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Gene Expression of Kallikreins in Breast Cancer Cell Lines

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Abstract. *Background/Aim: This study analyzed the gene expression of the "classic" KLK1 and "new" kallikreins KLK4-KLK15, in relation to the molecular characteristics and in vitro invasiveness of 21 breast cancer (BC) and three normal breast-derived cell lines (CLs). Materials and Methods: Gene expression of KLKs was determined by using real-time polymerase chain reaction (PCR). The invasiveness of the CLs was examined using a fibroblast-collagen-based in vitro cell culture assay. Results: KLK5 and KLK7–KLK11 were down-regulated in several BCCLs. In contrast, KLK4, KLK8, KLK12 and KLK15 demonstrated strikingly high expression in two BCCLs, UACC 812 and MDA-MB 330. The KLK expression differed frequently according to the presence of androgen receptor (KLK1 and KLK5–KLK9), and occasionally according to estrogen receptor (KLK9) and EGFR (KLK7). Two KLK clusters were detected (first: KLK1, 4, 12, 15; second: all other KLKs), with two subclasses within the second cluster (KLK5–9 and KLK10, 11, 13, and 14). The CLs that expressed at least six KLKs belonged predominantly to basal or HER2 intrinsic subtypes. No KLK predicted the in vitro invasiveness of CLs. Conclusion: Gene expression of KLKs was altered in BCCLs. This change was mostly down-regulation and often related to the presence of androgen receptor. The observed clusters point to a possible functional interplay of selected KLKs in BCCLs.*

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Breast cancer (BC) is the most common – and increasingly frequent – diagnosed malignancy in women worldwide, with an estimated 2.1 million cases and 626,679 deaths in 2018 (1). BC accounts for 24.2% of new cancer diagnoses and 15% of all cancer-related deaths in women (1). Traditionally, BC has been classified histologically according to type, *e.g.*, ductal or luminal, and grade, and according to the expression of estrogen receptor (ER), progesterone receptor (PR), or the overexpression of human epidermal growth factor receptor 2 (ERBB2, Her2/neu). ER and Her2/neu are established targets for the medical treatment of BC. The absence of ER, PR, and Her2/neu [triple-negative breast cancer (TNBC)] is associated with a poor prognosis. More than a decade ago, this traditional classification – based on gene expression analysis – was further refined into five molecular subtypes: luminal A, luminal B, Her-2-enriched (HER2), basal-like (overlaps with TNBC in approximately 80% of cases) and normal-like (2, 3). Subsequently, some alternative molecular classifications have been proposed (3). However, BC presents much more genetic and phenotypic heterogeneity, as outlined by five or six molecular subtypes, especially within the basal-like group.

The human kallikrein (*KLK*) gene and protein family was discovered at the end of the twentieth century (4, 5). Previously, only three "classic" kallikrein-related peptidases, KLK1-3, were sufficiently recognized. KLK3 (also known as "prostate specific antigen", PSA) quickly became a widely used marker for prostate cancer (4, 6). The role of the twelve "new" kallikrein-related peptidases (KLK4-KLK15) in physiology and disease remains under investigation (7). All 15 members of the human *KLK* gene family encode secreted serine proteases with trypsin-like (KLK2, KLK4-KLK6, KLK8, KLK10, KLK12, KLK13), chymotrypsin-like (KLK3, KLK7, KLK9), or both (KLK1, KLK11, KLK14) activities (5). They share a similar genomic organization, are often co-expressed, and have significant homology at both the nucleotide and protein level (5, 8). All KLK genes are located at the same chromosomal locus, namely 19q13.4; they represent the largest contiguous cluster of

proteases in the human genome (4, 5). Their gene expression is controlled by steroid hormones (androgens, gestagens, estrogens, or corticoids) and epigenetically through DNA methylation (4, 9-11).

KLKs are secreted as proenzymes in the intra- and extracellular space and eventually cleaved to active enzymes by auto-cleavage (*e.g.*, KLK2, KLK6, KLK13), cross-cleavage (*e.g.*, KLK5 activates itself and pro-KLK2, -KLK3, -KLK6, -KLK7, -KLK11, -KLK12, and -KLK14) or by other proteases (*e.g.*, trypsin and matrix metalloproteinases [MMPs], both of which activate a broad range of pro-KLKs) (5, 6, 11, 12). Typically, the coordinated action of KLKs occurs *via* proteolytic cascades or "activomes" (6, 13). Some examples for those proteolytic cascades are: KLK2, KLK3, and KLK5 in semen liquefaction or KLK5, KLK7, KLK8, and KLK14 in skin desquamation processes (5, 6). The most known KLK inhibitors are serpins and zinc ions (6, 7). Physiologically, KLK2 and KLK3 are almost exclusively expressed in prostate (5, 14) and detectable at only low levels in other tissues. The wide distribution of other KLKs in body tissues and fluids (14) mirrors the broad spectrum of their physiological roles, but makes the precise delineation of their individual functions more difficult. The action of KLKs can roughly be "mechanistic", *e.g.*, by degradation of major components of extracellular matrix (ECM) such as laminin, fibronectin and different types of collagen (5, 15, 16), or regulatory, through cleaving and thus activating pro-hormones, cell-surface receptors, growth factors, and their binding proteins, *e.g.*, epidermal growth factor (EGF), insulin-like growth factor (IGF), or transforming growth factor beta (TGFβ) binding proteins (7, 8, 11). Physiologically, KLKs play a role in semen liquefaction, skin desquamation, angiogenesis, cellular and humoral immunity, synaptic plasticity, tooth enamel formation, and blood coagulation, among others (4-8, 11). Their dysregulation has been observed in a range of pathological conditions, including respiratory diseases, neurodegeneration, anxiety, schizophrenia, skin-barrier dysfunction, and cancer (5, 7, 11). Nevertheless, we are still far from understanding the entire spectrum of the physiological roles and *in vivo* KLK targets (7, 11).

The up- or down-regulation of KLKs at the gene and protein levels have been observed in various malignancies, especially those of the prostate, breast, ovary, and kidney (5-11, 17-19). Some KLKs, *e.g.*, KLK5 or KLK10, are recognized as tumor suppressors, with down-regulation and activity loss during cancer progression (8, 17-20). Others are involved in cancer growth, invasion, and metastasis through degradation of extracellular matrix, promotion of angiogenesis, and processing of growth factors and adhesion molecules (5, 8, 17-18, 20).

Most KLKs are expressed in normal breast tissue or milk (4, 14). Their down- or up-regulation is a hallmark of BC development (8, 16-18, 20-24). For instance, KLK5 overexpression occurs frequently in premenopausal, more advanced, and ER-negative BCs and is associated with unfavorable prognosis (21, 22). On the contrary, KLK5 and KLK7 under-expression is correlated with postmenopausal tumors and ER-positive status (21, 22). Furthermore, KLK5 and KLK7 are tandemly down-regulated when comparing cancerous to benign tissues as well as metastases to primary tumors (21). KLK6 messenger RNA (mRNA) is significantly down-regulated in the majority of BCs, but its overexpression occurs in 27% of BC cases. This overexpression is associated with poor differentiation, basal or HER2 subtype, and dismal clinical prognosis (16, 24). Similarly, basal and HER2 tumors demonstrate higher KLK8 expression compared to luminal subtypes. Furthermore, KLK8 overexpression is proposed to be an independent predictor of adverse disease-free survival (DFS) (25). The observations about synchronous down-regulation of multiple KLKs in BC is not limited to KLK5 and KLK7. Yousef *et al.* (20) have demonstrated a simultaneous down-regulation of KLK5, KLK6, KLK10, and KLK12 in BC, with KLK10 under-expression as the most significant. This finding supports the role that KLKs, especially KLK10, serve as tumor suppressors in healthy breast (14, 17, 18). However, higher KLK14 expression can occur in advanced BC indicating cancer-promoting properties of KLK14 (18). KLKs can exhibit differential expression during various stages of BC as well as depending on the particular cell line derived from a primary or metastatic tumor (18). Taken together, the relationship of KLKs to initiation and development of BC is multifaceted and still not sufficiently elucidated.

In the present study, we aimed to analyze the gene expression of the "classic" KLK1 and the "new" kallikreins (KLK4-KLK15) in relation to the molecular characteristics and *in vitro* invasiveness in a broad panel of 21 breast cancer cell lines (BCCLs) and three CLs that originated from benign breast tissues. According to the research question, the term "KLK expression" is used for the mRNA – not the protein – expression.

Materials and Methods

Cell lines. The breast cancer cell lines BT 20, BT 474, BT 549, CAMA-1, Du 4475, Hs 578T, MCF 7, MDA-MB 134-VI, MDA-MB 175-VII, MDA-MB 231, MDA-MB 330, MDA-MB 361, MDA-MB 436, MDA-MB 453, MDA-MB 468, SKBR 3, T 47D, UACC 812, UACC 893, ZR 75-1, ZR 75-30, as well as the normal mammary gland cell lines HMEC, MCF 12F, MCF 10F were purchased from American Type Culture Collection (ATCC; Rockville, MD, USA) and cultivated according to the manufacturer's instructions.

In vitro invasiveness assay. The invasive capacity of the cell lines was analyzed by a previously reported method (26). Briefly, human foreskin fibroblasts were suspended in minimal essential medium containing 10% fetal calf serum (FCS) and 0.05% rat-tail collagen and immediately transferred into a well of the tissue culture cluster

plate (Costar, Cambridge, MA, USA). A glass plate insert was inserted into the mixture to form chambers of collagen matrix, where the cell suspension was added. After certain time of incubation, collagen matrix chambers were fixed and embedded in paraffin. Sections were stained and the invasiveness of the cells was determined by microscopy. Cell lines, which grew into the collagen matrix either as single cells or as clusters of cells were defined as invasive, whereas cell lines staying at the surface of the matrix were defined as non-invasive.

Total RNA preparation. Total RNA was extracted from cell lysates by isopycnic centrifugation as described previously (27) followed by a DNA digestion step of incubation with RNase-free DNase I (Roche Diagnostic, Mannheim, Germany) at 37˚C for 15 minutes. Quality of RNA was examined by 2100 Bioanalyzer together with RNA 6000 Nano Chips and RNA 6000 Nano Reagent & Supplies (Agilent Technologies, Waldbronn, Germany). The concentrations were determined spectrophotometrically.

Reverse transcription (RT). RT was carried out using Omniscript Reverse Transcriptase kit (Qiagen, Hilden, Germany). The total reaction volume was 20 μl including 500 ng RNA. The reaction mixture was incubated at 37˚C for 60 min, heated at 95˚C for 10 min and then cooled on ice. The cDNA was diluted 1:5 in water. Four μl were aliquoted for further analysis.

Real-time PCR. The primers and probes for the quantitation of the KLK1 and KLK4-KLK15 RNA and beta-2-microglobulin cDNA were designed using Sequence Detection System software (Version 1.3, Perkin-Elmer Biosystems, Foster City, CA, USA). The primers and probes for beta-2-microglobulin were included in TaqMan® PDAR beta-2-microblobulin RNA control reagent (Applied Biosystems, Branchburg, NJ, USA). The 5700 Sequence Detection System (Applied Biosystems) was used for real-time analysis. Four microliters of cDNA aliquot were used as the template for PCR in a total volume of 25 μl, including TaqMan® Universal PCR Master Mix and the corresponding probes and primers. The mixture was pre-incubated at 90˚C for 10 min followed by 50 cycles of two-step incubations at 95˚C for 15 sec and 60˚C for 1 min. All samples were measured in duplicates.

Quantitation of gene expression. The relative quantitation method with standard curves was used for the calculation of the relative amounts of mRNAs. The sample with a high expression level of a certain gene was chosen as calibrator. Its expression was defined as 1. Standard curve using serial dilutions of the calibrator was used to calculate the amount of RNAs in other samples. Target quantities of all other samples were expressed as n-fold in relation to the calibrator. To correct the quantity differences in the starting RNA samples, the target quantity of certain mRNA was normalized to that of the constitutively expressed housekeeping gene beta-2-microglobulin in the same sample.

Statistical analysis. To compare relative expression of KLKs in malignant and non-malignant cell lines in relation to common parameters (receptor status, histological and intrinsic subtype) we used the non-parametric, distribution-independent Kruskal-Wallis test with the Steel-Dwass-Critchlow-Fligner post hoc procedure for adjusting multiple pairwise comparisons. We investigated and visualized the cluster formation of the KLKs in our cell line panel by generating a heat map. Expression data was log-transformed and standardized before plotting. Using a logistic regression model, we examined whether KLK expression could predict the invasiveness of CLs in our *in vitro* assay. The statistical analyses were performed with the software package XL Stat 2019 Test Version (Dell Inc., Tulsa, OK, USA) and by the R Project for Statistical Computing (R Foundation for Statistical Computing, Vienna, Austria) (28). Two-sided p-values <0.05 were considered statistically significant.

The study was approved by the Institutional Review Board of the Medical University of Vienna, Vienna, Austria (Reference: EK/109/2003).

Results

Table I shows the characteristics of all investigated CLs [obtained from the manufacturer (29) and literature reviews (30, 31)] and the relative KLK expression in relation to selected parameters. Considering all investigated CLs, KLK5, KLK 7, KLK8, KLK9, and KLK10 (and a trend for KLK 11, with p=0.057) were significantly down-regulated in BCCLs compared to non-malignant CLs. However, there were striking, contrasting KLK expression profiles in particular BCCLs (Table I). For example, UACC 812 and MDA-MB 330 demonstrated very high KLK4, KLK8, KLK11, KLK12, and KLK15 expression compared to normal CLs or the majority of other BCCLs (*e.g.*, the expression of KLK4 and KLK12 in UACC 812 was roughly 150-fold and 300-fold higher as in non-cancerous MCF 12F and HMEC, respectively).

As shown in Table II, significant differences in KLK1, KLK5, KLK6, KLK 7, KLK8, and KLK9 gene expression among BCCLs were related to the androgen receptor (AR) presence; significantly higher KLK expression was observed in AR-negative BCCLs and normal breast CLs. On the contrary, the KLK expression often remained low or absent in AR-positive BCCLs. Regarding ER, the only significant difference was for KLK9, although KLK7 and KLK8 also nearly approached the significance level (p=0.053 for both). A singular significant difference, as determined by epidermal growth factor receptor (EGFR) expression, was noted for KLK7.

The heat map and dendrogram in Figure 1 show the cluster formation based on KLK expression in the investigated cell lines. We observed KLK clustering into two main classes: first: KLK1, 4, 12, 15; second: all other KLKs. The second cluster formed two subclasses: KLK5-9 and KLK10, 11, 13, and 14.

Otherwise, CLs strongly differed according to KLK expression. Table II and the vertical dendrogram in Figure 1 allow to delineate two main classes of CLs according to the distribution and intensity of KLK expression. The first class (expression of multiple KLKs) includes all non-cancerous CLs (MCF 12F, HMEC, MCF 10F) and six out of 21 BCCLs (Du 4475, MDA-MB 330, MDA-MB 468, UACC 812, UACC 893, BT 20) – all of them being negative for AR, ER,

Table I. *Breast cell line characteristics and gene expression of KLK 1 and KLK4-KLK15 (normalized to the expression of housekeeping gene beta-2-microglobulin; in bold).*

Name	Invasive growth	ER	PR	ERBB2	AR	EGFR	Histology	Common subtype	Intrinsic subtype	KLK1	KLK4	KLK5	KLK6	KLK7	KLK8	KLK9	KLK10	KLK11	KLK12	KLK13	KLK14	KLK15
MCF 12 F*	+						N/FCD			0.000	**0.053**	**2.869**	**0.143**	**0.763**	**14.086**	**19.111**	**15.393**	**5.741**	0.000	**0.654**	**0.061**	0.000
HMEC*	+						Normal			0.000	0.000	**3.105**	**0.026**	**2.746**	**1.161**	**1.281**	**8.226**	**5.784**	**0.045**	**0.193**	**0.250**	0.000
Du 4475	–	–	–	–	–	–	IDC	TNBC	Basal	0.000	**0.545**	**0.050**	**0.119**	**0.222**	0.000	**2.112**	**3.203**	**11.149**	0.000	**6.781**	**3.437**	0.000
MDA-MB 330	+	–	–	+	–	+	ILC	ER–/ HER2+	HER2	**2.086**	**7.236**	**0.139**	**0.040**	**1.333**	**1.058**	**0.292**	**7.326**	**21.839**	**1.248**	**5.211**	**4.344**	**0.708**
MDA-MB 468	+	–	–	–	–	+	AC	TNBC	Basal	**0.967**	**0.027**	**4.010**	**3.245**	**0.281**	**5.090**	**13.818**	**3.193**	0.000	0.000	**0.037**	**0.115**	0.000
UACC 893	–	–	–	+	–	–	IDC	ER–/ HER2+	HER2	0.801	0.000	**0.371**	**0.312**	**0.206**	**3.387**	**0.242**	**0.128**	0.000	0.000	**0.023**	**0.016**	0.000
MCF 10 F*	+						Normal			0.000	0.000	**3.451**	**0.350**	**8.807**	**1.186**	**3.696**	**3.651**	0.000	0.000	0.000	**0.014**	0.000
BT 20	+	–	–	–	–	+	IDC	TNBC	Basal	0.000	0.000	**2.021**	**0.793**	**0.046**	**0.485**	**0.823**	**0.014**	0.000	0.000	0.006	**0.012**	0.000
UACC 812	–	–	–	+	–	–	IDC	ER–/ HER2+	HER2	0.000	**7.903**	**0.576**	**0.605**	0.000	**6.701**	**1.132**	**0.629**	0.000	**14.277**	**0.233**	0.000	**2.265**
MDA-MB 436	+	–	–	–	–	–	IDC	TNBC	Basal	**7.190**	**0.021**	0.000	**0.317**	0.000	0.000	0.000	0.000	0.000	0.339	0.000	**0.009**	**7.470**
CAMA-1	–	+	+	–	+	–	IDC	ER+	Luminal A	0.000	**0.978**	**0.101**	0.000	0.000	0.000	0.000	**0.589**	0.000	**4.365**	**0.208**	**0.149**	0.000
MCF 7	–	+	+	–	+	–	IDC	ER+	Luminal A	0.000	0.000	0.000	**0.030**	0.000	0.000	0.000	**0.042**	**4.551**	**4.106**	**0.076**	0.000	0.000
T 47 D	–	+	+	–	+	–	IDC	ER+	Luminal A	0.000	0.109	0.000	0.000	0.000	0.000	0.000	**0.137**	0.000	0.000	0.030	0.000	0.000
BT 474	+	+	+	+	+	–	IDC	ER+/ HER2+	HER2	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	**2.714**	0.000	0.000	0.000
MDA-MB 361	–	+	–	+	+	–	AC	ER+/ HER2+	HER2	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	**1.138**	0.000	0.000	0.000
MDA-MB 175 VII	–	+	–	–	+	–	IDC	ER+	Luminal A	0.000	0.000	0.000	**0.210**	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000
BT 549	+	–	–	–	–	–	IDC	TNBC	Basal	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000
ZR 75-30	–	–	–	+	–	–	IDC	ER–/ HER2+	HER2	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000
MDA-MB 134 VI	+	+	–	–	+	–	ILC	ER+	Luminal A	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000
SKBR 3	–	–	–	+	+	–	IDC	ER–/ HER2+	HER2	0.000	**0.007**	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	**0.010**	0.000
MDA-MB 231	+	–	–	–	+	+	IDC	TNBC	Basal	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	**0.008**	0.000
ZR 75-1	–	+	+	–	+	–	IDC	ER+	Luminal A	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.089	0.000
MDA-MB 453	–	–	–	–	+	–	Apo	TNBC	Basal	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	**0.032**	0.000
HS 578 T	+	–	–	–	–	+	IDC	TNBC	Basal	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	**0.025**	0.000

*Non-malignant cell lines; Column "Invasive growth": (+) invasive growth *in vitro*, (–) non-invasive growth *in vitro*; Other columns: (+) positive, (–) negative. IDC: Invasive ductal carcinoma (NOS); Apo: apocrine carcinoma; ILC: invasive lobular carcinoma; AC: adenocarcinoma (further information lacking in literature and manufacturer's specification); ER: estrogen receptor; HER2: human epidermal growth factor receptor 2; TNBC: triple negative breast cancer; N/FCD: normal with fibrocystic disease. The characteristics of BCCLs was obtained from the manufacturer (29) and selected literature (30, 31).

Table II. *Differences in relative KLK expression according to selected parameters. The significance of differences is shown as p-values (in bold if p<0.05).*

Kallikrein	Malignant *vs.* non-malignant	Invasive *vs.* non-invasive	Basal *vs.* Luminal A *vs.* HER2	ER	PR	AR	ERBB2	EGFR
KLK1	0.420	0.230	0.395	0.091	0.228	0.024	0.514	0.185
KLK4	0.802	0.550	0.876	0.407	0.850	0.227	0.608	0.850
KLK5	**0.005**	0.156	0.507	0.092	0.376	**0.012**	0.534	0.077
KLK6	0.208	0.339	0.543	0.108	0.143	**0.009**	0.934	0.234
KLK7	**0.002**	0.054	0.284	0.053	0.167	**0.010**	0.653	**0.027**
KLK8	**0.008**	0.149	0.198	0.053	0.167	**0.010**	0.134	0.060
KLK9	**0.004**	0.144	0.211	**0.029**	0.121	**0.004**	0.513	0.078
KLK10	**0.004**	0.497	0.955	0.495	0.647	0.086	0.804	0.314
KLK11	0.057	0.569	0.984	0.766	0.786	0.418	0.902	0.588
KLK12	0.716	0.680	0.165	0.196	0.094	0.706	0.120	0.431
KLK13	0.318	0.706	0.903	0.659	0.464	0.138	0.869	0.522
KLK14	0.139	0.441	0.320	0.097	0.731	0.213	0.437	0.059
KLK15	0.494	0.547	0.396	0.154	0.309	0.056	0.244	0.786

ER: Estrogen receptor; PR: progesterone receptor; AR: androgen receptor; ERBB2 (=HER2): human epidermal growth factor receptor 2; EGFR: epidermal growth factor receptor.

and PR and belonging predominantly to basal or HER2 intrinsic subtypes. The second class comprises all other BCCLs with low or absent KLK expression.

None of the KLKs, as ascertained by the logistic regression analysis (Table III), predicted BCCL invasiveness in our collagen-fibroblast-based assay. Similarly, KLK expression did not significantly differ between invasive and non-invasive CLs. Notably, all benign CLs, which showed a high expression of almost all KLKs, presented invasive growth in the collagen-based *in vitro* invasiveness assay.

Discussion

The role of KLKs in the initiation of carcinogenesis is increasingly evident. Indeed, dysregulated KLK function results in proteolytic activation and aberrant activity of several proteases [other pro-KLKs, MMPs, urokinase-type plasminogen activator (uPA)], cell surface receptors (*e.g.*, protease-activated receptors, uPA receptor), growth factors [IGF binding proteins (IGFBPs), latent TGFβ, and hormones (parathyroid hormone-related protein)], all of which result in an accumulation of tumorigenic stimuli and trigger cancer development (8). KLK-dependent dysregulation of the IGF axis results in pro-mitogenic and anti-apoptotic effects on normal and breast tumor cells (4, 8). The migration of tumor cells into the surrounding tissues and entry into the circulation are fundamental for cancer dissemination. This process requires proteolysis of ECM proteins, where KLKs are involved both directly by cleaving the ECM compounds and indirectly by activation of other ECM-degrading proteases (4). We observed that the majority of KLKs (at least 8 out of 13 studied) were expressed in CLs derived from normal breast tissues, but otherwise most were expressed in six out of 21 malignant BCCLs (MDA-MB 468, UACC 812, UACC 893, BT 20, DU 4475, and MDA-MB 330). The observed higher KLK expression in non-malignant CLs reached statistical significance for KLK5 and KLK7-KLK10. These data are consistent with their presumed role as tumor suppressors, which are subsequently down-regulated during cancer progression (8, 17). Therefore, we were not surprised that two thirds of BCCLs in our assay expressed KLKs at a low level or not at all. However, there were exceptions. For instance, we observed strong KLK10 expression in all normal CLs, as well as three aggressive BCCLs (Du 4475, MDA-MB 330, and MDA-MB 468). Similarly, KLK5 expression was significantly up-regulated in non-malignant CLs and two basal-like BCCLs, BT 20 and MDA-MB 468. KLK15 has been reported to be almost undetectable in normal breast tissues (14, 18). In the present study, KLK1 and KLK15 were not expressed in benign and the majority of malignant CLs. Nevertheless, the basal-like BCCL MDA-MB 436 had very high expression of both KLK1 and KLK15, while two HER2 BCCLs, UACC 812 and MDA-MB 330, expressed KLK15 along with multiple other KLKs. The latter observation is of interest because all three BCCLs were AR-negative, while KLK15 has been previously reported as an androgen-regulated KLK, associated with favorable BC prognosis (32). In the studied BCCLs, KLK expression (KLK1 and KLK5-KLK9) differed significantly with regards to AR, and – to much lesser degree – ER (only KLK9) and EGFR (only KLK7). These results are consistent with the knowledge that KLK expression remains under the control of steroid hormones, especially androgens (4, 9, 10, 17, 32). Notably, in our study all BCCLs with high KLK expression were negative for ER, PR, and AR. This

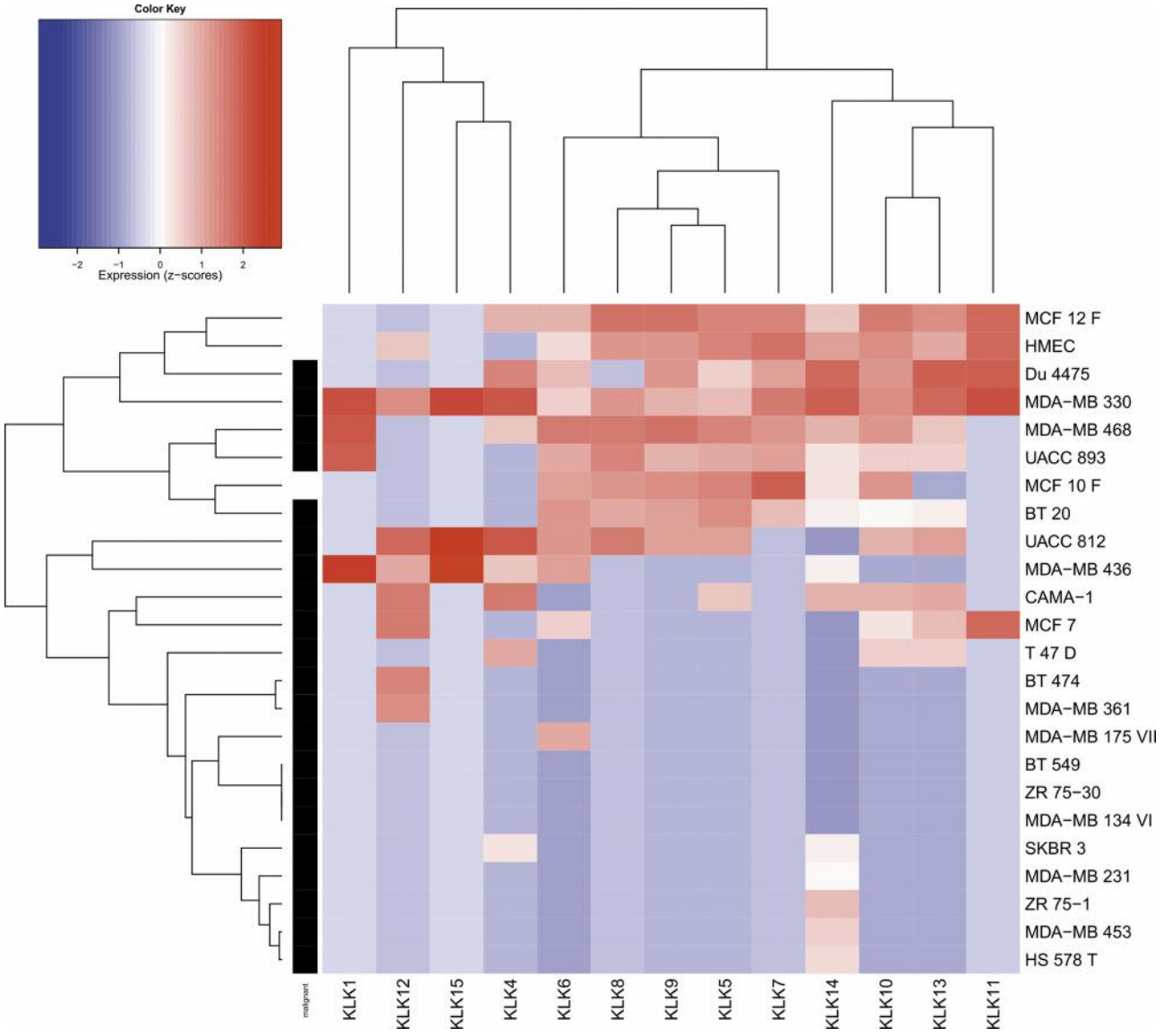

Figure 1. *KLK expression in breast cancer cell lines. KLK expression relative to beta-2-microglobulin expression is shown. Red indicates higher relative expression of a gene compared to the other cell lines while blue indicates lower relative expression. CLs are arranged in rows while KLK expression data are arranged in columns.*

finding is important because AR has recently been proposed as a parameter for further subclassification of TNBC into "AR+ TNBC" and "quadruple negative" BC (33). AR has been identified as a mediator and biomarker of radioresistance in BC (34), and anti-AR therapies emerge as promising treatment options (33, 35). We did not investigate KLK expression under hormonal stimulation, so their expression in the hormone-receptor positive BCCLs was rare or completely absent (*e.g.*, MDA-MB 134 VI, ZR 75-1, and BT 474). Nevertheless, in almost every BCCL, KLK expression did not show any association with the hormone-receptor status.

Some BCCLs demonstrated contrastingly high KLK expression patterns that were independent from the intrinsic subtypes to which they were attributed. BT 549, ZR 75-30, and MDA-MB 134-VI, each representing another intrinsic BC subtype (basal, HER2 and luminal A, respectively), presented virtually no expression of any KLK, while Du 4475, MDA-MB 330, and MDA-MB 468 (belonging to basal or HER2 subtypes) showed similar and unusually high (for BCCLs) KLK expression. Such paradoxes have been observed by other authors, *e.g.*, for KLK8, which is generally down-regulated in BC, but its higher expression in

TNBC (as compared to luminal A and B subtypes) correlates with advanced tumor stage and poor prognosis (25). There were striking differences within the HER2 subtype (*e.g.*, ZR 75-30 with no expression versus MDA-MB 330 and UACC 812, both of which showed high KLK expression) and basal subtype (*e.g.*, BT 549 with null KLK expression, followed by MDA-MB 231, MDA-MB 453, and HS 578T with low expression of singular KLK14, in contrast to Du 4475, MDA-MB 468, BT 20, and MDA-MB 436, all of which presented moderate to very high levels of multiple KLKs). Only the luminal BCCLs showed uniformly no or low KLK expression, with few exceptions concerning singular BCCLs, like CAMA-1 with high expression of KLK12, and MCF-7, that expressed high KLK11 and KLK12 levels.

The original histological type of BCCLs – ductal or lobular – did not influence KLK expression. The underreporting and the difficulty in obtaining reliable results with cultured lobular BCCLs is known (36, 37). In our study, two "classical" lobular BCCLs, MDA-MB 330 and MDA-MB 134 VI, constituted two extremes concerning KLK expression: While MDA-MB 134 VI expressed no KLKs, MDA-MB 330 was the only CL in our set (including the non-cancerous ones) that expressed all studied KLKs, and some of them – KLK4, KLK10, KLK11, KLK13, and KLK14 – at a very high level. Both aforementioned lobular BCCLs are derived from pleural effusions in premenopausal patients. However, they differ according to molecular characteristics: MDA-MB 330, attributed to the HER2 intrinsic subtype, is an ER- and AR-negative and ERBB2- and EGFR-positive BCCL, while MDA-MB 134 VI, part of the luminal A intrinsic subtype, is positive for ER and AR, and negative for ERBB2 and EGFR (29-31).

These findings indicate differentiated KLK functions within the cellular and molecular heterogeneity of BCCLs. At this point, one should address the shortcomings of CL-based research. Although approximately 100 BCCLs have been available for the last half decade, 70% of all reported studies have been limited to three cell lines: MCF 7, T47D, and MDA-MB 231 (31, 38). We used one of the largest panels of CLs reported in the KLK literature to reflect "the intratumoral heterogeneity of individual breast tumors, regardless of their molecular classification", as previously postulated (39). In turn, we obtained results that represent the diversity of BC with regards to KLK expression. Another methodological difficulty is that CL characteristics – as defined by manufacturer and replicated in the literature – are often restricted to few parameters (expression of hormone receptors, key mutations, *etc*.) and are not routinely revised. In fact, during decades of cultivation, accumulating mutations and epigenetic modifications can lead to decreasing similarity of BCCLs with the tumors they are supposed to represent (3, 31, 38). Furthermore, normal breast CLs (*e.g.*, MCF 10A and MCF 10F) can change their benign phenotype under laboratory conditions (39). Having said that, we cautiously interpret the results of our logistic regression analysis, which demonstrated that none of the KLKs could predict cell invasiveness *in vitro*. All normal breast CLs (HMEC, MCF 12F, and MCF 10F), which constitutively show high KLK expression, grew "invasively" in the assay, probably as the result of the physiological ability of KLKs to cleave collagen. Invasive growth is not limited to malignant cells; for example, trophoblast or ectopic endometrial cells invade the surrounding tissues in physiological (placentation) or pathological conditions (*e.g.* placenta increta, deep infiltrating endometriosis). Otherwise, invasion constitutes one of the hallmarks of cancer. Therefore, it is intriguing why BCCLs with high KLK gene expression, namely UACC 812, UACC 893, and Du 4475, did not invade the medium. We hypothesize that this observation could be due to components of the invasiveness assay (*e.g.*, fibroblasts) that could modify the invasive capacity of selected BCCLs, but we were unable to find the "missing link" to explain this confusing observation.

An important finding of our study is the identification of KLK clusters according to their *in vitro* expression. The first cluster comprised KLK1, KLK4, KLK12, and KLK15; the second cluster consisted of all other KLKs, with two additional explicit subclasses within the second main cluster: KLK5-KLK9 versus KLK10, KLK11, KLK13, and KLK14. Co-expression of KLKs, resulting from their functional networking, has been repeatedly reported in physiological and pathological conditions, including cancer (4-8, 20). Our results contribute to the still incomplete knowledge about coordinately dysregulated KLK expression in selected BCCLs. The identification of simultaneously up- or down-regulated KLKs illuminates their functional synergy in normal and cancerous CLs. For instance, our results indicated a synergistic action of KLK 5-KLK10 in normal breast cell lines and their down-

Table III. *Prediction of the in vitro invasiveness of CLs by KLK gene expression - results of the logistic regression analysis.*

Predictor variable	Estimate	Std. error	z value	Pr(>|z|)
KLK1	1.233	0.234	0.005	0.996
KLK4	0.043	0.119	0.357	0.721
KLK5	–1.822	1.871	–0.974	0.330
KLK6	–0.190	0.144	–1.321	0.186
KLK7	–1.416	0.991	–1.428	0.153
KLK8	–0.336	0.236	–1.425	0.154
KLK9	–0.537	0.398	–1.348	0.178
KLK10	–1.570	1.187	–1.322	0.186
KLK11	–0.144	0.083	–1.744	0.081
KLK12	0.048	0.107	0.454	0.650
KLK13	–0.099	0.107	–0.928	0.353
KLK14	–0.170	0.141	–1.203	0.229
KLK15	1.224	0.266	0.005	0.996

regulation (or silencing) in cancer cells. The use of 21 BCCLs and three non-malignant CLs enabled us to expose dramatic differences in KLK expression among various BCCLs, which were discussed in detail above. Otherwise, our cluster analysis revealed similarities in KLK expression between normal breast cells and some BCCLs. Besides the fact that "normal-like" features at gene and protein expression are not contradictory to the aggressive BCCL nature, this finding should stimulate further identification of KLK functional similarities in both normal and disease states.

Some limitations of our study were already addressed within the discussion. Furthermore, we consider the exclusion of KLK2 and KLK3 from the study as its main shortcoming. We relied on the dogma that "KLK2 and KLK3 are only expressed in the prostate" (5). However, KLK2 and KLK3 are expressed in the breast, albeit at low levels (17, 18). KLK3 has been identified within the orchestrated action of KLKs during BC progression, and its expression is associated with favorable prognosis (17, 18).

Notwithstanding these limitations, we conclude that our work revealed some useful insights into the landscape of KLK expression in BCCLs. Intriguing – and deserving of further research – was the delineation of KLK expression clusters, which probably mirrors their functional relationships within the studied BCCLs. Secondary, we confirmed that the KLK expression significantly differed according to AR presence. We observed the down-regulation (KLK 5 and KLK7-KLK10) or absence of KLK expression in the majority of malignant CLs, but at the same time, there was a striking overexpression of selected KLKs in individual BCCLs. Possibly due to overlapping with its nonspecific proteolytic properties, we could not confirm any statistically significant association of KLK expression with the *in vitro* invasiveness of BCCLs. The functions of KLKs in particular BCCLs appear to be individual. One should be aware of the limitations and pitfalls of CL-based studies.

Conflicts of Interest

All Authors declare that they have no conflicts of interest related to this study.

Authors' Contributions

All Authors contributed to the study conception and design. RW analyzed the data and wrote the manuscript. DCC-T performed the experiments and collected the data. EO performed the experiments. RZ supervised the experiments, data collection and manuscript preparation. All authors read and approved the final manuscript.

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References

1 Bray F, Ferlay J, Soerjomataram I, Siegel RL, Torre LA and Jemal A: Global cancer statistics 2018: GLOBOCAN estimates of incidence and mortality worldwide for 36 cancers in 185 countries. CA Cancer J Clin *68*: 394-424, 2018. PMID: 30207593. DOI: 10.3322/caac.2149

2 Russnes HG, Lingjærde OC, Børresen-Dale AL and Caldas C: Breast Cancer Molecular Stratification: From Intrinsic Subtypes to Integrative Clusters. Am J Pathol *187*: 2152-2162, 2017. PMID: 28733194. DOI: 10.1016/j.ajpath.2017.04.022

3 Holliday DL and Speirs V: Choosing the right cell line for breast cancer research. Breast Cancer Res *13*: 215, 2011. PMID: 21884641. DOI: 10.1186/bcr2889

4 Borgoño CA and Diamandis EP: The emerging roles of human tissue kallikreins in cancer. Nat Rev Cancer *4*: 876-890, 2004. PMID: 15516960. DOI:10.1038/nrc1474

5 Prassas I, Eissa A, Poda G and Diamandis EP: Unleashing the therapeutic potential of human kallikrein-related serine proteases. Nat Rev Drug Discov *14*: 183-202, 2015. PMID: 25698643. DOI: 10.1038/nrd4534

6 Pampalakis G and Sotiropoulou G: Tissue kallikrein proteolytic cascade pathways in normal physiology and cancer. Biochim Biophys Acta *1776*: 22-31, 2007. PMID: 17629406. DOI: 10.1016/j.bbcan.2007.06.001

7 Kalinska M, Meyer-Hoffert U, Kantyka T and Potempa J: Kallikreins - The melting pot of activity and function. Biochimie *122*: 270-282, 2016. PMID: 26408415. DOI: 10.1016/j.biochi.2015.09.023

8 Avgeris M, Mavridis K and Scorilas A: Kallikrein-related peptidases in prostate, breast, and ovarian cancers: from pathobiology to clinical relevance. Biol Chem *393*: 301-317, 2012. PMID: 22505514. DOI: 10.1515/hsz-2011-0260

9 Shaw JL and Diamandis EP: Regulation of human tissue kallikrein-related peptidase expression by steroid hormones in 32 cell lines. Biol Chem *389*: 1409-1419, 2008. PMID: 19031603. DOI: 10.1515/BC.2008.158

10 Paliouras M and Diamandis EP: Coordinated steroid hormone-dependent and independent expression of multiple kallikreins in breast cancer cell lines. Breast Cancer Res Treat *102*: 7-18, 2007. PMID: 16897430. DOI: 10.1007/s10549-006-9312-y

11 Kontos CK and Scorilas A: Kallikrein-related peptidases (KLKs): a gene family of novel cancer biomarkers. Clin Chem Lab Med *50*: 1877-1891, 2012. PMID: 23093268. DOI: 10.1515/cclm-2012-0247

12 Yoon H, Blaber SI, Li W, Scarisbrick IA and Blaber M: Activation profiles of human kallikrein-related peptidases by matrix metalloproteinases. Biol Chem *394*: 137-147, 2013. PMID: 23241590. DOI: 10.1515/hsz-2012-0249.

13 Sotiropoulou G, Pampalakis G and Diamandis EP: Functional roles of human kallikrein-related peptidases. J Biol Chem *284*: 32989-32994, 2009. PMID: 19819870. DOI: 10.1074/jbc.R109.027946

14 Shaw JL and Diamandis EP: Distribution of 15 human kallikreins in tissues and biological fluids. Clin Chem *53*: 1423-1432, 2007. PMID: 17573418. DOI: 10.1373/clinchem.2007.088104

15 Kryza T, Parent C, Pardessus J, Petit A, Burlaud-Gaillard J, Reverdiau P, Iochmann S, Labas V, Courty Y and Heuzé-Vourc'h N: Human kallikrein-related peptidase 12 stimulates endothelial cell migration by remodeling the fibronectin matrix. Sci Rep *8*: 6331, 2018. PMID: 29679011. DOI: 10.1038/s41598-018-24576-9

16 Haritos C, Michaelidou K, Mavridis K, Missitzis I, Ardavanis A, Griniatsos J and Scorilas A: Kallikrein-related peptidase 6 (KLK6) expression differentiates tumor subtypes and predicts clinical outcome in breast cancer patients. Clin Exp Med *18*: 203-213, 2018. PMID: 29435805. DOI: 10.1007/s10238-018-0487-4

17 Schmitt M, Magdolen V, Yang F, Kiechle M, Bayani J, Yousef GM, Scorilas A, Diamandis EP and Dorn J: Emerging clinical importance of the cancer biomarkers kallikrein-related peptidases (KLK) in female and male reproductive organ malignancies. Radiol Oncol *47*: 319-329, 2013. PMID: 24294176. DOI: 10.2478/raon-2013-0053

18 Figueroa CD, Molina L, Bhoola KD and Ehrenfeld P: Overview of tissue kallikrein and kallikrein-related peptidases in breast cancer. Biol Chem *399*: 937-957, 2018. PMID: 29885274. DOI: 10.1515/hsz-2018-0111

19 Girgis AH, Bui A, White NM and Yousef GM: Integrated genomic characterization of the kallikrein gene locus in cancer. Anticancer Res *32:* 957-963, 2012. PMID: 22399617.

20 Yousef GM, Yacoub GM, Polymeris ME, Popalis C, Soosaipillai A and Diamandis EP: Kallikrein gene downregulation in breast cancer. Br J Cancer *90*: 167-172, 2004. PMID: 14710225. DOI: 10.1038/sj.bjc.6601451

21 Yousef GM, Scorilas A, Kyriakopoulou LG, Rendl L, Diamandis M, Ponzone R, Biglia N, Giai M, Roagna R, Sismondi P and Diamandis EP: Human kallikrein gene 5 (KLK5) expression by quantitative PCR: an independent indicator of poor prognosis in breast cancer. Clin Chem *48*: 1241-1250, 2002. PMID: 12142380. DOI: 10.1093/clinchem/48.8.1241

22 Li X, Liu J, Wang Y, Zhang L, Ning L and Feng Y: Parallel underexpression of kallikrein 5 and kallikrein 7 mRNA in breast malignancies. Cancer Sci *100*: 601-607, 2009. PMID: 19453546. DOI: 10.1111/j.1349-7006.2009.01090.x.

23 Talieri M, Devetzi M, Scorilas A, Prezas P, Ardavanis A, Apostolaki A and Karameris A: Evaluation of kallikrein-related peptidase 5 expression and its significance for breast cancer patients: association with kallikrein-related peptidase 7 expression. Anticancer Res *31*: 3093-3100, 2011. PMID: 21868565.

24 Pampalakis G, Zingkou E, Sidiropoulos KG, Diamandis EP, Zoumpourlis V, Yousef GM and Sotiropoulou G: Biochemical pathways mediated by KLK6 protease in breast cancer. Mol Oncol *13*: 2329-2343, 2019. PMID: 30980596. DOI: 10.1002/1878-0261.12493

25 Michaelidou K, Ardavanis A and Scorilas A: Clinical relevance of the deregulated kallikrein-related peptidase 8 mRNA expression in breast cancer: a novel independent indicator of disease-free survival. Breast Cancer Res Treat *152*: 323-336, 2015. PMID: 26099606. DOI: 10.1007/s10549-015-3470-8

26 Tong D, Czerwenka K, Sedlak J, Schneeberger C, Schiebel I, Concin N, Leodolter S and Zeillinger R: Association of *in vitro* invasiveness and gene expression of estrogen receptor, progesterone receptor, pS2 and plasminogen activator inhibitor-1 in human breast cancer cell lines. Breast Cancer Res Treat *56*: 91-97, 1999. PMID: 10517346. DOI: 10.1023/A:1006262501062

27 Tong D, Schneeberger C, Leodolter S and Zeillinger R: Quantitative determination of gene expression by competitive reverse transcription-polymerase chain reaction in degraded RNA samples. Anal Biochem *251*: 173-177, 1997. PMID: 9299013. DOI: /10.1006/abio.1997.2280

28 R Core Team. R: a language and environment for statistical computing. R Foundation for Statistical Computing, Vienna, Austria, 2013. Available at: http://www.r-project.org [Last Accessed July 10, 2019]

29 ATCC Breast cancer and normal cell lines, 2019. Available at: https://www.atcc.org [Last Accessed May 20, 2019]

30 Riaz M, van Jaarsveld MT, Hollestelle A, Prager-van der Smissen WJ, Heine AA, Boersma AW, Liu J, Helmijr J, Ozturk B, Smid M, Wiemer EA, Foekens JA and Martens JW: miRNA expression profiling of 51 human breast cancer cell lines reveals subtype and driver mutation-specific miRNAs. Breast Cancer Res *15*: R33, 2013. PMID: 23601657. DOI: 10.1186/bcr3415

31 Dai X, Cheng H, Bai Z and Li J: Breast cancer cell line classification and its relevance with breast tumor subtyping. J Cancer *8*: 3131-3141, 2017. PMID: 29158785. DOI: 10.7150/jca.18457

32 Yousef GM, Scorilas A, Magklara A, Memari N, Ponzone R, Sismondi P, Biglia N, Abd Ellatif M and Diamandis EP: The androgen-regulated gene human kallikrein 15 (KLK15) is an independent and favourable prognostic marker for breast cancer. Br J Cancer *87*: 1294-1300, 2002. PMID: 12439720. DOI: 10.1038/sj.bjc.6600590

33 Barton VN, D'Amato NC, Gordon MA, Christenson JL, Elias A and Richer JK: Androgen receptor biology in triple negative breast cancer: A case for classification as AR+ or quadruple negative disease. Horm Cancer *6*: 206-213, 2015. PMID: 26201402. DOI: 10.1007/s12672-015-0232-3

34 Speers C, Zhao SG, Chandler B, Liu M, Wilder-Romans K, Olsen E, Nyati S, Ritter C, Alluri PG, Kothari V, Hayes DF, Lawrence TS, Spratt DE, Wahl DR, Pierce LJ and Feng FY: Androgen receptor as a mediator and biomarker of radioresistance in triple-negative breast cancer. NPJ Breast Cancer *3*: 29, 2017. PMID: 28840192. DOI: 10.1038/s41523-017-0038-2

35 Christopoulos PF, Vlachogiannis NI, Vogkou CT and Koutsilieris M: The role of the androgen receptor signaling in breast malignancies. Anticancer Res *37*: 6533-6540, 2017. PMID: 29187427. DOI: 10.21873/anticanres.12109

36 Christgen M and Derksen P: Lobular breast cancer: molecular basis, mouse and cellular models. Breast Cancer Res *17*: 16, 2015. PMID: 25757734. DOI: 10.1186/s13058-015-0517-z

37 Tasdemir N, Bossart EA, Li Z, Zhu L, Sikora MJ, Levine KM, Jacobsen BM, Tseng GC, Davidson NE and Oesterreich S: Comprehensive phenotypic characterization of human invasive lobular carcinoma cell lines in 2D and 3D Cultures. Cancer Res *78*: 6209-6222, 2018. PMID: 30228172. DOI: 10.1158/0008-5472.CAN-18-1416

38 Cifani P, Kirik U, Waldemarson S and James P: Molecular portrait of breast-cancer-derived cell lines reveals poor similarity with tumors. J Proteome Res *14*: 2819-2827, 2015. PMID: 26055192. DOI: 10.1021/acs.jproteome.5b00375

39 Keller PJ, Lin AF, Arendt LM, Klebba I, Jones AD, Rudnick JA, DiMeo TA, Gilmore H, Jefferson DM, Graham RA, Naber SP, Schnitt S and Kuperwasser C: Mapping the cellular and molecular heterogeneity of normal and malignant breast tissues and cultured cell lines. Breast Cancer Res *12*: R87, 2010. PMID: 20964822. DOI: 10.1186/bcr2755

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