Kallikreins as Markers of Disseminated Tumour Cells in Ovarian Cancer – A Pilot Study

Katerina Oikonomopoulou[a, b]   Andreas Scorilas[d]   Iacovos P. Michael[a, b]
Linda Grass[b]   Antoninus Soosaipillai[b]   Barry Rosen[c]   Joan Murphy[c]
Eleftherios P. Diamandis[a, b]

[a]Department of Laboratory Medicine and Pathobiology, University of Toronto, [b]Department of Pathology and Laboratory Medicine, Mount Sinai Hospital, [c]Gynecologic Oncology Clinic, Princess Margaret Hospital, Toronto, Canada; [d]Department of Biochemistry and Molecular Biology, Faculty of Biology, University of Athens, Athens, Greece

Abstract

Background: Kallikreins are a family of secreted serine proteases, encoded by 15 genes which all localize in tandem on chromosome 19q13.4. Several members of this family have been previously associated with ovarian cancer. Kallikreins 6 (KLK6) and 10 (KLK10) are elevated in tumour cells, serum and ascites fluid of ovarian cancer patients and correlate with disease prognosis. Other kallikreins that have been related to ovarian cancer include KLK4, 5, 7, 8, 9, 11, 13, 14 and 15. We hypothesized that KLK6 and KLK10 can be utilized to monitor dissemination of ovarian cancer cells in blood and ascites fluid of ovarian cancer patients. *Methods:* RNA was isolated by immunomagnetic separation of cancer cells and was amplified by RT-PCR. *Results:* Screening for disseminated cancer cells in blood from 24 ovarian cancer patients, with RT-PCR for KLK6 mRNA, resulted in 75% positivity; however, this was not different from the positivity of normal controls. By utilizing KLK10 as a marker, the positivity of patients was 40% versus 20% of controls. Screening of ascites fluid of ovarian cancer patients revealed 90% positivity for KLK6 and KLK10 mRNA compared with 33% for other cancer types. Significant correlations were identified among mRNA of KLK4, 5, 6, 7, 8, 9, 10, 11, 13, 14 and 15 in cancer cells isolated from ascites fluid. *Conclusion:* Kallikrein expression by ovarian cancer cells is not specific enough for detecting disseminated disease. Kallikrein expression may have some value for differentiating ovarian cancer from other types of cancer or from non-malignant diseases that lead to ascites accumulation.

Copyright © 2006 S. Karger AG, Basel

Introduction

Ovarian cancer is the most lethal gynaecological malignancy, with a survival of less than 50% [1]. The lifetime probability of epithelial ovarian carcinomas, which constitute approximately 90% of ovarian cancer cases, is 1.7% [1]. The high mortality rate of ovarian cancer is mainly ascribed to late diagnosis, and 75% of women with the disease have tumour spread beyond the pelvis at the time of diagnosis.

The International Federation of Gynecology and Obstetrics stage at diagnosis represents the major prognostic factor for ovarian cancer. Other conventional prognostic markers include tumour grade, age, residual tumour after

Dr. E.P. Diamandis
Mount Sinai Hospital, Department of Pathology and Laboratory Medicine
600 University Avenue
Toronto, Ontario M5G 1X5 (Canada)
Tel. +1 416 586 8443, Fax +1 416 586 8628, E-Mail ediamandis@mtsinai.on.ca

surgery, presence or absence of ascites and histology [2]. The 5-year survival of patients with ovarian cancer decreases according to stage; stage I patients have a 5-year survival of around 80–90% compared with only 12–25% for patients with late-stage disease [3].

Dissemination of tumour cells occurs primarily in stage II when the tumour invades the surrounding organs within the abdominal cavity [4]. At a later stage, tumour cells spread within the abdominal cavity and the main indicator of intraperitoneal metastasis is the formation of ascites fluid. At later stages, tumour cells enter the lymphatic or the haematogenous circulation.

One way to assess metastasis is to determine the levels of disseminated tumour cells in the circulation. This test can also be used to monitor response to therapy or to detect cancer relapse [5, 6]. Thus far, detection of disseminated tumour cells has been studied mainly in blood, bone marrow and lymph nodes of patients with several types of cancers, such as ovarian, breast, colorectal and prostate cancer [7–11]. The methods are based on immunological or molecular detection of cancer-specific genes and proteins. Assays that utilize mRNA transcripts have a detection limit around 1–10 tumour cells admixed with 10^7 normal cells [7, 8, 10]. Recent studies focus on the flow cytometric identification of tumour cells [12, 13], as well as on the enrichment and molecular analysis of tumour cells isolated from fluids such as ascites fluid and blood [14, 15]. Both methods use antibodies against tumour or tissue-specific proteins. Up to 10^4- to 10^5-fold enrichment of tumour cells may be achieved and the detection limit can reach the level of 1 tumour cell per millilitre [14].

One of the major screening methods for ovarian cancer is the serum levels of CA125. However, specificity is poor due to high levels of CA125 expression in other malignant and non-malignant conditions, such as pregnancy, endometriosis, uterine fibroids and pelvic inflammatory disease [16]. CA125 remains an excellent tool for aiding diagnosis and for monitoring response to treatment [17]. Several other biomarkers such as lysophosphatidic acid, placental alkaline phosphatase and carcinoembryonic antigen, have been studied in parallel with CA125, in an effort to increase its diagnostic sensitivity for ovarian cancer [17, 18]. A family of proteases, human tissue kallikreins, are also under investigation for their clinical value in detecting ovarian cancer [19].

Kallikreins belong to the S1 family of serine proteases. In humans, the term 'tissue kallikreins' is used to describe a group of 15 structurally related and secreted serine proteases, hK1–hK15 (the genes are designated 'KLK1–

KLK15'). Among kallikreins, prostate-specific antigen (also known as hK3) is the best biomarker for prostate cancer and along with hK2 has been extensively studied [20]. Recent reviews provide detailed information on the genomic and structural organization of kallikreins, their tissue expression and mode of regulation [19, 21]. These proteases are abundantly expressed in groups in several cancer types and especially in hormone-dependent cancers [19, 22]. In addition, many alternative kallikrein transcripts and splice variants are differentially expressed in cancer and some are cancer specific [19].

In ovarian carcinoma, kallikreins (KLK) 4, 5, 6, 7, 8, 9, 10, 11, 13, 14 and 15 are up-regulated in tissues, serum or ovarian cancer cell lines at the mRNA and/or protein levels [19]. The utilization of kallikreins as cancer biomarkers has been the subject of several recent studies [19, 21–30]. In this present study, we examined if KLK6 and KLK10 expression can be used to detect circulating tumour cells in the blood of ovarian cancer patients. We also studied the expression of other kallikreins which have previously been associated with ovarian cancer in tumour cells isolated from ascites fluid of ovarian cancer patients.

Materials and Methods

Collection of Blood and Ascites Samples
Blood and ascites fluid samples from ovarian cancer patients were collected at the Gynecologic Oncology Clinic, Princess Margaret Hospital, Toronto, Canada. Blood and/or ascites fluid from liver, pancreatic, uterine and peritoneal cancer patients and patients with carcinoma of unknown origin, as well as blood from apparently healthy women were collected.

Histopathological staging, pre- and post-surgical levels of CA125, as well as information on the date of diagnosis, surgery and chemotherapy, were also available. Blood was collected in acid citrate dextrose (22 g/l trisodium citrate, 8 g/l citric acid, 24.5 g/l dextrose) tubes, while ascites samples were collected in 500-ml vacuum bottles. Samples were kept at 4°C and processed within 2 h to avoid cell lysis and mRNA degradation. Our procedures have been approved by the Ethics Review Board of Mount Sinai Hospital and Princess Margaret Hospital, Toronto, Canada.

Cell Lines
We identified Caov-3 (also known as HTB75), an ovarian adenocarcinoma cancer cell line that expresses KLK6 at the mRNA and protein level, and LNCaP, a prostatic carcinoma cell line that is KLK6 negative. Cell lines were obtained from the American Type Culture Collection (ATCC, Rockville, Md., USA). PC3 (AR6), a prostate cancer cell line that has been stably transfected with the androgen receptor gene and found to express KLK6, was kindly provided by Dr. Theodore Brown (Samuel Lunenfeld Research Institute, Mount Sinai Hospital) [31] and was used as a positive control. All cell lines were cultured in RPMI 1640 medium (Invi-

trogen, Carlsbad, Calif., USA) supplemented with fetal bovine serum (10%) and gentamicin (0.04 mg/ml) in 75-cm^2 flasks. Cultures were maintained at 37°C in a humidified 5% CO_2 atmosphere until near confluent, harvested by treatment with trypsin-EDTA (0.05%) (Invitrogen) for 10 min and washed at least once with cold PBS (4°C). All cells were counted prior to use. Cells used to spike blood from healthy women were cultured as above but were incubated with 10 m*M* EDTA, instead of trypsin. Cells were pelleted by centrifugation for 10 min at 1,200 rpm and washed with cold PBS.

Detection Limit of KLK6 Transcripts

Human KLK6 cDNA (GeneBank accession number NM_002774), cloned into a Bluescript plasmid vector and propagated in a DH5α *Escheria coli* host strain, was kindly provided by Dr. Georgia Sotiropoulou (Department of Pharmacy, University of Patras, Greece) and was stored at –20°C. Construct stock concentration was spectrophotometrically estimated at 7.5×10^{10} transcripts/μl. To evaluate the detection limit of our method, this stock was serially diluted down to 1 transcript/μl. All dilutions were done in salmon sperm DNA (0.1 μg/ml) (Sigma-Aldrich Canada Ltd., Oakville, Canada).

Isolation of Spiked Tumour Cells from Blood

Whole blood from 20 healthy volunteers was collected in acid citrate dextrose tubes, as previously described [32]. Ten samples were collected from women aged 30–60 years, 5 from women aged 19–30 years and 5 from men aged 25–35 years. HTB75 cells were spiked into 10 ml of blood (from the same donor) to obtain a series with 15,000–15 tumour cells per tube. Thousand LNCaP cells spiked to 10 ml of blood and a non-spiked blood sample served as negative controls.

Isolation of tumour cells from whole blood was performed using Dynabeads® Epithelial Enrich (Dynal Biotech Inc., Brown Deer, Wisc., USA), according to the manufacturer's instructions. This method utilizes magnetic beads, coated with a monoclonal antibody (Ber-EP4) specific for two glycoprotein antigens (34 and 39 kDa) of the cell surface. Briefly, the enrichment step involves incubating the sample with the magnetic beads and placing the tube inside a magnetic separator for capture of the magnetically labeled cells. Ber-EP4 antigens are present in the majority of malignant cells and some non-malignant cells; however, the expression is limited to cells of epithelial origin [33]. Blood samples from healthy volunteers were similarly analyzed. Cells isolated with the beads were subjected to RNA extraction.

Isolation of Tumour Cells from Blood and Ascites of Cancer Patients

We analyzed blood samples from (1) histologically confirmed ovarian cancer patients (n = 24), (2) ovarian cancer patients before (n = 8) or after (n = 13) surgery, (3) ovarian cancer patients before (n = 5), during (n = 5) or after (n = 11) chemotherapy, and (4) patients in remission (n = 6), treatment (n = 9) or relapse (n = 9). We also analysed blood samples from 3 patients with other gynaecological cancers (2 uterine and 1 of unknown origin). We included 2 blood samples from patients with non-gynaecological tumours (1 pancreatic and 1 metastatic breast cancer of unknown primary origin). Tumour cells were isolated as above.

We additionally isolated cancer cells from ascites fluid of 7 ovarian cancer patients, for which we also had blood samples, and 3 ovarian cancer patients with no matched blood samples. We further analyzed ascites fluid from 2 patients with gynaecological tumours (1 uterine and 1 of unknown origin) and 4 patients with other types of cancer (2 pancreatic, 1 liver and 1 metastatic breast cancer of unknown primary origin). For 1 case of gynaecological cancer of unknown origin and 2 cases of pancreatic and metastatic breast cancer of unknown primary origin, we had available blood samples as well. Ascites fluid was centrifuged for 10 min at 4,000 rpm (4°C). Cells were pelleted and used for RNA extraction.

RNA Extraction

Total RNA from HTB75 and LNCaP cells, tumour cells isolated from spiked blood and cells isolated from blood or ascites of ovarian cancer patients was extracted using Trizol reagent (Invitrogen), according to the manufacturer's instructions. In the case of cells obtained from human blood, we additionally used 250 μg/ml glycogen (Invitrogen) as carrier [34]. All total RNA samples were quantified spectrophotometrically.

RT-PCR

In the case of cell lines and cells isolated from ascites, 2 μg of total RNA were reverse transcribed into the first cDNA strand using the Superscript™ First-Strand system (Invitrogen), according to the manufacturer's instructions. In the case of cells isolated from human blood, the maximum allowable quantity of RNA (as suggested by the manufacturer) was used in order to maximize sensitivity. The final volume of RT-PCR reactions was 21 μl. The cDNA originating from the cancer cell line HTB75 was serially diluted to simulate smaller number of cells. Dilutions were performed (1) in salmon sperm DNA and (2) in cDNA extracted from 10^5 LNCaP cells. In the latter case, ratios ranging from 1:10 to 1:10^5 HTB75:LNCaP cells were used.

Based on the nucleotide sequence of the human *KLK6* gene (GenBank accession number NM_002774), two gene-specific primers were designed. The forward primer was designed to flank cDNA exon 1 and the reverse primer to flank exons 3 and 4 to avoid amplification of genomic DNA. Primers were also designed accordingly for KLK4, 5, 7, 8, 9, 10, 11, 13, 14 and 15. PCR was carried out in a mixture containing 2 μl of cDNA from cell lines and cells that originated from ascites samples, 2.5 μl 10 × reaction buffer (containing 15 m*M* $MgCl_2$), 200 μ*M* dNTPs, 0.4 μ*M* of primers and 2.5 units of HotStar Taq DNA polymerase (Qiagen, Mississauga, Canada) in a final volume of 25 μl on an Eppendorf thermal cycler (Brinkmann, Westbury, N.Y., USA). In the case of total RNA isolated from spiked blood samples or blood samples from ovarian cancer patients, the reaction mixture contained 10.5 μl of cDNA in a final volume of 25 μl. In the case of KLK6 plasmid transcripts, 1 μl of KLK6 cDNA was used in a final volume of 25 μl. cDNA obtained from all samples (tumour cells from blood and ascites, cell lines and cells from normal blood) was also subjected to β-actin amplification as a control. In this reaction, 1 μl of cDNA was subjected to PCR, using the reaction mixture described above, in a final volume of 25 μl. The cycling conditions were 94°C for 15 min to activate the Taq polymerase, followed by 25–40 cycles of 94°C for 30 s, annealing at 60–65°C for 30 s and extension at 72°C for 30 s. Final extension was performed at 72°C for 7 or 10 min. The primer sequences, PCR product lengths, annealing temperatures and number of PCR cycles used are summarized in table 1. Equal amounts of PCR products were electrophoresed on 1.5% agarose gels and visualized with ethidium bromide staining.

Oikonomopoulou et al.

Table 1. PCR amplification of kallikrein genes and β-actin

KLK gene	Primer sequence[a]	Length of product bp	Annealing temperature, °C	Number of PCR cycles	Cell source
KLK4	F: GCGGCACTGGTCATGGAAAACG R: AACATGCTGGGGTGGTACAGCGG	437	65	35, 25	ascites
KLK5	F: GTCACCAGTTTATGAATCTGGGC R: GGCGCAGAACATGGTGTCATC	328	60	35, 25	ascites
KLK6	F: GAAGCTGATGGTGGTGCTGAGTCTG R: GTCAGGGAAATCACCATCTGCTGTC	454	60	40 40 35, 25	cell lines blood ascites
KLK7	F: CCGCCCACTGCAAGATGAATGAG R: AGCGCACAGCATGGAATTTTCC	454	65	35, 25	ascites
KLK8	F: GCCTTGTTCCAGGGCCAGC R: GCATCCTCACACTTCTTCTGGG	416	65	35, 25	ascites
KLK9	F: TCTTCCCCCACCCTGGCTTCAAC R: CGGGGTCTGGAGCAGGGCTCAG	409	65	35, 25	ascites
KLK10	F: GGAAACAAGCCACTGTGGGC R: GAGGATGCCTTGGAGGGTCTC	468	60	40 35, 25	blood ascites
KLK11	F: CTCTGGCAACAGGGCTTGTAGGG R: GCATCGCAAGGTGTGAGGCAGG	461	60	35, 25	ascites
KLK13	F: GGAGAAGCCCCACCCACCTG R: CACGGATCCACAGGACGTATCTTG	441	65	35, 25	ascites
KLK14	F: CACTGCGGCCGCCCGATC R: GGCAGGGCGCAGCGCTCC	485	65	35, 25	ascites
KLK15	F: CTACGGACCACGTCTCGGGTC R: GACACCAGGCTTGGTGGTGTTG	459	65	35, 25	ascites
β-Actin	F: ATCTGGCACCACACCTTCTA R: CGTCATACTCCTGCTTGCTG	835	62	35 35 35	cell lines blood ascites

[a] 5′→3′ orientation.

Statistical Evaluation

Statistical evaluation was performed with SAS (SAS Institute Inc., Cary, N.C., USA) and SPSS software (SPSS Inc., Chicago, Ill., USA). Relationships among the different kallikreins, as well as between kallikrein transcripts and serum CA125 levels, were assessed by the Spearman correlation coefficient.

Results

Detection Limit

We were able to detect 1 KLK6 transcript cloned in a plasmid vector (fig. 1a) with 40 amplification cycles. With 35 cycles, the detection limit was 75 KLK6 tran-

scripts (data not shown). We also detected KLK6 transcripts from 0.03 HTB75 cells (fig. 1b). Similar data were obtained when KLK6 cDNA was mixed with cDNA from LNCaP cells at a ratio down to $1:10^5$ cells (fig. 1c).

Isolation of Spiked Tumour Cells from Blood

After immunomagnetic enrichment, we were able to detect down to 15 HTB75 cells spiked in 10 ml of anticoagulated blood (fig. 2a), which represent an approximate ratio of 1 tumour cell per 10^7 white blood cells. Unspiked blood, as well as blood spiked with 1,000 LNCaP cells, served as negative controls.

Fig. 1. Detection limit of *KLK6* gene transcripts. **a** Serial dilutions of KLK6 transcripts cloned in Bluescript plasmid. The detection limit is 1 KLK6 transcript. Lane 1: molecular weight marker. Lanes 2–7: number of KLK6 transcripts, ranging from 75,000 to 1 transcript. Lane 8: positive control (cDNA derived from 10^6 HTB75 cells). Lane 9: negative control (no cDNA). **b** Serial dilutions of cDNA reverse transcribed from total RNA isolated from HTB75 cells. The detection limit is 0.03 HTB75 cells. Lane 1: molecular weight marker. Lanes 2–6: number of HTB75 cells, ranging from 300 to 0.03 cells. Lane 7: positive control (cDNA derived from 10^6 HTB75 cells). Lane 8: negative control (no cDNA). β-Actin data are also shown for comparison. **c** Serial dilutions of cDNA from HTB75 cells mixed with cDNA from LNCaP cells. The detection limit is 0.03 HTB75 cells mixed with LNCaP cells, corresponding to a ratio of $1:10^5$ LNCaP cells (lane 6). cDNA from LNCaP cells was also amplified as negative control. Lane 1: molecular weight marker. Lanes 2–6: number of HTB75 cells, ranging from 300 to 0.03 cells. Lane 7: positive control (cDNA derived from 10^6 HTB75 cells). Lane 8: 10^3 LNCaP cells. Lane 9: negative control (no CDNA). β-Actin data are also shown for comparison. bp = Base pairs.
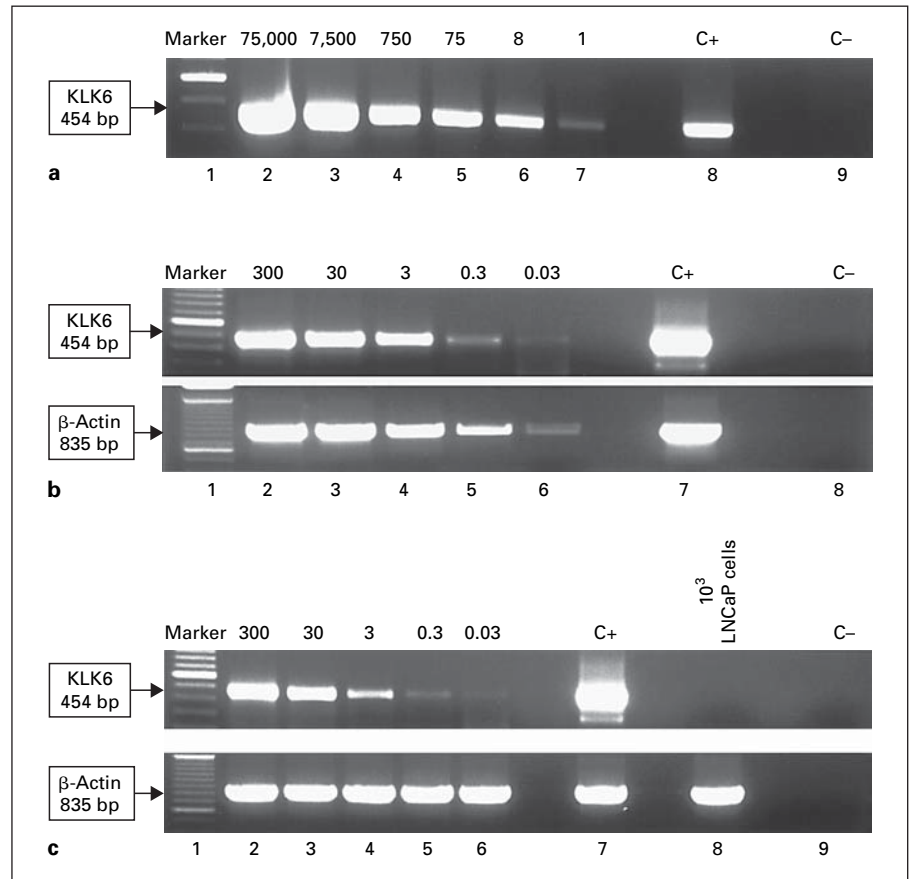

Fig. 2. a Isolation of spiked HTB75 tumour cells from blood. 15,000, 1,500, 150 and 15 HTB75 cells were spiked into 7 ml of whole blood and isolated by immunomagnetic cell separation as described under 'Materials and Methods'. The detection limit is 15 HTB75 cells (lane 5). A blood sample spiked with 1,000 LNCaP cells served as negative control (lane 9). Lane 1: molecular weight marker. Lanes 2–5: number of HTB75 cells. Lane 6: non-spiked blood. Lane 7: positive control (cDNA derived from 10^6 HTB75 cells). Lane 8: negative control (no cDNA). β-Actin was used to check the integrity of the cDNA. **b** Expression pattern of KLK6 in the blood of female healthy volunteers. Note the weak expression of KLK6 in most of the samples. For more data and discussion, see text. **c** KLK6 expression in blood of cancer patients. Lanes 1–2: uterine cancer. Lanes 3–10: ovarian cancer. Note the weak expression of KLK6 in most of the samples, similarly to **b**.
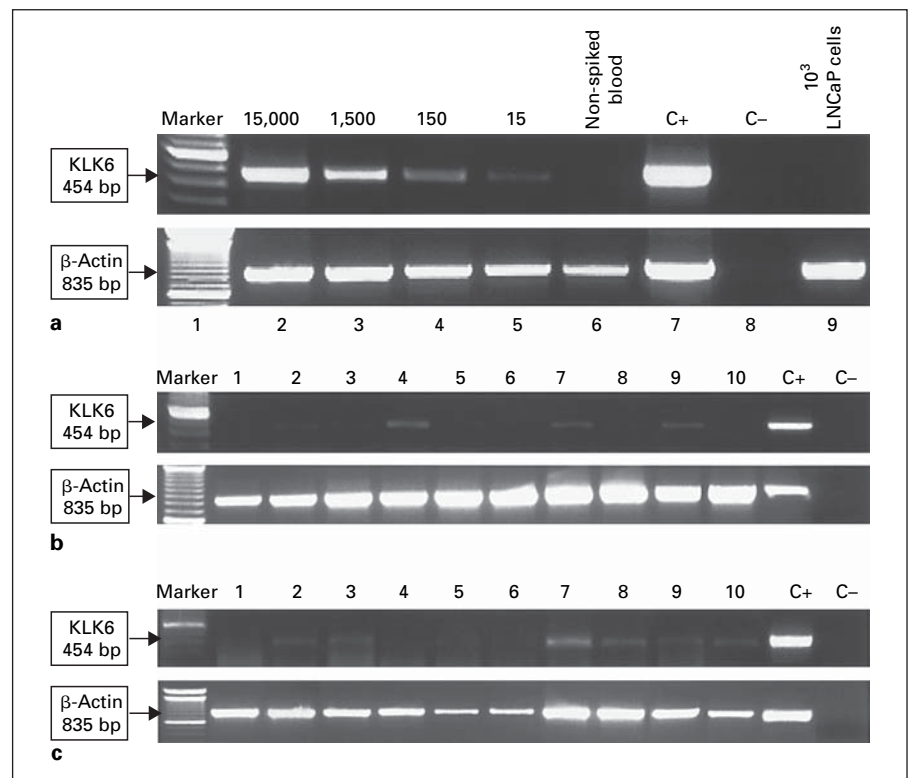
Fig. 3. a Expression pattern of KLK10 in the blood of female healthy volunteers. The blood of 10 female healthy volunteers was analyzed to determine the expression pattern of KLK10. Two samples were weakly positive (lanes 3 and 9; not shown in picture). **b** Expression pattern of KLK10 in blood of 10 ovarian cancer patients. Four of the patients are positive. β-Actin was amplified to check the integrity of the cDNA.
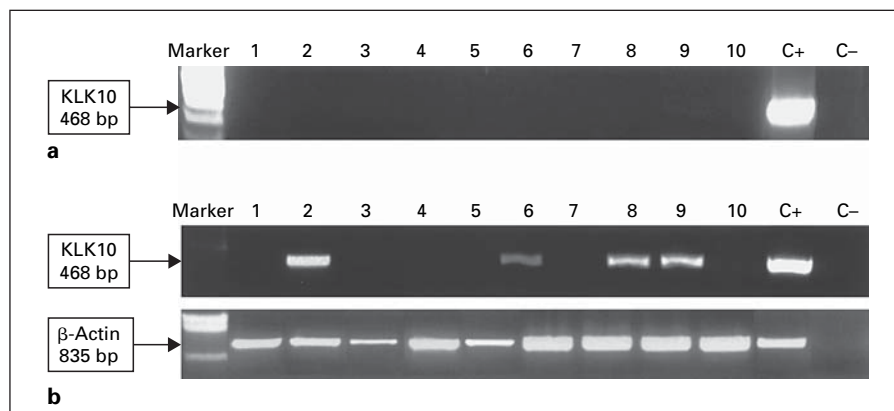
Table 2. Positivity rate for kallikrein mRNAs isolated from cells derived from ascites fluid of cancer patients

mRNA positivity	KLK4	KLK5	KLK6	KLK7	KLK8	KLK9	KLK10	KLK11	KLK13	KLK14	KLK15
All patients (n = 16)											
Ovarian cancer	6/10	9/10	9/10	9/10	9/10	9/10	10/10	10/10	10/10	7/10	7/10
Other gynaecological cancers	1/2	1/2	1/2	1/2	1/2	1/2	1/2	1/2	1/2	0/2	2/2
Non-gynaecological cancers	1/4	2/4	1/4	2/4	0/4	2/4	2/4	3/4	4/4	3/4	2/4
Ovarian cancer patients (n = 10)											
Surgery											
Before	3/6	5/6	5/6	5/6	5/6	5/6	6/6	6/6	6/6	4/6	4/6
After	3/4	4/4	4/4	4/4	4/4	4/4	4/4	4/4	4/4	3/4	3/4
Chemotherapy											
Before	1/1	1/1	1/1	1/1	1/1	1/1	1/1	1/1	1/1	1/1	0/1
During	3/5	4/5	4/5	4/5	4/5	4/5	5/5	5/5	5/5	3/5	3/5
After	2/4	4/4	4/4	4/4	4/4	4/4	4/4	4/4	4/4	3/4	4/4
Condition											
Treatment	2/4	3/4	3/4	3/4	3/4	3/4	4/4	4/4	4/4	3/4	1/4
Relapse	4/6	6/6	6/6	6/6	6/6	6/6	6/6	6/6	6/6	4/6	6/6
Surgery + chemotherapy											
Before	1/1	1/1	1/1	1/1	1/1	1/1	1/1	1/1	1/1	1/1	0/1
After	2/3	3/3	3/3	3/3	3/3	3/3	3/3	3/3	3/3	2/3	3/3
Before surgery											
During chemotherapy	2/4	3/4	3/4	3/4	3/4	3/4	4/4	4/4	4/4	2/4	3/4
After chemotherapy	0/1	1/1	1/1	1/1	1/1	1/1	1/1	1/1	1/1	1/1	1/1
After surgery											
During chemotherapy	1/1	1/1	1/1	1/1	1/1	1/1	1/1	1/1	1/1	1/1	0/1

KLK6 Expression in Blood of Healthy Volunteers and Ovarian Cancer Patients

Sixteen out of 20 (80%) blood samples from healthy volunteers were weakly positive for KLK6 mRNA (fig. 2b). Eighteen blood samples from the 24 ovarian cancer patients (75%) were also KLK6 positive (fig. 2c). One out of 3 (33%) non-ovarian gynaecological tumours, as well as 2/2 (100%) blood samples obtained from non-gynaecological cancer patients were positive as well.

KLK10 Expression in Blood of Healthy Volunteers and Ovarian Cancer Patients

Out of 10 tested samples from healthy controls, 2 were weakly positive for KLK10 mRNA (fig. 3a), while 4/10 samples from ovarian cancer patients were also positive (fig. 3b).
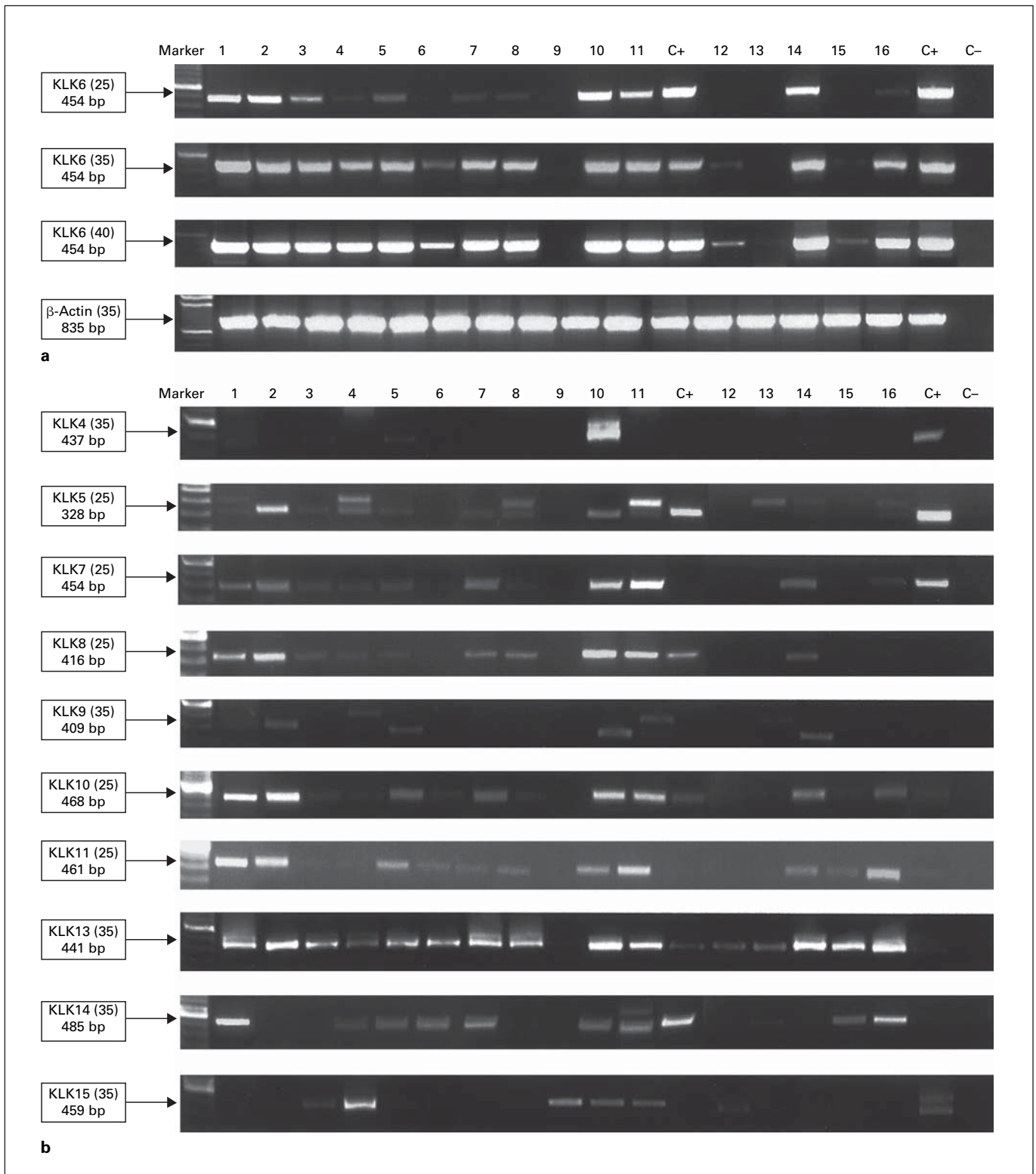
Fig. 4. Expression pattern of various kallikreins in ascites fluid of cancer patients. Lanes 1–8 and 10–11: ovarian cancer. Lane 9: gynaecological cancer of unknown origin. Lanes 12 and 16: pancreatic cancer. Lane 13: liver cancer. Lane 14: uterine cancer. Lane 15: metastatic breast cancer of unknown primary origin. **a** Data for KLK6 and β-actin at amplification cycles shown in brackets. **b** Data for other kallikreins, as shown at various amplification cycles (in brackets). Double bands represent splice variants [19].

Table 3. Correlations between kallikrein mRNAs from cells isolated from ascites fluid of ovarian cancer patients

	KLK5	KLK6	KLK7	KLK8	KLK9	KLK10	KLK11	KLK13	KLK14	KLK15
KLK4										
rs	0.27	0.75	0.45	0.61	0.59	0.57	0.41	0.38	0.13	−0.20
p	0.42	**0.008**	0.17	**0.047**	0.055	0.066	0.22	0.25	0.69	0.55
KLK5										
rs	1.00	0.76	0.84	0.88	0.52	0.78	0.78	0.81	0.19	0.14
p		**0.007**	**0.001**	**<0.001**	0.098	**0.005**	**0.005**	**0.003**	0.58	0.67
KLK6										
rs		1.00	0.72	0.94	0.67	0.78	0.79	0.69	0.09	−0.09
p			**0.012**	**<0.001**	**0.023**	**0.004**	**0.003**	**0.018**	0.79	0.79
KLK7										
rs			1.00	0.85	0.73	0.88	0.78	0.76	0.31	−0.26
p				**0.001**	**0.01**	**<0.001**	**0.005**	**0.006**	0.35	0.43
KLK8										
rs				1.00	0.75	0.84	0.80	0.77	0.16	−0.16
p					**0.008**	**0.001**	**0.003**	**0.006**	0.64	0.64
KLK9										
rs					1.00	0.81	0.69	0.64	0.27	−0.44
p						**0.002**	**0.017**	**0.032**	0.43	0.18
KLK10										
rs						1.00	0.89	0.89	0.37	−0.08
p							**<0.001**	**<0.001**	0.26	0.81
KLK11										
rs							1.00	0.80	0.42	−0.006
p								**0.003**	0.20	0.99
KLK13										
rs								1.00	0.21	0.14
p									0.53	0.69
KLK14										
rs									1.00	0.07
p										0.84

p values <0.05 are shown in bold. rs = Spearman's correlation coefficient.

Kallikrein Expression in Tumour Cells Isolated from Ascites Fluid of Cancer Patients

We tested for KLK6 transcripts with 25, 35 and 40 amplification cycles. At 25 cycles, 9/10 (90%) ovarian cancer patients were KLK6 positive (table 2). One out of 2 (50%) patients with other gynaecological malignancies was also positive and 1/4 (25%) non-gynaecological cancer patients was KLK6 positive (fig. 4a). With 35 and 40 cycles, ovarian cancer positivity was 100%, but the positivity of other types of cancer increased to 67 and 83%, respectively.

We further amplified mRNA from ascites fluid-isolated cells (16 patients) for KLK4, 5, 7, 8, 9, 10, 11, 13, 14 and 15 at 25 and 35 cycles (fig. 4b). Nine out of 10 patients (90%) were positive for KLK5, 7, 8 and 9, 10/10 (100%) for KLK10, 11 and 13, 7/10 (70%) for KLK14 and 15, and 6/10 (60%) for KLK4. For gynaecological tumours other than ovarian cancer, the positivity for all kallikreins was 1/2 (50%), with the exception of KLK14 and KLK15 (0/2 and 2/2 positive samples, respectively). Finally, the positivity for these kallikreins was >50% for non-gynaecological tumour cases (2–4/4), with the exception of 0/4 (0%) and 1/4 (25%) for KLK8 and KLK4, respectively. These data are summarized in table 2.

Correlations

Correlations between different kallikrein transcripts, between kallikrein transcripts of cells isolated from blood and from ascites fluid, as well as between kallikrein transcripts and serum levels of CA125 were examined. All kallikrein transcript levels were semi-quantitatively assessed as absent, low, medium or high, based on band intensities at different PCR amplification cycles. Data were also correlated with various clinicopathological parameters.

We found many statistically significant correlations among kallikrein transcript levels in ascites fluid of ovarian cancer patients (table 3). KLK6, for example, was correlated with KLK4 (p = 0.008), KLK5 (p = 0.007), KLK7 (p = 0.012), KLK8 (p < 0.001), KLK9 (p = 0.023), KLK10 (p = 0.004), KLK11 (p = 0.003) and KLK13 (0.018). No significant correlation between gene expression of each kallikrein and pre- or post-treatment CA125 levels was observed. The time course between the surgery or the chemotherapy treatment and the sample collection, as well as the rounds of chemotherapy, did not correlate with the levels of kallikrein transcripts (data not shown).

Discussion

Intraperitoneal spread is the primary mode of dissemination of ovarian cancer [16]. A common finding in ovarian cancer, co-existing with peritoneal metastasis, is the formation of ascites fluid. However, a limitation of ascites fluid as an indicator of ovarian neoplasms is that it is common in non-malignant conditions (e.g., liver cirrhosis), as well as in abdominal malignancies other than ovarian cancer [35, 36]. Even though it is considered a rare finding in ovarian cancer, some studies support the dissemination of tumour cells through the lymphatic and haematogenous circulation [4]. The small number of cells entering the circulation and their intermittent shedding pose limitations for early ovarian cancer detection, based on circulating cancer cells [5, 37].

RT-PCR for the detection of mRNA expressed by disseminated tumour cells is a highly sensitive method, allowing detection of 1 tumour cell in the presence of 10^7 background cells [7, 8]. Blood-based detection of cancer is an example of a minimally invasive method. Here, we preliminarily examine if the *KLK6* gene, which is over-expressed in ovarian cancer, can be utilized to detect disseminated ovarian cancer cells for the purpose of early-disease diagnosis, monitoring or prognosis. We found that KLK6 transcripts are detected in blood of normal subjects and cancer patients with approximately the same frequency, precluding the use of KLK6 as a cancer-specific marker in blood. A recent study identified KLK6 mRNA in disseminated tumour cells from the blood of patients with colorectal cancer, but detection was also limited by background gene expression [38]. Expression of the gene of interest by non-malignant cells has also been reported for other genes and is a common problem in establishing a diagnostic method based on gene expres-

sion [14]. This can be due to illegitimate transcription of the gene [39] or may be the result of other conditions that could alter gene expression, such as inflammation [40]. KLK6 and its rat ortholog, myelencephalon-specific protease, have been previously identified in inflammatory lesions of multiple sclerosis and have been related to the inflammatory process in these sites [41]. KLK10 mRNA was found to be less sensitive but slightly more specific in comparison with KLK6 but not suitable for clinical use either.

Many kallikreins have been associated with either unfavourable or favourable ovarian cancer prognosis [21]. The expression patterns of these genes in tumour cells isolated from ascites fluid of ovarian cancer patients, as well as their correlations, have not been previously reported. Using a highly specific RT-PCR method for detecting KLK6 mRNA transcripts, we found expression of this gene in 90% (9/10) of ovarian cancer patients (table 2). However, the positivity of this method for detecting non-gynaecological cancers (2 pancreatic, 1 liver and 1 metastatic breast cancer of unknown primary origin) was 25% and for detecting other gynaecological cancers 50%. These data suggest that KLK6 is not a sufficiently specific ascites fluid marker to differentiate between ovarian cancer and other gynaecological or non-gynaecological malignances. KLK10, 11 and 13 seem to be even more sensitive and KLK8 more ovarian cancer specific compared with the other kallikrein genes. Recent studies support the use of marker panels to identify circulating tumour cells or as diagnostic tests [42, 43]. In two of our previous studies, serum levels of KLK6 and KLK10 were combined with CA125 to increase the diagnostic sensitivity in both early and late stages of disease [44, 45].

The mechanisms through which kallikreins may promote or inhibit carcinogenesis remain to be elucidated. Of interest is the recent finding that tumour cells shed vesicles into the circulation, which are loaded with proteases that are responsible for increased invasiveness [46, 47]. According to another study, the chemopreventive agent alpha-difluoromethylornithine blocked the expression of many tumorigenesis-related genes but left unaffected the expression of some others, including KLK6 [48]. It is possible that KLK6 may play a role in chemotherapy-resistant tumours, as it has been proposed for KLK3 and KLK10 in breast cancer [19] and KLK4 in ovarian cancer [27]. Kallikreins have also been reported to cleave extracellular matrix proteins, have been associated with tissue remodelling [19] and neo-vascularization [49] and can effectively activate pro-urokinase-type plasminogen activator [19], a protease involved in invasion

and metastasis. They have also been co-localized with several hormones [19], indicating a possible role in hormone activation. It has been speculated that kallikreins are part of a cascade enzymatic pathway that is activated in ovarian cancer [19]. In this regard, in the future, kallikreins may serve as potential therapeutic targets.

Acknowledgement

References

1 American Cancer Society: Facts and Figures 2004. Atlanta, American Cancer Society, 2004.
2 Holschneider CH, Berek JS: Ovarian cancer: epidemiology, biology, and prognostic factors. Semin Surg Oncol 2000;19:3–10.
3 Nguyen HN, Averette HE, Hoskins W, Sevin BU, Penalver M, Steren A: National survey of ovarian carcinoma. 6. Critical assessment of current International Federation of Gynecology and Obstetrics staging system. Cancer 1993;72:3007–3011.
4 Kawamoto S, Urban BA, Fishman EK: CT of epithelial ovarian tumors. Radiographics 1999;19:S85–S102.
5 Vlems FA, Ruers TJ, Punt CJ, Wobbes T, van Muijen GN: Relevance of disseminated tumour cells in blood and bone marrow of patients with solid epithelial tumours in perspective. Eur J Surg Oncol 2003;29:289–302.
6 Smith BM, Slade MJ, English J, Graham H, Luchtenborg M, Sinnett HD, et al: Response of circulating tumor cells to systemic therapy in patients with metastatic breast cancer: comparison of quantitative polymerase chain reaction and immunocytochemical techniques. J Clin Oncol 2000;18:1432–1439.
7 Corey E, Corey MJ: Detection of disseminated prostate cells by reverse transcription-polymerase chain reaction (RT-PCR): technical and clinical aspects. Int J Cancer 1998;77:655–673.
8 Raj GV, Moreno JG, Gomella LG: Utilization of polymerase chain reaction technology in the detection of solid tumors. Cancer 1998;82:1419–1442.
9 Suzuki M, Ohwada M, Saga Y, Kohno T, Takei Y, Sato I: Micrometastatic p53-positive cells in the lymph nodes of early stage epithelial ovarian cancer: prognostic significance. Oncology 2001;60:170–175.
10 Roddiger SJ, Renneberg H, Martin T, Tunn UW, Zamboglou N, Kurek R: Human kallikrein 2 (hK2) mRNA in peripheral blood of patients with thyroid cancer: a novel molecular marker? J Cancer Res Clin Oncol 2003;129:29–34.
11 Pantel K, Brakenhoff RH: Dissecting the metastatic cascade. Nat Rev Cancer 2004;4:448–456.
12 Fadlon EJ, Rees RC, McIntyre C, Sharrard RM, Lawry J, Hamdy FC: Detection of circulating prostate-specific antigen-positive cells in patients with prostate cancer by flow cytometry and reverse transcription polymerase chain reaction. Br J Cancer 1996;74:400–405.

13 Racila E, Euhus D, Weiss AJ, Rao C, McConnell J, Terstappen LW, et al: Detection and characterization of carcinoma cells in the blood. Proc Natl Acad Sci USA 1998;95:4589–4594.
14 O'Hara SM, Moreno JG, Zweitzig DR, Gross S, Gomella LG, Terstappen LW: Multigene reverse transcription-PCR profiling of circulating tumor cells in hormone-refractory prostate cancer. Clin Chem 2004;50:826–835.
15 Agarwal R, Reis-Filho J, Choy S, Krishnan B, Gore M, Dowsett M, et al: Isolation of tumour cell from ascites in ovarian cancer (OC) for expression analysis (abstract 4459). Proc Am Assoc Cancer Res 2004:45:1028.
16 Hoskins WJ, Perez CA, Young RC (eds): Principles and Practice of Gynecologic Oncology. Philadelphia, Lippincott, 1992, pp 71–86, 715–781.
17 Bast RC Jr: Status of tumor markers in ovarian cancer screening. J Clin Oncol 2003;21:200–205.
18 Shen Z, Wu M, Elson P, Kennedy AW, Belinson J, Casey G, et al: Fatty acid composition of lysophosphatidic acid and lysophosphatidylinositol in plasma from patients with ovarian cancer and other gynecological diseases. Gynecol Oncol 2001;83:25–30.
19 Borgono CA, Michael IP, Diamandis EP: Human tissue kallikreins: physiologic roles and applications in cancer. Mol Cancer Res 2004;2:257–280.
20 Stephan C, Jung K, Diamandis EP, Rittenhouse HG, Lein M, Loening SA: Prostate-specific antigen, its molecular forms, and other kallikrein markers for detection of prostate cancer. Urology 2002;59:2–8.
21 Borgono CA, Diamandis EP: The emerging roles of human tissue kallikreins in cancer. Nat Rev Cancer 2004;4:876–890.
22 Clements JA, Willemsen NM, Myers SA, Dong Y: The tissue kallikrein family of serine proteases: functional roles in human disease and potential as clinical biomarkers. Crit Rev Clin Lab Sci 2004;41:265–312.
23 Shvartsman HS, Lu KH, Lee J, Lillie J, Deavers MT, Clifford S, et al: Overexpression of kallikrein 10 in epithelial ovarian carcinomas. Gynecol Oncol 2003;90:44–50.
24 Yousef GM, Polymeris ME, Yacoub GM, Scorilas A, Soosaipillai A, Popalis C, et al: Parallel overexpression of seven kallikrein genes in ovarian cancer. Cancer Res 2003;63:2223–2227.

25 Santin AD, Zhan F, Bellone S, Palmieri M, Cane S, Bignotti E, et al: Gene expression profiles in primary ovarian serous papillary tumors and normal ovarian epithelium: identification of candidate molecular markers for ovarian cancer diagnosis and therapy. Int J Cancer 2004;112:14–25.
26 Diamandis EP, Yousef GM: Human tissue kallikreins: a family of new cancer biomarkers. Clin Chem 2002;48:1198–1205.
27 Xi Z, Kaern J, Davidson B, Klokk TI, Risberg B, Trope C, et al: Kallikrein 4 is associated with paclitaxel resistance in ovarian cancer. Gynecol Oncol 2004;94:80–85.
28 Ni X, Zhang W, Huang KC, Wang Y, Ng SK, Mok SC, et al: Characterisation of human kallikrein 6/protease M expression in ovarian cancer. Br J Cancer 2004;91:725–731.
29 Lu KH, Patterson AP, Wang L, Marquez RT, Atkinson EN, Baggerly KA, et al: Selection of potential markers for epithelial ovarian cancer with gene expression arrays and recursive descent partition analysis. Clin Cancer Res 2004;10:3291–3300.
30 Diamandis EP, Borgono CA, Scorilas A, Harbeck N, Dorn J, Schmitt M: Human kallikrein 11: an indicator of favorable prognosis in ovarian cancer patients. Clin Biochem 2004;37:823–829.
31 Heisler LE, Evangelou A, Lew AM, Trachtenberg J, Elsholtz HP, Brown TJ: Androgen-dependent cell cycle arrest and apoptotic death in PC-3 prostatic cell cultures expressing a full-length human androgen receptor. Mol Cell Endocrinol 1997;126:59–73.
32 Burin M, Dutra-Filho C, Brum J, Mauricio T, Amorim M, Giugliani R: Effect of collection, transport, processing and storage of blood specimens on the activity of lysosomal enzymes in plasma and leukocytes. Braz J Med Biol Res 2000;33:1003–1013.
33 Latza U, Niedobitek G, Schwarting R, Nekarda H, Stein H: Ber-EP4: new monoclonal antibody which distinguishes epithelia from mesothelial. J Clin Pathol 1990;43:213–219.
34 Bracete AM, Mertz LM, Fox DK: Isolation of total RNA from small quantities of tissues and cells. Focus 1999;21:38–39.
35 Garcia-Tsao G: Cirrhotic ascites: pathogenesis and management. Gastroenterologist 1995;3:41–54.
36 Ringenberg QS, Doll DC, Loy TS, Yarbro JW: Malignant ascites of unknown origin. Cancer 1989;64:753–755.

37 Rappl G, Hasselmann DO, Rossler M, Ugurel S, Tilgen W, Reinhold U: Detection of tumor-associated circulating mRNA in patients with disseminated malignant melanoma. Ann NY Acad Sci 2001;945:189–191.

38 Schuster R, Max N, Mann B, Heufelder K, Thilo F, Grone J, et al: Quantitative real-time RT-PCR for detection of disseminated tumor cells in peripheral blood of patients with colorectal cancer using different mRNA markers. Int J Cancer 2004;108:219–227.

39 Chelly J, Hugnot JP, Concordet JP, Kaplan JC, Kahn A: Illegitimate (or ectopic) transcription proceeds through the usual promoters. Biochem Biophys Res Commun 1991;178:553–557.

40 Jung R, Kruger W, Hosch S, Holweg M, Groger N, Gutensohn K, et al: Specificity of reverse transcriptase polymerase chain reaction assays designed for the detection of circulating cancer cells is influenced by cytokines in vivo and in vitro. Br J Cancer 1998;78:1194–1198.

41 Blaber SI, Ciric B, Christophi GP, Bernett MJ, Blaber M, Rodriguez M, et al: Targeting kallikrein 6 proteolysis attenuates CNS inflammatory disease. FASEB J 2004;18:920–922.

42 Taback B, Chan AD, Kuo CT, Bostick PJ, Wang HJ, Giuliano AE, et al: Detection of occult metastatic breast cancer cells in blood by a multimolecular marker assay: correlation with clinical stage of disease. Cancer Res 2001; 61:8845–8850.

43 Miyashiro I, Kuo C, Huynh K, Iida A, Morton D, Bilchik A, et al: Molecular strategy for detecting metastatic cancers with use of multiple tumor-specific MAGE-A genes. Clin Chem 2001;47:505–512.

44 Diamandis EP, Scorilas A, Fracchioli S, van Gramberen M, de Bruijn H, Henrik A, et al: Human kallikrein 6 (hK6): a new potential serum biomarker for diagnosis and prognosis of ovarian carcinoma. J Clin Oncol 2003;21: 1035–1043.

45 Luo LY, Katsaros D, Scorilas A, Fracchioli S, Bellino R, van Gramberen M, et al: The serum concentration of human kallikrein 10 represents a novel biomarker for ovarian cancer diagnosis and prognosis. Cancer Res 2003;63: 807–811.

46 Ginestra A, Miceli D, Dolo V, Romano FM, Vittorelli ML: Membrane vesicles in ovarian cancer fluids: a new potential marker. Anticancer Res 1999;19:3439–3445.

47 Graves LE, Ariztia EV, Navari JR, Matzel HJ, Stack MS, Fishman DA: Proinvasive properties of ovarian cancer ascites-derived membrane vesicles. Cancer Res 2004;64:7045–7049.

48 Ignatenko NA, Zhang H, Watts GS, Skovan BA, Stringer DE, Gerner EW: The chemopreventive agent alpha-difluoromethylornithine blocks Ki-ras-dependent tumor formation and specific gene expression in Caco-2 cells. Mol Carcinog 2004;39:221–233.

49 Aimes RT, Zijlstra A, Hooper JD, Ogbourne SM, Sit ML, Fuchs S, et al: Endothelial cell serine proteases expressed during vascular morphogenesis and angiogenesis. Thromb Haemost 2003;89:561–572.