogen receptor positive ovarian carcinoma cell line OVCAR-3, >8–24 h. Our results show that the level of *KLK4* expression and expression of *KLK4* mRNA variants are associated with progression of ovarian cancer, particularly late stage SER adenocarcinomas. Moreover, hK4 may be a candidate marker for the diagnosis and/or monitoring of ovarian epithelial carcinomas.

INTRODUCTION

Ovarian carcinoma is the leading cause of death from gynecological malignancy. The overall 5-year survival rate of ovarian cancer patients is <50%, as the majority of these patients are diagnosed at an advanced stage (III or IV) of the disease, at which time the primary tumor has metastasized (1). Recent studies have revealed that proteolytic enzymes, such as Ser proteases, are very important in the processes of tumor invasion and metastases in ovarian cancer (2). The Ser proteases, protease M (3, 4), stratum corneum chymotryptic enzyme (5), and neuropsin (also known as tumor-associated differentially expressed gene-14; Ref. 6), were found previously to be highly expressed in ovarian carcinomas. Recently, these proteases have been shown to be members of the tissue *KLK*[3] gene family and have been renamed *KLK6*, *KLK7*, and *KLK8*, respectively (7–9). *KLK4* (also known as prostase, *KLK-L1*, and PRSS17), another member of this gene family (10–13), was shown to be expressed in the prostate cancer cell line LNCaP (10), the breast cancer cell line BT-474 (11), and endometrial carcinoma cell lines (14). Moreover, the levels of *KLK4* mRNA (10, 11) and its protein (hK4; Ref. 14) were up-regulated by androgens, progestins, and estrogen in these cell lines, respectively. However, the expression of *KLK4* and hK4 in the normal ovary and ovarian cancers remains to be described. Thus, the aims of this study were to examine *KLK4*/hK4 expression in the normal ovary and ovarian tumors of different histology, stage, and differentiation and to examine the hormonal regulation of hK4 in an ovarian cancer cell line.

MATERIALS AND METHODS

Tumor Samples and Cell Culture. Normal ovaries and ovarian tumor samples were obtained at surgery from women who underwent laparotomy for benign and malignant conditions in the Department of Obstetrics and Gynecology at the Royal

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[3] The abbreviations used are: *KLK*, kallikrein; hK4, *KLK4* protein product; NOE, normal ovarian epithelial; SER, serous; MUC, mucinous; END, endometrioid; CCC, clear cell carcinoma; GCT, granulosa cell tumor; DIG, digoxigenin; RT-PCR, reverse transcription-PCR; ECM, extracellular matrix; Asp, aspartic acid; Ser, serine; PSA, prostate-specific antigen.

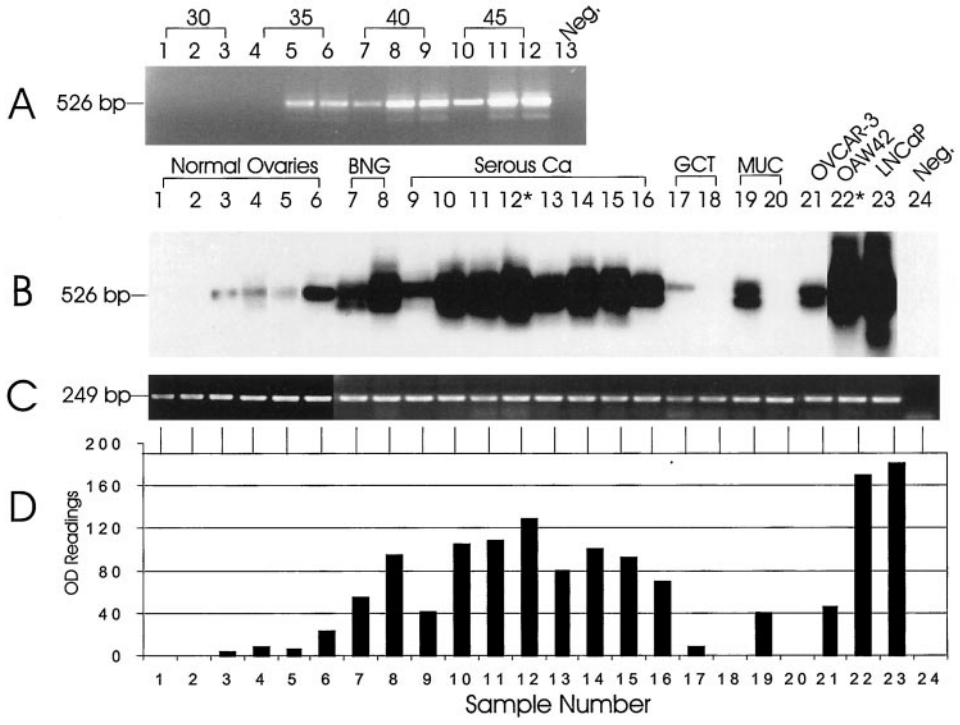

Fig. 1 *KLK4* expression in normal ovaries and ovarian tumors. *A,* analysis of amplification of *KLK4* in ovarian epithelial cells for different PCR cycles. *Lanes 1–3,* 30 cycles; *Lanes 4–6,* 35 cycles; *Lanes 7–9,* 40 cycles; *Lanes 10–12,* 45 cycles; *Lanes 1, 4, 7,* and *10,* NOE; *Lanes 2, 5, 8,* and *11,* SER carcinoma cells; *Lanes 3, 6, 9,* and *12,* OVCAR-3 cell line; and *Lane 13,* negative control (no cDNA). *B,* Southern blot analysis of the *KLK4* RT-PCR products with the DIG-labeled exon 3 *KLK4* probe. *C,* ethidium bromide-stained agarose gel of the RT-PCR for β2-microglobulin as an internal control. *D,* densitometry analysis of the above Southern blot, showing the *KLK4* mRNA expression level (in *A*) in different ovarian samples. *Lanes 1–3,* normal ovarian tissues; *Lanes 4–6,* NOE; *Lanes 7* and *8,* primary cultured cells from SER adenomas of ovary (*BNG*: benign); *Lanes 9* and *10,* primary cultured cells from stage II SER carcinomas of ovary; *Lanes 11–13,* primary cultured cells from stage III and IV SER carcinomas of ovary; *Lanes 14–16,* SER ovarian carcinoma tissues; *Lanes 17* and *18,* GCT tissues (*GCT*); *Lanes 19* and *20,* MUC adenoma, MUC carcinoma tissues (*MUC*); *Lanes 21* and *22,* SER ovarian carcinoma cell lines OVCAR-3 and OAW42; *Lane 23,* prostate cancer cell line LNCaP; and *Lane 24,* negative control (no cDNA). The tumor cells marked with ∗ were used for DNA sequencing analysis of the alternate spliced forms. The size of the PCR products is indicated in bp on the left of the figure.

Women's Hospitals and Monash Medical Center. Ethics approval was obtained from the respective institutional Ethics Committees, and informed consent was obtained from all patients. Epithelial cells from normal, benign, and malignant ovaries were isolated from some of these tissue samples, and the primary cultured cells were grown in M199 (Sigma Chemical Co., St. Louis, MO) and MCDB 105 (Sigma Chemical Co.) media supplemented with 10% FCS and 10 ng/ml human epidermal growth factor (Boehringer, Mannheim, Germany; Ref. 15). The ovarian cancer cell lines used in this study were derived from late stage SER carcinomas with well (PEO14 and OAW42), moderate (SKOV-3 and OVCAR-3), or poor (JAM, CI-80-13S, PEO1, and PEO4) differentiation. SKOV-3 and OVCAR-3 were from American Type Culture Collection, and the remainders have been described previously (16, 17). These cell lines were grown in RPMI (Life Technologies, Inc., Gaithersburg. MD) supplemented with 10% FCS. For the estrogen regulation study, OVCAR-3 cells were grown to 50% confluency. Twenty-four h before the experiments, the culture medium was replaced with phenol red-free RPMI containing 0.05% BSA, and 17β-estradiol (Sigma Chemical Co.) was added into the culture media at a final concentration of 100 nM. The cells were cultured for 8, 16, 24, and 30 h, respectively, and then harvested for protein extraction.

RT-PCR, Southern Blot, and DNA Sequencing Analysis. Total RNA was isolated from tumor cells or tissues using TRIzol reagent (Life Technologies, Inc.) following the manufacturer's instructions. Two μg of total RNA was reverse transcribed into first-strand cDNA using Superscript II in a 20-μl reaction. Fifty ng of *KLK4*-specific primers (5′-GCGGCACTGGTCATGGAAAACG-3′ and 5′-CAAGGCCCTGCAAGTACCCG-3′) and Platinum Taq (Life Technologies, Inc.) were used for PCR. PCR was performed with 1 μl of cDNA for three different ovarian samples (NOE cells, SER cancer cells, and the OVCAR-3 cell line) for 30, 35, 40, and 45 cycles to determine that amplification was in the linear range (Fig. 1*A*). The final chosen optimum cycling conditions were 94°C for 5 min, followed by 40 cycles of 94°C, 62°C, and 72°C for 1 min each, and a final extension at 72°C for 7 min. PCR for β2-microglobulin (5′-TGAATTGCTATGTGTCTGGGT-3′ and 5′-CCTCCATGATGCTGCTTACAT-3′), which was used as an internal control, was performed for 35 cycles with similar PCR conditions except for the annealing temperature (56°C). The PCR products were electrophoresed on a 1.5% agarose gel and visualized by

ethidium bromide staining. The resulting amplicons were analyzed by Southern blot hybridization using a DIG 3′ end-labeled *KLK4* oligonucleotide probe (5′-CTCCTACACCATCGGGCTGGGC-3′) in Easyhyb solution (Roche, Mannheim, Germany) overnight at 37°C. Washes with 0.2 × SSC/0.1% SDS were performed at 37°C. The membrane was blocked with anti-DIG antibody, and signals were detected by CDP-star (Roche) using X-ray film. The intensity of bands was determined by densitometry (GS-690 Imaging; Bio-Rad) using the Bio-Rad Multi-Analyst program. PCR products were also gel purified (Qiagen Pty, Ltd., Hilden, Germany) and sequenced. DNA sequences were analyzed using tBLASTN.

***In Situ* Hybridization.** Formalin fixed paraffin blocks from two normal ovaries and four SER ovarian tumors were sectioned (4 μm), deparaffinized, rehydrated, and pretreated for *in situ* hybridization as described previously (18). Hybridization was performed with DIG-labeled cRNA probes overnight at 50°C. *KLK4* probes were generated from a *KLK4* RT-PCR product (526 bp) cloned in p-GEM-T (Promega, Madison, WI) and confirmed by sequencing analysis to verify *KLK4* identity and orientation within p-GEM-T. Antisense and sense probes were generated using T7 and SP6 RNA polymerase (Boehringer Mannheim) after *Nco*I and *Sal*I digestion, respectively. After hybridization, sections were washed at 50°C in 2 × SSC, then at room temperature in 0.5 × SSC. Sections were blocked in 1% (w/v) blocking reagent (Roche), then incubated (2 h) with anti-DIG-alkaline phosphatase conjugated antibody (1/500 dilution; Roche). The signals were detected with 5-bromo-4-chloro-3-indolyl phosphate/nitroblue tetrazolium (Roche) and counterstained with fast nuclear red.

Immunohistochemistry. Immunohistochemical staining was performed on sections obtained from the same tissue blocks as above using a Zymed kit (Zymed Laboratories, Inc., San Francisco, CA). Paraffin sections (4 μm) were deparaffinized, and antigen retrieval was performed by microwave heat treatment in 10 mM sodium citrate buffer (pH 6.0). After H_2O_2 treatment and blocking, the sections were incubated (2 h) with a combination of affinity-purified anti-hK4 peptide antibodies (1/250 dilution) at room temperature, then biotinylated goat antirabbit immunoglobulins and streptavidin-horseradish peroxidase conjugate following the manufacturer's instructions. The hK4 antibodies were generated by immunization of New Zealand rabbits with peptides from three different regions of the hK4 protein, respectively. The production, specificity, and characterization of the hK4 antibodies will be described elsewhere[4]. Peroxidase activity was detected using 3,3′-diaminobenzidine (Sigma Chemical Co.) as the chromogen with H_2O_2 as the substrate. The sections were counterstained with Mayer hematoxylin. Negative controls were performed by using normal rabbit serum or primary antibody preabsorbed with hK4 peptides instead of the primary antibody.

Western Blot Analysis. Cytoplasmic extracts (150 μg of protein) from cultured tumor cells were electrophoresed on 12% SDS-polyacrylamide gels, and the protein was then transferred to a Protran membrane (Schleicher & Schüll, Dassel, Germany). The membrane was stained with ponceau S (Sigma Chemical Co.) to determine equivalent protein loading in each lane, blocked with 5% skim milk in Tris-buffered saline/Tween 20 overnight at 4°C, and then incubated with the same affinity-purified anti-hK4 peptide antibodies as used above (1/500 dilution, 2 h) at room temperature. The blot was washed and then incubated (1 h) with a horseradish peroxidase goat antirabbit IgG (Dako, Glostrup, Denmark; 1/2000 dilution) at room temperature. The signals were visualized on X-ray film by enhanced chemiluminescence. Densitometry analysis was performed to determine any changes in signal intensity on the Western blot.

RESULTS

Expression of *KLK4* in Normal Ovaries and Ovarian Tumors. The results of optimization of the *KLK4* PCR are shown in Fig. 1*A* and indicate that 40 PCR cycles are within the linear amplification range. Thus, six normal ovaries, 31 different ovarian tumors, and eight SER ovarian cancer cell lines were examined for their expression of *KLK4* by RT-PCR over 40 cycles, Southern blot, and densitometry analyses. The results of Southern blot analysis of the *KLK4* expression pattern in representative samples are shown in Fig. 1*B* with the densitometry analysis of the Southern blot for these samples shown in Fig. 1*D*. β2-microglobulin, which was used as an internal control (Figs. 1*C* and 2*B*), showed a consistent pattern of expression in all samples indicating the integrity of the RNA. Clinical information of all tumor tissues and cell lines are summarized in Table 1. *KLK4* expression was detected in normal ovaries (four of six), as well as epithelial-derived SER (benign: two of two; malignant: 11 of 11; cell lines: eight of eight), MUC (benign: one of one; malignant: two of three), END (four of five), clear cell tumors (two of three), and GCTs (three of six; Table 1). The level of *KLK4* expression was determined from the absorbance (*A*) reading of bands obtained from densitometry analysis. A higher level of *KLK4* expression (>40 *A*) was observed in the two benign adenomas, 10 of 11 SER carcinomas, and all eight SER-derived cancer cell lines compared with the normal ovaries, MUC, END, and clear cell and GCTs that are *KLK4* positive (Fig. 1, *B* and *D* and Table 1). Of these latter tumor types or normal ovaries, only four exhibited a level of *KLK4* expression >40 *A*, and indeed, *KLK4* expression was not observed in many of these samples, although 40 cycles of amplification were performed. These results suggest that *KLK4* is most highly expressed by late stage SER epithelial ovarian carcinomas.

In addition to the expected wild-type *KLK4* mRNA amplicon (526 bp), three alternate splicing forms of *KLK4* were observed in the ovarian cancer lines and ovarian tumor cells, as well as the LNCaP control, but not in normal ovaries (Fig. 1*B* and 2*A*). These three *KLK4* variants were noted in all SER epithelial ovarian tumors, including SER adenomas (two of two), SER carcinomas (11 of 11), and SER carcinoma cell lines (eight of eight), whereas three of four MUC tumors, four of five END carcinomas, two of three CCCs, and only one of six GCTs showed expression of these variants (Fig. 1 and Table 1). To

[4] T. Harvey, Y. Dong, J. Hooper, and J. A. Clements. Production and characterisation of antipeptide kallikrein 4 antibodies, manuscript in preparation.

Table 1 Patient characteristics and expression patterns of *KLK4*

No.	Histology	Stage[a]/grade[b]	Survival months	*KLK4* expression (variants)[c]	Summary of *KLK4* positive tumors with different histologies
1	NOT[d]			0	
2	NOT			0	
3	NOT			3 (−)	
4	NOE			8 (−)	
5	NOE			6 (−)	
6	NOE			23 (−)	4/6
7	SER adenoma			54 (+)	
8	SER adenoma			94 (+)	2/2
9	SER Ca	IIb/2	20D[e]	41 (+)	
10	SER Ca	IIc/3	2D	104 (+)	
11	SER Ca	IIIc/1	66	108 (+)	
12	SER Ca	IV/2–3	22D	129 (+)	
13	SER Ca	IIIc/3	25D	80 (+)	
14	SER Ca	IIIc/3	19D	100 (+)	
15	SER Ca	IIIb/3	18D	92 (+)	
16	SER Ca	III/1	162	39 (+)	
17	SER Ca Tissue	III/3	14	100 (+)	
18	SER Ca Tissue	III/2–3	16	92 (+)	
19	SER Ca Tissue	III/3	19	70 (+)	11/11
20	JAM (SER)	Xenograft/3		92 (+)	
21	CI-80-13S (SER)	IV/3		84 (+)	
22	SKOV-3 (SER)	III/1		48 (+)	
23	OVCAR-3 (SER)	III/NA		45 (+)	
24	PEO1 (SER)	III/3		60 (+)	
25	PEO4 (SER)	Recurrent		88 (+)	
26	PEO14 (SER)	III/1		92 (+)	
27	OAW42 (SER)	III/NA		169 (+)	8/8
28	MUC adenoma			40 (+)	
29	MUC Ca	I/NA	NA	0	
30	MUC Ca	I/NA	NA	75 (+)	
31	MUC Ca	II/NA	NA	41 (−)	3/4
32	END Ca	IIb/2	84	0	
33	END Ca	III/2–3	10D	92 (+)	
34	END Ca	Ic/LMP	48	22 (+)	
35	END Ca	Ia/NA	69	28 (+)	
36	END Ca	IIIb/3	84	18 (+)	4/5
37	CCC	Ia/2	124	38 (+)	
38	CCC	IIc/NA	74	0	
39	CCC	IIIb/2	14D	95 (+)	2/3
40	GCT	I/NA	NA	8 (+)	
41	GCT	I/NA	NA	0	
42	GCT	I/NA	NA	9 (−)	
43	GCT	Ia/NA	NA	0	
44	GCT	Unstaged/NA	NA	0	
45	GCT	Recurrent/NA	NA	50 (−)	3/6

[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 *KLK4* Southern blot bands determined by densitometric analysis (absorbance units); presence (+) or absence (−) of KLK4 mRNA variants.
[d] NOT, normal ovarian tissues; NOE, normal ovarian epithelial cells; SER, serous; MUC, mucinous; END, endometrioid; CCC, clear cell carcinoma; GCT, granulosa cell tumor; and NA, not available.
[e] D, died from this cancer; uninitialed numbers indicate still alive or died from other causes.

examine the three alternate forms of *KLK4*, PCR products from NOE cells, primary cultured cells from a SER ovarian carcinoma and from the ovarian cancer cell line OAW42 (Fig. 2*A*), were sequenced. Three alternate sequences (variants 1–3 in Fig. 2*A*) were successfully identified. Variant 1 (Fig. 2*A*, 609 bp) has an 83-bp insertion of intronic sequence from intron 3, and variant 2 (401 bp) has a 12-bp insertion of intronic sequence from intron 2 and exon 4 deletion. The variant 2 sequence is similar to a variant with 2 12-bp insertions reported in prostate tissue (19) but also includes an exon 4 deletion. Variant 3 (389 bp) has only the region corresponding to exon 4 deleted (Fig. 2*C*) and is similar to the sequence reported previously in an endometrial carcinoma cell line (14). All these variants exhibit a frame shift of the coding region that generates a premature stop codon giving rise to a truncated protein product that does not contain the Asp^{116}, variant 2 only or Ser residue (Ser^{207}) of the catalytic triad (Fig. 2*C*). This result was confirmed by RT-PCR using

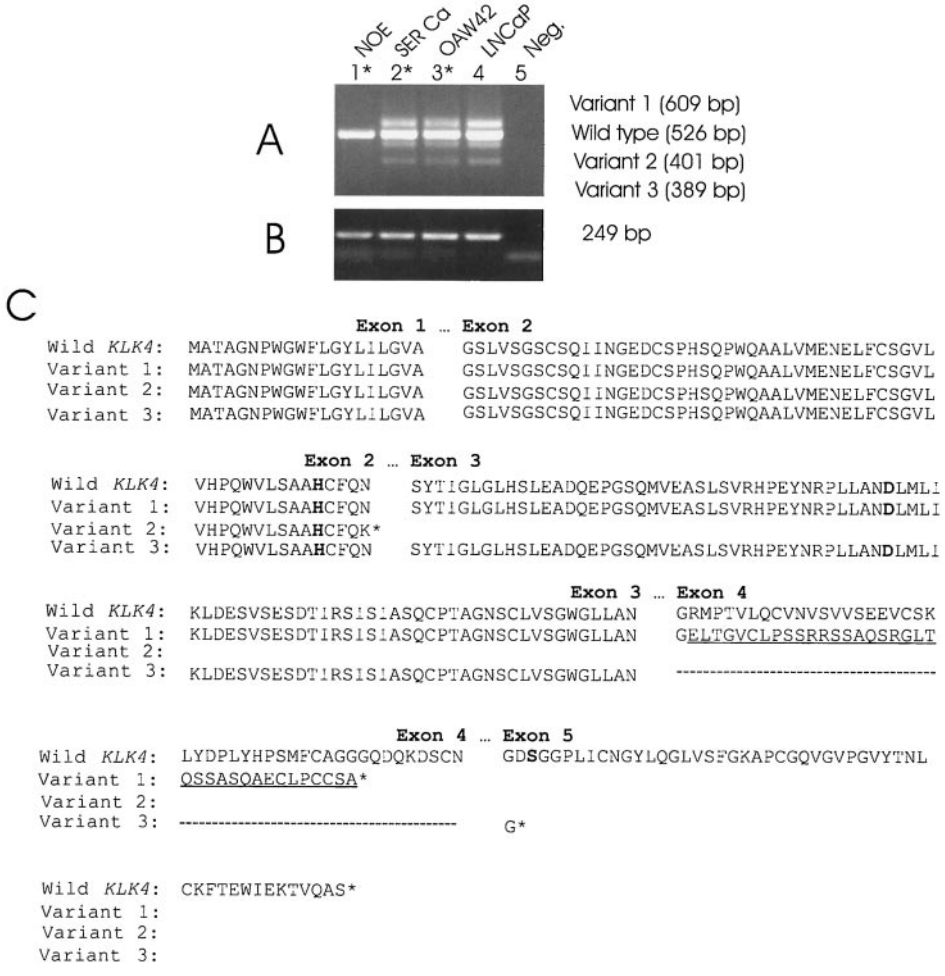

Fig. 2 KLK4 mRNA variant expression in normal ovaries and ovarian tumors. *A*, ethidium bromide-stained agarose gel of the RT-PCR for *KLK4* with exon 2 and exon 5 PCR primers. *Lane 1*, NOE; *Lane 2*, primary cultured SER ovarian carcinoma cells (*SER Ca*); *Lane 3*, ovarian carcinoma cell line OAW42; *Lane 4*, LNCaP as positive control; and *Lane 5*, negative control (no cDNA). *B*, RT-PCR for β2-microglobulin as an internal control. The sizes of the variant and wild-type PCR products are indicated to the right. DNA sequencing was performed on the PCR products marked with *. *C*, amino acid sequence of the *KLK4* putative product from the wild type and three variants. The five exons of the coding region are marked, and the introns are indicated by a dotted line (····). The intronic insertion (intron 3) is indicated by underline (_). The exon 4 deletion is indicated as a dashed line (----). The amino acids that constitute the catalytic triad, Histidine (***H***), Asp (***D***), and Ser (***S***), are marked in bold. *, the end of the predicted protein sequence.

DNase I-treated RNA, indicating that this finding is not attributable to genomic DNA contamination.

Expression of *KLK4* Transcripts and hK4 in Ovarian Cancer Tissues. The *in situ* hybridization and immunohistochemistry results shown in Fig. 3, *A–E* are representative of the patterns observed for the two normal ovaries and all four ovarian cancer samples. On *in situ* hybridization with a DIG-labeled *KLK4* antisense cRNA probe, *KLK4* expression was detected in the ovarian adenocarcinoma cells of moderately differentiated SER carcinomas (Fig. 3*A*). Ovarian carcinoma sections hybridized with a *KLK4* sense cRNA probe were negative (Fig. 3*B*). On immunohistochemistry, using a combination of the affinity-purified hK4 antipeptide antibodies, essentially no hK4 staining was observed in the normal ovary (Fig. 3*C*). In SER ovarian carcinoma cells, hK4 staining was primarily found in the cytoplasm, with some focal membrane localization observed (Fig. 3*D*), whereas only focal and equivocal staining was seen in stroma. No staining was seen in the negative control with normal rabbit serum instead of the primary antibody (Fig. 3*E*) or preabsorption of the hK4 antibodies with K4 peptides (data not shown).

Western Blot Analysis and Estrogen Regulation of hK4. The affinity-purified antipeptide hK4 antibodies recognized a protein of $M_r \approx 40{,}000$ in the prostate cancer cell line LNCaP, eight different ovarian cancer cell lines (see "Materials and Methods"), and primary cultured ovarian carcinoma cells. Fig. 3*F* (*Lanes 1–4*) shows a representative Western blot with one hK4 antipeptide antibody (COOH-terminal directed) of the ovarian cancer cell line, OAW42, and primary cultured SER ovarian carcinoma cells, N12 and N15, with LNCaP as the positive control. Similar expression patterns were observed with the other two antibodies to peptides from different regions of the hK4 protein (Fig. 3*F*, *Lanes 10* and *11*), although these bands were fainter as these antibodies are not as avid. The estrogen receptor-positive ovarian carcinoma cell line OVCAR-3 was used to evaluate if hK4 expression is under estrogen regulation. As shown in Fig. 3*E* (*Lanes 5–9*), a 1.5–4-fold up-regulation of hK4 intracellular levels by 100 nM estrogen was found, and this regulation was time dependent (Fig. 3*G*).

DISCUSSION

Ovarian cancer is the leading cause of death in all gynecological malignancies in part because of an inability to diagnose this disease at an early stage. Several Ser proteases are known to be involved in the tumor invasion and metastases indicative of advanced disease (2). In this study, we have investigated the expression of the *KLK* Ser protease, *KLK4*, in

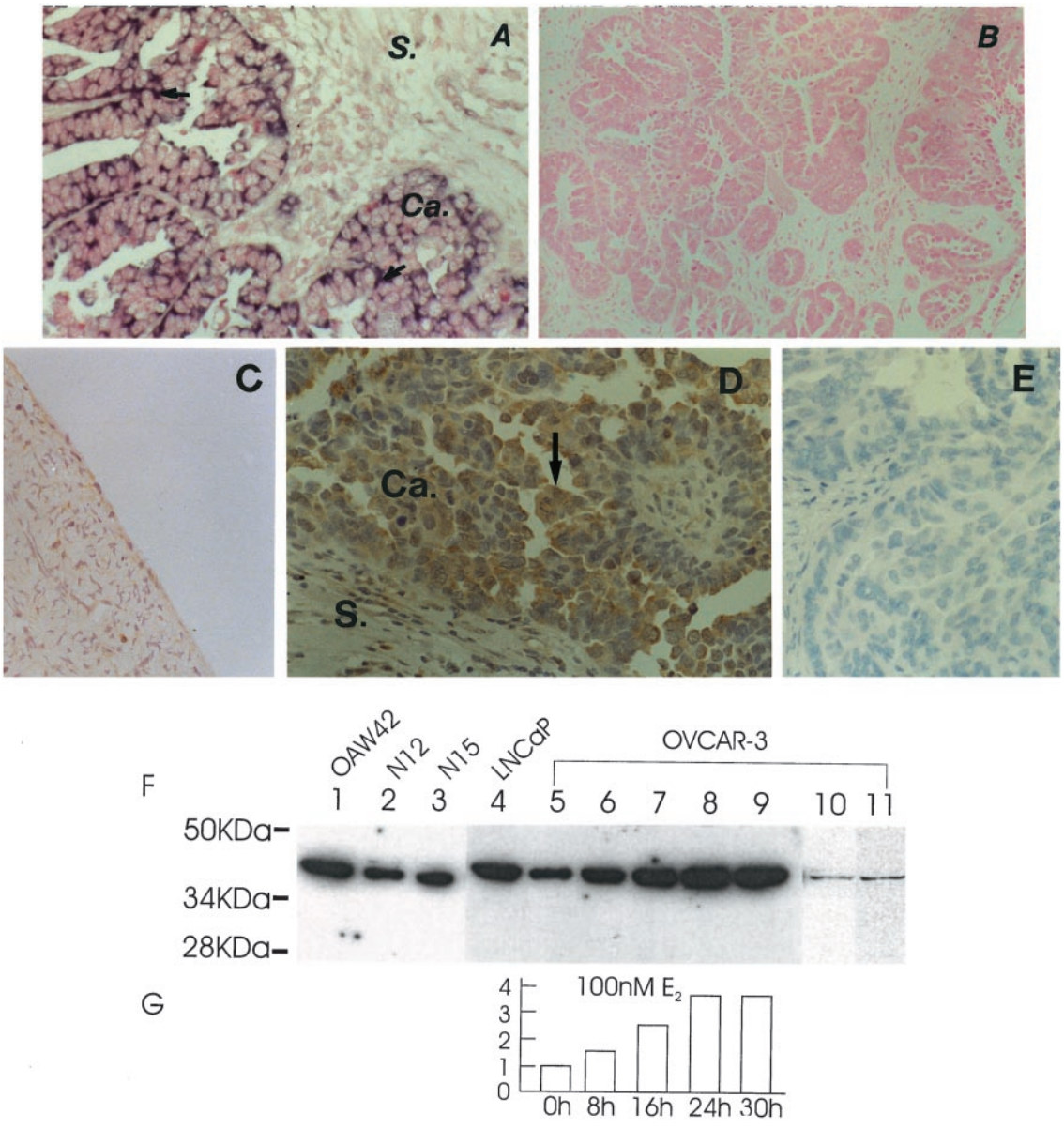

Fig. 3 *KLK4* mRNA and hK4 protein expression in ovarian cancer. *A,* moderately differentiated SER ovarian carcinoma showing *KLK4* mRNA transcript expression (*arrows*), as detected by *in situ* hybridization with DIG-labeled antisense *KLK4* cRNA probe. *B,* hybridization with the DIG-labeled *KLK4* sense cRNA probe as the negative control. *C,* normal ovaries showing no hK4 expression, as detected by affinity purified antipeptide hK4 antibody. *D,* moderately differentiated SER ovarian carcinoma showing hK4 cytoplasmic and membrane localization (*arrow*), as detected by affinity purified antipeptide hK4 antibodies. *E,* negative control with 10% normal goat serum instead of primary antibody (*S.* = stroma and *Ca.* = cancer; magnifications of *A, D,* and *E*: ×125; *B* and *C*: ×60). *F,* Western blot analysis with an affinity purified antipeptide hK4 antibody of cytoplasmic extract (~150 μg of protein) from the ovarian cancer cell line (*OAW42*), primary cultured SER ovarian carcinoma cells (*N12* and *N15*), prostate cancer cell line LNCaP, and β-estradiol treatment on the ovarian cancer cell line OVCAR-3. *Lanes 1–9,* anti-COOH-terminal peptide antibody; *Lane 10,* anti-NH_2-terminal peptide antibody; and *Lane 11,* anti-mid region peptide antibody. The size of the protein molecular weight markers is shown at the left. A hK4 protein of $M_r \approx 40{,}000$ was observed. *G,* densitometry analysis of the above Western blot, showing the up-regulation of intracellular hK4 levels after 17β-estradiol treatment (100 nM) on OVCAR-3 >30 h.

normal ovaries, ovarian cancer cell lines, and ovarian tumors. Our results showed the differential expression of *KLK4* in ovarian tumors compared with normal ovaries, with high expression of *KLK4* in SER carcinomas, especially in late stage disease and two benign adenomas. *KLK4* was expressed at low levels in the majority of ovarian tumors of MUC and granulosa cell origin. Of interest, three variant *KLK4* mRNA transcripts were detected in ovarian tumors but not in normal ovaries. In addition, we have detected a hK4 protein of $M_r \approx 40{,}000$ and report that the intracellular level of hK4 is up-regulated by estrogen treatment in the estrogen receptor-positive ovarian carcinoma cell line OVCAR-3.

Previous studies have shown that *KLK6*, *KLK7*, and *KLK8* are overexpressed by ovarian carcinomas compared with normal ovaries (3–6, 20). In addition, the expression of *KLK5* was reported recently to be associated with poor prognosis of ovarian cancer patients (21). We now report a similar pattern of expression for the fourth *KLK* gene, *KLK4*, in benign and malignant ovarian tissues. In contrast, it is interesting to note that Bicher *et al.* (22) reported that loss of heterozygosity on chromosome 19q13.2–q13.4, a region spanning the human *KLK* gene locus, was present in 53% of ovarian cancers. Indeed, the expression of another *KLK* gene, *KLK10* (normal epithelial cell-specific 1 gene NES1), is down-regulated in breast cancer (23). These data demonstrate that this region is an important area of genomic activity with respect to hormone-dependent cancers.

In the present study, using RT-PCR, Southern blot, and densitometry analyses, we have shown that the expression of *KLK4* and its variants may be related to the histology and/or stage of ovarian tumors. RT-PCR analysis, performed for 40 amplification cycles, showed clear expression in many samples compared with no expression in other samples, allowing a comparative analysis. All of the stage III and stage IV SER ovarian carcinomas showed the highest *KLK4* expression (as indicated by the intensity of the hybridization signal), whereas only four of eight of the MUC or GCTs showed high *KLK4* expression, although all of the MUC tumors and five of six GCTs used in this study were early stage tumors. Indeed, three of six GCT samples showed no *KLK4* signal, although they were clearly positive for β2-microglobulin. Both adenomas also exhibited a higher level of *KLK4* expression, although these findings will need to be confirmed on a larger group of samples. All of the ovarian cancer cell lines were also epithelial derived from late stage SER carcinomas and showed high *KLK4* expression. These cell lines covered a spectrum from well to poorly differentiated, but no correlation between *KLK4* expression and differentiation state could be drawn from this study. However, MUC ovarian tumors and GCTs have relatively reduced proliferative rates, when compared with SER tumors, and therefore, the expression of *KLK4* may be related to the proliferative status of a tumor. In addition, although all late stage ovarian cancers have poor outcomes, the prognosis of early stage SER and CCCs is worse than MUC, END, and GCTs. In this context, it is of interest to note that one stage III SER carcinoma (number 16, Table 1), three END carcinomas (numbers 32, 35, and 36, Table 1), and two CCCs (numbers 37 and 38; Table 1) had a better survival than the other tumors, and these tumors did not show high *KLK4* expression.

We also observed that three *KLK4* variants were detected in different ovarian tumors but not in normal ovaries. These variants had premature stop codons that would lead to a truncated hK4 protein if translated. All of these variants would not contain Ser^{207} of the catalytic triad (indeed, variant 2 would also not contain Asp^{116} of the catalytic triad), and therefore, they are unlikely to encode proteins with enzymatic activity. Previous studies from our laboratory have identified the *KLK4* variant 3 mRNA splice form in endometrial carcinoma cell lines (14). A similar variant to our variant 2 with a 12-bp insertion has also been reported in the prostate (19). Moreover, mRNA variants have been demonstrated for other *KLK*s, such as *KLK1* (24), *KLK2* (25), *KLK3* (26, 27), and *KLK13* (*KLK-L4*; Ref. 28). Thus, variant mRNA transcripts are a common feature of the human *KLK* family. Overall, we have shown increased expression of the wild-type *KLK4* transcript in late stage ovarian tumors and that several *KLK4* mRNA variants are expressed by ovarian tumors but not by normal ovaries. It will be important to now determine whether, like PSA in prostate and breast cancer (7, 25), *KLK4*/hK4, or the *KLK4* variant forms, could be a useful diagnostic or prognostic marker for some ovarian cancers or monitor this disease.

In situ hybridization and immunohistochemical staining of ovarian tumor sections from four different patients revealed that *KLK4* mRNA and the hK4 protein are detected in the cytoplasm and occasionally on the cell membrane of the SER epithelial-derived adenocarcinoma cells of the tumor tissues. However, no hK4 immunostaining was observed in normal ovaries. Consistent with our immunohistochemical staining results, the cell lysates from the ovarian carcinoma cell lines and carcinoma cells showed immunoreactivity to the hK4 antibody. The difference between the Western blot determined molecular weight ($M_r \approx 40{,}000$) and predicted ($M_r \approx 30{,}000$) molecular weight is probably because of a post-translation modification, as the predicted hK4 amino acid sequence contains *N*-glycosylation sites. Given the consistent results with three antipeptide antibodies to three different regions of the hK4 protein (Fig. 3*E*), it is likely that these antibodies are detecting the endogenous hK4 protein. The cell membrane staining was a surprising finding, as other *KLK*s, such as PSA, are secreted enzymes and usually localized to the cytoplasm. However, there are five predicted myristoylation sites in the hK4 sequence that may indicate a cell membrane function.

KLK4 expression was found previously in the breast cancer cell line BT-474 (11) and endometrial carcinoma cell lines (14), as well as in the prostate cancer cell line LNCaP (10). Moreover, *KLK4* mRNA expression was up-regulated by androgen, progestin, and estrogen (10, 11), and the intracellular levels of hK4 were induced by estrogen and progestin treatment (14) in these cell lines. Consistent with these findings, the present study showed that hK4 protein levels were induced by estrogen in the estrogen receptor-positive ovarian carcinoma cell line OVCAR-3 and that the induction was time dependent. Ovarian tumors (>50%) were found to be estrogen and progesterone receptor-positive, with some authors suggesting that SER and END tumors are more frequently positive (29). Although it is still controversial as to the possible role of estrogen or progesterone regulation in ovarian tumorigenesis, there is some suggestion that a subgroup of patients with aggressive ovarian tumors refractory to conventional chemotherapy may benefit from hormonal therapy (29). It will be interesting to determine whether any association exists between estrogen receptor status and *KLK4*/hK4 expression in advanced SER and/or other ovarian epithelial tumors.

The function of hK4 is not yet known, but hK4 shows 72% protein identity to pig enamel matrix seine proteinase 1, which degrades the ECM in preparation for enamel maturation (30). This suggests that the function of hK4 may be similar to enamel matrix seine proteinase 1, and hK4 may be involved in the degradation of ECM. Therefore, hK4 may play a similar role to other *KLK*s and be involved in the progression and metastasis of

several cancers. Both *KLK* 2 protein (hK2; Ref. 31) and PSA can degrade the ECM glycoprotein fibronectin (32), and PSA can also degrade laminin (32). PSA degrades insulin growth factor binding protein-3 and activates the pro-forms of epidermal growth factor, nerve growth factor, and transforming growth factor-β, thus regulating the bioavailability of these growth factors (25). Whether *KLK4* will perform similar function(s) is yet to be elucidated, but a role in ECM degradation and/or growth factor activation would be consistent with its expression in the highly proliferative and invasive late stage SER carcinomas.

In summary, the expression patterns observed in the present study suggest that *KLK4* mRNA and its variant forms are highly expressed in late stage ovarian cancer, particularly SER carcinomas. Additional studies are required to determine the precise function and role of *KLK4* in ovarian tumorigenesis and its usefulness in the diagnosis and/or monitoring of these tumors.

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