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Down-regulation of hK7 in the sera of breast cancer and benign breast disease patients

Samina Ejaz^a, Faiz-ul-Hassan Nasim^{b,*}, Muhammad Ashraf^b, Gulzar Ahmad^c

^a Department of Biochemistry and Biotechnology, The Islamia University of Bahawalpur, Pakistan

^b Department of Chemistry, The Islamia University of Bahawalpur, Bahawalpur, Pakistan

^c Department of Surgery, Quaid-i-Azam Medical College, Bahawal Victoria Hospital, Bahawalpur, Pakistan

* Corresponding author.

E-mail address: faiz.nasim@iub.edu.pk (F.-u.-H. Nasim).

Abstract

Introduction: Breast cancer is known as a leading cause of cancer-related death among women all over the world. Biomarkers facilitate diagnosis at the earliest possible stage and better prognosis of the disease. Hence, may help to improve the overall survival rate among breast cancer patients. To find a better diagnostic/prognostic marker we evaluated human tissue kallikrein 7 (hK7) as biomarker of breast cancer. hK7 is a secreted serine protease having chymotrypsin like activity. Serum hK7 is known to have aberrant expression in ovarian and prostate cancer but has not been yet studied in breast cancer. However, the expression level of KLK7 mRNA in breast cancer tissues has been indicated as a better prognostic marker for the unfavorable prognosis of breast carcinoma.

Materials and methods: In this study a time-resolved immunofluorometric indirect back titration ELISA (bt-ELISA) was employed for the quantification of hK7 in serum of breast cancer patients (n = 47), benign breast disease patients (n = 13) alongwith the gender and age group specific controls (n = 99).

Results: hK7 was significantly down-regulated in the sera of female breast cancer patients ($p < 0.0001$; Mean 0.704 ± 0.533 $\mu\text{g/L}$) and benign breast disease patients ($p = 0.0008$; Mean 0.651 ± 0.584) as compared to normal controls (Mean 1.665 ± 1.174 $\mu\text{g/L}$).

Conclusions: Down regulation of hK7 suggests the possible role of this protein in natural course of breast cancer and benign breast diseases. Study should be

extended on large-scale to confirm the potential of hK7 as biomarker of breast cancer.

Keywords: Cancer research, Oncology, Medicine

1. Introduction

Globally, breast cancer is known as the third most common type of cancer and is a leading cause of death among women. According to the WHO report annually more than one million cases of breast cancer are reported globally. Of which about 58% (580,000) cases are detected in developed countries and 42% (420,000) in developing countries [1]. North America, Europe and Australia have been identified as high risk areas in world [2, 3]. Based on breast cancer incidence rate in 2000–2014, American Cancer Society has estimated 255,180 new cases of breast cancer and 41,070 deaths due to breast cancer in 2017 [4]. Among Asian countries Pakistan has the highest prevalence of breast cancer with the annual death rate of about 40,000 women die per year [5, 6]. It is reported that only a small fraction (i.e., 10%) of the total annually reported cases of breast cancer (i.e., 90,000) are diagnosed and treated while most (75%) of the patients do not go for treatment and after diagnosis survive for less than five years [5, 7].

Currently diagnosis of breast cancer is being carried out by conventional non-invasive (mammography, palpation, and MRI scan) and invasive methods (biopsy and histopathological examination). All these methods have their own limitations like small lesions (i.e., <0.5 cm) cannot be detected using available imaging techniques and mammogram is not useful for diagnosis in younger women having higher breast density. Histopathological examination is only beneficial when the normal cells are completely transformed into morphologically distinct malignant cells [8, 9].

Before the onset of disease oncogenic changes occurring inside the cells can be identified at the earliest possible stage through molecular methods involving studying genes, transcripts and proteins [10]. Prior to the onset of the disease, certain changes take place inside the cells that are measureable at the molecular level and are known as biomarkers. A biomarker is a molecular entity like a gene, protein or a metabolite present in the blood or tissues. Biomarkers are useful for the early detection, diagnosis and prognosis of the diseases [11, 12]. Blood-based molecular tests are preferred for screening due to the involvement of non-invasive ways of sample collection and minimum patient participation. Moreover, overall cost and possible patient risk are also reduced [13, 14].

Early detection of breast cancer significantly contributes to enhance the survival rate among breast cancer patients. Overall survival rate of breast cancer patients is influenced by the stage at which breast cancer is diagnosed. According to

American Cancer Society 99% of the women diagnosed with localized breast cancer survive for at least 5 years. However, survival rate is declined to 84% in patients suffering from regional stage (with involved nearby tissues or lymph nodes) and 24% in distant stage (cancer spread to distant lymph nodes or organs) breast cancer patients [15]. Due to the absence of reliable serum/plasma biomarker majority of the breast cancer patients are diagnosed at the stage when the tumor has been metastasized [8, 16].

Human tissue Kallikreins (KLKs) is a family of 15 genes (KLK1-KLK15) encoding serine proteases (hK1-hK15). Except KLK1 and KLK2, all KLKs have been implicated in the pathogenesis of breast cancer [17, 18, 19]. KLK7 encodes hK7, stratum corneum chymotryptic enzyme (SCCE), was originally purified from the stratum corneum of human skin. It is known to catalyze the degradation of intercellular cohesive structures present in the outermost layer of the skin and thus contributes to the cell shedding process at the skin surface [20]. It is known that the degradation of cell-cell adhesion molecules and components of extracellular matrix facilitate invasion of tumor cells. Due to the ability to cleave cell-cell adhesion proteins including extracellular matrix molecules like fibronectin and E-cadherin hK7/hSCCE is suggested to promote cancer progression [21, 22, 23]. The suggested role of hK7 is supported by the report that hK7 promotes invasion of pancreatic tumor cells by shedding E-cadherin [23].

According to GenBank record total 11 alternative transcripts of KLK7 gene have been reported and only three (i.e., AY646152.1, AK295313.1 and AF411215.1) can be categorized as alternatively spliced transcripts/isoforms. Protein coding region of KLK7 mRNA consists of last 73 nucleotides of E2, E3, E4 and 156 nucleotides of E5).

Higher expression level of hK7 has been noticed in the serum of normal males (i.e., range: 1.9–5.4 µg/L, mean (SD): 2.9(0.9)) as compared to the normal healthy female individuals (range: 1.5–4.0 µg/L, mean (SD): 2.3(0.7)). A weak negative correlation was identified between serum hK7 concentration and age of healthy subjects (both male and female). It was further proposed that androgens might be responsible for the down regulated expression of hK7 in males [24].

Differential expression of hK7 has been observed in many types of carcinomas. There is a report of hK7 up-regulation in late stage serous ovarian cancer [25] and cervical adenocarcinoma [26]. Conversely down regulation of hK7 has been noticed in prostate cancer [27] and adenocarcinoma of lung [28]. To our knowledge in the published scientific literature there is no information about level of hK7 in the sera of breast cancer patients. However, KLK7 mRNA expression level in breast cancer tissues has been estimated using RT-PCR and declared as a better prognostic marker for the unfavorable prognosis of breast carcinoma. KLK7 mRNA was considerably decreased in either stage I or stage II breast cancer

patients and raised level of KLK7 mRNA exhibited association with better prognosis [29, 30].

We conducted this study with the aim to evaluate the potential of serum hK7 as biomarker of breast cancer.

2. Materials and methods

2.1. Ethical approval and consent to participate

The study was approved by proper institutional technical committees including the departmental Ethics and Research Committee and by the Advanced Studies and Research Board (ASRB) of the Islamia University of Bahawalpur, Pakistan. Written Consent to take part in the study was taken on a prescribed form from all patients included in the study. The experimental work involving humans was in complete compliance with the Helsinki Declaration.

2.2. Sample collection

Samples were collected from breast cancer and benign breast disease patients presented at BVH (Bahawalpur Victoria Hospital), Pakistan after getting due consent of patients on Performa designed to record the demographic information of patients. Similarly blood samples were collected from apparently healthy normal individuals from urban and rural areas of Bahawalpur after the consent was granted. Standard procedure was followed to obtain sera from the collected blood samples and sera obtained were aliquoted and stored at -70°C . Patients found positive for HBV/HCV/HIV or any other infectious disease were excluded from the main cohort of the patients to evaluate the potential of hK7 as biomarker of breast cancer.

2.3. Quantification of human serum hK7

Sera of patients suffering from breast cancer ($n = 31$), and other benign breast lesions or complications ($n = 13$) along with apparently healthy female ($n = 47$) and male ($n = 51$) subjects were analyzed to estimate hK7. Patients were grouped into non-chemotherapeutically treated ($n = 31$) and chemotherapeutically treated ($n = 8$) patient groups. Few breast cancer patients found positive for hepatitis B or C infection ($n = 7$) were excluded from the patient group and handled as a separate group. All these precautions helped to document the impartial expression behavior of hK7 in breast carcinoma and other benign breast complications and exclude the possible contribution of any other disease or reason if any towards the altered expression level of hK7. One breast cancer patient was HBV positive and had undergone chemotherapeutic treatment. Hence, it was neither placed in the chemotherapeutically treated patient group nor in the HCV/HBV positive breast

cancer patients' group. Results of the patients group were compared with gender and age group specific controls.

For quantification of hK7 in the sera of normal controls and patients (breast cancer and benign breast disease patients) an already optimized, highly specific, sensitive and an anti-hK7 monoclonal antibody (KLK7 (1407), sc-80148, Lot # 11610, Santa Cruz Biotechnology Inc., USA) based time resolved immunofluorometric (TRIF) back titration ELISA was employed. Principal and procedure of the method has been discussed in detail somewhere else [31]. KLK7 (1407), sc-80148 mouse monoclonal antibody was raised against 23–252 amino acids of human hK7. As a result of alternative splicing only a part of this region alters (data not shown), hence it can be used to detect all known alternate isoforms of hK7.

Calibration curve was drawn using variable concentrations (i.e., ranging from 1–1000 pg/100 μ L) of the purified hK7 peptide (Cat # ab41385, Lot # 275205) purchased from Abcam. Calibration curve was drawn using values of the 6 parallel runs of reproducible results (Fig. 1). All estimations of serum hK7 were carried out in triplicate.

2.4. Statistical analysis of data

Statistical analysis was carried out by employing SPSS 18.0 (with Confidence Interval CI = 0.95) and MedCalc 12.2.0.0. To find the normality distribution data was subjected to D'Agostino-Pearson test. Depending upon the Gaussian or non-Gaussian nature of the data appropriate parametric or non-parametric test was

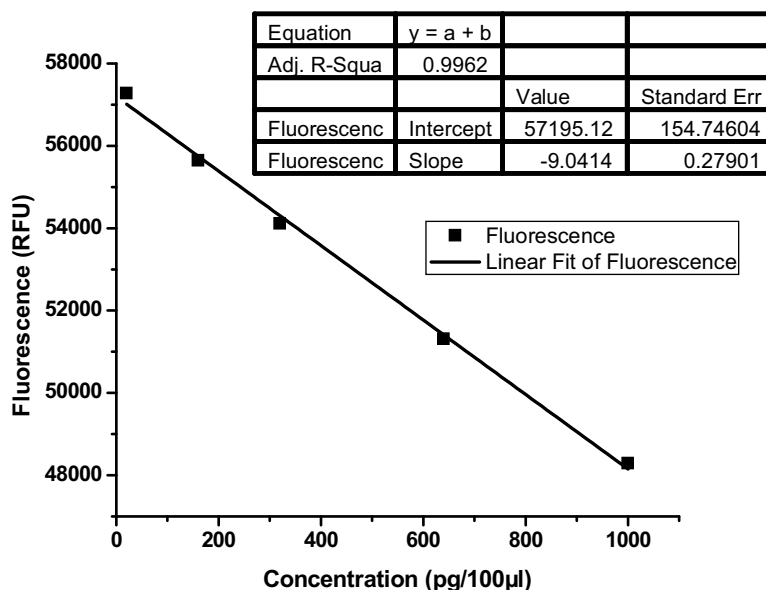


Fig. 1. Calibration curve for the quantification of hK7 by TRIF competitive ELISA (RFU = Relative Fluorescence Units).

selected for the calculation of p value. For normally distributed/Gaussian data p value was calculated through independent sample t test whereas Mann-Whitney U test was employed to compute the p value of non-Gaussian data.

Extended statistical analysis including ROC (Receiver Operating Characteristics) curve analysis and logistic regression analysis (both univariate and multivariate) helped to find out the predictive power of hK7 expression level to predict the risk of developing the particular disease (i.e., breast cancer or asthma). Significance level of AUC (Area Under the Curve) in ROC curve analysis was used to evaluate the discriminating power of KLK protein expression level to discriminate diseased subjects from normal healthy individuals.

Scatter plots were drawn to study the distribution pattern of hK7 expression level in patients as well as normal controls (data not shown). Nature of association between hK7 expression level and age of the subject either diseased or normal was accessed through correlation and regression coefficients. For non-Gaussian data Spearman's rho (ρ), and Kendall's tau (τ) rank correlation coefficients were determined but for Gaussian or normally distributed data Pearson correlation coefficient was documented.

3. Results

Expression of hK7 has been detected in all the sera of breast cancer ($n = 47$) and benign breast diseases patients ($n = 13$). However, only one normal female control was found to have hK7 below the detection limit of the assay.

3.1. Diagnostic potential and disease prediction power of serum hK7 expression level

To reduce the gender specific variations, standard approach of comparing the pathological parameters with gender specific controls was preferred and data of hK7 expression level in breast cancer/benign breast diseases patients was compared with gender specific normal controls.

Result of D'Agostino-Pearson test for normal distribution was 'reject normality' ($p = 0.0002$) for hK7 expression level data. Therefore hK7 data subjected to non-parametric statistical analysis test. In the present study serum hK7 concentration of apparently healthy normal female controls ($n = 47$) was found to be 0.0030–3.247 $\mu\text{g/L}$ (range) with median 1.021 $\mu\text{g/L}$. Our results are far below and above the earlier reported serum hK7 concentration (range 1.5–4.0, median 2.1 $\mu\text{g/L}$) in normal female individuals [24]. hK7 was down-regulated (p value < 0.005 ; Table 1 and Fig. 2) in the sera of female breast cancer patients (mean 0.0656 ± 0.477 , median 0.624 $\mu\text{g/L}$) and patients with other breast diseases like inflammatory lesions or benign tumors (mean 0.651 ± 0.584 , median 0.335 $\mu\text{g/L}$).

Table 1. Serum hK7 concentration in breast cancer and benign breast disease patients.

Serum	Tested samples (n)	Range ($\mu\text{g/L}$)	Mean (SD) ($\mu\text{g/L}$)	Median ($\mu\text{g/L}$)	<i>p</i> value
Breast Cancer Patients					
Females	31	0.029–1.965	0.656 (0.477)	0.624	0.0061 ^a
Other breast complications					
Female	13	0.004–1.962	0.651 (0.584)	0.335	0.0238 ^a 0.6996 ^b
Normal Controls					
Females	47	0.0030–3.247	1.237 (0.929)	1.066	
Total	91				

Data was evaluated at CI = 0.95 *p* value was calculated by Mann-Whitney U test.

^aWith respect to gender specific normal controls.

^bWith respect to breast cancer patients who have not been treated chemotherapeutically.

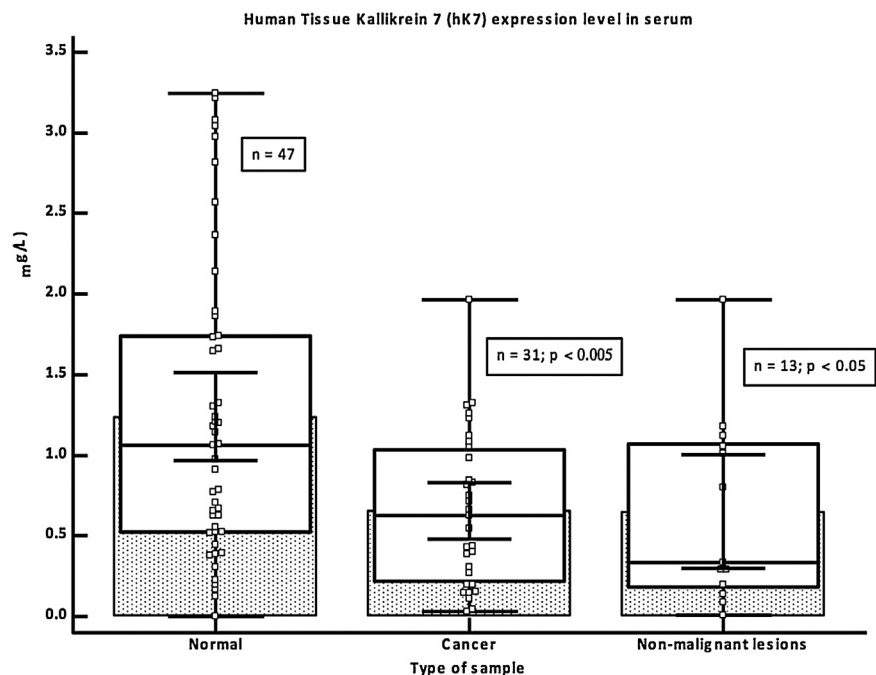


Fig. 2. A comparison of hK7 concentration in the serum of normal female and females suffering from breast cancer and other non-malignant breast lesions (Box and Whisker plot). In this graph horizontal line extends from the minimum to maximum, central box represents the values from lower to upper quartile (i.e., 25 to 75 percentile), middle line of the central box represents the median, horizontal line inside the central box represents 95% CI for mean. Dotted area indicates error bars for the mean, small box along the horizontal line directly correspond to the distribution of samples w.r.t serum hK7 concentration.

L, $p < 0.05$) as compared to normal controls (mean 1.211 ± 0.936 , median $1.021 \mu\text{g/L}$).

The focus of the current study was to search a better diagnostic marker for breast cancer. We were interested to know if hK7 quantification can be used as a clinical test for the discrimination of normal females from breast cancer patients? For this it was necessary to find out the predictive value of hK7 expression down-regulation. The clinical value of the down-regulated expression of hK7 for the prediction of breast cancer was determined by ROC (receiver operating curve) analysis combined with logistic regression analysis (both univariate and bivariate). AUC (Area under the curve) in ROC analysis was found to be 0.684 with $p = 0.0021$ indicating that hK7 quantification is a good diagnostic test with potential for the discrimination of normal females from female breast cancer patients. Univariate logistic regression analysis further validated that there is a negative correlation ($p = 0.0323$) between the expression level of hK7 and risk of breast cancer. But the results of bivariate analysis showed that hK7 expression level cannot be a better diagnostic test ($p = 0.0556$) in relation to the age of the patient (Table 2, Fig. 3).

The diagnostic potential of hK7 expression level for the prediction of non-malignant breast lesions, ROC and logistic regression analysis were carried out. In ROC analysis AUC was 0.706 with p value 0.0114. Both, univariate and bivariate logistic regression analysis has predicted that hK7 expression level does not help for better discrimination of the patients with non-malignant breast lesions from normal healthy individuals (Table 3 and Fig. 4).

Similarly ROC curve analysis and logistic regression analysis showed that hK7 down-regulation as a test is not suitable for discrimination of breast cancer patients from patients having non-malignant breast lesions (Table 4 and Fig. 5). Age is negatively correlated with the risk of breast cancer ($p < 0.0001$) but no such association was observed in case of non-malignant breast lesions patients (Tables 2, 3, 4).

Table 2. Logistic regression analysis of hK7 expression level for the prediction of breast cancer in females.

Variables	Crude Odds Ratio	SE	95% CI	p	Coefficient	Overall Model Fit
<i>Univariate Analysis</i>						
Log [hK7]	0.311	0.546	0.1067–0.9062	0.0323	-1.168	$p = 0.0195$
Age	1.076	0.019	1.0361–1.1181	0.0001	0.073	$p < 0.0001$
<i>Bivariate Analysis</i>						
Log [hK7]	0.313	0.606	0.0955–1.0282	0.0556	-1.603	$p < 0.0001$
Age	1.075	0.019	1.035–1.1173	0.0002	0.073	

Data was evaluated at $CI = 0.95$.

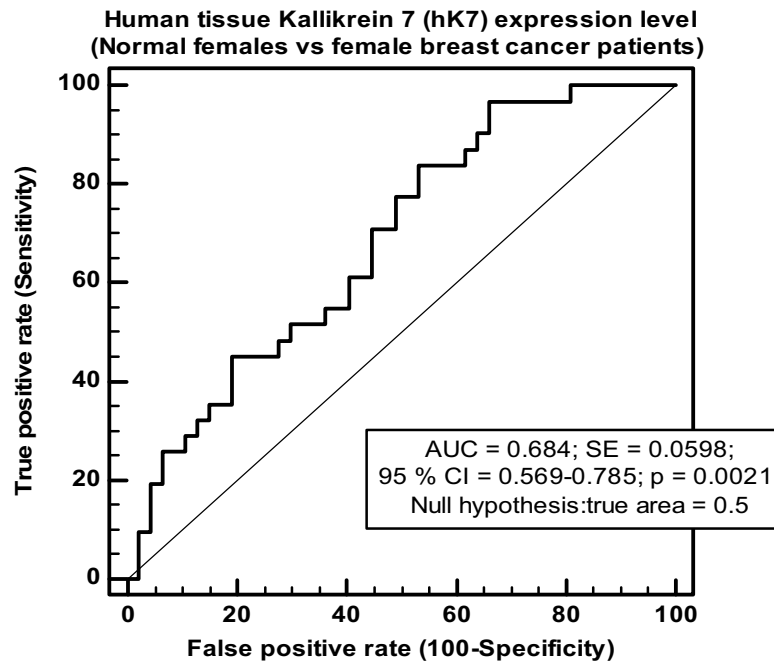


Fig. 3. ROC curve analysis of hK7 quantification for discrimination between normal females and female breast cancer patients. AUC was analyzed by Hanley and McNeil (1982) method, CI is binomial exact confidence interval.

3.2. Correlation of hK7 expression levels with various clinico-pathological features

The expression level of hK7 was significantly lower in the female breast cancer patients as compared to the normal female controls (Table 1). It was important to further explore the correlation of hK7 expression level with different clinico-pathological features of breast cancer and benign breast diseases to understand the behavior of hK7 in the natural course of breast cancer and other breast diseases.

Table 3. Logistic regression analysis of hK7 expression level for the prediction of benign breast diseases in females.

Variables	Crude Odds Ratio	SE	95% CI	p	Coefficient	Overall Model Fit
<i>Univariate Analysis</i>						
Log [hK7]	0.344	0.550	0.1171–1.0098	0.0521	-1.068	p = 0.0376
Age	1.038	0.022	0.9948–1.0839	0.0850	0.038	p = 0.0834
<i>Bivariate Analysis</i>						
Log [hK7]	0.341	0.574	0.1108–1.0515	0.0611	-1.075	p = 0.0182
Age	1.037	0.023	0.9913–1.0845	0.1141	0.036	

Data was evaluated at CI = 0.95.

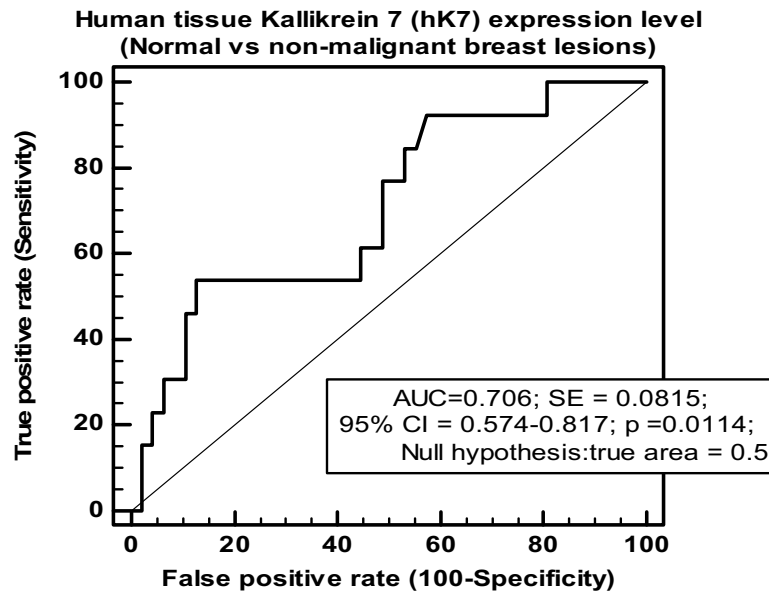


Fig. 4. ROC curve analysis of hK7 quantification for discrimination between normal females and non-malignant breast lesions. AUC was analyzed by Hanley and McNeil, 1982 method, CI is binomial exact confidence interval.

Female breast cancer patients included in the study had different types and stage of breast cancer. To evaluate the effect of breast cancer type on expression level of hK7, the patient group was further categorized or fractionated according to type of breast carcinoma. As the number of representative samples for each type of breast cancer was low it was not preferred to subdivide the group of each breast cancer type into stage specific groups.

hK7 exhibited significant down-regulation in the patient group lacking information regarding type of carcinoma ($p = 0.0077$). Moreover, hK7 down-regulation also seems to be quite significant in case of invasive ductal carcinoma patients ($p =$

Table 4. Logistic regression analysis to evaluate the potential use of hK7 expression level for the discrimination of female breast cancer patients from females having non-malignant breast lesions.

Variables	Crude Odds Ratio	SE	95% CI	p	Coefficient	Overall Model Fit
<i>Univariate Analysis</i>						
Log [hK7]	1.579	0.606	0.4814–5.1775	0.4511	0.457	$p = 0.4531$
Age	1.039	0.025	0.9881–1.0923	0.1356	0.038	$p = 0.1200$
<i>Bivariate Analysis</i>						
Log [hK7]	1.595	0.622	0.4713–5.3984	0.4529	0.467	$p = 0.2251$
Age	1.039	0.026	0.9881–1.0934	0.1348	0.039	

Data was evaluated at CI = 0.95.

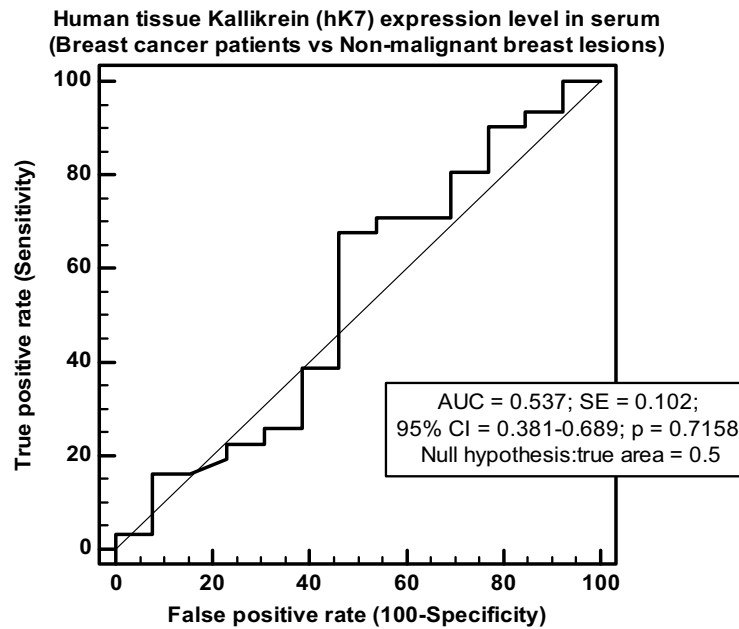


Fig. 5. ROC curve analysis of hK7 quantification for discrimination between breast cancer patients and non-malignant breast lesions. AUC was analyzed by Hanley and McNeil, 1982 method, CI is binomial exact confidence interval.

0.0541). But hK7 expression down-regulation was insignificant ($p > 0.05$) in all other cases of breast cancer and benign breast diseases (Table 5).

A study of variations in hK7 serum concentration ($\mu\text{g/L}$) in breast cancer patients with respect to age revealed significantly lowered expression level ($p < 0.05$) in sera of patients belonging to age group 31–40 years ($p = 0.0128$) and 51–60 years ($p = 0.0404$). For all other age group variation was insignificant with $p > 0.05$ (Table 6). As the data was not Gaussian p value was calculated by Mann-Whitney U test. To further check the effect of age on hK7 expression level data was plotted in the form of scatter graph and linear regression coefficient helped to access the behavior of hK7 serum level with increasing age. Scatter graphs were plotted for hK7 expression level in normal females, breast cancer patients and patients of benign breast diseases taking age along x-axis as independent variable (Data not shown). Correlation between hK7 expression level and age was weak in all cases as it was evident by Spearman's rho (ρ) and Kendall's tau (τ) rank correlation coefficients. Both rho and tau have either negative or very low values and p value is greater than 0.05 in all cases including normal females, breast cancer patients and patients of benign breast diseases. Regression equation and coefficient of regression (R^2) has further confirmed the correlation results.

Both post- and pre-menopausal patient groups have shown down regulation of hK7 but the down regulation is slightly more significant in post-menopausal women ($p = 0.0182$) as compared to pre-menopausal women ($p = 0.0370$, Table 7).

Table 5. Serum level variations of hK7 in patients with different types of breast carcinoma.

Serum	Tested sample (n)	Range (µg/L)	Mean (SD) (µg/L)	Median (µg/L)	p value
Breast Cancer Types					
Infiltrating/Invasive Ductal Carcinoma	16	0.029–1.309	0.679 (0.468)	0.761	0.0541 ^a
Invasive Lobular Carcinoma	3	0.428–1.965	1.048 (0.810)	0.751	0.8541 ^a
Duct Carcinoma Breast (<i>In situ</i>)	1	—	0.398	—	—
Spindle Cell Neoplasm	1	—	1.055	—	—
Unknown	10	0.043–1.322	0.489 (0.372)	0.410	0.0077 ^a
Total breast cancer patients	31				
Other Breast diseases					
Phylloid Tumor	3	0.086–1.962	1.035 (0.938)	1.056	0.728 ^a 0.564 ^b
Mammary dysplasia	3	0.141–0.797	0.424 (0.337)	0.335	0.090 ^a 0.379 ^b
Chronic severe acute non- specific Inflammation	1	—	0.291	—	—
Fibroadenoma	3	0.004–1.177	0.458 (0.630)	0.194	0.102 ^a 0.379 ^b
Chronic Mastitis	3	0.291–1.117	0.807 (0.450)	1.012	0.475 ^a 0.504 ^b
Total benign breast diseases patients	13				
Normal Controls					
Females	47	0.0030–3.247	1.237 (0.929)	1.066	
Breast cancer patients (females)	31	0.029–1.965	0.656 (0.477)	0.624	
Total	122				

Data was evaluated at CI = 0.95 p value was calculated by Mann-Whitney U test.

^a With respect to gender specific normal controls.

^b With respect to breast cancer patients who have not been treated chemotherapeutically.

Breast cancer patients who have been treated chemotherapeutically (number of cycles varying from 2–6) have shown insignificant variation ($p > 0.05$) when compared with the normal female controls and breast cancer patients who have not received any chemotherapeutic treatment (Table 8).

Variations in hK7 serum concentration of HCV/HBV positive breast cancer patients was found to be insignificant ($p > 0.05$) when compared with normal females, HCV/HBV positive females, and female breast cancer patients with no HCV/HBV infection (Table 9).

4. Discussion

In this study hK7 was significantly down-regulated in the sera of breast cancer and benign breast disease patients as compared to the normal healthy female

Table 6. Variations in hK7 serum concentration in breast cancer and benign breast disease patients with respect to age.

Serum	Tested sample (n)	Range ($\mu\text{g/L}$)	Mean (SD) ($\mu\text{g/L}$)	Median ($\mu\text{g/L}$)	<i>p</i> value
Breast Cancer Patients (females)					
21-30 years	4	0.624–1.309	0.868 (0.306)	0.770	0.1879 ^a
31-40 years	7	0.110–0.546	0.265 (0.160)	0.197	0.0128 ^a
41-50 years	7	0.029–1.322	0.816 (0.522)	1.055	0.9491 ^a
51-60 years	7	0.143–1.085	0.642 (0.381)	0.813	0.0404 ^a
61-70 years	5	0.043–1.965	0.738(0.744)	0.663	0.4561 ^a
71-80 years	1	—	1.117	—	—
Total breast cancer patients	31				
Other breast diseases (females)					
11-20 years	1	—	1.117	—	—
21-30 years	1	—	0.335	—	—
31-40 years	8	0.004–1.962	0.723 (0.689)	0.651	0.650 ^a 0.418 ^b
51-60 years	1	—	0.797	—	—
61-70 years	2	0.141–0.291	0.216 (0.106)	0.216	0.083 ^a 0.439 ^b
Total benign breast diseases patients	13				
Normal Controls (female)					
11-20 years	4	0.123–1.068	0.450 (0.436)	0.304	
21-30 years	20	0.226–3.215	1.675 (0.974)	1.735	
31-40 years	11	0.0030–1.238	0.752 (0.384)	0.774	
41-50 years	7	0.200–3.247	1.021 (1.050)	0.668	
51-60 years	2	1.325–3.076	2.200 (1.238)	2.200	
61-70 years	3	0.626–1.206	1.010 (0.333)	1.199	
Total normal controls	47				
Total	91				

Data was evaluated at CI = 0.95 *p* value was calculated by Mann-Whitney U test.

^aWith respect to gender and age group specific normal controls.

^bWith respect to gender & age group specific breast cancer patients who have not been treated chemotherapeutically.

individuals. However, patients having benign breast diseases exhibited non-significant variation in serum hK7 concentration when compared with the serum concentration of hK7, 9 and 10 in breast cancer patients. No study has been conducted regarding hK7 expression level in the sera of breast cancer and benign breast diseases patients. This study is the first to report the under expression of hK7 in breast carcinoma and benign breast diseases. However, down-regulation of KLK7 mRNA in breast tumors has been reported earlier [30, 32].

Table 7. Effect of menopausal status on hK7 serum concentration in breast cancer patients.

Serum	Tested sample (n)	Range ($\mu\text{g/L}$)	Mean (SD) ($\mu\text{g/L}$)	Median ($\mu\text{g/L}$)	<i>p</i> value
Breast Cancer Patients					
Pre-Menopausal	16	0.029–1.322	0.629 (0.478)	0.511	0.0370
Post-Menopausal	15	0.043–1.965	0.685 (0.492)	0.663	0.0182
Total breast cancer- patients	31				
Normal Controls					
Pre-Menopausal	40	0.030–3.247	1.218 (0.955)	0.943	
Post-Menopausal	7	0.626–3.076	1.343 (0.818)	1.206	
Total normal controls	47				
Total	78				

Data was evaluated at CI = 0.95 *p* value was calculated by Mann-Whitney U test.

Reduced level of hK7 does not seem to be associated with the type of breast carcinoma. As least significant variation of hK7 was observed in case of infiltrating/invasive ductal breast carcinoma patients. hK7 exhibited non-significant variation in all other types of breast carcinomas and benign breast diseases. Down-regulation of hK7 may be the characteristic feature of either type or stage of the breast carcinoma as significantly lowered gene expression of KLK7 mRNA in breast cancer patients of either grade I or II [30]. For the present study, it was not possible to find such correlation of hK7 expression down regulation with any particular type of breast carcinoma and benign breast diseases due to low number of samples in each representative group. Although it can be concluded that under

Table 8. Evaluation of hK7 as prognostic biomarker.

Serum	Tested sample (n)	Range ($\mu\text{g/L}$)	Mean (SD) ($\mu\text{g/L}$)	Median ($\mu\text{g/L}$)	<i>p</i> value
Breast Cancer Patients					
After chemotherapy	9	0.057–1.447	0.788 (0.568)	0.964	0.233 ^a 0.447 ^b
	8*	0.057–1.447	0.842 (0.583)	1.023	0.390 ^a 0.313 ^b
Controls					
Normal Females	47	0.003–3.247	1.237 (0.929)	1.066	
Breast cancer patients (No chemotherapy)	31	0.029–1.965	0.656 (0.477)	0.624	
Total	87/86				

Data was evaluated at CI = 0.95 *p* value was calculated by Mann-Whitney U test.

^a With respect to normal female controls.

^b With respect to breast cancer patients who have not been treated chemotherapeutically.

* Results excluding HCV positive breast cancer patient treated chemotherapeutically.

Table 9. hK7 serum concentration in HCV positive breast cancer patients.

Serum	Tested sample (n)	Range (µg/L)	Mean (SD) (µg/L)	Median (µg/L)	p value
<i>Patients of Breast Cancer and other lesions</i>					
HCV positive	8	0.291–2.447	0.748 (0.721)	0.434	0.086 ^a 0.057 ^b 0.944 ^c
<i>Breast Cancer</i>					
HCV positive	7*	0.291–2.447	0.804 (0.760)	0.475	0.160 ^a 0.106 ^b 0.778 ^c
<i>Controls</i>					
Normal Females	47	0.003–3.247	1.237 (0.929)	1.066	
HCV positive Females	5	0.792–1.681	1.155 (0.360)	1.043	
Breast cancer patients	31	0.029–1.965	0.656 (0.477)	0.624	
Total	91/90				

Data was evaluated at CI = 0.95 p value was calculated by Mann-Whitney U test.

^aWith respect to normal female controls.

^bWith respect to HCV +ive females.

^cWith respect to breast cancer female patients(no chemotherapy).

*Results excluding HCV positive mammary dysplasia patient.

expression of hK7 might play a role in the pathogenesis of infiltrating ductal carcinoma and some other types for which information is lacking in the present study

Considering age of the patients, it was observed that expression level of hK7 was particularly lower in 31–40 and 51–60 years age groups. Weak negative but statistically non-significant correlation has been observed between age of breast cancer patients and hK7. Earlier studies have shown lower expression of KLK7 mRNA in patients older than 50 years as compared to younger patients [32]. However, in the current study down regulation of hK7 was more significant in the younger patients (age below 40 years) than older patients (of age 51–60 years). A weak negative correlation between serum hK7 concentration and age in case of normal males and females has been reported earlier [24].

Menopausal status seems to have a profound effect on expression of hK7 because hK7 expression level was more significantly lowered in post- menopausal patients as compared to peri/pre- menopausal patients. Results are in accordance with the earlier studies in which significant down-regulation of KLK7 mRNA has been observed in post-menopausal breast cancer patients as compared to the pre/peri-menopausal patients [32]. However, our results are contrary to the earlier study

indicating that there exists no association between KLK7 mRNA expression level and menopausal status [30].

Chemotherapeutically treated breast cancer patients exhibited non-significant variation of hK7 expression as compared to the normal as well as breast cancer patients who have not undergone any chemotherapeutic treatment. Apparently it seems that chemotherapeutic treatment helps to regain the normal hK7 level but conclusively it cannot be said unless controlled study involving quantification of serum hK7 concentration before and after chemotherapy treatment of the same patients is conducted. Results of the follow-up study before and after chemotherapy will help to explore the nature of the association between hK7 and chemotherapy treatment. The results of present study may not be truly the response of chemotherapeutic treatment but the outcome of individual genetic variations. No study has been conducted so far to monitor the effect of chemotherapeutic treatment on serum hK7 concentration in breast cancer patients although KLK7 mRNA expression level is suggested to be better prognostic marker for breast cancer particularly associated with unfavorable prognosis of disease [29, 30].

HBV/HCV positive breast cancer patients were found to have non-significant variation of hK7 expression level as compared to normal healthy females, HBV/HCV positive non-cancerous females and breast cancer patients with no HBV/HCV infection. HBV/HCV infection has added another level of complexity and it may be related with type and stage of breast cancer along with type of HBV/HCV infection. Although HCV infection has not been proved to play any role in the pathogenesis of breast cancer [33] and does not affect the outcome of chemotherapeutic treatment [34] but the effect on hK7 expression level in HCV positive breast cancer patients indicates HCV infection might add an extra layer of complexity towards the natural course of breast cancer. There is no earlier report about either solitary effect of HBV/HCV infection or combined effect of HBV/HCV infection on serum hK7 concentration.

5. Conclusions

Down-regulation of hK7 seems to be associated with the pathogenesis of breast cancer. ROC curve analysis indicated that hK7 can be a good diagnostic tool that can be used to differentiate the breast cancer patients from normal subjects and prediction of breast cancer. Moreover, it was shown by the ROC curve analysis that hK7 expression levels may further help to differentiate benign breast disease patients from the normal female controls but cannot be used for the differentiation of breast cancer patients from patients of benign breast diseases. There is need to further extend the study on large scale to further validate the biomarker potential of hK7.

Declarations

Author contribution statement

Samina Ejaz: Performed the experiments; Wrote the paper.

Faiz-ul-Hassan Nasim: Conceived and designed the experiments; Wrote the paper.

Muhammad Ashraf: Analyzed and interpreted the data; Wrote the paper.

Gulzar Ahmad: Contributed reagents, materials, analysis tools or data.

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Competing interest statement

The authors declare no conflict of interest.

Additional information

No additional information is available for this paper.

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