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Original Article

Elevated levels of both microRNA 378 (miR-378) and kallikrein-related peptidase 4 (KLK4) mRNA are associated with an unfavorable prognosis in triple-negative breast cancer

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Abstract: Triple-negative breast cancer (TNBC) patients have the worst outcome among all breast cancer subtypes. In oral squamous carcinoma cells, miR-378 was reported to target the mRNA of kallikrein-related peptidase 4 (KLK4), resulting in inhibition of cell proliferation, migration and invasion, induction of apoptosis, and reduction of tumor growth *in vivo*. Similarly, a miR-378/KLK4 axis has been proposed in prostate cancer. Here, we analyzed the correlation between miR-378 and KLK4 mRNA expression and determined the prognostic impact of both factors in TNBC. miR-378 and KLK4 mRNA expression levels were determined by quantitative PCR in tumor tissue of TNBC patients (n=103) and correlated with clinical parameters and patients' survival. There was no significant correlation between miR-378 and KLK4 mRNA expression. In univariate Cox regression analysis, elevated miR-378 expression was significantly associated with shortened disease-free survival (DFS, P=0.047) and overall survival (OS, P=0.031), high KLK4 mRNA levels were linked to a worse DFS (P=0.033). Combination of KLK4 mRNA and miR-378 (KLK4+miR-378, low/low versus high and/or high) allowed even better discrimination between favorable and unfavorable prognosis (DFS, P=0.008; OS, P=0.025). In multivariable analysis, miR-378 and KLK4+miR-378 expression remained independent predictive factors for DFS (P=0.014, P=0.010, respectively) and OS (P=0.016, P=0.049, respectively), while KLK4 mRNA only showed a trend towards significance for DFS (P=0.061). Our findings suggest that in TNBC there is no significant impact of miR-378 on KLK4 expression. Both factors, miR-378 and, to a lesser extent, KLK4 mRNA represent unfavorable prognostic markers in TNBC patients.

Keywords: Triple-negative breast cancer, miR-378, kallikrein-related peptidase, KLK4, prognostic marker, clinical relevance

Introduction

Breast cancer is the most common tumor in women worldwide [1]. Triple-negative breast cancer (TNBC) is defined by the absence of the estrogen receptor (ER), progesterone receptor (PR) as well as low expression of the human epidermal growth factor receptor 2 (HER2) and accounts for about 15% of all breast cancer subtypes [2, 3]. Compared to the other breast cancer subtypes, TNBC is characterized by increased aggressiveness, early metastasis and recurrence after surgery, as well as poor outcome [4, 5]. Moreover, TNBC does not respond to hormonal or targeted immune-therapies, such as trastuzumab. Therefore, there is an urgent need to search for more specific molecular targets for TNBC therapies.

The human kallikrein-related peptidase (KLK) family, comprising 15 serine proteases, is located on chromosome 19q13.3-q13.4 and modu-

lates various physiological and pathological processes [6, 7]. Many KLKs, including KLK4, have been demonstrated to be clinically relevant cancer biomarkers [8]. Elevated KLK4 protein and mRNA levels have been observed in various cancer types including prostate cancer, renal cell carcinoma, and thyroid cancer and were shown to be associated with poor prognosis [9-12]. KLK4 protein and mRNA overexpression have been detected in breast cancer tissue and cell lines as well [13, 14]. An association between KLK4 overexpression and poor prognosis has been shown for overall survival (OS) in oral squamous carcinoma (OSC) [15], especially in terms of increased metastasis formation [16]. In colorectal adenocarcinoma, patients with elevated KLK4 mRNA levels were more frequently found to have a short-term relapse [17] and shorter OS [18]. Moreover, elevated KLK4 mRNA is considered as an unfavorable prognostic marker for OS in ovarian cancer [19], which was recently validated by a study of our group in a homogenous patient cohort encompassing advanced high grade serous ovarian cancer patients only [20].

MicroRNAs (miRNAs), encompassing approximately 21-23 nucleotides, are small noncoding RNAs that regulate post-transcriptional gene expression through binding to the 3'untranslated regions (UTRs) of mRNAs [21, 22]. One single miRNA can target mRNAs from multiple genes, and one mRNA species can be targeted by multiple miRNAs, generating a complex regulatory network affecting gene expression. Accumulating studies have demonstrated aberrant miRNA expression levels in various cancer types, including breast cancer, and provided evidence that miRNAs regulate cell proliferation, differentiation, apoptosis, migration, invasion, and metastasis to act as either oncogenes or tumor suppressors [23].

Previously, high miR-378 expression was reported to be associated with prolonged disease-free survival (DFS) in high-recurrence risk prostate cancer patients. These beneficial effects were suggested to be mediated via targeting both KLK2 and KLK4 mRNA and, thus, suppressing protein expression of these two factors [24]. Furthermore, in an *in vitro* oral squamous carcinoma model, it was shown that KLK4 is, in fact, a target gene of miR-378: overexpression of miR-378 led to a distinct decrease of KLK4 mRNA levels resulting in inhibition of cell migration and invasion. Re-expression of KLK4 mRNA in these cells reversed these effects by up-regulating matrix metalloproteinase 2 and 9 (MMP-2, MMP-9), and N-cadherin and down-regulating the E-cadherin levels [25]. Moreover, miR-378-mediated suppression of KLK4 mRNA expression resulted in inhibition of angiogenesis [26], reduced cell proliferation, and increased apoptosis [27]. Finally, in an *in vivo* nude mouse model, miR-378 overexpression in the OSC cells resulted in significantly smaller tumors after subcutaneous injection of the tumor cells and reduced lung colonization after injection of the tumor cells into the tail vein [25, 27]. These results strongly indicate that there is a direct miR-378/KLK4 mRNA interaction in OSC, whereby increased miR-378 expression results in distinctly reduced KLK4 expression. Thus, at least in particular cancer entities, miR-378 may act as a tumor suppressor, whereas its target KLK4 can be considered as a tumor-supporting factor. The latter statement is in line with the above mentioned, previously published studies on the predictive value of KLK4 in several types of cancer.

Both miR-378 and KLK4 are expressed at low levels, if at all, in normal breast tissue, whereas, in general, elevated levels of miR-378 and KLK4 are observed in tumor tissue [28, 29]. In the present study, we aimed at examining whether there is a miR-378/KLK4 axis in TNBC as well. For this, the expression patterns of both KLK4 mRNA and miR-378 in tumor tissue from TNBC patients were analyzed by quantitative PCR (qPCR) and their relationship evaluated. Furthermore, their association with established clinical and histomorphological parameters as well as with prognosis was assessed in the TNBC patient cohort.

Methods

Breast cancer samples

The present study analyzed tumor tissue specimens from 103 cases of TNBC diagnosed in the Department of Obstetrics and Gynecology, Klinikum Rechts der Isar, Technical University of Munich (TUM), between 1988 and 2012. All samples were collected from pathology archives and selected based on the confirma-

tion by pathologists for the lack of expression of ER, PR, and the absence of overexpression of HER2. The screened cases were then subjected to qPCR assay and statistical analyses were performed.

Tumor staging was performed according to the TNM-staging system, which based on the primary tumor size (T), regional lymph node status (N), and distant metastasis (M) [30]. Medical treatment of patients comprised mastectomy or breast-conserving surgery, chemotherapy, and radiotherapy. The follow-up information of DFS (median follow-up period 72 months) was available for 99 patients and the available data of OS (median follow-up period 82 months) was for 101 patients. Written informed consent for utilization of tissue specimens for research purposes was obtained from all patients.

Assessment of KLK4 expression

Concerning RNA extraction, reverse transcription, first-strand cDNA synthesis, primers design, and quantitative PCR, a comprehensive description has been previously published [20]. Purified RNA was stored at -80°C until further use. Hypoxanthine-guanine phosphoribosyltransferase 1 (HPRT1) was used as reference gene [31].

Assessment of miR-378 expression

Total RNA (harboring both mRNA and miRNA) was isolated from the tissues using the automated QIAcube sample preparation machine (Qiagen), together with the All Prep DNA/RNA/miRNA Universal Kit (Qiagen) following the manufacturer's instructions [20]. After measurement of the total RNA concentration by spectrophotometry, reverse transcription of the isolated RNA (1 µg) was performed using 5x miScript HiFlex Buffer and the miScript II Reverse Transcription kit, as prescribed by the manufacturer (Qiagen). The final elution of samples in RNAse-free H_2O resulted in a final cDNA concentration of 1.66 ng/µl for samples. Then, all cDNA samples were stored at -20°C until further use.

For miR-378, gene-specific primers were designed based on the miRNA sequences in the miRBase database (http://microrna.sanger.ac.uk/). The assay was optimized for a SYBR-Green-based real-time PCR assay using QuantiTect SYBR Green PCR Mastermix and miScript SYBR Green PCR kit (assay ID: Hs_miR-422b_1) according to the manufacturer's instructions (Qiagen). All amplification reactions were performed in triplicates (input: 5 ng/well) in 96-well plates. The following cycling program was performed: 3 min for initial activation step at 95°C, followed by 3-step cycling (94°C, 15 sec [denaturation]; 55°C, 30 sec [annealing]; 70°C, 30 sec [extension]). The expression levels of miR-378 were normalized to miR-16 (assay ID: Hs_miR-16_2) [32] and were calculated utilizing the 2exp-ΔΔCt method.

Statistical analysis

Data analyses were carried out by employing the SPSS statistical analysis software (version 20.0; SPSS Inc.). Expression levels of KLK4 mRNA and miR-378 were compared using the Mann-Whitney U test and Spearman rank correlation (r_s). A detailed description of survival analyses and the association of tumor biological factors with clinical characteristics of patients have been previously published [20].

Results

KLK4 mRNA and miR-378 expression levels in tumor tissues of triple-negative breast cancer patients and relation to clinical characteristics

KLK4 mRNA and miR-378 levels were determined by qPCR in tumor tissues of 103 patients afflicted with TNBC. The relative KLK4 mRNA levels (normalized to the housekeeping gene (HPRT1) were in the range of 0.00 to 8.19 (median: 0.09) and relative miR-378 expression levels (normalized to miR16) ranged from 0.07 to 54.95 (median: 1.75). Based on previous observations in oral squamous carcinoma cells indicating that miR-378 efficiently targets KLK4 mRNA and by this reduces its mRNA levels [25-27], an inverse correlation of these two factors was expected. However, in Spearman rank correlation analysis, no correlation was observed (r_s=0.189, **Figure 1A** and **1B**). For further statistical analysis, the expression levels of both factors were classified into a low- versus high-expressing group by the median. Again, no indication for an inverse relationship of miR-378 versus KLK4 expression was observed. There was rather a trend towards significance concerning a positive association

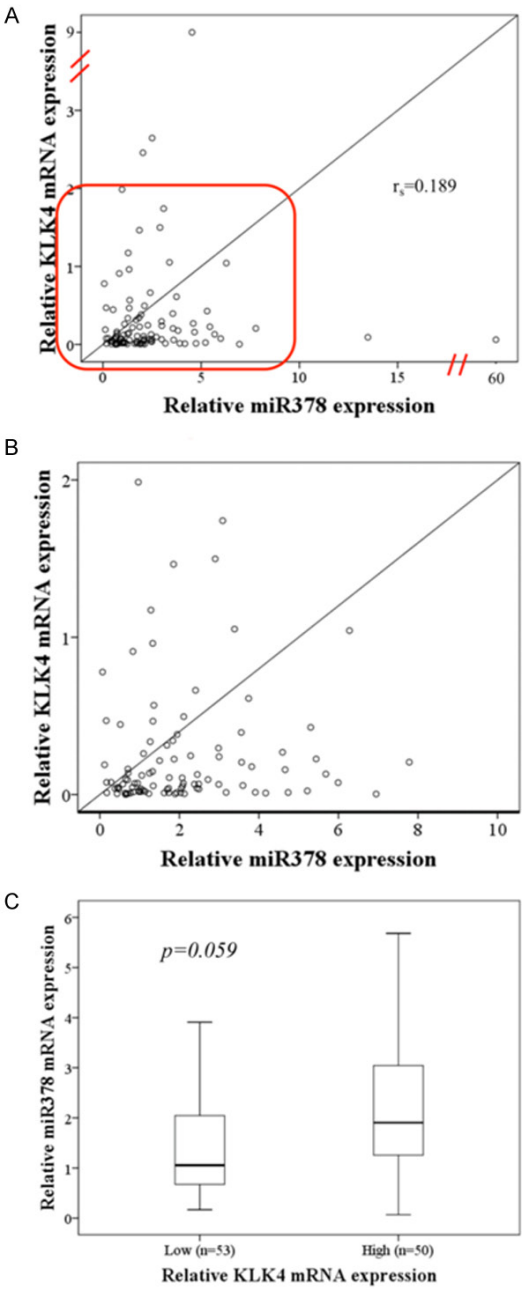

Figure 1. Correlation of KLK4 mRNA and miR-378 expression levels in tumor tissues of triple-negative breast cancer patients. A. In tumor tissue of TNBC patients (n=103), no significant correlation of KLK4 mRNA with miR-378 is observed (Spearman rank correlation analysis, r_s=0.189, P > 0.05). B. Enlargement of the inset of (A), with relative KLK4 mRNA expression from 0-2 and miR-378 from 0-9. Omitted cases: relative KLK4 mRNA expression > 2, n=3; relative miR-378 expression > 9, n=2. C. In Mann-Whitney test analysis, KLK4 mRNA expression shows a trend towards significance concerning a positive correlation with miR-378 levels in tumor tissue (P=0.059). Both factors were dichotomized into low and high by the median.

between the two factors (Mann-Whitney U-test, P=0.059, **Figure 1C**).

Table 1 depicts the correlation of expression levels of KLK4 mRNA, miR-378, as well as the combination of KLK4 mRNA and miR-378 with established clinical variables, including age (≤ 60 years vs. > 60 years), lymph node status (N0 vs. N+), tumor size (≤ 20 mm vs. > 20 mm), and histological grade (grade II vs. grade III). KLK4 mRNA expression is significantly associated with tumor grade (P=0.002), the combination of KLK4 and miR-378 expression (low/low versus high and/or high; KLK4+miR-378) shows a trend towards significance for tumor grade (P=0.053). miR-378 did not correlate with any clinical parameters.

High KLK4 mRNA and miR-378 expression levels are associated with recurrence and survival in triple-negative breast cancer

Kaplan-Meier survival analyses indicated that higher KLK4 mRNA levels are significantly associated with shorter DFS (P=0.030, **Figure 2A**) and show a trend towards significance for OS (P=0.063, **Figure 2B**). Patients with elevated miR-378 levels display both a worse DFS (P= 0.043, **Figure 2C**) and OS (P=0.027, **Figure 2D**), compared to those with low miR-378 levels. Combination of KLK4 mRNA and miR-378 (KLK4+miR-378, low/low versus high and/or high) allows to even better discriminate between patients with favorable and unfavorable prognosis for DFS (P=0.008, **Figure 2E**) and OS (P=0.025, **Figure 2F**).

These observations were further supported by univariate Cox regression analysis, depicted in **Table 2**. Here, patients with elevated KLK4 mRNA expression have a worse DFS (P=0.033) than those with low KLK4 levels, while patients with high miR-378 levels display a significantly increased risk of disease progression (P=0.047) and cancer-related death (P=0.031), compared to cases with low miR-378 levels. Regarding the combination of KLK4 and miR-378, high KLK4+miR-378 values represent a significant predictor for DFS (P=0.011) and OS (P=0.032). As expected, age is a significant predictive factor of DFS (P=0.002) and OS (P<0.001), while the lymph node status is a significant indicator of OS (P=0.031) only.

Table 1. Association between KLK4 mRNA and/or miR-378 expression levels with clinical or histomorphological parameters in TNBC patients

Clinico-pathological parameters	KLK4 mRNA[a]	miR-378[a]	KLK4 mRNA +miR-378[b]
Age	p=0.639	p=0.615	p=0.713
≤ 60 years	30/26	27/29	16/40
> 60 years	23/24	25/22	15/32
Lymph node status	p=0.392	p=0.775	p=0.125
N0	32/26	30/28	21/37
N+	21/24	22/23	10/35
Tumor size	p=0.254	p=0.363	p=0.656
≤ 20 mm	11/15	15/11	7/19
> 20 mm	42/34	36/40	24/52
Histological grade	p=0.002	p=0.444	p=0.053
Grade II	0/8	3/5	0/8
Grade III	53/42	49/46	31/64

Cut-off point (Chi-square test): [a]KLK4 mRNA as well as miR-378 = median; [b]combination of KLK4 mRNA and miR-378: low/low versus high and/or high. Due to one missing value, for tumor size only n=102 data are available.

Next, the independent relationship of KLK4, miR-378, and KLK4+miR-378 with DFS and OS was evaluated by the multivariable Cox regression analysis, which was adjusted by age, lymph node status, and tumor size in the base model (**Table 3**). As expected, age was found to be the strongest independent indicator of DFS (P=0.001) as well as OS (P<0.001). Lymph node status represents an independent predictive marker for OS (P=0.044). When added separately to the base model, the prognostic value of KLK4 is no longer statistically significant, but still shows a trend towards significance for DFS (P=0.061). Both miR-378 and KLK4+miR-378 expression represent independent predictive factors for DFS (P=0.014, P=0.010, respectively) and OS (P=0.016, P=0.049, respectively).

Discussion

In the present study, miR-378 and KLK4 mRNA expression levels via qPCR were quantified, for the first time, in a homogenous cohort of 103 patients afflicted with triple-negative breast cancer. Elevated miR-378 expression was demonstrated to be associated with both shorter OS and DFS. Also, elevated KLK4 mRNA levels turned out to be a marker for poor prognosis of TNBC patients, albeit to a lesser extent. Combination of both factors indicated that this allowed to even better discriminate between favorable and unfavorable patient outcome.

Numerous publications strongly suggest that miR-378 is a tumor-associated key regulator in different cancer types. Previous *in vitro* cell culture-based studies, including non-small cell lung cancer (NSCLS) [33] or chronic myeloid leukemia [34] cells, indicated that miR-378 supports tumor-promoting characteristics including cell proliferation, migration, invasion and angiogenesis. Furthermore, miR-378 expression was shown to be overexpressed, to stimulate migration and invasion, and to correlate with lymph node metastasis in cervical cancer [35]. In ovarian cancer, miR-378 expression is upregulated [36] and high miR-378 expression was reported to be associated with poor progression-free survival (PFS) [37]. This unfavorable prognosis was linked to miR-378-mediated dysregulation of genes involved in apoptosis and cell cycle regulation [38]. Moreover, miR-378 overexpression promoted cell proliferation in patients with osteosarcoma [39]. Tan and co-workers [40] suggested increased migration and invasion of cervical tumors when miR-378 expression was increased. Similar effects were detected in melanoma, where miR-378 correlates with metastasis formation in an epithelial-to-mesenchymal transition (EMT)-dependent matter [41].

However, in contrast to all of these results, in prostate cancer high miR-378 expression levels were found to be associated with a significant reduction in tumor size, suggesting that miR-378 may act as a tumor suppressor by decreasing proliferation, migration, and invasion [42]. In line with this, Li and colleagues [43] showed that high miR-378 expression was a favorable predictive marker for OS in glioma. Downregulation of miR-378 was detected in colorectal cancer and low expression was identified as an independent unfavorable prognostic factor for OS in colorectal cancer patients [44]. Functional studies in different cancer cell lines, including colon, gastric, prostate, glioma, and glioblastoma cells, showed that miR-378 upregulation also promotes tumor-suppressive properties such as reduced cell proliferation, migration, invasion, and increased apoptosis

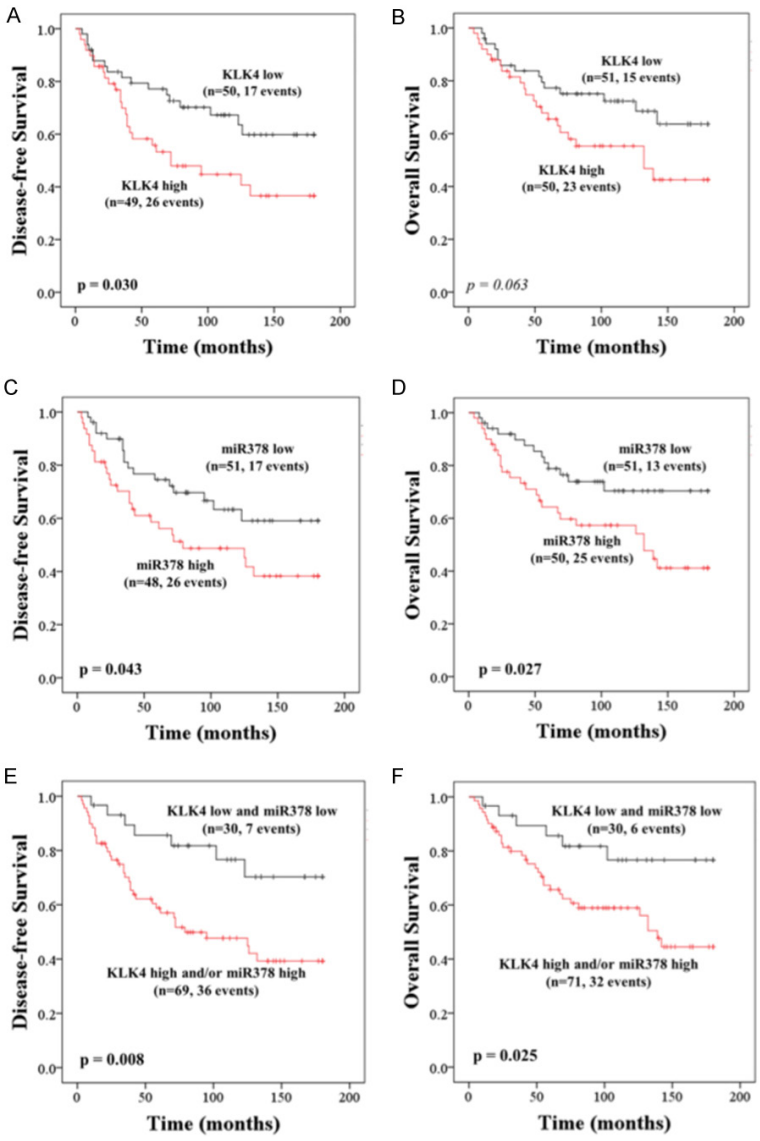

Figure 2. Disease-free and overall survival of TNBC patients as a function of KLK4 mRNA and/or miR-378 expression. Univariate Kaplan-Meier survival analysis reveals that TNBC patients with KLK4 mRNA status high have a significantly increased risk of relapse (A). Furthermore, a trend towards significance concerning death is observed (B). Patients displaying high expression of miR-378 display a significantly worse prognosis for both DFS (C) and OS (D) compared to patients with low miR-378 expression levels (Kaplan-Meier analysis). Patients with high KLK4 and/or high miR-378 expression display a significantly shorter DFS (E) and OS (F) compared to patients with low expression levels of both factors (Kaplan-Meier analysis). KLK4 mRNA and miR-378 levels were dichotomized into low and high by the median.

[42, 45-50]. All in all, overexpression of miR-378 may have different, either tumor-supporting or -suppressing, effects in different types of cancer.

Although expression and function of miR-378 have been studied in various cancer types, its potential role in breast cancer has not been fully elucidated. Using formalin-fixed paraffin-embedded tissue, miR-378 overexpression was observed by qPCR in breast cancer [51]. Yin et al. [52] found that miR-378 was upregulated but not associated with the clinicopathological status of breast cancer patients, *i.e.* age, grading or receptor status. In agreement with the latter study, our results revealed the lack of any association of miR-378 expression with clinical and histomorphological parameters in the subgroup of TNBC patients, including age, lymph node status, tumor size and histological grade. Another group identified that miR-378 expression leads to a metabolic shift from oxidative phosphorylation to glycolysis and promotes cell proliferation in breast cancer [28], thus indicating that ERBB2-induced miR-378 expression correlates with progression of breast cancer. These data are also supported by Winsel and others [53], who reported a link of miR-378 overexpression with breast cancer tumorigenesis as well. Indeed, regarding the clinical relevance, our present study demonstrates that miR-378 represents an independent predictive marker in TNBC, whereby its elevated expression is associated with a shorter OS and DFS of patients.

In other cancer entities, several reports are underlining the oncogenic potential of miR-378. In cholangiocarcinoma, miR-378 overexpression is not only an independent poor prognostic predictor for OS, but *in vitro* induces cell proliferation, migration, and invasion [54]. miR-378 was shown to prevent cell apoptosis and promote cell proliferation by inhibiting FOXG1 expression

Table 2. Univariate Cox regression analysis of clinical outcome in TNBC patients with respect to clinical and histomorphological parameters as well as KLK4 mRNA and/or miR-378 expression levels

Clinico-pathological parameters	DFS			OS		
	No[a]	HR (95% CI)[b]	p	No[a]	HR (95% CI)[b]	P
Age			**0.002**			**<0.001**
≤ 60 years	54	1		54	1	
> 60 years	45	2.63 (1.42-4.88)		47	3.56 (1.78-7.11)	
Lymph node status			*0.063*			0.031
N0	55	1		56	1	
N+	44	1.77 (0.97-3.24)		45	2.04 (1.07-3.92)	
Tumor size			0.450			0.418
≤ 20 mm	26	1		26	1	
> 20 mm	72	1.33 (0.64-2.78)		74	1.41 (0.62-3.20)	
Histological grade			0.667			0.481
Grade II	8	1		8	1	
Grade III	91	0.80 (0.28-2.24)		93	0.69 (0.24-1.95)	
KLK4 mRNA[c]			**0.033**			*0.068*
low	50	1		51	1	
high	49	1.95 (1.06-3.60)		50	1.84 (0.96-3.52)	
miR-378[c]			**0.047**			**0.031**
low	51	1		51	1	
high	48	1.86 (1.01-3.43)		50	2.10 (1.07-4.10)	
KLK4 mRNA+miR-378			**0.011**			**0.032**
low/low	30	1		30	1	
high and/or high	69	2.87 (1.27-6.45)		71	2.60 (1.09-6.22)	

Significant *p*-values (P<0.05) are indicated in bold, trends towards significance in italics. Due to one missing value, thenumber for tumor size does not add up to n=99 (DFS) and n=101 (OS). [a]Number of patients. [b]HR: hazard ratio (CI: confidence interval) of univariate Cox regression analysis. [c]Dichotomized into low and high levels by the median.

in lung cancer cells [55]. Another study showed that miR-378 overexpression in lung cancer cells leads to loss of E-cadherin accompanied by induction of the intermediate filament protein vimentin, *i.e.* to an EMT-like phenotype, resulting in tumor cell migration and invasion [56]. In an NSCLC model, Skrzypek and coworkers [57] found that miR-378 modulates vascular tube formation through enhancing expression of vascular endothelial growth factor (VEGF), interleukin-8 (IL-8) and Ang-1, thereby significantly modulating NSCLC progression and angiogenesis. Finally, in glioblastoma U87 cells, miR-378 was confirmed to regulate tumor growth and angiogenesis by targeting expression of the two tumor suppressors, SuFu and Fus-1, *in vitro* and *in vivo* [58]. Altogether, these studies strengthen the hypothesis that miR-378 overexpression is not only associated with tumor growth and metastasis, but also with inhibition of apoptosis and induction of angiogenesis, which can well be related to its association of high expression with poor prognosis in TNBC.

In breast cancer, the expression of the members of the KLK family is generally down-regulated compared to normal breast tissue. KLK4, however, is up-regulated both at mRNA and antigen levels in cancerous breast tissues compared to normal and benign breast tissues [13, 59, 60]. Elevated KLK4 mRNA expression is detectable in undifferentiated versus well-differentiated tumors, in higher stages and progesterone-negative tumors [13]. Moreover, KLK4 antigen levels were found to be higher in invasive breast carcinoma than in normal and ductal carcinoma *in situ* [29]. Besides, Yang et al. [14] investigated KLK4 protein expression by immunohistochemistry in 188 TNBC patients, illustrating that KLK4 overexpression in stromal cells, but not in tumor cells, was associated with poor DFS. In line with these findings, we observed that elevated KLK4 mRNA

Table 3. Multivariable Cox regression analysis of clinical outcome in TNBC patients with respect to clinical and histomorphological parameters as well as KLK4 mRNA and/or miR-378 expression levels

Clinico-pathological parameters	DFS			OS		
	No[a]	HR (95% CI)[b]	p	No[a]	HR (95% CI)[b]	p
Age			**0.001**			**<0.001**
≤ 60 years	53	1		53	1	
> 60 years	45	2.84 (1.51-5.34)		47	4.02 (1.96-8.21)	
Lymph node status			0.093			**0.044**
N0	55	1		56	1	
N+	43	1.71 (0.92-3.18)		44	2.00 (1.02-3.94)	
Tumor size			0.644			0.691
≤ 20 mm	26	1		26	1	
> 20 mm	72	1.20 (0.56-2.54)		74	1.19 (0.51-2.77)	
KLK4 mRNA[c]			*0.061*			0.146
low	50	1		51	1	
high	48	1.82 (0.97-3.42)		49	1.64 (0.84-3.19)	
miR-378[c]			**0.014**			**0.016**
low	50	1		50	1	
high	48	2.22 (1.18-4.18)		50	2.37 (1.18-4.76)	
KLK4 mRNA+miR-378			**0.010**			**0.049**
low/low	30	1		30	1	
high and/or high	68	2.97 (1.30-6.81)		70	2.44 (1.00-5.95)	

Significant *p*-values (P<0.05) are indicated in bold, trends towards significance in italics. Biological factors (KLK4 mRNA, miR-378, and KLK4 mRNA+miR-378) were separately added to the base model of clinical parameters: age, lymph node status, and tumor size. [a]Number of patients. [b]HR: hazard ratio (CI: confidence interval) of univariate Cox regression analysis. [c]Dichotomized into low and high levels by the median.

expression was significantly associated with shortened DFS (and showed a trend towards significance concerning shortened OS), further indicating that KLK4 may display a tumor-promoting function in TNBC. Elevated KLK4 mRNA expression was also found to be related to unfavorable prognosis in other cancer types such as prostate or ovarian cancer [20, 24].

KLK4 was previously found to stimulate tumor cell proliferation and metastasis by initiating proteinase-activated receptor (PAR) 1 and/or 2-mediated signaling [61-63] and/or activating urokinase-type plasminogen activator (uPA) [64, 65], which have been reported to be involved in breast cancer progression and associated with patient prognosis [66, 67]. In addition, KLK4 has been shown to activate the proform of MMP-1, again a protease promoting tumor growth and metastasis, as well as cleave off an N-terminal fragment of thrombospondin-1 displaying potential angiogenic activity [68, 69]. Moreover, KLK4 may regulate several pathways controlled by growth factors, *e.g.* by degrading members of the insulin-like growth factor (IGF) binding protein (IGFBP) family. This leads to an increased tissue availability of IGF [70]. Another example is activation of the proform of hepatocyte growth factor activator zymogen, which in turn is the major activator of the tumor-promoting pro-hepatocyte growth factor/scatter factor (pro-HGF/SF) [71]. In prostate tissues, an alternative form of KLK4, lacking exon 1, has been described, whereby its intracellular overexpression was proposed to result in sustained activation of both the androgen receptor and mTOR signaling pathways, crucial for cancer progression [72]. Taken together, all these observations delineate that KLK4 might represent a potential multifunctional modulator for supporting tumorigenesis.

Previous studies have indicated that an axis composed of miR-378 targeting the KLK4 mRNA may play an important role in inhibiting cancer progression. Initially, KLK4 was identified as a target of miR-378 in prostate cancer by *in silico* analysis [24]. In fact, when studying

whether miR-378 targets any of the mRNAs encoding members of the KLK family, Samaan and co-workers [73] demonstrated that prostate cancer cells transfected with the miR-378 precursor inhibited KLK4 and KLK14 expression. Moreover, a luciferase reporter assay confirmed that miR-378 binds to the 3'UTR of KLK4, confirming that miR-378 can modulate KLK4 mRNA expression through a post-transcriptional process [25]. In the present study, however, we did not find any evidence for the existence of such a miR-378/KLK4 axis in TNBC. Moreover, we also analyzed miR-378 and KLK4 mRNA expression in another type of cancer, namely in a cohort of patients afflicted with advanced high grade serous ovarian cancer. Again, there was no significant, inverse relationship observed between the expression levels of both factors. One possible explanation for the lack of a strong correlation between miR-378 and KLK4 mRNA *in vivo* may be that both factors are embedded in rather complex networks. Thus, a single miRNA will rarely lead to a dramatic impact on targeted mRNA expression, probably due to many further interactions involved in the tumor-associated miR- and/or proteolytic networks. In the study performed by Samaan and co-workers [73], who analyzed 23 miRNAs which are differentially expressed in patients of high- versus low-risk biochemical failure in prostate cancer, 8 of these, including miR-378, were predicted to target the KLK4 mRNA, whereas miR-378 was also able to target KLK2 and KLK14. Certainly, KLK4 expression may be regulated by further processes, such as promoter regulation, epigenetic modification, RNA splicing and stabilization.

Conclusion

In the present study, we analyzed the expression of both miR-378 and KLK4 mRNA in a homogenous TNBC patient cohort. Elevated miR-378 expression was identified as an independent unfavorable prognostic factor for DFS and OS, respectively. Increased KLK4 mRNA expression, to a lesser extent, was associated with poor patient prognosis. Especially, the combination of low miR-378 and low KLK4 mRNA expression allowed better identification of patients with an about 2.5- to 3-fold decreased risk of death and relapse. These findings support the potential role of miR378 and KLK4 as novel biomarkers in breast cancer and may also represent attractive targets for therapeutic applications. As the present study is the first to report simultaneous expression of miR-378 and KLK4 mRNA in TNBC, further cell biological assays are necessary with respect to this breast cancer subtype to obtain more insights into the involvement of miR-378 and KLK4 in tumor biological functions.

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Disclosure of conflict of interest

None.

Abbreviations

DFS, disease-free survival; ER, estrogen receptor; EMT, epithelial-mesenchymal transition; HER2, human epidermal growth factor receptor 2; HPRT1, hypoxanthine-guanine phosphoribosyl transferase 1; IGF, insulin-like growth factor; IGFBP, insulin-like growth factor-binding protein; IL-8, interleukin-8; KLK, kallikrein-related peptidase; N, regional lymph node status; NSCLS, non-small cell lung cancer; M, distant metastasis; miR-378, microRNA 378; miRNAs, microRNAs; MMP, matrix metalloproteinase; OS, overall survival; OSC, oral squamous carcinoma; PAR, proteinase-¬activated receptor; PFS, progression-free survival; PR, progesterone receptor; pro-HGF/SF, pro-hepatocyte growth factor/scatter factor; qPCR, quantitative PCR; T, primary tumor size; TNBC, triple-negative breast cancer; TUM, Technical University of Munich; uPA, urokinase-type plasminogen activator; UTRs, untranslated regions; VEGF, vascular endothelial growth factor.

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