

Kallikrein 10 (*KLK10*) methylation as a novel prognostic biomarker in early breast cancer

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Background: We evaluated the prognostic significance of *KLK10* exon 3 methylation in patients with early-stage breast cancer since it has been shown to have a significant impact on biological characteristics of breast tumors.

Materials and methods: Using methylation-specific PCR, we evaluated the specificity of *KLK10* methylation in 10 breast tumors and matching normal tissues, 10 breast fibroadenomas, 11 normal breast tissues and in a testing group of 35 patients. The prognostic significance of *KLK10* methylation was validated in an independent cohort of 93 patients.

Results: *KLK10* was not methylated in normal breast tissues and fibroadenomas while it was in 5 of 10 breast tumors and in 1 of 10 matching normal tissues. In the testing group of 35 patients, *KLK10* methylation was detected in 70.0% of patients who relapsed ($P = 0.001$) and in 77.8% of patients who died ($P = 0.025$). In the independent cohort, 53 of 93 (57.0%) patients were found positive for *KLK10* methylation. During the follow-up period, 24 of 93 (25.8%) patients relapsed and 19 of 93 (20.4%) died. Disease-free interval (DFI) and overall survival (OS) were significantly associated with *KLK10* methylation ($P = 0.0025$ and $P = 0.003$). Multivariate analysis revealed that *KLK10* methylation was an independent prognostic factor for DFI and OS.

Conclusion: *KLK10* exon 3 methylation provides important prognostic information in early breast cancer patients.

Key words: DNA methylation, early breast cancer, epigenetic markers, *KLK10*, methylation-specific PCR, prognostic biomarker

Introduction

During the last years, early detection and novel treatments have improved survival rates in breast cancer patients. Although the most important prognostic factor for patients with early breast cancer is still the presence of axillary lymph node involvement, there is no absolute biomarker as yet that can reliably predict which patients will present disease progression; therefore, practically all patients with early-stage breast carcinoma receive adjuvant treatment. Accurate prognosis based on reliable prognostic biomarkers may spare patients from receiving unnecessary adjuvant chemotherapy and expensive medical costs [1].

DNA methylation is one of the most frequently occurring epigenetic events taking place in the mammalian genome [2]. The best characterized epigenetically mediated transcriptional-silencing events are associated with increases in DNA methylation, particularly at promoter regions of genes that regulate important cell functions. Recent evidence indicates that epigenetic changes might 'addict' cancer cells to altered

signal transduction pathways during the early stages of tumor development. Dependence on these pathways for cell proliferation or survival allows them to acquire genetic mutations in the same pathways, providing the cell with selective advantages that promote tumor progression [3, 4]. Methylation of tumor suppressor genes plays a fundamental role in cancer development and progression and is considered to be a promising biomarker for early detection and prognosis estimation in cancer patients [5].

KLK10 is a member of the human tissue kallikrein family of secreted serine proteases, encoded by a family of 15 genes clustered in a tandem on chromosome 19q13.3–4 [6]. *KLK10* was first identified by subtractive hybridization between normal and radiation-transformed mammary epithelial cells [7] and further studies have shown that this gene was downregulated in breast tumors [7, 8]. It has been suggested that *KLK10* may function as a tumor suppressor gene since its over expression in nude mice was shown to suppress tumor formation [9]. Moreover, stable expression of *KLK10* in the *KLK10*-negative MDA-MB-231 breast cancer cell line suppressed their oncogenicity [9]. By using a luciferase reporter system, it was demonstrated that loss of *KLK10* expression was due to a promoter independent mechanism, while sequence analysis

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revealed that *KLK10* promoter is not CpG rich. In contrast, a strong correlation was found between *KLK10* exon 3 hypermethylation and loss of *KLK10* messenger RNA (mRNA) expression in a panel of breast cancer cell lines and in primary breast tumors. *KLK10* exon 3 was found to be CpG rich and satisfied the formal criteria for CpG islands [10]. Subsequently, exon 3 CpG island methylation as a basis for tumor-specific loss of *KLK10* expression has been found in breast, ovarian, prostate, acute lymphoblastic leukemia (ALL) and gastric cancers [10–13].

However, despite the fact that *KLK10* exon 3 methylation has been shown to have a significant impact on the biological characteristics of breast tumors, the relationship between CpG island methylation of this gene and prognosis in breast cancer has not been reported as yet. In this report, we evaluated for the first time the prognostic significance of *KLK10* exon 3 methylation in early-stage breast cancer.

materials and methods

cell lines and patients' samples

The study material consisted of a total of 169 breast formalin-fixed paraffin-embedded tissues: 128 paraffin-embedded breast carcinomas, obtained from patients with early-stage breast cancer with a known clinical outcome and a long follow-up period, 10 paraffin-embedded breast carcinomas with paired adjacent normal tissues, 10 breast fibroadenomas and 11 specimens from reduction mammoplasty (histologically cancer free), which were used as normal breast tissue controls. All patients were enrolled to adjuvant chemotherapy research protocols of the Hellenic Oncology Research Group [i.e. FEC regimen or sequential docetaxel (Taxotere, Sanofi-Aventis) followed by epirubicin in combination with cyclophosphamide (D/EC regimen) or docetaxel in combination with epirubicin (DE regimen)]; patients with breast conservative surgery also received radiation treatment and those with hormone receptor-positive tumors received adjuvant tamoxifen for 5 years. All patients gave their informed consent and the study has been approved by the Ethical and Scientific Committees of our institution. Tissue sections of 10 μm containing >80% of tumor cells were cut and were used for DNA extraction and methylation-specific PCR (MSP) analysis. The breast cancer cell line MDA-MB-231 was used as positive control in MSP reactions for the detection of *KLK10* exon 3 methylation.

isolation of genomic DNA

Genomic DNA (gDNA) was isolated from paraffin tissues and breast cancer cell lines with the High Pure PCR Template Preparation Kit (Roche, Mannheim, Germany), according to the manufacturer's protocol. DNA concentration was determined by using the Nanodrop ND-1000 spectrophotometer (Nanodrop Technologies, Willmington, USA).

sodium bisulfite conversion

One microgram of extracted DNA was modified with sodium bisulfite, converting all unmethylated, but not methylated, cytosines to uracil. Bisulfite conversion was carried out using the EZ DNA Methylation Gold Kit (ZYMO Research Co., Orange, CA), according to the manufacturer's instructions. The converted DNA was stored at -70°C until use. In each sodium bisulfite conversion reaction, dH_2O as a negative control and MDA-MB-231 gDNA as a positive control were included.

methylation-specific PCR

Each MSP reaction was carried out in duplicate in a total volume of 25 μl . One microliter of sodium bisulfite-converted DNA was added into a 24 μl reaction mixture that contained 0.1 μl of *Taq* DNA polymerase (5 U/ μl , platinum DNA polymerase; Invitrogen, Carlsbad, USA), 2.5 μl of the supplied 10 \times PCR buffer, 1.0 μl of MgCl_2 (50 mmol/l), 0.5 μl of dNTPs (10 mmol/l; Fermentas, Burlington, Ont, Canada) and 1 μl of the corresponding forward and reverse primers (10 $\mu\text{mol/l}$); finally, dH_2O was added to a final volume of 25 μl . Thermocycling conditions used were as follows: one cycle at 95°C for 5 min, followed by 40 cycles of 95°C for 30 s, 58°C for 60 s and 72°C for 45 s, with a final extension cycle of 72°C for 4 min. MSP products were fractionated on 2% agarose gels containing 40 mM Tris-acetate/1.0 mM EDTA (pH = 8.0) and visualized by ethidium bromide staining. The sequences of all primers used in this study were the same as previously described [10]. Human placental gDNA (Sigma-Aldrich, Milwaukee, USA) methylated *in vitro* with *SssI* methylase (NEB, Ipswich, MA) was used, after sodium bisulfite conversion, as a fully methylated (100%) positive control; the same placental gDNA, that is not methylated, was used, after sodium bisulfite conversion, as an unmethylated DNA control.

statistical analysis

Correlation between methylation status and clinicopathological features of the patients was assessed by the chi-square test. Disease-free interval (DFI) and overall survival (OS) curves were calculated by using the Kaplan–Meier method and comparisons were carried out using the log-rank test. A Cox regression analysis was carried out in order to determine the relative contribution of various variables to the assessment of DFI and OS.

P values ≤ 0.05 were considered statistically significant. Statistical analysis was carried out by using the SPSS Windows version 11.0 (SPSS Inc., Chicago, IL).

results

sensitivity of *KLK10* MSP

The sensitivity of the MSP assay for *KLK10* was evaluated by using 1 μg of a fully methylated DNA sample as a positive control (100% methylation) serially diluted (10-fold dilutions) in 1 μg of sodium bisulfite-converted unmethylated DNA. The MSP assay for *KLK10* was carried out in triplicate and it was sensitive enough to detect 1 ng of the methylated *KLK10* sequence in the presence of 1 μg of unmethylated *KLK10* sequence (sensitivity 1 : 1000, Figure 1A).

specificity of *KLK10* MSP

To validate the specificity of the primers used for the methylated sequence of *KLK10*, we first tested these primers *in silico* and then in PCRs, using as a template bisulfite-modified human placental gDNA that is not methylated: no amplification of *KLK10* was observed, while the same primers clearly recognized the methylated sequence when we used our fully methylated (100%) positive control (Figure 1B, lane 14). The specificity of *KLK10* methylation in tumor tissues was further confirmed by the absence of any detectable *KLK10* methylation in all normal breast tissues from reduction mammoplasty (0 of 11), nor in any breast fibroadenoma (0 of 10); conversely, *KLK10* exon 3 was methylated in 5 (50%) of 10 breast tumors studied and in only 1 (10%) of their 10 matching normal tissues (Figure 1B, Table 1).

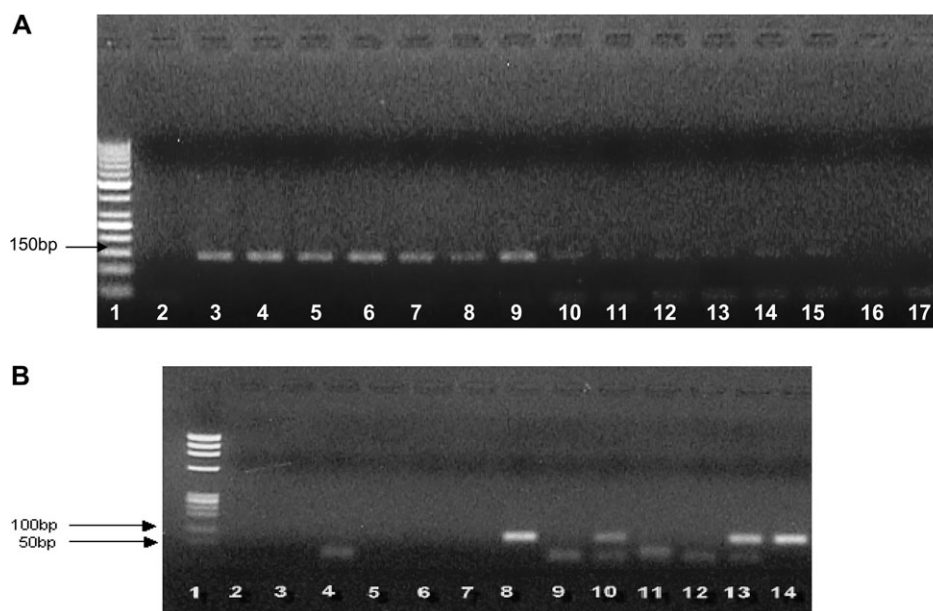


Figure 1. (A) Sensitivity of methylation-specific PCR for *KLK10*: lane 1, DNA marker 50 bp; lane 2, negative control; lane 3, 100% methylated DNA; lanes 4–6, 50% methylated DNA; lanes 7–9, 5% methylated DNA; lanes 10–12, 0.5% methylated DNA; lanes 13–15, 0.1% methylated DNA (1 : 1000); lanes 16 and 17, 0.05% methylated DNA. (B) Agarose gel electrophoresis of methylation-specific PCR products for *KLK10* exon 3 methylation in breast tumors: lane 1, DNA marker 50 bp; lane 2, negative control; lanes 3–5, normal breast tissues; lanes 6 and 7, breast fibroadenomas; lanes 8 and 9, pair of breast tumor and adjacent normal tissue; lanes 10 and 13, breast tumors (methylated); lanes 11 and 12, breast tumors (unmethylated); lanes 14, positive control.

Table 1. Specificity of *KLK10* methylation-specific PCR assay

Group	<i>n</i>	<i>KLK10</i> methylation (%)
Healthy (normal breast from reduction mammoplasty)	11	0 (0)
Fibroadenomas	10	0 (0)
Breast tumors	10	5 (50)
Adjacent normal tissues from breast tumors	10	1 (10)

KLK10 methylation in early breast cancer patients

The methylation status of *KLK10* was firstly assessed in an initial testing group consisting of 35 primary tumors from early breast cancer patients. Among them, 20 patients developed metastasis and nine died of metastatic disease. *KLK10* exon 3 was found methylated in 14 (70.0%) of the 20 patients who relapsed and in only 2 (13.3%) of 15 who remained metastasis free (chi-square test for association $P = 0.001$); similarly, *KLK10* exon 3 was found methylated in 7 (77.8%) of 9 patients who died and in 9 (34.6%) of 26 patients who remained alive (chi-square test for association $P = 0.025$).

evaluation of the prognostic significance of *KLK10* methylation in an independent cohort of early breast cancer patients

The methylation status of *KLK10* was further evaluated in an independent cohort that consisted of 93 early breast cancer patients whose clinicopathological characteristics and clinical outcome was not known when the analysis was carried out.

KLK10 was found methylated in 53 (57.0%) of 93 breast tumor samples. As can be seen in Table 2, there was no correlation between the *KLK10* exon 3 methylation and the major clinicopathological characteristics of the patients.

disease relapse and DFI

After a median follow-up period of 76 months, 24 (25.8%) of the 93 patients relapsed. The incidence of relapses was significantly higher in patients with methylated (35.8%) than in patients with unmethylated *KLK10* exon 3 (12.5%; Table 3; $P = 0.011$). The Kaplan–Meier estimates of the cumulative DFI for patients with methylated and nonmethylated *KLK10* exon 3 were significantly different in favor of patients with nonmethylated *KLK10* exon 3 ($P = 0.0025$; Figure 2A).

overall survival

During the follow-up period, 19 (20.4%) patients died as a consequence of disease progression. Table 3 shows that the incidence of disease-related deaths was higher in patients with methylated (28.3%) than in patients with unmethylated *KLK10* exon 3 (10.0%; $P = 0.030$). The Kaplan–Meier estimates of the cumulative OS for patients with methylated and nonmethylated *KLK10* exon 3 were significantly different in favor of patients with nonmethylated *KLK10* exon 3 ($P = 0.003$; Figure 2B).

univariate and multivariate analysis

KLK10 exon 3 methylation, menopausal and axillary lymph node status, tumor size, stage and grade, estrogen and progesterone receptor status and HER2 score were tested in univariate analysis for association with DFI. *KLK10* exon 3 methylation was significantly associated with decreased DFI

($P = 0.028$); in addition, tumor stage and grade, number of involved axillary lymph nodes and HER2 score were significantly associated with decreased DFI ($P = 0.038$, $P = 0.025$, $P = 0.002$ and $P = 0.038$, respectively). The same clinical and epigenetic variables were also tested in univariate analysis for OS. *KLK10* exon 3, methylation, estrogen receptor-negative tumors, number of involved axillary lymph nodes and tumor grade were significantly associated with a decreased OS ($P = 0.003$, $P = 0.013$, $P = 0.037$ and $P = 0.001$, respectively).

Multivariate analysis demonstrated that methylation of *KLK10* exon 3, as well as the number of involved axillary lymph nodes were independently associated with a high risk of relapse [hazard ratio (HR) = 3.492, $P = 0.026$ and HR = 5.982,

Table 2. *KLK10* methylation in relation to patient's characteristics ($n = 93$)

Patient's characteristics	n^a	<i>KLK10</i> methylation (%)	P^b
Pre-chemotherapy	38	23 (60.5)	0.567
Post-chemotherapy	55	30 (54.5)	
Tumor size (cm)			0.184
0–2.0	26	12 (46.1)	
2.1–5.0	56	32 (57.1)	
>5.0	10	8 (80.0)	
Axillary lymph node			0.062
0	27	11 (40.7)	
1–3	30	16 (53.3)	
≥4	34	24 (70.6)	
Tumor grade			0.369
I and II	42	21 (50.0)	
III	42	25 (59.5)	
Lobular	5	4 (80.0)	
Tumor stage			0.078
I	24	10 (41.7)	
II	69	43 (62.3)	
Estrogen receptor			0.642
Positive	56	33 (58.9)	
Negative	37	20 (54.0)	
Progesterone receptor			0.394
Positive	30	19 (63.3)	
Negative	63	34 (54.0)	
HER2 score			0.501
0–2+	72	38 (52.8)	
3+	11	7 (63.6)	

^aIn cases that the total number of patients is <93, this is due to non available clinical information.

^bChi-square test.

Table 3. Incidence of disease relapse and disease-related death according to the methylation status of *KLK10* exon 3

Gene	Methylation status	Relapses (%)	P^a	Median DFI (range)	Deaths (%)	P^a	Median OS (range)
<i>KLK10</i>	M ^b ($n = 53$)	19 (35.8)	0.011	81 (71–90)	15 (28.3)	0.030	98 (89–106)
	U ^c ($n = 40$)	5 (12.5)		110 (103–117)	4 (10.0)		117 (111–123)

^aChi-square test.

^bMethylated.

^cUnmethylated.

DFI, disease-free interval; OS, overall survival. Values in bold typeface are statistically significant.

$P = 0.019$, respectively; Table 4]. Similarly, *KLK10* exon 3 methylation, as well as the absence of estrogen receptor positivity (HR = 6.874, $P = 0.011$ and HR = 3.406, $P = 0.020$, respectively; Table 4) were independently associated with an increased risk of death due to the disease.

discussion

A strong correlation between exon 3 methylation and loss of *KLK10* mRNA expression in a panel of breast cancer cell lines and in a small number of primary breast tumors has already clearly been shown [7], suggesting an important role of DNA methylation in *KLK10* gene silencing. Till now, the loss of *KLK10* expression has been demonstrated to correlate with disease progression in a small number of patients with solid tumors, including breast cancer [10, 11, 13]. Furthermore, Roman-Gomez et al. [12] have shown that *KLK10* methylation is a poor prognostic factor in both childhood and adult ALL. *KLK10* expression was found to be elevated in normal breast tissue and benign lesions, while loss of *KLK10* expression during tumor progression was detected, thus suggesting *KLK10* as a molecular tool in the study of breast cancer progression [14]. Despite the fact that the performance of studies with a larger series of specimens to help assess whether *KLK10* expression can be used as a diagnostic and/or prognostic marker in breast cancer has already been suggested many years ago [14], our study is the first carried out since then on this subject.

In the current study, we have evaluated for the first time *KLK10* methylation as a prognostic biomarker in early-stage breast cancer, by using MSP, a methodology which has been shown to be highly specific and sensitive [15]. Our data demonstrate that *KLK10* methylation provides important prognostic information in early breast cancer patients. It is important to note the fact that this conclusion is not only based on our results taken through a small number of early breast cancer patients with a well-known clinical outcome but also confirmed through an independent cohort of 93 patients, the largest reported so far for *KLK10* methylation, whose clinical outcome was not known to us when the analysis was carried out. The fact that we did not detect any *KLK10* methylation in the group of normal breast tissues as well as in benign fibroadenomas, and in only 1 of 10 (10%) paired nontumorous breast tissue adjacent to tumors make these results even more promising and worth to be announced and further validated in an even larger number of patients.

During the last years, there is a tremendous search for novel biomarkers based on the methylation status of tumor

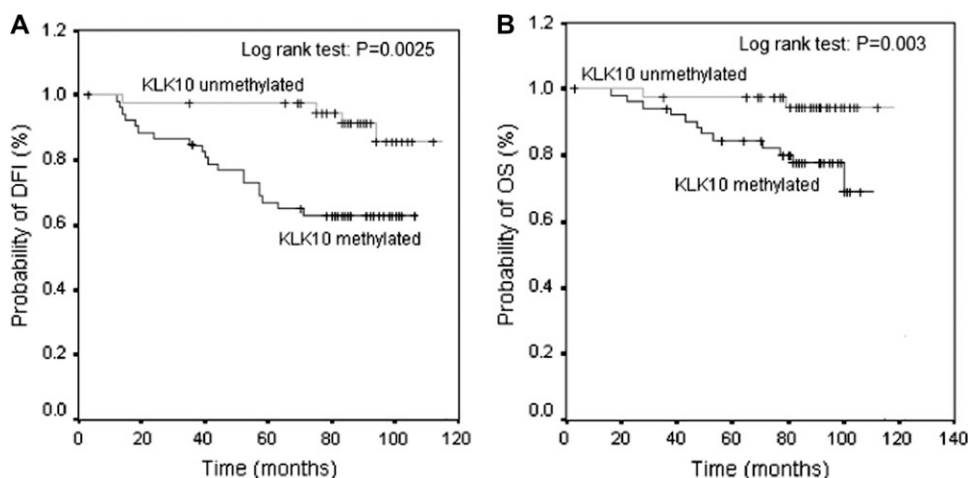


Figure 2. (A) Kaplan–Meier estimates of disease-free interval (DFI) for early breast cancer patients with or without *KLK10* methylation ($P = 0.0025$). (B) Kaplan–Meier estimates of overall survival (OS) for early breast cancer patients with or without *KLK10* methylation ($P = 0.003$).

Table 4. Multivariate analysis for DFI and OS of patients with early breast cancer

Parameter	Hazard ratio	95% CI	P^a
DFI			
<i>KLK10</i> (methylated versus unmethylated)	3.492	1.165–10.465	0.026
Axillary lymph node (<4 versus ≥ 4)	5.982	1.338–26.739	0.019
OS			
<i>KLK10</i> (methylated versus unmethylated)	6.874	1.561–30.275	0.011
Estrogen receptor (negative versus positive)	3.406	1.215–9.547	0.020

^aCox regression analysis.

CI, confidence interval; DFI, disease-free interval; OS, overall survival. Values in bold typeface are statistically significant.

suppressor genes since through this mechanism gene silencing occurs. In this way, DNA methylation can be seen as one hit in the well-known Knudson's model for tumorigenesis [16]. The potential of DNA methylation as a novel area of biomarkers research is really tremendous. There is plenty of interesting tumor suppressor genes, whose silencing through DNA methylation has been evaluated in many types of cancer and especially in breast cancer [17–19].

The results reported here demonstrate that *KLK10* exon 3 methylation provides important prognostic information in early breast cancer patients. *KLK10* was identified as a target of methylation and silencing in a significant number of tumors of early breast cancer patients, suggesting that inactivation of this gene is a frequent event in the process of mammary tumorigenesis. Whether methylation of *KLK10* exon 3 is also an early event during breast cancer development remains unclear. According to our data, patients with *KLK10* exon 3 methylation had shorter DFI and OS than those without and this was also validated in an independent cohort of patients. Moreover, the detection of *KLK10* exon 3 methylation emerged in the

multivariate analysis to be an independent prognostic factor for disease relapse and disease-related death. It is probable that *KLK10* gene silencing due to exon 3 methylation deactivates its tumor suppressor role and can thus possibly contribute to a shorter survival in breast cancer patients. Whereas additional studies are mandated to define the biological function of *KLK10*, our results support the notion that *KLK10* exon 3 methylation maybe useful as a novel biomarker in the study of breast cancer progression.

In conclusion, our data demonstrate for the first time that *KLK10* exon 3 methylation provides important prognostic information in early breast cancer patients and that this methylation plays an important role in the clinical behavior of breast tumors. Nevertheless, the methylation status of this gene should be prospectively evaluated as a promising prognostic biomarker in a larger cohort of patients with early breast cancer.

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