IFITM1 influences natural killer cell-mediated cytotoxicity by modulation of HLA-class I expression in triple-negative breast cancer cells

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Introduction

Triple-negative breast cancer (TNBC) remains the subtype with the most dismal prognosis. There has been an unmet need for new therapeutic strategies to improve the clinical outcome of patients with TNBC. Recently, immune checkpoint inhibitors (ICIs) such as programmed cell death protein 1 or programmed cell death ligand 1 (PD-L1) inhibitors have been introduced for the treatment of TNBC; however, their efficacy has been modest. Therefore, various strategies, including combination therapies, are being explored to enhance the efficacy of immunotherapy.

The Janus kinase (JAK)/signal transducer and activator of transcription (STAT)/interferon (IFN) signaling induces the expression of numerous IFN-stimulated genes, including IFN-induced transmembrane proteins (IFITMs). Recent studies have shown that IFITM proteins are upregulated in several types of cancers, including breast cancer, and are associated with a poor prognosis and with drug resistance. However, the precise molecular mechanisms of IFITM proteins in cancer are not yet fully understood. Therefore, we investigated the mechanism by which IFITM1 drives tumorigenesis in TNBC.

Methods and Materials

Cell lines: The TNBC cell lines BT-20 and MDA-MB-231 (MB-231), were purchased from the Korean Cell Line Bank (Seoul, Korea). BT-20 cells were cultured in RPMI-1640 medium (Gibco, Grand Island, NY, USA), while MB-231 cells were maintained in Dulbecco's modified eagle medium (DMEM; Gibco, USA). MDA-MB-231 cells were stably transfected with a pcDNA3.1 expression vector encoding full-length human IFITM1 cDNA fused with a Flag epitope tag and selected using neomycin to generate IFITM1-overexpressing cells. As a control, cells were transfected with empty pcDNA3.1 vector and subjected to identical antibiotic selection. For knockdown studies, short hairpin RNA (shRNA) targeting IFITM1 was cloned into the pLKO.1-puro vector (Sigma-Aldrich, St. Louis, MO, USA), and BT-20 cells were stably transfected with the construct. Puromycin was used for selection to establish IFITM1-knockdown cells.

T-cell factor (TCF)/lymphocyte enhancer factor (LEF) luciferase reporter assay: TCF/LEF transcriptional activity was measured using the TCF/LEF Reporter Assay Kit (#60500; BPS Biosciences, San Diego, CA, USA).

NK cell activity assay: Target cells (BT-20 or MB-231) were seeded in 96-well plates at a density of 1×10^4 cells/well and incubated overnight. NK-92cells (effector cells) were added to the target cells at various effector-to-target (E:T) ratios (5:1 and 10:1) and co-cultured for 4h at 37°C in a 5% CO2 incubator. The cytotoxic activity of NK cells against target cells was evaluated using the lactate dehydrogenase (LDH) Glo™ Cytotoxicity Assay (#J2380; Promega, USA).

RNA-seq analysis: RNA-seq analysis was performed using IFITM1-overexpressing MDA-MB-231 cells and IFITM1-knockdown BT-20 cells along with their respective control cells (empty vector and scrambled shRNA). Raw sequencing reads in FASTQ format were assessed for quality using FastQC (v0.11.9). To identify differentially expressed genes, the count data generated by StringTie were analyzed with DESeq2 (v1.38.3).

Results

1. IFITM1 overexpression enhances cell proliferation of TNBC cells. The cell viability assay showed that knockdown of IFITM1 in BT-20 cells (BT-20-IFITM1-KD) reduced cell proliferation and that ectopically expressed IFITM1 in MB-231 cells (MB-231-IFITM1-OE) increased cell proliferation compared with each control cell line. IFITM1-overexpressing cells exhibited a modest reduction in the G1 population and a sustained proportion of cells in the S phase throughout the time course, suggesting an enhanced progression through the G1/S checkpoint.

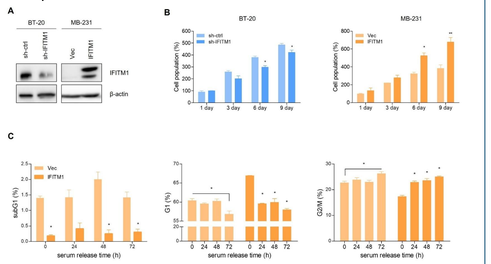


Fig 1. Effect of IFITM1 on cell viability and cell cycle distribution. (A) Western blot analysis of IFITM1 expression in BT-20 cells transduced with scrambled control (Scr) or IFITM1 short hairpin RNA (shRNA, sh-IFITM1), and in MB-231 cells transduced with empty vector (Vec) or IFITM1-expressing vector (IFITM1). β-actin was used as a loading control. (B) Cell viability assays were performed in BT-20 (left) and MB-231 (right) cells transduced with the indicated constructs, measured at 3, 6, and 9 days. (C) Cell cycle distribution was assessed in MB-231 cells overexpressing IFITM1 and vector control (Vec) using flow cytometry. Cells were synchronized by serum starvation in 1% FBS for 24 h and then released with complete medium for the indicated time points (0, 24, 48, and 72 h) *Data represent the mean ± standard deviation. Statistical significance was assessed using the Student's t-test. *p < 0.05, **p < 0.01, **p < 0.001. The figure shown is representative of three independent experiments.

2. IFITM1 overexpression is associated with increased Wnt signaling and NK cell-mediated cytotoxicity in TNBC cells. Among the pathways that were upregulated in IFITM1-overexpressing cells and downregulated in IFITM1-knockdown cells, Wnt signaling pathway and NK cell-mediated cytotoxicity were commonly observed.

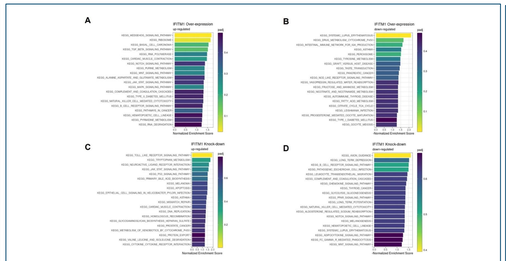


Fig 2. KEGG pathway analysis of differentially expressed genes in IFITM1 overexpression and knockdown cell lines. Top 20 KEGG pathways among (A) upregulated and (B) downregulated genes in the IFITM1-overexpressing cell lines, MB-231-IFITM1-OE cells compared with the control cells. Top 20 KEGG pathways among (C) upregulated and (D) downregulated genes in the IFITM1-knockdown cell lines, BT-20-IFITM1-KD cells compared with the control cells.

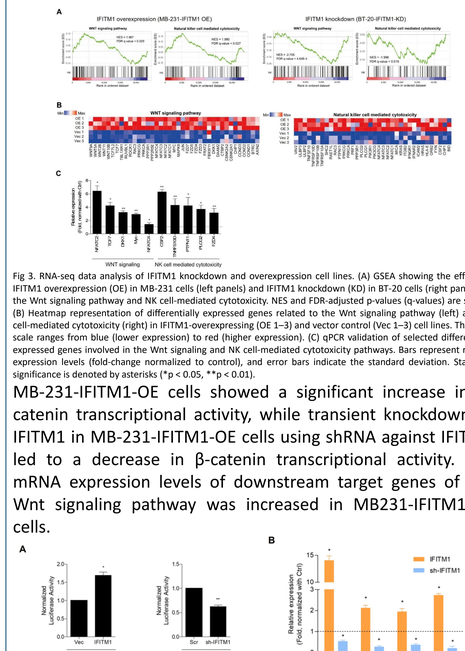


Fig 3. RNA-seq data analysis of IFITM1 knockdown and overexpression cell lines. (A) GSEA showing the effects of IFITM1 overexpression (OE) in MB-231 cells (left panels) and IFITM1 knockdown (KD) in BT-20 cells (right panels) on the Wnt signaling pathway and NK cell-mediated cytotoxicity. NES and FDR-adjusted p-values (q-values) are shown. (B) Heatmap representation of differentially expressed genes related to the Wnt signaling pathway (left) and NK cell-mediated cytotoxicity (right) in IFITM1-overexpressing (OE 1–3) and vector control (Vec 1–3) cell lines. The color scale ranges from blue (lower expression) to red (higher expression). (C) qPCR validation of selected differentially expressed genes involved in the Wnt signaling and NK cell-mediated cytotoxicity pathways. Bars represent relative expression levels (fold-change normalized to control), and error bars indicate the standard deviation. Statistical significance is denoted by asterisks (*p < 0.05, **p < 0.01).

MB-231-IFITM1-OE cells showed a significant increase in β-catenin transcriptional activity, while transient knockdown of IFITM1 in MB-231-IFITM1-OE cells using shRNA against IFITM1 led to a decrease in β-catenin transcriptional activity. The mRNA expression levels of downstream target genes of the Wnt signaling pathway was increased in MB231-IFITM1-OE cells.

Fig 4. IFITM1 modulates Wnt/β-catenin signaling activity and Wnt target gene expression.(A) TCF/LEF luciferase reporter assays were conducted to assess Wnt/β-catenin transcriptional activity in MB-231 cells overexpressing IFITM1 (left) and MB-231-IFITM1-OE cells with IFITM1 knockdown (right). (B) qPCR was used to measure expression levels of Wnt target genes (Axin 2, CD44, MMP7) in MB-231 cells following IFITM1 overexpression or knockdown. *Data represent the mean ± standard deviation. *p < 0.05, **p < 0.01, **p < 0.001. The figure shown is representative of three independent experiments.

MB-231-IFITM1-OE cells showed increased NK cell cytotoxic activity compared with controls, while knockdown of IFITM1 in MB-231-IFITM1-OE cells using shRNA against IFITM1 led to a decrease in NK cell cytotoxicity.

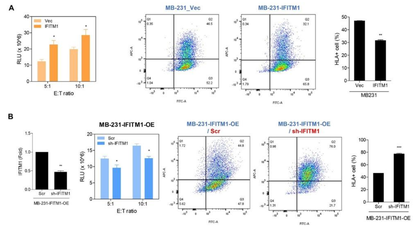


Fig 5. IFITM1 suppresses immune recognition and HLA class I expression in breast cancer cells. (A) NK cell-mediated cytotoxicity assays were performed using MB-231 cells overexpressing IFITM1 compared to vector control (Vec) at E:T ratios of 5:1 and 10:1. HLA-I surface expression was assessed by flow cytometry and quantified. (B) qPCR was used to verify IFITM1 knockdown in MB-231-IFITM1-OE cells. NK cell cytotoxicity and HLA-I expression were assessed by cytotoxicity assays and flow cytometry. *Data represent the mean ± standard deviation. *p < 0.05, **p < 0.01, ***p < 0.001. The figure shown is representative of three independent experiments.

3. IFITM1 overexpression leads to low expression of HLA class I in TNBC. Activation of NK cells is regulated by the HLA class I molecules of target cells. The HLA class I of target cells are recognized by NK cell inhibitory receptors, and this interaction inhibits the activation of NK cells. Therefore, we investigated the surface expression of HLA class I on TNBC cells according to IFITM1 expression using flow cytometry. We found that there was an inverse correlation between IFITM1 and HLA class I expression. MB-231-IFITM1-OE cells exhibited a decrease in HLA class I expression, which may contribute to their increased susceptibility to NK cell-mediated lysis. To confirm the specific role of IFITM1 in modulating NK cell-mediated cytotoxicity and HLA class I expression, we knocked down IFITM1 in MB-231-IFITM1-OE cells using shRNA against IFITM1. When IFITM1 expression was knocked down in these cells, we observed a decrease in NK cell-mediated cytotoxicity and an increase in HLA class I expression, reversing the effects seen in IFITM1-overexpressing cells.

4. IFITM1 overexpression shows a trend for a negative HLA class I expression in TNBC patients. We evaluated the expression of IFITM1, β-catenin, and HLA class I in the tissues of 57 patients with TNBC who underwent curative surgery. In patients with IFITM1 overexpression, there was a trend toward increased β-catenin positivity and HLA class I negativity.

Valuable	N (%)	IFITM1		p-value
		Positive	Negative	
Total patients	57 (100)	16 (28.0)	41 (72.0)	
β-catenin				0.128
Positive	30 (52.6)	11 (68.7)	19 (46.3)	
Negative	27 (47.4)	5 (31.3)	22 (53.7)	
HLA class I				0.205
Positive	33 (57.9)	6 (37.5)	23 (56.1)	
Negative	24 (42.1)	10 (62.5)	18 (43.9)	

5. β-catenin or JAK inhibitor can affect NK cell-mediated cytotoxicity by modulating the expression of HLA class I in TNBC cells.

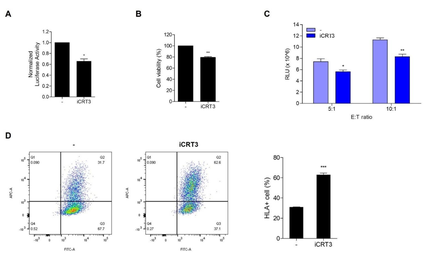


Fig 6. Inhibition of Wnt/β-catenin signaling by iCRT3 enhances immune recognition and HLA class I expression. (A) TCF/LEF luciferase assays were used to evaluate Wnt/β-catenin signaling activity in MB-231-IFITM1-OE cells treated with the Wnt inhibitor iCRT3. (B) Cell viability was measured after iCRT3 treatment. (C) NK cell-mediated cytotoxicity assays were conducted at E:T ratios of 5:1 and 10:1. (D) HLA-I expression was analyzed by flow cytometry and quantified. *Data represent the mean ± standard deviation. *p < 0.05, **p < 0.01, ***p < 0.001. The figure shown is representative of three independent experiments.

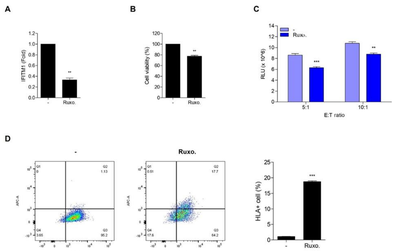


Fig 7. JAK/STAT pathway inhibition by ruxolitinib reduces IFITM1 expression and enhances HLA class I expression. (A) qPCR was performed to assess IFITM1 mRNA expression in MB-231-IFITM1-OE cells treated with the JAK1/2 inhibitor ruxolitinib. (B) Cell viability was measured after ruxolitinib treatment. (C) NK cell-mediated cytotoxicity assays were conducted at E:T ratios of 5:1 and 10:1. (D) HLA-I surface expression was evaluated by flow cytometry and quantified. *Data represent the mean ± standard deviation. *p < 0.05, **p < 0.01, ***p < 0.001. The figure shown is representative of three independent experiments.

Conclusion

The overexpression of IFITM1 was associated with increased β-catenin activity and NK cell-mediated cytotoxicity via downregulation of HLA class I. The interplay between tumor and immune cells within the TME is complex and could result in either tumor progression or antitumor activity. Therefore, it is crucial to consider not only the influence of diverse cellular signaling pathways on the tumor cells itself but also the impact of these pathways on the TME. Our findings suggest that IFITM1 can affect not only cancer cells itself but also immune regulation in the TME and be a potential therapeutic target in the era of cancer immunotherapy.

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