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RESEARCH ARTICLE

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Detection of IKK ϵ by immunohistochemistry in primary breast cancer: association with EGFR expression and absence of lymph node metastasis

Virginie Williams^{1,2}, Andrée-Anne Grosset^{1,3,4}, Natalia Zamorano Cuervo¹, Yves St-Pierre³, Marie-Pierre Sylvestre^{1,5}, Louis Gaboury⁴ and Nathalie Grandvaux^{1,2*} 

Abstract

Background: IKK ϵ is an oncogenic kinase that was found amplified and overexpressed in a substantial percentage of human breast cancer cell lines and primary tumors using genomic and gene expression analyses. Molecular studies have provided the rationale for a key implication of IKK ϵ in breast cancer cells proliferation and invasiveness through the phosphorylation of several substrates.

Methods: Here, we performed immunohistochemical detection of IKK ϵ expression on tissue microarrays constituted of 154 characterized human breast cancer tumors. We further determined the association with multiple clinicopathological parameters and 5-years overall, disease-free and distant disease free survival.

Results: We observed expression of IKK ϵ in 60.4% of the breast cancer tumors. IKK ϵ expression status showed no association with a panel of markers used for molecular classification of the tumors, including ER/PR/HER2 status, or with the molecular subtypes. However, IKK ϵ expression was inversely associated with lymph node metastasis status ($p = 0.0032$). Additionally, we identified a novel association between IKK ϵ and EGFR expression ($p = 0.0011$).

Conclusions: The unexpected observation of an inverse association between IKK ϵ and lymph node metastasis advocates for larger scale immunohistochemical profiling of primary breast tumors to clarify the role of IKK ϵ in metastasis. This study suggests that breast cancer tumors expressing EGFR and IKK ϵ may be potential targets for drugs aiming at inhibiting IKK ϵ activity or expression.

Keywords: IKK ϵ , Breast, Cancer, EGFR, Metastasis, Immunohistochemistry, Biomarker, Prognosis

Background

Breast cancer remains a leading cause of cancer-related mortality in women [1]. Improved outcome and survival of patients have resulted from the identification of Estrogen Receptor (ER), Progesterone Receptor (PR), and HER2 biomarkers that have been used to stratify tumors and define targeted therapies [2]. However, the morphological, clinical and molecular complexity and heterogeneity of breast carcinomas argues for the use of

additional specific target genes and pathways as additional biomarkers to define personalized prognostic and predictive therapeutic approach [3]. Initiation and progression of breast cancer relies on the deregulation of a complex network of pathways and genes that control cell proliferation and survival [4]. Knowledge of these pathways provides opportunities for identification of new biomarkers in primary tumors.

The inhibitor of NF- κ B kinases (IKK) ϵ is a member of the IKK family of kinases [5, 6]. IKK ϵ is well recognized for its role in the regulation of distinct NF- κ B pathways [7–9] and of the interferon-mediated innate immunity through phosphorylation of Interferon regulatory factors (IRFs) and signal transducer and

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activator of transcription (STAT) 1 [10–12]. Additional studies have unveiled a key role of IKK ϵ in mammary epithelial cell transformation and invasiveness. Suppression of IKK ϵ by shRNA or transfection of a dominant negative form results in inhibition of anchorage-independent growth and invasiveness of breast cancer cell lines [13, 14]. IKK ϵ -mediated oncogenesis relies on the phosphorylation of multiple substrates, cylindromatosis tumor suppressor (CYLD), estrogen receptor α (ER α), tumor necrosis factor receptor-associated factor 2 (TRAF2), Forkhead box O 3a (FOXO3a) and Akt, and on the regulation of the expression of genes, such as *CCND1*, *MMP-9* and *Bcl-2* [13–19]. In Triple Negative Breast Cancer cells (TNBC), IKK ϵ is involved in the coordinated activation of NF- κ B, STAT, and cytokine signaling [20]. IKK ϵ is also involved in the development of resistance to tamoxifen (Tam) treatment. Silencing of IKK ϵ expression sensitizes ER $^+$ T47D breast cancer cell line expressing high level of IKK ϵ cells to Tam-induced cell death and apoptosis and to Tam-mediated inhibition of focus formation. Conversely, overexpression of IKK ϵ protects the MCF-7 breast cancer cell line from Tam-induced cell death and apoptosis and reduced Tam-mediated inhibition of focus formation [21].

Analysis of epithelial breast cancer cell lines and primary breast tumors showed copy-number gain or amplification of the 1q32 region resulting in up to 10 copies of the *IKBKE* locus encoding for IKK ϵ [13]. Gene and protein expression studies performed in epithelial breast cancer cell lines, primary breast tumors and in chemically-induced murine mammary breast tumors demonstrated that increased IKK ϵ levels can also result from aberrant expression without gene amplification suggesting that analysis at genomic levels is not appropriate to fully characterize IKK ϵ status in breast cancer [13, 14, 20]. To the best of our knowledge, very limited information is available regarding the relationship between IKK ϵ protein expression and clinicopathological status of primary breast tumors.

Here, we studied IKK ϵ expression by immunohistochemistry (IHC) using tissue microarrays (TMA) of 154 human breast cancer tissues and analyzed the association with clinicopathological parameters and with a panel of biomarkers used for molecular classification of tumors.

Methods

Tissue microarrays

High-density tissue microarrays (TMAs) were constructed from formalin-fixed paraffin-embedded material isolated from 154 primary tumor samples and normal adjacent tissues. Tissues were fixed with 10% neutral buffered formalin and paraffin embedded according to usual methods. Samples were cut into 5 μ m slices. Three

cores were used for each patient. Tumor samples were obtained from patients diagnosed with primary breast cancer at the Centre Hospitalier de l'Université de Montréal. Tumors contained in TMAs were previously characterized on the basis of the histological diagnosis according to the classification of Nottingham modified by Elston and Ellis. The cohort consists of low- and high-grade ductal carcinomas and of medullary carcinomas (typical and atypical). The tumors were previously characterized immunohistochemically for ER α , PR, ErbB2 (Her-2/neu), Ki67 and EGFR among others [22, 23]. Molecular subtypes of patients from the cohort were obtained from the clinical chart and presented the following characteristics: Luminal A: ER $^+$ /HER2 $^-$, Ki-67 < 14%; Luminal B: ER $^+$ /HER2 $^-$, Ki-67 \geq 14% or ER $^+$ /PR $^+$ /HER2 $^+$; HER2: ER $^-$ /PR $^-$ /HER2 $^+$; Triple negative: ER $^-$ /PR $^-$ /HER2 $^-$.

Immunohistochemistry (IHC)

IHC was assessed according to manufacturer recommendations on an immunostainer (Discovery XT system, Ventana Medical Systems, Tucson, AZ). Antigen retrieval was performed with proprietary reagents (cell conditioner 1 for 60mn, Ventana Medical Systems). Monoclonal rabbit anti-IKK ϵ D20G4 (1/50, Cell Signaling #2905) or control Rabbit DA1E mAb IgG XP isotype control (Cell Signaling #3900) antibodies were applied on every sample at room temperature for 4 h. Sections were then incubated with a specific secondary biotinylated antibody for 30 mn. Streptavidin horseradish peroxidase, and 3,3'-diaminobenzidine were used according to the manufacturer's instructions (DABmap detection kit, Ventana Medical Systems). Finally, sections were counterstained with hematoxylin. Each section was scanned at a high resolution (Nanozo-omer, Hamamatsu Photonics K.K.).

Scoring of IHC staining

IKK ϵ expression was classified according to the following grading system. Two independent observers, including the expert pathologist who made the initial assessment of tissue pathology, scored the intensity of IKK ϵ staining, the percentage of positive cells and the subcellular localization (cytoplasmic and nuclear). IKK ϵ staining intensity and percentage of positive cells were categorized on 0–3 arbitrary scales (Intensity: 0 = absence, 1 = weak, 2 = moderate, 3 = high; Percentage of positive cells: 0 \leq 1%, 1 \leq 30%, 2 \leq 70%, 3 > 70%). The individual categories were multiplied to give an IHC score ranging between 0 and 9 (actual values were 0–4 and 6 and 9) so that the final IHC score reflects the number of cells effectively stained in the tumors tissue and the staining intensity. Localization of IKK ϵ was categorized as cytoplasmic or nuclear. The staining of IKK ϵ corresponds to the mean of staining performed on 3 different cores from a single tumor.

RNAi transfection and immunoblot

ZR75.1 and MCF7 breast cancer cell lines (obtained from Dr. S. Mader, University of Montreal, Canada) were cultured in RPMI 1640 medium supplemented with 10% heat-inactivated FBS (HI-FBS) and MEM medium supplemented with non-essential amino acids, sodium pyruvate and 10% HI-FBS, respectively. All media and supplements were obtained from Life Technologies. RNAi transfection in ZR75.1 and MCF-7 cells was performed with TransiT siQuest (Mirus) and Dharmafect 1 reagent, respectively, according to the manufacturer's instructions for 72 h. The sequences of control (siCTRL) and IKK ϵ -specific (siIKK ϵ) RNAi oligonucleotide sequences (Dharmacon) have been previously described [10]. For IKK ϵ immunoblot, cells were lysed as described in [24]. For EGFR immunoblot, cells were lysed by sonication after incubation for 30 min on ice in Triton-X100 lysis buffer (50 mM HEPES pH 7.4, 1 mM EDTA, 250 mM NaCl, 1.5 mM MgCl₂, 10% Glycerol and 1% Triton-X100) containing 1 mM PMSF, 10 μ g/mL aprotinin and 10 μ g/mL leupeptin, and quantified using the BCA protein assay (Pierce). Whole cell extracts (WCE), were subjected to SDS-PAGE electrophoresis followed by immunoblot analysis using monoclonal rabbit anti-IKK ϵ D20G4 (1/50, Cell Signaling #2905), monoclonal rabbit anti-EGFR EP38Y (1/4000, Abcam #ab52894) and anti-actin (Chemicon International MAB1501) antibodies. Antibodies were diluted in PBS containing 5% Tween and 5% non-fat dry milk or BSA. Immunoreactive bands were visualized by enhanced chemiluminescence using the Western Lightning Chemiluminescence Reagent Plus (Perkin Elmer Life Sciences) and a CCD-camera LAS400 mini apparatus (GE Healthcare).

Statistical analysis

Associations between IKK ϵ expression status (positive vs. negative) and both molecular markers and clinicopathological parameters were tested using chi-squared tests. *P*-values <0.05 were considered statistically significant. Kaplan Meier curves were used to estimate overall survival (OS), disease-free survival (DFS) and distant disease free survival (DDFS) over 5 years starting at time of surgery. Survival curves were compared using log-rank test. *P*-values <0.05 were considered statistically significant.

Results

Validation of anti-IKK ϵ antibody

To study the expression of IKK ϵ at the protein level, the specificity of the commercially available monoclonal rabbit anti-IKK ϵ D20G4 antibody used for TMA IHC staining was first evaluated by immunoblot. WCE derived from the epithelial breast cancer cell line ZR75.1 transfected with control RNAi or RNAi specifically designed to target IKK ϵ

were examined (Fig. 1). A single band at 79 kDa corresponding to the expected size of the full length IKK ϵ was detected. This band was dramatically decreased upon transfection of IKK ϵ -specific RNAi, validating the specificity of the antibody and its application for IHC staining of TMA.

IHC staining of IKK ϵ

IKK ϵ expression was evaluated on TMA containing triplicates from 154 breast cancer patients and from normal tissues. TMA were stained with the monoclonal rabbit anti-IKK ϵ D20G4 or control rabbit isotype antibodies. Representative IHC photomicrographs are shown in Fig. 2. No significant specific staining was observed with the control rabbit isotype antibody in normal mammary gland or tumor breast tissues (Fig. 2b–h). Normal breast tissues exhibited no detectable IKK ϵ staining (Fig. 2a). Amongst breast tumor samples, 93 out of 154 (60.4%) showed positive IKK ϵ staining of epithelial cancer cells (Fig. 2c–g). In line with previous reports showing IKK ϵ expression in immune cells [5], IKK ϵ was detected both in cancer epithelial cells and invading immune cells in tumor with immune infiltrate (data not shown). An IHC score was determined for 148 out of the 154 samples. Six positive samples had undetermined scores because of discrepancies between the triplicate slides (Fig. 3). The scores of epithelial cells IKK ϵ staining amongst the positive

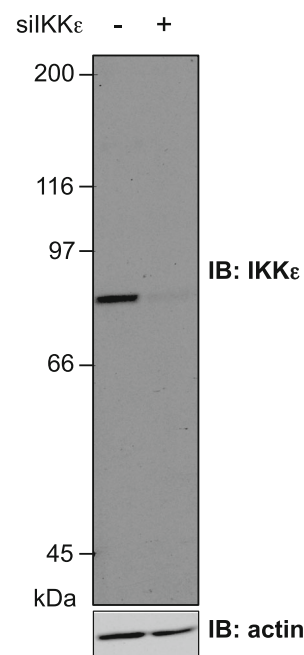
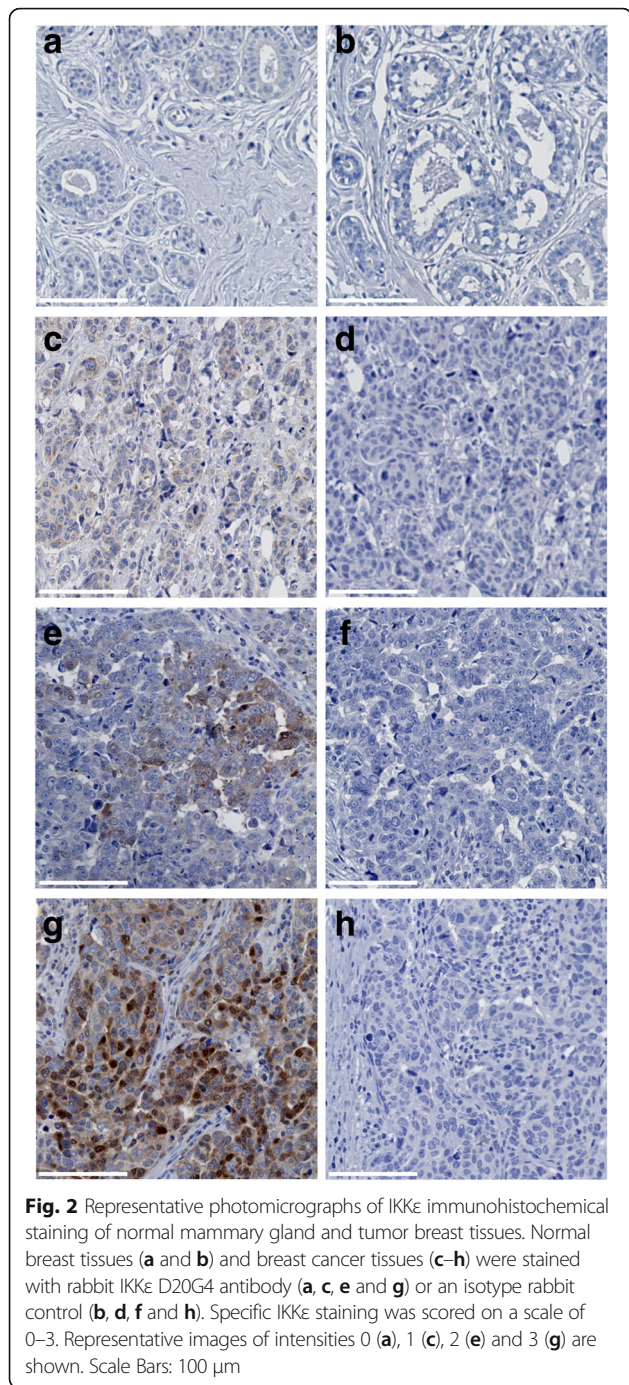
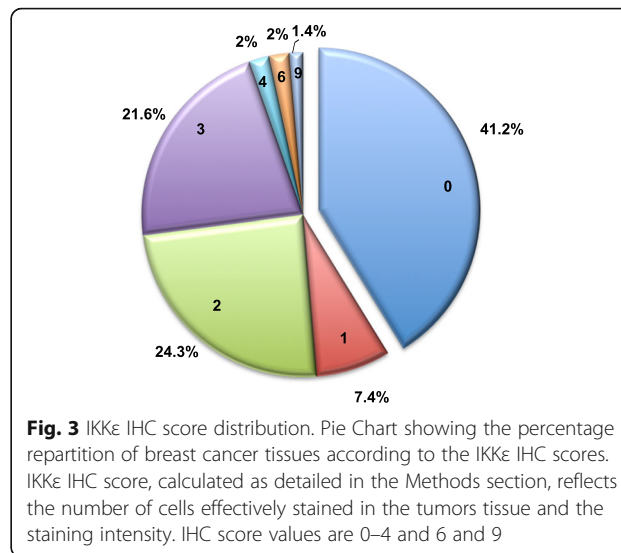


Fig. 1 Specificity of the IKK ϵ antibody. ZR75.1 epithelial breast cancer cells expressing high levels of IKK ϵ were transfected with control RNAi (non-targeting) or IKK ϵ -specific RNAi. WCE were resolved by SDS-PAGE and analyzed by immunoblot using anti-IKK ϵ D20G4 and anti-actin antibodies



tumors varied significantly, but the vast majority exhibited low (1–3) IHC scores (79/87; 90.8%). As recent reports described substantial nuclear localization of IKKε [25, 26], the localization of IKKε staining in our cohort of breast tumors was carefully assessed. IKKε was restricted to the cytoplasm in the vast majority of tumors. Only 4 tumors out of the 93 positive for IKKε expression exhibited both cytoplasmic and nuclear staining. Although the low number of tumors with nuclear staining prevented correlation



studies, it is noteworthy that they were distributed throughout a wide range of IKKε IHC scores. However, the only two tumors with the highest IKKε IHC score of 9 showed cytoplasmic and nuclear staining (Table 1).

Association between IKKε expression and breast cancer clinicopathological parameters

The fact that not all patients with breast cancer expressed IKKε suggests that IKKε might be associated with specific characteristic(s). Analysis of the association between IKKε expression and a panel of markers used for molecular classification of tumors and clinicopathological parameters (Table 2) did not reveal significant association with age, specific breast tumor pathological or molecular subtype or expression of the classical markers ER, PR and HER2. Expression of IKKε showed a significant inverse association with the lymph node metastasis status ($p = 0.0032$), as 75.3% of breast tumors expressing IKKε were found negative for lymph node metastasis implicating that IKKε⁺ breast tumors are less likely to present metastasis (Table 2). Amongst the molecular markers tested, epidermal growth factor receptor

Table 1 IKKε localization in breast cancer tumors

IKKε IHC score	Cytoplasmic only	Cytoplasmic and nuclear
0	61	0
1	11	0
2	35	1
3	31	1
4	3	0
6	3	0
9	0	2
Total	144	4

Table 2 Molecular and clinicopathological parameters correlation according to IKKε expression in breast cancer patients

Parameters		IKKε positive <i>n</i> (%)	IKKε negative <i>n</i> (%)	<i>P</i> -value	
Age	<45	11 (11.8)	4 (6.6)	0.4231	
	≥45	82 (88.2)	57 (93.4)		
Pathological type	Ductal	63 (67.8)	47 (77)	0.2855	
	Medullary	30 (32.3)	14 (23)		
Grade	I	12 (12.9)	3 (4.9)	0.1561	
	II	8 (8.6)	3 (4.9)		
	III	73 (78.5)	55 (90.2)		
	unknown	0	0		
Molecular subtypes	Triple negative	54 (58)	40 (65.6)	0.4439	
	other	39 (42)	21 (34.4)		
	HER2	7 (7.5)	6 (9.8)		0.8707
	Luminal A	8 (8.6)	8 (13.1)		
	Luminal B	2 (2.2)	3 (4.9)		
	unknown	22	4		
Lymph Node Metastasis	positive	23 (24.7)	30 (49.2)	0.0032	
	negative	70 (75.3)	31 (50.8)		
	unknown	0	0		
ERα	positive	20 (21.5)	15 (24.6)	0.8872	
	negative	70 (75.3)	46 (75.4)		
	unknown	3	0		
PR	positive	13 (14)	6 (9.8)	1	
	negative	71 (76.3)	50 (82)		
	unknown	9	1		
HER2	positive	18 (19.4)	6 (9.8)	0.1196	
	negative	67 (72)	54 (88.5)		
	unknown	8	1		
EGFR	positive	47 (50.5)	16 (26)	0.0011	
	negative	38 (40.9)	44 (72)		
	unknown	8	1		
Ki67	positive	83 (89.2)	51 (83.6)	0.1061	
	negative	5 (5.4)	9 (14.8)		
	unknown	5	1		
p53	positive	30 (32.2)	10 (16.4)	0.5735	
	negative	42 (45.2)	20 (32.8)		
	unknown	21	31		
CK5/6	positive	46 (49.5)	21 (34.4)	0.0568	
	negative	42 (45.2)	39 (64)		
	unknown	5	1		
CK14	positive	34 (36.6)	16 (26.2)	0.0869	
	negative	48 (51.6)	45 (73.8)		
	unknown	11	0		

Table 2 Molecular and clinicopathological parameters correlation according to IKKε expression in breast cancer patients (Continue)

Parameters		IKKε positive <i>n</i> (%)	IKKε negative <i>n</i> (%)	<i>P</i> -value
GATA3	positive	17 (18.3)	8 (13.1)	0.797
	negative	55 (59.1)	20 (32.8)	
	unknown	21	33	
CD276	positive	37 (39.8)	13 (21.3)	0.3007
	negative	22 (23.7)	14 (23)	
	unknown	34	34	
Galectin 7	positive	38 (40.9)	12 (19.7)	0.4758
	negative	38 (40.9)	18 (29.5)	
	unknown	17	31	

P-values were computed using a chi-squared test. Observations with parameter values 'unknown' were omitted from the test

P-values <0.05 were considered statistically significant and indicated as bold italicized

(EGFR) expression was significantly associated with IKKε expression ($p = 0.0011$), as 72% of the IKKε⁻ breast tumors were also negative for EGFR expression (Table 2 and Fig. 4a–d). To evaluate the possibility that EGFR expression could be placed under the control of IKKε-dependent signalling, the impact of IKKε silencing on EGFR expression levels was tested in MCF-7 epithelial breast cancer cell line. MCF-7 were transfected with control or IKKε-specific RNAi and EGFR expression was monitored by immunoblot. As shown in Fig. 4e and f, silencing of IKKε resulted in the significant inhibition of EGFR expression levels.

Prognostic significance of IKKε expression in breast cancer

To further assess the clinical relevance of IKKε expression in breast tumors, we analyzed the 5-year after surgery overall survival (OS), disease-free survival (DFS) and distant disease free survival (DDFS) follow-up information available from IKKε negative ($n = 26$) and IKKε positive ($n = 60$) tumors (Fig. 5). Comparison of survival curves by log-rank test in Kaplan-Meier survival analyses showed that patients in the IKKε⁺ subgroup exhibit no significant differences of OS, DFS or DDFS compared to IKKε⁻ subgroup.

Discussion

The implication of IKKε in breast cancer tumorigenesis provides opportunities for targeted therapies. However, the relationship between IKKε expression in primary breast carcinomas and clinicopathological markers remained to be established. Previous genomic and gene expression analyses have highlighted increased IKKε expression, accompanied or not by gene amplification, in 30% of breast tumors [13, 14, 20]. This same percentage was also observed by IHC staining of a very limited number of primary breast tumors ($n = 20$) [13]. Here, IHC staining of 154 tumor breast tissues revealed a

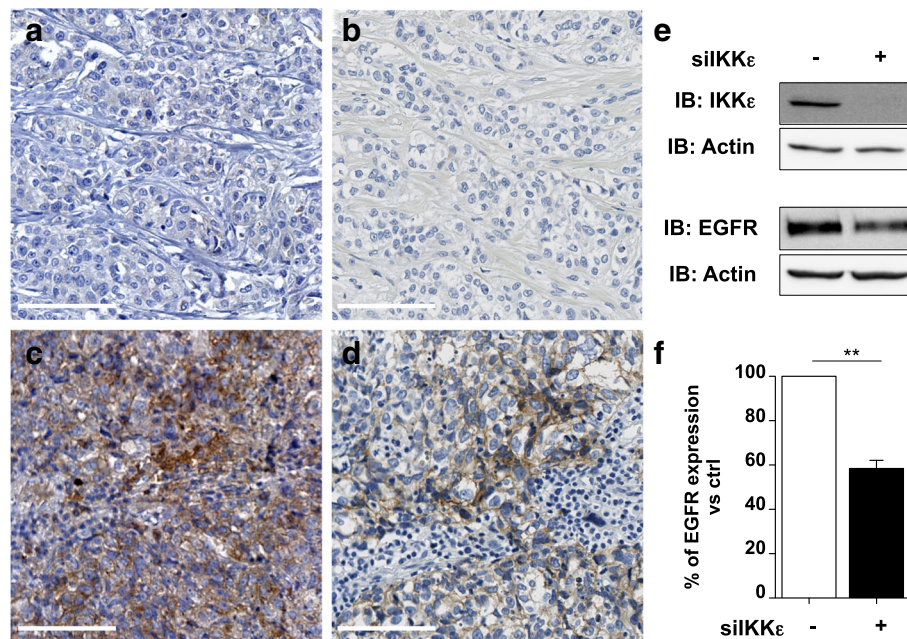


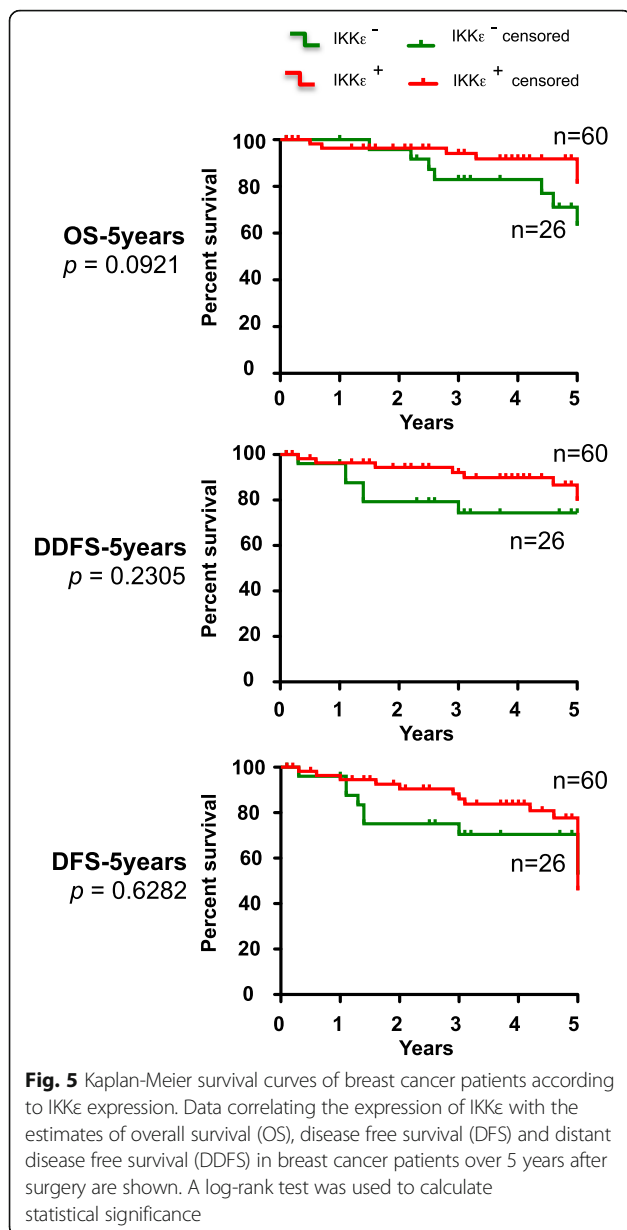
Fig. 4 Association between IKKε and EGFR expression. Representative photomicrographs of IKKε (a, c) and EGFR (b, d) immunohistochemical staining of tumor breast tissues are shown. Representative images of staining in IKKε⁻/EGFR⁻ (a and b) and IKKε⁺/EGFR⁺ (c and d) breast cancer tissues are shown. Scale Bars: 100 μm. In (e and f), control RNAi (non-targeting) or IKKε-specific RNAi were transfected into MCF-7 cells. Efficiency of IKKε silencing and the impact on EGFR expression were analyzed by immunoblot (IB) using specific antibodies. Actin was used as loading control (e). EGFR levels normalized over actin levels were quantified by densitometric analysis using the ImageQuant software. In (f), Quantification data are expressed as mean ± SEM from $n = 4$ independent experiments and analyzed using a t-test (** $p < 0.01$)

substantially higher percentage (60.4%) of tumor exhibiting IKKε protein expression. The vast majority of breast tumors in our cohort exhibited low staining levels (IHC score: 1–3) and the detection was restricted to the cytoplasm. In contrast to previous reports showing prominent nuclear staining of IKKε in human prostate cancer and Kaposi sarcoma tumors [25, 26], only 4 out of the 93 IKKε⁺ tumors showed localization of IKKε in the nucleus. The molecular mechanisms and functional significance of the nuclear localization of IKKε still remains elusive, but would benefit from further molecular studies and larger scale correlation analyses.

Corroborating previous observations made by gene expression studies [13, 20], we failed to find an association with tumor subtypes or with ER/PR/HER2 status. However, IKKε protein expression exhibited a significant inverse association with lymph node metastasis. This result differs from the absence of correlation previously observed between *IKBKE* copy-number gain and the presence of lymph node involvement at diagnosis [13]. However, in the latter study, only 30 breast tumor specimens were analyzed and thus the lack of correlation may be due to the small number of specimens studied. The discrepancy with our study might also reflect the previous observation that IKKε levels in breast cancer does not solely results from gene amplification, but also

occurs as a result of aberrant expression due to yet to be fully characterized mechanisms [13, 14, 20]. Thus, breast tumors harboring *IKBKE* copy-number gain studied by Boehm and coll. represent only a subset of IKKε-positive tumors [13]. The inverse association between IKKε and lymph node metastasis was unexpected considering previously published molecular studies performed in cell lines, which were paradoxically suggestive of a potential association between IKKε expression and invasiveness. Indeed, silencing of IKKε or expression of a dominant negative form of IKKε in the SK-BR-3 or NF639 breast cancer cell lines resulted in a defect of cell migration and invasion abilities, two properties essential for the spreading of cancer cells and metastasis [14, 18]. Although these in vitro assays performed using single cell types provide information regarding cell autonomous mechanisms contributing to metastasis, they do not take into account the in vivo microenvironment of the tumor, which is also important in the metastatic process. Further in vivo mechanistic studies will be required to clarify the role of IKKε in the metastasis process. Additionally, larger scale IHC profiling of primary breast tumors including TNM stage classification will allow assessment of the association with distant metastases that were not evaluated in our cohort.

To the best of our knowledge our study is the first to report an association between IKKε and expression of



the EGFR marker in breast tumors. The observation that silencing of IKK ϵ in epithelial breast cancer cells significantly diminishes EGFR expression levels, suggest that the association between IKK ϵ and EGFR might result at least in part from EGFR expression levels being placed under the control of IKK ϵ -dependent signalling. EGFR is a tyrosine kinase receptor in the HER family, which is either overexpressed or mutated in breast cancer cells [27, 28] and is involved in cancer pathogenesis and progression [29]. EGFR is overexpressed in all subtypes of breast cancer, but is more frequently associated with aggressive TNBC and inflammatory breast tumors [30, 31]. Here, we did not observe an association between IKK ϵ and TNBC. Several EGFR-targeting

therapies have been developed, but have shown limited benefit and resistance has been observed, leading to the search of additional biomarkers that could be targeted simultaneously [32, 33]. Interestingly, a functional relationship between IKK ϵ and EGFR has also been described in the context of non-small lung cancer cells harboring activating EGFR mutations [34]. EGFR directly interacts with and phosphorylates IKK ϵ leading to activation of downstream Akt pathway. Silencing of IKK ϵ or treatment with the IKK ϵ inhibitor amlexanox selectively decreased cell survival, providing rational support to target IKK ϵ as a therapeutic strategy for non-small lung cancer [34]. Our observation warrants further studies to determine the functional relationship between IKK ϵ and EGFR in breast cancer. Particularly, mechanistic studies will be necessary to determine how IKK ϵ -dependent signaling cascade(s) contributes to EGFR expression and if IKK ϵ expression correlates with mutated EGFR. Alternatively, based on the positive association between IKK ϵ and EGFR expression it would also be interesting to determine whether EGFR activation is required for IKK ϵ expression in IKK ϵ ⁺/EGFR⁺. This knowledge will help determine if therapeutic strategies targeting IKK ϵ are relevant for IKK ϵ ⁺/EGFR⁺ breast tumors.

In ovarian cancer, IKK ϵ expression was found to be a relatively strong predictor of poor clinical outcome [35]. In contrary, expression of IKK ϵ and the closely related kinase TBK1 in gastric cancer was not associated with difference in survival when compared to IKK ϵ ⁻/TBK1⁻ subgroup [36]. Although Kaplan-Meier curves show a tendency of IKK⁺ subgroup to have a better OS, analysis of our cohort did not show statistically significant relationship between IKK ϵ expression and the clinical outcome. However, we cannot exclude that the absence of statistical significance could be due to a limitation of our follow-up study in term of number of patients examined and low number of events in the subgroups. Therefore, additional studies including larger cohort of patients will be required to verify the association of IKK ϵ expression with breast cancer prognosis.

Compelling evidence of the involvement of IKK ϵ in the pathophysiology of breast cancer and other diseases provided the rational for the search of therapeutic inhibitor of IKK ϵ [37]. IKK ϵ and TBK1 share an overall 65% sequence similarity and 72% identity in the kinase domain [38]. A series of dual TBK1/IKK ϵ inhibitor compounds have been identified with relative specificities amongst other kinase [37, 39–42]. Some of these compounds exhibit antiproliferative activity in breast cancer cell lines [42]. Recently, the antiallergic small molecule amlexanox was found to be a selective inhibitor of TBK1/IKK ϵ [43, 44]. Additionally, we showed that the redox-regulating compounds triphenylmethane dyes, Gentian Violet and

Brilliant Green, and nitroxide Tempol inhibit IKK ϵ , but not TBK1, expression in breast cancer cells [24]. This finding offers alternative therapeutic avenue to target IKK ϵ in breast cancer.

Conclusions

Immunohistochemical analysis of IKK ϵ expression in our cohort of primary breast tumors revealed an unexpected inverse association with lymph node metastasis and a positive association with EGFR status. Both observations advocate additional studies, including larger scale IHC profiling of primary breast tumors, to determine the functional relationship between EGFR and IKK ϵ and importantly to clarify the role of IKK ϵ in metastasis. Additionally, these studies will be essential to confirm if IKK ϵ can be used as a biomarker to define personalized prognostic and the potential of targeting IKK ϵ for therapeutic opportunities, particularly in EGFR+ breast tumors.

Abbreviations

CYLD: Cylindromatosis tumor suppressor; DDFS: Distant disease free survival; DFS: Disease-free survival; EGFR: Epidermal growth factor receptor; ER: Estrogen Receptor; FBS: Fetal bovine serum; FOXO3a: Forkhead box O 3a; HI: Heat-inactivated; IHC: Immunohistochemistry; IKK: Inhibitor of NF- κ B kinase; IRF: Interferon regulatory factor; OS: Overall survival; PR: Progesterone receptor; STAT: Signal transducer and activator of transcription; Tam: Tamoxifen; TBNC: Triple negative breast cancer cell; TMA: Tissue microarrays; TRAF: Tumor necrosis factor receptor-associated factor; WCE: Whole cell extracts

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Availability of data and materials

The data that support the findings of this study are available from the corresponding author on reasonable request.

Authors' contributions

VW and NG: conceived and designed the experiments; AA, YSP and LG were involved in TMA construction and characterization of clinicopathological parameters including biomarkers staining and scoring. VW and LG scored the IKK ϵ IHC staining. NZC performed EGFR immunoblot analysis experiments. VW, AA, NZC and NG analyzed the data; MPS carried out the statistical analyses. NG wrote the manuscript. All authors contributed edit and approved the final manuscript.

Authors' information

Not applicable.

Competing interests

The authors declare that they have no competing interests.

Consent for publication

Not applicable.

Ethics approval and consent to participate

The study was approved by the research ethics committee of the research center at the Centre Hospitalier de l'Université de Montréal (project 09-096) in accordance with the Tri-Council Policy statement on Research with Human subjects. Consents directly from patients were not required in this study as per Ethics Board guidelines.

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References

1. Siegel RL, Miller KD, Jemal A. Cancer statistics, 2016. *CA Cancer J Clin*. 2016;66(1):7–30.
2. Toss A, Cristofanilli M. Molecular characterization and targeted therapeutic approaches in breast cancer. *Breast Cancer Res*. 2015;17:60.
3. Thiery JP, Sastre-Garau X, Vincent-Salomon B, Sigal-Zafarani X, Pierga JY, Decraene C, et al. Challenges in the stratification of breast tumors for tailored therapies. *Bull Cancer*. 2006;93(8):E81–9.
4. Hanahan D, Weinberg RA. The hallmarks of cancer. *Cell*. 2000;100(1):57–70.
5. Shimada T, Kawai T, Takeda K, Matsumoto M, Inoue J, Tatsumi Y, et al. IKK-i, a novel lipopolysaccharide-inducible kinase that is related to I κ B kinases. *Int Immunol*. 1999;11(8):1357–62.
6. Peters RT, Liao SM, Maniatis T. IKKepsilon is part of a novel PMA-inducible I κ B kinase complex. *Mol Cell*. 2000;5(3):513–22.
7. Adli M, Baldwin AS. IKK-i/IKKepsilon controls constitutive, cancer cell-associated NF-kappaB activity via regulation of Ser-536 p65/RelA phosphorylation. *J Biol Chem*. 2006;281(37):26976–84.
8. Mattioli I, Geng H, Sebald A, Hodel M, Bucher C, Kracht M, et al. Inducible phosphorylation of NF-kappa B p65 at serine 468 by T cell costimulation is mediated by IKK epsilon. *J Biol Chem*. 2006;281(10):6175–83.
9. Harris J, Olieri S, Sharma S, Sun Q, Lin R, Hiscott J, et al. Nuclear accumulation of cRel following C-terminal phosphorylation by TBK1/IKKepsilon. *J Immunol*. 2006;177(4):2527–35.
10. Sharma S, tenOever BR, Grandvaux N, Zhou GP, Lin R, Hiscott J. Triggering the interferon antiviral response through an IKK-related pathway. *Science*. 2003;300(5622):1148–51.
11. Fitzgerald KA, McWhirter SM, Faia KL, Rowe DC, Latz E, Golenbock DT, et al. IKKepsilon and TBK1 are essential components of the IRF3 signaling pathway. *Nat Immunol*. 2003;4(5):491–6.
12. Tenover BR, Ng SL, Chua MA, McWhirter SM, Garcia-Sastre A, Maniatis T. Multiple functions of the IKK-related kinase IKKepsilon in interferon-mediated antiviral immunity. *Science*. 2007;315(5816):1274–8.
13. Boehm JS, Zhao JJ, Yao J, Kim SY, Firestein R, Dunn IF, et al. Integrative genomic approaches identify IKKBE as a breast cancer oncogene. *Cell*. 2007;129(6):1065–79.
14. Eddy SF, Guo S, Demicco EG, Romieu-Mourez R, Landesman-Bollag E, Seldin DC, et al. Inducible I κ B kinase/I κ B kinase epsilon expression is induced by CK2 and promotes aberrant nuclear factor-kappaB activation in breast cancer cells. *Cancer Res*. 2005;65(24):11375–83.
15. Hutti JE, Shen RR, Abbott DW, Zhou AY, Spratt KM, Asara JM, et al. Phosphorylation of the tumor suppressor CYLD by the breast cancer oncogene IKKepsilon promotes cell transformation. *Mol Cell*. 2009;34(4):461–72.

16. Shen RR, Zhou AY, Kim E, Lim E, Habelhah H, Hahn WC. IkkappaB kinase epsilon phosphorylates TRAF2 to promote mammary epithelial cell transformation. *Mol Cell Biol*. 2012;32(23):4756–68.
17. Guo JP, Tian W, Shu S, Xin Y, Shou C, Cheng JQ. IKKε phosphorylation and inhibition of FOXO3a: a mechanism of IKKε oncogenic function. *PLoS One*. 2013;8(5):e63636.
18. Qin B, Cheng K. Silencing of the IKKε gene by siRNA inhibits invasiveness and growth of breast cancer cells. *Breast Cancer Res*. 2010;12(5):R74.
19. Guo JP, Coppola D, Cheng JQ. IKKε protein activates Akt independent of phosphatidylinositol 3-kinase/PDK1/mTORC2 and the pleckstrin homology domain to sustain malignant transformation. *J Biol Chem*. 2011;286(43):37389–98.
20. Barbie TU, Alexe G, Aref AR, Li S, Zhu Z, Zhang X, et al. Targeting an IKKε cytokine network impairs triple-negative breast cancer growth. *J Clin Invest*. 2014;124(12):5411–23.
21. Guo JP, Shu SK, Esposito NN, Coppola D, Koomen JM, Cheng JQ. IKKε phosphorylation of estrogen receptor alpha Ser-167 and contribution to tamoxifen resistance in breast cancer. *J Biol Chem*. 2010;285(6):3676–84.
22. Demers M, Rose AA, Grosset AA, Biron-Pain K, Gaboury L, Siegel PM, et al. Overexpression of galectin-7, a myoepithelial cell marker, enhances spontaneous metastasis of breast cancer cells. *Am J Pathol*. 2010;176(6):3023–31.
23. Grosset AA, Labrie M, Vladoiu MC, Yousef EM, Gaboury L, St-Pierre Y. Galectin signatures contribute to the heterogeneity of breast cancer and provide new prognostic information and therapeutic targets. *Oncotarget*. 2016;7(14):18183–203.
24. Mukawera E, Chartier S, Williams V, Pagano PJ, Lapointe R, Grandvaux N. Redox-modulating agents target NOX2-dependent IKKε oncogenic kinase expression and proliferation in human breast cancer cell lines. *Redox Biol*. 2015;6:9–18.
25. Peant B, Forest V, Trudeau V, Latour M, Mes-Masson AM, Saad F. IkkappaB-kinase-epsilon (IKKε/IKKi/IkkappaBepsilon) expression and localization in prostate cancer tissues. *Prostate*. 2011;71(10):1131–8.
26. Wang Y, Lu X, Zhu L, Shen Y, Chengedza S, Feng H, et al. IKK epsilon kinase is crucial for viral G protein-coupled receptor tumorigenesis. *Proc Natl Acad Sci U S A*. 2013;110(27):11139–44.
27. Ro J, North SM, Gallick GE, Hortobagyi GN, Gutterman JU, Blick M. Amplified and overexpressed epidermal growth factor receptor gene in uncultured primary human breast carcinoma. *Cancer Res*. 1988;48(1):161–4.
28. Weber F, Fukino K, Sawada T, Williams N, Sweet K, Brena RM, et al. Variability in organ-specific EGFR mutational spectra in tumour epithelium and stroma may be the biological basis for differential responses to tyrosine kinase inhibitors. *Br J Cancer*. 2005;92(10):1922–6.
29. Normanno N, De Luca A, Bianco C, Strizzi L, Mancino M, Maiello MR, et al. Epidermal growth factor receptor (EGFR) signaling in cancer. *Gene*. 2006;366(1):2–16.
30. Guerin M, Gabillot M, Mathieu MC, Travagli JP, Spielmann M, Andrieu N, et al. Structure and expression of c-erbB-2 and EGF receptor genes in inflammatory and non-inflammatory breast cancer: prognostic significance. *Int J Cancer*. 1989;43(2):201–8.
31. Tischkowitz M, Brunet JS, Begin LR, Huntsman DG, Cheang MC, Akslen LA, et al. Use of immunohistochemical markers can refine prognosis in triple negative breast cancer. *BMC Cancer*. 2007;7:134.
32. Lluch A, Eroles P, Perez-Fidalgo JA. Emerging EGFR antagonists for breast cancer. *Expert opinion on emerging drugs*. 2014;19(2):165–81.
33. Masuda H, Zhang D, Bartholomeusz C, Doihara H, Hortobagyi GN, Ueno NT. Role of epidermal growth factor receptor in breast cancer. *Breast Cancer Res Treat*. 2012;136(2):331–45.
34. Challa S, Guo JP, Ding X, Xu CX, Li Y, Kim D, et al. IKKε is a substrate of EGFR and a therapeutic target in non-small cell lung cancer with activating mutations of EGFR. *Cancer Res*. 2016;76(15):4418–29.
35. Guo JP, Shu SK, He L, Lee YC, Kruk PA, Grenman S, et al. Deregulation of IKKε is associated with tumor progression, poor prognosis, and cisplatin resistance in ovarian cancer. *Am J Pathol*. 2009;175(1):324–33.
36. Lee SE, Hong M, Cho J, Lee J, Kim KM. IKKε and TBK1 expression in gastric cancer. *Oncotarget*. 2016;
37. Niederberger E, Moser CV, Kynast KL, Geisslinger G. The non-canonical IkkappaB kinases IKKε and TBK1 as potential targets for the development of novel therapeutic drugs. *Curr Mol Med*. 2013;13(7):1089–97.
38. Grandvaux N. The IKKε kinase in breast cancer: from oncogenesis to treatment resistance. *Médecine/Sciences*. 2011;27:619–25.
39. Clark K, Peggie M, Plater L, Sorcek RJ, Young ER, Madwed JB, et al. Novel cross-talk within the IKK family controls innate immunity. *Biochem J*. 2011;434(1):93–104.
40. McIver EG, Bryans J, Birchall K, Chugh J, Drake T, Lewis SJ, et al. Synthesis and structure-activity relationships of a novel series of pyrimidines as potent inhibitors of TBK1/IKKε kinases. *Bioorg Med Chem Lett*. 2012;22(23):7169–73.
41. Deng T, Liu JC, Chung PE, Uehling D, Aman A, Joseph B, et al. shRNA kinome screen identifies TBK1 as a therapeutic target for HER2+ breast cancer. *Cancer Res*. 2014;74(7):2119–30.
42. Li J, Huang J, Jeong JH, Park SJ, Wei R, Peng J, et al. Selective TBK1/IKKi dual inhibitors with anticancer potency. *Int J Cancer*. 2014;134(8):1972–80.
43. Makino H, Saijo T, Ashida Y, Kuriki H, Maki Y. Mechanism of action of an antiallergic agent, amlexanox (AA-673), in inhibiting histamine release from mast cells. Acceleration of cAMP generation and inhibition of phosphodiesterase. *Int Arch Allergy Appl Immunol*. 1987;82(1):66–71.
44. Reilly SM, Chiang SH, Decker SJ, Chang L, Uhm M, Larsen MJ, et al. An inhibitor of the protein kinases TBK1 and IKK-ε improves obesity-related metabolic dysfunctions in mice. *Nat Med*. 2013;19(3):313–21.