



Promotion on NLRC5 upregulating MHC-I expression by IFN- γ in MHC-I-deficient breast cancer cells

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Abstract

Breast cancer is the most dominant cancer in women and the second most frequent cancer in the general population worldwide. NLRC5 critically transactivates MHC class I (classically HLA-ABC in human) which is crucial for cancer immunosurveillance. But the expressional and functional impairments of NLRC5 have been found in many cancers as a major mechanism of immune evasion. Promotion of NLRC5 with the enhancement of MHC class I contributes to cancer immunotherapy and counteraction against cancer immune evasion. In many cancers, IFN- γ promotes the expression of MHC class I involving NLRC5; however, it is unclear in breast cancer cells. In this study, qRT-PCR, western blot, and flow cytometry were used to detect the mRNAs and proteins of NLRC5, β 2m, and HLA-ABC in MHC class I-deficient human SKBR3 breast cancer cells after IFN- γ treatment. It was shown that the relative levels of NLRC5 mRNA, β 2m mRNA, and HLA-ABC α heavy chain mRNA, in concentrations of 50 U/ml and 100 U/ml IFN- γ groups, were statistically increased ($p < 0.05$) with dose dependent tendency compared with the control group. The protein levels of NLRC5 and β 2m in concentrations of 50 U/ml and 100 U/ml IFN- γ groups, HLA-ABC (positive rates) in different concentrations of IFN- γ groups, were statistically increased ($p < 0.05$), with dose dependent tendency for NLRC5 and HLA-ABC, compared with the control group. Promotion of NLRC5 by IFN- γ with upregulation of MHC class I (HLA-ABC) in SKBR3 breast cancer cells, suggesting the contribution to counteracting cancer evasion from immunosurveillance and benefiting cancer immunotherapy.

Keywords Breast cancer · NLRC5 · β 2m · MHC class I · Immunotherapy

Introduction

Breast cancer is known as the most dominant type of cancer in women and the second most frequent cancer in the general population worldwide [1]. Besides various present treatments available for breast cancer therapy, among which surgery is the most common one, new strategies are under development [2]. With the close relationship between cancer and immunology [3–8], it has been demonstrated that immunotherapy is valuable to treat various types of cancers, among which anti-PD1/PD-L1 (programmed cell death-1/programmed death-ligand 1) agents used in breast cancer have demonstrated promising results both in monotherapy and in combination with conventional therapies [9]. NLRC5 (NLR family, CARD domain containing

protein 5), as the largest member of the nucleotide-binding oligomerization domain-like receptor (NLR) family, has recently attracted a growing level of attention for its close relationship with various cancers pathogenesis involving immunology indicated with increasing evidence [10].

NLRC5 was involved in innate and adaptive immune responses such as inflammatory and type I interferon responses beyond the transactivation of major histocompatibility complex (MHC) class I [11]. The key role in the transcriptional activation of MHC class I and related genes [12] makes NLRC5 crucial for cancer immunosurveillance because sufficient expression of MHC class I which is critically transactivated by NLRC5 is essential for the recruitment and activation of tumor killing CD8+ T cells [13]. Expressional and functional impairments of NLRC5 have been found in many cancers as a major mechanism of immune evasion [14].

The importance of NLRC5 for anti-PD1/PD-L1 immunotherapy is attributed to the necessity of sufficient expression of MHC class I on cancer cells for anti-PD1/PD-L1 immunotherapy [15]. MHC, also known as human leukocyte antigen

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(HLA) in human, is critical for the activation of the adaptive immune system because they present antigens to CD8+ or CD4+ T cells. MHC class I presents endogenous antigenic peptides to CD8+ T cells, whereas MHC class II presents exogenous antigenic peptides to CD4+ T cells [16, 17]. Cancer antigen is endogenous antigen. In many cancers, including breast cancer [18], however, deficient MHC class I has been found as a dominant immune evasion strategy because successful tumor growth requires cancer cells evading immune surveillance [19]. Blocking PD1/PD-L1 pathway has shown remarkable clinical responses as a new treatment paradigm in many different malignancies because blocking PD-L1 binding to PD1, an inhibitory receptor expressed on the activated T and B cells, blocks its delivery of an inhibitory signal into the activated T cells [20]. Deficient expression of MHC class I on cancer cells, however, disturbs anti-PD1/PD-L1 immunotherapy and other adaptive immunotherapies because the resultant failure in antigen presentation results in deficient activation of CD8+ effector T cells with invalid cancer killing function, even if PD1/PD-L1 has been inhibited by anti-PD1/PD-L1 immunotherapy.

Expressional promotion of NLRC5 with the enhancement of MHC class I contributes to cancer immunotherapy, such as anti-PD1/PD-L1. It has been demonstrated that interferon- γ (IFN- γ) promotes the expression of MHC class I with the involvement of NLRC5 in many types of cancer cells [21, 22]; however, it is unclear in breast cancer cells. The present study was designed to demonstrate the effect of IFN- γ on expressional promotion of NLRC5 and MHC class I in MHC class I-deficient SKBR3 breast cancer cells.

Materials and methods

Culture and preparation of breast cancer cells

Human SKBR3 breast cancer cells deficient in MHC class I expression [18, 23–25] and MCF-7 breast cancer cells more positive in MHC class I expression [25] were maintained under standard cell culture conditions. The cells were seeded at a density of 6×10^5 /well in 6-well culture plates and cultured to settle for 24 h at 37 °C in a humidified atmosphere containing 5% CO₂ in DMEM medium supplemented with 10% FBS. Then, after 12-h preculture of the cells in the medium containing 1% FBS, IFN- γ was added into to the indicated final concentration in the medium containing 10% FBS for a further 48-h incubation. Cells were harvested for assay. All experiments were individually performed three times.

Western blot

The total cellular proteins in the IFN- γ treated and untreated cells grown in 6-well culture plates were extracted by lysis

with radioimmunoprecipitation assay (RIPA) buffer (Beijing Solarbio Science & Technology Co., Ltd., Beijing, China) and quantified with BCA Protein Assay Kit (Beijing Solarbio Science & Technology Co., Ltd., Beijing, China). The total cellular proteins extracted from the cells were subjected to 10% (for NLRC5) or 12% (for β 2m) sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and electrotransferred to polyvinylidene fluoride (PVDF) membranes. After 2-h incubation with 5% skim milk at room temperature for blockade, the indicated primary antibodies, polyclonal rabbit anti-NLRC5 antibody (Abcam, Cambridge, England), recombinant monoconal rabbit anti- β 2m antibody (HuaBio Inc., Cambridge, USA), and mouse monoclonal anti-GAPDH antibody (OriGene Technologies Inc., Rockville, USA), were added for overnight incubation at 4 °C, followed by three washes with TBST and 2-h incubation with horseradish peroxidase-conjugated secondary antibodies. ECL was used for visualization, and Image J software (US National Institutes of Health, Bethesda, MD, USA) was used for quantification with normalization by GAPDH in relative intensity manner compared with the control.

Quantitative real-time PCR

The total RNAs in the cells after 24-h treatment with IFN- γ were extracted using TRIzol reagent (Invitrogen, Carlsbad, USA) according to manufacturer's instruction under RNase-free condition, followed by reverse transcription of the RNAs to cDNAs by a FastQuant RTkit (Tiangen Biotech Co., Ltd., Beijing, China). Quantitative real-time PCR (qPCR) was executed with the responsible primers (all the primers used for qRT-PCR were obtained from GeneCopoeia or Invitrogen), NLRC-5 (GeneCopoeia Inc., Germantown, Maryland, USA), β 2m (GeneCopoeia Inc.), HLA-ABC α heavy chain (Invitrogen, Carlsbad, USA), and GAPDH (GeneCopoeia Inc.), using SuperReal PreMix Plus kit (Tiangen Biotech Co., Ltd., Beijing, China) on Roche Cobas z 480 Real-Time PCR Detection System (Roche, Basel, Switzerland). The expression of the mRNAs was normalized by expression of GAPDH mRNA as a control and analyzed by using the $2^{-\Delta\Delta C_t}$ method.

Flow cytometry

The cells after 24-h treatment were collected by trypsinization and centrifugation, and then the washed cells were resuspended with binding buffer (1×10^7 cells/ml). The cell suspensions (100 μ L) were incubated with 5- μ L HLA-ABC-FITC (Tianjin Sungene Biotech Co., Ltd., Tianjin, China) or isotype control antibody at room temperature for 40 min in the dark. A total of 5000 cells from each tube were detected for data collection, and expression of the HLA-ABC on the cell surface was

analyzed by using Cytomics FC 500 MCL flow cytometer (Beckman Coulter, Miami, FL, USA) and CXP software.

Statistical analysis

The data were shown as mean ± standard deviation (SD) and Tukey test was used after one-way ANOVA to determine the statistical significance. Three independent experiments were performed. $P < 0.05$ was statistically considered significant.

Results

The expression of NLRC5 mRNA and protein

Compared with MCF-7 breast cancer cells which are more positive in MHC class I (HLA-ABC) expression, SKBR3 breast cancer cells showed the downregulation of NLRC5 ($p < 0.05$) (Fig. 1a). After 24-h treatment of SKBR3 breast cancer cells with IFN- γ , the relative levels of NLRC5 mRNA in the groups treated with 50 U/ml and 100 U/ml IFN- γ were increased with dose dependent tendency compared with the control group with statistical significance ($p < 0.05$) (Fig. 1b). The NLRC5 protein levels in the groups treated with 50 U/ml and 100 U/ml IFN- γ were increased with dose dependent tendency as well compared with the control group with statistical significance ($p < 0.05$) (Fig. 1c).

The expression of β 2-microglobulin mRNA and protein

Beta 2-microglobulin (β 2m) is a component of MHC class I molecules and associated with MHC class I deficiency and its expression may be promoted by NLRC5. It was shown that after 24-h treatment of SKBR3 breast cancer cells with IFN- γ , the relative levels of β 2m mRNA were increased with dose dependent tendency in the groups treated with 50 U/ml and 100 U/ml IFN- γ compared with the control group with statistical significance ($p < 0.05$) (Fig. 2a). The β 2m protein levels in the groups treated with different concentrations of IFN- γ were increased with statistical significance ($p < 0.05$) compared with the control group as well (Fig. 1b).

The expression of MHC class I (HLA-ABC) mRNA and protein

MHC class I expression may be promoted by NLRC5. It was shown that, after 24-h treatment of SKBR3 breast cancer cells with IFN- γ , the relative levels of HLA-ABC α heavy chain mRNA were increased with dose dependent tendency in the groups treated with 50 U/ml and 100 U/ml IFN- γ compared with the control group with statistical significance ($p < 0.05$) (Fig. 3a). The protein levels of HLA-ABC (positive rates) in

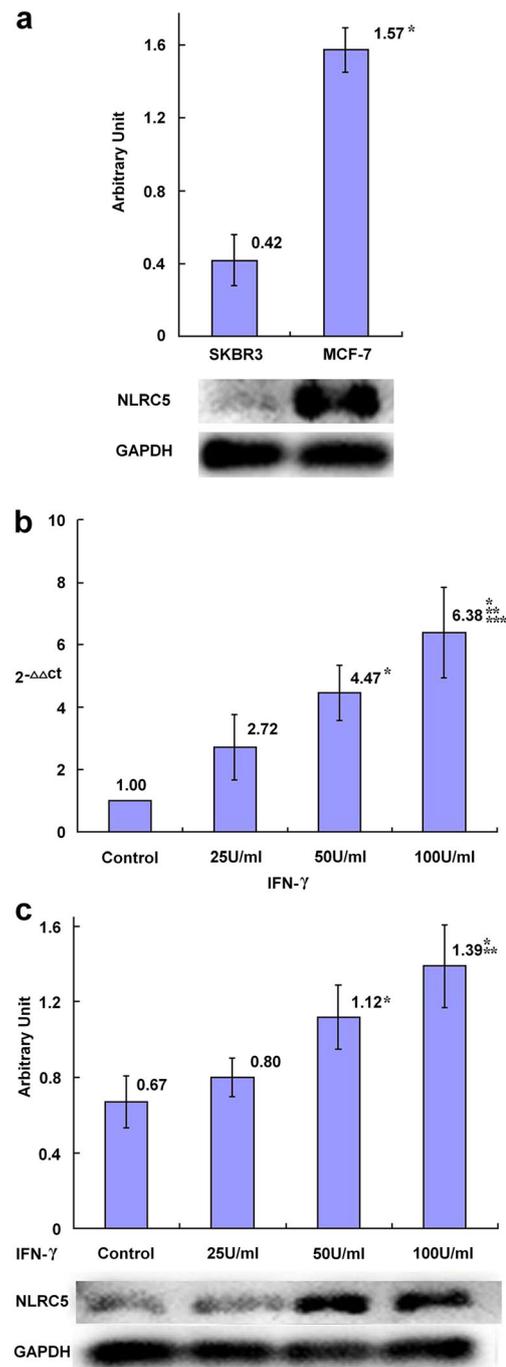


Fig. 1 The expression of NLRC5 mRNA and protein. Downregulation of the expression of NLRC5 protein in SKBR3 breast cancer cells was detected with western blot in comparison with MCF-7 breast cancer cells which is more positive in MHC class I (HLA-ABC) expression (a). After 24-h treatment of SKBR3 breast cancer cells with IFN- γ , the expression of NLRC5 mRNA and protein was detected by qRT-PCR (b) and western blot (c), respectively. Error bars indicate standard deviation of the means. Asterisks indicate p values < 0.01 compared with the SKBR3 cells (a), or LSD p values < 0.05 , compared with the control after one-way anova (b, c). Double asterisks indicate LSD p values < 0.05 , compared with the 25 U/ml IFN- γ group after one-way anova. Triple asterisks indicate LSD p values < 0.05 , compared with the 50 U/ml IFN- γ group after one-way anova

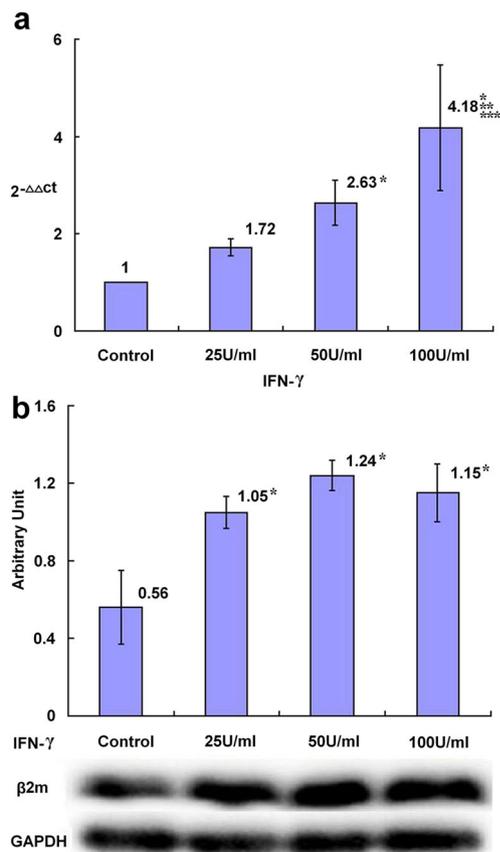


Fig. 2 The expression of $\beta 2$ -microglobulin mRNA and protein. After 24-h treatment of SKBR3 breast cancer cells with IFN- γ , the expression of $\beta 2$ -microglobulin mRNA and protein was detected by qRT-PCR (a) and western blot (b), respectively. Error bars indicate standard deviation of the means. Asterisks indicate LSD p values < 0.05 , compared with the control after one-way anova. Double asterisks indicate LSD p values < 0.05 , compared with the 25 U/ml IFN- γ group after one-way anova. Triple asterisks indicate LSD p values < 0.05 , compared with the 50 U/ml IFN- γ group after one-way anova

the groups treated with different concentrations of IFN- γ were statistically increased with dose dependent tendency compared with the control group as well ($p < 0.05$) (Fig. 3b, c).

Discussion

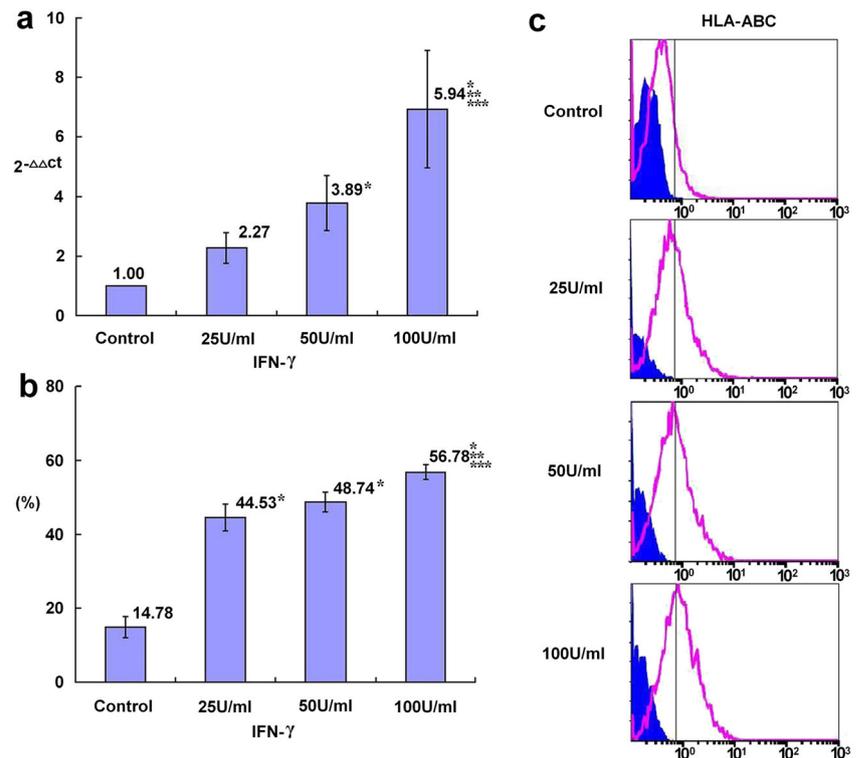
Breast cancer is a considerable cancer in women. The fact that cancer cells bear specific epitopes which can be recognized by host adaptive immune system in MHC class I restricted manner brings the opportunity to develop immunotherapies targeting these cancer-specific epitopes (neoepitopes) [26]. Among the recent revolutions of cancer immunotherapies to eradicate malignant cells by improvement of the host immune system, anti-PD1/PD-L1 agents of immune checkpoint inhibitors have exhibited the most encouraging outcomes [27–30]. PD1 is a protein expressed on cell surface of T lymphocytes, and its ligands, PD-L1 and PD-L2 expressed on cell membrane of several cell

types, bind to PD1 to make peripheral immune tolerance which can be employed by cancer cells to evade the immune rejection via the expression of the ligands on cancer cells [31]. PD1/PD-L1 blockade blocks cancer evasion from the immune rejection. It has been demonstrated, however, that the effectiveness of the immune checkpoint blockade antibody were severely weakened by loss of MHC class I expression [15, 32], and the loss of MHC class I expression on cancer cells account for another mechanism employed by cancer cells to evade the immune rejection as well.

One of the key regulators of MHC class I expression is NLRC5 which is first cloned and characterized in 2010 [33–36]. NLRC5, as a member of NLR protein family, is a large protein with a structure of tripartite domain containing 1866 amino acids and the molecular weight of NLRC5 is approximately 200 kD [33, 34, 37, 38]. The tripartite structure of NLRC5 is composed of amino terminal location of atypical caspase activation and recruitment domain (CARD) containing six α -helix bundle with a general death fold (specific structural features strikingly different from the typical CARD make its atypical categorizing), center location of nucleotide-binding domain (NBD), and carboxy terminus location of a long leucine-rich repeats (LRRs) [39]. NLRC5 expression induces the expression of genes sharing similar promoter architecture as MHC class I promoters, including classical MHC class I genes such as HLA-A, HLA-B, and HLA-C, non-classical MHC class I genes such as HLA-E and HLA-F, and MHC class I-related genes, $\beta 2m$, TAP1, and PSMB9 (LMP2) [40], without induction on expression of any MHC class II or related genes [41]. In most, albeit not all cell types, NLRC5 transfers to the nucleus where MHC class I genes are transactivated by NLRC5 [14, 42, 43]. Promotion of NLRC5 expression contributes to enhancement of MHC class I expression for counteraction against the evasion from the immune rejection which is mediated by deficient expression of MHC class I in cancer cells. IFN- γ is well known for its effect on promotion of MHC class I expression. It was shown in this study that IFN- γ enhanced the expression of NLRC5 and its mRNA in SKBR3 breast cancer cells.

Beta 2-microglobulin ($\beta 2m$), a constant 11.6-kD light chain, along with 45-kD variable α heavy chain of the MHC class I molecule, which combines an antigenic peptide to the groove of the heavy chain, forms the trimeric complex by which the antigenic peptide derived from cancer or pathogen is presented to a cytotoxic T cell [44, 45]. The best characterized function of the $\beta 2m$ is to interact with and stabilize the tertiary structure of the α chain [46]. It is well demonstrated that $\beta 2m$ plays an important role in deficiency of MHC class I expression, and the capacity to synthesize the $\beta 2m$ protein, which is essential to produce a functional active MHC class I molecule, can be downregulated by different types of mutation [47–49]. Absence of $\beta 2m$ results in most less expression of MHC class I molecules on the cells surface [50]. In different cancers, it is convincingly evidenced that the deficiency of

Fig. 3 The expression of HLA-ABC mRNA and protein. After 24-h treatment of SKBR3 breast cancer cells with IFN- γ , the expression of mRNA of HLA-ABC α heavy chain and HLA-ABC protein was detected by using qRT-PCR (a) and flow cytometry (b), respectively. (c Shows histograms of HLA-ABC protein expression on the cells evaluated by flow cytometry. Open histograms represent HLA-ABC protein and filled histograms represent isotype control). Error bars indicate standard deviation of the means. Asterisks indicate LSD p -values < 0.05, compared with the control after one-way anova. Double asterisks indicate LSD p -values < 0.05, compared with the 25 U/ml IFN- γ group after one-way anova. Triple asterisks indicate LSD p values < 0.05, compared with the 50 U/ml IFN- γ group after one-way anova



β 2m generates immune escape [44, 51]. The deficiency of functional MHC class I can be restored by transfection with ER-retained β 2m in β 2m-deficient cells [52]. Promotion of the β 2m expression on cancer cells may counteract β 2m deficiency resultant cancer immune evasion, and NLRC5 may have the activity to promote β 2m expression. In this study, IFN- γ showed its effects on the enhancement of the expression of β 2m and its mRNA with the enhancement of NLRC5 expression in SKBR3 breast cancer cells.

The T cell adaptive immunity is driven by the MHC class I antigen processing and presentation pathway. MHC class I molecules are cell surface proteins ubiquitously expressed on the surface of all nucleated somatic cells and essential for the immune defense against intracellular pathogens and cancers for its antigen presentation by noncovalent combination with antigen peptides derived from intracellular proteins for CD8+ T cell recognition [53]. By virtue of antigen processing and presentation pathway, the MHC class I molecules are transported to the cell surface after loading empty MHC class I in the endoplasmic reticulum with the peptide fragments which are derived from the whole antigen by processing them in the proteasome, and once the peptides binding MHC class I are presented on the cell surface, CD8+ T cells recognize the presentation through interactions with highly specific T cell receptors (TCRs) [54, 55]. Therefore, sufficient expression of MHC class I on cancer cells is critical for successful adaptive immunotherapy, whereas deficient expression of MHC class I is often employed by cancer cells to evade immune eradication. Promotion of the MHC class I expression on cancer cells

may counteract this kind of cancer evasion from the immune rejection. Enhancement on NLRC5 as well as β 2m may promote the MHC class I expression. It was shown in this study that IFN- γ enhanced the expression of MHC class I and its α heavy chain mRNA with the enhancement of NLRC5 expression in SKBR3 breast cancer cells.

The Cancer Genome Atlas (TCGA) database-based bioinformatical analysis on the correlation between MHC class I and NLRC5 downregulation in breast cancer has been included in the detailed study on the correlation between MHC class I and NLRC5 downregulation in different cancers [14]. In this study, promotion of NLRC5 accompanying up-regulation of MHC class I (HLA-ABC) by IFN- γ in MHC class I-deficient human SKBR3 breast cancer cells was demonstrated. Besides IFN- γ [56], other cytokines and inflammatory factors, such as transforming growth factor beta (TGF- β) [57], and tumor necrosis factor alpha (TNF- α) [58], regulate MHC class I expression as well [59]. Functional promoter elements [21] and important cis-regulatory elements that regulate MHC class I expression including enhancer A [60], interferon (IFN)-stimulated regulatory element [61], and the SXY module [62, 63], which are bound by nuclear factor kappa-light-chain-enhancer of activated B cells (NF- κ B), interferon regulatory factor 1 (IRF1), and MHC class II (MHC-II) enhanceosome A, respectively, have been demonstrated with their roles in MHC class I regulation. STAT1 is involved in the IFN- γ signaling pathway [64] and plays its role in MHC class I regulation [65]. Post-translational modifications, such as peptide loading onto MHC class I [66, 67], ubiquitination

of MHC class I [68], are involved in MHC class I regulation as well. Therefore, the dependence instead of just coincidence for the role of NLRC5 in MHC class I induction by IFN- γ in SKBR3 cells remains to be further clarified. Some other mechanisms (like STAT1, post-translational modifications, etc.) may be involved in the induction of MHC class I by IFN- γ in SKBR3 cells as well.

In summary, NLRC5 was promoted by IFN- γ with upregulation of MHC class I (HLA-ABC) in SKBR3 breast cancer cells, suggesting the contribution to counteracting cancer evasion from immunosurveillance and benefiting cancer immunotherapy.

Compliance with ethical standards

Conflict of interest The authors declare that they have no conflict of interest.

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