Tumor Biol. (2015) 36:9677–9683
DOI 10.1007/s13277-015-3733-3

RESEARCH ARTICLE

Frequent methylation of the KLOTHO gene and overexpression of the FGFR4 receptor in invasive ductal carcinoma of the breast

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Received: 8 June 2015 / Accepted: 28 June 2015 / Published online: 8 July 2015

Abstract Invasive ductal carcinoma of the breast is the most common cancer affecting women worldwide. The marked heterogeneity of breast cancer is matched only with the heterogeneity in its associated or causative factors. Breast cancer in Saudi Arabia is apparently an early onset with many of the affected females diagnosed before they reach the age of 50 years. One possible rationale underlying this observation is that consanguinity, which is widely spread in the Saudi community, is causing the accumulation of yet undetermined cancer susceptibility mutations. Another factor could be the accumulation of epigenetic aberrations caused by the shift toward a Western-like lifestyle in the past two decades. In order to shed some light into the molecular mechanisms underlying breast cancer in the Saudi community, we identified KLOTHO (KL) as a tumor-specific methylated gene using genome-wide methylation analysis of primary breast tumors utilizing the MBD-seq approach. KL methylation was frequent as it was detected in 55.3 % of breast cancer cases from Saudi Arabia (n=179) using MethyLight assay. Furthermore, KL is downregulated in breast tumors with its expression induced following treatment with 5-azacytidine. The involvement of KL in breast cancer led us to investigate its relationship in the context of breast cancer, with one of the protagonists of its function, fibroblast growth factor receptor 4 (FGFR4). Overexpression of FGFR4 in breast cancer is frequent in our cohort and this overexpression is associated with poor overall survival. Interestingly, FGFR4 expression is higher in the absence of KL methylation and lower when KL is methylated and presumably silenced, which is suggestive of an intricate relationship between the two factors. In conclusion, our findings further implicate "metabolic" genes or pathways in breast cancer that are disrupted by epigenetic mechanisms and could provide new avenues for understanding this disease in a new context.

Keywords Breast cancer · Methylation · KLOTHO · FGFR4 · FGF19

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Introduction

Breast cancer is the most common cancer type worldwide [1]. Marked reductions in its incidence rates are observed in the developed countries; however, breast cancer incidence rates are increasing in developing countries such as the Kingdom of Saudi Arabia [2, 3]. A notable feature of breast cancer in the Kingdom of Saudi Arabia is the relatively young age of onset

of its sufferers where most of the affected females are below 50 years of age [2] in contrast to over 50 in Western countries [4]. The relatively young age of onset in this population could be attributed to the interplay between common genetic susceptibility background substantiated by consanguinity and epigenetic aberrations caused by the shift in lifestyle experienced in this region in the past two decades. We have recently shown that there are possible breast cancer risk haplotypes; however, those haplotypes are unlikely to be common or widespread in the breast cancer sufferers from this region [5]. Genome-wide methylation events could therefore indicate further routes to carcinogenesis independently of DNA sequence changes.

We have recently shown that fibroblast growth factor 19 (FGF19) is overexpressed in invasive ductal carcinoma and this increase is associated with poor prognosis [6]. FGF19 is a member of a signaling pathway that includes klotho (KL) and fibroblast growth factor receptor 4 (FGFR4) as well as FGF21 and FGF23 that function to regulate various metabolic processes [7]. Our efforts to map the genome-wide methylation events of breast cancer in breast cancer samples from Saudi Arabia have revealed the methylation of KL in a tumor-specific manner. KL involvement in breast cancer is intriguing as it was originally identified as an anti-aging effector since KL-mutant mice develop signs of premature aging syndrome that is ameliorated by restoring KL expression [8]. KL is a primarily type I transmembrane protein that is further processed to produce a secreted form with wide spectrum of tissue expression and functions [9]. KL has overlapping functions including the regulation of FGF23 signaling and subsequently affecting the regulation of calcium homeostasis and nitric oxide production [10], suppression of the insulin/insulin-like growth factor 1 (IGF-1) signaling [11], suppression of Wnt signaling and oxidative stress [12, 13], oligodendrocyte maturation, and developmental myelination of the CNS and inhibition of cancer development [14]. The relationship of KL and human cancers is suggested by the observation of its down-regulation in several cancer types through methylation of its promoter region [15–20]. FGFR4 is a potential effector for KL with the latter protein could be required for the regulation of FGFR4-FGF interactions [21, 22]. FGFR4 variants have recently been suggested to play a role in carcinogenesis along with observed overexpression of the receptor in several human cancers [23–26].

In this study, we report the detection of KL methylation using the methyl binding domain protein-sequencing (MBD-seq) approach for the genome-wide methylation analysis of primary breast cancer. We demonstrate a significant downregulation and widespread methylation of KL coupled with the overexpression of FGFR4 in primary breast tumors with potential consequences on patients' prognosis for survival.

Patients and methods

Formalin-fixed and paraffin-embedded (FFPE) breast cancer tissues were obtained from female breast cancer patients, diagnosed with invasive ductal carcinoma, at the Department of Pathology, King Abdulaziz University Hospital, Jeddah, Saudi Arabia, and the National Oncology Institute, Sabratha, Libya, between 2000 and 2009 [6]. For the MBD-seq and expression analysis, genomic DNA or total RNA was extracted from surgically resected breast tumors from female breast cancer patients, diagnosed with invasive ductal carcinoma, at King Abdulaziz University Hospital, Jeddah, Saudi Arabia. Sample collection procedures followed were in accordance with the local ethical guidelines. The mean age at the time of diagnosis was 48 years (range 26–94 years).

MBD-seq

Whole-genome methylation analysis was performed using the MethylMiner™ kit from Invitrogen according to the manufacturer's protocol. Briefly, genomic DNA (3 μg) extracted from fresh frozen surgically resected breast tumors and matching non-tumorous tissues was fragmented using the Covaris S2 system to produce DNA fragments 150 bp. Enrichment of the methylated DNA was achieved by incubation with MBD-coated magnetic beads overnight at 4 °C. Methylated DNA was eluted from the beads using 1 M NaCl followed by ethanol precipitation. The enriched DNA was processed for SOLiD™ 5500xl sequencing using the manufacturer's protocol for fragment library sequencing. The methylated fragments were mapped to the hg19 edition of the human genome sequence by using the MethylMiner™ mapping module within the LifeScope™ software (Applied Biosystems). Methylated peaks were then identified using MACS v1.4 software [27] followed by visualization and annotation using PeakAnalyzer [28] and Integrated Genome Viewer software [29] applications.

Analysis of KLOTHO methylation

Hypermethylation of the KL promoter region was performed using the MethyLight assay as previously described [30]. Briefly, 0.5 μg of DNA prepared from fresh frozen surgically resected breast tissues or FFPE archival tissues. Bisulfite conversion was achieved using the Epitect Bisulfite Conversion kit from Qiagen. The MethyLight assay for KL methylation was performed using the probe FAM-CGGTTGGGTTAATC GCGTTTT-BHQ and amplification oligonucleotide primers KL-MLF 5′-AGCGTTTGTAGGACGTTTAC-3′ and KL-

MLR 5′-TAACGAAAACAAAACTCCGC-3′. For normalization, a probe (VIC-CCTTCATTCTAACCCAATACCTATCCCACCTCTAAA-BHQ) targeting methylation-independent and bisulfite-conversion-dependent COL2A1 sequence was used with the amplification primers COL2A1-CTRL-F (5′-TCTAACAATTATAAACTCCAACCACCAA-3′) and COL2A1-CTRL-R (5′-GGGAAGATGGGATAGAAGGGAATAT-3′). Samples exhibiting percentage of methylated ratio (PMR) cutoff of ≥10 was considered positive for KL methylation [30].

Analysis of KLOTHO expression

KL expression in fresh frozen surgically resected tumors was analyzed using TaqMan® approach. KL expression was detected using the TaqMan® gene expression assay Hs00183100_m1 from Life Technologies. A probe assaying GAPDH expression was used as a control (Hs03929097GL). Differential expression of KL in tumors (RQ) was determined using the formula: $2^{(\Delta C_{T[tumor]} - \Delta C_{T[normal]})}$ where the expression of KL was normalized to the expression of GAPDH in every sample (ΔC_T). 5-Aza-2′-deoxycytidine was used at 10-μM concentration to treat MCF7 for up to 3 days. RNA was extracted at different time points and KL expression was analyzed as detailed above.

Analysis of FGFR4 expression

Immunohistochemistry (IHC) analysis of FGFR4 expression was performed by modifying the protocol previously described in [31]. Briefly, antigen was retrieved in sodium citrate pH 6.0 for 20 min followed by peroxidase blocking in H_2O_2. After blocking in serum, 1:30 dilution of FGFR4 rabbit polyclonal IgG (Santa Cruz; sc-124) was applied overnight at 4 °C. Secondary labeling was achieved using 1:100 dilution of ImmunoCruz™ rabbit ABC Staining System (sc-2018). Color was developed using DAB Chromogen. The evaluation of staining of all tissue slides was performed in a blind fashion to the patients' clinicopathological parameters with an upright light microscope at ×40 magnification. Staining was graded into two categories: (i) no/weak (low) expression and (ii) moderate/strong (high) expression. The staining index was calculated as described previously [6].

Statistical analysis

Statistical analyses were performed using the IBM SPSS® Statistics (IBM Company, New York, NY, USA) software package (IBM SPSS Statistics for Mac, version 21). Fischer's exact test (two-sided) was used to assess the significance of the associations between the categorical variables.

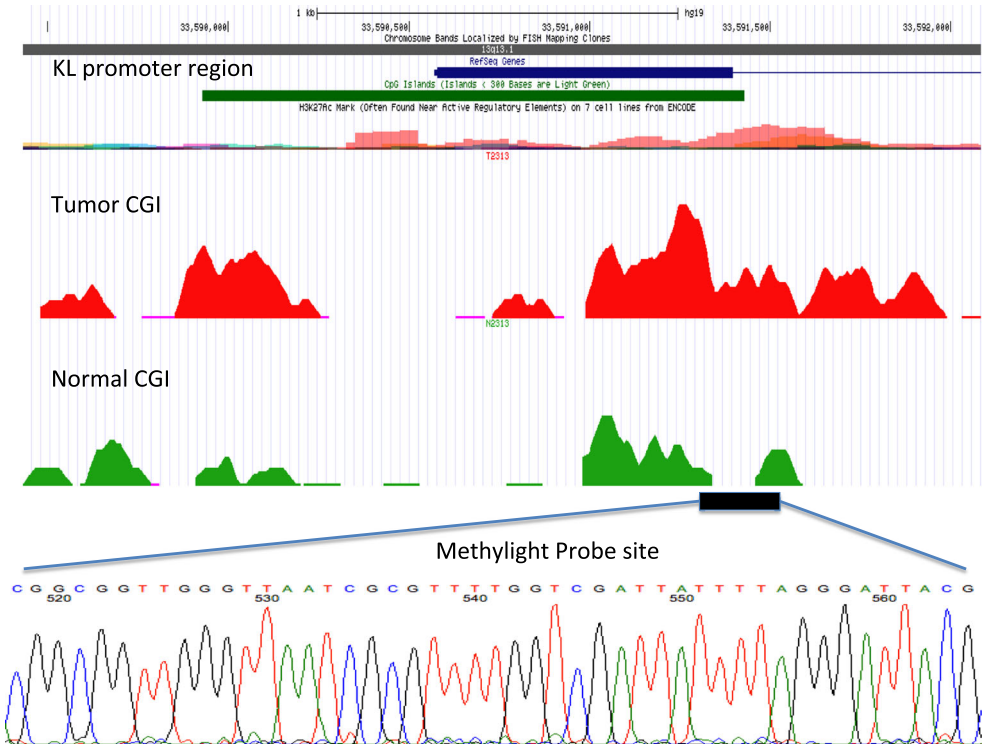

Fig. 1 Genome-wide methylation analysis of primary breast cancer using MBD-seq. Methylated DNA from tumor (*red*) and matching normal sample (*green*) is represented by sequenced reads modeled into peaks using MACS v1.4 software. The peaks are visualized using the UCSC Genome Browser overlapping klotho CpG island and promoter region. Bisulfite sequencing of the area of interest in the klotho promoter shows the differential methylation at those particular CpG dinucleotides. Microarray expression analysis of breast cancer demonstrates the downregulation of klotho (*bottom panel*)

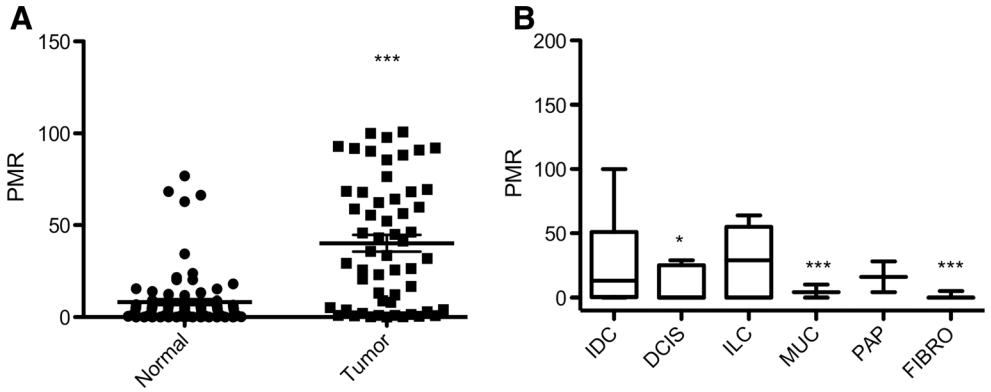

Fig. 2 Tumor-specific KLOTHO methylation. **a** Methylation analysis of the KLOTHO promoter by MethyLight shows the increased methylation of klotho in the tumors relative to their matching normal. **b** Methylation analysis of klotho promoter in different breast cancer subtypes. *IDC* invasive ductal carcinoma, *DCIS* ductal carcinoma in situ, *ILC* invasive lobular carcinoma, *MUC* mucinous carcinoma, *PAP* papillary carcinoma, *FIBRO* fibroadenoma, *PMR* percentage of methylated ratio. An *asterisk* indicates statistically significant differences between IDC and that particular subtype

Univariate survival analysis was based on Kaplan-Meier method, with log-rank (Mantel-Cox) comparison test. In all tests, the values $p<0.05$ were regarded as statistically significant.

Results

Analysis of KLOTHO methylation in invasive ductal carcinoma

The MBD-seq approach was used to identify new sites of breast-tumor-specific DNA methylation and the associated gene promoters. KL promoter region was found to be hypermethylated in a tumor-specific manner by this method (Fig. 1). The genomic region of the best enrichment of tumor-specific DNA methylation was determined, and its sequence was utilized to design a custom MethyLight assay for KL methylation in fresh frozen surgically resected breast tumors as well as archival FFPE tissues. In order to investigate the tumor-specific nature of KL methylation, DNA from 65 normal/tumor pairs of surgically resected breast tissues was interrogated for KL methylation using the MethyLight approach (Fig. 2a). Forty-three out of the 65 (66.15 %) surgically resected tumors showed significant KL methylation (PMR≥ 10) compared to 10 out of 65 surgically resected matching non-tumorous breast tissues (15.38 %). The overall KL methylation levels, as measured by the PMR value, were significantly higher in surgically resected tumors relative to non-tumorous breast tissues ($p<0.001$, Fig. 2a). The high frequency of KL methylation in breast tumors was maintained when archival FFPE tissues were analyzed as 56 out of 114 (49.12 %) archival FFPE breast cancer samples exhibited significant KL methylation. Overall, the average KL methylation frequency in fresh frozen surgically resected and archival FFPE breast tumors is 99 out of 179 samples (55.30 %). Interestingly, KL methylation was widespread and does not exhibit any significant association with clinicopathological parameters such as age, hormonal status, or lymph node metastasis.

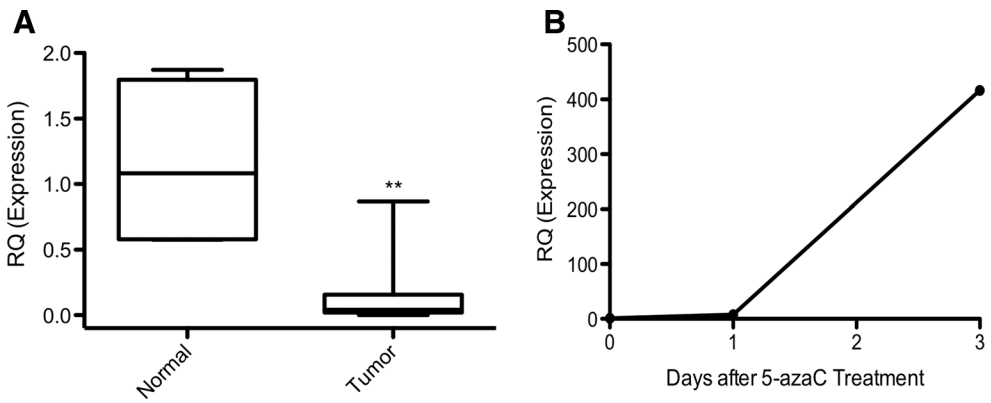

Fig. 3 Loss of expression of klotho in breast cancer. **a** Quantitative PCR (qPCR) analysis of KL expression in malignant breast tissues (*Tumor*) compared to a cohort of non-malignant breast tissues (*Normal*). **b** qPCR analysis of KLOTHO expression in the MCF7 cell line before and after the addition of the methylation-reversing drug, 5-aza-2′-deoxycytidine. An *asterisk* indicates statistically significant differences

KLOTHO is downregulated in breast cancer

In order to investigate the relationship between KL methylation and its expression, we analyzed RNA from 48 fresh frozen surgically resected breast tumors and 4 non-tumorous tissues using TaqMan® gene expression assays (Fig. 3a). KL was downregulated in the majority of the tumors investigated relative to its level in the non-tumorous controls. Furthermore, MCF7 breast cancer cell line, where KL expression is weak, was treated with 10 μM 5-azacytidine for 1 or 3 days followed by RNA extraction analysis of KL expression using TaqMan®. As shown in Fig. 3b, KL expression increases significantly following 3 days of azacytidine treatment confirming the role of promoter methylation in silencing KL expression.

KLOTHO expression may be required for high FGFR4 expression

We have previously shown that FGF19, an important effector in the KL pathway, is upregulated in breast cancer [6]. In order to further elucidate the role the FGF-KL in breast cancer, we investigated the expression of the FGFR4 receptor in the same cohort of FFPE tissues using immunohistochemistry. FGFR4 was found to be overexpressed in 41 % of the FFPE cases examined (n=112, where KL methylation data are available; Fig. 4). The overexpression of FGFR4 correlated negatively with KL methylation (Pearson's R=−0.137, p=0.149). Furthermore, high FGFR4 expression is associated with lower PMR value for KL methylation, whereas the low FGFR4 expression is associated with higher PMR values for KL methylation (p<0.01; Fig. 5).

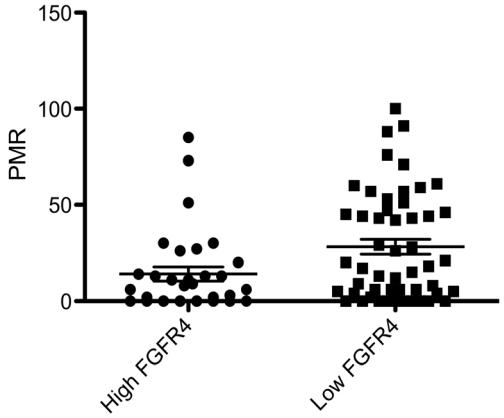

Fig. 5 The relationship between klotho methylation and FGFR4 expression in breast cancer. *PMR* percentage of methylated ratio

In order to shed some light into the role of FGFR4 in breast cancer, its expression was analyzed in 69 additional archival FFPE cases using immunohistochemistry (total n=181). FGFR4 expression was elevated in 43.6 % of the total cases examined. A trend toward an association between high FGFR4 expression and poor overall survival can be shown using univariate Kaplan-Meier analysis (p=0.082). However, there is a statistically stronger association between high FGFR4 levels and poor overall survival where KL is also methylated (Fig. 6; p=0.038). Multivariate Cox's regression analysis supports the potential role of FGFR4 as an independent poor prognosis marker in lymph-node-positive breast cancer (Table 1).

We have previously shown that high FGF19 expression is a marker for poor prognosis in breast cancer [6]. In this study, high FGFR4 expression is significantly associated with high FGF19 expression in our cohort of breast cancer samples (p=0.009). High expression of both FGFR4 and FGF19 is

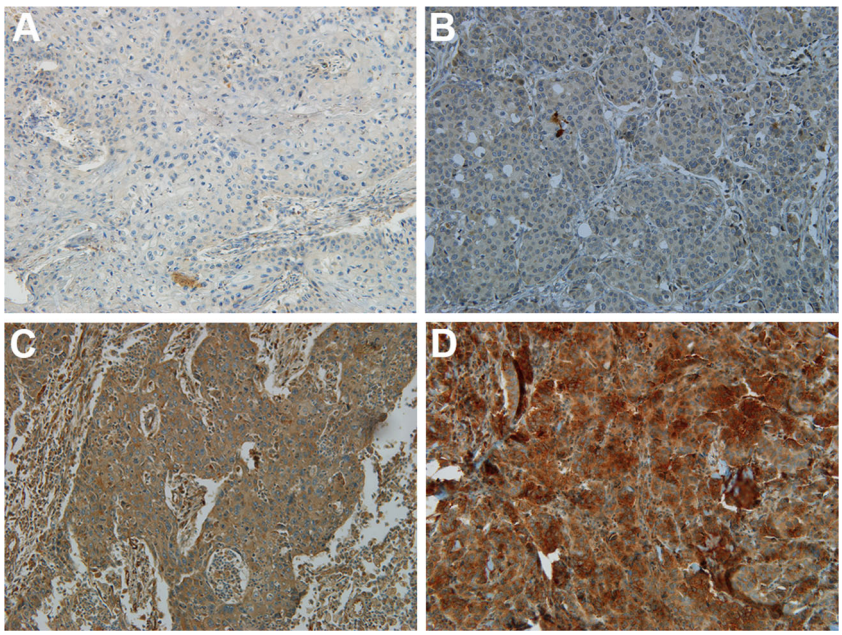

Fig. 4 FGFR4 expression pattern in a breast cancer cohort. **a** No or weak expression, **b** weak FGFR4 expression, **c** moderate level of FGFR4 expression, and **d** strong FGFR4 expression. Magnification is ×40

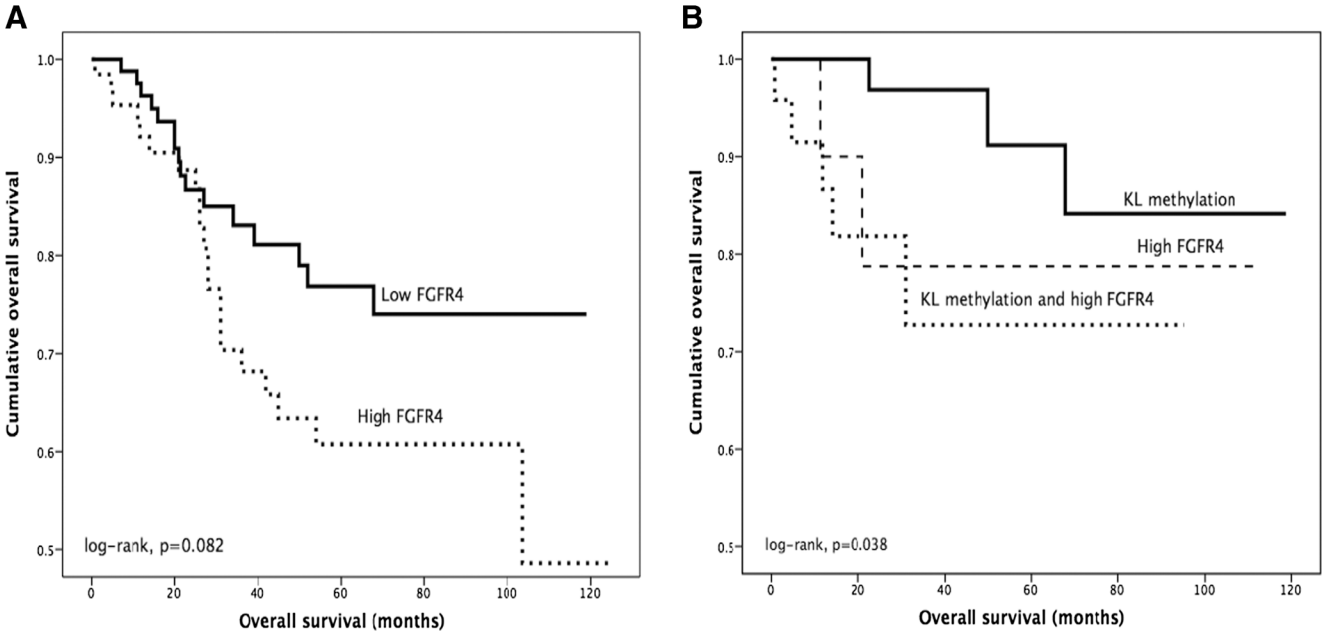

Fig. 6 The effect of FGFR4 expression on overall survival of breast cancer patients as determined by univariate Kaplan-Meier analysis (**a**) or when stratified according to klotho methylation status (**b**)

associated with poor overall survival ($p=0.023$). Furthermore, perturbation of FGFR4 and FGF19 pathway correlates with young age of onset (below 50 years old, $p=0.014$) particularly in patients exhibiting high FGF19 expression in the absence of elevated FGFR4 expression.

Discussion

We have identified KLOTHO independently as a tumor-specific methylated gene through genome-wide methylation analysis of primary breast cancer samples (22 primary breast tumors and 6 non-malignant breast tissues). The approach that we adopted utilizes the high affinity of the methyl domain binding protein (MBD) toward methylated cytosines in the genome. High-throughput sequencing on the SOLiD 5500xl platform then identified enriched DNA fragments. Further analysis of KL methylation was driven by the observation that breast cancer sufferers in the Kingdom of Saudi Arabia are often diagnosed at a relatively young age. KL was first identified as an aging suppressor where mice lacking KL exhibit symptoms of premature aging. In this study, we show that KL methylation was identified in 55 % of analyzed cases with no clear association with known clinicopathological parameters. This percentage is higher than the one reported in breast cancer by Rubinek et al. [18] where 8 out of 23 breast tumor samples showed significant methylation at the KL promoter. Methylation of KL promoter is also reported to occur frequently in other cancer types including colon, pancreas, liver, stomach, and cervix.

We also show that KLOTHO expression is downregulated in the majority of the breast tumors examined and this loss of expression is reversible by treatment with 5-azacytidine. This loss of expression indicates an increasing likelihood of a role of this gene in breast carcinogenesis. However, the nature of this role remains unknown as it is unlikely that KL has direct tumor suppressor activities despite several reports showing that it can cause apoptosis and blocks cellular proliferation. It is possible to postulate that KL may affect breast cancer through its interaction with the fibroblast growth factor receptors (FGFRs) and modulate their control over proliferation. We have previously shown that an important FGFR ligand, FGF19, is overexpressed in breast cancer and this overexpression is a potential biomarker for poor prognosis in this disease. A likely receptor for FGF19 is FGFR4 which is also shown to be overexpressed in breast cancer.

We have analyzed the expression of FGFR4 in our cohort and show that this receptor is overexpressed in 43.6 % of the breast cancer cases examined by immunohistochemistry.

Table 1 Multivariate Cox regression analysis of various clinicopathological parameters and their use as prognostic markers for overall survival in lymph-node-positive BC patients in our cohort

	p value	SE value	Relative risk	95 % CI
Age	0.128	0.362	1.602	0.936–3.869
ER status	0.000	0.428	0.587	0.225–0.897
Grade 1	0.040	0.713	0.682	1.069–17.54
Grade 2	0.162	0.407	1.757	0.794–3.921
High FGFR4 expression	0.029	0.396	1.153	1.094–5.173

Interestingly, FGFR4 overexpression associated with low KL methylation could indicate that KL expression may be required to maintain FGFR4 expression through an unknown mechanism. KL methylation is a characteristic of many breast cancer cases regardless of age, lymph node metastasis, grade, or hormonal status. However, the resulting or associated perturbation in FGFR4 expression, similar to FGF19, could be utilized as a biomarker for poor prognosis in our cohort or even targeted therapeutics in a similar fashion to ERBB2/HER-2.

Acknowledgments The authors would like thank the Ministry of Education and King Abdulaziz City for Science and Technology (KACST) for their financial support to this research project (ARP-29-292).

Conflicts of interest None

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