Published in final edited form as:

Genes Chromosomes Cancer. 2013 October ; 52(10): 961–975. doi:10.1002/gcc.22091.

A Novel Isoform of the B Cell Tyrosine Kinase BTK Protects Breast Cancer Cells from Apoptosis

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Abstract

Tyrosine kinases orchestrate key cellular signaling pathways and their dysregulation is often associated with cellular transformation. Several recent cases in which inhibitors of tyrosine kinases have been successfully used as anticancer agents have underscored the importance of this class of proteins in the development of targeted cancer therapies. We have carried out a large-scale loss-of-function analysis of the human tyrosine kinases using RNA interference to identify novel survival factors for breast cancer cells. In addition to kinases with known roles in breast and other cancers, we identified several kinases that were previously unknown to be required for breast cancer cell survival. The most surprising of these was the cytosolic, nonreceptor tyrosine kinase, Bruton's tyrosine kinase (BTK), which has been extensively studied in B cell development. Down regulation of this protein with RNAi or inhibition with pharmacological inhibitors causes apoptosis; overexpression inhibits apoptosis induced by Doxorubicin in breast cancer cells. Our results surprisingly show that BTK is expressed in several breast cancer cell lines and tumors. The predominant form of BTK found in tumor cells is transcribed from an alternative promoter and results in a protein with an amino-terminal extension. This alternate form of BTK is expressed at significantly higher levels in tumorigenic breast cells than in normal breast cells. Since this protein is a survival factor for these cells, it represents both a potential marker and novel therapeutic target for breast cancer.

INTRODUCTION

Tyrosine kinases (TKs) catalyze the reversible process of tyrosine phosphorylation, a key step in most signal transduction pathways that govern cellular proliferation, survival, differentiation, and motility. Dysregulation of TKs, as occurs through inappropriate expression, activation, or both, is commonly associated with human cancers (Blume-Jensen and Hunter, 2001; Giamas et al., 2010). As a result, TKs, as a class, are the most commonly

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found dominant oncogenes (Blume-Jensen and Hunter, 2001; Vassilev and Uckun, 2004; Krause and Van Etten, 2005; Baselga, 2006).

Small molecules that directly inhibit the catalytic activity of tyrosine kinases have been sought as potential cancer chemotherapeutics. Recent successes with a few well-studied tyrosine kinases have proven the value of these proteins as drug targets. Imatinib mesylate (Gleevec) has proven hugely successful in treating chronic myelogenous leukemia (CML). The EGFR inhibitors Gefitinib (Iressa) and erlotinib (Tarceva), are currently used on a variety of solid tumors (Kris et al., 2003; Krause and Van Etten, 2005; Shepard et al., 2008). Trastuzumab (Herceptin), a humanized monoclonal antibody that specifically inhibits ERBB2, is widely used in the treatment of breast cancers. Each of these treatments, however, has significant limitations related to tissue spectrum, acquired resistance, and efficacy in advanced disease (Nahta and Esteva, 2006). The identification of additional TK genes and pathways that contribute to the survival of distinct cancer cell types, so that they can be effectively targeted, would be of great value.

We have performed a large-scale RNA interference (RNAi) screen to identify additional tyrosine kinases as potential therapeutic targets for breast cancer. We find that targeting 30% of the TKs with RNAi results in a 50% or greater decrease in a reporter of cell health, which may reflect decreased cellular proliferation or increased cell death, in BT474 breast cancer cells. Unexpectedly, a novel isoform of a member of the Tec family of non-receptor tyrosine kinases, Bruton's tyrosine kinase (BTK), which is known primarily for its critical role in B cell maturation, is among the TKs that exhibited the strongest impact on cellular proliferation. The expression of this novel BTK isoform is elevated in a number of breast cancer cell lines compared to non-tumorigenic breast cell lines. These results indicate that an alternative BTK protein contributes to breast cancer cell survival.

MATERIALS AND METHODS

Cell Culture

Cell lines NAMALWA, BT474, MCF-7, SK-BR-3, MDA-MB-361, and MCF10a were obtained from the ATCC. Human mammary epithelial cells (HMEC) were obtained from Cambrex. HEK 293FT cells were obtained from Invitrogen (Carlsbad, CA). BT474, MCF-7 and HEK 293FT cells were cultured in DMEM (Hyclone, Logan, UT) supplemented with 10% FBS (Hyclone) and 100 U/μl of penicillin-streptomycin (Cellgro, Manassas, VT). NAMALWA were cultured in RPMI-1640 medium (ATCC) supplemented with 10% FBS and 100 U/μl of penicillin-streptomycin. MDA-MB-361 were cultured in RPMI-1640 medium (ATCC) supplemented with 20% FBS and 100 U/μl of penicillin-streptomycin. HMECs were cultured in MEGM medium (Cambrex). MCF10a were cultured in DME/F12 1:1 medium supplemented with 5% Horse serum, 20 ng/ml EGF, 0.5 μg/ml hydrocortisone, 100 ng/ml cholera toxin, 10 μg/ml insulin, and 100 U/μl of penicillin-streptomycin.

RNAi Methods

A collection of short hairpin RNAs (shRNAs) targeting each of the TKs were assembled from the pSHAG-MAGIC 2 (pSM2) shRNA library (Silva et al., 2005). Plasmid DNA was

isolated, in 96-well format, from bacterial stocks containing each of the shRNAs using the Perfectprep Plasmid 96 Vac Direct Bind kit (Eppendorf, Hamburg, Germany). ShRNAs targeting the firefly (*Photinus pyralis*) luciferase gene were used as controls. Transfection efficiency was monitored by co-transfection with a modified MSCV-Puro vector expressing green fluorescent protein (GFP). The alamarBlue (Biosource, Carlsbad, CA) assay was performed 96 h post-transfection. Quantification was done on a BioTek HT Synergy plate reader. Transfections were performed using FuGENE 6 (Roche, Indianapolis, IN) according to the manufacturer's protocol. High-throughput transfections were performed using an EpMotion 5070 fluidics station (Eppendorf). Z-scores were calculated using the following formula: (normalized sample value – normalized data set mean)/data set standard deviation. BTK was also knocked down using the siGEMOME SMART pool duplex (Dharmacon, Lafayette, CO) transfected with Oligofectamine Reagent (Invitrogen, Gaithersburg, MD) according to the manufacturer's instructions. BTK-C specific siRNAs were custom synthesized (Dharmacon, Lafayette, CO): siRNA1 sense: GGUUAUUGGAUGCCCAUUAUU, antisense: U AAUGGGCAUCCAAUAACCUU; siRNA2 sense: CAACAAUGGUUAUUGGAUUU; antisense: A UCCAAUAACCAUUUGUUGUU.

Cell Viability and Apoptosis Assays

For high-throughput experiments, cells grown on 96-well plates were washed once with 1× PBS, fixed with 2.5% formaldehyde, and stained with Hoechst 33342 (Molecular Probes-Invitrogen). Cell images were acquired using an In Cell Analyzer 1000 (GE Healthcare, Piscataway, NJ) high content imaging system, with a 20× objective. At least 50 fields were imaged per single experiment. Cell counts and statistics were then performed using In Cell Investigator 3.4 high-content image analysis software (GE Healthcare). Apoptosis was detected by cleaved Caspase-3 after 48 hr of siRNA treatments or treatment with the BTK specific inhibitor LFM-A13. BT474 cells were treated with 35 μM LFM-A13. Control cells were treated with DMSO. For the cleaved caspase-3 assay, cells were fixed with 2.5% formaldehyde, washed with 1xPBS, permeabilized with 0.1% Triton-X 100, blocked with 3% normal goat serum (Sigma-Aldrich, St. Louis, MO), incubated with a 1:200 dilution of the primary antibody, washed with 1xPBS, incubated with a 1:800 dilution of the secondary antibody, washed again with 1xPBS and finally stained with Hoechst 33342 (Molecular Probes-Invitrogen). Cells were imaged with an In Cell Analyzer 1000 (GE Healthcare). At least 500 cells were counted for cleaved caspase-3. Apoptotic cells were calculated as a percentage of the total cellular population. Antibodies used: cleaved caspase-3 (Asp175, #9661; Cell Signaling Technology Danvers, MA), Alexa Fluor 568 goat anti-rabbit IgG (#A-11011; Invitrogen).

Immunoblotting

Cell extracts for immunoblots were prepared using RIPA buffer (1% Triton X-100, 40 μM NaCl, 0.1% SDS, 10 μM Tris pH 8.0) or non-denaturing lysis buffer: (20 μM Tris (pH 8.0), 137 mM NaCl, 10% glycerol, 1% Triton X-100, 2 μM EDTA), supplemented with cComplete cocktail of proteinase inhibitors (Roche). BT474 cells containing the stably integrated BTK-A-flag tag MarxIV, the BTK-C-flag tag MarxIV, and the β-galactosidase MarxIV vectors were incubated with 100 μM LFM-A13 for 45 min. For detection of phosphorylated

epitopes, the PhosSTOP cocktail of phosphatase inhibitors (Roche) was added in the lysis buffer. Tyrosine-phosphorylated BTK was assessed by immunoprecipitation (IP) using anti-Flag and Western blot (WB) analysis using anti-BTK Phospho (pY223) and anti-BTK. Protein extracts were separated by SDS-PAGE, transferred to Immobilon-P (Millipore, Billerica, MA) membranes and immunoblotted according to standard protocols. Blots were imaged using a FluorChem HD (Alpha Innotech) imaging system. A polyclonal anti-BTK antibody (C-20), the monoclonal anti-BTK antibody (E-9) and a polyclonal anti-GAPDH antibody (V-18) were obtained from Santa Cruz Biotechnology (Santa Cruz, CA). A polyclonal anti-BTK antibody raised against residues 2-172 of BTK was obtained from Becton Dickinson (BD Transduction Laboratory, 611116). A polyclonal anti-BTK antibody used for immunofluorescence was obtained from ProSci Incorporated (Poway, CA). The monoclonal anti-FLAG M2 antibody was from Stratagene (Cedar Creek, TX). The polyclonal anti-caspase-3 antibody (Asp175) was obtained from Cell Signaling Technology. The protease inhibitor cocktail was obtained from Roche and the phosphatase inhibitor cocktail was from Pierce (Rockford, IL, USA). Secondary antibodies included an anti-rabbit IgG-HRP (sc-2204, Santa Cruz Biotechnology), anti-goat IgG-HRP (sc-2768, Santa Cruz Biotechnology), and anti-mouse IgG-HRP (#31430; Pierce Biotechnology).

RNA Isolation and qRT-PCR

Total RNA was extracted from cells using TRizol (Invitrogen) according to the manufacturer's instructions, followed by the addition of DNaseI (Roche) for 20 min at 37°C and purified using the RNeasy column (Qiagen, Valencia, CA) cleanup protocol. cDNA was amplified using a modified version of the NIH/NCI Reid Lab cDNA synthesis protocol. qRT-PCR reactions using *SYBR Green* Master Mix (Applied Biosystems), Taq SYBR Green Super Mix (BioRad), or TaqMan qPCR using TaqMan Gene Expression Master Mix (Applied Biosystems) were performed on a ABI PRISM 7900HT Sequence Detection System (Applied Biosystems). The primer pairs used were designed using ABI's Primer Express software and are shown in Supporting Information Table 1. After the initial denaturation step (95°C for 2.5 min), PCR reactions consisted of 40 cycles of a 95°C – 15 sec step, and a 60°C - 1 min step. Analysis was conducted using ABsystems Real-Time Analysis software Version 2.2 The RT-PCR amplification mix consisted of 1X Taq polymerase buffer (Fisher), 0.2 µM dNTPs, 0.2 µM each primer (Table 1), 10 µl cDNA, and 5 u Taq polymerase (Roche) in a 100 µl total volume. After the initial denaturation step (5°C for 2 min), PCR reactions consisted of 40 cycles of a 95°C - 30 sec step, 55°C - 30 sec step and a 72°C - 3 min step. Aliquots of each PCR reaction were electrophoresed on 1% agarose gels. The GeneRacer Kit (Invitrogen) was used, according to the manufacturer's specifications, for amplification of the N-terminal portion of the BTK message.

Measurement of BTK mRNA expression in normal and tumor tissues was performed using an array of first-strand complementary DNA (cDNA) from human breast tissues contained in the TissueScan Cancer Survey Panel in 384-well plates from OriGene (Rockville, MD) (CSRT302). The cDNAs were prepared from normal breast tissues or breast adenocarcinoma biopsy samples. The cDNAs from one plate were used for measurement of BTK mRNA levels by real-time RT-PCR analysis. The same cDNAs in another plate were used for measurement of actin. The data presented are relative BTK isoform mRNA levels

normalized to actin. This experiment was conducted twice with a representative dataset shown.

BTK-A and BTK-C Expression Vector Construction

A sequence encoding a triple flag tag was amplified using standard conditions with 100 ng of the pCMV-3Tag-3a Vector (Stratagene) as template and the pCMV-3X Flag forward and reverse primers (Table 1). The PCR amplified flag tag product was cloned into the MarxIV vector (ref) using ApaI and XhoI (NEB) restriction sites. DNA sequence analysis of plasmid DNA from selected clones confirmed the clones. The BTK-A and BTK-C sequences were amplified using a proofreading Taq polymerase (Phusion DNA polymerase, NEB), according to the manufacturer's specifications. BTK-A was amplified using Namalwa cDNA as template with the BTK-Flag primer set (Table 1). The BTK-C sequence was amplified using over-lap extension PCR. BTK-C (N-terminus) was amplified using 10 ng pCR2.1-TOPO plasmid DNA (Invitrogen), containing the N-terminal BTK-C sequence (constructed in the 5' RACE experiment) as template with the N-term-BTK-C primer set (Table 1). BTK-C (C-terminus) was amplified using Namalwa cDNA as template with the BTK-C-terminus primer set (Table 1), in 100 µl total volume. BTK-C (full-length) was amplified using the BTK-C (N-terminus) and BTK-C (C-terminus) PCR DNAs as template with the BTK-C-Flag primer set. The BTK-C (full-length) PCR product was cloned into the MarxIV-Flag vector using BamHI and XhoI (NEB) restriction sites. The BTK-A PCR product was cloned into the MarxIV-Flag vector using MfeI and XhoI (NEB) restriction sites. Selected clones were sequence-verified.

BTK Immunolocalization

Cells were immunostained on cover slips with anti-BTK antibody (ProSci), anti-Flag antibody (Stratagene) and Alexa568 conjugated 2° antibody, with Hoechst to stain nuclei, and were imaged using a Leica TCS SP5 confocal microscope system (Leica Microsystems Inc., Bannockburn, IL). Breast cancer tissue arrays were obtained from Biomax.us (BRC-961) and contained 96 breast cancer cases with a range of disease stages and patient ages. Arrays were processed with standard immunohistochemical procedures. Briefly, slides were baked at 65°C for 1 h, then de-paraffinized in HistoChoice clearing agent, and rehydrated through a series of decreasing concentrations of ethanol (100, 95, 70, 50%) and finally into PBS. Slides were washed with PBS/0.3% Triton X-100 for 10 min and epitopes retrieved in a pressure cooker for 20 min. Sections were then blocked in 10% donkey serum/3% BSA in PBS and then incubated O/N at 4°C with BTK antibody (1:200 dilution in 3% BSA/PBS). Slides were washed in PBS and primary antibody detected with Cy5 donkey anti-mouse secondary antibody (1:250 dilution in 3% BSA/PBS, Jackson Immunologicals). Slides were washed, stained in DAPI to visualize the nuclei, rinsed, and mounted with DABCO anti-fade in 90% glycerol. TMA cores were imaged on a Zeiss AxioImager Z1 equipped for epifluorescence with a 300W Xenon excitation source, 20X 0.8 n.a. objective, filter sets optimized for DAPI and CY5 (Semrock), and a Hamamatsu ORCA digital CCD camera. Exposure for DAPI and CY5 was fixed across the tissue array sample, and auto-focusing was used after pre-setting coordinates for image acquisition using the AxioVision software (Carl Zeiss).

RESULTS

BTK Identified as a Survival Factor in an RNAi Knockdown Screen of the TKs in BT474 Breast Cancer Cells

A functional genomic approach was taken to evaluate the functional contribution of each TK to breast cancer cell viability. Short hairpin-based RNA interference was used to knockdown the genomic complement of human TKs in the ERBB2-positive breast cancer cell line BT474. 236 short hairpin RNAs (shRNAs) (Silva et al., 2005) targeting 82 of the 90 human PTK genes were used, such that, on average, each PTK was targeted with three independent shRNA constructs. Each shRNA was cotransfected with a plasmid that directs the expression of GFP so that differences in transfection could be normalized. Effects of each shRNA on cells were monitored using Alamar Blue (Biosource), a fluorimetric indicator of cell viability and proliferation that has proven useful in RNAi screens (Kourtidis et al., 2007). Twenty-five of the 82 genes (30%) when silenced by shRNAs result in a decrease in BT474 cellular proliferation of fifty percent or greater when compared to controls (Fig. 1A). EGFR, ERBB2, ABL2, FES, NTRK2 (TRK-B), PTK2B, FGFR2, LYN (V-yes-1), EphA1, and BTK are among the kinases that when knocked down cause the greatest reduction in BT474 cellular proliferation levels (Table 1). Many of these TKs have previously described roles in breast tumors (EGFR, ERBB2, FGFR2, PTK2B, NTRK 2/TRK-B, EphA1, ABL2) (Ogawa et al., 2000; Brantley-Sieders et al., 2005; Chan et al., 2006; Srinivasan and Plattner 2006; Behmoaram et al., 2008). These results support the validity of the screen in identifying kinases that are important for breast cancer cell proliferation or survival.

Bruton's Tyrosine Kinase (BTK) is among those genes whose knockdown causes the most significant reduction in BT474 cellular proliferation (Fig. 1A, Table 1). This is surprising since BTK is thought to function primarily in cells derived from the hematopoietic cell lineage (de Weers et al., 1993; Smith et al., 1994). Mutations in the human BTK gene cause heritable X-linked agammaglobulinemia, which is characterized by a virtual absence of B-lymphocytes due to a block between the pro- and pre-B cell stages of B cell maturation (Mohamed et al., 2009). Since no function has been described for it in either normal or malignant breast cellular processes, we examined BTK's role in breast cancer cells.

The shRNA screen and additional siRNA silencing experiments reveal a significant decrease in cellular proliferation after knockdown of BTK in BT474 cells (Fig. 1). Knockdown of BTK in BT474 cells with siGENOME SMART pool duplex siRNAs, which are transfected into these cells more efficiently, leads to widespread cell death. In these cells, BTK transcript levels are specifically knocked down to 37.5% of control at 48 hr (not shown) prior to the significant loss of cells that occurs between 72 and 96 hr and that is due to increased apoptosis. BT474 cells transfected with siRNAs targeting BTK have an 11-fold increase in apoptotic cells compared to control as evidenced by increased cleaved caspase 3 levels (Figs. 1B and 1C). Similarly, knockdown of BTK has the same effect increasing levels of apoptosis in MCF-7 cells. This indicates that the decrease in proliferative activity found in BTK-silenced BT474 cells is due, at least in part, to increased apoptosis. Since BTK knockdown with both shRNAs and several siRNAs all result in a similar apoptotic effect, we are confident this result is due to silencing of BTK and is not an off-target effect.

BT474 Breast Cancer Cells Express a Novel Variant of the BTK Message from an Alternative Promoter

PCR performed with primers designed to amplify the sequence located between the published Ref seq BTK start and stop codons (BTK internal; Supporting Information Table 1, Supporting Information Fig. 1, and data not shown), produces a product of the expected size from BT474 cDNA. Quantitative PCR (qPCR) analysis detects the *BTK* transcript in round 34 of 40 total cycles suggesting that *BTK* levels in BT474 cells are significantly lower than *GAPDH* reference transcripts. Interestingly, however, RT-PCR analysis of the full length *BTK* transcript indicates that the *BTK* mRNA expressed in BT474 cells is missing a PCR primer binding site in the 5' UTR when compared to that expressed in B-cells.

Although internal primer pairs yield products of the expected size, no product is amplified from BT474 cDNA when a forward primer designed to hybridize within the 5' UTR is used (BTK 5' UTR; Table 1, Supporting Information Fig. 1). This 5' UTR primer does, however, generate a product of the expected size from a positive control cDNA sample generated from Namalwa B-cells (Supporting Information Fig. 1). This result suggests that the N-terminal sequence of the BTK transcript in BT474 breast cancer cells differs from the published sequence deduced from B-cell mRNA. The 5' end of the BTK transcript expressed in BT474 cells was obtained using RACE-PCR and subsequently analyzed by DNA sequencing. When aligned to the 5' end of the published BTK message (BTK-A; accession # U13399) the nucleotide sequence of the transcript isolated from BT474 cells is identical downstream from position 307; however, the sequences upstream from position 307 diverge significantly (Fig. 2A) indicating that the BTK transcript in BT474 cells has an alternative first exon (Fig. 2B).

Since the BTK transcript found in BT474 cells is identical to an automated computationally predicted (GNOMON) sequence named BTK-cra-C, we refer to this isoform as BTK-C and the alternative exon, exon 1C (Figs. 2B and 2C). This transcript has been predicted in genome databases (Levy et al., 2007) (Griffiths-Jones 2004) and isolated as an expressed sequence tag (EST) from human pheochromocytoma tissue (Yang et al., 2000, unpublished; accession # AV733045) and peripheral blood mononuclear cells (Kimura et al., 2006). The portion of the sequence that is specific to the *BTK* message expressed in BT474 cells is located on the long arm of the X-chromosome, 4,416 bp distal from the start site of exon 1 of the published *BTK* gene (BTK-A). The 5' end of BTK-C is 255 bp from the start site of the ribosomal protein L36a gene which is transcribed in the opposite direction (Fig. 2C). A putative transcription start site exists within a CpG island 200 bp upstream from the start of exon 1C (Down and Hubbard, 2002). In addition, predicted transcription factor binding sites are also present, including ETS, AP2, AHR and HOXA7 binding sites (Matys et al., 2003) (Supporting Information Table 2). Taken together, these data indicate that expression of the BTK-C transcript in BT474 breast cancer cells is driven by an alternate promoter located upstream from the published BTK-A promoter. The first exons from the BTK-A and BTK-C isoforms utilize different donor sites to splice into a common acceptor site, located within exon 2, to yield the mature BTK-A and BTK-C mRNA isoforms (Fig. 2B).

Due to the additional sequence, the BTK-C message encodes a product that contains an amino-terminal 34 amino acid extension to the BTK-A protein (Fig. 3A). This extension is

phylogenetically conserved, since DNA sequence encoding it appears in most mammalian species upstream of the BTK start site in each organism (data not shown). A product consistent with this size is observed on immunoblots as an 80 kD product in cellular lysates from several breast cell lines using a polyclonal antibody raised against the pleckstrin homology domain, residues 2-172, of BTK (BD Transduction Laboratory, 611116). The observed size agrees with the BTK-C predicted size of 79.9 kDa and is larger than the 76.3 kDa BTK-A product which is also detected in the breast lines but at significantly lower levels than in the control NAMALWA B cell lysate (Fig. 3B). These results indicate that initiation of translation occurs from the initial methionine codon found in the 5' end of the BTK-C transcript in breast cells. Consistent with this, BTK-A and BTK-C sequences cloned into a retroviral vector containing a CMV promoter and a C-terminal triple flag tag sequence (BTK-A-flag and BTK-C-flag vectors, respectively) produce different sized products. 293FT cells transiently transfected with the BTK-A flag vector yield a 79.5 kDa molecular weight product, which is in agreement with the predicted size of the BTK-A protein containing a triple flag tag (Supplementary Fig. 3A). When 293FT cells were transiently transfected with the BTK-C flag vector, however, two products are detected of approximately 79 kDa and 83 kDa, respectively (Fig. 3C, Supporting Information Figs. 3A and 3B). These translation product sizes could result from the BTK-C transcript if translation was initiated from the N-terminal methionine (83 kDa) of BTK-C, as well as the methionine start codon of the BTK-A message which is retained as the 35th codon of BTK-C (79.5 kDa) (Fig. 2B).

shRNAs targeting internal exons of the BTK gene effectively silence the BTK gene decreasing protein expression, as would be predicted. 293FT cells overexpressing either the BTK-A or BTK-C proteins, when transfected with the BTK shRNA, contained significantly less BTK protein compared to cells transfected with the control shRNA (Supporting Information Fig. 3A). Additionally, transient transfection of BT474 cells stably overexpressing BTK-C with siRNAs targeting BTK resulted in an approximate 70% decrease in BTK protein compared to control (Supporting Information Fig. 3B). That the BTK isoform is important to breast cells was confirmed by designing siRNAs that would specifically target this isoform. As shown in Figure 3B, siRNAs corresponding to exon 1C reduce BTK-C-flag protein expression in transfected HEK 293T cells. Importantly, these siRNAs also decrease the viability of BT474 cells, indicating that this isoform is important for cell viability. Taken together these results confirm that the BTK shRNA and siRNA are strong effectors of BTK gene silencing and that the expression of this isoform is important for breast cancer cell survival.

To assess BTK activation in BT474 cells, the phosphorylation status of tyrosine 223, which becomes auto-phosphorylated after activation, was assessed. BT474 cells stably overexpressing either the BTK-A-flag or BTK-C-flag proteins were subjected to immunoprecipitation using a flag specific antibody and the immunoprecipitates were separated by SDS-PAGE electrophoresis. Blots were probed with an anti-phospho Tyr²²³-BTK antibody (Santa Cruz) or a total BTK antibody (Santa Cruz, E-9) to control for loading. The specific phosphorylation of the BTK-A protein in addition to both forms of BTK produced from the BTK-C vector indicate that the BTK-C protein is activated in BT474 cells (Fig. 4A) under standard growth conditions. Autophosphorylation of BTK was tested by the addition of the BTK specific inhibitor LFM-A13 which has an IC₅₀ for BTK of 17

μM (Vassilev and Uckun, 2004). Treatment with 100 μM LFM-A13 for 45 min significantly impedes phosphorylation of both the BTK-A and BTK-C proteins, indicating that autophosphorylation of the elongated BTK-C protein is inhibited to a similar level as BTK-A under these conditions (Fig. 4A). Consistent with this finding, inhibition of BTK autophosphorylation in BT474 cells using 35 μM LFM-A13 increases apoptosis levels six fold compared to control, further establishing the protective role that BTK plays in BT474 cell survival (Fig. 4B).

BTK over-expression has been implicated in imatinib resistance to chronic myeloid leukemia (CML) and acute lymphoblastic leukemia (ALL) (Hofmann et al., 2002; Villuendas et al., 2006). Its constitutive activation due to deregulated B cell receptor (BCR) engagement is an integral component to certain B cell lymphomas (Irish et al., 2006); (Kuppers, 2005) and it has been shown to serve a protective role through inhibition of Fas/APO-1 mediated apoptosis (Vassilev et al., 1999; Qiu and Kung, 2000). For this reason we determined whether BTK-C inhibits apoptosis induced by doxorubicin in breast cancer cells. The BTK-C isoform is expressed at relatively low levels in MCF-10A cells (Figs. 4C and 5B). Overexpression of BTK-C in MCF-10A cells using Flag-tagged MCF-10-vector (10A-Vec) or MCF-10A-Btk-C (10A-Btk-C) constructs reveals that BTK-C counteracts the effects of doxorubicin. The number of apoptotic cells after doxorubicin treatment decreases nearly threefold in cells overexpressing BTK-C as assayed by cleaved caspase-3 signal (Figs. 4D and 4E). Treatment of MCF-10A cells overexpressing BTK-C with 35 μM LFM-A13 for 24 h abolishes the acquired resistance to doxorubicin (1 μM). These results suggest that BTK expression in tumor cells may lead to chemotherapeutic resistance.

BTK-C Expression is Elevated in Breast Cancer Cells

The data from RACE-PCR and RT-PCR (Fig. 2A and Supporting Information Fig. 2) suggest that BTK-C may be preferentially expressed in breast cancer cells compared to non-tumorigenic cells. Immunohistochemical staining of breast tissue microarray samples (Biomax.us BRC-961) shows increased expression of BTK in clinical breast cancer tissues compared to matched, non-tumorigenic, breast tissues using a BTK specific antibody. Significant levels of anti BTK staining are observed in most of the tumor samples (80.2%). Representative images are shown in Figure 5A. This confirms that at least some form of BTK is unexpectedly expressed in cells of this tissue type, although isoform-specific protein level determination is not currently possible. So that we could discriminate between BTK-A and BTK-C levels in normal and cancer cells, isoform specific qPCR primer sets were designed to the heterogeneous regions of the two sequences located within the 5'UTRs (BTK-A_5'UTR and BTK-C_5'UTR, respectively). cDNA from the BTK-A positive B-cell line Namalwa, the breast cancer cell lines BT474, MCF7, and MDA-MB-361, the non-tumorigenic breast cell line MCF10a, and human mammary epithelial cells (HMEC), was independently amplified with each primer set using SYBR Green. A product is detected only for the BTK-A positive malignant B cell line Namalwa using the BTK-A specific primer set (data not shown). However, products are detected in all tested breast samples using the BTK-C specific primer set. Both the BT474 and MCF7 breast cancer cell lines have 4-fold more BTK-C transcript compared to either the non-tumorigenic MCF10a and HMEC breast cells

or to the malignant B-cell line Namalwa, while MDA-MB-361 cells had approximately 2 fold more expression compared to MCF10a or HMEC cells (Fig. 5B).

Although BTK-C expression was higher in each of the cancer cell lines tested, isoform-specific expression determinations in tissue sample RNA were more complex. Ten of 23 breast cancer samples had significantly higher expression of BTK-C compared to BTK-A (Fig. 5C). Most of the other breast cancer samples had relatively low levels of both isoforms, with only one tumor sample showing higher levels of BTK-A compared to BTK-C. These results agree with mRNA expression profiling studies performed on clinical breast cancer tissues and made available in the Oncomine database (Oncomine, 2004). In these studies, BTK-A and BTK-C isoforms were not considered independently. In the TCGA data set, the median BTK expression was upregulated 2-fold in invasive breast carcinoma, 1.85 fold in invasive breast ductal carcinomas, and 9-fold in invasive lobular breast carcinoma compared to normal breast tissue (The Cancer Genome Atlas, 2011). In a second study, total BTK levels are elevated 6.86 fold (P value = 6.50E-6) in the stroma of invasive ductal cancers (7 samples) compared to all matched, cancer-free, breast tissue samples analyzed (15 samples) (Karnoub et al., 2007). In another study that profiled the mRNA expression levels of 54 breast cancer samples to 9 non-pathogenic tissue samples, BTK expression was 5.4 fold higher in breast carcinomas (5 samples), 3.2 fold higher in invasive ductal breast carcinoma samples (32 samples), and 4.2 fold higher in invasive lobular breast carcinoma samples (7 samples) compared to non-pathogenic tissue samples (9 samples) (Radvanyi et al., 2005). Taken together, these results indicate that BTK-C expression is enhanced in breast cancer cells compared to non-tumorigenic breast cells, further supporting the notion that expression of the BTK-C transcript, through use of an alternative promoter, contributes to the survival of these cells.

Despite decades of study in hematopoiesis, relatively little is understood about the effectors of BTK. To explore potential mechanisms of BTK-C in breast cell survival, overexpression constructs were used to determine if the BTK-A and BTK-C isoforms are functionally distinct. Assessments of growth rate, morphology, or resistance to apoptotic agents in BT474 cells stably over-expressing either the BTK-A or the BTK-C form proteins, under either standard growth conditions or serum-free conditions, reveal no significant differences (data not shown). Furthermore, a bioinformatic analysis (ExPASy Proteomics Tools) of exon 1C to identify conserved motifs, including cleavage sites, phosphorylation, N-glycosylation, or N-myristoylation sites, does not identify any putative functional roles.

Since BTK activity has been shown to affect the nuclear localization and activation of a number of transcription factors in hematopoietic cells, we performed microarray analysis of MCF-10A cells overexpressing the BTK-C isoform. In these experiments, BTK-C expression from a retrovirus CMV promoter is increased approximately eightfold compared to vector (data not shown). Microarray analysis of MCF-10A expressing either the BTK-A or BTK-C isoforms indicates that BTK may also affect transcriptional targets in breast cells. In these experiments, expression from a retrovirus CMV promoter of either BTK isoform was increased approximately eightfold more than vector. One gene upregulated in cells overexpressing BTK-C is STEAP4 which participates in a wide range of biologic processes (Gomes et al., 2012; Grunewald et al., 2012), such as control of cell proliferation and

apoptosis, and glucose uptake (Qin et al., 2011). Since BTK-C also has similar effects on proliferation and apoptosis resistance, we tested whether BTK-C activity was correlated with glucose uptake by assaying 2-NBDG fluorescence. LFM-A13 inhibits glucose uptake in those breast cancer cell lines that express BTK-C (Figs. 6A and 6B). Overexpression of BTK-C in MCF-10A cells also results in increased glucose uptake that is inhibited by LFM-A13 (Figs. 6C and 6D). These results are consistent with the notion that BTK, by altering the expression of STEAP4, has the potential to influence both tumor chemoresistance and energy metabolism, both of which are key features of the cancer cell phenotype.

DISCUSSION

Using an unbiased RNAi approach to screen the human TKs, we have found that 30% of the TKs examined strongly contributed to the viability of BT474 breast cancer cells. Among these, 54% were receptor TKs and 46% were nonreceptor cytoplasmic TKs and, with few exceptions, were distinct from those identified as survival kinases in an RNAi screen carried out in HeLa cells (MacKeigan et al., 2005). The validity of the breast screen is supported by the identification of several kinases with known transformative properties. ERBB-2, in particular was expected to be required for BT474 proliferation since it is amplified and constitutively activated in this breast cancer cell line. Other TK's identified in the screen have established roles in breast cancer. FGFR2 is amplified or overexpressed in 5–10% of breast tumors (Adnane et al., 1991; Penault-Llorca et al., 1995; Cha et al., 2008), and has been the focus of several genome-wide association studies covering thousands of unique breast tumors (Hunter et al., 2007). NTRK2/TRKB is the brain-derived neurotrophic factor (BDNF) receptor and is expressed in a subset of high-grade human breast tumors (Cameron and Foster 2008).

The major and largely unexpected discovery of the screen was the identification of a functional role for the cytosolic, nonreceptor tyrosine kinase BTK in breast cancer cell survival. BTK is thought to function primarily in cells derived from the hematopoietic cell lineage, where it is crucial for B cell maturation. Although a recent study has indicated that the expression of this kinase is elevated in a subset of basal breast tumors due to the presence of tumor-infiltrative lymphocytes (Sabatier et al., 2011), our study focused on tumor cells. Inhibition of BTK using RNAi or pharmacological inhibitors results in increased apoptosis in BT474 and MCF-7 breast cancer cells (Figs. 1B and 1C; and 4B). Importantly, RNAi-triggers specific to the alternate isoform, BTK-C, which is expressed in breast cancer cells compromise cell survival; BTK-C overexpression inhibits apoptosis induced by doxorubicin (Figs. 4D and 4E), both indicating that at least some of the pro-survival effects of BTK are due to this isoform.

Although no role has previously been described for BTK in epithelial cell cancers, dysregulated BTK expression is associated with the formation and maintenance of leukemias and lymphomas and is currently being targeted therapeutically. BTK overexpression has been implicated in imatinib resistance to chronic myeloid leukemia and acute lymphoblastic leukemia (Villuendas et al., 2006); (Hofmann et al., 2002). Its constitutive activation due to deregulated B cell receptor (BCR) engagement contributes to the genesis of B cell lymphomas (Irish et al., 2006); (Kuppers 2005) and new inhibitors are

currently in clinical trials for lymphoid malignancies (Honigberg et al., 2010; Winer et al., 2012). It is worth noting that a number of tyrosine kinase genes related to B cell signaling were also identified in the screen as being important for the proliferation or survival of BT474 cells. These include LYN which induces BTK phosphorylation and ABL2 (Lin et al., 2009) which may regulate BTK (Backesjo et al., 2002) among others.

The BTK protein expressed in breast cells has an amino-terminal 34 amino acid extension, which we have termed BTK-C. Higher levels of this BTK-C mRNA are found in breast cancer cells compared to non-cancerous breast cells. Early experimentation has not detected functional differences between the BTK-A and BTK-C proteins. It may be that deregulation of an alternative promoter in breast cancer cells causes increased expression of BTK-C and that this provides an essential function for these cells. In this sense, BTK-C is similar to other cancer related genes. Recent work has shown that alternative promoter usage in genes involved in cancer initiation and progression are significantly more likely to have multiple promoters than are non-cancer causing genes (Davuluri et al., 2008). For example, the aberrant use of an alternative promoter in the *TGFB3*, *LEF1*, and *CYP19A1* genes have been directly linked to cancer cell growth (Harada et al., 1993; Archey et al., 1999; Li et al., 2006). More recently, the expansion of genomic platforms has led to genome-wide views of promoter usage that have revealed increased alternative promoter usage in tumor cells compared to normal cells (Thorsen et al., 2011).

At present, the mechanism for BTK supporting breast cancer cell survival is unknown. BTK, like several kinases identified in the screen, has multiple protein-protein/protein-lipid interaction domains, enabling the formation of numerous and diverse signal complexes. This complexity has made its mechanism of action in B cells, where it is relatively well studied, poorly understood. In breast cells, microarray analysis indicates that BTK may affect transcription of specific targets. Among transcripts more than 2.5-fold upregulated by BTK-C compared to BTK-A were the calcium handling proteins calbindin (CALB1) and troponin (TNNT2) which may suggest a role for this kinase in calcium signaling as occurs in B cells, and STEAP4 which implicates BTK-C in the increased glucose uptake found in a number of breast cancer cells (Fig. 6). In many ways, these findings are similar to recent studies that indicate that other B cell kinases, including Abl (Srinivasan and Plattner 2006) (Srinivasan et al., 2008), BMX (Dai et al., 2006; Jiang et al., 2007) and Syk (Ruschel and Ullrich 2004), have critical functions in solid tumors and not just haematological malignancies. In these cases the mechanisms are incompletely understood and may involve pathways different from those operating in B cells. Further experimentation is necessary for the elucidation of how BTK and the other B cell kinases become integrated in epithelial cell signal transduction pathways.

Acknowledgments

Supported by: U.S. Army Medical Research Acquisition Activity grant DAMD17-02-1-0729 and NCI grant R01CA136658 to DSC.

The authors would like to thank members of the Conklin lab for helpful suggestions.

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Figure 1.

An RNAi screen targeting tyrosine kinase genes in an ERBB2 positive breast cancer. A: BT474 breast cancer cells were transfected with 234 shRNA constructs targeting 83 protein tyrosine kinase genes. Three transfection mixes were produced for each shRNA and each was transfected into triplicate wells of BT474 cells for 96 hr. AlamarBlue was used to monitor cell proliferation and viability. The averages of the nine parallel cultures were calculated for each shRNA, normalized to transfection efficiency, presented as the % of control shRNA and sorted on the basis of effect. shRNAs that produced a greater than 50% decrease in AlamarBlue signal are shown in red and are presented in Table 1. B: siRNA knockdown of BTK in BT474 cells (48 hr) results in increased cleaved caspase-3 (Caspase-3) staining indicative of apoptosis. C: Degree of apoptosis due to BTK knockdown in BT474 and MCF-7 cells was calculated as a percentage of the total cellular population. The data were expressed as the mean of triplicate of the samples transfected with the BTK siRNA relative to scrambled siRNA control samples. Error bars represent standard deviation from the average of three replicates. Statistical significance between samples was calculated using the student's *t* test, where (*) indicates a *P* value of <0.0001. [Color figure can be viewed in the online issue, which is available at wileyonlinelibrary.com.]

Figure 2.

An alternative form of the BTK transcript is present in BT474 breast cancer cells. A: Nucleotide sequence 1 – 395 bp from the published BTK sequence (accession # U13399) was aligned to the nucleotide sequence obtained from BT474 cells using 5' RACE. Identical sequence is highlighted in grey. The BT474 sequence obtained using 5' RACE encodes an additional 34 amino acid open reading frame (ORF) and contains two additional methionine codons, highlighted in grey, that are in frame with the methionine start codon from the published BTK gene (highlighted in grey and with an arrow). B: Schematic representation showing alternate splicing of alternative first exons from both isoforms inferred from sequence analysis. Sequences are identical from exons 2 through 19. C: Map of BTK on the X-chromosome. BTK-C exon 1 transcription initiates divergently 255 bp from the start site of the ribosomal protein L36a gene.

Figure 3.

The BTK-C gene produces an 80 kD product. A: A schematic representation showing the domains of the BTK-A and predicted BTK-C protein. B: Total lysate from breast lines and Namalwa B-cells subjected to immunoblotting and probed with an anti-BTK antibody (BD Transduction Laboratory, 611116). C: HEK293 cells cotransfected with a BTK-C flag vector and either BTK-C siRNAs or Non-Target siRNA. Total lysate was prepared 96 hr post transfection and was used for immunoblotting with anti-Flag antibody. D: BT474 cells were transfected with two BTK-C specific siRNAs, non-target siRNA as a control and cotransfected with GFP to mark transfected cells. Transfected cells were counted at 24 and 96 hr and the 96 to 24 hr ratio was calculated and expressed as % of the control.

Figure 4.

BTK-C is activated in BT474 cells. In BT474 cells both forms of the over-expressed BTK-C proteins are phosphorylated on tyrosine residue 223, which becomes autophosphorylated after activation. BT474 cells containing the stably integrated BTK-A-flag, the BTK-C-flag or control flag vector were treated with 100 μ M LFM-A13 for 45 min. Tyrosine-phosphorylated BTK was assessed by immunoprecipitation (IP) using anti-Flag (Stratagene) and immunoblot analysis using anti-BTK Phospho (pY223) and anti-BTK antibody (BTK-E9 Santa Cruz). B: Inhibition of BTK autophosphorylation using LFM-A13 results in increased apoptosis. BT474 cells incubated with 35 μ M LFM-A13 for 48 hr results in increased cleaved caspase-3 (Caspase-3) compared with control cells treated with DMSO. Apoptotic cells were calculated as a percentage of the total cellular population as in Figure 1C. C: BTK-C inhibits apoptosis induced by doxorubicin in MCF-10A cells. BTK-C expression in vector control (10A-Vec) and MCF-10A-BTK-C (10A-Btk-C) cells using anti-Flag antibody. GAPDH is used as a loading control. D: BTK-C expression reduces Doxorubicin-induced apoptosis as monitored by Cleaved caspase-3 signal. 10A-Vec or 10A-

BTK-C cells were either treated with DMSO (Con) or with 35 μ M LFM-A13 for 24 hr, after that the cells were washed with PBS for three times and added fresh medium with doxorubicin(1 μ M) for 24 hr. Immunofluorescence was performed for cleaved caspased-3 signal; cell nuclei were stained with Hoechst 33342. E: Apoptotic cells were calculated as a percentage of the total cellular population, as indicated B. Error bars indicate the standard deviation from three individual experiments, * $P < 0.01$. [Color figure can be viewed in the online issue, which is available at wileyonlinelibrary.com.]

Figure 5.

BTK is more abundant in breast cancer cells compared to nontumorigenic breast cells. A: BTK protein levels were examined in normal, matched breast tissues and breast carcinoma tissue in tissue microarrays using immunofluorescence microscopy. DAPI staining of nuclei is shown in cyan false color; anti-BTK (ProSci) staining is red. Tissue samples and BTK classifications were (i) normal-low level; (ii) benign hyperplasia-low level; (iii) cancer-low-moderate/heterogenous; (iv) cancer-heterogenous with strong positives; (v) cancer-homogenous moderate with nuclear; (vi) cancer-negative. B: BTK-C message is more abundant than the BTK-A isoform in cancer cell lines. qPCR primers designed to target specifically the BTK-C message and cDNA from the breast cancer cell lines BT474, MCF7, MDA-MB-361, and two nontumorigenic breast cell lines, HMEC and MCF10a, were amplified using SYBR Green. Fold change was calculated using the delta, delta Ct method. Error bars represent standard deviation from the average of four replicates. Statistical significance between samples was calculated using the student's t test, where (*) indicates a *P* value of < 0.005 and (**) indicates a *P* value of < 0.0005. C: BTK-C message is more abundant than the BTK-A isoform in breast tumors. cDNA prepared from RNA isolated from human breast tissue was subjected to qPCR using primers specific for BTK-A and BTK-C isoforms. The same set of samples in another plate was used for detection of actin mRNA. The data represent relative mRNA levels of each BTK isoform normalized to actin. [Color figure can be viewed in the online issue, which is available at wileyonlinelibrary.com.]

Figure 6.

BTK-C promotes glucose uptake. A: LFM-A13 inhibits glucose uptake in breast cancer cell lines. MCF-7, MDA-MB-361, and BT474 cell lines. Cells were either treated with DMSO (Control) or with 35 μ M LFM-A13 for 24 hr, at which point cells were washed with PBS three times and 100 μ M 2-NBDG added for 15 min. Immunofluorescence images were acquired with an INCELL-1000; (B) Fluorescence intensity quantification. Error bars indicate standard deviation from three individual experiments, $*P < 0.01$. C: The effect of BTK-C on glucose uptake in breast cells. 10A-Vec and 10A-BTK-C cells cell lines were treated as in A. Fluorescence intensity is shown in (D), $*P < 0.01$. [Color figure can be viewed in the online issue, which is available at wileyonlinelibrary.com.]

TABLE 1

Tyrosine Kinase Genes which when Silenced Result in Greater than 50% Reduction in Normalized Proliferation

Symbol	Name	z-score
ABL2	V-abl Abelson murine leukemia viral oncogene homolog 2	-5.68
BTK	Bruton agammaglobulinemia tyrosine kinase	-4.95
FES	Feline sarcoma oncogene	-4.35
NTRK2	Neurotrophic tyrosine kinase, receptor, type 2	-3.95
PTK2B	Protein tyrosine kinase 2 beta	-3.95
Ptk9	Protein tyrosine kinase 9	-3.38
FGFR2	Fibroblast growth factor receptor 2	-3.20
EPHA1	EPH receptor A1	-3.00
LYN	V-yes-1 Yamaguchi sarcoma viral related oncogene homolog	-2.93
FLT3	Fms-related tyrosine kinase 3	-2.78
IGF1R	Insulin-like growth factor 1 receptor	-2.68
EGFR	Epidermal growth factor receptor	-2.45
JAK1	Janus kinase 1 (a protein tyrosine kinase)	-2.25
DDR1	Discoidin domain receptor family, member 1	-2.15
TXK	TXK tyrosine kinase	-2.08
EPHB2	EPH receptor B2	-2.08
EPHA4	EPH receptor A4	-2.05
RYK	RYK receptor-like tyrosine kinase	-1.95
PDGF	RBPlatelet-derived growth factor receptor, beta polypeptide	-1.83
ERBB2	V-erb-bZ erythroblastic leukemia viral oncogene homolog 2	-1.80
ZAP70	Zeta-chain (TCR) associated protein kinase 70 kDa	-1.70
JAK2	Janus kinase 2 (a protein tyrosine kinase)	-1.45
BMX	BMX non-receptor tyrosine kinase	-1.43
TEC	Tec protein tyrosine kinase	-1.30
EPHB6	EPH receptor B6	-1.15