

Quantitative analysis of human kallikrein gene 14 expression in breast tumours indicates association with poor prognosis

GM Yousef^{1,2}, CA Borgoño^{1,2}, A Scorilas³, R Ponzzone⁴, N Biglia⁴, L Iskander², M-E Polymeris², R Roagna⁴, P Sismondi⁴ and EP Diamandis^{*,1,2}

¹Department of Pathology and Laboratory Medicine, Mount Sinai Hospital, Toronto, Ontario, Canada; ²Department of Laboratory Medicine and Pathobiology, University of Toronto, Toronto, Ontario, Canada; ³National Center of Scientific Research 'Demokritos', IPC, Athens, 153 10, Greece; ⁴Academic Division of Gynecological Oncology, University of Turin, Maurizio Umberto Hospital and Institute for Cancer Research and Treatment (IRCC) of Candiolo, Turin, Italy

KLK14 (formerly known as *KLK-L6*) is a recently identified member of the human kallikrein gene family. This family harbours several genes aberrantly expressed in various cancers as well as established (PSA/hK3, hK2) and potential (hK6, hK10) cancer markers. Similar to other kallikrein genes, *KLK14* was found to be regulated by steroid hormones, particularly androgens and progestins, in breast and ovarian cancer cell lines. Preliminary studies indicated that *KLK14* is differentially expressed in breast, ovarian, prostatic and testicular tumours. Given the above, we determined the prognostic significance of *KLK14* expression in breast cancer. We studied *KLK14* expression in 178 histologically confirmed epithelial breast carcinomas by quantitative reverse transcription–polymerase chain reaction and correlated with clinicopathological variables (tumour stage, grade, histotype etc.) and with outcome (disease-free survival and overall survival), monitored over a median of 76 months. *KLK14* mRNA levels ranged from 0 to 1219 arbitrary units in breast cancer tissues, with a mean \pm s.e. of 136 ± 22 . An optimal cutoff value of 40.5 arbitrary units was selected, to categorise tumours as *KLK14*-positive or negative. Higher concentrations of *KLK14* mRNA were more frequently found in patients with advanced stage (III) disease ($P=0.032$). No statistically significant association was found between *KLK14* and the other clinicopathological variables. *KLK14* overexpression was found to be a significant predictor of decreased disease-free survival (hazard ratio of 2.31, $P=0.001$) and overall survival (hazard ratio of 2.21, $P=0.005$). Cox multivariate analysis indicated that *KLK14* was an independent prognostic indicator of disease-free survival and overall survival. *KLK14* also has independent prognostic value in subgroups of patients with a tumour size ≤ 2 cm and positive nodal, oestrogen receptor and progestin receptor status. We conclude that *KLK14* expression, as assessed by quantitative reverse transcription–polymerase chain reaction, is an independent marker of unfavourable prognosis for breast cancer.

British Journal of Cancer (2002) 87, 1287–1293. doi:10.1038/sj.bjc.6600623 www.bjcancer.com

© 2002 Cancer Research UK

Keywords: serine proteases; breast cancer; cancer genes; tumour markers; prognostic; predictive factors; kallikreins; *KLK14*

Breast cancer is the most prevalent malignancy among women, accounting for 21% of all female cancers and ranking third overall when both sexes are considered (Parkin *et al*, 1999). Although the increased use of screening for early disease diagnosis and the widespread administration of systemic adjuvant therapies have led to a decline in mortality rates, breast cancer is still the leading cause of death from cancer in women, causing over 39 500 deaths in the US annually (Peto *et al*, 2000; Jemal *et al*, 2002).

Given the heterogeneous nature of breast carcinomas, much attention has been focussed on the identification of tumour associated molecular markers that reveal the biological profile of each tumour and ultimately aid in determining cancer risk, diagnosis, screening, prognosis, monitoring, management and prediction of therapeutic response in breast cancer patients (Duffy, 2001). Among the multitude of markers discovered are serine proteases,

which participate in many aspects of carcinogenesis, including stimulating cellular growth, angiogenesis and the degradation of the extracellular matrix (Gottesman, 1990; Duffy, 1991). These functions are in accord with clinical studies demonstrating that the aberrant expression of certain serine proteases correlates with the invasiveness and metastasis of cancer cells and predicts poor prognosis in various malignancies (Herszenyi *et al*, 1999; Kuhn *et al*, 1999).

Human kallikreins are a subset of secreted serine proteases found in a wide range of tissues and biological fluids and implicated in diverse physiological and pathological processes (Diamandis *et al*, 2000b; Yousef and Diamandis, 2001). The kallikrein genes, denoted *KLK1–KLK15*, are located on chromosome 19q13.4 and encode for corresponding kallikrein enzymes, hK1–hK15 (Diamandis *et al*, 2000a; Yousef *et al*, 2000b). Accumulating evidence indicates that many members of this family are differentially expressed in certain malignancies, including prostate (Rittenhouse *et al*, 1998; Magklara *et al*, 1999; Barry, 2001; Yousef *et al*, 2001c; Diamandis *et al*, 2002), testicular (Luo *et al*, 2001c), breast (Yousef *et al*, 2000a,c) and ovarian (Anisowicz *et al*, 1996; Diamandis *et al*, 2000c; Kim *et al*, 2001; Luo *et al*, 2001b; Magklara *et al*, 2001; Obiezu *et al*, 2001; Yousef *et al*, 2001a) cancers. Also,

*Correspondence: Dr EP Diamandis, Mount Sinai Hospital, Department of Pathology and Laboratory Medicine, 600 University Avenue, Toronto, Ontario M5G 1X5, Canada; E-mail: ediamandis@mtsina.on.ca

Received 29 May 2002; revised 3 September 2002; accepted 4 September 2002

many kallikrein genes examined thus far are under steroid hormone regulation, further suggesting a role for these enzymes in endocrine-related tissues (Yousef and Diamandis, 2002). Additionally, PSA/hK3, is the best tumour marker available in clinical medicine for diagnosing and managing prostate cancer (Diamandis, 1998; Barry, 2001), hK2 is useful for certain subgroups of prostate cancer patients (Magklara *et al*, 1999), hK6, 10 and 11, have recently emerged as potential serological epithelial ovarian cancer markers (Luo *et al*, 2001a; Diamandis *et al*, 2002), and several others possess clinical utility as prognostic/predictive markers (Diamandis and Yousef, 2001).

Human kallikrein gene 14 (*KLK14*), formerly known as *KLK-L6*, is a recently identified member of the human kallikrein gene family (Yousef *et al*, 2001b). Structurally, this gene is formed of five coding exons and four intervening introns. The encoded protein, hK14, is a trypsin-like serine protease, translated as an inactive 251 amino acid preproenzyme precursor of about 27.5 kDa, of which 18 amino acids constitute the signal peptide and six amino acids the activation peptide. hK14 harbours the conserved catalytic triad characteristic of serine proteases and is highly homologous to other kallikreins, including PSA/hK3. *KLK14* has a restricted tissue expression pattern and is found in the central nervous system as well as in endocrine-related tissues such as the uterus, ovary, thyroid and testis. Additionally, *in situ* hybridisation studies demonstrated that *KLK14* is expressed by the secretory epithelial cells of benign prostate gland, prostatic intraepithelial neoplasia and malignant prostate cells (Hooper *et al*, 2001). Preliminary studies have shown that *KLK14* is down-regulated at the mRNA level in prostatic, testicular, ovarian and breast cancer tissues and in two breast cancer cell lines (Yousef *et al*, 2001b). Hormonal regulation studies in breast and ovarian cancer cell lines indicate that *KLK14* expression is controlled by the androgen receptor in response to steroid hormones, particularly androgens and progestins (our unpublished data). Based on these collective findings, we hypothesised that *KLK14* expression in malignant breast tissues may have prognostic/predictive value for patients with breast carcinomas.

MATERIALS AND METHODS

Study population

Tumour specimens from 178 consecutive patients undergoing surgical treatment for primary breast carcinoma at the Department of Gynecologic Oncology, University of Turin, Turin, Italy were analysed in this study. Patient age ranged from 25 to 87 years, with a median of 58 years (Table 1). Follow-up information (median follow-up period of 76 months) was available for 164 patients, among whom 60 (36%) had relapsed and 51 (31%) died. All tissue specimens were histologically confirmed and frozen in liquid nitrogen immediately after surgery.

Clinical and pathological information documented at the time of surgery included clinical stage, grade, histology and size of the tumour, number of positive axillary nodes, steroid hormone receptor status and treatment strategy (Table 2). Tumour sizes ranged from 0.1 to 15 cm, with a median of 2.2 cm. Out of the 178

Table 1 Distribution of numerical variables in the study

Variable	No. of patients	Mean \pm s.e. ^a	Median	Range
<i>KLK14</i> (arbitrary units)	178	136 \pm 22	4.28	0.00–1219
Age (years)	175	57.5 \pm 0.98	58.0	25–87
Tumour size (cm)	175	2.53 \pm 0.12	2.20	0.01–15.00
Lymph nodes ^b	160	3.6 \pm 0.5	1	0–35

^aSE, standard error. ^bNumber of lymph nodes positive for malignancy.

Table 2 Associations between *KLK14* status^a and other variables in 178 patients with epithelial breast carcinomas

Variable	No. of patients (%)			P value
	Total	<i>KLK14</i> -negative	<i>KLK14</i> -positive	
Age (years)				
<45	34	22 (64.7)	12 (35.3)	
45–55	46	34 (73.9)	12 (26.1)	0.63 ^d
>55	95	64 (67.4)	31 (32.6)	
X	3			
Menopausal status				
Pre/peri	62	44 (71.0)	18 (29.0)	0.73 ^e
Post	113	76 (67.3)	37 (32.7)	
X	3			
Tumour size (cm)				
<2	82	60 (73.2)	22 (26.8)	0.25 ^e
≥ 2	93	60 (64.5)	33 (35.5)	
X	3			
Nodal status				
Negative	67	51 (76.1)	16 (23.9)	0.17 ^e
Positive	93	61 (65.6)	32 (34.4)	
X	18			
Stage ^c				
I	74	58 (78.4)	16 (21.6)	
II	68	46 (67.6)	22 (32.4)	0.032 ^d
III	22	11 (50.0)	11 (50.0)	
X	14			
Grade ^b				
I	72	48 (66.7)	24 (33.3)	
II	51	36 (70.6)	15 (29.4)	0.89 ^e
III	49	33 (67.3)	16 (32.7)	
X	6			
Histology				
Ductal	109	73 (67.0)	36 (33.0)	
Lobular	28	17 (60.7)	11 (39.3)	0.27 ^d
Other	37	29 (78.4)	8 (21.6)	
X	4			
ER status				
Negative	62	45 (72.6)	17 (27.4)	0.49 ^e
Positive	107	71 (66.4)	36 (33.6)	
X	9			
PR status				
Negative	78	52 (66.7)	26 (33.3)	0.74 ^e
Positive	92	64 (69.6)	28 (30.4)	
X	8			
Adjuvant treatment				
None	33	26 (78.8)	7 (21.2)	
Tamoxifen	83	56 (67.5)	27 (32.5)	0.35 ^d
Chemotherapy \pm tamoxifen	59	38 (64.4)	21 (35.6)	
X	4			

^aCutoff point: 40.5, equals to 69th percentile. ^bBloom-Scarff-Richardson grading system. ^cTNM system. ^d χ^2 test. ^eFisher's Exact Test. X Status unknown.

patients, 109 (61%) had ductal carcinoma, 28 (16%) lobular carcinoma while 37 (21%) possessed other histological types. The histotype was unknown for four patients. Patients with disease of all three Stages (I–III) and tumour grades (I–III) were represented in this study, with staging determined according to the TNM classification and grading in accordance to the Bloom-Richardson grading system (Bloom and Richardson, 1957). Oestrogen and progesterone receptor status was established as described by the European Organization for Research and Treatment of Cancer (EORTC, 1980). With respect to treatment, 33 (19%) received no adjuvant treatment, 83 (47%) received tamoxifen, while 59 (33%) received chemotherapy with or without tamoxifen. This study has been approved by the Institutional Review Board of the University of Turin.

Total RNA extraction and cDNA synthesis

Tumour tissues were minced with a scalpel, on dry ice, and transferred immediately to 2 ml polypropylene tubes. They were then homogenized and total RNA was extracted using Trizol™ reagent (Gibco–BRL) following the manufacturer’s instructions. The concentration and purity of mRNA were determined spectrophotometrically. Two µg of total RNA was reverse-transcribed into first-strand cDNA using the Superscript™ pre-amplification system (Gibco–BRL). The final volume was 20 µl.

Quantitative real-time polymerase chain reaction (PCR) and continuous monitoring of PCR products

Based on the published genomic sequence of *KLK14* (GenBank accession #AF161221), two gene-specific primers were designed (6F5: 5'-AGT GGG TCA TCA CTG CTG CT-3' and 6R5: 5'-TCG TTT CCT CAA TCC AGC TT-3'). These primers spanned more than two exons to avoid contamination by genomic DNA.

Real-time monitoring of PCR reaction was performed using the LightCycler™ system (Roche Molecular Systems, Indianapolis, USA) and the SYBR green I dye, which binds preferentially to double-stranded DNA. Fluorescence signals are proportional to the concentration of the product and are measured at the end of each cycle and immediately displayed on a computer screen, permitting real time monitoring of the PCR reaction. The reaction is characterised at the point during cycling when amplification of PCR products is first detected, rather than the amount of PCR product accumulated after a fixed number of cycles. The higher the starting quantity of the template, the earlier a significant increase in fluorescence is observed. The threshold cycle is defined as the fractional cycle number at which fluorescence passes a fixed threshold above baseline.

Endogenous control

For each sample, the amount of *KLK14* cDNA and of an endogenous control (β actin, a housekeeping gene) were determined using a calibration curve (see below). The amount of *KLK14* was then divided by the amount of the endogenous reference, to obtain a normalised *KLK14* value.

Standard curve construction

The full-length mRNA sequence of the *KLK14* gene was amplified by PCR using gene-specific primers, and the PCR product was cloned into a TOPO TA cloning vector (Invitrogen, Carlsbad, CA, USA) according to the manufacturer’s instructions. A plasmid containing β -actin cDNA, was similarly prepared. Plasmids were purified using a mini-prep kit (Qiagen Inc., Valencia, CA, USA). Different standard curves for actin and *KLK14* were constructed using serial dilutions of the plasmid. These standards were included in each run. The LightCycler software automatically calculates the standard curve by plotting the starting dilution of each standard sample vs the threshold cycle, and the sample concentrations are then calculated accordingly. Standards for both *KLK14* and actin RNAs were defined to contain an arbitrary starting concentration, since no primary preparations exist. Hence, all calculated concentrations are relative to the concentration of the selected standard. Each sample was repeated twice to ensure reproducibility.

PCR amplification

The PCR reaction was carried out on the LightCycle™ system. For each run, a master mixture was prepared on ice, containing 1 µl of cDNA, 2 µl of LC DNA Master SYBR Green 1 mix, 50 ng of primers and 1.2 µl of 25 mM MgCl₂. After the reaction mixture

was loaded into the glass capillary tube, the cycling conditions were carried out as shown in Table 3.

Melting curve

For distinguishing specific from non-specific products and primer dimers, a melting curve was obtained after amplification by holding the temperature at 70°C for 30 s followed by a gradual increase in temperature to 98°C at a rate of 0.2°C per s, with the signal acquisition mode set at step. To verify the melting curve results, representative samples of the PCR products were run on 1.5% agarose gels, purified, and cloned into the pCR 2.1-TOPO vector (Invitrogen, Carlsbad, CA, USA) according to the manufacturer’s instructions. The inserts were sequenced from both directions using vector-specific primers with an automated DNA sequencer.

Statistical analysis

Patients were subdivided into groups based on different clinical or pathologic parameters (Table 2) and statistical analyses were performed using SAS software (SAS Institute, Cary, NC, USA). An optimal cutoff value was defined by χ^2 analysis based on the ability of *KLK14* values to predict the disease-free survival (DFS) and overall survival (OS) of the study population. According to this value, tumours were categorised as *KLK14*-positive or *KLK14*-negative and associations between *KLK14* status and other qualitative variables were analysed using the χ^2 or the Fisher’s Exact test, where appropriate. The cutoff value for tumour size was 2 cm. Lymph node status was either positive (any positive number of nodes) or negative. Age was categorised into three groups: less than 45 years, 45–55 years and greater than 55 years. Survival analyses were performed by constructing Kaplan–Meier disease free survival (DFS) and overall survival (OS) curves and differences between curves were evaluated by the log-rank test (Mantel, 1966), as well as by estimating the relative risks for relapse and death using the Cox proportional hazards regression model (Cox, 1972). Cox analysis was conducted at both univariate and multivariate levels. Only patients for whom the status of all variables was known were included in the multivariate regression models, which incorporated *KLK14* and all other variables for which the patients were characterised. The multivariate models were adjusted for *KLK14* expression in tumours, patient age, tumour size, stage, grade, histological type and oestrogen receptor (ER) and progesterin receptor (PR) status.

Table 3 Experimental protocol used for quantitative PCR amplification of the *KLK14* gene

Segment number	Temperature target (°C)	Hold time (s)	Slope (°C/s)	Application mode
Program:	Denaturation		Cycles:	1
1	95	600	20	None
Program:	PCR		Cycles:	35
1	95	0	20	None
2	62	5	20	None
3	72	40	20	None
4	85	5	20	Single
Program:	Melting		Cycles:	1
1	95	0	20	None
2	72	30	20	None
3	97	0	0.2	Step
Program:	Cooling		Cycles:	1
1	40	30	1	None

RESULTS

Relationship between *KLK14* expression and other parameters

KLK14 mRNA levels ranged from 0 to 1219 arbitrary units in breast cancer tissues, with a mean \pm s.e. of 136 ± 22 . The cutoff point (40.5 arbitrary units; 69th percentile) indicated that 55 (31%) of the 178 breast tumour tissues were positive for *KLK14* expression (Figure 1). Table 2 depicts the distribution of *KLK14* expression, positive or negative, in breast tumour tissues in relation to other established prognostic factors such as menopausal status, tumour size, stage, grade, histological type, nodal status, steroid receptor status and adjuvant therapy. Patients with *KLK14*-positive breast tumours more frequently had advanced stage (III) disease ($P=0.032$). Significant associations between *KLK14* expression and tumour size, grade and histology, or menopausal, nodal and steroid receptor status were not observed.

Univariate and multivariate survival analysis

The strength of association between each clinicopathological variable and DFS and OS is displayed in Table 4. In univariate analysis, patients with *KLK14*-positive tumours had a significantly increased risk of relapse (decreased DFS) and death (decreased OS) (hazards ratios of 2.31 and 2.21; $P=0.001$ and 0.005, respec-

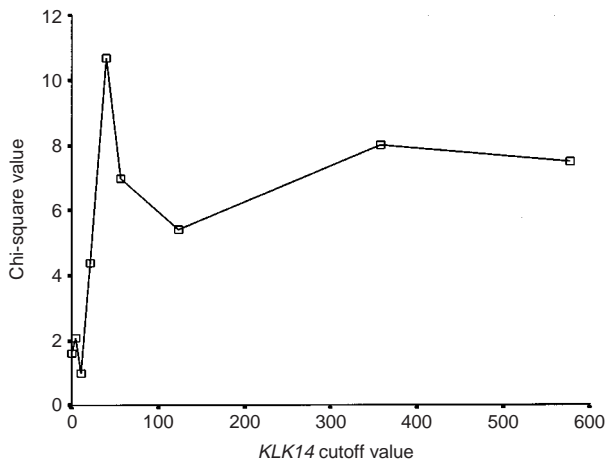


Figure 1 Determination of the optimal cutoff value for *KLK14* expression. For details, see text.

tively). Importantly, when treated as a continuous variable, *KLK14* expression retained its statistically significant association with decreased DFS and OS ($P<0.001$). Further, when survival outcomes were adjusted for all other variables in the multivariate analysis (i.e. Cox proportional hazard regression model), the adverse effects of *KLK14* positivity on DFS and OS were preserved (hazards ratios of 2.14 and 1.99; $P=0.009$ and 0.029, respectively), implying that *KLK14* expression is an independent prognostic indi-

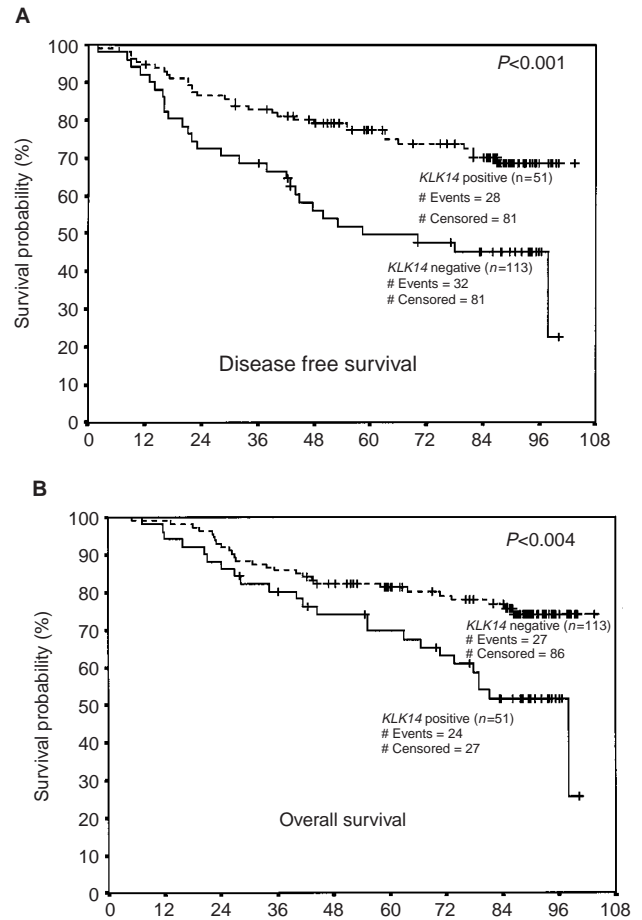


Figure 2 Kaplan–Meier survival curves for disease-free survival (A) and overall survival (B) in patients with *KLK14*-positive and negative breast tumours. *n*=number of samples.

Table 4 Univariate and multivariate analysis of *KLK14* expression in relation to DFS and OS

<i>KLK14</i> status	Disease-free survival (DFS)			Overall survival (OS)		
	HR ^a	95% CI ^b	P value	HR ^a	95% CI ^b	P value
Univariate analysis (n=164)						
Categorical variable ^c						
Negative	1.00			1.00		
Positive	2.31	1.39–3.85	0.001	2.21	1.27–3.83	0.005
Continuous variable	1.001	1.00–1.002	<0.001	1.001	1.00–1.002	<0.001
Multivariate analysis ^d (n=148)						
Categorical variable ^c						
Negative	1.00			1.00		
Positive	2.14	1.21–3.80	0.009	1.99	1.07–3.70	0.029

^aHazard ratio (HR) estimated from Cox proportional hazard regression model. ^bConfidence interval of the estimated HR. ^c*KLK14* status based on a cutoff point equal to the 69th percentile of the distribution of *KLK14* values. ^dMultivariate models were adjusted for lymph node status, tumour size, patient age, grade, histologic type, ER and PR expression.

cator. As expected, Kaplan–Meier survival curves (Figure 2) demonstrate that patients with *KLK14*-positive tumours have shorter DFS ($P<0.001$) and OS ($P=0.004$) compared to those who are *KLK14*-negative.

Univariate and multivariate survival analysis in subgroups of patients

We further examined the associations between *KLK14* expression levels and survival outcomes in subgroups of patients stratified by tumour size, nodal, OR and PR status (Table 5). *KLK14* expression significantly impacted survival in subgroups of patients with a tumour size ≤ 2 cm and positive nodal, OR and PR status. Univariate analysis revealed that *KLK14*-positive patients with a tumour size ≤ 2 cm were about three times more likely to suffer disease progression and four times more likely to die than *KLK14*-negative patients ($P=0.004$ and 0.007 , respectively). These survival differences remained significant after the data were subjected to multivariate analysis ($P=0.021$ and 0.014 , respectively). Among patients with positive nodal status, high *KLK14* expression was associated with approximately two-fold greater risk of relapse and death in both univariate ($P=0.007$ and 0.017 , respectively) and multivariate analyses ($P=0.010$ and 0.025 , respectively). Similarly, there was a tendency for an increased risk of disease progression and death in both OR and PR positive patients with *KLK14*-positive tumours. In univariate analysis, hazard ratios derived from the Cox regression model in relation to DFS and OS were 3.40 ($P=0.001$) and 3.47 ($P=0.004$) respectively, for ER positive and 3.61 ($P=0.001$) and 3.17 ($P=0.014$) for PR positive patients. Under multivariate analysis, *KLK14* retained its independent prognostic value in these subgroups of patients.

DISCUSSION

The optimal management of women with breast cancer involves a multidisciplinary approach, including the use of biological markers. Ultimately, the goal is to select the best marker or panel of markers that are most informative in terms of their ability to predict relapse, disease progression, survival and response to therapy. Traditional prognostic/predictive factors in breast cancer include tumour size, grade, nodal status, hormone receptor status, vascular invasion and age (Denley *et al*, 2001). However, only hormone receptor status has predictive value in selecting patients who are likely to respond to therapy, and is the only marker recommended for routine use by the American Society of Clinical Oncology (Smith *et al*, 2001) and the College of American Pathologists Consensus Statement (Fitzgibbons *et al*, 2000).

Many other potential biological markers have been identified as prognostic factors including p53, *c-myc*, BCL-2, HER-2, vascular endothelial growth factor (VEGF), urokinase plasminogen activator (uPA), CA 15-3, BR 27.29 and cathepsin B and D (Maguire *et al*, 1998; Duffy *et al*, 1999; Scorilas *et al*, 1999; Hamilton and Piccart, 2000; Bundred, 2001). Some markers are also predictive. For instance, expression of HER-2 has value in determining response to treatment and in selecting patients for Herceptin therapy (Ross and Fletcher, 1998). The multitude of new candidate biomarkers will likely lead to insights into the cellular changes that correlate with, or determine the different biological properties and diverse behaviour of individual breast tumours. Furthermore, stratifying patients based on the presence or absence of such markers may help to tailor different therapeutic strategies to meet individual needs (Clark *et al*, 1994).

Table 5 Associations between *KLK14* and DFS and OS in subgroups of patients stratified by tumour size, nodal status, oestrogen and progesterone receptor status

Variable	Disease-free survival (DFS)			Overall survival (OS)		
	HR ^a	95% CI ^b	P value	HR ^a	95% CI ^b	P value
Tumor size ≤ 2 cm (n=79)						
KLK14 unadjusted	3.48	1.47–8.29	0.004	4.13	1.47–11.64	0.007
KLK14 adjusted ^c	3.52	1.22–11.31	0.021	6.26	1.46–26.87	0.014
Tumor size > 2 cm (n=85)						
KLK14 unadjusted	1.66	0.88–3.13	0.11	1.51	0.77–2.92	0.22
KLK14 adjusted ^c	1.58	0.77–4.09	0.17	1.90	0.84–4.27	0.12
Node negative (n=65)						
KLK14 unadjusted	2.27	0.64–8.06	0.20	1.51	0.27–8.24	0.63
KLK14 adjusted ^d	2.01	0.46–8.71	0.35	1.89	0.29–12.15	0.49
Node positive (n=88)						
KLK14 unadjusted	2.24	1.24–4.06	0.007	2.12	1.14–3.95	0.017
KLK14 adjusted ^d	2.31	1.21–4.34	0.010	2.13	1.09–4.14	0.025
ER negative (n=59)						
KLK14 unadjusted	1.83	0.84–3.98	0.12	1.97	0.90–4.33	0.087
KLK14 adjusted ^e	0.89	0.33–2.34	0.82	0.92	0.33–2.52	0.88
ER positive (n=99)						
KLK14 unadjusted	3.40	1.65–6.99	0.001	3.47	1.49–8.05	0.004
KLK14 adjusted ^e	3.14	1.26–7.79	0.014	2.88	1.01–8.18	0.047
PR negative (n=72)						
KLK14 unadjusted	1.68	0.85–3.34	0.13	1.83	0.91–3.69	0.088
KLK14 adjusted ^f	1.27	0.59–2.74	0.53	1.31	0.06–2.89	0.49
PR positive (n=87)						
KLK14 unadjusted	3.61	1.63–7.97	0.001	3.17	1.25–8.06	0.014
KLK14 adjusted ^f	6.04	2.19–16.61	0.001	4.45	1.37–14.39	0.012

^aHazard ratio (HR) estimated from Cox proportional hazard regression model. ^bConfidence interval of the estimated HR. ^cMultivariate models were adjusted for tumor grade, nodal status, ER, PR, histologic type and age. ^dMultivariate models were adjusted for tumour size, grade, ER, PR, histologic type and age. ^eMultivariate models were adjusted for tumour size, grade, nodal status, PR, histologic type and age. ^fMultivariate models were adjusted for tumour size, grade, nodal status, ER, histologic type and age.

In this paper, we identify *KLK14* as a new independent marker of unfavourable prognosis in breast cancer. Patients with *KLK14*-positive breast tumours were more likely to have advanced stage (III) disease. When assessing *KLK14* expression in terms of predicting survival outcomes, we found an increased risk of relapse and death for patients with *KLK14*-positive tumours. This was also observed in subgroups of patients with a tumour size ≤ 2 cm, positive nodal, OR and PR status. Hence, *KLK14* expression may aid in predicting relapse, disease progression and/or survival in breast cancer patients.

Interestingly, the results obtained for *KLK14* in this study are comparable to those obtained for *KLK3*, *KLK6* and *KLK15* in breast cancer (Yu et al, 1995; Anisowicz et al, 1996) and *KLK4*, *KLK5* and *KLK10* in ovarian cancer (Kim et al, 2001; Luo et al, 2001b; Obiezu et al, 2001), in that high expression of these kallikrein genes also correlated with patient prognosis. Similar to *KLK14*, these genes are also under steroid hormone regulation (Yousef and Diamandis, 2001). These observations allow us to speculate that multiple kallikreins may participate in a common enzymatic pathway that plays a role in the normal physiology of the breast. This pathway may be deregulated in breast carcinogenesis. As is the case with many other serine proteases, certain kallikreins may degrade the extracellular matrix promoting tumour invasiveness and metastasis.

It was recently realised that *KLK14* is under steroid hormone regulation, particularly androgens and progestins, and that these effects are mediated through the androgen receptor (our unpublished data). It is also known that breast cancer is a hormone-

dependent malignancy (Russo and Russo, 1998) and that androgen receptors are present in 70–90% of primary breast tumours (Soreide et al, 1992), and 75% of breast cancer metastases (Lea et al, 1989). Thus, it is likely that the androgen receptor, acting as a ligand-activated transcription factor, upregulates *KLK14* gene expression during breast carcinogenesis.

The role of androgens in the aetiology of breast cancer is ill-defined. *In vitro*, they both stimulate (MCF-7, MDA-453) or inhibit (T-47D, ZR-75-1, MFM-223) the growth of AR-positive breast cancer cell lines (Hackenberg et al, 1991; Birrell et al, 1995). Animal studies have demonstrated that androgens shorten the latency period, enhance tumour size and increase the incidence of breast tumours, by promoting rather than initiating carcinogenesis in rodents (Liao et al, 1998; Xie et al, 1999). These effects may be mediated by androgen-regulated genes that directly function in cell growth regulation and through interaction of the AR with other transcription regulators, allowing for cross-talk with other growth pathways (Brys, 2000). Thus, the identification of androgen regulated genes, such as *KLK14*, may help to define new targets for breast cancer treatment. Such new treatments may be particularly important in metastatic disease, where the AR is often the sole steroid receptor expressed.

In summary, we quantified *KLK14* expression in breast tumours and found that high *KLK14* expression is associated with decreased DFS and OS in both univariate and multivariate analysis. Additional basic and clinical studies are required to delineate the activity of *KLK14* in both the normal and malignant breast and to further define the clinical value of this biomarker.

REFERENCES

- Anisowicz A, Sotiropoulou G, Stenman G, Mok SC, Sager R (1996) A novel protease homolog differentially expressed in breast and ovarian cancer. *Mol Med* **2**: 624–636
- Barry MJ (2001) Clinical practice. Prostate-specific-antigen testing for early diagnosis of prostate cancer. *N Engl J Med* **344**: 1373–1377
- Birrell SN, Bentel JM, Hickey TE, Ricciardelli C, Weger MA, Horsfall DJ, Tilley WD (1995) Androgens induce divergent proliferative responses in human breast cancer cell lines. *J Steroid Biochem Mol Biol* **52**: 459–467
- Bloom HJG, Richardson WW (1957) Histological grading and prognosis in breast cancer. *Br J Cancer* **11**: 359–377
- Brys M (2000) Androgens and androgen receptor: do they play a role in breast cancer? *Med Sci Monit* **6**: 433–438
- Bundred NJ (2001) Prognostic and predictive factors in breast cancer. *Cancer Treat Rev* **27**: 137–142
- Clark GM, Hilsenbeck SG, Ravdin PM, De Laurentiis M, Osborne CK (1994) Prognostic factors: rationale and methods of analysis and integration. *Breast Cancer Res Treat* **32**: 105–112
- Cox DR (1972) Regression models and life tables. *R Stat Soc B* **34**: 187–202
- Denley H, Pinder SE, Elston CW, Lee AH, Ellis IO (2001) Preoperative assessment of prognostic factors in breast cancer. *J Clin Pathol* **54**: 20–24
- Diamandis EP (1998) Prostate-specific antigen – its usefulness in clinical medicine. *Trends Endocrinol Metab* **9**: 310–316
- Diamandis EP, Okui A, Mitsui S, Luo LY, Soosaipillai A, Grass L, Nakamura T, Howarth DJ, Yamaguchi N (2002) Human kallikrein 11: a new biomarker of prostate and ovarian carcinoma. *Cancer Res* **62**: 295–300
- Diamandis EP, Yousef GM (2001) Human tissue kallikrein gene family: a rich source of novel disease biomarkers. *Expert Rev Mol Diagn* **1**: 182–190
- Diamandis EP, Yousef GM, Clements J, Ashworth LK, Yoshida S, Egelrud T, Nelson PS, Shiosaka S, Little S, Lilja H, Stenman UH, Rittenhouse HG, Wain H (2000a) New nomenclature for the human tissue kallikrein gene family. *Clin Chem* **46**: 1855–1858
- Diamandis EP, Yousef GM, Luo LY, Magklara A, Obiezu CV (2000b) The new human kallikrein gene family: implications in carcinogenesis. *Trends Endocrinol Metab* **11**: 54–60
- Diamandis EP, Yousef GM, Soosaipillai AR, Bunting P (2000c) Human kallikrein 6 (zyme/protease M/neurosin): a new serum biomarker of ovarian carcinoma. *Clin Biochem* **33**: 579–583
- Duffy MJ (1991) The role of proteolytic enzymes in cancer invasion and metastasis. *Clin Exp Metast* **10**: 145–155
- Duffy MJ (2001) Biochemical markers in breast cancer: which ones are clinically useful? *Clin Biochem* **34**: 347–352
- Duffy MJ, Maguire TM, McDermott EW, O'Higgins N (1999) Urokinase plasminogen activator: a prognostic marker in multiple types of cancer. *J Surg Oncol* **71**: 130–135
- EORTC (1980) Revision of the standards for the assessment of hormone receptors in human breast cancer; report of the second EORTC Workshop, held on 16–17 March, 1979, in the Netherlands Cancer Institute. *Eur J Cancer* **16**: 1513–1515
- Fitzgibbons PL, Page DL, Weaver D, Thor AD, Allred DC, Clark GM, Ruby SG, O'Malley F, Simpson JF, Connolly JL, Hayes DF, Edge SB, Lichter A, Schnitt SJ (2000) Prognostic factors in breast cancer. College of American Pathologists Consensus Statement 1999. *Arch Pathol Lab Med* **124**: 966–978
- Gottesman M (1990) The role of proteases in cancer. *Semin Cancer Biol* **1**: 97–160
- Hackenberg R, Luttmann S, Hofmann J, Kunzmann R, Holzel F, Schulz KD (1991) Androgen sensitivity of the new human breast cancer cell line MFM-223. *Cancer Res* **51**: 5722–5727
- Hamilton A, Piccart M (2000) The contribution of molecular markers to the prediction of response in the treatment of breast cancer: a review of the literature on HER-2, p53 and BCL-2. *Ann Oncol* **11**: 647–663
- Herszenyi L, Plebani M, Carraro P, De Paoli M, Roveroni G, Cardin R, Tulasay Z, Naccarato R, Farinati F (1999) The role of cysteine and serine proteases in colorectal carcinoma. *Cancer* **86**: 1135–1142
- Hooper JD, Bui LT, Rae FK, Harvey TJ, Myers SA, Ashworth LK, Clements JA (2001) Identification and characterization of *klk14*, a novel kallikrein serine protease gene located on human chromosome 19q13.4 and expressed in prostate and skeletal muscle. *Genomics* **73**: 117–122
- Jemal A, Thomas A, Murray T, Thun M (2002) Cancer statistics, 2002. *CA Cancer J Clin* **52**: 23–47
- Kim H, Scorilas A, Katsaros D, Yousef GM, Massobrio M, Fracchioli S, Piccinno R, Gordini G, Diamandis EP (2001) Human kallikrein gene 5 (*KLK5*) expression is an indicator of poor prognosis in ovarian cancer. *Br J Cancer* **84**: 643–650

- Kuhn W, Schmalfeldt B, Reuning U, Pache L, Berger U, Ulm K, Harbeck N, Spathe K, Dettmar P, Hofler H, Janicke F, Schmitt M, Graeff H (1999) Prognostic significance of urokinase (uPA) and its inhibitor PAI-1 for survival in advanced ovarian carcinoma stage FIGO IIIc. *Br J Cancer* **79**: 1746–1751
- Lea OA, Kvinnsland S, Thorsen T (1989) Improved measurement of androgen receptors in human breast cancer. *Cancer Res* **49**: 7162–7167
- Liao DZ, Pantazis CG, Hou X, Li SA (1998) Promotion of estrogen-induced mammary gland carcinogenesis by androgen in the male Noble rat: probable mediation by steroid receptors. *Carcinogenesis* **19**: 2173–2180
- Luo LY, Bunting P, Scorilas A, Diamandis EP (2001a) Human kallikrein 10: a novel tumor marker for ovarian carcinoma? *Clin Chim Acta* **306**: 111–118
- Luo LY, Katsaros D, Scorilas A, Fracchioli S, Piccinno R, Rigault de la Longrais IA, Howarth DJ, Diamandis EP (2001b) Prognostic value of human kallikrein 10 expression in epithelial ovarian carcinoma. *Clin Cancer Res* **7**: 2372–2379
- Luo LY, Rajpert-De Meyts ER, Jung K, Diamandis EP (2001c) Expression of the normal epithelial cell-specific 1 (NES1; KLK10) candidate tumour suppressor gene in normal and malignant testicular tissue. *Br J Cancer* **85**: 220–224
- Magklara A, Scorilas A, Catalona WJ, Diamandis EP (1999) The combination of human glandular kallikrein and free prostate-specific antigen (PSA) enhances discrimination between prostate cancer and benign prostatic hyperplasia in patients with moderately increased total PSA. *Clin Chem* **45**: 1960–1966
- Magklara A, Scorilas A, Katsaros D, Massobrio M, Yousef GM, Fracchioli S, Danese S, Diamandis EP (2001) The human KLK8 (neuropsin/ovasin) gene: identification of two novel splice variants and its prognostic value in ovarian cancer. *Clin Cancer Res* **7**: 806–811
- Maguire TM, Shering SG, Duggan CM, McDermott EW, O'Higgins NJ, Duffy MJ (1998) High levels of cathepsin B predict poor outcome in patients with breast cancer. *Int J Biol Markers* **13**: 139–144
- Mantel N (1966) Evaluation of survival data and two new rank order statistics arising in its consideration. *Cancer Chemother Rep* **50**: 163–170
- Obiezu CV, Scorilas A, Katsaros D, Massobrio M, Yousef GM, Fracchioli S, Rigault de la Longrais IA, Arisio R, Diamandis EP (2001) Higher human kallikrein gene 4 (KLK4) expression indicates poor prognosis of ovarian cancer patients. *Clin Cancer Res* **7**: 2380–2386
- Parkin DM, Pisani P, Ferlay J (1999) Estimates of the worldwide incidence of 25 major cancers in 1990. *Int J Cancer* **80**: 827–841
- Peto R, Boreham J, Clarke M, Davies C, Beral V (2000) UK and USA breast cancer deaths down 25% in year 2000 at ages 20–69 years. *Lancet* **355**: 1822
- Rittenhouse HG, Finlay JA, Mikolajczyk SD, Partin AW (1998) Human Kallikrein 2 (hK2) and prostate-specific antigen (PSA): two closely related, but distinct, kallikreins in the prostate. *Crit Rev Clin Lab Sci* **35**: 275–368
- Ross JS, Fletcher JA (1998) The HER-2/neu oncogene in breast cancer: Prognostic factor, predictive Factor, and target for therapy. *Oncologist* **3**: 237–252
- Russo IH, Russo J (1998) Role of hormones in mammary cancer initiation and progression. *J Mammary Gland Biol Neoplasia* **3**: 49–61
- Scorilas A, Yotis J, Pateras C, Tragas T, Talieri M (1999) Predictive value of c-erbB-2 and cathepsin-D for Greek breast cancer patients using univariate and multivariate analysis. *Clin Cancer Res* **5**: 815–821
- Smith RA, Von Eschenbach AC, Wender A, Levin B, Byers T, Rothenberger D, Brooks D, Creasman W, Cohen C, Runowicz C, Saslow D, Cokkinides V, Eyre H (2001) American Cancer Society guidelines for early detection of cancer: update of early detection guidelines for prostate, colorectal, and endometrial cancers, and update 2001: testing for early lung cancer detection. *CA Cancer J Clin* **51**: 7–38
- Soreide JA, Lea OA, Varhaug JE, Skarstein A, Kvinnsland S (1992) Androgen receptors in operable breast cancer: relation to other steroid hormone receptors, correlations to prognostic factors and predictive value for effect of adjuvant tamoxifen treatment. *Eur J Surg Oncol* **18**: 112–118
- Xie B, Tsao SW, Wong YC (1999) Sex hormone-induced mammary carcinogenesis in female noble rats: the role of androgens. *Carcinogenesis* **20**: 1597–1606
- Yousef GM, Chang A, Diamandis EP (2000a) Identification and characterization of KLK-L4, a new kallikrein-like gene that appears to be down-regulated in breast cancer tissues. *J Biol Chem* **275**: 11891–11898
- Yousef GM, Chang A, Scorilas A, Diamandis EP (2000b) Genomic organization of the human kallikrein gene family on chromosome 19q13.3-q13.4. *Biochem Biophys Res Commun* **276**: 125–133
- Yousef GM, Magklara A, Diamandis EP (2000c) KLK12 is a novel serine protease and a new member of the human kallikrein gene family – differential expression in breast cancer. *Genomics* **69**: 331–341
- Yousef GM, Diamandis EP (2001) The new human tissue kallikrein gene family: structure, function, and association to disease. *Endocr Rev* **22**: 184–204
- Yousef GM, Kyriakopoulou LG, Scorilas A, Fracchioli S, Ghiringhello B, Zarghooni M, Chang A, Diamandis M, Giardina G, Hartwick WJ, Richiardi G, Massobrio M, Diamandis EP, Katsaros D (2001a) Quantitative expression of the human kallikrein gene 9 (KLK9) in ovarian cancer: a new independent and favorable prognostic marker. *Cancer Res* **61**: 7811–7818
- Yousef GM, Magklara A, Chang A, Jung K, Katsaros D, Diamandis EP (2001b) Cloning of a new member of the human kallikrein gene family, KLK14, which is down-regulated in different malignancies. *Cancer Res* **61**: 3425–3431
- Yousef GM, Scorilas A, Jung K, Ashworth LK, Diamandis EP (2001c) Molecular cloning of the human kallikrein 15 gene (KLK15). Up-regulation in prostate cancer. *J Biol Chem* **276**: 53–61
- Yousef GM, Diamandis EP (2002) Human kallikreins: common structural features, sequence analysis and evolution. *Curr Genom In Press*
- Yu H, Giai M, Diamandis EP, Katsaros D, Sutherland DJ, Levesque MA, Roagna R, Ponzzone R, Sismondi P (1995) Prostate-specific antigen is a new favorable prognostic indicator for women with breast cancer. *Cancer Res* **55**: 2104–2110