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High IKKα expression is associated with reduced time to recurrence and cancer specific survival in oestrogen receptor (ER)-positive breast cancer.

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Novelty and Impact

Results from the present study support a role for IKKα in the progression of ER-positive breast cancer. For the first time this study demonstrates that increased levels of IKKα are associated with reduced recurrence-free survival on tamoxifen and reduced cancer specific survival. Furthermore, in ER-positive cells reduction of IKKα induced apoptosis and decreased cell viability. This study provides for the first time, evidence for IKKα as a possible target in endocrine treated breast cancer.

Abstract

The aim of the current study was to examine the relationship between tumour IKKα expression and breast cancer recurrence and survival. Immunohistochemistry was employed in a discovery and a validation tissue microarray to assess the association of tumour IKKα expression and clinico-pathological characteristics. Following siRNA-mediated silencing of IKKα, cell viability and apoptosis were assessed in MCF7 and MDA-MB-231 breast cancer cells. In both the discovery and validation cohorts, associations observed between IKKα and clinical outcome measures were potentiated in oestrogen receptor (ER) positive Luminal A tumours. In the discovery cohort, cytoplasmic IKKα was associated with disease-free survival (P=0.029) and recurrence-free survival on tamoxifen (P<0.001) in Luminal A tumours. Nuclear IKKα and a combination of cytoplasmic and nuclear IKKα (total tumour cell IKKα) were associated with cancer-specific survival (P=0.012 and P=0.007, respectively) and recurrence-free survival on tamoxifen (P=0.013 and P<0.001, respectively) in Luminal A tumours. In the validation cohort, cytoplasmic IKKα was associated with cancer-specific survival (P=0.023), disease-free survival (P=0.002) and recurrence-free survival on tamoxifen (P=0.009) in Luminal A tumours. Parallel experiment with breast cancer cells *in vitro* demonstrated the non-canonical NF-κB pathway was inducible by exposure to lymphotoxin in ER-positive MCF7 cells and not in ER-negative MDA-MB-231 cells. Reduction in IKKα expression by siRNA transfection increased levels of apoptosis and reduced cell viability in MCF7 but not in MDA-MB-231 cells. IKKα is an important determinant of poor outcome in patients with ER-positive invasive ductal breast cancer and thus may represent a potential therapeutic target.

Introduction

Breast cancer is the most common female cancer in the UK and remains the second most common cause of cancer death in women despite earlier detection and improved treatments. Oestrogen receptor (ER)-positive tumours can be effectively treated with tamoxifen and other endocrine therapies, however endocrine resistance remains a major clinical problem [1]. The Gap Analysis Working Group identified molecular mechanisms driving resistance to treatment as one of the top 10 gaps in research that if addressed would make the most clinical impact [2]. Studies have demonstrated that 30% of patients with Luminal A disease and 90% of patients with Luminal B exhibit high recurrence scores [3, 4].

The Nuclear Factor kappa B (NF-κB) family of transcription factors has five members (p105/50 (NF-κB1), p100/52 (NF-κB2), p65 RELA, RelB, c-REL) involved in two main pathways, the canonical and non-canonical pathways [5]. Upon activation of the non-canonical NF-κB pathway, the NF-κB-inducing kinase (NIK) phosphorylates IKKα which in turn phosphorylates p100 to identify it for ubiquitination and targeted proteolytic processing subsequently generating p52 and allowing the liberation of active p52/RelB dimers [6]. These complexes translocate to the nucleus and regulate transcription of a variety of genes important in apoptosis, proliferation, invasion and adaptive immunity [7-9]. Therefore it is not surprising that studies have demonstrated dysregulation of the non-canonical NF-κB pathway in many solid tumours [10, 11]. More recently the non-canonical NF-κB pathway has been implicated in the development and progression of breast cancer [12]. Oestrogen inhibits the Notch pathway and application of anti-oestrogens result in the activation of Notch [13] and the kinase activity of IKKα has been found to be associated with Notch in the activation of ER-dependent genes [14]. In mice a delay in mammary gland development is observed when p100/52 is overexpressed and these mice developed multiple tumours [15], however other information is lacking. The current study aims to assess if members of the non-canonical

NF-κB pathway are associated with clinical outcome measures in breast cancer patients.

Materials and methods

Patient cohorts

This retrospective study used previously constructed tissue microarrays (TMAs) made from formalin-fixed paraffin-embedded tissue (FFPE) blocks retrieved from pathology archives. A consultant pathologist identified tumour rich areas, and three different 0.6mm^2 cores were lifted from these areas and placed into new blocks. Ethical approval for the use of this tissue was granted by the Research Ethics Committee of the North Glasgow University Hospitals NHS Trust (NHS GG&C rec no 10/50704/60).

The discovery cohort TMA included 362 breast cancer patients presenting with invasive ductal breast cancer in the West of Scotland (at Glasgow Royal Infirmary, Glasgow Western Infirmary and Stobhill Hospital), between 1995 and 1998. Patients were excluded if follow-up was incomplete or tumour tissue was insufficient. Clinico-pathological data available included age, tumour grade, tumour size, lymph node status, therapy, ER, PgR and HER2 status and Ki67 proliferation index. Information on inflammatory infiltrate and tumour microenvironment had previously been established for the cohort [16-28].

IHC for IKKα was performed on a validation cohort of ER-positive patients. This TMA and clinical database included 266 ER-positive patients presenting with invasive ductal breast cancer between 1980 and 1995 from Glasgow Royal Infirmary. Clinico-pathological data available included age, tumour grade, tumour size, lymph node status, therapy, PgR and HER2 status and Ki67 proliferation index [29-32]. All patients in this cohort were treated with adjuvant tamoxifen.

Immunohistochemistry

Immunohistochemistry (IHC) was performed to assess protein levels of RelB, NIK and IKKα; components of the non-canonical NF-κB pathway. TMA sections (2.5µm thick) were dewaxed by immersion in xylene and rehydrated through a series of graded alcohols. Heat

induced antigen retrieval was performed in a solution of citrate buffer pH6. Tissue was then incubated in 3% (v/v) hydrogen peroxide before non-specific binding was blocked by incubation in either 5% (v/v) normal horse serum solution (Vector Laboratories; IKKα, RelB) or 1x caesin solution (Vector Laboratories; NIK). Slides were then incubated in primary antibody overnight at 4°C at optimal concentration of antibody diluted in antibody diluent (Dako). The primary antibodies and concentrations used are as follows: anti-RelB (#4954, Cell Signaling) was used at 1:75, anti-NIK (S2622, Epitomics) at 1:250, and anti-IKKα (GWB-662250, Genway) at 1:1000. Staining was developed using EnVision™ (Dako) and 3,3'-diaminobenzidine (DAB, Vector Laboratories). Harris Haematoxylin counterstaining was performed and tissue was dehydrated and mounted using DPX. Supplementary Figure 1 provides examples of high and low IKKα staining. Antibodies were validated by western blotting and IHC, for the anti- IKKα antibody, a single band of the predicted molecular weight (85kDa) was observed on a western blot. Cells were pre-treated with 200 nM siRNA against either IKKα or IKKβ to check specificity for IKKα. A reduction in expression was observed in IKKα silenced but not IKKβ silenced cells as assessed by western blotting of cell lysates and IHC of cell pellets (supplementary figure 2). Stained TMA sections were scanned using a Hamamatsu NanoZoomer (Welwyn Garden City, Hertfordshire, UK) at x20 magnification and visualization was carried out using Slidepath Digital Image Hub, version 4.0.1 (Slidepath, Leica Biosystems, Milton Keynes, UK). Protein expression was assessed using the weighted histoscore method [LB], with a second independent observer [JE] scoring 10% of cores and the interclass corRelation coefficient (ICCC) calculated to ensure no observer bias [33]. Cytoplasmic and nuclear expression were calculated separately.

Cell culture

MCF7 (ER-positive) and MDA-MB-231 (ER-negative) breast cancer cells were routinely

cultured in 5% CO_2 at 37°C in Dulbecco's Modified Eagle Medium (DMEM, Life Technologies) with 10% (v/v) Fetal Bovine Serum (FBS, Sigma-Aldrich), 10 Units/ml Penicillin/Streptomycin (Life Technologies) and 1x GlutaMAX™ (Life Technologies).

Ligand exposure

Cells were seeded in 12 well plates at $1x10^5$ cells per well and once 70% confluent were rendered quiescent by incubation in serum free media for 24hours and exposed to 20ng/ml lymphotoxin $\alpha_1\beta_2$ (LTx, Sigma-Aldrich) for 4 hours, 8 hours and 24 hours to stimulate the non-canonical pathway.

Western blotting

After exposure to the LTx or siRNA, cells were lysed in pre-heated Laemmli's sample buffer and SDS-Polyacrylamide Gel Electrophoresis (SDS-PAGE) was performed using resolving gels. Proteins were then transferred to nitrocellulose membranes by electrophoretic blotting in wet conditions and non-specific binding blocked by incubation in a solution of 3% (w/v) BSA in NaTT buffer. Membranes were incubated overnight, either at room temperature or 4°C, in primary antibody specific to the target protein diluted to optimal concentration in NaTT buffer containing 0.3% (w/v) BSA. Antibodies and dilutions used were as follows: IKKα (1:3500, OP133, Calbiochem), p100/52 (1:3000, 05-361, Millipore), phospho-p100 (1:2000, #4810, Cell Signaling). β-tubulin was used (1:5000, ab21058, Abcam) as a loading control.

Membranes were washed for 1h 30min with NaTT, changed every 15 min, and then were incubated in secondary HRP-conjugated antibody (either rabbit or mouse, depending on primary antibody) diluted 1:10000 for 1h 30min and then washed again as per previous. Enhanced chemiluminescence (ECL) reagent (1:1 mixture of solution 1 [1M Tris pH8.5, 250mM luminol, 250mM p-cymuric acid and water] and solution 2 [1M Tris pH8.5, 0.19% H_2O_2 and water]) was used to develop and X-ray films developed using an X-OMAT

machine (Kodak) or the SynGene imaging system. Images were quantified using ImageJ with protein expression normalised to the β-tubulin loading control and expression compared to untreated cells, calculated as fold change to control. Statistical analysis was performed using a one-way ANOVA with Bonferroni correction and Dunnett's test to compare to control levels.

siRNA-mediated Silencing of IKKα

In order to observe the impact of loss of cellular IKKα expression on cellular viability, MCF7 and MDA-MB-231 cells were transfected with ON-TARGETplus siRNA (Thermo Scientific) against sequences for IKKα (*CHUK,* #J-003473-09) and non-targeting sequence (NT) was used as a negative control (Non-targeting #1, #D-001810-01-20). Lipofectamine® RNAiMAX (Life Technologies) diluted in Opti-MEM® (Life Technologies) was used to deliver the siRNA to the cells. After 6 hours media was replaced with normal DMEM containing 10% (v/v) FCS and after 48 hours protein lysis and analysis via Western blotting was performed, or a functional assay was applied to assess apoptosis or viability.

Apoptosis and cell viability assays

We employed the use of RNA interference to observe the effect a decrease in expression of IKKα had on cell growth and viability in MCF7 and MDA-MB-231 cells. MCF7 and MDA-MB-231 cells were transfected with 200nM NT siRNA or 200nM IKKα siRNA. These cells were then seeded for use in apoptotic assays, wst-1 assays and western blotting. The western blot was used to ensure a decrease in expression of IKKα had been achieved in the cells used for the apoptotic and cell viability assays. Forty eight hours post treatment, apoptotic and cell viability assays were performed and cells lysed for western blot analysis. This method ensured that the cells used in both the assays were from the same population. Cells were assessed for apoptosis levels using a Cell Death Detection enzyme-linked immunosorbent

assay kit (Roche) and for cell viability using the water-soluble tetrazolium salt (WST-1) reagent (Roche). Cells were seeded in 96 well plates at a density of $5x10^3$ cells/well in 100μL of standard culture medium. Once a confluency of 60-70% was reached cells were exposed to siRNA for 48 hours and the assays then performed following manufacturer's instructions. Assays were performed in triplicate and error bars representing standard deviation added to graphs.

Cell viability via xCELLigence

The xCELLigence machine (ACEA Biosciences, San Diego) was used to display cell growth and viability in real time following silencing of IKKα. With this method, measurements are continuously sent to the computer, allowing for real time growth curves to be plotted using "Cell Index" which represents the number and viability of the cells. Cells were seeded in a 96 well *E-plate*™ (ACEA Biosciences, San Diego) at $3x10^3$ cells/well with 200μl of media in each well, grown for two days to ensure log phase growth before treatment with siRNA and after 72 hours graphs showing cell index over time were drawn.

Taqman low density arrays (TLDA)

As the biggest effect on phenotypic output was observed for apoptosis in ER-positive MCF7 cells, 384 TLDA gene signature apoptosis arrays containing 93 genes Related to apoptosis and 3 candidate endogenous controls were employed to investigate the change in gene profile following siRNA-mediated silencing (NT or IKK α). After 48 hour silencing incubation, the media was replaced with serum free media for 12 hours to rendered quiescent followed by LTx stimulation for 12 hours. Thus, the total time for silencing and LTx treatment was 72 hours. RNA was extracted using an RNeasy mini kit following manufacturer's protocol with DNase digestion performed on the column (Qiagen). Preparation of cDNA and RT-PCR were performed using TaqMan RT-PCR methodology and reagents (Perkin-Elmer Applied Biosystems). TLDA gene arrays containing 93 genes

and 3 candidate endogenous controls were employed to determine if IKKα silencing induced change in expression of genes (supplementary Table 1). Each sample from 3 independent silencing experiments on MCF7 cells was run in duplicate. All results were analyzed simultaneously by RQ Manager Software (ABI, UK) and the threshold cycle (Ct) values for the genes of interest were calibrated against 18S Ct (dCt).

Statistical Analysis

Statistics were performed using IBM SPSS version 21. Kaplan-Meier curves were constructed for cancer-specific survival and the log rank test was employed to compare high and low expression. Hazard ratios were calculated using Cox regression with 95% confidence intervals. Cox regression multivariate analysis was also performed with the inclusion of known predictive factors. Inter-Relationships between variables were assessed using contingency tables with the chi-squared test for trend as appropriate. Values of $P<0.05$ were considered statistically significant. Statistical analysis for Western blots, apoptosis and cell viability assays was performed using a one-way ANOVA with Bonferroni correction and Dunnett's test. P values were considered significant if $P<0.05$ and highly significant if $P<0.001$. TLDA card raw data were analysed using Quantstudio 7 with the following settings: 1) automatic baseline 2) threshold = 0.2. Ct values over 32 were defined as undetectable. Significant changes in gene expression were defined with a P-value boundary of 0.05. Results are presented as a volcano plot.

Results

IKKα expression is associated with poor prognosis in breast cancer

Discovery cohort:

A total of 362 patients who presented with invasive ductal breast cancer were included in the study. ER, PgR, HER2 and Ki67 status were available allowing determination of the tumour subtype, 46% (165) of patients had Luminal A disease (ER or PgR-positive, HER2-negative and low Ki67 <14%), 22% (81) had Luminal B disease (ER- or PgR- and HER2-positive or ER, PgR and high Ki67 (>14%)), 20% (72) had triple negative disease (ER-, PgR- and HER2-negative) and 10% (38) had HER2 enriched (ER- and PgR-negative, HER2-positive). We were unable to define subtype in 2% (6) of cases due to missing data.

Expression of IKKα was observed in the tumour cell cytoplasm and nucleus (median expression 100, interquartile range 69-140 and median expression 126, interquartile range 73-115, respectively). NIK expression was observed in the tumour cell cytoplasm (median expression 100, interquartile range 69-140) and RelB expression was observed in the tumour cytoplasm and the nucleus (median expression 113, interquartile range 90-146 and median expression 0, interquartile range 0-7, range 0-190, respectively). No correlations were observed between NIK expression and expression of IKKα in any cellular location investigated. Expression of cytoplasmic IKKα correlated with cytoplasmic and total tumour cell RelB (P=0.005 and P=0.003, respectively), nuclear IKKα correlated with nuclear and total tumour cell RelB (P<0.001 and P<0.001, respectively) and total tumour cell IKKα correlated with cytoplasmic, nuclear and total tumour cell RelB expression (P=0.010, P=0.004 and P<0.001, respectively).

Eighty four patients had local or distant recurrence, the median follow-up of survivors was 164 months with 76 cancer-associated deaths and 77 non-cancer deaths. Expression of NIK or RelB was not associated with cancer-specific survival, disease-free survival or recurrence-

free survival on tamoxifen, in the full patient cohort or when stratified by tumour subtype (Table 1 and Table 2).

High cytoplasmic IKKα was not associated with cancer-specific survival in the full cohort (Table 1), however was significantly associated with shorter disease-free survival (Table 1)(P=0.045) and recurrence-free survival on tamoxifen (Table 1)(P=0.003). 5 year disease-free survival was reduced from 88% (low) to 79% (high) (P=0.039) and recurrence-free survival on tamoxifen was reduced from 73% (low) to 25% (high) (P=0.019).

When stratified by tumour subtype, high cytoplasmic IKKα expression showed a non-significant trend towards shorter cancer-specific survival (Figure 1A; P=0.076), a significant association with shorter disease-free survival (Figure 1B; P=0.029) and a significant association with shorter recurrence-free survival on tamoxifen (Figure 1C; P<0.001) in Luminal A tumours. 5 year disease-free survival was reduced from 99% (low) to 89% (high) (P=0.011) and recurrence-free survival on tamoxifen was reduced from 94% (low) to 30% (high) (P<0.001).

The relationship between cytoplasmic IKKα and tumour characteristics was examined. High expression of cytoplasmic IKKα was associated with tumour size (P=0.032), tumour grade (P=0.009), ER-positive disease (P=0.002), HER2-positive disease (P=0.023) and tumour subtype (P<0.001). When associations with the tumour microenvironment were investigated, cytoplasmic IKKα was not significantly associated with tumour stroma percentage (TSP) or tumour budding. However an association was observed with necrosis (P=0.009) and local inflammatory cell infiltrate as assessed by Klintrup-Makinen grade (P=0.005).

High nuclear IKKα was not associated with cancer-specific survival, disease-free survival or recurrence-free survival on tamoxifen in the full cohort (Table 1). However when stratified by tumour subtype, high nuclear IKKα expression showed a significant association with

shorter cancer-specific survival (Figure 1D; P=0.012), a non-significant trend towards shorter disease-free survival (Figure 1E; P=0.066) and a significant association with recurrence-free survival on tamoxifen (Figure 1F; P=0.013) in Luminal A tumours. 10 year cancer-specific survival was reduced from 97% (low) to 87% (high) (P=0.013) and recurrence-free survival on tamoxifen was reduced from 100% (low) to 55% (high) (P=0.015).

The relationship between nuclear IKKα and tumour characteristics was examined. High expression of nuclear IKKα was associated with tumour grade (P=0.014), ER-positive disease (P=0.047), tumour subtype (P=0.042) and patient survival (P=0.008). When associations with the tumour microenvironment were investigated, nuclear IKKα was not significantly associated with TSP or tumour budding. However an association was observed with local inflammatory cell infiltrate as assessed by Klintrup-Makinen grade (P=0.016) and density of $CD8^{+}$ T-lymphocytes (P=0.035).

To examine the relationship of cancer-specific survival and total tumour cell expression of IKKα, a cumulative prognostic score of cytoplasmic and nuclear IKKα was examined (total tumour cell IKKα). Patients with both high cytoplasmic and nuclear expression were classified as the high expression group and patients with either low cytoplasmic or nuclear expression were classified as the low expression group. High expression of total tumour cell IKKα, was not associated with shorter cancer-specific survival or disease-free survival in the full cohort, but was associated with recurrence-free survival on tamoxifen (Table 1; P=0.011). Recurrence-free survival on tamoxifen was reduced from 79% (low) to 54% (high) (P=0.029).

When stratified by tumour subtype, high total tumour cell IKKα expression showed a significant association with shorter cancer-specific survival (Figure 1G; P=0.007), shorter disease-free survival (Figure 1H; P=0.013) and recurrence-free survival on tamoxifen

(Figure 1I)(P<0.001) in Luminal A tumours. 10 year cancer-specific survival was reduced from 96% (low) to 83% (high) (P=0.006), 5 year disease-free survival was reduced from 99% (low) to 86% (high)(P=0.001) and recurrence-free survival on tamoxifen was reduced from 94% (low) to 30% (high) (p<0.001).

High expression of total tumour cell IKKα was only associated with density of $CD8^{+}$ T-lymphocytes (P=0.034) and not with any other tumour or microenvironment characteristics.

In Luminal A tumours, when cytoplasmic, nuclear and total tumour cell IKKα were entered into a multivariate model using a backwards conditional method with clinic-pathological parameters, only nuclear IKKα remained independently associated with cancer-specific survival (HR=9.4, 95% CI=2.02-22.67, P=0.004, Table 2).

Validation cohort:

As the strongest associations were observed between IKKα and clinical outcome measures in patients with Luminal A tumours in the discovery cohort, this was investigated in an independent ER-positive validation cohort. A total of 266 patients who presented with ER-positive invasive ductal breast cancer were included in the study. ER, PgR, HER2 and Ki67 status were available for these patients allowing us to determine the tumour subtype. 76% (201) of patients had Luminal A disease and 21% (56) had Luminal B disease. We were unable to define subtype in 3% (9) of cases due to missing data.

Expression of IKKα was observed in the tumour cell cytoplasm within a similar range to that observed for the ER-positive tumours in the discovery cohort (discovery cohort: median expression, 96 interquartile range 61-130, validation cohort: median expression 96, interquartile range 73-120). However expression in the nucleus between the cohorts was not similar (discovery cohort: median expression 133, interquartile range 80-180, validation cohort: median expression 33, interquartile range 15-60).

Seventy four patients had local or distant recurrences, the median follow-up of survivors was 96 months with 69 cancer-associated deaths and 58 non-cancer deaths. As was previously observed in discovery cohort, high cytoplasmic IKKα was not associated with cancer-specific survival and was significantly associated with shorter disease-free survival (Figure 2A; P=0.024) and recurrence-free survival on tamoxifen (Figure 2B; P=0.038) in the validation cohort.

When stratified by tumour subtype, high cytoplasmic expression of IKKα showed a significant association with shorter cancer-specific survival (Figure 2C; P=0.023), shorter disease-free survival (Figure 2D; P=0.002) and recurrence-free survival on tamoxifen (Figure 2E; P=0.009) in Luminal A tumours. 10 year cancer-specific survival was reduced from 80% (low) to 62% (high) (P=0.005), 5 year disease-free survival was reduced from 87% (low) to 64% (high) (P=0.004) and recurrence-free survival on tamoxifen was reduced from 84%(low) to 66% (high) (P=0.027).

High expression of cytoplasmic IKKα was associated with tumour grade (P=0.009) as previously observed for discovery cohort. We were unable to assess associations with IKKα and the tumour microenvironment as these parameters were not available for analysis for the validation cohort.

In Luminal A tumours, when cytoplasmic IKKα was entered into a multivariate model using a backwards conditional method with size, grade and nodal status, parameters, cytoplasmic IKKα remained independently associated with cancer-specific survival (HR=2.03, 95% CI=1.06-3.87, P=0.031).

In the validation cohort nuclear IKKα and total tumour cell IKKα were not associated with cancer-specific survival, disease-free survival or recurrence-free survival on tamoxifen in the full cohort or in Luminal A tumours.

IKKα silencing is associated with reduced cell viability and induction of apoptosis in ER-positive breast cancer cells

We observed that IKKα expression is associated with poor prognosis in breast cancer patients, and this is potentiated in the ER-positive Luminal A subtype. Therefore the next aim of the study was to establish if IKKα could offer a novel therapeutic target for treatment of breast cancer. MCF7 cells were chosen to represent ER-positive breast tumours and MDA-MB-231 were chosen to represent ER-negative breast tumours.

Both cell lines were treated with lymphotoxin (LTx), a ligand known to stimulate the non-canonical NF-κB pathway. In ER-positive MCF7 cells LTx significantly increased p52 expression on average 2.1 fold after 24 hours (Figure 3A)(P=0.049). An increase in p100 phosphorylation was clearer with an increase at 8 hours LTx exposure (Figure 3A) (P=0.024) and peak expression at 24 hours exposure with an average 8.8 fold increase compared to untreated MCF7 cells (Figure 3A) (P=0.005).

In ER-negative MDA-MB-231 cells constitutive expression of phospho-p100 was observed, with little difference between control and cells exposed to LTx (Figure 3A). Additionally, no significant increase in p52 was evident with LTx exposure at any time point (Figure 3A).

Using a lipofection based method, siRNA was delivered to the cells to reduce expression of IKKα. After 48 hours incubation siRNA targeted to IKKα effectively reduced expression of IKKα at all concentrations in both cell lines (Figure 3B).

MCF7 and MDA-MB-231 cells were transfected with 200nM IKKα siRNA, as well as non-targeting (NT) siRNA, and 48 hours post treatment the apoptosis assay was performed. Serum starvation, which induces apoptosis, was used as an additional control. ER-positive MCF7 cells treated with NT siRNA did not show a significant difference in apoptosis when compared to untreated control cells (Figure 3C) (P=0.992). A significant increase was observed in levels of apoptosis when cells were treated with siRNA to silence expression of

IKKα (Figure 3C) ($P=2.5x10^{-5}$). ER-negative MDA-MB-231 cells treated with either NT or IKKα siRNA did not show a significant difference in apoptosis when compared to untreated control cells (Figure 3C) (both P=1.0).

Cell viability was also assessed in both MCF7 and MDA-MB-231 cells following siRNA-mediated silencing of IKKα for 48 hours. In MCF7 cells treated with NT siRNA, cell viability was not significantly different to untreated control cells (Figure 4A; P=0.550). A decrease in cell viability was observed when cells were treated with siRNA to silence expression of IKKα (Figure 4A; P=0.001). In MDA-MB-231 cells, cell viability was not significantly different in cells treated with NT or IKKα siRNA in comparison to untreated control cells (Figure 4A; P=0.952 and P=0.889, respectively).

Cell viability was also measured using the xCELLigence platform. In MCF7 ER-positive cells NT siRNA did not appear to be different to that observed for untreated cells but siRNA to silence expression of IKKα reduced cell viability (Figure 4B). In MDA-MB-231 cells neither NT nor IKKα siRNA displayed any difference in cell viability compared to untreated cells (Figure 4B).

As the associations with cell viability were only observed in ER-positive MCF7 cells and the strongest association being for apoptosis, TLDA apoptotic signature gene cards were employed to examining changes in gene expression as a result of IKKα silencing in these cells. The results are displayed as a volcano plot (Figure 4C). Fourteen genes induced by LTx treatment were noted to change significantly in response to IKKα siRNA (CHUK, BAK1, BBC 3, CRADD, BCL 10, RIPK2, TNFSF10, FAS, BIRC3, CASP8, NFKBIA, CASP3, CFLAR and TNFRSF2) (Figure 4C). CHUK (IKKα) was used as an internal control to demonstrate that silencing had been effective. The pro-apoptotic genes BAK and BBC3 showed the greatest fold change increase across the 3 experiments.

Discussion

In the current study immunohistochemistry was employed to assess expression/levels of NIK, RelB and IKKα in a discovery cohort and establish associations with clinico-pathologic parameters. NIK and RelB were not associated with cancer-specific survival, disease-free survival or recurrence-free survival on tamoxifen in the full cohort or when stratified by tumour subtype as determined by ER, Pgr, HER2 and Ki67 status. Breast cancer can be stratified into 4 molecular subtypes (Luminal A, Luminal B, triple negative and HER2 enriched) bases on gene profiling, however it is now widely accepted that tumours can now be classified into tumour subtypes using a panel of 4 biomarkers (ER, PgR, Ki67 and HER2), and although not true representatives of molecular subtype, do represent tumour subtypes with different patient prognosis, therefore in this study although when tumour subtype is referred to it is on the basis of ER, PgR, HER2 and Ki67 status and not molecular subtypes as defined by gene profiling.

In the current study although NIK and RelB are not associated with patient recurrence or survival, in contrast levels of IKKα expression were associated with disease-free survival in the full cohort and when the cohort was stratified by subtype. Associations between patient outcome measures were completely negated in the Luminal B subtype, but potentiated in the ER-positive Luminal A subtype. Luminal A subtype is the most common subtype in this patient cohort, therefore it could be possible that this observation is due to the Luminal A group being the only appropriately powered subtype in this cohort. However no trends were observed for patients with Luminal B, triple negative or HER2 enriched subtypes, providing weight to the argument that the associations observed were only observed in patients with Luminal A tumours.

As it is only IKKα that is associated with patient outcome and not expression levels of NIK or RelB, it is probable that activation status of NIK and RelB are required to be measured to

demonstrate activation of the pathway, however as there are inherent problems associated with phosphorylated antibodies we propose IKKα is a more robust marker to be employed. An alternative explanation is IKKα has a role independent of the non canonical pathway, however the current study has no conclusive evidence to support this theory. Recently work in colorectal cancer and cutaneous squamous cell carcinoma has demonstrated a role for IKKα independent of the non-canonical NF-□B pathway [10, 34-36] and this been suggested to be via a truncate form of IKKα (p45 IKKα) that is constitutively active and specifically resides in the nucleus [10]. In the discovery cohort, when Luminal A tumours were considered only nuclear IKKα remained independently associated with cancer-specific survival. This suggests that nuclear IKKα expression had a stronger predictive power than total tumour cell IKKα, and could be due to detection of the truncated activated form of IKKα. However, this observation was not upheld in the validation cohort, as it was only cytoplasmic IKKα that remained associated with prognosis, suggesting it is more likely that cytoplasmic IKKα is a better marker of non canonical pathway activation, and nuclear truncated IKKα does not play a strong role.

Taken in combination, these results suggest irrespective of cellular location that IKKα plays an important role in the progression of breast cancer, as cytoplasmic, nuclear and total tumour cell expression of IKKα were all associated with clinical outcome measures in patients with Luminal A disease.

Due to the associations of IKKα expression with clinical outcome measures in patients with Luminal A disease, IKKα was assessed in a second validation cohort, of 266 women with ER-positive breast cancers, all of whom received tamoxifen therapy. This cohort, provided greater power for investigating associations with recurrence-free survival on tamoxifen. In the validation cohort, cytoplasmic IKKα expression levels were observed at very similar levels to that of the discovery cohort. However nuclear IKKα expression in the validation

cohort was at a much lower level than that of the discovery cohort. The reason for this was unclear as the same antibody and immunohistochemical protocol were employed across both studies. One possible explanation is that the second cohort was older therefore may have been subject to different fixation methods or storage or that nuclear IKKα is more prone to degradation than cytoplasmic IKKα. As a result of the differing nuclear expression levels, the same cut offs for nuclear IKKα to determine high and low expression could not be employed across both cohorts. Even when median values of nuclear IKKα from the validation cohort were used, the results obtained in the discovery cohort were not replicated.

In contrast we were able to validate the results observed in the discovery cohort for cytoplasmic IKKα expression in the validation cohort. Once again cytoplasmic IKKα was observed to be associated with disease-free survival and recurrence-free survival on tamoxifen in the full validation cohort, and this observation was potentiated in patients with Luminal A disease. Taken together the results from both cohorts suggest that cytoplasmic IKKα is a more robust marker than nuclear IKKα and may be employed as a prognostic marker in Luminal breast cancer to predict patients likely to develop resistance to tamoxifen or IKKα targeted therapies.

Preliminary experiments were performed to investigate if targeting IKKα offered a possible therapeutic target in breast cancers. IKKα expression was suppressed using siRNA and cell viability assessed. Decreased cell growth and viability can reflect either a reduction in proliferation rates or an increase in apoptosis, or a combination of both mechanisms. Cell viability was measured through various methods including assessment of apoptosis using an ELISA, viability using a WST-1 assay, real-time growth and viability using xCELLigence. The effect of silencing IKKα on phenotypic outputs was examined in both ER-positive MCF7 cells and ER negative MDA-MB-231 cells, to establish if a difference was observed between ER status as was observed in the patient tissue samples. In keeping with results

from the clinical specimens, IKKα silencing only had an impact in ER-positive MCF7 breast cancer cells and not on ER-negative MDA-MB-231 cells. In addition, gene card experiments demonstrated that reducing IKKα expression had a significant impact on increased expression of genes associated with induction of apoptosis, in particular BAK1 and BBC3. This is in keeping with previous reports that demonstrate that both BAK1 and BBC3 are associated with prognosis and or response to therapy in breast cancer [37-39]. It therefore appears that IKKα selective inhibitors would be most beneficial to patients with ER-positive tumours and may function via induction of apoptosis and inhibition of proliferation. Studies from the literature demonstrate that IKKα is involved in regulation of oestrogen-dependent genes such as *cyclin D1* and *c-myc,* resulting in increased proliferation [6]. However future studies in a large panel of ER positive and ER negative cell lines are required to confirm this observation. Future studies should also investigate impact of tamoxifen treatment and resistance to tamoxifen in cell line studies.

The results from both the tissue and *in vitro* work from the current study demonstrate that IKKα is associated with regulation of ER-positive breast cancer, suggesting inhibitors of IKKα would be beneficial for breast cancer patients. Exploiting IKKα as a target may offer additional therapeutic options following development of endocrine resistance [2]. Additional thorough investigation, including both larger tissue studies and mechanistic work, is required to further elucidate the role of IKKα in the progression of breast cancer, though involvement of IKKα in breast cancer recurrence is clearly apparent. Once novel compounds are found to be selective and efficient in preclinical models, inhibitors of IKKα may therefore provide a promising therapeutic approach in the future for breast cancer, particularly in patients who develop endocrine resistance.

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Table 1:
The relationship between clinico-pathological characteristics, members of the non canonical NF−κB and cancer specific survival, disease-free survival and recurrence on tamoxifen in patients with invasive ductal breast cancer

Full cohort	**Cancer-specific survival**	**Disease-free s survival**	**Recurrence on Tamoxifen**
	***P*-value (n=362)**	***P*-value (n=344)**	***P-value* (n=65)**
Age (≤50/>50 years)	0.275	0.436	0.743
Size (≤20/21-50/>50 mm)	**0.001**	**0.009**	0.733
Grade (I/II/III)	**<0.001**	**<0.001**	**0.020**
Involved lymph node (no/yes)	**<0.001**	**<0.001**	**0.043**
ER status (no/yes)	**0.002**	**<0.001**	NA
PR status (no/yes)	**<0.001**	**<0.001**	0.154
HER2 status (no/yes)	**0.002**	**<0.001**	0.065
Tumour subtype(Luminal A/Luminal B/triple negative, HER2 enriched)	**<0.001**	**<0.001**	**0.001**
Tumour necrosis (low/high)	**<0.001**	**<0.001**	**0.003**
Lymph vessel invasion (no/yes)	**<0.001**	**<0.001**	**0.001**
Blood vessel invasion (no/yes)	**<0.001**	**0.003**	0.719
Klintrup–Mäkinen grade (week/strong)	0.180	0.223	0.775
CD68+ (low/moderate/high)	0.321	0.325	0.096
CD4+ (low/moderate/high)	0.428	0.942	0.823
CD8+ (low/moderate/high)	**0.005**	**0.028**	**0.046**
CD138+(low/moderate/high)	0.555	0.117	0.533
Angiogenesis (low/moderate/high)	0.094	**0.025**	0.574
Tumour stroma percentage (low/high)	**<0.001**	**0.001**	0.777
Tumour budding (low/high)	**0.002**	0.164	0.535
Cyto IKKα (low/high)	0.264	**0.045**	**0.003**
Nuclear IKKα (low/high)	0.143	0.555	0.123
Total tumour cell IKKα(one low/both high)	0.883	0.504	**0.011**
Cyto NIK(low/high)	0.155	0.390	0.765
Cyto RELB (low/high)	0.511	0.495	0.319
Nuclear RELB (low/high)	0.304	0.254	0.972

Total tumour cell RELB (one low/both high)	0.349	0.500	0.774

Bold indicates a significant association

Table2:
The relationship between clinico-pathological characteristics, members of the non-canonical NF−κB and cancer specific survival in patients with Luminal A invasive ductal breast cancer

	Univariate analysis		Multivariate analysis	
Patients (n=165)	**Hazard ratio (95% CI)**	***P*-value**	**Hazard ratio (95% CI)**	***P*-value**
Age (≤50/>50 years)	2.44(0.54-10.93)	0.227		
Size (≤20/21-50/>50 mm)	1.38(0.53-3.61)	0.555		
Grade (I/II/III)	1.41(0.61-3.25)	0.262		
Involved lymph node (no/yes)	1.24(0.41-3.71)	0.692		
PgR status (no/yes)	0.30(0.10-0.88)	**0.020**	0.31(0.10-0.92)	**0.035**
Tumour necrosis (low/high)	3.01(1.09-9.2)	0.042		
Lymph vessel invasion (no/yes)	1.59(0.53-4.74)	0.401		
Blood vessel invasion (no/yes)	4.02(1.26-12.83)	**0.011**	6.3(1.89-21.3)	**0.003**
Klintrup–Mäkinen grade (week/strong)	0.04(0-56.64)	0.117		
CD68+ (low/moderate/high)	0.81(0.39-1.67)	0.375		
CD4+ (low/moderate/high)	0.78(0.40-1.51)	0.682		
CD8+ (low/moderate/high)	0.65(0.32-1.30)	0.365		
CD138+(low/moderate/high)	0.89(0.47-1.69)	0.937		
Angiogenesis (low/moderate/high)	1.84(0.90-3.72)	0.211		
Tumour stroma percentage (low/high)	4.02(1.25-12.88)	**0.011**	4.98(1.53-16.21)	**0.008**

Tumour budding (low/high)	2.04(0.71-5.90)	0.175		
Cytoplasmic IKKα (low/high)	2.59(0.87-7.74)	0.076		
Nuclear IKKα (low/high)	5.48(1.22-24.53)	**0.012**	9.4(2.02-22.67)	**0.004**
Total tumour cell IKKα (one low/both high)	1.99(1.15-3.44)	**0.007**		
Cytoplasmic NIK (low/high)	0.86(0.27-2.73)	0.810		
Cytoplasmic RELB (low/high)	0.80(0.25-2.49)	0.703		
Nuclear RELB (low/high)	0.66(0.180-2.45)	0.536		
Total tumour cell RELB (one low/both high)	0.68(0.24-1.91)	0.541		

Bold indicates a significant association

Supplementary Table 1:

Genes on TLDA Card

Assay	Gene Symbol
Hs00236911_m1	BIRC2
Hs00832876_g1	BAK1
Hs00180403_m1	BCL3
Hs00354836_m1	CASP1
Hs00892481_m1	CASP2
Hs00362072_m1	CASP5
Hs00169152_m1	CASP7
Hs01018151_m1	CASP8
Hs00154260_m1	CASP9
Hs00395088_m1	IKBKB
Hs99999901_s1	**18S**
Hs00242739_m1	LTB
Hs00172036_m1	MCL1
Hs00765730_m1	NFKB1
Hs00174517_m1	NFKB2
Hs00182115_m1	NFKBIB
Hs00234431_m1	NFKBIE
Hs00560402_m1	PMAIP1
Hs00232399_m1	RELB
Hs00153550_m1	TNFRSF1B
Hs00269492_m1	TNFRSF10A
Hs00196075_m1	CARD4
Hs00248187_m1	NALP1
Hs00201637_m1	CASP14
Hs00209789_m1	BCL2L13
Hs00205419_m1	TNFRSF21
Hs00376860_g1	HTRA2
Hs00179410_m1	TBK1
Hs00215973_m1	ESRRBL1
Hs00388035_m1	LRDD
Hs00223394_m1	CARD15
Hs00364485_m1	CARD9
Hs00230071_m1	NFKBIZ
Hs00373302_m1	BCL2L14
Hs00223384_m1	BIRC7
Hs00261581_m1	CARD6
Hs01057786_s1	BIRC8
Hs00370206_m1	DEDD2
Hs00559441_m1	APAF1
Hs00985031_g1	BIRC3
Hs00745222_s1	BIRC4
Hs00977611_g1	BIRC5
Hs00236330_m1	FAS
Hs00181225_m1	FASLG
Hs00188930_m1	BAD

Assay	Gene Symbol
Hs00608023_m1	BCL2
Hs00187845_m1	BCL2A1
Hs00169141_m1	BCL2L1
Hs00187848_m1	BCL2L2
Hs00154189_m1	BIK
Hs00188949_m1	BNIP3L
Hs00261296_m1	BOK
Hs00263337_m1	CASP3
Hs00154250_m1	CASP6
Hs01017902_m1	CASP10
Hs00234480_m1	DAPK1
Hs00193477_m1	HIP1
Hs01847653_s1	BIRC1
Hs00153283_m1	NFKBIA
Hs00153294_m1	RELA
Hs00174128_m1	TNF
Hs00175318_m1	IKBKG
Hs00269428_m1	PEA15
Hs00601065_g1	TRADD
Hs00169407_m1	RIPK1
Hs00705213_s1	HRK
Hs00234356_m1	TNFSF10
Hs00538709_m1	FADD
Hs00366272_m1	TNFRSF10B
Hs00153439_m1	CFLAR
Hs00172768_m1	DEDD
Hs00368095_m1	BCL2L10
Hs00708019_s1	BCL2L11
Hs00248075_m1	BBC3
Hs00203118_m1	PYCARD
Hs00219876_m1	DIABLO
Hs00212288_m1	BIRC6
Hs99999905_m1	**GAPDH**
Hs99999903_m1	**ACTB**
Hs00989502_m1	CHUK
Hs00968436_m1	REL
Hs01042313_m1	TNFRSF1A
Hs01572688_m1	RIPK2
Hs01063858_m1	IKBKE
Hs01036137_m1	BCAP31
Hs01043258_m1	ICEBERG
Hs01076336_m1	TA-NFKBH
Hs00609632_m1	BID
Hs00969291_m1	BNIP3
Hs01031947_m1	CASP4
Hs99999086_m1	LTA
Hs00980365_g1	TNFRSF25
Hs01011159_g1	CRADD
Hs00961847_m1	BCL10
Hs01594281_m1	CASP8AP2

Figure Legend

Figure 1A shows the relationship between cytoplasmic IKKα expression and cancer specific survival in patients with Luminal A primary operable invasive ductal breast cancer (P=0.076).

Figure 1B shows the relationship between cytoplasmic IKKα expression and disease-free survival in patients with Luminal A primary operable invasive ductal breast cancer (P=0.029).

Figure 1C shows the relationship between cytoplasmic IKKα expression and recurrence-free survival on tamoxifen in patients with Luminal A primary operable invasive ductal breast cancer (P<0.001).

Figure 1D shows the relationship between nuclear IKKα expression and cancer specific survival in patients with Luminal A primary operable invasive ductal breast cancer (P=0.012).

Figure 1E shows the relationship between nuclear IKKα expression and disease-free survival in patients with Luminal A primary operable invasive ductal breast cancer (P=0.066).

Figure 1F shows the relationship between nuclear IKKα expression and recurrence-free survival on tamoxifen in patients with Luminal A primary operable invasive ductal breast cancer (P=0.013).

Figure 1G shows the relationship between total tumour cell IKKα expression and cancer specific survival in patients with Luminal A primary operable invasive ductal breast cancer (P=0.007).

Figure 1H shows the relationship between total tumour cell IKKα expression and disease-free survival in patients with Luminal A primary operable invasive ductal breast cancer (P=0.009).

Figure 1I shows the relationship between total tumour cell IKKα expression and recurrence-free survival on tamoxifen in patients with Luminal A primary operable invasive ductal breast cancer (P<0.001).

Figure 2A shows the relationship between cytoplasmic IKKα expression and disease-fee survival in patients with ER-positive primary operable invasive ductal breast cancer (P=0.024).

Figure 2B shows the relationship between cytoplasmic IKKα expression and recurrence-free survival on tamoxifen in patients with ER-positive primary operable invasive ductal breast cancer (P=0.038).

Figure 2C shows the relationship between cytoplasmic IKKα expression and cancer specific survival in patients with Luminal A primary operable invasive ductal breast cancer (P=0.023).

Figure 2D shows the relationship between cytoplasmic IKKα expression and disease-free survival in patients with Luminal A primary operable invasive ductal breast cancer (P=0.002).

Figure 2E shows the relationship between cytoplasmic IKKα expression and recurrence-free survival on tamoxifen in patients with Luminal A primary operable invasive ductal breast cancer (P=0.009).

Figure 3A shows Western blot and plots when expression was quantified by image-J for MCF7 cells and MDA-MB-231 cells stimulated with LTx.

Figure 3B shows Western blot for MCF7 and MDA-MB-231 cells treated with lipofectamine (C), non-targeting siRNA (NT) and IKKα (IKKα) siRNA.

Figure 3C shows plots for fold change in apoptosis levels in MCF7 cells and MDA-MB-231 cells treated with lipofectamine (C), non-targeting siRNA (NT) and IKKα (IKKα) siRNA. The western blot displayed demonstrates the decrease in expression of IKKα achieved in the cells used for the apoptotic assay shown in Figure 3C and also for the cells used in the wst-1 assays Figure 4A.

Figure 4A shows plots for fold change in cell viability levels in MCF7 cells and MDA-MB-231 cells treated with lipofectamine (C), non-targeting siRNA (NT) and IKKα (IKKα) siRNA. Refer to Figure 3C for western blot demonstrating level of IKKα knock down achieved.

Figure 4B shows xCELLigence cell index plots for MCF7 cells and MDA-MB-231 cells treated with lipofectamine (C), non-targeting siRNA (NT) and IKKα (IKKα) siRNA.

Figure 4C shows volcano plot for change in gene expression in MCF7 cells pretreated with non-targeting siRNA (NT) or IKKα (IKKα)siRNA and stimulated with LTx (20ng/ml) for 12 hours.

Supplementary Figures

S.Figure 1A and B shows an example of low cytoplasmic and low nuclear IKKα expression.

S.Figure 1C and D shows an example of high cytoplasmic and high nuclear IKKα expression.

S.Figure 1E and F shows an example of a negative control.

S.Figure 2A shows western blot displaying a single band of appropriate size (85kDa). A reduction in expression was observed in lysates from IKKα silenced cells but not from IKKβ silenced cells.

S.Figure 2b shows paraffin embedded cell pellets treated with siRNA. A reduction in expression was observed in cell pellets for IKKα silenced cells but not for IKKβ silenced cells compared to the control cells.

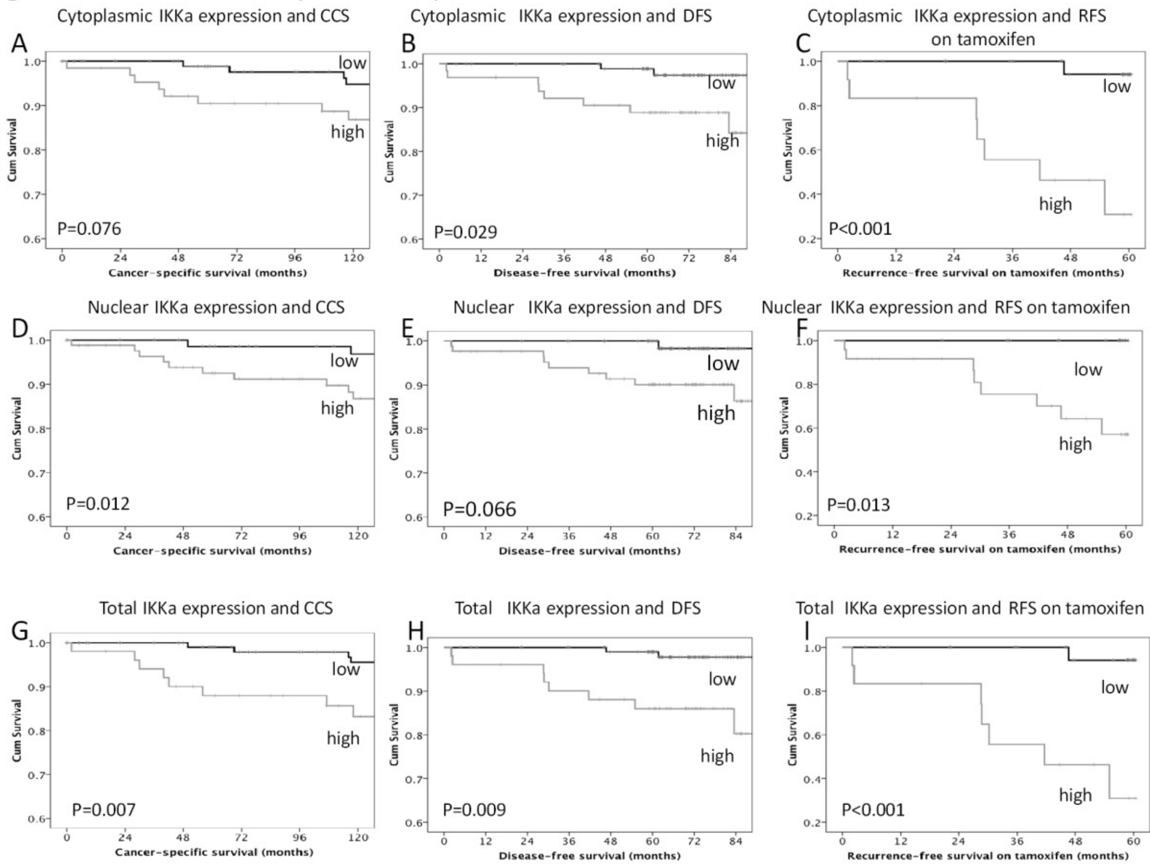
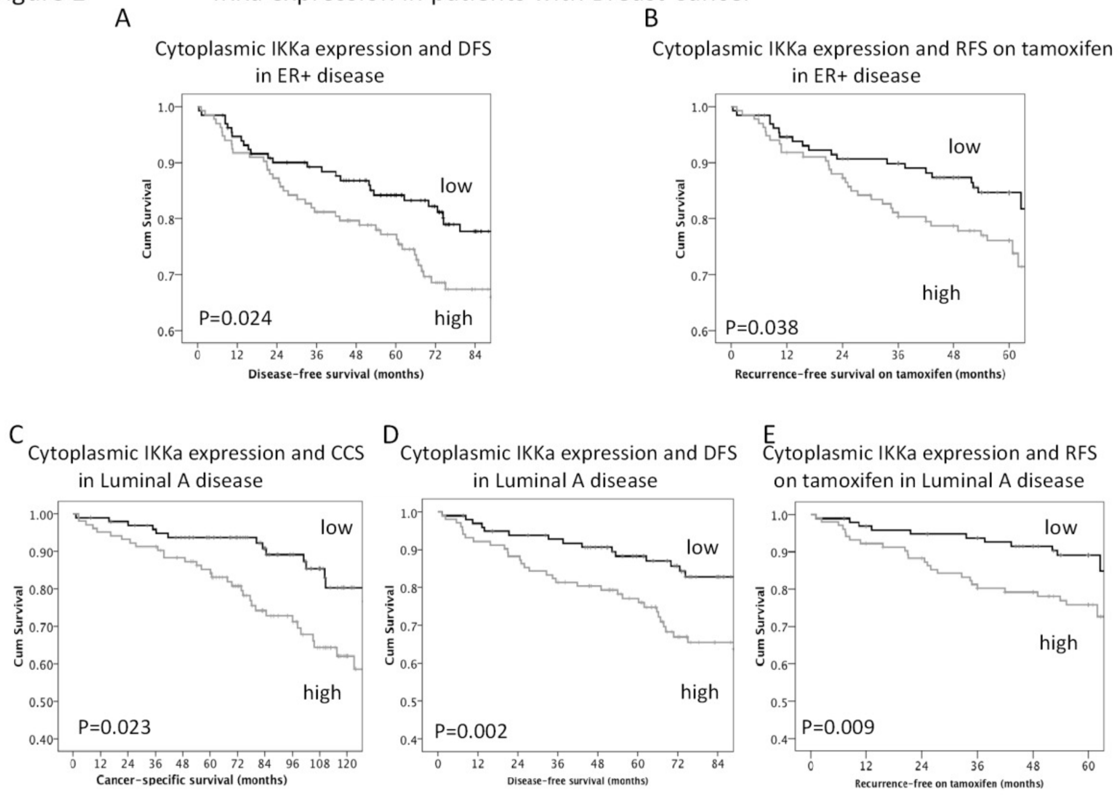


Figure 1 IKKa expression in patients with Luminal A Breast Cancer
Cytoplasmic IKKa expression and CCS
A
low
high
P=0.076
Cum Survival
Cancer-specific survival (months)
Cytoplasmic IKKa expression and DFS
B
low
high
P=0.029
Cum Survival
Disease-free survival (months)
Cytoplasmic IKKa expression and RFS on tamoxifen
C
low
high
P<0.001
Cum Survival
Recurrence-free survival on tamoxifen (months)
Nuclear IKKa expression and CCS
D
low
high
P=0.012
Cum Survival
Cancer-specific survival (months)
Nuclear IKKa expression and DFS
E
low
high
P=0.066
Cum Survival
Disease-free survival (months)
Nuclear IKKa expression and RFS on tamoxifen
F
low
high
P=0.013
Cum Survival
Recurrence-free survival on tamoxifen (months)
Total IKKa expression and CCS
G
low
high
P=0.007
Cum Survival
Cancer-specific survival (months)
Total IKKa expression and DFS
H
low
high
P=0.009
Cum Survival
Disease-free survival (months)
Total IKKa expression and RFS on tamoxifen
I
low
high
P<0.001
Cum Survival
Recurrence-free survival on tamoxifen (months)

Figure 2 IKKa expression in patients with Breast Cancer



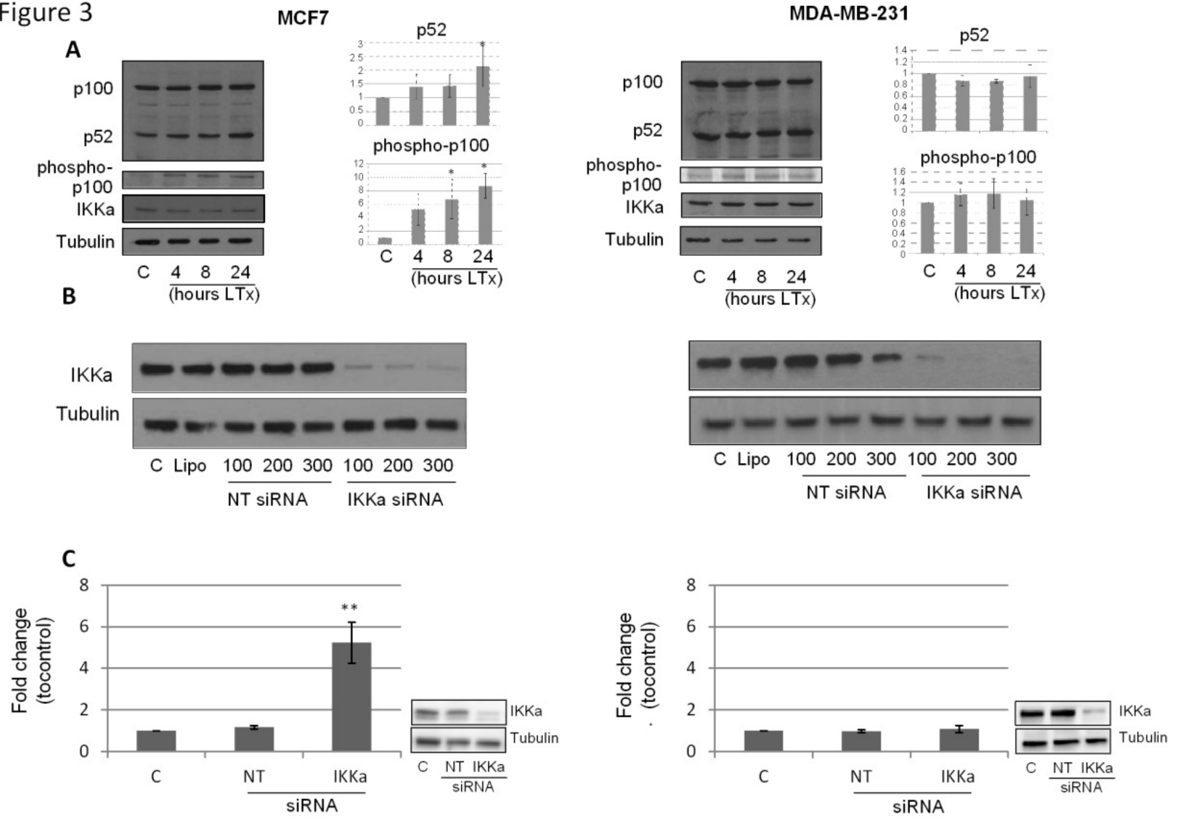
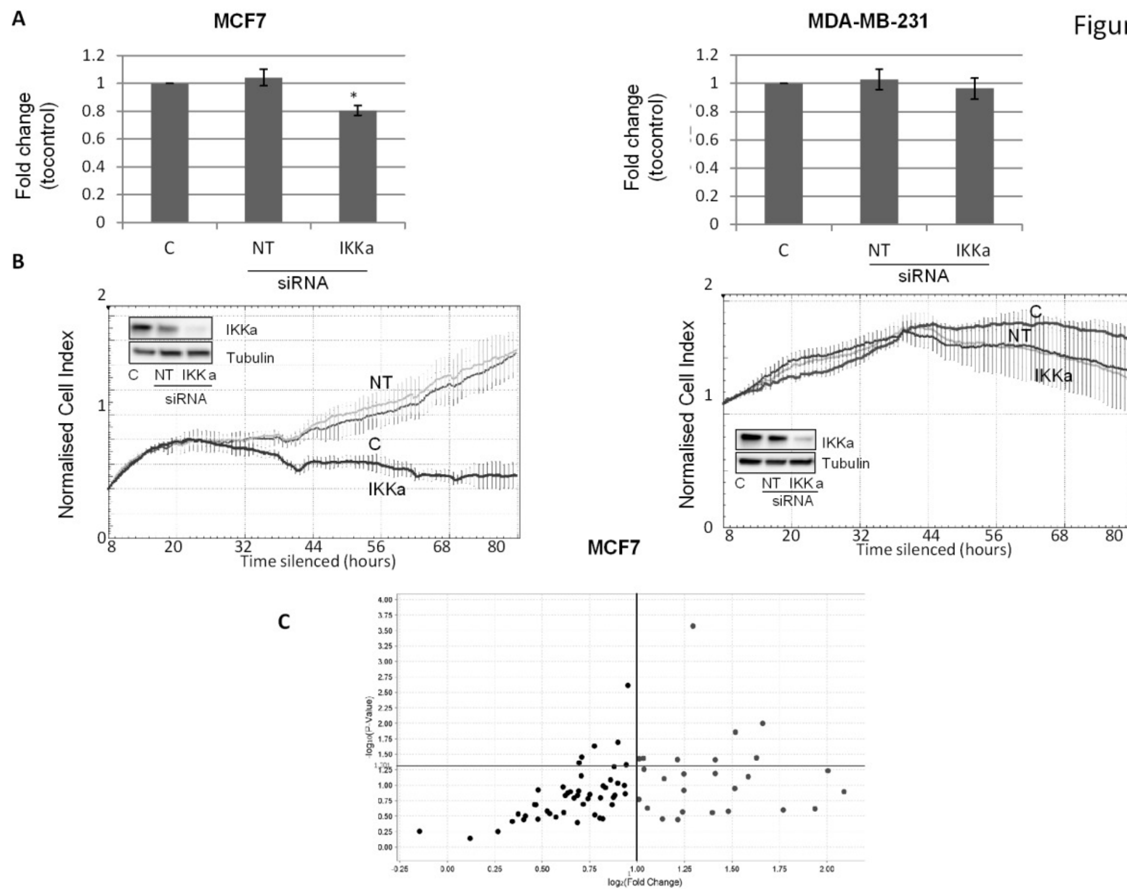
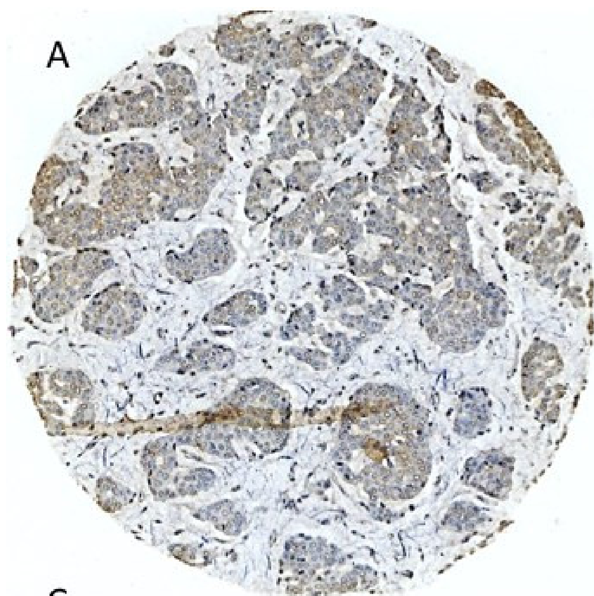


Figure 3
MCF7
MDA-MB-231
A
p100
p52
phospho-
p100
IKKa
Tubulin
C 4 8 24
(hours LTx)
p52
phospho-p100
B
IKKa
Tubulin
C Lipo 100 200 300 100 200 300
NT siRNA
IKKa siRNA
C
Fold change
(tocontrol)
**
C NT IKKa
siRNA
IKKa
Tubulin
C NT IKKa
siRNA

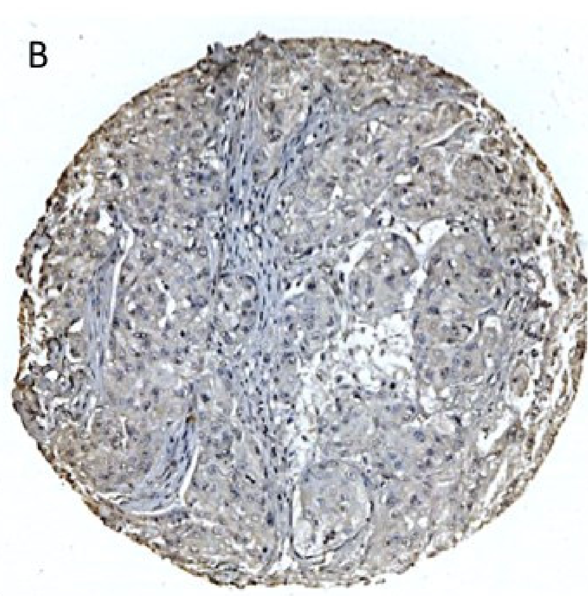
Figure 4



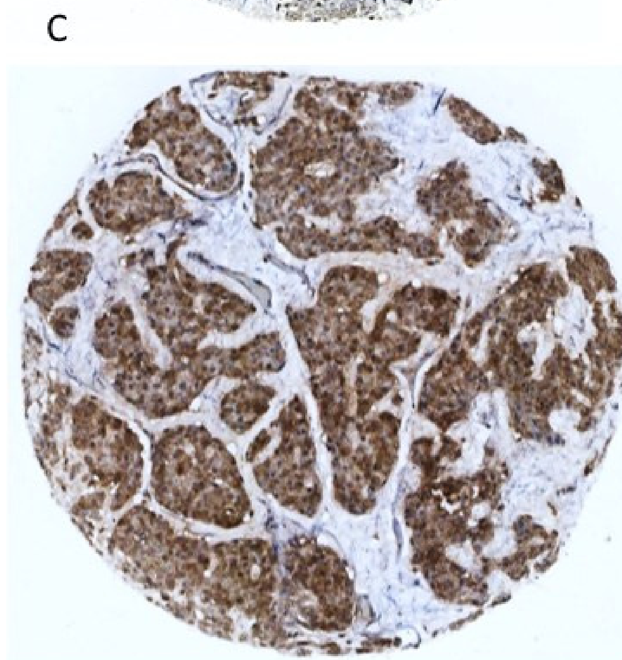
A



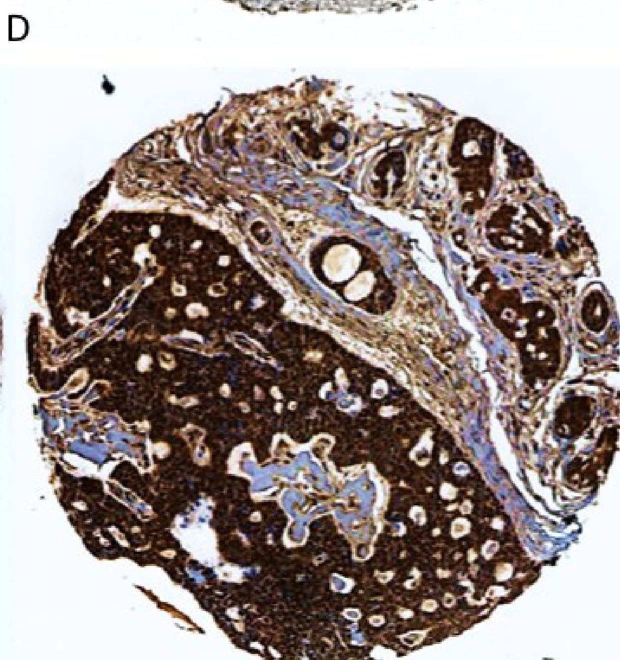
B



C



D



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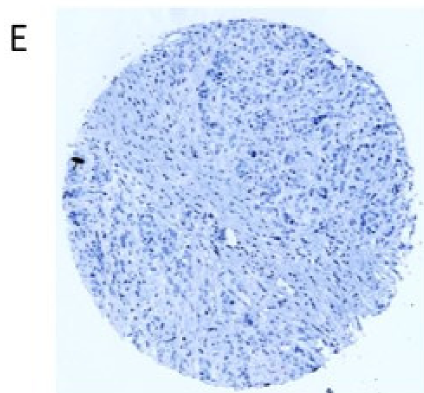
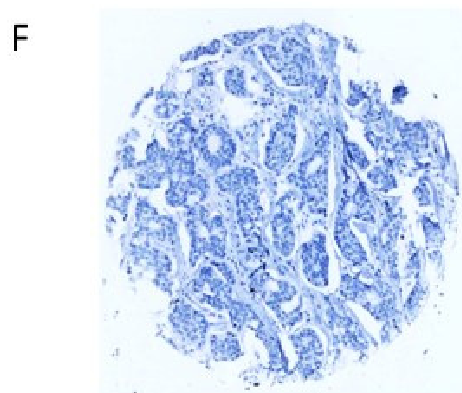
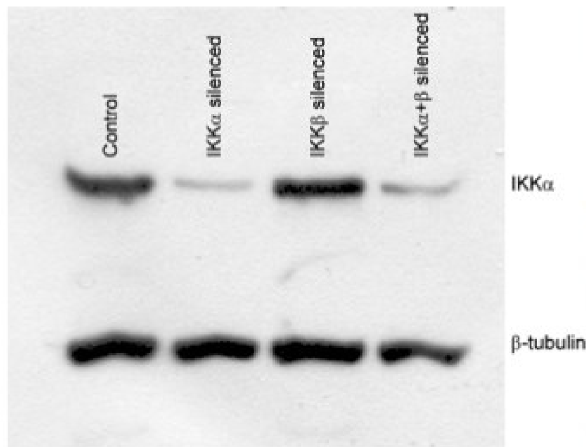


F



A



B