

ABSTRACT

Introduction: Kallikreins are a family of 15 recently discovered secreted serine proteases that have been associated with several types of cancer.^(1,2,3) The genomic locus of these proteases lies at chromosome 19q13.4. Kallikreins 4, 5, 6, 7, 8, 9, 10, 11, 13, 14 and 15 are elevated in tumour cells, serum or ascites fluid of ovarian cancer patients, at the mRNA or protein levels, and correlate with disease prognosis.

Hypothesis: We hypothesized that kallikreins 4, 5, 6, 7, 8, 9, 10, 11, 13, 14 and 15 could be utilized to monitor dissemination of cancer cells in blood and/or ascites fluid of ovarian cancer patients.

Objectives: This study aims to utilize KLK gene transcripts to detect disseminated ovarian cancer cells. Previous studies on the subject showed a sensitivity of around 1 cancer cell admixed in 106 cells not expressing the marker gene.⁽⁴⁾

Methods/Results: We established a sensitive RT-PCR method for detection of KLK6,10 mRNA by utilizing positive or negative kallikrein cell lines. We detected mRNA transcripts in 10^6 HTB75 cells, an ovarian cancer cell line. We were also able to detect 1 HTB75 cell in a background of 105 kallikrein-negative cells or 106 blood cells. RT-PCR for KLK6 mRNA in disseminated cancer cells from 24 ovarian cancer patients' blood, resulted in 75% positivity, which was not different from the positivity rate of the normal controls (80%). Utilizing KLK10 as a marker, we obtained 40% positivity for ovarian cancer patients vs. 20% for controls. Screening of ascites fluid of 10 ovarian cancer patients revealed 90% positivity for KLK6 and 10 mRNA, compared to 33% for other cancer types. Significant correlations were identified among the mRNA transcripts of kallikreins 4,5,6,7,8,9,10,11,13,14,15 in cancer cells isolated from ascites fluid of ovarian cancer patients.

Conclusions: We conclude that KLK6 cannot be utilized as a marker for blood dissemination of ovarian cancer cells. KLK expression in cells isolated from ascites fluid may aid in differentiating ovarian cancer from other types of cancer or non-malignant diseases that lead to ascites accumulation.

INTRODUCTION

General Information on Human Tissue Kallikreins (hK)

- Kallikreins are a family of homologous secreted serine proteases that localize to chromosome 19q13.3-13.4^(1,2,3)
- Certain kallikreins are candidate biomarkers for the prognosis, detection and monitoring of ovarian cancer (Table 1)
- Several kallikreins are potential biomarkers for breast and prostate cancer (Tables 2 and 3)

Table 1: Ovarian cancer biomarkers

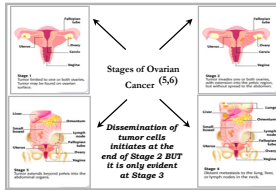
KLK	Prognosis	hK2, hK3	Diagnosis, Favourable prognosis and monitoring
KLK4	Unfavourable prognosis	hK2, hK3	Diagnosis, Favourable prognosis and monitoring
KLK5	Unfavourable prognosis	KLK5	Unfavourable prognosis
KLK6/9/10	Unfavourable prognosis	hK6	Diagnosis
hK6	Diagnosis	KLK7	Unfavourable prognosis
KLK6/9/10	Unfavourable prognosis	KLK9	Favourable prognosis
hK6	Unfavourable prognosis, Diagnosis and monitoring	hK10	Resistance to therapy
KLK7	Unfavourable prognosis	KLK13	Favourable prognosis
KLK7/hK7	Unfavourable prognosis	KLK14	Unfavourable prognosis
KLK8	Favourable prognosis	hK14	Diagnosis
hK8	Diagnosis, prognosis and monitoring	KLK15	Favourable prognosis
KLK9	Favourable prognosis		
hK10	Unfavourable prognosis, Diagnosis and monitoring		
KLK10	Diagnosis and prognosis		
hK11	Favourable prognosis and diagnosis		
hK12	Favourable prognosis		
KLK14	Unfavourable prognosis		
hK14	Diagnosis		
KLK15	Unfavourable prognosis		

Table 2: Breast cancer biomarkers

KLK	Prognosis	hK2, hK3	Diagnosis, Favourable prognosis and monitoring
KLK4	Unfavourable prognosis	hK2, hK3	Diagnosis, Favourable prognosis and monitoring
KLK5	Unfavourable prognosis	KLK5	Unfavourable prognosis
KLK6/9/10	Unfavourable prognosis	hK6	Diagnosis
hK6	Diagnosis	KLK7	Unfavourable prognosis
KLK6/9/10	Unfavourable prognosis	KLK9	Favourable prognosis
hK6	Unfavourable prognosis, Diagnosis and monitoring	hK10	Resistance to therapy
KLK7	Unfavourable prognosis	KLK13	Favourable prognosis
KLK7/hK7	Unfavourable prognosis	KLK14	Unfavourable prognosis
KLK8	Favourable prognosis	hK14	Diagnosis
hK8	Diagnosis, prognosis and monitoring	KLK15	Favourable prognosis
KLK9	Favourable prognosis		
hK10	Unfavourable prognosis, Diagnosis and monitoring		
KLK10	Diagnosis and prognosis		
hK11	Favourable prognosis and diagnosis		
hK12	Favourable prognosis		
KLK14	Unfavourable prognosis		
hK14	Diagnosis		
KLK15	Unfavourable prognosis		

Table 3: Prostate cancer biomarkers

KLK	Prognosis	hK2, hK3	Diagnosis, Favourable prognosis and monitoring
KLK4	Unfavourable prognosis	hK2, hK3	Diagnosis, Favourable prognosis and monitoring
KLK5	Unfavourable prognosis	KLK5	Unfavourable prognosis
KLK6/9/10	Unfavourable prognosis	hK6	Diagnosis
hK6	Diagnosis	KLK7	Unfavourable prognosis
KLK6/9/10	Unfavourable prognosis	KLK9	Favourable prognosis
hK6	Unfavourable prognosis, Diagnosis and monitoring	hK10	Resistance to therapy
KLK7	Unfavourable prognosis	KLK13	Favourable prognosis
KLK7/hK7	Unfavourable prognosis	KLK14	Unfavourable prognosis
KLK8	Favourable prognosis	hK14	Diagnosis
hK8	Diagnosis, prognosis and monitoring	KLK15	Favourable prognosis
KLK9	Favourable prognosis		
hK10	Unfavourable prognosis, Diagnosis and monitoring		
KLK10	Diagnosis and prognosis		
hK11	Favourable prognosis and diagnosis		
hK12	Favourable prognosis		
KLK14	Unfavourable prognosis		
hK14	Diagnosis		
KLK15	Unfavourable prognosis		



Spreading pattern of Metastasis

- Direct extension by contact
- Metastatic spread within the abdominal cavity (ascites fluid)
- Hematogenous or Lymphatic metastasis

We hypothesized that:

- The number of kallikrein transcripts in tumour cells isolated from blood and
- tumour cells isolated from ascites fluid of ovarian cancer patients can be utilized as a biomarker for the early diagnosis and prognosis of ovarian cancer

HYPOTHESIS

OBJECTIVES

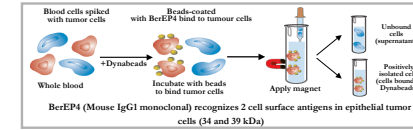
- Identify a cell line that strongly expresses KLK6 and KLK10
- Determine the sensitivity of the method, using KLK6 and KLK10 as markers
- Check the expression pattern of KLK6 and KLK10 in healthy donor's blood
- Determine the detection limit of spiked cancer cells in healthy donor's blood
- Use the method to identify circulating tumor cells in blood of ovarian cancer patients
- Detect KLK 4, 5, 6, 7, 8, 9, 10, 11, 13, 14, 15 in cancer cells isolated from ascites fluid of ovarian cancer patients.

MATERIALS AND METHODS

- We performed serial dilutions of KLK6,10 –pBluescript plasmid, diluted in Salmon Sperm DNA
- The detection limit of KLK transcripts was determined by PCR
- We isolated total RNA from 10⁶ HTB75 cells (positive for KLK6,10) and LNCaP cells (negative for KLK6,10) Isolated with Trizol (phenol and guanidine isothiocyanate method) Glycogen was used as an RNA carrier^(5,6)
- We performed Reverse Transcription to convert total RNA to cDNA
- We serially diluted the cDNA of HTB75 cells (to simulate reduced numbers of cells)
- We mixed the cDNA of HTB75 cells with cDNA from 10⁵ LNCaP cells

The detection limit of KLKs was determined by PCR

- We spiked 7ml of blood with HTB75 cells and we used Dynabeads⁽⁹⁾ to isolate the tumor cells

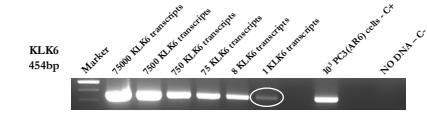


- We isolated tumour cells from ascites fluid of ovarian cancer patients

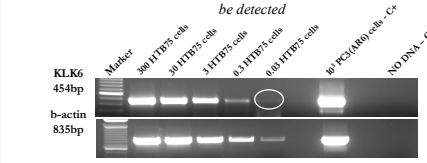
We extracted total RNA from the isolated cells and performed RT-PCR to check the expression pattern of KLKs

RESULTS

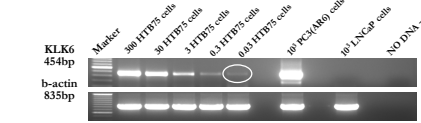
- Serial dilutions of KLK6 transcripts cDNA as little as 1 KLK6 and KLK10 transcript per reaction can be detected



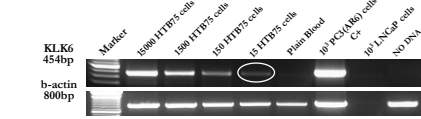
- HTB75 cDNA dilutions cDNA corresponding to as little as 0.03 cells per reaction can easily be detected



- HTB75 cDNA mixed with cDNA from 10⁵ LNCaP cells cDNA corresponding to 0.03 cells per reaction can easily be detected (RATIO: ~ 1:10⁶)



- Spiking the blood with HTB75 cells As few as 15 tumour cells per 7ml of blood can easily be detected



STATISTICAL ANALYSIS (ascites fluid)

DETECTION OF TUMOUR CELLS IN ASCITES FLUID

- Ovarian cancer patients (n=10)
- 90% of samples were positive for KLK 5, 7, 8 and 9
- 100% of samples were positive for KLK 10, 11 and 13
- 70% of samples were positive for KLK14 and 15
- 60% of samples were positive for KLK4

Gynecological tumours other than ovarian cancer (n=2)

- 50% of samples were positive for KLK4,5,6,7,8,9,10,11,13
- 0% of samples were positive for KLK14
- 100% of samples were positive for KLK15

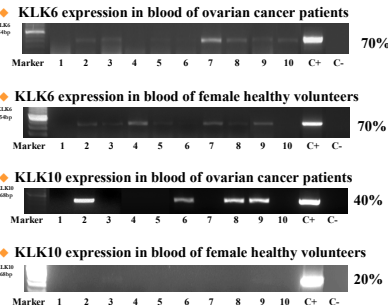
Non-gynecological tumours (n=4)

- >50% of samples were positive for KLK5,7,9,10,11,13,14,15
- with the exception of KLK6 (25%), KLK8 (0%) and KLK4 (25%)

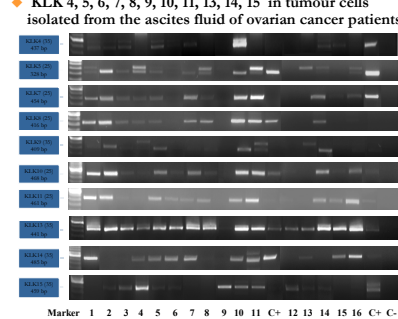
Table 4: Correlations between kallikreins in cells isolated from ascites fluid

KLK4	KLK5	KLK6	KLK7	KLK8	KLK9	KLK10	KLK11	KLK13	KLK14	KLK15
(p)	0.27	0.78	0.46	0.61	0.59	0.57	0.41	0.38	0.13	-0.2
(p)	0.42	0.008	0.17	0.047	0.056	0.066	0.22	0.25	0.69	0.55
(p)	1	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	<0.001	0.098	0.005	0.005	0.003	0.58	0.67
KLK6										
(p)	1	0.72	0.44	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										
(p)	1	0.85	0.73	0.88	0.78	0.78	0.76	0.31	-0.26	
(p)	0.001	0.01	<0.001	<0.001	0.005	0.006	0.35	0.43		
KLK8										
(p)	1	0.75	0.64	0.8	0.77	0.16	-0.16			
(p)	0.008	0.001	0.003	0.006	0.64	0.64				
KLK9										
(p)	1	0.81	0.69	0.64	0.27	-0.44				
(p)	0.002	<0.017	0.032	0.43	0.18					
KLK10										
(p)	1	0.89	0.89	0.37	-0.08					
(p)	<0.001	<0.001	0.001	0.26	0.81					
KLK11										
(p)	1	0.8	0.42	-0.006						
(p)	0.003	0.2	0.99							
KLK13										
(p)	1	0.21	0.14							
(p)	0.53	0.69								
KLK14										
(p)	1	0.07								
(p)	0.84									

DETECTION OF TUMOUR CELLS IN BLOOD (representative results, total n=24)



DETECTION OF TUMOUR CELLS IN ASCITES FLUID (representative results)



CONCLUSIONS

- Kallikrein 6 cannot be utilized for monitoring ovarian tumour cell dissemination in blood
- KLK transcript numbers from tumor cells that circulate in blood, ascites fluid and lymph nodes may be candidate markers:
 - for early detection of ovarian cancer
 - for monitoring metastasis or re-occurrence of the ovarian tumor after surgery
- Kallikreins that are correlatively expressed in ascites fluid may be the subject of a multiparametric analysis for the detection of ovarian cancer

FUTURE STUDIES

- Examine other Kallikreins as possible biomarkers to detect ovarian tumor cells in blood⁽¹⁰⁾
- We aim to use a multimer approach to improve the sensitivity of the method
- Study a large number of patients

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