Loss of Neuroligin 4X Induces an Intrinsic Innate Immune Response in TNBC

Alehegne Yirsaw

Tuskegee University

Muhammad G Omar

Tuskegee University

Isra A Elhussin

Tuskegee University

Dequarius King

Tuskegee University

Henry J Henderson

Vanderbilt University

Maninder Sandhey

Auburn University

Jason White

Tuskegee University

Temesgen Samuel

Tuskegee University

Balusubramanyam Karanam

Tuskegee University

Clayton Yates

Tuskegee University

Deepa Bedi (✉ dbedi@tuskegee.edu)

Tuskegee University <https://orcid.org/0000-0003-4403-2680>

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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.

Full-text

Due to technical limitations, full-text HTML conversion of this manuscript could not be completed. However, the manuscript can be downloaded and accessed as a PDF.

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 negative), T2N1miMo (Stage 2, node metastasis positive), T2N3aM0 (stage 3, node metastasis positive), Lymph node metastasis (pair of primary tumor, T2N3aM0). Scale bar 1 mm.

Figure 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.

Figure 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

Figure 4

NLGN4X KD enhances MAVS-IRF signaling. Western blot analysis of RIG1, MDA5, MAVS, IRF7, IFIT3 and GAPDH in scrambled and neuroigin 4X MDA-MB-231 (a) and MBA-MB-468 (b) respectively.

Figure 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.

Figure 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.

d.

e.

Figure 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 coes (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 Files

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- [SupplementaryTable1.pdf](#)