# α-Her2-CAR *CD38* knockin enhance the persistence CAR expression and augment the anti-tumor capacity of NK cells against gastric cancer cells

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**Abstract:**

**Aim:**

This study aims to develop an efficient site-specific gene transduction method to generate natural killer (NK) cells stably expressing anti-Her2 chimeric antigen receptor (CAR) for the immunotherapy of gastric cancer, thereby aiming to improve patient survival rates.

**Methods:**

Primary NK cells from peripheral blood were expanded using K562-mIL-21/4-1BBL feeder cells and IL-2. On Day 7, the anti-Her2 second generation CAR cassette was integrated by homologous recombination into the CD38 gene locus of NK cells via Cas9-RNP electroporation combined with AAV6-mediated CAR delivery. CAR

expression on NK cells and its phenotyping were analyzed by flow cytometry. Furthermore, CAR-NK cells' cytotoxicity against Her2+ gastric cancer cell lines, cytokine secretion, and activation markers were also analyzed by flow cytometry. A gastric cancer organoid model was employed to test cytotoxicity, and a xenograft NSG mouse model was used to evaluate the anti-tumor activity of CD38KI-Her2-CAR-NK cells *in vivo*.

**Results:**

The site-specific integration of the CAR into the CD38 locus via Cas9-RNP and AAV6 may avoided the risks associated with random viral gene insertion. CD38KI-Her2-CAR-NK cells exhibited higher proliferation (221.5 ± 30.35 expansion fold than 156 ± 18.02 for LV-CAR-NK on day 15, n = 6) and comparable CAR+ expression to lentiviral transduction, LV-Her2-CAR (31.88% CD38KI-Her2-CAR-NK v.s. 32.32% LV- Her2-CAR-NK, n = 6). *In vitro,* CD38KI-Her2-CAR-NK cells showed increased activation molecule expression (NKp44, NKp46, CD107a), enhanced secretion of cytokines (IFN-γ, TNF-α), and superior antigen-specific cytotoxicity against $Her2^{+}$ gastric cancer cells (MKN-28, AGS) when compared to LV-Her2-CAR NK cells. In the gastric cancer organoid model, CD38KI-Her2-CAR-NK cells demonstrated stronger anti-tumor efficacy compared to lentiviral-transduced CAR-NK cells. In xenograft models, CD38KI-Her2-CAR-NK treatment resulted in improved survival rates and reduced tumor growth compared to the lentiviral-transduced LV-Her2-CAR NK cells group. Flow cytometry revealed that tumor-infiltrating lymphocytes NK cells from the CD38KI-Her2-CAR-NK group expressed more activation markers and secreted higher levels of cytokines.

**Conclusion:**

This study successfully developed CD38KI-Her2-CAR-NK cells using Cas9-RNP electroporation with AAV6, confirming the method is efficiency and safety. The engineered NK cells displayed enhanced proliferation and potent anti-tumor activity against Her2+ gastric cancer cells, presenting a promising strategy for gastric cancer immunotherapy that may extend to other solid tumors.

**Keywords**: Tumor Immunotherapy; CAR-NK cells; Gastric Cancer; Gene Editing; CRISPR/Cas9 technology.