Monalizumab efficacy correlates with HLA-E surface expression and NK cell activity in head and neck squamous carcinoma cell lines

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Research Article

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Abstract

Purpose

NKG2A, an inhibitory receptor expressed on NK cells and T cells, leads to immune evasion by binding to HLA-E expressed on cancer cells. Here, we investigated the relationship between HLA-E surface expression on head and neck squamous cell carcinoma (HNSCC) cell lines and the efficacy of monalizumab, an NKG2A inhibitor, in promoting NK cell activity.

Methods

Six HNSCC cell lines were used as target cells. After exposure to IFN-γ, HLA-E surface expression on HNSCC cell lines was measured by flow cytometry. Peripheral blood mononuclear cells (PBMCs) from healthy donors and isolated NK cells were used as effector cells. NK cells were stimulated by treatment with IL-2 and IL-15 for 5 days, and NK cell-induced cytotoxicity was analyzed by CD107a degranulation and ⁵¹Cr release assays.

Results

We confirmed that HLA-E expression was increased by IFN-γ secreted by NK cells and that HLA-E expression was different for each cell line upon exposure to IFN-γ. Cell lines with high HLA-E expression showed stronger inhibition of NK cell cytotoxicity, and efficacy of monalizumab was high. Combination with cetuximab increased the efficacy of monalizumab. In addition, stimulation of isolated NK cells with IL-2 and IL-15 increased the efficacy of monalizumab, even in the HLA-E low groups.

Conclusion

Monalizumab efficacy was correlated with HLA-E surface expression and was enhanced when NK cell activity was increased by cetuximab or cytokines. These results suggest that monalizumab may be potent against HLA-E-positive tumors and that monalizumab efficacy could be improved by promoting NK cell activity.

Introduction

Patients with head and neck squamous carcinoma (HNSCC) have a poor prognosis (Johnson, Burtness, et al. 2020). Although therapies that modulate immune checkpoint molecules on T cells, such as programmed death protein-1 and programmed death ligand-1, show promising clinical responses for some cancer types (Herbst, Soria, et al. 2014, Powles, Eder, et al. 2014), the response rate to T-cell-based immunotherapy is generally low (Chen, Li, et al. 2018, Gotwals, Cameron, et al. 2017). Therefore, research
focusing on other types of immune cells or immune checkpoint molecules is needed to develop new treatment strategies.


The objective of this study was to evaluate the association between HLA-E surface expression on HNSCC cell lines and the efficacy of monalizumab on NK cells. We also investigated the correlation between NK cell activity and monalizumab efficacy after stimulation of NK cells by cytokines ex vivo.

Materials And Methods

Cell lines and cell culture
The human HNSCC cell lines SNU-1041, SNU-1066, SNU-1076 (purchased from the Korean Cell Line Bank, Seoul, Korea) were cultured in Roswell Park Memorial Institute (RPMI) 1640 medium, and Detroit-562 and FaDu (purchased from the American Type Culture Collection, Manassas, Virginia, USA) were cultured in Minimum Essential Media containing 10% fetal bovine serum (FBS) and 1% penicillin/streptomycin. Recombinant human IFN-γ (R&D Systems, Minneapolis, MN, USA) was used to increase HLA-E surface expression.

**Establishment of β2M KO SNU-1041 cells by CRISPR**

Guide RNA, scrambled control, and GFP-puro donor in beta 2 microglobulin (β2M) Human Gene Knockout CRISPR Kit (ORIGENE, Rockville, MD, USA) was used to establish SNU-1041 MHC-I knockout (KO) cells. Lipofectamine and PLUS™ reagent (Invitrogen, Carlsbad, CA, USA) were used as transfection reagents, and transfection was performed according to the manufacturer's instructions. Forty-eight hours after transfection, one-tenth of cells were split and grown again for 3 days, which was repeated for 3 weeks. β2M KO SNU-1041 cells were selected by puromycin (2 µg/ml), and non-expression of MHC-I was verified by flow cytometry.

**Preparation of peripheral blood NK cells, NK cells isolated from peripheral blood mononuclear cells, and NK cell conditioned medium**

Using Ficoll density gradient sedimentation, peripheral blood mononuclear cells (PBMCs) from healthy donors were isolated using leukoreduction system chambers. PBMCs were activated in RPMI 1640 medium supplemented with 10% FBS and 1 ng/ml rhIL-15 (PeproTech, Rocky Hill, NJ, USA) for 5 days.

For the 51 chromium (Cr) release assay and preparation of NK cell conditioned medium (CdM), NK cells were isolated from PBMCs using MACS columns and an NK cell isolation kit (Miltenyi Biotec, Bergisch Gladbach, Germany). Purified NK cells were cultured in NK MACS® Medium (Miltenyi Biotec) supplemented with 10% FBS, 500 IU/ml rhIL-2, and 10 ng rhIL-15. The purity of isolated NK cells was confirmed by flow cytometry. Purified NK cells were cultured in RPMI with 500 IU/ml rhIL-2 for 3 days to generate CdM. Cell culture supernatants were harvested by centrifugation and passed through a 0.45-µm Acrodisc syringe filter (Pall Corporation, Port Washington, NY, USA). IFN-γ mAb (NIB42; Thermo Fisher Scientific, Waltham, MA, USA) was used to neutralize IFN-γ in CdM.

**Reagents and antibodies**

To evaluate the efficacy of monalizumab to promote NK cell activity and ADCC, monalizumab and cetuximab were used. Monalizumab (purchased from Creative Biolabs, Shirley, NY, USA) is a humanized IgG4 mAb that targets NKG2A. Cetuximab is a chimeric IgG1 mAb targeting EGFR that induces ADCC.

Cell surface staining was performed using the following fluorescently labeled mAbs: isotype control-PE (MOPC-2, RRID:AB_396091), anti-HLA-E-PE (3D12, RRID:AB_2869949) (eBioscience, San Diego, CA, USA),
anti-CD107a-PE (H4A3, RRID:AB_396135), CD16-FITC (3G8 RRID: AB_395806, BD Biosciences, San Jose, CA, USA), anti-CD3-eFlour450 (17A2. AB_1272193, Invitrogen), anti-CD56-PE-Vio770 (REA196, RRID:AB_2726091), and anti-NKG2A-APC (REA110, RRID:AB_2726447, Miltenyi Biotec). To exclude dead cells, eFluor 506 (#65-0866-14, Invitrogen) staining was performed. Golgi-Stop (#554724, BD Biosciences) was used in the CD107a degranulation assay as a protein transport inhibitor.

**Analysis of cell surface protein expression using flow cytometry**

Cell surface expression of HLA-E was measured by flow cytometry. Cells were incubated with or without 10 ng/ml IFN-γ for 24 hours and stained with fluorochrome-conjugated HLA-E antibody or isotype control for 20 min at 4°C. FACS Calibur (BD Biosciences) was used for data acquisition, and data were analyzed using FlowJo software (LCC, Ashland, OR, USA). mAb staining was measured as geometric mean fluorescence intensity (gMFI). We defined the difference between control and HLA-E-stained cancer cell lines as delta (Δ)gMFI.

**CD107a degranulation assay**

CD107a degranulation assay was performed using PBMCs or isolated NK cells from healthy donors with an effector-to-target (E:T) ratio of 1:1 using a previously described method (Park, Kim, et al. 2020). PBMCs (2 × 10^5) or isolated NK cells were incubated with HNSCC cells for 1 hour at 37°C in U-bottom 96-well plates. Next, GolgiStop solution was added followed by incubation for 3 hours. All cells were transferred to a FACS tube, washed with FACS buffer, and stained with fluorescently labeled mAbs. Data were acquired and analyzed using a FACS Canto II (BD Biosciences) and FlowJo software.

**51Cr release assay**

51Cr release assay methodology is described in our previous study (Park, Ahn, et al. 2019). Target cells were labeled with 50 µCi for 1 hour at 37°C, and then 2.5 × 10^5 cells were washed three times with growth media. Target cells were cultured with 10 µg/ml of the indicated antibodies, seeded at 5 × 10^3 cells/well into U-bottom 96-well plates, and co-incubated with effector cells at a 10:1 E:T ratio for 4 hours at 37°C. Following co-culture, 75 µl supernatant from each well was transferred to 96-well PP 1.2-ml cluster tubes for gamma counting. To calculate radioactivity, a WIZARD2 Automatic Gamma Counter (PerkinElmer, Waltham, MA, USA) was used.

**Statistical analysis**

GraphPad Prism v7.0 (GraphPad Software, San Diego, CA, USA) was used to analyze data, which is reported as mean ± standard deviation (SD). Two-tailed paired Student’s t-tests were used to compare groups, and a two-sided P<0.05 was considered statistically significant.

**Results**
Surface expression of HLA-E in HNSCC cell lines

We measured the surface expression of HLA-E in HNSCC cell lines using flow cytometry. In normal conditions, all cell lines showed low HLA-E surface expression except for MHC-I KO cells. However, when IFN-γ was added, an increase in HLA-E expression was observed in all cell lines (Fig. 1A). Depending on the HLA-E expression level after IFN-γ treatment, cell lines with a change in mean fluorescence intensity (ΔMFI) ≥ 20 were classified into the HLA-E high group (SNU-1041, SNU-1066, and FaDu), whereas cell lines with ΔMFI < 20 were classified into the HLA-E negative or low group (SNU-1041 MHC-I KO, SNU-1076, and Detroit-562). There were no significant correlations between the HLA-E mRNA expression or genotype of each cell line and its level of HLA-E surface expression (Supplementary Fig. S1).

To confirm that HLA-E surface expression was increased due to IFN-γ secreted from NK cells, we prepared NK cell CDM. When SNU-1041 cells were cultured in CDM, the surface expression of HLA-E increased to a level similar to that following IFN-γ treatment, whereas neutralization with IFN-γ mAb attenuated this increase (Fig. 1B).

Correlation between HLA-E surface expression in HNSCC cell lines and monalizumab efficacy

To investigate the correlation between HLA-E surface expression in HNSCC cell lines and monalizumab efficacy, we measured the cytotoxicity of primary NK cells in PBMCs by CD107a degranulation assay. PBMCs were activated with a low concentration of IL-15 (1 ng/ml) for 3 days, and NK cells were identified by flow cytometry (Supplementary Fig. S2). There were no statistical significant effects of monalizumab among HNSCC cell lines in the negative or low HLA-E group either under control conditions or after IFN-γ treatment (Fig. 2A). However, among HNSCC cell lines in the HLA-E high group, monalizumab showed significant effects, with even larger effects when monalizumab was combined with cetuximab (Fig. 2B).

The level of HLA-E surface expression was correlated with the degree of inhibition of cytotoxic NK cells, including ADCC induced by cetuximab (Fig. 3A, B). In addition, the recovery of cytotoxic NK cells by monalizumab was correlated with HLA-E surface expression (Fig. 3C). Taken together, these results suggest that HLA-E surface expression in HNSCC cell lines predicts the efficacy of monalizumab in promoting NK cell activity.

Ex vivo stimulation of NK cells increases the efficacy of monalizumab

To measure the efficacy of monalizumab upon stimulation of NK cells with a cytokine, we performed a CD107a degranulation assay after isolating NK cells from PBMCs. When the assay was performed with NK cells incubated in growth medium for 1 day, the effect of monalizumab was not significant in any HNSCC cell line, even when combined with cetuximab (Supplementary Fig. S3). By contrast, after stimulation with IL-2 (500 IU/ml) and IL-15 (10 ng/ml) for 5 days, effects of monalizumab alone and
monalizumab combined with cetuximab were observed for SNU-1041 cells with high HLA-E surface expression as well as SNU-1076 cells with negative or low HLA-E surface expression (Fig. 4A). The effect of monalizumab alone was comparable to that of cetuximab in SNU-1076 cells and was higher than that of cetuximab in SNU-1041 cells.

We also performed a $^{51}$Cr release assay to confirm whether *ex vivo* stimulation with a cytokine improves the effect of monalizumab. As with the CD107a degranulation assay results, effects of monalizumab alone and monalizumab in combination with cetuximab were observed in all HNSCC cell lines including those in the HLA-E negative or low group, although larger effects were observed in the HLA-E high group (Fig. 4B, C). These results suggest that stimulation of NK cells by IL-2 and IL-15 enhances the efficacy of monalizumab.

**Discussion**

We found a correlation between HLA-E surface expression on HNSCC cell lines and monalizumab efficacy in promoting NK cell activity. We also showed that combination of cetuximab and stimulation of NK cells with IL-2 and IL-15 improved the efficacy of monalizumab, even in cell lines with low HLA-E surface expression (Fig. 5). These findings suggest that the antitumor effect of monalizumab is stronger in tumors with higher HLA-E expression and depends on NK cell activity.

By measuring levels of HLA-E surface expression on HNSCC cell lines, we showed that HLA-E expression was increased by IFN-$\gamma$ secreted from NK cells. The surface expression of HLA-E requires binding to the MHC-I leader sequence, and HLA-E surface expression and affinity for NKG2A vary depending on the type of leader peptide that is bound (Battin, Kaufmann, et al. 2022, Matsunami, Miyagawa, et al. 2002). Antibodies that bind HLA-E leader peptide complexes enhance NK cell cytotoxicity (Li, Brackenridge, et al. 2022). HLA-E surface expression is also affected by tapasin, TAP, and $\beta_2$M, which are involved in movement to the cell surface (Braud, Allan, et al. 1998, Lee, Goodlett, et al. 1998, Lo Monaco, Sibilio, et al. 2008). We found that the surface expression of HLA-E in HNSCC cell lines increased upon exposure to IFN-$\gamma$, consistent with previous studies (Derré, Corvaisier, et al. 2006, Nguyen, Beziat, et al. 2009), likely due to activation of the STAT-1 pathway by IFN-$\gamma$ (Lee and Benveniste 1996, Zhou 2009). As HLA-E expression in the TME is also increased by IFN-$\gamma$, expression of IFN-$\gamma$ and STAT-1 pathway-related genes in the TME may also be affected. Furthermore, HLA-E surface expression is high in acute leukemia patients with the genotype HLA-E*01:03 (Lauterbach, Wieten, et al. 2015, Xu, Wieten, et al. 2019), suggesting that the genotype of HLA-E could affect HLA-E surface expression. However, in the present study, neither the surface expression of HLA-E nor the level of HLA-E mRNA was significantly associated with genotype. Together, these findings suggest that the surface expression of HLA-E is affected by complex interactions among multiple factors, which requires clarification through future studies.

Using CD107a degranulation assay, we observed correlations between HLA-E expression and monalizumab efficacy, with stronger correlations observed when monalizumab was combined with cetuximab. The higher efficacy of monalizumab, when used in combination with cetuximab, may be due
to an increase in NK cell activity through the CD16 receptor, which also mediates ADCC (Capuano, Pighi, et al. 2021). Monalizumab has been demonstrated to have potency in combination with other treatments (van Hall, André, et al. 2019), and clinical trials of monalizumab combination therapies are ongoing (NCT02643550, NCT02671435, NCT05221840). Our results suggest that HLA-E surface expression is a tumor biomarker for monalizumab efficacy and that the use of monalizumab in combination with other cancer therapies that promote NK cell or T cell activity could be more effective. INTERLINK-1 phase 3 trial (NCT04590963) which compared cetuximab with/without monalizumab in HNSCC had terminated because of futility issue. We speculate that better outcomes could be obtained when they apply molecular enrichment based on HLA-E-positivity in patient selection.

In unstimulated NK cells, even the combination of monalizumab and cetuximab did not show efficacy, whereas stimulation of NK cells with IL-2 and IL-15 increased the efficacy of monalizumab in both CD107a degranulation and $^{51}$Cr release assays, even within the HLA-E low group. *In vitro* stimulation of NK cells with IL-2 and/or IL-15 increases NK cell-activating receptors and NK cell cytotoxicity (Croxatto, Martini, et al. 2017, Horng, Bezbradica, et al. 2007, Lehmann, Zeis, et al. 2001, Sanchez-Correa, Bergua, et al. 2017, Wang, Frank, et al. 2000). Also, *in vitro* stimulation increases the ratio of NKG2A$^+$ NK cells (André, Denis, et al. 2018, Hromadnikova, Pirkova, et al. 2013), and NKG2A$^+$ NK cells may induce more cytotoxicity (Mahaweni, Ehlers, et al. 2018). Taken together, these findings suggest that the efficacy of monalizumab increases when NK cell activity is promoted by cytokine treatment and that NK cell activity in the TME is important. In addition, considering that adoptive NK cell transfer is an attractive therapeutic candidate for solid tumors and that NKG2A is an interfering factor (Cichocki and Miller 2019), these findings imply that adoptive NK cell therapy may exert greater antitumor activity when combined with monalizumab after *ex vivo* stimulation.

Our study has several limitations. As our study employed only *in vitro* experiments, verification of our results using *in vivo* experiments is required. Our experiment did not take into account the infiltration of NK cells into the TME or interactions with other immune cells that may affect the efficacy of monalizumab. Furthermore, it is necessary to determine the major factors that affect HLA-E surface expression *in vivo*. However, this study is meaningful in that it firstly identifies a potential biomarker and effective therapeutic strategy involving monalizumab.

In conclusion, our study reveals that HLA-E surface expression on HNSCC cell lines is correlated with monalizumab efficacy and that increasing NK cell activity by combined treatment with cetuximab or cytokines enhances the efficacy of monalizumab. Our findings suggest that monalizumab has potency against tumors with higher HLA-E surface expression and that the efficacy of monalizumab is improved when NK cell activity is increased by other treatments.

**Declarations**

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**Competing Interests:** None declared

**Author Contributions:** Conception and design: JL and BK. Development of methodology: JL, BK, H-RP, J-EP, SK and TMK. Acquisition of data (eg, provided cells and provided facilities, etc.): JL, H-RP, J-EP and SK. Analysis and interpretation of data: JL, BK, SK and TMK, Study supervision: BK, SK, MK, TMK, D-WK and DSH, Writing, review and/or revision of the manuscript: all authors

**Data Availability:** Data are available upon reasonable request. Contact email: bhumsuk@snu.ac.kr.

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Figures

Figure 1

Surface expression of HLA-E on HNSCC cell lines. (A) Surface expression of HLA-E on HNSCC cell lines with or without treatment with IFN-γ for 24 hours was measured by flow cytometry. (B) SNU-1041 and SNU-1041 MHC-I KO cells were exposed to NT, IFN-γ, NK cell CdM, or NK cell CdM with anti-IFN-γ mAb, and HLA-E surface expression was measured after 24 hours by flow cytometry. Data are shown as mean
± SD. ΔgMFI, difference in geometric mean fluorescence intensity; NT, no treatment; CdM, conditioned medium.

Figure 2

Increased NK cell cytotoxicity after monalizumab treatment of HNSCC cell lines. The cytotoxicity of primary NK cells was measured by CD107a degranulation assay after coculture of PBMCs and tumor
cells with an E:T ratio of 1:1. PBMCs from healthy donors were used for effector cells and were activated by IL-15 (1 ng/ml) for 3 days before coculture. NK cells in PBMCs were identified using flow cytometry after fluorescence staining. The experiment was conducted by dividing the control and IFN-γ pretreated groups for 24 hours. The effects of monalizumab alone and monalizumab in combination with cetuximab were measured. HNSCC cell lines were treated with monalizumab (gray), cetuximab (red), or monalizumab plus cetuximab (blue) or were not treated (NT, black). (A) CD107a degranulation assay results for SNU-1041 MHC-IKO, SNU-1076, and Detroit-562, which had negative or low surface HLA-E expression. (B) CD107a degranulation assay results for SNU-1041, SNU-1066, and FaDu, which had high surface HLA-E expression. Data are shown as mean ± SD. *P < 0.05, ***P < 0.001, ****P < 0.0001. Statistical significance was determined by two-tailed paired Student’s t-tests. neg/lo, negative or low; hi, high; Ctrl, control; NT, no treatment; Mo, monalizumab; Ctx, cetuximab; ns, not significant.

Figure 3

Correlations between surface expression of HLA-E and monalizumab efficacy. (A) Monalizumab efficacy (measured by the percentage of CD107a+ NK cells) in HNSCC cell lines with or without treatment with IFN-γ (left) and correlation between HLA-E surface expression and monalizumab efficacy (right). (B) Monalizumab plus cetuximab efficacy in HNSCC cell lines with or without treatment with IFN-γ (left) and correlation between HLA-E surface expression and monalizumab plus cetuximab efficacy (right). (C)
Correlation between HLA-E surface expression and efficacy of monalizumab (left) and monalizumab plus cetuximab (right). NT, no treatment; Ctx, cetuximab.

**Figure 4**

*Ex vivo* stimulation of NK cells increased the efficacy of monalizumab. To determine whether stimulation with NK cells increases the efficacy of monalizumab. NK cells were isolated from PBMCs. And then, NK
cells were stimulated with IL-2 (500 IU/ml) and IL-15 (10 ng/ml) for 5 days. HNSCC cells were treated with IFN-γ for 24 hours. (A) Results of CD107a degranulation assay for SNU-1041 MHC-KO (HLA-E negative), SNU-1076 (HLA-E low), and SNU-1041 (HLA-E high) cell lines. $^{51}$Cr release assay was performed using stimulated NK cells. HNSCC cells were treated with IFN-γ or not for 24 hours before the assay and labeled with $^{51}$Cr. NK cells and tumor cells were cocultured for 4 hours at an E:T ratio of 10:1. Results of $^{51}$Cr release assay for (A) HLA-E negative or low cell lines and (B) HLA-E high cell lines Data are shown as mean ± SD. **P < 0.01, ****P < 0.0001. Statistical significance was determined by two-tailed paired Student’s t-tests. neg/lo, negative or low; hi, high; Ctrl, control NT, no treatment; Mo, monalizumab; Ctx, cetuximab; ns, not significant.

**The efficacy of monalizumab is better in high surface HLA-E expression HNSCC cells**

![Diagram](image)

High decrease in NK cell cytotoxicity by HLA-E & High monalizumab efficacy

Low decrease in NK cell cytotoxicity by HLA-E & Low monalizumab efficacy

**Figure 5**

**Schematic representation of a model representing monalizumab efficacy depending on HLA-E expression and NK cell activity.** HLA-E$^{\text{high}}$ cells strongly inhibits NK cell cytotoxicity, and have higher efficacy of monalizumab than HLA-E$^{\text{low}}$ cells. Increasing NK cell activity by stimulating NK cells with cytokines or...
inducing ADCC with IgG1 mAb such as cetuximab can improve the efficacy of monalizumab. In particular, stimulation of NK cells using IL-2 and IL-15 enhances monalizumab efficacy even in HLA-E^low cells.

**Supplementary Files**

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