Am J Cancer Res 2013;3(2):230-239
www.ajcr.us /ISSN:2156-6976/ajcr0000176

Original Article

Correlation of Notch1, pAKT and nuclear NF-κB expression in triple negative breast cancer

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Received February 8, 2013; Accepted March 15, 2013; Epub April 3, 2013; Published April 13, 2013

Abstract: Gene expression profiling reveals elevated Notch1 mRNA expression in triple negative breast cancers (TNBC), both basaloid and claudin-low subtypes. Notch ligands, Jagged1 and Jagged2, have been correlated with poor prognosis in TNBC. AKT, an oncogenic protein kinase family that is activated downstream of Notch in breast cancer cell lines, is frequently activated in breast cancer. Recent publications suggest that inhibition of cell growth, migration, invasion, and induction of apoptosis caused by Notch1 or Jagged1 inhibition may be attributed in part to inactivation of the AKT signaling pathway. There is significant evidence that Notch1 activates NF-κB in several models, and that AKT can mediate NF-κB activation. In this study, we evaluated Notch1 protein expression by immunohistochemistry (IHC) and correlated this with expression of pAKT and nuclear NF-κB p65 (RelA) in TNBC. A tissue microarray (TMA) containing 32 formalin-fixed, paraffin-embedded (FFPE) TNBC tumor specimens was constructed from the archival tissue database of the Department of Pathology at UMMC and IHC for Notch1 protein, pAKT 1/2/3 (Ser473), and NF-κB, p65 subunit was performed on the TMA with appropriate positive and negative controls. Of the 32 TNBC in our cohort, 100% expressed Notch1 protein by IHC: 24 (75%) showed cytoplasmic expression, 25 (78%) showed membranous expression, and 17 (53%) showed both cytoplasmic and membranous expression. Overall, 29 (91%) expressed pAKT by IHC: 28 (97%) showed cytoplasmic expression, 14 (48%) showed nuclear expression and 13 (45%) showed both cytoplasmic and nuclear expression. Nuclear staining for NF-κB p65 was detected in all 32 TNBC specimens with variable intensities. On bivariate analysis, cytoplasmic Notch1 was significantly correlated with cytoplasmic pAKT ($r = 0.373$, $P = 0.035$) and nuclear NF-κB ($r = 0.483$, $P = 0.005$); both cytoplasmic and nuclear pAKT significantly correlated with nuclear NF-κB ($r = 0.391$, $P = 0.027$; $r = 0.525$, $P = 0.002$, respectively). These results suggest that 1) the cross-talk between Notch1, AKT and NF-κB identified in preclinical models may operate in a significant fraction of human TNBC, and 2) combination therapy with agents targeting these pathways warrants further investigation.

Keywords: Triple negative breast cancers (TNBC), Notch1, AKT, NF-κB, immunohistochemistry (IHC), tissue microarray (TMA)

Introduction

Triple-negative breast cancers (TNBC) lack expression of estrogen receptor α (ERα) and progesterone receptor (PR) and do not have genomic amplification of human epidermal growth factor receptor-2 (HER2/Neu). TNBC account for approximately 15% of all breast cancers, and are more prevalent among young, African-American, and Latino patients. Recent genomics studies suggest that TNBC include six subgroups with different gene expression profiles: basal-like1, basal-like2, immunomodulatory, mesenchymal, mesenchymal stem-like, and luminal androgen-receptor [1, 2]. A different classification scheme subdivides TNBC into basal-like, Her2-enriched, and claudin-low [3]. IHC markers for basal-like tumors include cytokeratins (CK) 5 and 6 and c-Kit. Claudin-low tumors are characterized by loss of expression of several claudins, which are adhesion molecules involved in intercellular junctions. A recent multi-platform classification of breast cancers based on genomic sequencing, gene expression profiling, and proteomics indicated that 80% of TNBC fall within the basal-like subgroup, 9% fall within the Her2-enriched subgroup, and the remainder have mixed characteristics.

Basal-like tumors are also characterized by frequent mutations in the PI3K-AKT pathway and elevated expression of phospho-AKT [4]. Due to its aggressive behavior, poor prognosis, and insensitivity to currently available ER-targeted and Her2-based therapies, there is increasing research interest in the clinical, biologic, and epidemiologic features of TNBC [5-8]. A better understanding of the pathological mechanisms underlying TNBC development and progression may allow improved classification, risk stratification, and individualized treatment for this breast cancer subgroup.

The Notch signaling pathway regulates cell fate decision, proliferation, and death. Emerging evidence suggests that the Notch signaling pathway is likely to be involved in the pathogenesis of TNBC. Cell line data show that basal-like TNBC have elevated Jagged-1 levels and BRCA-1 mutant breast cancers, which are typically basal-like TNBC subtype, show elevated Jagged-1 expression compared with their BRCA2 (predominantly luminal) counterparts [9]. Resection specimens from TNBC show a statistically significant association between increased expression of Notch ligands/receptors and basal-like TNBC [10, 11]. Notch1 is highly expressed in breast cancer, compared with normal tissue, and segregates with basal-like TNBC [12]. Recently, it has been reported that Notch receptor mRNAs are differentially expressed in breast cancer subgroups. Hormone receptor positive cancers express Notch4, whilst Notch1 and Notch3 receptors are involved in the development of TNBC [13]. Further, more than half of primary ER (+) PR (+) breast cancers contain an ER (-) PR (-) CK5 (+) "luminobasal" subpopulation exceeding 1% of cells. Luminobasal cell expansion in response to hormone therapies is regulated by Notch1 signaling and can be blocked by γ-secretase inhibitors [14]. An immunohistochemical analysis of Notch1 and Notch4 expression in 29 TNBC cases revealed that both Notch1 and Notch4 receptors are overexpressed in tumor and vascular endothelial cells with subcellular location different from that of hormone receptor-positive breast cancer [15]. Recently, activating mutations in Notch1 and Notch2 were described in a small subset of TNBC [16]. Additionally, Xu et al. have described loss of Notch negative regulator Lunatic Fringe (LFNG) in the majority of basal-like breast cancers. Targeted deletion of LFNG in the mammary gland of mice was sufficient to cause TNBC-like mammary tumors, which are Notch-driven and contain high levels of activated Notch1, 2, 3, and 4. The same authors demonstrated that Notch1 mRNA expression is highest in basal-like TNBC, followed by claudin-low TNBC and other breast cancer subtypes [17].

The PI3K/AKT/mTOR pathway plays a key regulatory function in cell survival, proliferation, migration, metabolism, angiogenesis, and apoptosis. The pathway is constitutively activated in various malignancies, including breast cancer [18]. The PI3K pathway is up-regulated in basal-like carcinomas, as shown by a significantly increased activation of downstream targets, AKT and mTOR [19]. López-Knowles E et al. found that PI3K pathway activation was significantly associated with ER/PR negative status, high tumor grade, and a "basal-like" phenotype, where 92% of tumors had an altered PI3K/AKT pathway [20]. These results were confirmed by recent multi-platform classification efforts [4]. TNBC may also harbor recurrent MAGI3-AKT3 fusion, leading to constitutive AKT activation, which can be abolished by treatment with an ATP-competitive small-molecule AKT inhibitor [21]. Together, these data suggest that the PI3K/AKT pathway may have a role in the pathological mechanisms of TNBC.

There is growing evidence that Notch regulates the AKT pathway in several normal and cancer cell types [22-43]. Notch1 activation apparently enhances melanoma cell survival via activation of the AKT pathway [44]. Palomero et al. report that Notch1 induces up-regulation of the PI3K-AKT pathway via Hes-1, which negatively controls the expression of PTEN in T-cell acute lymphoblastic lymphoma [45]. Notch signaling induces an autocrine signaling loop that activates AKT in breast epithelial cells, and the inhibition of Notch signaling in breast cancer cells induces a decrease in AKT activity [33]. Cytoplasmic, non-canonical Notch signaling through PI3K and/or mTORC2 has been shown to activate AKT in normal and neoplastic T-cells [42, 46]. Recent publications suggest that inhibition of cell growth, migration, invasion, and induction of apoptosis caused by Jagged-1 or Notch1 inhibition may be attributed, in part, to inactivation of the AKT signaling pathway [32, 47].

Table 1. List of primary antibody information

	Source	Cat. #	Isotype	Dilution ratio	Incubation condition
Notch1 (C-20)	Santa Cruz Biotechnology	sc-6014	Goat IgG	1/100	30 minute at R.T
p-Akt 1/2/3 (Ser 473)	Santa Cruz Biotechnology	sc-7985-R	Rabbit IgG	1/200	One hour at R.T.
Anti-NFκB p65	Millipore	MAB3026	Mouse IgG3	1/300	Overnight at 4°C

The NF-κB pathway, another major regulator of cell survival, proliferation, and differentiation, is also regulated by Notch signaling in human cancer cells [48-54]. NF-κB is widely involved in the initiation and progression of breast cancer, and activation of this pathway is commonly detected in TNBC of several subtypes [1, 2]. Moreover, AKT pathway activation can result in NF-κB activation via multiple mechanisms, including direct phosphorylation of IKKα at Ser23 [55]. NF-κB inhibitors are attractive candidates for TNBC treatment. Genistein, a phytochemical originally isolated from soybean, was reported to inhibit the growth of MDA-MB-231 TNBC cells by inhibiting NF-κB activity via the Notch1 pathway [56]. However, the precise molecular mechanisms whereby activation of Notch signaling contributes to TNBC development and progression remain poorly understood.

In this study, we evaluated Notch1 protein expression in TNBC by IHC and correlated this with the expression of pAKT, nuclear NF-κB p65 (RelA), and basal-like markers, cytokeratin 5/6 and c-Kit. Our results demonstrate a significant correlation between Notch1, pAKT, and NF-κB expression in TNBC. These novel results suggest that the cross-talk between Notch1, AKT, and NF-κB identified in preclinical models may also operate in a significant proportion of human TNBC.

Materials and methods

Tissue collection and tissue microarray (TMA) construction

The University of Mississippi Medical Center (UMMC) Institutional Review Board approved the study protocol. We retrieved clinical information and pathology reports from our institutional database, and included 32 formalin-fixed paraffin-embedded (FFPE) TNBC tissue blocks from the pathology archives. Two pathologists confirmed the histologic features of TNBC in hematoxylin- and eosin-stained slides and provided topographic correlation with corresponding paraffin blocks. We used a Beecher MTA1 Manual Tissue Arrayer to transfer a 2 mm cylindrical core from each specified site of the primary FFPE blocks to a composite paraffin block to construct a tissue microarray (TMA). The resulting TMA block was sectioned at 5 μm intervals prior to immunohistochemical (IHC) studies.

Immunohistochemical staining

TMA sections were placed in a 60°C oven overnight for tissue adherence. The sections were deparaffinized in xylene, rehydrated through graded ethanol, and washed with PBS before being treated with 1X Reveal in a Decloaking Chamber (Biocare Medical, CA) for antigen retrieval following the manufacturer's protocol. After rinsing in PBS for 15 min, the sections were soaked in 3% H_2O_2 in PBS for 20 minutes to quench endogenous peroxidase activity. We then applied 3% normal rabbit serum (for goat primary antibody), horse serum (for mouse primary antibody), and goat serum (for rabbit primary antibody) to block non-specific binding sites. The dilution ratios and incubation conditions of primary antibodies were determined in pilot experiments and are shown in **Table 1**. Species-matched normal non-immune IgG used at the same concentration as primary antibodies served as negative controls. Following extensive washing in PBS, antigen-antibody complexes were detected using the ABC Elite kit from Vector Laboratories and staining was performed with ImmPact™ DAB peroxidase substrate kit. Sections were then counterstained in Gill's hematoxylin and dehydrated in ascending grades of ethanol before clearing in xylene and mounting under a coverslip using Cytoseal XYL. Two investigators evaluated the staining results of Notch1, pAKT and NF-κB according to subcellular localization, and scored the intensity of immunolabeling as negative (0), weakly positive (1+), moderately positive (2+), or strongly positive (3+). To explore the relationship between Notch1 expression and basal-like markers, we also evaluated the expression of cytokeratin 5/6 and c-Kit in our

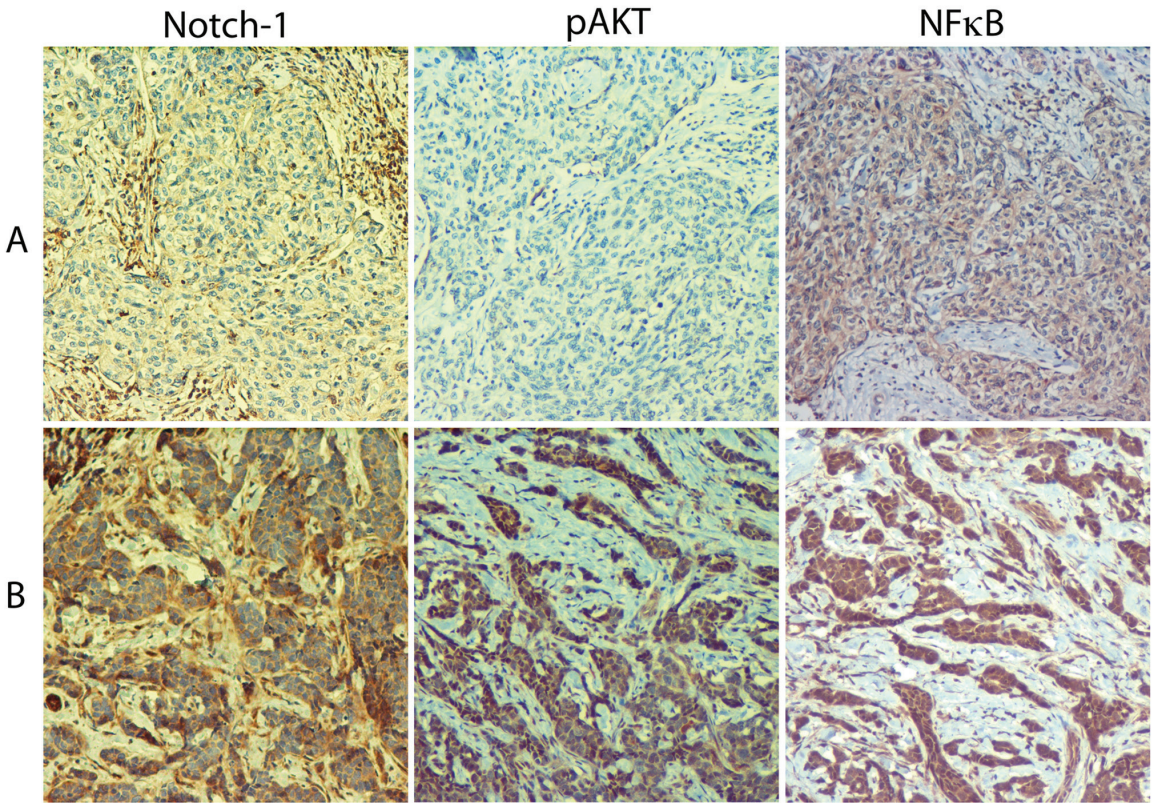

Figure 1. Expression of Notch1, pAKT and NFκB in triple negative breast cancer (TNBC) determined by IHC. A: A representative TNBC case showing low expression of Notch1, pAKT and nuclear NFκB; B: A representative TNBC case showing high expression of Notch1, pAKT and unclear NFκB.

TNBC cohort. Due to exhaustion of the TMA block, the expression of CK5/6 and c-Kit was evaluated in only 30 of the 32 cases. For each case, we defined (i) cytokeratin 5/6 expression as unequivocal cytoplasmic staining in >50% of tumor cells; and (ii) c-Kit expression as strong and diffuse cytoplasmic staining in the majority of tumor cells.

Statistical analysis

Spearman correlation test was used to evaluate correlation between Notch1, pAKT, and nuclear NF-κB p65 expression. Fisher's exact test was employed to explore associations between Notch1 expression and cytokeratin 5/6 expression or c-kit expression. The level of statistical significance was set at $P < 0.05$ and all P values were 2-sided. Statistical analysis was performed using the statistical software SPSS version 19.0 (SPSS Inc., Chicago, IL).

Results

Representative images of TMA-based IHC staining are shown in **Figure 1**. We detected Notch1 expression in all 32 TNBC specimens: 24 (75%) showed cytoplasmic expression, 25 (78%) showed membranous expression, and 17 (53%) showed both cytoplasmic and membranous expression. Additionally, nuclear staining was detected in 7 TNBC specimens (22%), of which 5 cases had <5% nuclear staining for individual cancer cells. Because this staining pattern is consistent with the short half-life of nuclear Notch1 and with our previous experience, we did not enter nuclear Notch1 expression into the correlation analysis. We detected pAKT expression in 29 of 32 (91%) TNBC specimens: 28 (97%) showed cytoplasmic signal, 14 (48%) showed nuclear signal, and 13 (45%) showed both cytoplasmic and nuclear signals. All 32 TNBC specimens showed moderate cytoplasmic staining and varying intensities of nuclear staining for NF-κB p65. Given the similar cytoplasmic staining intensity and the uncertain significance thereof, we excluded cytoplasmic expression of NF-κB p65 from statistical analysis. Statistical analysis revealed significant correlation of cytoplasmic Notch1 expression with cytoplasmic pAKT expression (r

Table 2. Correlation of Notch1 expression with pAKT and NFκB in TNBC

		Notch1 expression		pAKT expression		NFκB expression
		Cytoplasmic	Membranous	Cytoplasmic	Nuclear	Nuclear
Notch1	Cytoplasmic	--------	--------	r = 0.373, P = 0.035	r = 0.136, P = 0.457	r = 0.483, P = 0.005
	Membranous	--------	--------	r = 0.293, P = 0.104	r = 0.217, P = 0.233	r = 0.208, P = 0.254
pAKT	Cytoplasmic	--------	--------	--------	--------	r = 0.391, P = 0.027
	Nuclear	--------	--------	--------	--------	r = 0.525, P = 0.002

= 0.373, P = 0.035) and nuclear NF-κB expression (r = 0.483, P = 0.005). Further analysis revealed significant correlation of nuclear NF-κB expression with cytoplasmic (r = 0.391, P = 0.027) and nuclear pAKT expression (r = 0.525, P = 0.002). The associations between membranous Notch1 expression and either cytoplasmic pAKT (r = 0.293, P = 0.104) or nuclear NF-κB (r = 0.208, P = 0.254) were not statistically significant (**Table 2**). Of the 30 TNBC with Notch1 protein expression, 6 (20%) expressed cytokeratin 5/6, 17 (57%) expressed c-Kit, 4 (13%) expressed both cytokeratin 5/6 and c-Kit, and 11 (37%) expressed neither cytokeratin 5/6 nor c-Kit. There was no significant association (P > 0.05) between Notch1 expression (cytoplasmic or membranous) and cytokeratin 5/6 expression or c-Kit expression.

Discussion

In this study, we analyzed the subcellular localization of Notch1 and pAKT in TNBC specimens. The subcellular localization of Notch1 and pAKT expression may provide us with important clues for the activity status of relevant pathways, and therefore is potentially more informative than simple staining intensity and/or fraction of positive cells. Previous studies from our group have shown that (1) estrogen-treated ERα-positive breast cancer cells have high levels of Notch1 membranous expression and relatively lower levels of Notch1 cytoplasmic/nuclear expression [57]; (2) there is significantly more membranous Notch1 expression in ERα-positive breast cancers compared with ERα-negative breast cancers (73% vs. 27%), suggesting that tumors with active estrogen pathways tend to accumulate Notch1 at cell surfaces [58]. Conversely, in our study of 32 TNBC specimens, Notch1 staining was evenly distributed between membrane and cytoplasm (78% and 75%, respectively). Also, nuclear staining was demonstrated in 7 of the 32 (22%) TNBC. The rate of nuclear staining in our study is higher than previously reported from an IHC study of mostly ERα-positive breast cancers (22% vs. 6%) [58]. Therefore, our results suggest that both cytoplasmic and nuclear Notch signaling may be active in TNBC. Recently, Speiser et al. reported that in 25 TNBC cases, the subcellular localization of Notch1 was predominantly nuclear and cytoplasmic, with simultaneous nuclear and cytoplasmic immunoreactivity in 64% of cases, cytoplasmic immunoreactivity in 36%, and no membranous expression in any case [15]. Likewise, our results show a relatively higher nuclear expression of Notch1 in TNBC when compared with what has been reported in ERα-positive breast cancer [58]. Our demonstration of membranous Notch1 expression in 25 (78%) TNBC may reflect TNBC heterogeneity or variation in experimental conditions.

The serine/threonine kinase AKT is a multifunctional kinase that is activated in response to a variety of extracellular signals. Three paralogs exist: AKT1, 2, and 3. Inactive cytosolic AKT is recruited to the plasma membrane in the presence of phosphoinositide triphosphate, where it is activated by phosphorylation of residues T308 by phosphoinositide-dependent kinase 1 (PDK1) and S473 by mTOR/rictor (mTORC2) [59, 60]. Activated AKT, as measured mostly by antibodies that recognize phosphorylated AKT at S473 (pAKT), can be found in the plasma membrane, the cytoplasm, and the nucleus [59]. The PI3K/AKT is one of the most frequently activated pathways in breast cancer. In basal-like TNBC, loss of function of negative regulator PTEN is the most common alteration, followed by constitutively activating AKT3 mutations [4]. Thus far, the clinical importance of pAKT localization and the mechanism(s) underlying this compartmentalization are not fully understood. Some authors suggest that

the oncogenic activity of AKT arises exclusively from the cytoplasm, possibly through regulation of cell size, energy metabolism, and translational control [59, 61]. However, Badve et al. recently reported that nuclear pAKT expression is associated with luminal type A breast cancers (as defined by ERα+ and/or PR+ phenotype), and nuclear-pAKT expression in ERα-positive breast cancer is significantly associated with better survival, suggesting that AKT activity and/or localization may be different in ERα-positive and ERα-negative breast cancers [62]. Our study showed cytoplasmic AKT expression in 28 (88%) of our TNBC cases and nuclear expression in 14 (44%). Furthermore, cytoplasmic Notch1 expression was significantly correlated with cytoplasmic pAKT expression, but not with nuclear pAKT expression. This unique correlation between cytoplasmic Notch1 and cytoplasmic pAKT in TNBC is consistent with the model of cytoplasmic Notch signaling originally proposed by the Sarin group [46]. The clinical significance of this correlation warrants further investigation.

The active form of NF-κB is a heterodimeric protein, consisting of p50 and p65 subunits. Once activated, NF-κB translocates from the cytoplasm to the nucleus, where it binds to specific DNA sequences and initiates transcription [63]. To detect nuclear NF-κB, we used a specific anti-NF-κB p65 subunit (RelA) monoclonal antibody, which recognizes an epitope overlapping the nuclear localization signal (NLS) in the p65 subunit of the most abundant NF-κB heterodimer (p50-p65). This epitope is masked when p65 is in an inactive complex with cytoplasmic IκB, and is only revealed upon IκB dissociation. Thus, the antibody selectively binds to the activated form of NF-κB [64]. Using this antibody, positive nuclear staining with varying intensities was detected in all 32 TNBC. Bivariate correlation analysis revealed a significant correlation between cytoplasmic Notch1 expression and nuclear NF-κB expression, which was consistent with our previous observations [49-51, 53, 65, 66]. Further analysis revealed significant correlation between nuclear NF-κB expression and both cytoplasmic pAKT and nuclear pAKT, which supports a possible biological link between PI3K/AKT and NF-κB pathways in TNBC.

Although basal-like TNBC cases showed high Notch1 expression in this study, no significant correlation between Notch1 protein expression and basal-like markers, cytokeratin 5/6 and c-Kit was observed. The lack of correlation between Notch1 and either CK5/6 or c-kit expression suggests that while Notch1 mRNA expression is highest in basal-like tumors followed by claudin-low tumors, Notch1 protein expression is frequently elevated in both basal-like and non-basal-like TNBC. A significant proportion (37%) of our cases did not express basal-like markers, CK5/6 and c-Kit, and it is possible that such non-basal-like cancers may have different Notch1 subcellular expression patterns compared to basal-like TNBC.

Many dysregulated signaling pathways have the potential for cross-talk, thereby forming functional networks that increase the aggressiveness of various cancers [52, 67, 68]. Such cross-talk often allows cancer cells to escape death in response to different pro-apoptotic signals, ultimately resulting in unregulated proliferation and the emergence of more aggressive and drug-resistant phenotypes [68]. Emerging evidence demonstrates that a complex cross-talk exists among Notch, PI3K/AKT, and NF-κB signaling pathways in various cancers [32, 44, 45, 47, 51, 53, 69, 70]. In our study, we demonstrate novel significant associations between the expression of Notch1, pAKT, and NF-κB in TNBC. These findings suggest that the cross-talk between Notch1, AKT, and NF-κB in several preclinical models may also operate in a significant fraction of human TNBC. Therefore, combination therapy with agents targeting Notch, PI3K/AKT, and/or NF-κB pathways may provide novel therapeutic approaches to the treatment of TNBC with co-activation of these pathways.

Acknowledgements

This work was supported by a grant (NIH P01 AG025531) from the National Institute of Health.

Conflict of interests

The authors declared no conflict of interests with respect to the research, authorship and/or publication of this article.

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