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Original Article

High levels of KLK7 protein expression are related to a favorable prognosis in triple-negative breast cancer patients

Xiaocong Geng[1], Lamiya Babayeva[1], Axel Walch[2], Michaela Aubele[3], Eva Groß[4], Marion Kiechle[1], Holger Bronger[1], Tobias Dreyer[1], Viktor Magdolen[1], Julia Dorn[1]

[1]*Clinical Research Unit, Department of Obstetrics and Gynecology, Technical University of Munich (TUM), German;* [2]*Research Unit Analytical Pathology,* [3]*Institute of Pathology, Helmholtz Zentrum München, Neuherberg, Germany;* [4]*Department of Obstetrics and Gynecology, University of Munich (LMU), Germany*

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Abstract: In normal physiology, kallikrein-related peptidase 7 (KLK7), together with other members of the kallikrein-related peptidase family, is mainly involved in skin desquamation and keratinization processes. Moreover, expression of KLK7 was shown in various tumor types to be dysregulated and to correlate to patients' survival time. However, there are contradictory reports in breast cancer whether KLK7 represents an unfavorable or favorable prognostic biomarker. In the present study, we examined the prognostic value of KLK7 protein expression in triple-negative breast cancer (TNBC), determined by immunohistochemistry (IHC). A cohort encompassing 133 TNBC specimens, present on tissue microarrays, was analyzed. For quantification of the staining intensity, an automated digital IHC image analysis algorithm was applied. In both Kaplan-Meier and univariate Cox analyses, elevated KLK7 protein levels were significantly linked with prolonged overall survival (OS). In multivariable Cox analysis, addition of KLK7 immunoreactivity scores to the base model (including the clinical parameters age, tumor size, and nodal status) demonstrated that KLK7 protein expression remained as a statistically significant, independent parameter for prolonged OS. These results strongly indicate that KLK7 is a favorable prognostic biomarker in triple-negative breast cancer.

Keywords: Triple-negative breast cancer, kallikrein-related peptidase, KLK7, prognostic marker, clinical relevance

Introduction

Breast cancer is the most commonly diagnosed malignancy in women [1]. In all breast cancer patients, intratumoral expression of three receptors is generally tested, namely estrogen receptor (ER), progesterone receptor (PR), and the human epidermal growth factor receptor 2 (HER2). Triple-negative breast cancer (TNBC) refers to a molecular subtype that is characterized by the absence or low levels of expression of ER and PR as well as lack of HER2 overexpression and accounts for approximately 12%-17% of all breast cancers [2]. TNBC usually is highly aggressive, displaying adverse clinicopathological tumor signatures and poor long-time prognosis of the patients. TNBC patients have a high recurrence risk within the first three years after initial treatment, often with visceral metastasis [3]. The lack of receptor expression renders this tumor subtype inaccessible to established targeted therapies (other than chemotherapy), such as endocrine treatment or specific anti-HER2 therapies, *e.g.,* with trastuzumab [4]. Therefore, the identification of new prognostic biomarkers and/or alternative therapeutic targets are major goals in TNBC research.

The kallikrein-related peptidases (KLKs) form a family of 15 homologous serine proteases clustered on chromosome 19q13.3-19q13.4. For decades, abnormal expression profiles and pathological implications of KLKs have been explored in various diseases, including malignancies [5-9]. In breast cancer, several members of the KLK family have been reported to be involved in cancer progression as prognostic biomarkers [5, 9]. In a cohort of 188 patients afflicted with TNBC, elevated KLK4 protein

expression levels in the tumor stroma were significantly correlated with worse disease-free survival [10]. In a group of 165 breast cancer patients, Haritos and co-workers [11] reported that higher KLK6 mRNA levels significantly predicted a worse disease-free survival (DFS) of patients. Michaelidou and co-workers [12] reported that higher KLK8 mRNA expression levels in tumor tissue were significantly associated with a shorter disease-free survival when analyzing a group of 150 breast cancer patients.

KLK7 has been first purified from human stratum corneum and displays chymotryptic activity. It is also expressed in distinct amounts in other tissues, *e.g.*, the central nervous system, kidney, mammary, and salivary glands [13]. KLK proteases are part of complex proteolytic networks. One of the best-studied KLK-networks is present in the epidermis where KLK7, together with KLK5 and, to a lesser extent, some other KLKs, are involved in the proteolysis of extracellular components and thereby in physiological skin desquamation processes [14]. In skin disorders with barrier abnormalities, *e.g.*, Netherton syndrome and atopic dermatitis, unrestricted activity of both KLK7 and KLK5 are observed due to the loss of the major endogenous inhibitor, lymphoepithelial Kazal-type-related inhibitor (LEKTI) [14].

Previous studies have reported dysregulated expression of KLK7 in various malignancies, like human melanoma [15, 16], pancreatic adenocarcinomas [17, 18], ovarian [19], and colorectal cancer [20]. KLK7 also has been described as a valuable biomarker in different malignancies, *e.g.*, as a favorable prognostic marker in pancreatic ductal adenocarcinomas (PDAC) [21] or as an unfavorable marker in colorectal cancer tissues [22]. There are, however, studies with contradictory results concerning the prognostic value in the same tumor type. Dorn and co-workers [23], on the one hand, found that ovarian cancer patients with high KLK7 antigen levels in tumor tissue extracts had a significantly lower risk of death. On the other hand, the results of another study by Dong et al. [24] suggested an association of high KLK7 levels with chemoresistance and poor prognosis for serous epithelial ovarian cancer (EOC) patients. In breast cancer, conflicting results were observed as well: in a study by Talieri and colleagues [25], survival analysis showed that breast cancer patients with KLK7 mRNA-expressing tumors displayed shorter DFS and OS than patients with KLK7 negative tumors. Conversely, Holzscheiter and co-workers [26] described a favorable association of high KLK7 mRNA expression levels with patients' outcome in breast cancer. On the protein level, up to now, no biomarker studies have been performed in breast cancer.

To get more insights into the clinical relevance and tumor biological role of KLK7 in breast cancer, especially in the most severe subtype, triple-negative breast cancer (TNBC), the present study aimed at analyzing KLK7 protein expression in tumor tissue of a homogenous cohort encompassing 133 TNBC patients. Tissue microarrays were stained with a KLK7-specific antibody and KLK7 expression levels determined by an automated digital algorithm allowing analysis of the association of KLK7 protein expression with clinical parameters and patients' outcome.

Materials and methods

Patients

In this retrospective study, 133 patients afflicted with triple-negative breast cancer (TNBC), treated between 1988 and 2006 at the Department of Obstetrics and Gynecology, Klinikum rechts der Isar, Technische Universität München, Germany, were enrolled. The study was approved by the local Ethics Committee (Faculty of Medicine, Technical University Munich, project 491/17 S). Informed consent was obtained from patients included in this study. Median patients' age at time of surgery was 55 years (range 30-90 years). All patients underwent initial resection surgery for breast tumors, with 13 patients (9.8%) receiving neoadjuvant therapy. Following surgery, all patients were treated with adjuvant chemotherapy according to the consensus recommendations at that time. Median time of follow-up for overall survival (OS) was 82 months (range 1-323 months), whereas median follow-up time for disease-free survival (DFS) was 65 months (range 2-323). Clinical factors documented at the time of surgery included: age, nuclear grade, and nodal status (**Table 1**). During 15 years of follow-up, 64 (48.1%) patients had died, and 48 (39.3%) patients had relapsed.

Table 1. Association between clinical characteristics of triple-negative breast cancer patients and KLK7 protein expression

Clinical parameters	KLK7[a] Low/high	p
Age (years)		0.064
≤ 50	33/17	
> 50	66/17	
Tumor size		0.512
pT0+pT1	30/10	
pT2+pT3+pT4	66/24	
Nodal status		0.142
pN0	50/22	
pN+	46/12	
Nuclear grade		0.493
G2	8/2	
G3	89/32	

Chi-square test. [a]Dichotomized into low and high levels by the 75th percentile; Due to missing values, numbers do not always add up to n = 133.

Immunohistochemistry (IHC)

Formalin-fixed, paraffin-embedded triple-negative breast cancer tissue specimens were retrieved from the archives of the Institute of Pathology of the Technische Universität München (Munich, Germany). The tissue microarrays were produced based on the protocol as previously described [23, 27]. Dewaxed and rehydrated tissue microarray sections were treated via pressure cooking for antigen retrieval (citrate buffer, pH 6.0, 4 min). Endogenous peroxidase activity was quenched with 3% hydrogen peroxide (room temperature, 20 min). The sections were incubated (4°C, overnight) with an established polyclonal goat antibody targeting human KLK7 (AF2624, R&D Systems, Abingdon, UK, 1:500) [23]. Following incubation with a mouse anti-goat IgG, the polymer one-step system reporter assay (DAB staining, room temperature, 8 min) was used to visualize the interaction of antibody and target KLK7 protein. Following that, nuclei were counterstained with hematoxylin [23].

Quantification of immunostaining

For image analysis, the free software ImageJ (Java 1.8.0, 64 bit, https://imagej.nih.gov/ij) was used. The NDP.View2 software was used to scan whole staining images of slides. Five selected images of each tumor tissue core were collected and loaded into the ImageJ platform for the quantitative analysis. A detailed description of this automatic analysis assay for KLK staining has been previously published [28].

Statistical analyses

The associations of KLK7 with clinical parameters were evaluated using the Chi-square test. For survival analyses, overall (OS) and disease-free survival (DFS) of triple-negative breast cancer patients were used as follow-up endpoints. Associations of KLK7 and clinical parameters with OS and DFS were analyzed by univariate and multivariable Cox proportional hazards regression models and are presented as hazard ratio (HR) and its 95% confidence interval (95% CI). For all survival analyses, the observation period was restricted to 180 months. The multivariable Cox regression model was adjusted based on established triple-negative breast cancer clinical factors including age, residual tumor mass, nuclear grade, and nodal status. Survival curves were depicted according to Kaplan-Meier using log-rank tests for testing differences. All statistical analyses were performed by the SPSS statistical analysis software (version 20.0; SPSS Inc., Chicago, IL, USA). *p*-values ≤ 0.05 were considered as statistically significant.

Results

KLK7 protein expression pattern in TNBC tumor tissue and its relation to patients' tumor characteristics

We analyzed the clinical impact of KLK7 protein expression in tumor tissue in a cohort of 133 patients afflicted with triple-negative breast cancer. IHC was performed on tumor tissue microarrays employing the KLK7-specific antibody AF2624. We evaluated the protein expression focusing on tumor cells using a quantitative immunoreactivity score (IRS). Overall, in tumor specimens, a differential staining pattern of KLK7 protein with varying intensities from case to case was observed (**Figure 1**). Tumor cells displayed cytoplasmic protein expression. KLK7 values range from 19.1 to 126.3 (median = 39.6). Based on the observed expression pattern, we categorized the protein

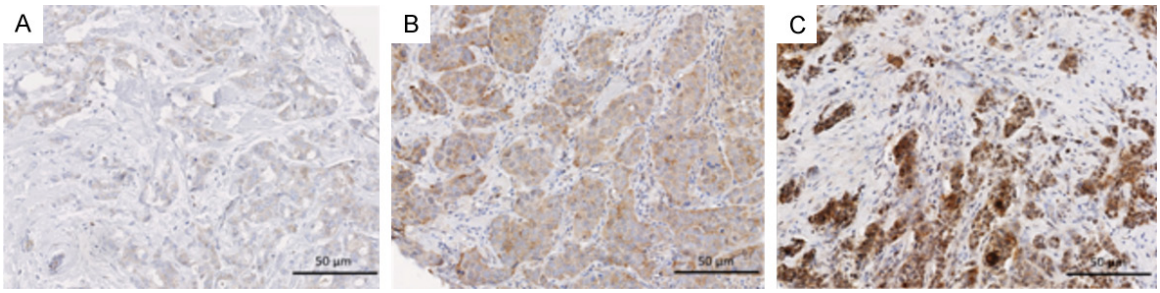

Figure 1. KLK7 immunoexpression in tumor tissue of triple-negative breast cancer patients. Tissue sections were stained with the KLK7-specific antibody AF2624, applying the polymer one-step system. A-C. Illustrate representative core punches corresponding to low, moderate, and high immunoexpression of KLK7 in tumor cells, respectively. Bar, 50 µm.

Table 2. Univariate Cox regression analysis of clinical outcome (overall survival and disease-free survival) in triple-negative breast cancer patients with respect to clinical parameters and the tumor biological factor KLK7

Clinical parameters	OS 180			DFS 180		
	No[a]	HR (95% CI)[b]	p	No[a]	HR (95% CI)[b]	p
Age (years)			**0.009**			0.184
≤ 50	50	1		46	1	
> 50	83	2.09 (1.20-3.64)		76	1.50 (0.82-2.75)	
Tumor size			0.350			0.985
pT0+pT1	40	1		37	1	
pT2+pT3+pT4	90	1.32 (0.74-2.36)		82	1.01 (0.54-1.89)	
Nodal status			**0.002**			**0.004**
pN0	72	1		69	1	
pN+	58	2.24 (1.34-3.74)		52	2.36 (1.32-4.23)	
Nuclear grade			0.652			0.575
G2	10	1		10	1	
G3	121	1.26 (0.46-3.48)		110	1.40 (0.43-4.50)	
KLK7 IRS[c]			**0.011**			0.365
low	99	1		88	1	
high	34	0.40 (0.20-0.81)		34	0.73 (0.37-1.44)	

Significant *p*-values (P < 0.05) are indicated in bold. [a]Number of patients; [b]HR: hazard ratio (CI: confidence interval) of univariate Cox regression analysis; [c]IRS: immunoreactive score, dichotomized into low and high levels by the 75th percentile; Due to missing values, numbers do not always add up to n = 133.

expression levels into low and high groups by the 75th percentile for KLK7 (quartiles 1+2+3 = low versus quartile 4 = high).

Table 1 shows the associations between KLK7 (low versus high) and established clinical parameters in TNBC, including age (≤ 50 versus > 50 years), tumor size (pT0+pT1 versus pT2+pT3+pT4), nuclear grade (G2 versus G3) and nodal status (pN0 versus pN+). KLK7 expression values are not significantly associated with these clinical parameters, yet, there is a trend towards significance with patients' age (P = 0.064).

Association of KLK7 and clinical parameters with overall (OS) and disease-free survival (DFS)

The impact of established clinicopathological parameters and KLK7 protein expression levels on patients' fifteen-year OS and DFS was analyzed by univariate Cox regression analysis and is summarized in **Table 2**. Among the clinical factors, patient's age and a positive lymph node status indicated a significantly shorter OS and, for lymph node status, also a shorter DFS. Conversely, elevated KLK7 protein expression levels were found to be significantly associated

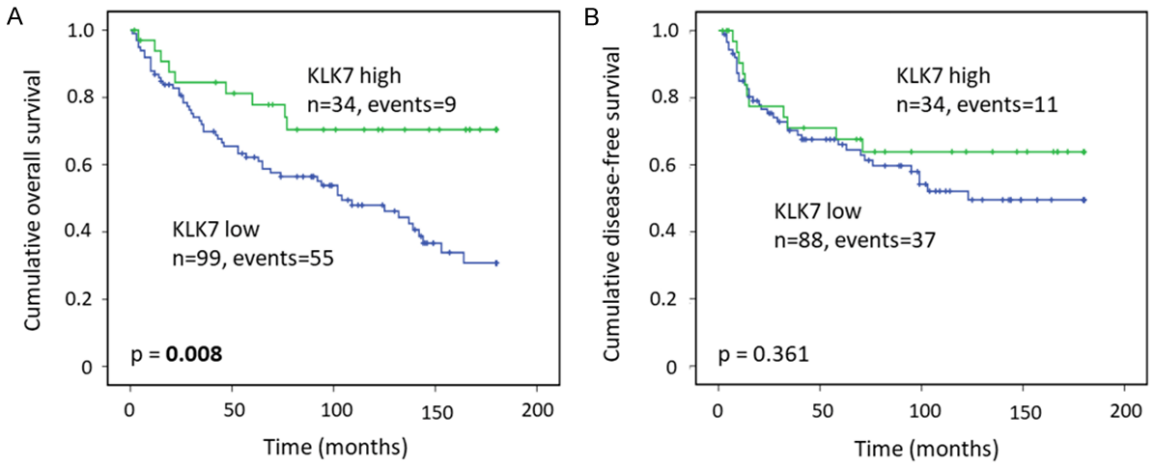

Figure 2. Probability of overall survival (OS) and disease-free survival (DFS) of patients afflicted with triple-negative breast cancer as stratified by KLK7 protein expression levels in primary tumor tissues. Patients with elevated KLK7 protein expression levels show a significantly better OS (Kaplan-Meier analysis, P = 0.008) (A), but not DFS (B), compared to patients dispaying a lower KLK7 protein expression.

Table 3. Multivariable Cox regression analysis of clinical outcome (overall survival) in triple-negative breast cancer (TNBC) patients with respect to clinical parameters and the tumor biological factor KLK7

Clinical parameters	OS 180		
	No[a]	HR (95% CI)[b]	p
Age (years)			0.078
≤ 50	47	1	
> 50	79	1.70 (0.94-3.05)	
Tumor size			0.942
pT0+pT1	40	1	
pT2+pT3+pT4	86	1.02 (0.56-1.87)	
Nodal status			**0.010**
pN0	71	1	
pN1+pN2+pN3+	55	2.01 (1.18-3.43)	
Nuclear grade			0.371
G2	10	1	
G3	116	1.60 (0.57-4.45)	
KLK7 IRS[c]			**0.040**
low	92	1	
high	34	0.47 (0.23-0.97)	

The biological marker KLK7 was added to a base model of the clinical parameters: age, tumor size, nodal status and nuclear grade. Significant *p*-values ($P < 0.05$) are indicated in bold. [a]Number of patients; [b]HR: hazard ratio (CI: confidence interval) of multivariable Cox regression analysis; [c]Dichotomized into low and high levels by the 75th percentile.

with longer OS (HR = 0.40, 95% CI = 0.20-0.81, P = 0.011). Patients with a high expression of KLK7 displayed an over two-fold decreased risk for death. No statistically significant association was observed between KLK7 expression and DFS (**Table 2**). The findings for KLK7 were confirmed by Kaplan-Meier survival analysis. The respective survival curves are depicted in **Figure 2**.

To test the independence of the prognostic value of KLK7 for OS, multivariable Cox hazard regression analysis was used. The base model was established by factors including age, tumor size, nuclear grade, and nodal status. In this base model, the nodal status (N0 versus N+) was the only clinical parameter displaying a significant association with OS (HR = 2.01, 95% CI = 1.18-3.43, P = 0.010), while age lost its prognostic significance for OS when adjusted to multivariable analysis. Added to the base model, KLK7 expression remained as a significant parameter and thus contributed as an independent factor to the multivariable Cox model for OS (HR = 0.47, 95% CI = 0.23-0.97, P = 0.040) (**Table 3**).

Discussion

In the present study, protein expression levels of KLK7 were examined in a homogeneous group of 133 triple-negative breast cancer (TNBC) patients. Here, we demonstrated that high KLK7 protein expression represents an independent, statistically significant favorable marker for increased 15 year-overall survival.

In fact, high KLK7 expression doubles the chance of long-time survival of the patients.

Based on its aberrant expression in malignant diseases, KLK7 has been previously suggested to be a useful unfavorable tumor marker in a variety of malignant diseases. Shan and coworkers [19] showed that positive KLK7 expression (KLK7 is not expressed in the normal ovary) was significantly associated with shorter progression-free survival and increased risk of relapse in women afflicted with ovarian cancer. Similarly, in a study including 125 ovarian tumor patients, higher KLK7 mRNA expression in cancer tissue was associated with poor prognosis, particularly in those cases with lower-grade disease [29]. In colorectal cancer, elevated KLK7 mRNA expression levels were reported to significantly indicate poor overall survival, especially in those patients displaying liver metastasis [20, 22].

However, although several studies have suggested that KLK7 may play a role in cancer progression as an unfavorable prognostic marker, the evaluation of KLK7 expression has also shown a protective role of KLK7 in different malignancies. Dorn et al. [23, 30] as well as Lizama et al. [31] reported that KLK7 antigen levels in tumor tissue extracts of ovarian cancer patients were significantly associated with better survival. Furthermore, KLK7 mRNA and protein overexpression were suggested to directly correlate with an early FIGO stage at the time of diagnosis [32].

In breast cancer, expression of KLK7 mRNA is downregulated compared to normal and benign tissue and is further decreased in metastases [25, 26, 33]. Likewise, significantly reduced KLK7 antigen levels have been reported in the sera of breast cancer patients and even already in benign breast disease patients as compared to normal controls [34]. Thus, this suggests that loss of KLK7 expression may be associated with the development and/or progression of breast cancer. In line with this notion, Holzscheiter et al. [26] observed that lower KLK7 mRNA levels were associated with a decreased risk of relapse or death. These findings are concordant with our analysis of KLK7 protein expression in a cohort exclusively consisting of TNBC patients. It should, however, be mentioned that, in a study conducted by Talieri et al. [25], an association between high KLK7 mRNA levels with poor patient prognosis was reported. The latter results may be due to the determination of a KLK7 splice variant in addition to the wild-type mRNA [26]. Moreover, different functional tumor biological roles of KLK7 in different subtypes or stages of breast cancer may also be a reason for divergent results when exploring the prognostic impact of KLK7.

Although the biological function of KLK7 has so far not been addressed in breast cancer, it is reasonable to connect it to the enzymatic activity of KLK7 embedded within a tumor-associated proteolytic network. In an ovarian cancer cell model, several substrates for KLK7 have been identified that might have an impact on tumor formation and progression [35]. For example, not only matrix metalloproteinases (MMP2 and MMP-10), which cleave extracellular matrix (ECM) and basement membrane components, were identified as substrates of KLK7, but also several ECM components themselves are cleaved by KLK7. Thus, cleavage of these substrates may result in ECM remodeling and, by this, considerably affect tumor cell adhesion and migration. In melanoma cells, KLK7 was demonstrated to cleave midkine, a heparin-binding growth factor [36]. This cleavage strongly reduces the pro-proliferative effects of midkine. When melanoma cells were stimulated with recombinant midkine in the presence or absence of KLK7, only full length but not KLK7-cleaved midkine led to enhanced proliferation. In line with this, overexpression of KLK7 by stable transfection of melanoma cells with a KLK7 expression vector resulted in a significant reduction in cell proliferation [15]. But not only direct cleavage of substrates might influence tumor progression. A regulatory function of KLKs, including KLK7, has been suggested by Wang et al. [37], who analyzed whether overexpression of KLK4, 5, 6, and 7 modulates gene expression of other cancer-related genes in ovarian cancer cells. In fact, several genes encoding proteins potentially affecting tumor-relevant processes were identified, which are strongly up- and downregulated upon KLK4-7 overexpression. Taken togther, KLK7 may well be involved in several ways in tumor progression of breast cancer. The identification of specific KLK7 substrates and pathways, especially in TNBC, seems to be indispensable to further understand its tumor bio-

logical role and to provide deeper insights into the prognostic and therapeutic value of this protease.

In conclusion, we validated an automated protocol for IHC analysis of KLK7 protein expression in tumor tissue of triple-negative breast cancer patients. Our results strongly suggest to consider high KLK7 protein expression levels as an independent favorable prognostic factor for patients' overall survival in this most aggressive breast cancer subtype.

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Disclosure of conflict of interest

None.

Address correspondence to: Viktor Magdolen, Clinical Research Unit, Department of Obstetrics and Gynecology, Technische Universität München, Ismaninger Str. 22, D-81576 Munich, Germany. Tel: +49 89 4140 2493; E-mail: viktor.magdolen@tum.de

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