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Loss of Neuroligin 4X induces an intrinsic innate immune response in TNBC

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Abstract

Background

Immune checkpoint blockade therapies, which act on T cell inhibitory receptors, including CTLA-4 and PD-1, induce durable responses across diverse cancers. However, most patients do not respond to these therapies, and initially responsive cancers may relapse. Identifying molecular mechanisms that influence therapeutic responses and resistance is critical to realize the full therapeutic potential of immune checkpoint inhibitors. The presence of immune infiltrates in the tumor microenvironment is associated with positive outcomes in breast cancer, specifically in triple-negative breast cancer (TNBC). The underlying mechanisms driving this response are unclear. We have previously identified Neuroligin 4X (NLGN4X) as a protein expressed in TNBC.

Methods

Bioinformatic analysis was used for pathway analysis of TCGA TNBC patient dataset. Immunohistochemistry was performed on breast cancer tissue microarray for NLGN4X protein expression. RNA-seq was performed on MDA-MB-231 breast cancer cells for differential gene expression upon gene knockdown. Cytokine array, western blot, cell adhesion array and Nanostring was performed to determine the role of NLGN4X in TNBC.

Results

In this study, we report that NLGN4X expression is lost in breast cancer with lymph node metastasis. Its expression negatively correlates with immune markers *in vitro*, The Cancer Genome Atlas (TCGA) TNBC patient dataset, and metastatic breast cancer tissues. RNA-seq analysis of the MDA-MB-231 breast cancer cell line, silenced for NLGN4X by siRNA showed more than 500 differentially regulated genes. GSEA analysis of these genes revealed upregulation of interferon signaling pathway, cytokine signaling, and downregulation of cholesterol metabolism and lipid metabolism pathways. NLGN4X knockdown induced loss of cell adhesion, epithelial to mesenchymal transition (EMT), and MAVS-IRF7 signaling in breast cancer cells. Interestingly, analysis of the TCGA dataset of 104 TNBC patients also showed interferon signaling (IFN) as one of the significant pathways downregulated in TNBC patients expressing NLGN4X.

Conclusion

Loss of NLGN4X leads to innate immune activation in breast cancer and coincides with an aggressive phenotype of cancer. This study identifies the role of NLGN4X in regulating interferon signaling and immune microenvironment in TNBC.

Keywords: breast cancer, immunotherapy, Neuroligin 4X, interferon signaling, immune activation.

Introduction

Clinical evidence has shown that some breast tumors, including the subset of TNBC, elicit a robust antitumor immune response [1, 31]. TNBC tumors have high infiltration of immune cells including CD8+ T, CD4, macrophages, B cells and neutrophils. Patients with high intratumoral infiltrations of CD8+ cells respond well to immune checkpoint inhibitors (ICIs) and have better disease-free survival demonstrating a better disease-free survival [4, 22, 32]. The attraction of these immune cells is due to chemokines which mediate immune cell trafficking towards the tumor [30]. Among these chemokines, elevated levels of the C-C motif chemokine ligand 5 (CCL5), C-X-C motif chemokine ligand 9 and 10 (CXCL9 & CXCL10), are frequently associated with increased recruitment of CD8+ T lymphocytes to tumor sites [23]. Cancer cell intrinsic mechanisms play an important role in shaping tumor immune microenvironment. Studies have indicated that activation of DNA sensor, cGAS-STING [7, 10] and RNA sensor, retinoic acid inducible gene I (RIG-I) (encoded by *DDX58*) [9, 11] in tumor cells contributes to regulating antitumor immunity and responsiveness to checkpoint blockade. Tumor subtype, aberrant expression of oncogenes and mutational load highly impact the tumor microenvironment. For example, a recent study identified a DNA damage response-deficient (DDR^D) molecular subtype in breast cancer. This subtype was characterized by upregulation of Interferon Stimulated Genes (ISG) and lymphocyte infiltration in part due to inherent defect in DNA repair mechanism and activation of S phase STING [26]. Similarly, another study demonstrated that loss of *DDX3X* leads to activation of antiviral innate immune response by aberrant cytosolic accumulation of endogenous dsRNAs in the breast cancer cells, which triggers intrinsic type I IFN production via activation of cytoplasmic dsRNA sensing pathway [6]. Activation of these sensors can enhance the immunogenicity of otherwise poorly immunogenic tumors. A recent study showed that RIG-I activation in breast cancer cells resulted in tumor cell-intrinsic expression of inflammatory cytokines, leukocyte-recruiting chemokines, and increased expression of major histocompatibility (MHC)-I components [12]. Grasso et al., identified and defined distinct tumor immune microenvironment (TIME) by integrating spatial resolution of immune cells with laser capture microdissection gene expression and characterized fully inflamed (FI) tumors by low cholesterol/high IFN signaling signature. Patients with FI

TNBC have the best outcomes with standard-of-care chemotherapies and immune checkpoint blockade (ICB) [17]. However, molecular mechanism regulating the recruitment of these immune effectors to TME as well as in the tumor epithelium are not well understood.

Neuroligins constitute a family of neuronal transmembrane synaptic proteins whose structural and biochemical characteristics are indicative of a role in heterotypic cell adhesion. The neuroigin gene family consists of five members (NLGN1 at 3q26, NLGN2 at 17p13, NLGN3 at Xq13, NLGN4 at Xp22, and NLGN4Y at Yq11) [18, 39]. Their large extracellular N-terminal domain is homologous to serine esterases. They are of great importance in mediating synapse formation in the central nervous system, and they interact with neuroligins from the opposite side (in trans) of the synaptic cleft in a calcium-dependent manner [24]. Neuroligins have also been implicated in vascular remodeling during angiogenesis [16, 29]. Recent studies have shown a mitogenic effect of NLGN3 in glioma proliferation and progression [36].

Our previous the first study to link the expression of neuronal cell adhesion molecules, neuroligins, in breast cancer [18]. NLGN4X plays a vital role in enhancing tumorigenesis and aggressiveness of breast cancer with MCPH1 loss/mutation [35]. Here we describe for the first time that loss of NLGN4X activates MAVS-IRF signaling that induces IFN signaling and cytokine secretion from TNBC. NLGN4X expression negatively correlates with ISG and T-cell attractant cytokines in the patient dataset. Nanostring and immunohistochemistry (IHC) analysis of tissue microarrays from breast cancer patients shows high immune infiltration in NLGN4X-low breast cancer samples. This suggest that NLGN4X may constitute a novel biomarker of “fully inflamed” tumors.

Materials and Methods

Cell lines and Reagents

Fetal calf serum (FCS) and cell culture media (Dulbecco's modified Eagle's medium, DMEM) was purchased from Sigma (USA). Human breast cancer cell lines MDA-MB-231 and MDA-MB-468 were purchased from the American Type Culture Collection. MDA-MB-231 and MDA-MB-468 were maintained in DMEM supplemented with 10% fetal bovine serum (Sigma) at 37°C in 5% CO₂. All the antibodies were obtained from Cell Signaling Technology and Abcam.

siRNA transfection

siRNAs for human NLGN4X and scrambled siRNA were purchased from Integrated DNA Technologies (IDT, Coralville, Iowa, USA). Human MDA-MB-231 and MDA-MB-468 cells were transfected with Lipofectamine RNAiMAX (Invitrogen) according to the manufacturer's protocol. To study NLGN4X gene knockdown, 10⁵ cells in 6-well culture plates were transfected with NLGN4X-specific siRNA(UGAGAGAUCCUACUGCAUGACATG,AAGUAUCCAAAUUGGCGGUAAACCAGA,CCAAUCGAUGUUUAGUGUGAUAGGA) (80 nM) or scrambled siRNA and mixed with Lipofectamine RNAiMAX (Invitrogen, Carlsbad, CA) in optiMEM media. The plates were rocked gently at room temperature (RT) and incubated at 37°C for 72 h.

Western Blot

Cells were lysed in ice-cold complete 1x RIPA buffer (PMSF solution, sodium orthovanadate solution, protease inhibitor cocktail solution, and 1x lysis buffer) (Santa Cruz Biotechnology, Santa Cruz, Ca). The proteins were then quantified using the BCA Protein Assay Kit (Pierce Biotechnology, Rockford, IL). 25 µg of protein from each sample was separated by a 4–12% SDS-PAGE gel and then transferred to a 0.2 µm polyvinylidene difluoride (PVDF) membrane. Membranes were first blocked with 5% nonfat dry milk in TBS-T and incubated overnight with primary antibodies (Rig 1, MDA5, MAVS, Anti IRF 7, IFIT3, Slug, P-SMAD2/3, Zeb1, Zeb2, Snail1) at a dilution of 1:1000). After washing, membranes were incubated with horseradish peroxidase (HRP)-conjugated secondary antibody for 1 hr. Membranes were washed and blots

were visualized using enhanced chemiluminescence. To verify that equal amounts of protein was loaded from each sample, the membrane was stripped with mild stripping buffer and reprobed with GAPDH (Abcam). Finally, the antibody signals were detected by the ECL system on Amersham imager 680.

Cell adhesion assay

Assay for ECM cell adhesion array was carried out as specified by the manufacturers (ECM540-Millipore). The plate was rehydrated and incubated for 10 minutes at RT. 1×10^6 cells/mL were added to the plate and incubated for 2 h at 37°C in a CO₂ incubator. After extensive washing with assay buffer, cells were stained with cell stain solution and incubated for 5 minutes at RT. 100 µL of extraction buffer was added and incubated for 10 minutes at RT by gently rotated on an orbital shaker after repeated wash with deionized water. Finally, the absorbance was read at 540 nm on a microplate reader.

Cytokine antibody arrays

Assay for cytokine antibody arrays was carried out as specified by the manufacturers (ab133997-Abcam). Briefly, conditioned media prepared from MDA-MB-231 and MDA-MB-468 SCR and NLGN4X-KD cells was collected and incubated with the detection antibody cocktail. The sample/antibody mixture was then added onto the blocked membrane, containing 42 different capture antibodies. After washing, the membrane was incubated with diluted Streptavidin-HRP and Chemi Reagent Mix was added. The cytokines signals were detected by the ECL system on Amersham imager 680.

IHC staining

The breast cancer tissue microarrays (TMA) were obtained from Novus Biological (Littleton, CO) and stained as described before [18]. These include 40 infiltrating ductal carcinoma breast cancer, 10 metastatic lymph node and 9 adjacent normal breast tissues cores. The use of these tissues was approved by the Institutional Review Board of Tuskegee University. Briefly, tissues were de-paraffinized and for

antigen retrieval of NLGN4X the slides were pressure-cooked for 10 minutes. After blocking in 3% goat serum for 1 hour in humidity chambers with NLGN4X antibody (1:100) (Gentex, CA), the slides were incubated with HRP conjugated goat anti-mouse/anti-rabbit secondary antibody (Jackson ImmunoResearch Laboratories Inc, West Grove, PA) for 40 minutes. The antigen-antibody reaction was visualized after treating with diaminobenzidine (Sigma-Aldrich, MO). The slides were counterstained with hematoxylin (Sigma-Aldrich, MO) for 1 minute.

TCGA data collection and analysis

All raw data with attached clinical data in various format (txt, fastq, or bam) of each selected samples from TCGA was downloaded and parse through QC pipeline for low quality before normalization and statistics. For the fastq format, data were processed by removing adaptor sequences using Trimmomatic tool [3], alignment with HISAT2 [19] algorithm, and removal of PCR replicates using SAMtools [21] before expression level quantification. The resulting BAM or SAM files, or directly downloaded txt files were analyzed through a workflow of Partek Genomics Suite (PGS, St Louis, MO, USA). Reads per kilobase of transcript per million mapped reads (RPKM) normalization [8] was performed. The resulting gene list was used for functional analysis such as signaling pathways by using Ingenuity Pathway Analysis (IPA, Redwood City, CA USA). Tumor samples were grouped into high, medium and low groups based on NLGN4X gene expression levels, then these groups were compared by using ANOVA.

Nanostring analysis

The DSP assay was performed on breast cancer TMA slide sourced from Superbiochips Laboratories through the Nanostring Technology Access Program. ROI selection was performed by Nanostring scientists under guidance of the principal investigator. The following main steps are required under Nanostrings GeoMx DSP protocol: (1) tissues are stained following standard procedures; (2) tissues are imaged on the GeoMx platform; (3) ROI are selected and antibody-bound oligonucleotides are recovered from the defined ROI; (4) recovered oligonucleotides are analyzed using Nanostrings nCounter platform.

For sample visualization, this study utilized pan-cytokeratin (NBP2-33200AF488, 1:1000), CD45 (NBP2-34528AF594, 1:20) and CD68 (sc-20060AF647, 1:400), Syto83 (1:20). Protein expression levels were determined using all available Protein IO modules available at the time except the Cell Death module (complete target list in Supplementary Table I). Each selected ROI measured 600µm and oligonucleotides released from the assay antibodies through UV-light exposure. Oligonucleotides released from individual ROI were aspirated, deposited into single wells of 96-well plates and hybridized to barcode-labelled probes [14]. Hybridized probes were processed and counted using the nCounter Prep Station and nCounter Digital Analyzer [2].

Statistics

The significance of difference between two variables was assessed by the Student's t test. The difference was considered significant if the p value was <0.05. Data from all experiments are expressed as mean ± standard error mean (SEM). All statistical calculations were performed using Microsoft Excel.

Results

Neuroigin 4X expression decreases in higher grades primary tumor

Bioinformatic analysis of TCGA dataset showed that shallow deletion of NLGN4X with increasing grade of tumor as shown in Fig. 1A. IHC analysis of the NLGN4X antibody in a TMA (all stages of breast cancer with lymph node metastasis and normal adjacent breast tissue) also revealed its decreased expression in tumors with increasing stage and nodal metastasis. More specifically, in Fig. 1B, NLGN4X expression was higher in T2N0M0 as compared to T2N1M0 or T2N3aM0. Interestingly, when we compared NLGN4X expression in the primary tumor and its matched lymph node metastasis, the loss NLGN4X in the higher-grade primary tumors, was re-expressed in the lymph node metastatic tissues as shown in Fig. 1C. Thus, the loss of NLGN4X in expression in higher grade primary tumor coincides with aggression and spread of cancer and its expression in lymph node metastasis suggests that its re-expression is required for subsequent cell adhesion and tumor progression.

Neuroigin 4x knockdown decreases adhesion and induces EMT

To determine if NLGN4X loss does promote dissemination, we used siRNA to silence NLGN4X in two aggressive TNBC cell lines, MDA-MB-231(EMT-type) and MDA-MB-468 (epithelial-type) (Fig. 2A) and analyzed the ability of the NLGN4X-siRNA treated cells to adhere to ECM as compared to NLGN4X-SCR using ECM adhesion assay kits. As shown in Fig. 2B, NLGN4X knockdown mediated loss of attachment of MDA-MB-468 cells to all the ECM matrix substrate including Col I, Col II, laminin, TN and vitronectin. NLGN4X knockdown (KD) caused a trend in loss of attachment of MDA-MB-231 cells to fibronectin and laminin. To determine if NLGN4X regulates EMT and hence invasion, we looked at the protein expression of EMT-mediated genes. As shown in Fig. 2D, there was an increase in Zeb1, Zeb2, Snail1, Slug expression in MDA-MB-468-NLGN4X-KD as compared to SCR but there was no change in MDA-MB-231 cells (Fig. 2C). TGF β has been shown to induce EMT in breast cancer [38]. To determine if induction of EMT-mediating genes is via the TGF β signaling pathway, smad2/3 phosphorylation was measured in the

NLGN4X-KD or SCR treated cells. Smad2/3 was highly phosphorylated in MDA-MB-468- NLGN4X-KD cells compared to SCR while there was no change in MDA-MB-231 cells.

Loss of Neuroligin 4X increases the expression of genes involved antiviral innate immune response in TNBC

To assess the impact of NLGN4X on the overall gene expression in breast cancer cells, the transcriptome of MDA-MB-231 NLGN4X-KD cells was analyzed using RNA sequencing. There were more than 500 significant differentially regulated genes between the MDA-MB-231-SCR siRNA and MDA-MB-231-NLGN4X siRNA (Fig. 3A). GSEA analysis of top 200 differentially expressed genes (DEGs) in NLGN4X-SCR vs KD MDA-MB-231 cells identified the upregulation of type I IFN, cytokine signaling in immune system and IFN- γ signaling (Fig. 3B), downregulation of cholesterol metabolism and sterol biosynthesis pathways in MDA-MB-231 NLGN4X-KD as shown in Fig. 3D and decrease in log fold change in genes regulating cholesterol biosynthesis (Fig. 3E). Various ISG including IFIT3, OASL, IFIT2, OAS1, IFI6, IFIT5, DDX58 (Fig. 3C) were significantly upregulated suggesting IFN-driven transcriptional activation in NLGN4X-KD cells.

Inhibition of NLGN4X activates MAVS-IRF7 signaling axis

Next, we investigated which signaling pathway is responsible for type I IFN production in the NLGN4X-KD breast cancer cells. Activation of pattern recognition receptors, Toll8 like receptor family proteins (TLR3, TLR7), melanoma differentiation associated gene 5 (MDA5) (encoded by *IFIH1*), RIG-1 have been shown to induce type I IFN production [25, 27]. We examined the expression RIG-1, MDA5 and their downstream effector, MAVS in the MDA-MB-231 and MDA-MB-468 SCR and KD cells. The protein expression of MDA5 and RIG-I were significantly increased in the MDA-MB-231 NLGN4X-KD cells as compared to SCR treated cells (Fig. 4, A). There was no change in the protein expression of RIG-1 and MDA5 in MDA-MB-468 NLGN4X-KD cells (Fig. 4, B). However, the proteins levels of MAVS were significantly increased in MDA-MB-231 NLGN4X- KD and MDA-MB-468 NLGN4X-KD as compared to SCR (Fig. 4). Next, we investigated if there were changes in the transcription factors (IRF3, IRF7, NF κ B, TBK1) responsible for IFN signaling in NLGN4X-KD cells. There was no change in the phosphorylation

of TBK1, NF κ B or IRF3 in NLGN4X-KD cancer cells (data not shown) but there was a significant increase in IRF7 levels in both MDA-MB-231 NLGN4X KD and MDA-MB-468 NLGN4X KD cells as compared to SCR. (Fig. 4). Consistent with the role of MAVS-IRF7 signaling in type I IFN induction [5, 20], as mentioned above the protein levels of IFIT3 (Fig. 4) (type I IFN gene) and the levels of OASL, IFIT2, OAS1, IFI6, IFIT5 were highly increased in MDA-MB-231 and MDA-MB-468 NLGN4X-KD as compared to SCR. Collectively, this data implies that MAVS is the critical sensor to initiate the type I IFN signaling in the NLGN4X-KD cells.

Inhibition of NLGN4X induces expression of immune cell attracting chemokines

Activation of IRF's induces the secretion of cytokines for immune cell attraction. To determine if NLGN4X-KD mediated IRF induction leads to an increase in cytokine secretion, we probed cytokine array kit (Abcam) with conditioned media from MDA-MB-231 and MDA-MB-468 NLGN4X-KD and SCR cells. Knockdown of NLGN4X by siRNA in MDA-MB-231 cells significantly increased expression of proinflammatory cytokines, IL-1 β , IL6, IL8, TNF β (Fig. 5 A, B). In contrast, knockdown of NLGN4X in MDA-MB-468 cells has a more profound effect on the secretion of IL3, IL4, CCL2, CCL8, CCL5 (Fig. 5 C, D).

Low NLGN4X level is associated with chronic activation of the innate antiviral immune response in TNBC patients.

We next assessed the transcriptomes of TCGA TNBC patients with varying NLGN4X expression and analyzed 2000 differentially expressed genes in NLGN4X hi versus NLGN4X low groups (adjusted $P < 0.05$) (Fig. 6A), and found that NLGN4X mRNA was negatively associated with genes controlling IFN- γ signaling, cytokine signaling, antigen presentation (Fig. 6B). Pearson correlation of NLGN4X negatively correlated with the ISG (IFITM1, IRF, DDX58) and chemokines (CCL2, CCL7, CCL5, CXCL11) (Fig. 6 C). NLGN4X negative correlation with ISG and chemokine expression in TCGA TNBC patient dataset is consistent with the above described *invitro* data where NLGN4X KD induces IFN signaling and chemokine secretion.

Low NLGN4X expressions corresponds to immune infiltration in breast cancer

To validate the above *invitro* and patient dataset findings, we explored the correlation of NLGN4X expression with the immune markers in breast cancer tissue on tissue microarray. For this, we employed nanostring DSP technology [33, 37] using 40-panel markers that included markers for T cells, B cells, macrophages, Treg cells, PI3K-Akt signaling to interrogate differential protein expression in tumor epithelial and stromal compartments between patients who had high NLGN4X expression vs low NLGN4X expression. Each core was divided into epithelium (panCK positive) and stroma (panCK negative). Cores with low/absent NLGN4X expression had significant more infiltration of immune population in the stroma (panCK negative) as well as in the tumor epithelium (panCK positive) (Fig. 6 C, D) as compared to NLGN4X-high cores (Fig. 6 A, B). The volcano map of these cores shows differential expression of the target genes. The genes that were significantly upregulated in the stroma of the NLGN4X low core were B cell marker (CD20), T cell markers (CD3, CD8, CD4, CD45), T cell activation marker (ICOS, CD27), Treg (CD25), myeloid marker (IDO1, CD80, CD40, CD14), macrophages (CD163, CD68), Beta-2-microglobulin, Memory T cell (CD45RO, CD127), checkpoints (TIM3, OX40L, PDL-1, 4-1BB, LAG3), HLA-DR, EMT marker (Fibronectin), natural killer cell marker (CD56) and borderline significant myeloid marker (CD11c), immune checkpoint (VISTA), Ki67 and EpCam. The significant upregulated genes in NLGN4X high core stroma were Smooth muscle actin (SMA) and ER-alpha. (Fig. 7E)

Highly significantly upregulated genes in tumor epithelium of NLGN4X low core were myeloid/macrophage (CD163, CD14, CD40) T cell (CD3, ICOS, CD45), CD44, Fibronectin and EGFR. Borderline significant genes were myeloid/macrophage (CD68, CD68b, CD11c, ARG1), B cell (CD20), memory T cell (CD45RO), pan-RAS and EpCAM while in epithelium of NLGN4X high core were ER-alpha and INPP4B (Fig. 7F). This data suggests that TNBC with low NLGN4X expression recruit large number of immune-regulatory cells in the stroma as well as in the tumor epithelium and becomes “fully inflamed”.

Discussion

As the cancer progresses to a higher stage, it becomes more mesenchymal, aggressive, invasive and metastatic. While a plethora of factors has been implicated in the induction of EMT and increase in invasiveness, the role of NLGN4X in this context in TNBC is unknown. Here, we show that NLGN4X loss in the TNBC enhances the invasive property of the cancer cells via induction of EMT genes as evident by the increase in expression of EMT genes in the aggressive MDA-MB-468 breast cancer cells. We did not see any EMT induction in MDA-MB-231 cells, probably because it has already undergone EMT upon partial NLGN4X loss as NLGN4X proteins levels were lower in MDA-MB-231 as compared to MDA-MB-468. The cancer cells with NLGN4X KD poorly adheres to the ECM matrix mimicking higher stage metastatic tumor phenotype with low NLGN4X expression.

The findings in this study provides insight into the intrinsic mechanism in TNBC that regulates IFN signaling and can shape the TIME. Studies have shown the role of DNA damage and cGAS-STING mechanism in the induction of IFN signaling in the TNBC [10]. Other studies have also shown the role of dsRNA sensors include TLR3, TLR7, RIG-I, MDA5, and protein kinase R (PKR) in antiviral innate immune response [34]. The activation of these DNA/RNA sensors has been shown to enhance anti-tumor immunity via induction of cancer-derived type I IFN. Tumor cell-intrinsic type I IFN response has been achieved through the intracellular dsRNA transcription by using a CDK4/6 inhibitor [15] or DNA methylation inhibitors [28]. Our data shows that reducing the expression of NLGN4X enhances cancer cell-intrinsic type IFN response. GSEA analysis of DEGs from the RNA-seq analysis data of NLGN4X-KD breast cancer cells revealed that NLGN4X-KD is related to the strong upregulation of antiviral immune signatures including type I IFN signaling, innate immune response and cytokine signaling pathway suggesting a role of NLGN4X in mediating intrinsic immunity. This was supported by the upregulation of IFIT3 as well as enhanced secretion of cytokines from NLGN4X-KD cancer cells. Though the secreted cytokines from the NLGN4X KD MDA-MB-231 and MDA-MB-468 cells were different, nevertheless

NLGN4X KD induced IFN signaling in both the cell lines which is consistent with the *invivo* TCGA TNBC patient and TMA data that shows higher immune infiltration in NLGN4X low patient groups. The mechanism by which NLGN4X regulates type I IFN signaling and cytokine secretion is likely via activation of MAVS which then activates IRF7, induces IFN signaling and cytokine secretion. Usually, MAVS is activated by RIG-1/ or MDA5, which is seen in MDA-MB-231 but MAVS activation was not dependent on RIG-1 or MDA5 in MDA-MB-468 breast cancer cell line. We also did not see TBK1, IRF3 or NF- κ B phosphorylation upon NLGN4X KD. So how does NLGN4X KD regulate MAVS-IRF7 activation and subsequent IFN signaling? Studies have shown that selectively limiting flux through the cholesterol biosynthetic pathway engages a type I IFN response and induces anti-viral immunity [40]. GSEA analysis of the RNA-seq data of the downregulated genes upon NLGN4X KD showed downregulation of cholesterol biosynthesis pathway, lipid metabolism, SREBF mediated lipid synthesis pathways. While we did not conduct any knockdown studies of lipid biosynthesis pathways to look at the effect on IFN signaling in the breast cancer cells, our RNA-seq data that shows upregulation of IFN signaling and down-regulation of cholesterol metabolism upon NLGN4X KD perfectly aligns in to the above-mentioned study by York et al.,. Notably, the NLGN4X expression level is inversely correlated with the immune cell activation, including T cell activation, IFN signaling as observed in the TNBC patient dataset in NLGN4X high population as compared to the low. This was also supported by the nanostring DSP analysis using a panel of 40 antibodies that included T cell, B cell, macrophages, Treg's and neutrophils showed that low levels of NLGN4X in metastatic breast cancer cores coincides with high immune infiltration not only in the stroma but also in the tumor epithelium, enabling the tumor as "fully inflamed" while the NLGN4X positive tumors had negligible immune infiltration. The data also suggest that while there was CD8+ infiltration, there were myeloid suppressor cells, Treg's and checkpoints that can be targeted in NLGN4X-low population to increase the effectiveness of ICB. Furthermore, the tumor epithelium was also enriched in myeloid/macrophage markers (CD163, CD14, CD68, ARG1) suggesting "myeloid mimicry" of tumors which has been shown to evade immune invasion in some tumors [13]. With this data, we are tempted to

speculate that NLGN4X loss enhances cell-intrinsic innate antiviral immunity that leads to IFN signaling and cytokine secretion and recruitment of immune cells to the tumor site.

Conclusion

In the summary, we have probed into the molecular mechanism and significance of NLGN4X on the immune microenvironment and breast cancer progressiveness. For the first time, we have showed the role of a neuronal cell-adhesion molecule in regulating IFN signaling in cancer cells. Loss of NLGN4X induces an anti-viral state by activating MAVS-IRF7 signaling pathway in TNBC cells. This data is supported by bioinformatic analysis of TNBC patient dataset and Nanostring analysis of breast cancer TMA using 40 panel of immune markers, suggesting that Neuroligin 4X is a negative regulator of IFN signaling in TNBC and its high expression creates an immunosuppressive environment. For further work, we will perform *in vivo* studies to determine how loss of NLGN4X affect immune signaling, immunotherapy response and metastasis and *in vitro* experimentation to identify the molecular mechanism by which loss of NLGN4X induces MAVS-IRF7 signaling. There is an unmet demand for the development of biomarkers that can predict immunotherapy response or enable enhance the current immunotherapy regimen. The absence/loss of NLGN4X can be studied further as a biomarker of “fully inflamed tumors” that can benefit from immunotherapy by ICB.

Availability of data and materials

The datasets used and/or analyzed during the current study are available from the corresponding author on reasonable request.

Abbreviations

TNBC: triple-negative breast cancer

NLGN4X: Neuroligin 4X

EMT: epithelial to mesenchymal transition

TCGA: The Cancer Genome Atlas

IFN: interferon signaling

ICIs: immune checkpoint inhibitors

CCL5: C-C motif chemokine ligand 5

CXCL9: C C-X-C motif chemokine ligand 9

CXCL10: C-X-C motif chemokine ligand 10

RIG-I: retinoic acid inducible gene I

DDRD: DNA damage response–deficient

ISG: Interferon Stimulated Genes

MHC-I: major histocompatibility

TIME: tumor immune microenvironment

FI: fully inflamed

ICB: immune checkpoint blockade

KD: knockdown

DEGs: differentially expressed genes

TLR3, TLR7: Toll8 like receptor family proteins

MDA5: melanoma differentiation associated gene 5

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Ethics declarations

Ethics approval and consent to participate

The use of TMA was approved by Tuskegee University Institutional Review Board.

Consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

Figure legends

Fig 1. NLGN4X protein expression in breast cancer TMA. **a.** NLGN4X expression in neoplasm histological grade ranging from 1 to 3 (METABRIC database from cBIOPORTAL). **b.** Representative IHC images of NLGN4X expression in breast cancer TMA in different stages of tumor including, T2N0M0 (Stage 2, metastasis negative), T2N1miMo (Stage 2, node metastasis positive), T2N3aM0 (stage 3, node metastasis positive), Lymph node metastasis (pair of primary tumor, T2N3aM0). Scale bar 1mm.

Fig 2. NLGN4X-KD induces loss of adhesion of cancer cells to ECM proteins. **a.** NLGN4X knockout in MDA-MB-231 and MDA-MB-468 cells by NLGN4X siRNA and scrambled siRNA assessed by western blot. **b.** Cell adhesion ability in MDA-MB-231 and MDA-MB-468 NLGN4X-KD cells as compared to SCR measured by ECM cell adhesion array. Data are shown as mean $\pm$ SE. *P. The ECM proteins were shown on the X-axis and their measurement of absorbance on the Y-axis. $p \leq 0.05$ and $p \leq 0.005$ were shown by * and ** respectively. **c.** Western blot analysis of Slug, p-SMAD2/3, and GAPDH in MDA-MB-231 SCR and NLGN4X-KD cells; **d.** Zeb 1, Zeb 2, Slug, Snail 1, p-SMAD2/3 and GAPDH MDA-MB-468 SCR and NLGN4X-KD cells. Data are representative of at least three independent experiments.

Fig 3. RNA-seq analysis of MDA-MB-231 SCR and NLGN4X-KD cells. **a.** Hierarchical clustering of genes in Scr vs NLGN4X-KD MDA-MB-231 breast cancer cells. **b.** Enrichment of GSEA analysis of genes upregulated upon NLGN4X in MDA-MB-231 cells from RNA-seq data. **c.** Log2 fold change of ISG upregulated in MDA-MB-231 cells upon NLGN4X-KD. Values are calculated from fragments per kilobase per million fragments mapped and are averaged from RNA-seq analysis of three samples per group. **d.** GSEA analysis of genes downregulated upon NLGN4X in MDA-MB-231 cells from RNA-seq data. Bars indicate statistical significance shown as $-\log_{10}$ of p value. **e.** Log2 fold change of genes regulating cholesterol biosynthesis downregulated in MDA-MB-231 cells upon NLGN4X-KD

Fig 4. NLGN4X KD enhances MAVS-IRF signaling. Western blot analysis of RIG 1, MDA5, MAVS, IRF7, IFIT3 and GAPDH in scrambled and neuroligin 4X MDA-MB-231 (**a**) and MDA-MB-468 (**b**) respectively.

Fig 5. NLGN4X KD induces cytokine secretion in breast cancer cells. Human cytokine antibody array was performed following the manufacturer's protocol to detect secreted chemokines from MDA-MB-231 cells that were **a.** treated with scramble or, **b.** NLGN4X- siRNA, and MDA-MB-468 cells, **c.** treated with scramble or, **d.** NLGN4X- siRNA. Red boxes designate the cytokines whose expression was increased by NLGN4X-siRNA treatment as compared to SCR. The experiments were performed three times. MCP1-CCL2, MCP2-CCL8, MCP3-CCL7.

Fig 6. TCGA TNBC dataset of 104 patients. **a.** Hierarchical clustering of genes in TNBC patients with NLGN4X high, medium vs low groups. **b.** Enrichment of DEG by GSEA analysis of 104 TNBC patients from TCGA dataset reveal significant downregulation of pathways related to IFN signaling, antigen processing in NLGN4X-high groups. Bars indicate statistical significance shown as $-\log_{10}$ of p value. **c.** Pearson correlation of NLGN4X with ISG and cytokines in 104 TNBC patients.

Fig 7. Nanostring DSP analysis of breast cancer TMA. Cytokeratin, CD45, CD68 and NLGN4X IHC staining of representative TMA cores of breast tumors at different stages of progression. NLGN4X low tumors are highly immune infiltrated and “fully inflamed” compared to NLGN4X high tumors. **a-d.** PanCk- (tumor stroma), PanCk+ (tumor epithelium) and NLGN4X staining of breast cancer cores. NLGN4X high cores (**a, b**) and NLGN4X low cores (**c, d**). PanCk (green staining), CD68 (yellow staining), CD45 (red staining), DAPI (blue staining). **e.** Volcano plot depicting upregulation of genes of T cell, B cell, macrophages in tumor stroma in NLGN4X low tumor cores as compared to NLGN4X high cores. **f.**

Volcano plot depicting upregulation of genes of T cell activation, B cell, macrophages in tumor epithelium in NLGN4X low tumor cores as compared to NLGN4X high cores.

Supplementary Table I. The table provides the list of antibodies assay targets and their respective Nanostring GeoMx Protein IO modules.

Figures

Figure 1

NLGN4X protein expression in breast cancer TMA. a. NLGN4X expression in neoplasm histological grade ranging from 1 to 3 (METABRIC database from cBIOPORTAL). b. Representative IHC images of NLGN4X expression in breast cancer TMA in different stages of tumor including, T2N0M0 (Stage 2, metastasis

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d.

e.

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Supplementary Files

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