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Specific increase of human kallikrein 4 mRNA and protein levels in breast cancer stromal cells

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ABSTRACT

The kallikrein family (*KLK*) has been implicated in cancer and may be useful as tumor markers. Here, we compared the 15 *KLK* genes' expression in malignant and normal breast tissues using real-time quantitative PCR. Most *KLKs* were expressed at lower levels in breast cancer compared to normal breast tissue. The only exception was the eightfold increase level of *KLK4* in breast cancer tissues ($P = 0.008$). *KLK4* level was strongly associated with tumor grade ($P = 0.0015$). Interestingly, based on laser cell microdissection analysis and immunochemistry, the up-regulation of kallikrein 4 occurred in the surrounding stromal cells. Our findings suggest that *KLK4* may be associated with the development and progression of breast cancer and suggest its potential use in breast cancer monitoring.

At every stage of carcinogenesis, the alteration of gene expression triggers disorders in the production and/or secretion of an important number of proteins including proteases. Among these different proteases, metallo-, serine-, and aspartic proteases have attracted clinical interest because of their potential implication in the progression of cancer. Some of these proteases have been implicated in the degradation of the extracellular matrix, tumor cell proliferation, and angiogenesis.

To date, prostate-specific antigen (PSA) is the only protease routinely used as a serum biomarker to screen for early disease diagnosis and monitor patients with cancer [1]. PSA is a member of the human gene kallikrein family. Human kallikreins (hKs) are a subset of serine proteases that are encoded by 15 structurally similar genes (*KLK*) that co-localize to chromosome 19q13.4 [2,3]. These genes share common characteristics such as similar exon/intron organization and conserved nucleotide and amino acid sequences with 30–50% sequence identity, respectively [3]. *KLK* genes are differentially expressed in many tissues, suggesting their involvement in a variety of physiological processes, but distinct biological functions have been established for only a few hKs [4,5]. Among all kallikreins, some are emerging as new potential diagnostic and prognostic markers, mostly for prostate and ovarian cancer [6–8].

Our study used real-time quantitative PCR analysis to monitor the expression of the 15 human *KLK* genes in normal and cancerous breast tissues, in contrast with the multiple studies examining only small set of *KLKs* at the mRNA level by RT-PCR or *in silico* analysis,. We provide evidence that most of *KLKs* are down-regulated in breast cancer, with the exception of *KLK4*. We describe, for the first time, an increase of *KLK4* gene and protein expression level in cancerous vs. normal breast tissues. Interestingly, we show by laser cell microdissection analysis and immunohistochemistry that this up-regulation is localized in surrounding stromal cells rather than the epithelial tumors cells.

Materials and methods

Patients and sample collection. Primary breast carcinomas tissues were collected surgically from 37 patients at the Val d'Aurelle Cancer Center, Montpellier, France. Clinicopathological data were described in Table 1. The control group was from 14 healthy women who underwent surgery for mammary reduction. Tumor and normal tissues were immediately frozen in liquid nitrogen after surgery and stored at −80 °C. Sera from five cancer patients and five healthy volunteers were stored at −80 °C. Informed consent was obtained from all patients.

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Table 1
Clinicopathologic data of healthy controls and breast cancer patients

Characteristics		Normal (n = 14)	Cancer (n = 37)
Median age (years)		51	60
Range (years)		32–68	34–90
Tumor stage	T1	–	15
	T2	–	10
	T3	–	12
Lymph node status	Absent	–	14
	Present	–	23
Metastasis		–	0
Histologic grade	I	–	5
	II	–	24
	III	–	8
Histology	Ductal	–	27
	Lobular	–	10
Steroid receptor	ER +	–	26
	PR +	–	18
NPI*	<3.4	–	5
	3.4–5.4	–	26
	>5.4	–	6

* NPI = [(0.2× size) + grade + Nodal status], where NPI < 3.4, 3.4–5.4, and >5.4 represented good, moderate, and poor prognosis, respectively [10].

Total RNA extraction and reverse transcription. Frozen samples were sectioned at 7 μm using a cryostat. For whole tissues, total RNA was extracted using the RNeasy/Mini-kit (Qiagen) following the manufacturer's instructions with an additional DNAse I digestion step (Roche-Diagnostics). For laser-capture microdissection (LCM) using the PixCell-II LCM System (Arcturus Engineering), tissue sections were stained with RNAse-free reagents. Total RNA was extracted from laser-captured cells using the RNeasy®/Micro-kit (Qiagen) and eluted in 20 μl of RNAse-free water. RNA integrity was assessed using the RNA/6000-Pico LabChip® kit with the Agilent Bioanalyzer™ (Agilent). Reverse transcription was carried out by using oligo-dT primers and the Omniscript Reverse-Transcriptase kit for whole tissues and the Sensiscript Reverse-Transcriptase kit for LCM tissues (Qiagen). Complementary DNA was synthesized from 2 μg of RNA from whole tissues and 12 μl of RNA from LCM tissues.

Real-time quantitative RT-PCR analysis. The cDNA products were analyzed by real-time PCR using the LightCycler™ system (Roche). Specific HPLC-purified primers were designed for each kallikrein gene by the Genomics Platform of the NCCR Frontiers in Genetics program. Gene-specific oligonucleotide primers, amplicon length, and PCR conditions for all primers tested are shown in Table S1. Real-time PCR was performed in a 20 μl volume using 5 μl of the cDNA template, 15 μl of a mixture containing primers, dNTP, polymerase enzyme, and SYBR green I. Thermal cycling conditions included an initial 5 min denaturation step at 94 °C followed by 45 cycles including denaturation at 94 °C for 20 s, annealing for 12 s, and extension at 72 °C for 20 s. As internal controls, we used four housekeeping genes including β-*2-microglobulin*, *TBP89*, *RS9*, and *HPRT*. *HPRT* was identified as the most stable control gene in breast tissue samples. The fit point method was used to determine the threshold cycle value (C_t), i.e., sample above the background fluorescence. Each assay was done in triplicate. Relative quantification of *KLK* mRNA amount was accomplished by comparative C_t [36]. Non-cancer tissues were chosen as calibration. After normalization, *KLK* mRNA were expressed relative to the calibrated value using the formula: N-fold difference = $2^{-[\Delta C_t(\text{tumor}) - \Delta C_t(\text{non-cancer})]}$, where $\Delta C_t = C_t(KLK) - C_t(HPRT)$. N-Fold difference represents the fold change in *KLK* mRNA expression between the non-cancer tissues and the tumor tissues.

Immunohistochemistry. Three micrometer thick paraffin-embedded tissue sections were dewaxed, rehydrated and endogenous peroxydase activity was blocked with 3% aqueous hydrogen peroxide. Antigen retrieval was performed by heat water bath treatment in 10 mM sodium citrate buffer, pH6. After blocking (Dako protein block serum free), the sections were incubated for one hour with a 1/30 dilution (6.6 μg/ml) of rabbit polyclonal anti-hK4 antibody (Santa Cruz Biotechnology, reference 20373) or 1/500 (2 μg/ml) rabbit polyclonal anti-hK14 antibody (Abcam, reference 2290) at room temperature. Biotinylated link and streptavidin-horseradish peroxidase were applied for 30 min (hK4) or 15 min (hK14) each. Peroxidase activity was revealed using 3.3′-diaminobenzidine with hydrogen peroxide. The samples were counterstained with hematoxylin, dehydrated, and mounted. Omission of the primary antibody was used as a negative control. Immunohistochemical semi-quantification was based on the intensity of the staining and the percentage of positive-staining structures lead to five characterization groups: none, very weak, weak, moderate and strong staining.

Statistical analysis. Statistical analysis was done using Student's t test and Mann–Whitney U test for comparison of two groups, and using one-way ANOVA with Bonferroni multiple comparison post-test and non-parametric ANOVA with Dunn's multiple comparison post-test for comparison amongst more than two groups using GraphPad InStat (version 3.06). A probability level of $P < 0.05$ was chosen for statistical significance.

Results

KLK expression in normal and cancerous breast tissues

We analyzed the expression of the 15 *KLK* genes in 51 tissues from 14 normal and 37 breast cancer patients. In our conditions, the threshold cycle values for *KLK5*, *13* and *15* mRNA were undetectable. We then compared mRNA expression levels of the other 12 *KLK* genes using relative quantification by comparative C_t (Table 2). When mRNA expression in tumor tissues was examined, nine *KLK* mRNAs showed statistically significant lower expression levels in tumors compared to normal breast tissues. There were twofold and threefold non-significant increases in cancer relative to normal breast tissue for *KLK3* and *8*. *KLK4* mRNA expression showed significantly higher expression levels in cancerous tissue than in normal tissue with an 8.5-fold increase.

The relation between *KLK* mRNA expression in tumor tissue and clinical or histo-morphologic variables (Table 3) were statistically analyzed. Expression levels of *KLK1* are significantly lower in the pT1 compare to pT2 and pT3 stages ($P = 0.008$) and in the grade 1 compared to grades 2 and 3 ($P = 0.03$). Higher *KLK7* mRNA expression in lymph node positive tumors compared to lymph node negative tumors ($P = 0.02$) was observed. Significantly higher *KLK4* mRNA expression in pT2 stage tumors compared to other stages was found ($P = 0.0015$). Fig. 1 shows the *KLK4* expression level in normal tissue vs. cancer tissues from differing tumor sizes. *KLK4* mRNA expression increased progressively in pT1 ($P = 0.05$) and pT2 ($P = 0.0005$) stages compare to normal tissues, while a less elevated mRNA expression level was observed in pT3 ($P = 0.08$). Finally, there were no significant relations between *KLK* mRNA expression and all other clinical or histo-morphological variables (Table 3).

Stromal expression of KLK4/hK4

Since tumor microenvironment plays a key role in carcinogenesis, we specifically evaluated *KLK4* expression in stromal and epithelial cells. Three breast cancer tissues (T1N0) were subjected to LCM analysis in order to extract epithelial cells from surrounding stromal cells. About 5000 laser microdissected cells from each

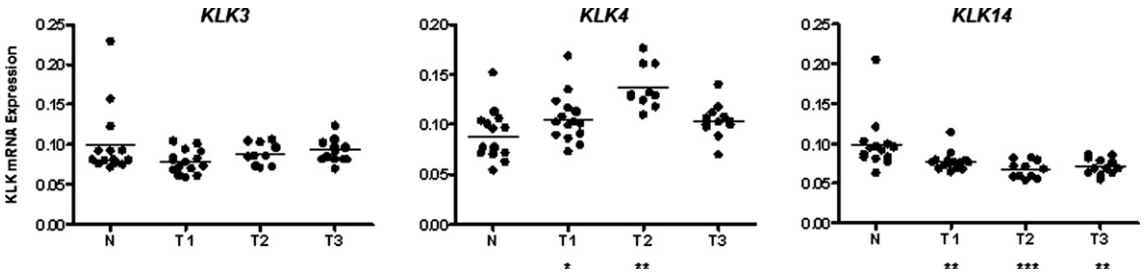

Fig. 1. *KLK3, 4, 14* mRNA expressions in normal and pT1N0, pT2N0, and pT3N0 cancerous breast tissues. The mean of *KLK* mRNA expression were indicated by horizontal lines.

sample were subjected to gene expression analysis for upregulated (*KLK4)* and downregulated (*KLK*14) mRNA expression (Fig. 2A). The same amount of laser microdissected normal epithelial cells obtained from the corresponding tissue was chosen as calibration. We observed that the decreased of *KLK14* mRNA expression could be partially explained by a moderate decrease in both the epithelial tumor cells (fourfold decrease), and the surrounding stromal cells (twofold decrease). Similar results were obtained for *KLK1, 6, 7*, and *10* (Fig. S2). In contrast, the increased of *KLK4* mRNA expression was mostly due to stromal (fivefold increase), but not to cancer epithelial cells (onefold increase). This result was confirmed in pT2N0 and pT3N0 breast cancer tissues (Fig. 2B).

Protein level was then analyzed by immunohistochemistry using anti-hK4 and -hK14 antibodies (Fig. 3A and B). There was a strong correlation between protein and mRNA expression levels. First, hK14 expression levels were high in the epithelial cells and weak in non-epithelial cells of normal breast tissues. The hK14 immunoreactivity was slightly lower in ductal carcinoma *in situ* (DCIS), in invasive carcinoma (with moderate expression), and was weak in stromal cells. Moreover, hK14 was localized in the cytoplasm. Second, hK4 expression levels were of similar intensity between normal, DCIS, and invasive carcinoma epithelial cells (very weak to weak). More interestingly, we also observed a strong hK4 expression in the stromal microenvironment of the invasive carcinoma, and more particularly in the extracellular compartment. This expression was higher in the invasive carcinoma than in normal and DCIS tissues. Focusing on the stromal microenvironment of the invasive carcinoma, we observed specific hK4 immunoreactivity in the fibroblasts (Fig. 3C). The increased hK4 immunoreactivity in the stromal invasive carcinoma was therefore mainly due to a higher percentage of fibroblasts expressing hK4, whereas the level of expression remained the same in normal, DCIS and invasive tissues.

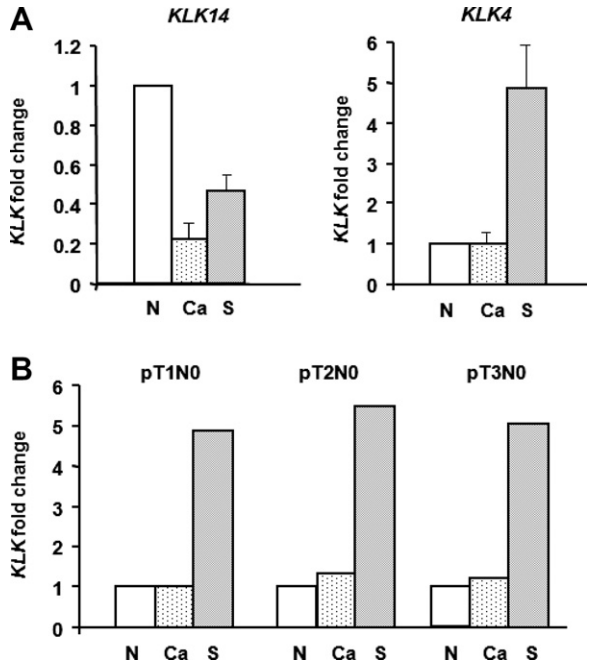

Fig. 2. KLK mRNA expression analysis on laser capture microdissected breast tumor tissues. (A) *KLK4* and *KLK14* mRNA expression in cancerous epithelial (Ca) and surrounding stromal (S) cells were compared to mRNA expression in normal adjacent epithelial (N) cells. (B) *KLK*4 mRNA expression in pT1N0, pT2N0, and pT3N0 cancerous epithelial and surrounding stromal cells were compared to *KLK*4 mRNA expression in normal adjacent epithelial cells.

Discussion

In contrast to the multiple studies examining a small set of *KLKs*, we quantitatively analyzed the expression pattern of 15 serine proteases in benign and malignant human breast cancer tissues by real-time PCR, in order to evaluate their usefulness as diagnostic tools. We observed a major down-regulation of most *KLK* genes in breast tumors compared to normal tissues, while *KLK4* was shown, for the first time, to be strongly up-regulated in breast cancer tissues. We observed concordant increase of kallikrein 4 at the protein level and showed that its expression in breast tissues was primarily due to the stromal cell components and not to cancer epithelial cells. Interestingly, the hK4 immunoreactive material was predominantly detected extracellularly in the surrounding tumor environment.

Dysregulated proteolytic balance is a hallmark of cancer. Tumor cells require a range of proteolytic activities for their survival, or more particularly, for their growth through degradation and remodelling of the extracellular matrix. Several *in vitro* studies have suggested the direct involvement of human kallikreins in cancer progression through extracellular matrix proteolysis, tumor-cell detachment and invasiveness [5,9,10]. Our results indicate that 11 *KLK* genes are down-regulated in breast cancer, which is consistent with earlier reports, in particular for KLK10, 13, and 14 [11–13]. Interestingly, some were previously shown to have anti-angiogenic (such as hK3) and tumor suppressor effects (such as hK3 and 10) on the growth of some breast cancer cell lines [14–16]. Concerning the up-regulation of kallikrein 4 expression, its biological function in breast cancer tissue samples remains poorly documented. Veveris-Lowe et al. showed *in vitro* that hK4, as well as PSA, could play an important functional role in prostate cancer progression through the loss of E-cadherin [17]. More recently, Klokk et al. showed that the overexpression of *KLK4* in prostate cell lines dramatically increased proliferation [18]. The reactive stromal microenvironment is associated to the cancer-related process through neo-angiogenesis and is involved as a substantial contributor to the overall secretion of extracellular degrading proteases. Among several extracellular proteolytic systems which are relevant in cancer, the urokinase plasminogen activation system (uPA), cathepsin D and matrix metalloproteinases such as MMP11, are the most studied in breast cancer [19–21].

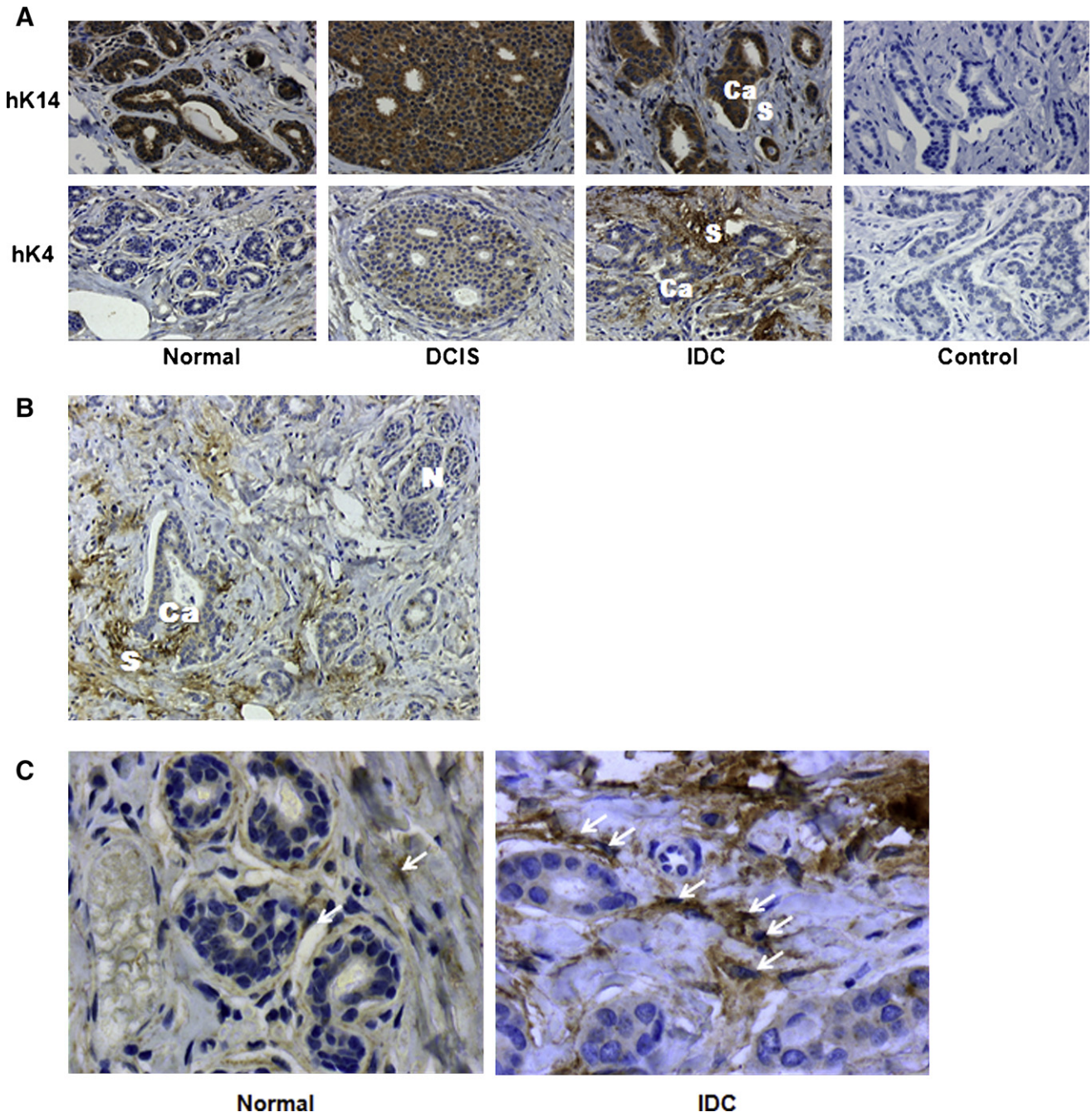

Fig. 3. Immunohistochemical analysis for hK4 and hK14 in normal and cancerous breast tissues. (A) Normal, high-grade ductal carcinoma *in situ* (DCIS) and invasive ductal carcinoma (IDC) were stained with anti-hK4 and anti-hK14 antibodies. Stromal (S) and cancer (Ca) cells were indicated. No staining was observed when primary antibodies were omitted (Control). (B) Magnification view of hK4 immunoreactivity in breast tumor tissue. A section of invasive ductal carcinoma tissue showing the stromal cells (S) and the cancerous epithelial cells (Ca), as well as a normal mammary gland (N) are indicated. (C) Magnification view of hK4 immunoreactivity in normal and invasive ductal carcinoma. Fibrosblastic hK4 expression is indicated by arrows.

The secreted serine-protease hK4 might facilitate tumor cell spreading by triggering a proteolytic cascade, as previously suggested with cathepsins. Indeed, hK4 has been shown to activate the pro-enzyme form of uPA and cleave its cell surface receptor uPAR [22,23]. More interestingly, recent data provide insight into hK4 regulation of intracellular signaling via members of the protease-activated receptor family (PARs) in prostate cancer [24]. The protease-activated receptors (PAR-1 to 4) are members of the G protein-coupled receptor subfamily and their activity is modulated by proteolytic cleavages [25,26]. Ramsay *et al.* suggested that hK4 could differentially regulate the cellular activity of PAR-1 and PAR-2 in prostate cancer, activating both at lower concentrations, whilst at higher concentrations activating PAR-2 and inactivating PAR-1 [24]. These receptors have distinct functions and elicit different response in several tissues. In breast cancer, PAR-1 expression inhibits migration and invasion of breast cancer cells [27], whereas PAR-2 contributes to angiogenesis by up-regulation of pro-angiogenic VEGF [28]. PAR-2 also leads to enhanced cell mobility [29]. Thus, our results on the deregulated expression of kallikrein 4 in breast tumors raise the possibility that hK4 could contribute to the angiogenesis, invasiveness and/or progression of breast cancer via the modulation of PAR activity.

The down-regulation of the *KLK4* gene observed here in pT3 stage tumors compared to pT1/T2 stage tumors may be evidence for the ability of hK4 to trigger angiogenesis and/or cancer development specifically in early breast cancer. Finally, hK5, hK6 and hK14 have also been shown to modulate *in vitro* the PAR activities, either activating and/or inactivating of PAR-1, PAR-2, or PAR-4 [30–32]. Thus, deregulation of the *KLK* gene family may be of particular relevance in breast cancer development via its complex and antagonist effects on the regulation of cellular signaling events. Further studies will be needed to investigate more specifically the kallikrein signaling via protease-activated receptors in breast cancer.

As for hK3 in prostate cancers, secreted kallikrein proteins may have potential clinical uses as tumor markers in breast cancer. The *KLK4* gene encodes for two proteins: a nuclear forms [33,34], and a cytoplasmically secreted forms [17,34,35], which have been described in prostate and ovarian cell lines or tissues, and in different biological fluids. We investigated the expression of hK4 in sera. In control cell line, we detected a 45 kDa band corresponding to the free hK4 (Fig. S1). However, although we analyzed crude or immunodepleted sera from breast cancer patients and healthy controls, we were unable to detect hk4 expression using Western blotting (Fig. S1) and immunoassay (data not shown). One explanation may be that hk4 could be complexed with unknown serine proteinase inhibitors, as previously suggested [17,34,35], inhibiting the immunoreactivity of these complexes. It is therefore premature to draw any conclusion on its significance as a circulating marker.

To summarize, we demonstrated predominant down-regulation of most of *KLK* genes with an exception for *KLK4* in breast cancer tissues. *KLK4*/hK4 is highly up-regulated in cancerous tissues and more precisely in the surrounding stromal cells. Therefore, this kallikrein may be particularly interesting to study in breast cancer, where it may be associated with breast carcinogenesis and may have a potential as a prognostic marker.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.bbrc.2008.07.138.

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