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Research Paper

Evaluation of kallikrein-related peptidase 5 [KLK5] and Survivin as Prognostic Markers in Breast Cancer

Medical Science

KEYWORDS : Breast cancer, KLK5 and survivin

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ABSTRACT

Background: Growing evidence suggests kallikrein-related peptidases (KLKs)*, a subgroup of serine proteases, to be implicated in many cascade reactions including apoptosis. So we aimed to explore the relation between kallikrein-related peptidase5 (KLK5) and survivin, an apoptotic inhibitor and to determine their association with breast cancer progression. Patients and methods: KLK5 and survivin were measured by ELISA in 56 breast cancer tissue lysates and 36 distant normal breast tissues. Levels were correlated to each others and to clinicopathological data of patients. Results: KLK5 and survivin levels were significantly high in breast cancer tissues compared to normal tissues (P<0.001). KLK5 levels were significantly correlated with lymph node involvement (P=0.007) and infiltrating duct carcinoma (IDC) (P=0.05). Increasing survivin levels were associated with advanced tumor stage (P<0.05) and high nuclear grade (P=0.00). Higher KLK5 level was associated with tumors which are negative for estrogen (P=0.002) and progesterone receptors (P=0.001), while survivin showed only significant elevated level in tumors with negative estrogen receptor (P=0.018). Meanwhile, A highly significant positive correlation was observed between KLK5 and survivin levels (r=0.62,P=0.000).Conclusion: Part of carcinogenic effects of KLK5 may be through its association with survivin. Moreover, the correlation of KLK5 and survivin with other unfavorable characteristics of breast cancer, highlights their potential value as prognostic biomarkers*

Introduction:

Breast cancer is the second most common malignant neoplasm after lung cancer in the world [1] and is a leading cause of cancer related deaths [2]. Although the significant advances have been made, the molecular mechanisms contributing to pathogenesis of breast cancer are poorly understood [3, 4].

Tissue kallikrein (KLK1) and kallikrein-related peptidases (KLK2-15) comprise a family of 15 highly conserved secreted serine proteases with similar structural characteristics [5]. Nevertheless, deregulated expression of KLK family members has been observed in several pathological conditions and, particularly, in endocrine-related human malignancies, including those of the prostate, breast, and ovary [6]. The prostate-specific antigen (PSA; encoded by the KLK3 gene); is one of these gene products; the best tumor marker for prostate cancer. Like the genes that encode PSA and other kallikreins, the KLK5 gene is a newly discovered human kallikrein gene [7].

KLK5 has the conserved catalytic triad of serine proteases and is regulated by steroid hormones in the BT-474 breast cancer cell line, that suspected it to be differentially expressed in breast cancer tissues and to have prognostic/predictive value as a breast cancer biomarker [6]. Reportedly, KLK5 displays reduced or activated expression in breast cancers and its role in tumor development and/or progression are still unknown/ yet to be described. Yousef et al. [8] reported that, KLK5 was overexpressed in breast cancer and was proved to be an independent indicator of poor prognosis in breast cancer. On the other hand Li et al. [9] found that KLK5 mRNA expression levels were down-regulated in breast carcinomas, and was correlated to limited disease free survival of breast cancer patients. The mechanism by which kallikreins might be involved in the pathogenesis and/or progression of cancer is not as yet fully understood. Preliminary reports indicate a possible role of kallikreins in controlling vital processes, like apoptosis, angiogenesis and tumor metastasis by cleavage of critical substrates such as growth factors, hormones or extracellular matrix [10].

Survivin, a unique member of the inhibitor of apoptosis protein (IAP) family, plays an important role in regulating both apoptosis and cell division. Overexpression of survivin is associated with increased risk of recurrence and poor outcome in cancer patients [11]. Survivin is widely expressed in fetal tissues and in human cancers, but generally not in normal adult tissue [12]. Previous reports demonstrated that, survivin expression is increased in tumor cells with perturbing normal cell cycle control and, therefore may allow cells with spindle defects or aberrant chromosome assembly to proceed through cell division [13]. Moreover, increased survivin levels is associated with drug resistance in cancer cells, and reduced patient survival after chemotherapy and radiotherapy. Agents that antagonize the function of survivin hold promise for treating many forms of cancer [14].

For predicting chemotherapy response in breast cancer, Papachristopoulou et al. [15] found that KLK5 levels increased 12 fold and tenfold in docetaxel- and methotrexate-treated cells, respectively, at 48 h. Whereas for the epirubicin treatment, a twofold decrease was the only noticeable alteration at the same time period. These data strengthen the hypothesis that the deregulation of KLK5 not only is connected to the mechanism of action of the drugs but also to the induced biochemical and/ or apoptotic pathways. Cheung et. al. [14] reported that serum levels of either survivin or KLK5 might be a useful immunological biomarker in patients with malignant disease. However, due to the diversity of disease and different evaluation criteria, we compared the prognostic significance of KLK5 and survivin as biological markers for their possible interplay and useful application in breast cancer follow up and management.

Patients and Methods.

Patients' database

Breast cancer tissue specimens were obtained from 56 patients (52.6 ± 10.2 years) treated for breast cancer at the Department of Surgery, Ain Shams University of Hospital between 2010 and 2012. The control group was as internal control samples from the same patients, obtained from distant breast tissues and diagnosed as completely normal at the Histopathology Department, National Cancer Institute. None of these patients received chemotherapy, radiotherapy or immunotherapy prior to surgery. Patients underwent either modified radical mastectomy or lumpectomy with complete axillary lymph node dissection followed by radiation therapy of residual breast tissue. All of the lymph node-positive patients (n=38) received adjuvant chemotherapy and/or tamoxifen therapy. Lymph node-negative

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patients (n=18) received adjuvant chemotherapy and/or tamoxifen therapy, only if adverse prognostic factors were present such as large primary tumors (>2 cm) or negative hormone receptor status. Tumor samples and clinical information were obtained after having consent from the patients. All laboratory work was done at Oncology Diagnostic Units, Ain Shams University.

Tissue extraction

Breast cancer tissue specimens and normal tissues were selected by the pathologist at the time of surgery and stored at -70°C until use. Tissues were homogenized and lysed on ice in an extraction buffer: 10 mmol/l HEPES buffer (pH 7.5 containing 10 mmol/l K2EDTA, 50 mmol/l NaCl, 5 mmol/l benzamidine, 10 mg/l Triton X-100, 10 mmol/l 2-mercaptoethanol, 0.39 mmol/l PMSF, and 5 mg/l aprotinin) and processed as previously described [16]. The homogenate was then centrifuged for 45 min at 100 000 X g at - 4C°. Supernatant fluid [tissue lysate] was then separated, aliquoted and stored at -70°C until used for further analysis.

Measurement of KLK-5 and survivin in cell lysates

Total proteins in cell lysates were quantified using Bradford assay kit using bovine serum albumin (17) a standard rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. Supernatant fluids of tissue homogenate from all subjects were subjected to measurement of Human Kallikrein-5 by using Enzyme-linked immunosorbent assay kit (R&D Systems, Oxon, UK) according to the instruction manual. Levels were expressed as µg/mg protein. Quantitative survivin protein levels were measured by immunoassay in the supernatant with a commercially available ELISA kit (R&D Systemes, Minneapolis, MN, USA) according to the instruction manual. Antigen concentrations for survivin were expressed as pg/mg protein.

Statistical analysis:

Data was expressed as Mean values ± SE and mean ranks. Mean values of investigated parameters were compared between groups using t-test or ANOVA. Mean ranks were compared using Mann-Whittney or Kruskal-Wallis tests. Correlation analysis [Pearson] between investigated parameters was also performed. SPSS, 16 computer software program was used for statistical analysis. The estimated parameters were correlated to each others and to clinicopathological parameters of patients.

Results

Malignant cases were pathologically diagnosed as 22 infiltrating duct carcinoma (IDC) and 34 non IDC. They were staged according to TNM classification of the American Joint Committee on cancer (AJCC) [18] into stage 1 (n= 14), stage II (n=15) and stage III (n=27) and graded by the Nottingham grading system [19] into grade 2 (n=39) and grade 3 (n=17).

KLK5 and survivin were higher in the malignant group (Mean rank = 57.4 and 62.5; respectively) as compared to normal control groups (Mean rank = 25.8 and 17.5; respectively), P = 0.000 (fig.1). KLK5 protein levels showed statistically significant difference between different subtypes (P= 0.016) with higher expression in infiltrating ductal carcinoma (IDC) than other subtypes. The higher levels of KLK5 were also associated with lymph node invasion, P= 0.009 (table 1). Moreover, a significant relation was found between survivin levels and tumor stages (P = 0.016) with higher expression in stage III compared to stage I (P= 0.016) and stage II (P= 0.031). While tumor grade III showed higher significant level compared to grade II (P= 0.00). On the other hand, survivin levels were higher in IDC tumors than none IDC but statistical significance was not in the reach, P= 0.07 [table 2]. There was a significant inverse relation between higher KLK5 levels and both negative estrogen and progesterone receptors in breast cancer (P=0.002 and P=0.001, respectively). While survivin showed higher level only in tumor with negative estrogen receptor, P= 0.018, table (3). Furthermore, there was a significant direct correlation between KLK5 and survivin levels (P: 0.000) , figure (2).

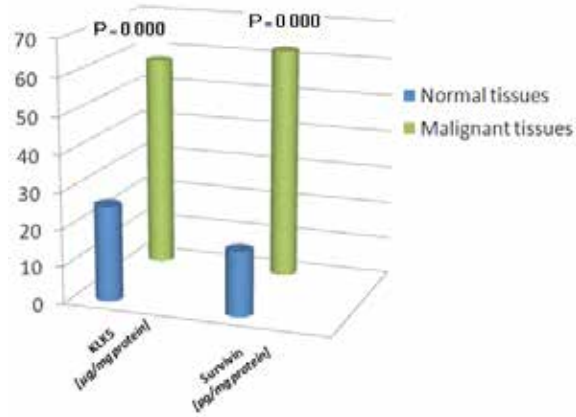

Figure (1): Mean rank of KLK5 and survivin in malignant and normal tissues.
***P≤0.05: is significant**

Table 1: Levels of KLK-5 [µg/mg protein] in normal and malignant tissues and its association with tumor characteristics.

Parameters	KLK-5 Mean ± SE	T for t-test or F for ANOVA [p]	KLK-5 Mean Rank [Median]	Z for Mann-Whitney & X for Kruskal-Wallis [p]
Normal [36]	188.1 ± 138	t=8.7	25.8 [120]	Z = 3 1 . 1 [0.000]*
Malignant [56]	416.2 ± 232	[0.004]*	57.4 [411]	
Tumor stage				
Tumor stage I [14]	363 ± 236	F= 0.52	15.07 [245]	X=9.4 [0.6]
Tumor stage II [15]	448.8 ± 262	[0.33]	28.57 [411]	
Tumor stage III [27]	425.6 ± 216	[0.42]	30.24 [425]	
Histological grade				
Grade II [39]	402.2 ± 233	T=0.013	27.0 [411]	Z=1.1 [0.29]
Grade III [17]	448.3 ± 233	[0.91]	31.94 [420]	
L.N.M				
Negative [18]	286.3 ± 153	T=7.88	230.2 [230]	Z=6.7 [0.009] *
Positive [38]	477.8 ± 239	[0.007]*	32.7 [425]	
Histological type				
IDC [22]	462.4 ± 241	T =3.8	32.68 [425]	Z=5.7 [0.016] *
None IDC [34]	344.9 ± 201	[0.05] *	22.05 [230]	

L.M.N: Lymph node metastasis, a group versus stage III., IDC: infiltrating ductal carcinoma, *P≤0.05: is significant .

Table 2: Levels of survivin [pg/mg protein] in normal and malignant tissues and its association with tumor characteristics.

Parameters	Survivin Mean ± SE	T for t-test or F for ANOVA [p]	Survivin Mean Rank [Median]	Z for Mann-Whitney & X for Kruskal-Wallis [p]
Normal [34]	101.9±40.9	t=5 [0.000]*	17.5 [123.4]	Z=63.1 [0.000]*
Malignant [56]	254±61.4		62.5 [257.2]	
Tumor stage				
Tumor stage I [14]	229.4±65.1a	F= 4.11	23.8 [257.1]	X=8.2 [0.016] *

Tumor stage II [15]	235.5±62.3a	[0.016]*	21.3 [257.2]	
Tumor stage III [27]	275.1±51.9	[0.031]*	34.8 [268.8]	
Histological grade				
Grade II [39]	232.2±54.7	t=3.2 [0.00]*	22.1 [257.2]	Z=20.4 [0.00]*
Grade III [17]	303.9±45.2		43.3 [332.2]	
L.N.M				
Negative [18]	256.5±70.4	t=0.21 [0.13]	29.9 [261.2]	Z=0.21 [0.6]
Posetive [38]	252.8±57.6		27.8 [257.2]	
Histological type				
IDC [34]	265.7±58.1	t=1.8 [0.075]	31.5 [266]	Z=3.03 [0.08]
None IDC [22]	235.8±63.2		23.8 [257]	

L.M.N: Lymph node metastasis, a group versus stage III., IDC: infiltrating ductal carcinoma, *P≤0.05: is significant

Table 3: relation between KLK5 and survivin and hormonal receptor status in breast cancer group

Parameters	KLK5 [µg/mg protein]		Survivin [pg/mg protein]	
	Mean ± SE	t [P]	Mean ± SE	t [P]
Estrogen receptor Negative [17] Positive [39]	639.3 ± 292 319 ± 154	6.1[0.002] *	283.1± 43.3 241.3± 64.2	2.4[0.018] *
Progesterone receptor Negative [13] Positive [43]	562.8± 264 362.8± 195	3.4[0.001] *	245.4± 49.5 264.5± 62.1	1.7[0.074]

***P≤0.05: is significant**

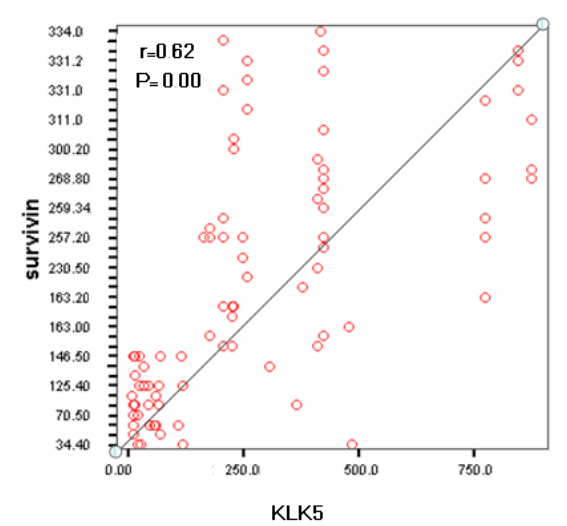

Figure (2): Correlation between tissue KLK5 and survivin concentrations.
r: Pearson Correlation coefficient, *P≤0.05: is significant.

Discussion

Breast cancer is a major health problem that threatens the lives of millions of women worldwide each year [20]. The revelation of new potential markers for diagnosis, prognosis and new potential predictive markers for treatment efficiency is of great importance. From that point of view, we measured KLK5 and survivin and correlated them with clinicopathological factors in breast cancer.

Our results revealed elevation of KLK5 in breast cancer tissues compared to normal tissues. These elevated KLK5 level was correlated to positive lymph node involvement and IDC breast cancer tumor type. The observed relationship between KLK5 status in this study with the clinical outcome of cancer patients and well established prognostic factors might identify it as a promising prognostic biomarker for breast cancer management. There were conflicting results from previous researches, some recorded that KLK5 was an independent indicator of poor prognosis in breast cancer [8] and was inversely correlated to disease free survival breast cancer patients [5]. Others reported that most of the KLKs, except KLK4 and KLK15, show reduced mRNA and/or protein expression levels compared to expression of the KLKs in normal breast tissue [21] and that KLK5 and KLK7 were reported to be overexpressed in several cancers, but underexpressed in prostate and breast cancers [22]. In our study, a significant association was found between KLK5 upregulation and both negative estrogen and progesterone receptor status in breast cancer group. Yousef et al. [8] found that high KLK5 expression was found more frequently in estrogen receptor-negative patients, indicating its unfavorable prognostic marker for breast carcinoma. Schmitt et al. [20] suspected that selective inhibitors to certain KLKs will be developed for future therapeutic application, that aim at blocking their enzymatic activity, in order to interfere with KLK-mediated degradation or activation of other proteins.

Trying to explore the role of human tissue kallikreins in cancer, Carla et al. [23] reported that human tissue kallikrein 1 (hK1), hK2 and hK3 might stimulate the growth and survival of tumor cells by liberating the mitogenic growth factor insulin-like growth factor 1 (IGF1), which induces cell proliferation and prevents apoptosis. We found in the current study a significant direct correlation between KLK5 and survivin. Zhang et al. [24] reported that expression of survivin and MMP2 was markedly decreased, while expression of caspase 9 and cleaved caspase 3 was markedly increased as a consequence of KLK14 inhibition in ovarian carcinoma.

Li et al. [25] reported that differential expression of survivin between normal and malignant breast tissue proves its role as inhibitor of apoptosis and regulator of mitosis. For further evaluating survivin as a biomarker in breast cancer, we found an increasing in its level in breast cancer tissues compared to control normal ones. These elevated levels were associated with advancing tumor stage and poor differentiated tumor grade, unmasking in this way its unfavorable prognostic value. These go in accordance with Span et al [26] and Ryan et al [27] who found a relationship between survivin levels and high tumor grade using real-time PCR and ELISA respectively. In contrast to our findings, other investigators who used immunohistochemistry [28, 12] or conventional PCR [29] failed to find a significant association between survivin and tumor grade. These findings suggest that the use of sensitive and quantitative assays, such as ELISA or real-time PCR, may provide more reliable data for survivin than the use of less sensitive and semi-quantitative methods such as immunohistochemistry or conventional PCR [13]. In this study, we found high survivin levels in IDC tumors than none IDC but statistical significance was not in the reach, while Ryan et al. [27] found more survivin expression in IDC than other pathological types in breast cancer, adding to its role as a marker for invasiveness and poor prognosis. Moreover, survivin was significantly high in negative estrogen receptor of the breast cancer group, revealing its association with more aggressive phenotypes in breast carcinoma. Kennedy et al. [12] and O'Driscoll et al. [29] recorded that no statistically significant relation was found between survivin expression and expression of both estrogen and progesterone receptors. On the other hand, Ryan et al. [13] recorded that survivin was significantly associated with both negative estrogen and progesterone receptors.

In conclusion, our preliminary data indicates that KLK5 may be implicated in the malignant behavior of breast cancer. One of its mechanism may be through its association with survivin, but functional experiments are needed to roll out their possible mechanism and justify their association to establish the clinical

value of these biomarkers.

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