The Future of Cancer Immunotherapy: Harnessing Next-Generation CAR-Ma through Innovative RNA Vector Multi-Modality

Research Report

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Student : Liem Kathleen Francisca
Supervisor : Dr. Rio Sugimura
Faculty : Li Ka Shing Faculty of Medicine
University : The University of Hong Kong

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Abstract

Cancer remains a leading global health challenge, with current therapies like chemotherapy and immunotherapy facing significant issues such as side effects, high costs, and ineffective targeting of solid tumors due to the tumor microenvironment (TME). This research, part of the HKU iGEM 2024 Project, endeavors to develop a novel CAR-Ma product—a chimeric antigen receptor expressed on macrophages instead of T-cells—powered by the world's first multi-modal RNA vector. The CAR-Ma design features replicative, transient, and release functions, enabling targeted immune responses while reducing the risks associated with traditional DNA-based therapies. By targeting immune evasion pathways such as CD47 and PD-L1, CAR-Ma enhances macrophage activity and phagocytosis. This project employs innovative engineering to synthesize a fifth-generation CAR, leveraging humanized nanobodies to target glypican-3 (GPC3), a marker prevalent in various cancers. The research methodology includes plasmid assembly, in-vitro transcription, and electroporation into target cell lines. Despite challenges in PCR and cloning trials, preliminary results confirmed successful plasmid verification and RNA synthesis. The CAR-Ma project offers a significant advancement in cancer immunotherapy, providing a versatile, controllable, and cost-effective solution to improve therapeutic efficacy and inform future research in fields like genomics and biotechnology among others. The next steps of this research could be to involve optimizing the design of primers that define the CAR-Ma fragments.

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1 Introduction

1.1 Target Problem

The National Cancer Institute (2024) emphasizes that cancer remains a leading cause of death worldwide. In 2022, nearly 20 million new cases and 9.7 million deaths were attributed to the disease. Projections indicate that by 2040, the annual incidence of new cancer cases could rise to 29.9 million, with six of the ten most prevalent cancers experiencing alarming annual increases of 0.6% to 3% (Siegel et al., 2024). In Hong Kong, where I am pursuing my undergraduate studies, cancer accounted for approximately 30% of deaths in 2020, according to the Hong Kong Cancer Registry (2020), underscoring the severity of the issue, even in a metropolis known for its cutting-edge advancements.

Despite these concerning figures, current cancer therapies—such as radiotherapy, chemotherapy, and immunotherapy—face significant challenges. These include debilitating side effects, high costs, limited accessibility, and ineffective cell signalling pathways (Chakraborty & Rahman, 2014; Mustaqeem & Rajkumar, 2012; Zugazagoitia et al., 2016). Furthermore, solid tumours, which account for over 90% of cancers, often resist these treatments due to the tumour microenvironment (TME), a formidable barrier that hinders effective drug penetration and contributes to the poor persistence of Chimeric Antigen Receptor (CAR)-T cells within the TME (Saggar et al, 2013; Sorkhabi et al., 2023). Recent advances in genomic technologies have further illuminated the complexities of cancer, revealing tumour heterogeneity and acquired resistance, which complicate precision in treatment (Zugazagoitia et al., 2016).

Overall, although existing therapies are powerful, they lack the sophistication necessary to effectively combat an adversary that operates on multiple levels. To address this multifaceted challenge in the form of cancer, we must adopt a comprehensive approach that understands and targets cancer in all its modalities, employing inherently multi-modal solutions.

1.2 Objective

This research project, under the HKU International Genetically Engineered Machine (iGEM) 2024 Team, aims to synthesize a CAR-Ma product—a chimeric antigen receptor expressed on macrophages instead of T-cells—powered by the world's first multi-modal RNA.

1.3 Engineering Components and Reasoning

Multi-Modal RNA

Designed and developed by the HKU iGEM 2024 team, the multi-modal RNA is intended to perform up to three distinct functions upon its activation: Replicative, Transience, and Release, which are explained as follows:

1. Replicative: This function, driven by a sequential code from the Venezuelan Equine Encephalitis Virus (VEEV) for the replication complex, ensures that the final CAR-Ma maintains a tightly regulated expression, thereby preserving cellular resources and longevity. Additionally, it replenishes CAR receptors lost during receptor-mediated phagocytosis.
    a. Synergy with 2nd Generation Self-Replicating RNA (srRNA): The system was enhanced by integrating high-fidelity RNA-dependent RNA polymerase proofreading domains—part of a transcriptional modulation and protein (TMP)-based expression control system developed by the HKU iGEM 2024 team. This integration minimizes mutations and acts as checkpoints within the reading frame of the plasmids, significantly extending the lifespan of the multi-modal RNA vector through a sustained self-replication mechanism.
2. Transience: This function seeks to disable tumour immune evasion by temporarily expressing specific genes, particularly targeting the signal regulatory protein alpha and cluster of differentiation 47 (SIRPa-CD47) signalling pathway and the CRISPR-associated protein 12a (CRISPR-Cas12a) knockout mechanisms that cancers commonly exploit to evade immune detection. By limiting gene expression to a short duration, this strategy enhances safety, applicability, and cost-effectiveness for future in vivo CRISPR-Cas9 or CRISPR-Cas12a products, while effectively disrupting oncogenic signalling pathways with high specificity for cancer cells.
3. Release: This function facilitates the release of functional non-coding RNA sequences, including guide RNAs—specifically, Cas9 or Cas12a guide RNA (gRNA) for Cas-related operations in this project—and small interfering RNA (siRNA) for SIRPa knockout and gene silencing through RNA interference (RNAi). In this research project, the RNAi system incorporated regulates gene expression by using short hairpin RNA (shRNA) to target and degrade complementary mRNA molecules (Moore et al., 2010).

As an RNA molecule that serves as a vector utilizing advanced, high-fidelity RNA replication circuit, it is inherently non-integrative, mitigating concerns associated with insertional mutagenesis—mutations in the

cellular genome due to the integration of exogenous DNA—and genetic permanence, which are prevalent issues in DNA-based therapies that can induce the deregulation of oncogenes and other harmful, cancer-promoting effects (Ranzani et al., 2013). Moreover, unlike DNA plasmids and viral strategies, the risk of cytotoxicity can be completely minimized. This is particularly beneficial in conjunction with the transient design of the multi-modal RNA product, which allows the cell to revert to its native state after therapy concludes, facilitated by an activation system.

Molecular Targeting of Immune Evasion Checkpoints in Cancer

Solid tumours frequently express immune evasion checkpoints like CD47 (a cell surface protein which binds SIRPa allowing cancers to generate a “don not eat me” signal) and Programmed Cell Death Ligand 1 (PD-L1) (a transmembrane protein that is considered to be a co-inhibitory factor of the immune response), which allow them to escape detection by immune cells and evade phagocytosis – oftentimes, in synergy with mechanisms such as the inducing of T-cell exhaustion, TME factors signalling, and the accumulation of metabolites (Han et al., 2020; Cho & Kim, 2022; Lau et al., 2023). By concurrently knocking out these checkpoints and additional proteins that inhibit macrophage activity and T-cell activation, this project greatly enhances phagocytosis and improves the coordination of immune cells.

Connecting back to the advantages of this project, which primarily stem from the feature of sustained transience in gene expression enabled by multi-modal RNA, this research also introduces a novel shRNA pipeline specifically designed for CAR-Ma, in conjunction with the previously mentioned CRISPR-based approaches. Rather than relying on a permanent knockout that alters the host genome and may lead to undesirable consequences, the shRNA facilitates the silencing of SIRPa, achieving a similar effect to CRISPR-mediated knockout while preserving a transient nature. As long as the multi-modal RNA system is active, SIRPa silencing can be sustained and regulated, allowing for the deactivation of the multi-modal RNA once the therapeutic process is complete – ultimately enabling the cell to return to its native state.

The Heart of CAR-Ma: 5th Generation CAR Design

The 5th generation CAR is powered by a humanized nanobody—derived from camelid antibodies, which offer lower immunogenicity and greater ease of humanization—that specifically targets glypican-3 (GPC3), a cell-surface glycoprotein highly expressed in various cancers, including hepatocellular carcinoma, the second leading cause of cancer deaths worldwide in 2020 (Guo et al., 2020). This makes GPC3 a reliable immunohistochemical marker. These nanobodies function as a modular domain that can be easily switched or multiplexed—allowing for the simultaneous transmission of multiple antigens—thereby enabling the potential to address a broad range of cancers.

Enhanced with upgraded co-stimulatory domains, this design further promotes phagocytosis, trogocytosis (the engulfment of large targets), and the coordination of the broader immune response. Additionally, the incorporation of interferon-gamma (IFN-γ) expression acts as a powerful polarization factor, driving transfected macrophages and tumour-associated macrophages (TAMs) towards an anti-cancer, proinflammatory state. As a final touch, the system has implemented a robust small-molecule-based variable control system that allows for precise regulation of CAR expression, ensuring that safe and controlled levels are consistently maintained.

The conceptualization of CAR-Ma, the next-generation CAR, and multi-modal RNA, along with the design of the plasmids and genetic constructs, was developed by Christian Vicera, the instructor of this year's HKU iGEM team.

2 Methodology and Protocols

2.1 Procedural Overview

The methodology and protocols include the amplification of DNA fragments, followed by plasmid assembly, cloning, minipreparation, and transformation of bacterial cultures (specifically DH5α and DH10B). Additionally, cell culturing techniques are employed for THP-1 (human monocytic cell line) and HEPG2 (human liver cancer cell line) cell lines.

The simplified procedural planned overview of this research project is as follows:

1. Plasmid assembly
2. Linearization of plasmids
3. In-vitro transcription (IVT) of DNA plasmids to RNA
4. Electroporation of the RNA product into target cell lines
5. Isolation of macrophage yield

2.2 Experimental Challenges

Several challenges encountered in the experiments of this research related to polymerase chain reaction (PCR) and cloning.

In PCR, issues such as non-specific amplification—where incorrect primer binding led to smearing or multiple bands on gels—complicated the results. Additionally, optimizing conditions—such as determining the appropriate annealing temperature and cycle number—often proved challenging and

involved considerable trial and error. The presence of inhibitors, such as impurities that interfered with the polymerase enzyme, was also discovered to decrease amplification efficiency. In cloning, difficulties included low transformation efficiency and the labour-intensive process of screening for positive clones.

Incomplete digestion or ligation have also further contributed to low cloning efficiency.

Addressing these challenges to improve specificity and yield required careful planning, optimization of procedures and conditions, and effective troubleshooting, which in turn, involved extensive research, reviewing online protocols, and seeking guidance from advisors throughout the PCR and cloning stages.

2.3 Possible Improvements

To enhance the success of this research project, several strategic improvements can be implemented:

1. Adjusting or Redesigning Primer Sequences:
   Optimizing primer sequences based on the specific characteristics of the target sequence—such as GC content, which indicates DNA stability and binding affinity, as well as melting temperature—is essential for minimizing non-specific amplification. This optimization enhances the efficiency of PCR reactions and ensures reliable annealing and amplification, among other benefits.
2. Utilizing Primer Design Software:
   To facilitate the creation of optimal primers, utilizing advanced primer design software and tools can analyse sequences and recommend primers that minimize background errors and potential interference, such as dimerization, as well as refine the screening process. This improvement can significantly increase the likelihood of successful amplification, ultimately leading to higher quality products.
3. Incorporating In Silico Tools for Validation:
   The implementation of in silico tools for validation, where software can perform multiple tasks, such as predicting the behaviour and characteristics of plasmids and assessing restriction site compatibility, can significantly enhance the accuracy and reliability of results. This approach saves time and resources by identifying potential issues before conducting actual experiments.

By adopting these possible improvements, expectations and standards can be elevated for more successful experimental outcomes.

3 Principal Results

The several key results present in this report offer valuable insights into both past and future outcomes of the research. All images included were sourced from the HKU iGEM 2024 documentation.

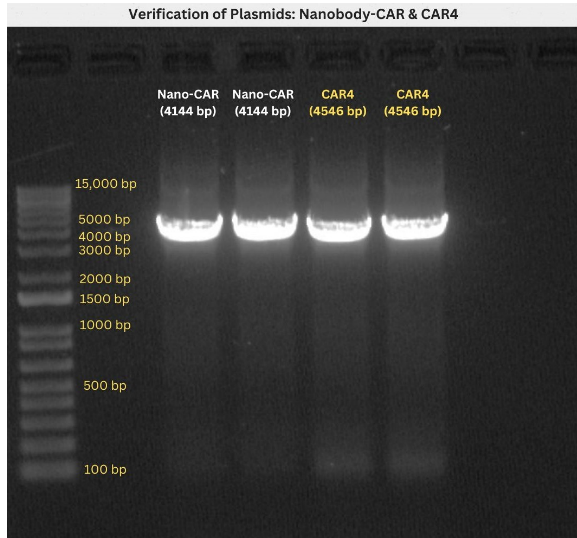

Figure 1 Annotated gel electrophoresis image, UV-visualized, demonstrating the verification of plasmids in Nanobody-CAR and CAR4. Band sizes are aligned with a standard DNA ladder for comparison.

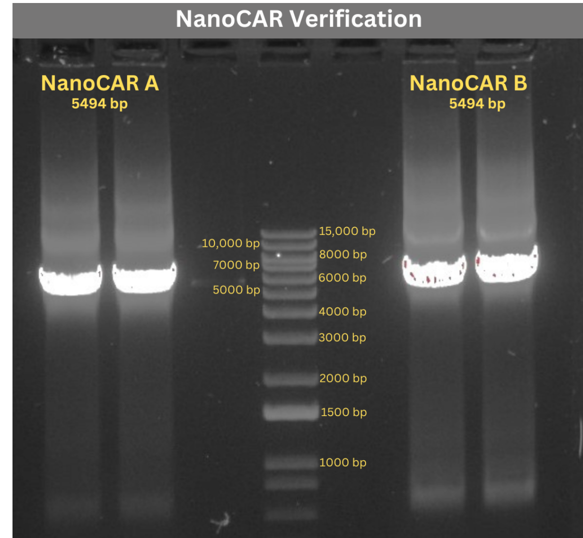

Figure 2 Annotated gel electrophoresis image, UV-visualized, demonstrating the verification of nanoCAR plasmids. Band sizes are aligned with a standard DNA ladder for comparison.

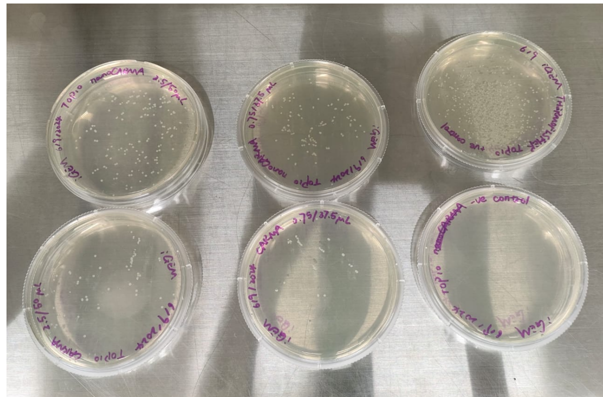

Figure 3 Image illustrating the successful cloning process of nanoCAR-Ma and CAR-Ma.

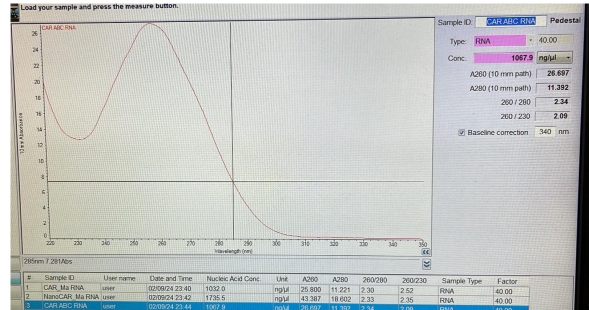

Figure 4 Image displaying the Nanodrop results, including a graph and numerical data that provide information on the nucleic acid concentration and purity levels of CAR-Ma RNA, nanoCAR-Ma RNA, and CAR ABC RNA.

Figure 5 Screen captures of plasmid sequence verification, used to confirm the presence of specific fragments.

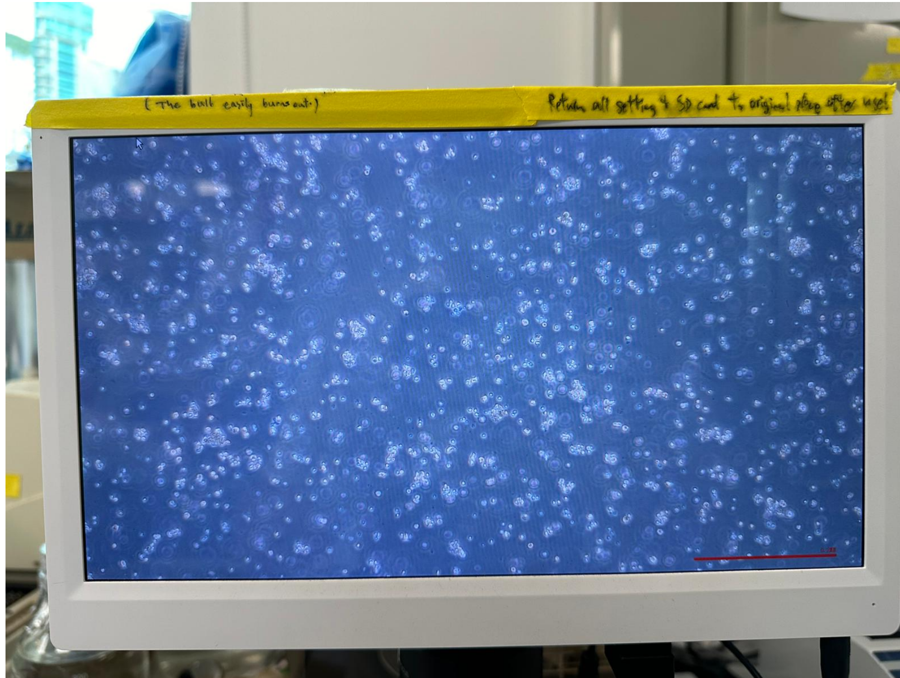

Figure 6 THP-1 cells prior to passage—a technique for transferring cells to a new culture vessel for continued growth—showing approximately 70% confluence, indicating that 70% of the culture surface is covered by cells.

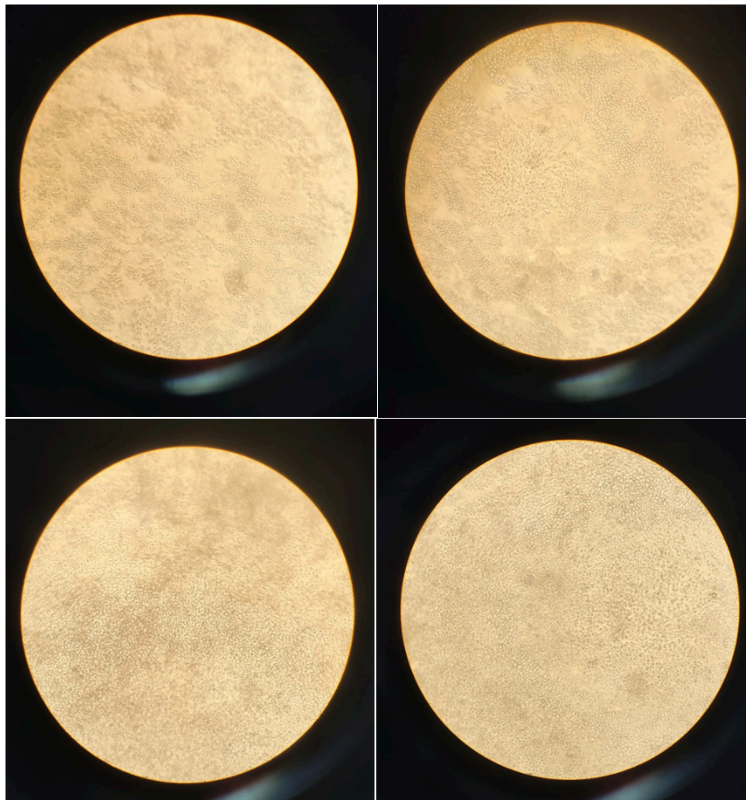

Figure 7 Microscope image of THP-1 cells treated with puromycin (a selection agent) at concentrations ranging from 0.2 to 5 µg/mL for cell culture visualization purposes and the selection of transfected cells; Puromycin is commonly used to identify and maintain cells that have successfully integrated a plasmid containing a puromycin-resistance gene, as only those cells expressing this resistance will survive in the presence of puromycin.

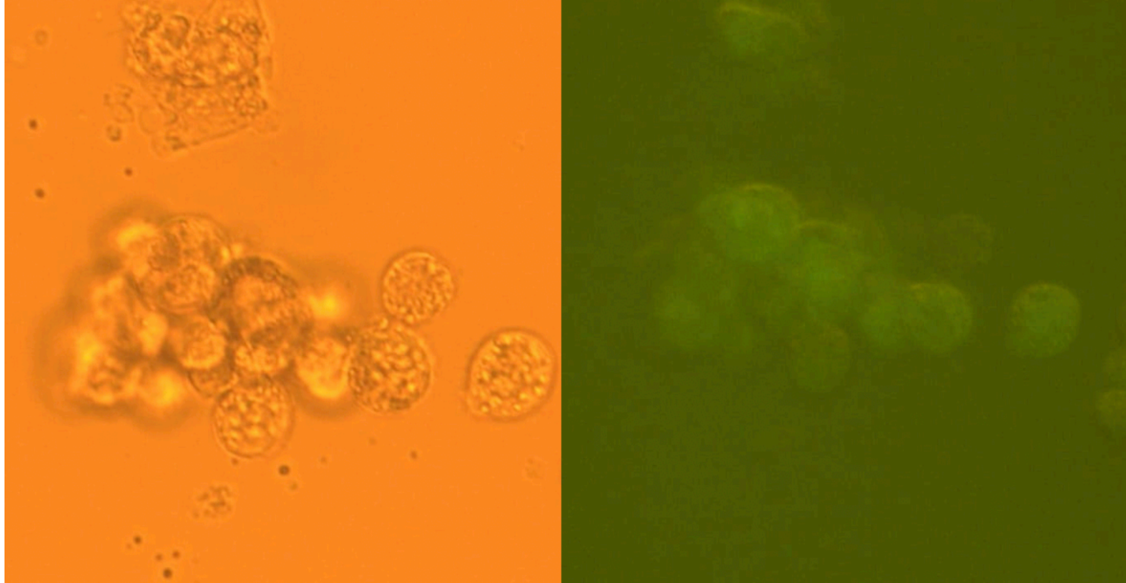

Figure 8 Microscope images of transfected THP-1: left without green fluorescent protein (GFP), right with GFP phagocytosing HEPG2 cells; Note: Confirmation is still needed.

4 Conclusion

This is CAR-Ma, a manifestation of multi-modality in RNA therapeutics—designed to be long-lasting, non-integrative, and controllable. Offering a versatile, cost-effective, and impactful solution for targeted therapies, CAR-Ma aims to achieve optimal therapeutic outcomes for cancer while minimizing the potential risks associated with traditional approaches.

Beyond the classroom, this research can have a significant impact by informing and being integrated into research and development initiatives, thereby contributing to advancements in fields such as genomics, biotechnology and medicine. Moreover, in regard to the social aspect of CAR-Ma, this project can inspire the next generation of scientists to explore synthetic biology with a fresh perspective, fostering an understanding that biological and health challenges are like puzzles, where solutions may reside within the intricacies of our biological systems. This awareness can encourage young scientists to enhance their critical thinking skills. Additionally, CAR-Ma can play a vital role in the global movement to foster innovation and entrepreneurship, paving the way for the development of new technologies and projects with significant potential for commercialization and widespread application.

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