RESEARCH ARTICLE

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Integrative Computational Immunology Applied to Identify and Characterize CD8+ T-cell Neoepitopes of Shared KRAS Neoantigen Oncogenic Driver Hotspot Mutations

Leana Rich Herrera-Ong*

Abstract

Background: Lung, pancreatic, and colorectal cancers continue to rank among the most lethal malignancies. Despite the availability of advanced treatments, these cancer types demand more effective immunotherapeutic solutions. Neoantigen formation is a common feature of cancer mechanisms, with the Kirsten rat sarcoma viral oncogene homolog (KRAS) being the most frequently mutated oncogene in various cancers. This study employed immunoinformatic tools and databases to identify and analyze potential cytotoxic T-cell neoepitopes derived from KRAS hotspot mutations. **Methods:** Experimentally validated neoepitopes were obtained from NEPdb. Key parameters such as IC_{50} values, differential agretopicity, mutation positions, and immunogenicity of the neoepitopes were determined. Results from this analysis, combined with additional factors recommended in the literature, were used to identify and characterize potential KRAS neoepitopes. Sequences with G12A, G12C, G12D, G12S, G12V, G12R, G13D, and A59T mutations were prepared. IC_{50} values and differential agretopicity were analyzed using NetMHCPan4.1. The epitopes' safety profiles and population coverage were assessed, and the top recommended neoepitopes were docked to calculate the dissociation constant (K_D) and Gibbs free energy of binding (ΔG_{bind}). **Results:** Twenty-one candidate KRAS CD8+ neoepitopes were identified. The recommended neoepitopes (KLVVVGAAGV, LVVVGAAGV, LVVVGACGV, KLVVVGADGV, LVVVGADGV, KLVVVGASGV, KLVVVGAV, KLVVVGAVGV, LVVVGAVGV, and VVGAVGVGK) exhibit TCR-facing mutated residues, significant differential agretopicity, and higher binding affinity compared to their wild-type counterparts. Safety and population coverage analyses suggest that these candidate KRAS neoepitopes are unlikely to cause allergy, toxicity, or cross-reactivity, and they demonstrate coverage across a substantial proportion of the population. **Conclusion:** This study introduced a preliminary integrative workflow for neoepitope identification. Findings indicate that the 21 candidate KRAS neoepitopes have the potential to be recognized by cytotoxic lymphocytes and trigger immune response. This positions them as promising elements for anti-cancer vaccine formulations, pending successful in vitro, animal, and clinical studies.

Keywords: KRAS- neoepitope- lung cancer- pancreatic cancer- colorectal cancer

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Introduction

Cancer ranks as the second leading cause of mortality worldwide, with lung cancer accounting for the majority of these deaths, making it the deadliest form of the disease. Other highly fatal cancers include colorectal and pancreatic cancer [1]. Despite advancements in treatments like surgery, radiation, passive immunotherapy, and chemotherapy, the global 5-year survival rates for lung (10-20%), pancreatic (12%), and colorectal (64-65%) cancer patients remain alarmingly low [2, 3, 4].

Cancers often generate neoantigens, which can include over 10 tumor-specific somatic mutations per megabase (Mb) [5]. These neoantigens, also known as tumor-specific antigens (TSA), arise from somatic DNA mutations, viral open reading frame (ORF) integrations, altered RNA splicing mechanisms, and disrupted post-translational modifications. Since neoantigens are unique to cancer cells, they serve as ideal targets for immunotherapy. Among oncogenes, the Kirsten rat sarcoma viral oncogene homolog (KRAS) has the highest mutation rate across all cancer types. As a member of the RAS family of GTPases, *KRAS* hydrolyzes guanosine triphosphate (GTP) into guanosine diphosphate (GDP). Other RAS family members include Harvey rat sarcoma viral oncogene (*HRAS*) and neuroblastoma rat sarcoma viral oncogene (*NRAS*). The KRAS structure consists of an effector-binding lobe, an allosteric region, and a carboxy-terminal region responsible for membrane anchoring, while the effector region contains the P loop

*Department of Biochemistry and Molecular Biology, College of Medicine, University of the Philippines Manila, Manila City, Philippines. *For Correspondence: lmherrera2@up.edu.ph*

and the switch I/II regions. Under normal circumstances, RAS acts as a molecular switch, alternating between an active GTP-bound state and an inactive GDP-bound state. This process induces conformational changes in the switch I and II regions, facilitating the transduction of extracellular signals to downstream effectors like PI3K in the PI3K–AKT pathway and RAF1 in the MAPK pathway. Through these mechanisms, activated *KRAS* plays a critical role in regulating cell proliferation, differentiation, and apoptosis [6].

KRAS neoantigens are linked to highly lethal cancers, including pancreatic ductal adenocarcinoma (PDAC), non-small cell lung cancer (NSCLC), and colorectal cancer [7]. The distribution of *KRAS* missense mutations varies among cancer types, with over 80% occurring at codon 12 (exon 2). This mutation alters the conformation of the GTP-binding site, decreasing GTP hydrolysis and leading to constant activation of RAS. The *KRAS* G12D substitution is present in 70% of PDAC and 50% of colorectal cancer (CRC) cases, while G12C is common in NSCLC which is 40% of metastatic lung adenocarcinoma cases [8]. Dominant mutations include G12D (67.6%) in pancreatic cancers, and G12D (39%) and G12V (24%) in colorectal carcinoma. G12C mutations are observed in 39%, 7%, and 1% of NSCLC, CRC, and PDAC cases, respectively. G12A appears in 11% of NSCLC cases [6], 0.48% of pancreatic cancers, and 2.13% of CRC cases [9]. The G12S mutation, although rare, is found in 1.84% of CRC patients, 0.5% of NSCLC cases, and 0.5% of pancreatic cancer cases [10]. In contrast, G12R is more prevalent in pancreatic cancer (13.54%) [9] and is present in around 1% of NSCLC and CRC cases [11]. Additionally, the G13D mutation, at exon 2 residue 13, occurs 7.31% of CRC patients, 0.8% of NSCLC cases, and 0.51% of pancreatic cancer cases. Lastly, the oncogenic *KRAS* hotspot mutation A59T in exon 3 is observed in 0.05% of AACR GENIE cases, predominantly in colorectal and lung adenocarcinoma [9].

Current cancer treatments such as radiotherapy, surgery, and chemotherapy have achieved progress in patient outcomes. However, highly lethal cancers like lung, colorectal, and pancreatic cancers still have unsatisfactory prognoses. Immunotherapy has emerged as a promising approach, utilizing the body's activated cytotoxic T cells to specifically target and destroy cancer cells that present specific neoantigens. Encouraging results from studies on *KRAS*-mutation-based cancer vaccines point to their potential as immunotherapeutic agents [7, 12]. These vaccines, which can be peptide-based, mRNA-based, or dendritic-cell-based, hold promise for addressing *KRAS*-driven oncogenesis [8]. Mutated wild-type peptides, called heteroclitic epitopes, are particularly effective in activating tolerant T cells. Tumor-specific CD8+ T cells have been isolated from cancer patients, and when stimulated, these cells can identify and target tumor cells expressing neoantigens through MHC I and T-cell receptor interactions [13]. A successful immunotherapy response is characterized by the release of interferon-gamma (IFN-γ) by CD8+ T cells upon encountering neoepitope-expressing cancer cells.

Recent research highlights that *KRAS* mutations are linked to poor outcomes and higher tumor aggressiveness in colorectal, lung, and pancreatic cancer cases [14]. Despite their significance, *KRAS*-specific vaccines for treating these deadly cancers are not yet available, emphasizing the need for innovative and targeted vaccine development strategies to combat *KRAS*-driven cancer growth. This study employed immunoinformatics, combining essential factors influencing neoepitope immunogenicity with the characteristics of experimentally validated neoepitopes. Through this approach, it identified and characterized CD8+ T-cell neoepitopes derived from eight common oncogenic mutations located in *KRAS* hotspots. With artificial intelligence playing an increasingly pivotal role in medicine, immunoinformatics is becoming a global tool for efficient and cost-effective epitope identification and vaccine development for various diseases, including cancers. The *KRAS* neoepitopes characterized in this study could serve as components for future vaccines—used individually or in combination—targeting lung, pancreatic, and colorectal cancers. Additionally, the research introduced an innovative workflow for identifying and analyzing neoepitopes from neoantigen sequences.

Materials and Methods

Retrieval and analysis of experimentally validated neoepitopes

The twenty most recently validated neoepitopes, comprising ten effective and ten ineffective ones along with their corresponding HLA binders, were retrieved from NEPdb (http://nep.whu.edu.cn), a comprehensive database containing over 17,000 experimentally confirmed immunogenic and non-immunogenic neoepitopes associated with human leukocyte antigen (HLA). These neoepitopes were evaluated using an integrative screening pipeline presented in this study. Key parameters analyzed include: the binding affinity (IC_{50}) comparison between mutant peptides (MP) and wild-type peptides (WP), the differential agretopicity between MP and WP, and the positioning of mutated residues interacting with the T-cell receptor (TCR) in the nonameric core sequence relative to their validated HLA binders. IC_{50} values of all validated MPs and their corresponding WPs were derived using NetMHCPan4.1 BA, as recommended by the Immune Epitope Database and Tools (IEDB). Additionally, the immunogenicity of effective neoepitopes was assessed using two advanced prediction tools: the IEDB Class I Immunogenicity tool (CIIm), which predicts epitope-MHC complex immunogenicity based on peptide properties and positions [15]; and the DeepImmuno-CNN 1.2 (DIm), a convolutional neural network-based tool that generates continuous immunogenic scores, where higher scores indicate higher immunogenic potential [16]. The DIm model identifies positions 4–6 in peptides as critical for immunogenicity [17].

Preparation of target peptides and mapping of cytotoxic T-cell KRAS neoepitopes

A curated amino acid sequence of the human GTPase KRAS protein (UniProt ID: P01116), encoded by the

KRAS gene, was sourced from UniProt and employed for neoepitope mapping. Specific *KRAS* residues (12, 13, and 59) were substituted with eight common oncogenic missense mutations: G12A, G12C, G12D, G12S, G12V, G12R, G13D, and A59T. Neoepitopes were mapped using NetMHCPan4.1 BA within IEDB. Only predicted neoepitopes with an IC_{50} below 500 nM were retained for further examination. The NetMHCPan4.1 model, trained on over 850,000 quantitative binding affinity (BA) measurements, utilizes an artificial neural network (ANN) to predict peptide binding affinities to major histocompatibility complexes (MHC) of known sequences. Recent advancements in its algorithm achieve over 90% accuracy in identifying naturally presented MHC peptides with 98% specificity [18]. The improved ability of NetMHCPan4.1 to differentiate positive from negative neoepitope data has also been demonstrated [19]. Neoepitopes involving post-translationally modified (PTM) residues in the original sequence were excluded from subsequent analysis.

Identification of potentially cross-reactive, allergenic and toxic neopitopes

BLASTp was utilized to identify epitopes with matches in the Reference Protein (RefSeq) database. Model sequences, non-redundant RefSeq proteins, and uncultured/environmental sample sequences were excluded to prevent the inclusion of unrelated sequences. Neoepitopes with an E-value < 0.1, 100% sequence coverage, and 100% sequence identity to any human protein sequence, excluding *KRAS*, were discarded. Potentially toxic peptides among the remaining epitopes were assessed using the Hybrid (ET+MERCI) method and a threshold value of 0.38 on the ToxinPred3.0 server (https://webs.iiitd.edu.in/raghava/toxinpred3/). The hybrid approach in ToxinPred3.0 demonstrated superior performance, achieving an AUROC of 0.98 and an MCC of 0.81 [20]. Potential allergens in the list were identified using AllerCatPro 2.0. On the validation dataset, AllerCatPro 2.0 outperformed other benchmark methods for predicting allergenic proteins, achieving a maximum performance of AUC 0.98 and MCC of 0.85 [21].

Identification of candidate neoepitopes and estimation of population coverage

Results from the preliminary analysis of 20 experimentally validated neoepitopes, along with factors identified from various studies in the literature to distinguish effective from ineffective neoepitopes, were integrated to establish a comprehensive set of criteria for selecting candidate *KRAS* neoepitopes. Additionally, two state-of-the-art CD8+ immunogenicity tools (CIIm and DIm) were utilized to evaluate the immunogenicity of the predicted neoepitopes. At present, there is no standard threshold for differentiating immunogenic from non-immunogenic peptides using these tools. Generally, higher scores suggest a greater likelihood of inducing immunogenicity [16]. To establish a positive reference threshold in this study, the immunogenicity scores of experimentally validated effective neoepitopes were determined. Predicted neoepitopes with immunogenicity scores lower than the lowest scores observed for CIIm and DIm were excluded from the pool of candidate neoepitopes. By combining the key parameters derived from experimentally validated neoepitopes with insights from various studies on effective neoepitopes, this study classified candidate neoepitopes using the following criteria: peptides with a binding strength fold (BSF) > 1.2, regardless of the mutation position and IC_{50} of the mutant peptide (MP); or peptides with a BSF < 1.2, MP IC_{50} < 500 nM, and a TCR-facing mutation. As highlighted above, immunogenicity scores were also factored into this classification.

The individual population coverage of 21 candidate epitopes was estimated using the Population Coverage tool in IEDB. More importantly, the population coverage in regions where pancreatic, lung, and colorectal cancers are predominant, was determined. These areas include: Europe, USA, East Asia, Northeast Asia, South Asia, Southwest Asia, and world [22, 23, 24].

The top candidate neoepitopes were chosen from the pool of candidate neoepitopes using stricter criteria. Specifically, neoepitopes with a TCR-facing mutated residue, a BSF exceeding 1.2, an IC_{50} below 500 nM for the MP sequence, and an IC_{50} above 500 nM for the corresponding WP [25] were identified as the most recommended neoepitopes.

Molecular docking of neoepitopes with HLA I allele binders

All recommended candidate neoepitopes for each mutation, or alternatively, those with the lowest IC_{50} value when recommendations were unavailable, along with 10 experimentally validated effective neoepitopes, were subjected to docking with their respective HLA allele binders using GalaxyPepDock (https://galaxy.seoklab.org/). The structural PDB files for the HLA allele binders were obtained from the Research Collaboratory for Structural Bioinformatics Protein Data Bank (RCSB PDB), with unrelated heteroatoms removed. The PROtein binDIng enerGY prediction (PRODIGY) webserver was utilized to estimate the Gibbs free energy of binding (ΔG_{bind}) and dissociation constant (K_D) for the docked MHC-epitope complexes at 37°C.

Results

Immunogenicity of the twenty experimentally validated neoepitopes

A total of ten effective (P1 to P10) and ten ineffective (N1 to N10) neoepitopes, validated through experimental methods, were sourced from NEPdb. Details for each neoepitope, including mutated peptide sequences, gene symbols, mutation positions, mutated residues, and the HLA I allele binder confirmed experimentally, are presented in Table 1.

Table 2 summarizes the findings from analyzing experimentally validated neoepitopes. Among the 10 effective neoepitopes, 7 (P2, P3, P4, P6, P7, P8, and P10) show IC_{50} values below 500 nM, indicating that many effective CD8+ neoepitopes exhibit high to intermediate binding affinity with MHC I. Specifically, P4 and P8 have

Table 1. Experimentally Validated Neoepitopes

Code	Mutated peptide	Gene symbol/ mutation	Residues	Known HLA I allele binder
P1	HMTEVVRHC	*TP53/ R175H*	9	A*02:01
P2	GVYPMPGTQK	*NCAPH2/ S181Y*	10	A*03:01
P3	SLFEGIDIYT	*HSPA1A/ F293I*	10	A*02:01
P4	KINKNPKYKK	*MYO1B/ E969K*	10	A*03:01
P5	ACDPHSGHFV	*CDK4/ R24C*	10	A*02:01
P6	FIASNGVKLV	*ACTN4/ K122N*	10	A*02:01
P7	AEPINIQTW	*LPGAT1/ D99N*	9	B*44:03
P8	RFLEYLPLRF	*DCAKD/ S199F*	10	A*24:02
P9	KLKFVTLVF	*ACPP/ E34K*	9	A*24:02
P10	DMKARQKALV	*FAM50B/ E78K*	10	B*08:01
N1	YSPSRNPPEVEAQ	*FBRSL1/ R1045Q*	13	A*02:01
N2	VVGACGVGK	*KRAS/ G12C*	9	C*03:03
N3	VVGACGVGK	*KRAS/ G12C*	9	A*24:02
N4	VVGACGVGK	*KRAS/ G12C*	9	B*40:01
N5	VVGACGVGK	*KRAS/ G12C*	9	B*40:02
N6	KLVVVGAVGV	*KRAS/ G12V*	10	DRB1*08:02
N7	KLVVVGAVGV	*KRAS/ G12V*	10	C*07:02
N8	KLVVVGAVGV	*KRAS/ G12V*	10	B*40:01
N9	KLVVVGAVGV	*KRAS/ G12V*	10	B*57:01
N10	KLVVVGAVGV	*KRAS/ G12V*	10	A*01:01

IC_{50} values under 500 nM, making them stronger HLA binders compared to their respective WP sequences, which have IC_{50} values above 500 nM. Conversely, 3 neoepitopes (P1, P5, and P9) have IC_{50} values greater than 500 nM. It is also noteworthy that the WP sequences of 5 effective neoepitopes (P2, P3, P6, P7, and P10) share IC_{50} values below 500 nM. In contrast, experimentally validated ineffective neoepitopes are characterized by either extremely high IC_{50} values (>5000 nM), classifying them as non-binders [26], or the absence of HLA allele binding

Table 2. TCR-Facing Mutation, Binding Affinity, Agretopicity, and Immunogenicity Scores of Experimentally Validated Neoepitopes

Code	Mutation Position	IC_{50} WT (nM)	IC_{50} MT (nM)	Differential Agretopicity	DIm	CIIm
P1	TCR-facing	5812.57	4543.17	1.27940843	0.873025179	0.43239
P2	Hidden	104.82	20.37	5.14580265	0.82218194	0.25499
P3	TCR-facing	8.35	19.61	0.42580316	0.919057488	0.17422
P4	Hidden	3353.99	113.42	29.571416	0.698031783	0.1393
P5	Hidden	23413.12	10969.07	2.13446719	0.867245317	0.09319
P6	Hidden	315.65	185.04	1.70584738	0.517387331	-0.00596
P7	TCR-facing	107.36	83.57	1.28467153	0.988803685	-0.19718
P8	Hidden	976.88	28.23	34.6043216	0.359784037	-0.26949
P9	Hidden	7976.35	1002.21	7.95876114	0.428799152	-0.27669
P10	TCR-facing	307.53	243.91	1.26083391	0.538710356	-0.56832
N1	-	Non-binder	Non-binder	-	-	-
N2	-	Non-binder	Non-binder	-	-	-
N3	TCR-facing	42170.89	39917.27	1.05645727	-	-
N4	TCR-facing	43724.63	43899.56	0.99601522	-	-
N5	-	Non-binder	Non-binder	-	-	-
N6	-	Non-binder	Non-binder	-	-	-
N7	-	Non-binder	Non-binder	-	-	-
N8	Hidden	36344.98	34477.97	1.05415081	-	-
N9	TCR-facing	25771.47	22633.5	1.13864272	-	-
N10	TCR-facing	34835.31	35550.39	0.97988545	-	-

as identified using the NetMHCPan tool. Immunogenicity assessments using CIIm and DIm tools revealed that effective neoepitopes scored lowest at 0.35978 (P8, via DIm) and –0.56832 (P10, via CIIm).

Candidate cytotoxic T-cell KRAS neoepitopes identified and characterized

The immunoinformatic workflow designed and implemented in this study initially identified 29 neoepitopes, corresponding to 8 common oncogenic *KRAS* missense mutations (G12A, G12C, G12D, G12S, G12V, G12R, G13D, and A59T) using the NetMHCPan tool. After filtering out peptides that were potentially allergenic, toxic, or cross-reactive, candidate neoepitopes were selected based on criteria derived from experimentally validated neoepitopes used in the study. These criteria were further reinforced using the findings from prior studies on effective neoepitopes. Table 3 highlights 21 KRAS CD8+ candidate neoepitopes (49 peptide-HLA pairs) identified in this study, with no candidates found for KRAS G12R. Additionally, two predicted candidate neoepitopes were experimentally validated as ineffective for specific HLA alleles. These include KLVVVGAVGV with DRB108:02 (N6), C07:02 (N7), B40:01 (N8), B57:01 (N9), and A01:01 (N10); and VVGACGVGK with A24:02 (N2), B40:01 (N3), B40:02 (N4), and C03:03 (N5). Nevertheless, the study suggests that KLVVVGAVGV could potentially serve as a *KRAS* neoepitope when presented by A02:01 (K36), A02:03 (K37), and A02:06 (K38), while VVGACGVGK might have similar potential when presented by A03:01 (K18) and A11:01 (K19).

The data in Table 4 highlights agretopicity, mutation position, and immunogenicity scores for candidate neoepitopes. Differential agretopicity (DA) values for these neoepitopes varies from 0.266 (K48) to 44.96 (K35), with 77.6% (38 out of 49) of peptide-HLA pairs presenting DA values over 1.2. Mutation position also plays a critical role in identifying candidate neoepitopes, as 17 out of 21 candidates (81%) exhibit TCR-facing mutations. Immunogenicity scores range from 0.148 (K19) to 0.942 (K4) in DIm, and from -0.196 (K1) to 0.22845 (K4) in CIIm. It's notable that the DIm tool did not accept the K35 peptide-HLA pair due to having fewer than 9 residues. Notably, the K4 peptide-HLA pair (KLVVVGAAGV:HLA-A*02:06) records the highest immunogenicity scores in both tools.

Candidate KRAS neoepitopes confer wider population coverage

The estimation of population coverage showed that the 21 candidate neoepitopes collectively encompass 78.47% of the global population (Table 5). Within specific regions, this set provides coverage of 87.23% in Europe, 75.62% in the USA, 52.67% in East Asia, 70.49% in Northeast Asia, 66.39% in South Asia, and 61.54% in Southwest Asia. Notably, the neoepitope VVVGAGDVGK achieves the highest regional coverage of 47.38% in Northeast Asia.

The top neoepitopes identified

This study strongly recommends ten candidate neoepitopes (K4, K7, K17, K25, K28, K34, K35, K38, K41, and K44). These neoepitopes exhibit specific characteristics: they possess TCR-facing mutated residues, display DA values greater than 1.2, have IC_{50} values below 500 nM for their MP sequences, and IC_{50} values exceeding 500 nM for their corresponding WP sequences (referenced in Table 4). Additionally, the immunogenicity scores of these recommended neoepitopes surpass the lowest scores observed among experimentally validated effective neoepitopes in this study, with the sole exception of K35, which lacks a measurable immunogenicity score according to the DIm tool.

Neoepitopes docked on the peptide-binding groove of HLA I

Figure 1 illustrates the recommended candidate neoepitopes (highlighted in yellow) situated on the peptide-binding groove of HLA I molecules (depicted in magenta and blue). The study calculated Gibbs free energy of binding (ΔG_{bind}) and dissociation constant (K_D) for candidate *KRAS* neoepitopes and experimentally validated effective neoepitopes (P1-P10), as detailed in Table 6. Binding affinity can be evaluated via equilibrium K_D, with a smaller K_D value signifying stronger ligand-target protein binding. A K_D value below 1.0e-07 M indicates high affinity in biochemical systems [27]. Among the experimentally validated neoepitopes, six (P1, P3, P5, P8,

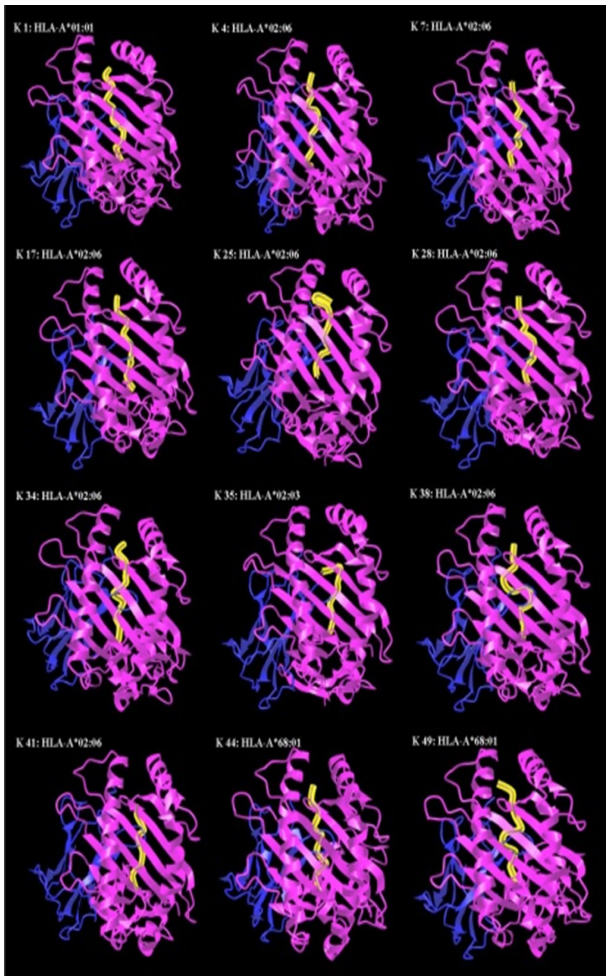

Figure 1. Structural Representations of HLA I Docked with the Selected Candidate CD8+ KRAS Neoepitopes

Table 3. Candidate KRAS Cytotoxic T-Cell Neoepitopes

Peptide-HLA Pair	Candidate Neoepitopes	Length	KRAS Mutation	HLA I Binders	IC_{50} WP (nM)	IC_{50} MP (nM)
K1	ILDTTGQEEY	10	A59T	HLA-A*01:01	85.26	91.82
K2	KLVVVGAAGV	10	G12A	HLA-A*02:01	346.13	180.48
K3		10		HLA-A*02:03	43.02	28.81
K4		10		HLA-A*02:06	538.7	289.1
K5	LVVVGAAGV	9		HLA-A*02:03	155.12	55.2
K6		9		HLA-A*68:02	230.81	38.78
K7		9		HLA-A*02:06	537.45	62.23
K8	VVGAAGVGK	9		HLA-A*03:01	247.59	225.09
K9		9		HLA-A*11:01	83.45	59.83
K10	VVVGAAGVGK	10		HLA-A*03:01	373.9	287.02
K11		10		HLA-A*11:01	153.55	104.17
K12		10		HLA-A*68:01	287.95	181.46
K13	KLVVVGACGV	10	G12C	HLA-A*02:01	346.13	166.86
K14		10		HLA-A*02:06	538.7	250.3
K15	LVVVGACGV	9		HLA-A*02:03	155.12	94.14
K16		9		HLA-A*68:02	230.81	131.35
K17		9		HLA-A*02:06	537.45	69.83
K18	VVGACGVGK	9		HLA-A*03:01	247.59	214.77
K19		9		HLA-A*11:01	83.45	72.99
K20	VVVGACGVGK	10		HLA-A*03:01	373.9	265.84
K21		10		HLA-A*11:01	153.55	122.12
K22		10		HLA-A*68:01	287.95	174.86
K23	KLVVVGADGV	10	G12D	HLA-A*02:01	346.13	280.47
K24		10		HLA-A*02:03	43.02	45.63
K25		10		HLA-A*02:06	538.7	391.28
K26	LVVVGADGV	9		HLA-A*02:03	155.12	346.43
K27		9		HLA-A*68:02	230.81	153.21
K28		9		HLA-A*02:06	537.45	164.12
K29	VVGADGVGK	9		HLA-A*11:01	83.45	172.2
K30	VVVGADGVGK	10		HLA-A*11:01	153.55	194.2
K31		10		HLA-A*68:01	287.95	231
K32	KLVVVGASGV	10	G12S	HLA-A*02:01	346.13	257.98
K33		10		HLA-A*02:03	43.02	26.71
K34		10		HLA-A*02:06	538.7	419.97
K35	KLVVVGAV	8	G12V	HLA-A*02:03	15516.38	345.15
K36	KLVVVGAVGV	10		HLA-A*02:01	346.13	174.89
K37		10		HLA-A*02:03	43.02	39.07
K38		10		HLA-A*02:06	538.7	261.91
K39	LVVVGAVGV	9		HLA-A*02:03	155.12	134.79
K40		9		HLA-A*68:02	230.81	50.51
K41		9		HLA-A*02:06	537.45	63.63
K42	VVGAVGVGK	9		HLA-A*03:01	247.59	134.21
K43		9		HLA-A*11:01	83.45	38.76
K44		9		HLA-A*68:01	1087.44	192.6
K45	VVVGAVGVGK	10		HLA-A*03:01	373.9	185.57
K46		10		HLA-A*11:01	153.55	68.99
K47		10		HLA-A*68:01	287.95	80.38
K48	VVGAGDVGK	9	G13D	HLA-A*11:01	83.45	313.98
K49	VVVGAGDVGK	10		HLA-A*68:01	287.95	229.58

Table 4. Candidate KRAS Cytotoxic T-Cell Neoepitopes with Agretopicity, Mutation Position and Immunogenicity Scores

Peptide-HLA Pair	Candidate Neoepitopes	HLA I Binders	Mutation Position	Differential Agretopicity	DIm	CIIm
K1	ILDTTGQEEY	HLA-A*01:01	TCR-facing	0.928556	0.292322	-0.19566
K2	KLVVVGAAGV	HLA-A*02:01	TCR-facing	1.91783	0.833501	0.13927
K3		HLA-A*02:03	TCR-facing	1.493232	0.822209	0.15102
K4		HLA-A*02:06	TCR-facing	1.863369	0.94214	0.22845
K5	LVVVGAAGV	HLA-A*02:03	TCR-facing	2.810145	0.532582	0.21779
K6		HLA-A*68:02	TCR-facing	5.951779	0.762563	0.21779
K7		HLA-A*02:06	TCR-facing	8.63651	0.802406	0.13927
K8	VVGAAGVGK	HLA-A*03:01	TCR-facing	1.09996	0.521402	0.20349
K9		HLA-A*11:01	TCR-facing	1.394785	0.266053	0.21779
K10	VVVGAAGVGK	HLA-A*03:01	Hidden	1.302697	0.700797	0.20349
K11		HLA-A*11:01	Hidden	1.474033	0.576229	0.13927
K12		HLA-A*68:01	Hidden	1.586851	0.754608	0.20349
K13	KLVVVGACGV	HLA-A*02:01	Hidden	2.074374	0.823307	0.21961
K14		HLA-A*02:06	Hidden	2.152217	0.903131	0.17759
K15	LVVVGACGV	HLA-A*02:03	TCR-facing	1.647759	0.572131	0.04515
K16		HLA-A*68:02	TCR-facing	1.757214	0.929435	0.21961
K17		HLA-A*02:06	TCR-facing	7.696549	0.851053	0.04515
K18	VVGACGVGK	HLA-A*03:01	TCR-facing	1.152815	0.460909	0.17759
K19		HLA-A*11:01	TCR-facing	1.143307	0.147856	0.04515
K20	VVVGACGVGK	HLA-A*03:01	Hidden	1.406485	0.722816	0.17759
K21		HLA-A*11:01	Hidden	1.25737	0.823232	0.16006
K22		HLA-A*68:01	Hidden	1.646746	0.887195	0.21961
K23	KLVVVGADGV	HLA-A*02:01	TCR-facing	1.234107	0.805391	0.18825
K24		HLA-A*02:03	TCR-facing	0.942801	0.817791	0.09907
K25		HLA-A*02:06	TCR-facing	1.376763	0.895555	0.00495
K26	LVVVGADGV	HLA-A*02:03	TCR-facing	0.447767	0.330562	0.00495
K27		HLA-A*68:02	TCR-facing	1.506494	0.616712	0.09907
K28		HLA-A*02:06	TCR-facing	3.274738	0.68126	0.09907
K29	VVGADGVGK	HLA-A*11:01	TCR-facing	0.484611	0.224263	0.16329
K30	VVVGADGVGK	HLA-A*11:01	TCR-facing	0.79068	0.82113	0.16329
K31		HLA-A*68:01	TCR-facing	1.246537	0.844393	0.16329
K32	KLVVVGASGV	HLA-A*02:01	TCR-facing	1.341693	0.768152	0.21024
K33		HLA-A*02:03	TCR-facing	1.610633	0.708859	0.18731
K34		HLA-A*02:06	TCR-facing	1.282711	0.892968	0.21024
K35	KLVVVGAV	HLA-A*02:03	TCR-facing	44.95547	Not acceptable	0.01104
K36	KLVVVGAVGV	HLA-A*02:01	TCR-facing	1.97913	0.785848	0.22254
K37		HLA-A*02:03	TCR-facing	1.101101	0.803435	0.11964
K38		HLA-A*02:06	TCR-facing	2.056813	0.890599	0.01104
K39	LVVVGAVGV	HLA-A*02:03	TCR-facing	1.150827	0.608027	0.22254
K40		HLA-A*68:02	TCR-facing	4.56959	0.820612	0.11964
K41		HLA-A*02:06	TCR-facing	8.446488	0.808952	0.19374
K42	VVGAVGVGK	HLA-A*03:01	TCR-facing	1.844795	0.581758	0.19412
K43		HLA-A*11:01	TCR-facing	2.152993	0.187601	0.11964
K44		HLA-A*68:01	TCR-facing	5.646106	0.641116	0.01104
K45	VVVGAVGVGK	HLA-A*03:01	TCR-facing	2.014873	0.88824	0.22254
K46		HLA-A*11:01	TCR-facing	2.225685	0.872597	0.19374
K47		HLA-A*68:01	TCR-facing	3.582359	0.910372	0.19412
K48	VVGAGDVGK	HLA-A*11:01	TCR-facing	0.265781	0.394399	0.21234
K49	VVVGAGDVGK	HLA-A*68:01	Hidden	1.254247	0.601694	0.21234

Table 5. Population Coverage of Candidate KRAS CD8+ T-Cell Neoepitopes

Cytotoxic Epitope	World	Europe	USA	East Asia	Northeast Asia	South Asia	Southwest Asia	Average
ILDTTGQEEY	17.34%	25.67%	12.94%	2.55%	3.44%	13.80%	14.66%	12.91%
KLVVVGAAGV	41.35%	47.37%	44.19%	35.98%	30.11%	19.20%	27.03%	35.03%
KLVVVGACGV	40.60%	47.37%	44.05%	35.65%	20.34%	17.37%	27.03%	33.20%
KLVVVGADGV	41.35%	47.37%	44.19%	35.98%	30.11%	19.20%	27.03%	35.03%
KLVVVGASGV	41.35%	47.37%	44.19%	35.98%	30.11%	19.20%	27.03%	35.03%
KLVVVGAV	0.97%	0.00%	0.19%	0.42%	10.99%	2.01%	0.00%	2.08%
KLVVVGAVGV	41.35%	47.37%	44.19%	35.98%	30.11%	19.20%	27.03%	35.03%
LVVVGAAGV	5.38%	1.73%	6.49%	15.72%	17.27%	10.48%	2.88%	8.56%
LVVVGACGV	5.38%	1.73%	6.49%	15.72%	17.27%	10.48%	2.88%	8.56%
LVVVGADGV	5.38%	1.73%	6.49%	15.72%	17.27%	10.48%	2.88%	8.56%
LVVVGAVGV	5.38%	1.73%	6.49%	15.72%	17.27%	10.48%	2.88%	8.56%
VVGAAGVGK	30.92%	34.68%	24.27%	17.76%	46.57%	33.60%	21.45%	29.89%
VVGACGVGK	30.92%	34.68%	24.27%	17.76%	46.57%	33.60%	21.45%	29.89%
VVGADGVGK	15.53%	11.17%	11.60%	16.42%	44.30%	22.81%	12.93%	19.25%
VVGAGDVGK	15.53%	11.17%	11.60%	16.42%	44.30%	22.81%	12.93%	19.25%
VVGAVGVGK	35.75%	39.79%	29.79%	18.87%	47.38%	42.69%	27.27%	34.51%
VVVGAAGVGK	35.75%	39.79%	29.79%	18.87%	47.38%	42.69%	27.27%	34.51%
VVVGACGVGK	35.75%	39.79%	29.79%	18.87%	47.38%	42.69%	27.27%	34.51%
VVVGADGVGK	20.88%	17.15%	17.58%	17.53%	45.13%	32.64%	19.06%	24.28%
VVVGAGDVGK	5.83%	6.35%	6.36%	1.22%	1.12%	11.23%	6.58%	5.53%
VVVGAVGVGK	35.75%	39.79%	29.79%	18.87%	47.38%	42.69%	27.27%	34.51%
Cumulative Coverage	78.47%	87.23%	75.62%	52.67%	70.49%	66.39%	61.54%	-

Table 6. Binding Free Energy and Dissociation Constants of Selected Candidate KRAS Neoepitopes and Experimentally Validated Effective Neoepitopes

Peptide-HLA Pair	Mutation	ΔGbind (kcal/mol)	K_D (M)
K1	*KRAS; A59T*	-9.5	1.90E-07
K4	*KRAS; G12A*	-10.3	5.60E-08
K7	*KRAS; G12A*	-9.6	1.60E-07
K17	*KRAS; G12C*	-9.3	2.90E-07
K25	*KRAS; G12D*	-9.4	2.30E-07
K28	*KRAS; G12D*	-10	8.60E-08
K34	*KRAS; G12S*	-11.2	1.30E-08
K35	*KRAS; G12V*	-9	4.30E-07
K38	*KRAS; G12V*	-10.2	6.60E-08
K41	*KRAS; G12V*	-9.4	2.50E-07
K44	*KRAS; G12V*	-10.5	4.20E-08
K49	*KRAS; G13D*	-10.1	7.20E-08
P1	*TP53; R175H*	-11.2	1.20E-08
P2	*NCAPH2; S181Y*	-9.9	1.10E-07
P3	*HSPA1A; F293I*	-11.1	1.50E-08
P4	*MYO1B; E969K*	-9.2	3.50E-07
P5	*CDK4; R24C*	-10.6	3.30E-08
P6	*ACTN4; K122N*	-9.6	1.60E-07
P7	*LPGAT1; D99N*	-9.6	1.80E-07
P8	*DCAKD; S199F*	-11.7	5.70E-09
P9	*ACPP; E34K*	-10.9	2.20E-08
P10	*FAM50B; E78K*	-10.5	4.00E-08

P9, and P10) meet this criterion. Out of 12 docked *KRAS* neoepitopes, six pairs (K4, K28, K34, K38, K44, and K49) show high binding affinity, while the remaining six exhibit K_D values close to the threshold, signaling favorable complex formation. Among the validated neoepitopes, P8 (RFLEYLPLRF, S199F) binds most spontaneously to HLA-A24:02, with ΔG_{bind} of -11.7 and K_D of 5.7E-09, the lowest in the group. Conversely, P4 (KINKNPKYKK, E969K) binds the least spontaneously to HLA-A03:01, with ΔG_{bind} of -9.2 and K_D of 3.5E-07. For candidate KRAS neoepitopes, K34 (ΔG_{bind} = -11.2) demonstrates the most favorable binding, while K35 (ΔG_{bind} = -9.0) represents the least, consistent with their K_D values of 1.3E-08 and 4.3E-07, respectively.

Discussion

Pancreatic, lung, and colorectal cancers rank among the most fatal cancer types globally. Despite advancements in medical strategies and enhanced anti-cancer treatments, the outlook for these cancers remains grim. Consequently, there is a pressing need for more effective and specialized therapies, like neoantigen-based cancer vaccines, to improve patient outcomes.

Genetic changes in cancer cells give rise to neoantigens, among which the KRAS neoantigen is linked to highly deadly cancers like pancreatic ductal adenocarcinoma (PDAC), non-small cell lung cancer (NSCLC), and colorectal cancer (CRC). Common missense mutations, such as KRAS G12D and G12C, are frequently found in PDAC, CRC, and metastatic lung adenocarcinoma patients

[8]. These mutations make KRAS an appealing target for developing anti-cancer vaccines and immunotherapy against these aggressive cancer types. Neoantigen-derived peptides can be presented by MHC molecules on tumor cells, enabling T-cell receptor (TCR) recognition and triggering an anti-tumor immune response. Tumor vaccines play a role in enhancing antigen presentation and activating TCR-specific T cells, helping to restore the effector function of CD8+ T cells in the immune system [5].

This study identified potentially immunogenic neoepitopes from eight common oncogenic KRAS missense mutations using immunoinformatics and databases. While machine learning tools for neoepitope prediction exist, many primarily focus on HLA-peptide binding and overlook other critical factors influencing T-cell responses, leading to high false-positive rates and less than 5% accuracy in predictions [17, 28]. To address this, an integrative screening workflow was developed. Experimentally validated neoepitopes were analyzed to determine essential factors for effective T-cell recognition and differentiation. These insights were combined with findings from prior studies to establish criteria for identifying KRAS cytotoxic T-cell neoepitopes in this research.

T cells that trigger immune responses against tumor cells can distinguish between self-peptides and mutant peptides containing single amino acid mutations. The peptide's binding affinity to its corresponding MHC molecule is mainly affected by specific anchor residues that fit into the MHC peptide-binding groove [29]. However, anchor residues are buried within the MHC binding pocket and thus, do not interact with the T-cell receptor (TCR); instead, non-anchor residues do. Studies have shown that TCR-facing residues of peptides presented by MHC I are typically found at positions 4, 5, 6, 7, and 8 of the peptide core sequence [30, 31, 32]. Interestingly, some experiments have demonstrated that TCRs can differentiate between wild-type and anchor-modified peptides, suggesting that anchor residue modifications influence TCR interactions [33, 34]. This indicates that identifying immunogenic neoepitopes based solely on anchor-mutated peptides may be insufficient [17, 35]. Consequently, this study considered additional factors like IC_{50} values and differential agretopicity, alongside the position of the mutant residue, to classify neoepitopes. It was found that peptide sequences with low binding affinity are not presented, whereas neoantigen peptides with significantly improved binding are distinctly recognized by the immune system.

Research indicates a link between outcomes in advanced lung and melanoma cancers and strong-binding neoepitopes with MHC I affinity under 500 nM [36]. Agretopicity, defined as the ratio of neoantigen to wild-type binding, helps measure immunogenicity [37]. Differences in HLA-binding affinity between MP and WP are recognized as reliable markers for neoepitope immunogenicity [38, 39]. Notably, immunogenic neoepitopes show over 20% improvement in binding (ratio > 1.2) compared to their non-immunogenic WP counterparts [39]. In this study, predicted *KRAS* neoepitopes were filtered via NetMHCPan4.1 BA, using IC_{50} values to identify peptide pairs exhibiting more than 20% enhanced binding.

Analysis of experimentally validated neoepitopes revealed that most effective neoepitopes exhibit high to intermediate HLA class I binding affinity. Supporting the earlier findings, WP sequences with IC_{50} values below 500 nM demonstrate that relying solely on IC_{50} values to identify effective neoepitopes is insufficient [17, 35]. This study found that most validated neoepitopes have a DA value exceeding 1.2 (see Table 2). Interestingly, while neoepitope P3 has a DA value below 1.2, its IC_{50} value is under 500 nM, and its mutation is in a TCR-facing position, factors that likely enable recognition by TCR as non-self, leading to immunogenicity. The data imply that, irrespective of the mutation's position, peptides with a BSF greater than 1.2 and an MP sequence IC_{50} value exceeding 500 nM, can be effectively recognized as non-self by TCR, triggering immunogenicity. However, for neoepitopes with a DA below 1.2, mutations must be in a TCR-facing position and exhibit high to intermediate HLA binding affinity (IC_{50} below 500 nM) to ensure recognition and immunogenicity. Notably, ineffective neoepitopes with TCR-facing mutations but DA below 1.2 and MP sequence IC_{50} values above 5000 nM are unlikely to bind effectively to MHC I, leading to poor presentation and reduced immunogenic potential. This may account for their inability to elicit an immune response in specific HLA alleles.

Given the absence of a standardized threshold for immunogenicity scores, effective neoepitopes were analyzed, and their scores were used as references to evaluate the potential immunogenicity of candidate KRAS cytotoxic neoepitopes. None of the candidate KRAS neoepitopes had immunogenicity scores below the minimum scores of experimentally confirmed effective neoepitopes in both immunogenicity tools, further supporting their potential. Overall, the 21 candidate KRAS neoepitopes assessed through these criteria, demonstrate a strong likelihood of binding to specific HLA I alleles and being recognized as foreign by TCRs, thereby triggering an immunogenic response.

The effectiveness of a peptide-based vaccine in stimulating T-cell responses greatly depends on its binding affinity to various HLA alleles. Epitopes with the ability to bind to two or more HLA alleles are generally favored, as they may enhance population coverage. Additionally, targeting dominant HLA alleles in regions where diseases are prevalent, is crucial for inducing immunogenicity in larger populations. Analysis of 21 candidate KRAS neoepitopes revealed a global population coverage of 78.47%. Among these, KLVVVGAV has the lowest individual coverage (2.08%). However, low-coverage neoepitopes shouldn't be dismissed, as they can be combined with other HLA alleles targeting the same mutation to expand coverage. Notably, KLVVVGAAGV, KLVVVGADGV, KLVVVGASGV, and KLVVVGAVGV achieve the highest average population coverage (35.03%). These neoepitopes can be employed alone for patients with G12A, G12D, G12S, and G12V KRAS missense mutations and expressing dominant alleles

like HLA-A02:01, HLA-A02:03, and HLA-A*02:06, or combined with other neoepitopes for broader coverage.

The study highlights 10 recommended neoepitopes (KLVVVGAAGV, LVVVGAAGV, LVVVGACGV, KLVVVGADGV, LVVVGADGV, KLVVVGASGV, KLVVVGAV, KLVVVGAVGV, LVVVGAVGV, and VVGAVGVGK) that feature TCR-facing mutated residues. These mutations enable TCRs to distinguish the neoepitopes from self. The neoepitopes encompass hotspot *KRAS* missense mutations, including G12A, G12C, G12D, G12S, and G12V. Additionally, the study's differential agretopicity analysis revealed that these 10 neoepitopes have stronger binding affinity to the same HLA allele compared to their corresponding wild-type peptide sequences (Table 4).

The study successfully identified 21 potential KRAS cytotoxic neoepitopes, corresponding to 49 peptide-HLA pairs, which encompass seven frequent oncogenic and hotspot KRAS mutations (G12A, G12C, G12D, G12S, G12V, G13D, and A59T) (Table 5). Key binding parameters, such as K_D and ΔG_{bind}, demonstrated that all the identified neoepitopes can spontaneously and favorably bind to specific HLA I alleles, as illustrated in Figure 1 and detailed in Table 6.

Beyond efficacy, the safety profile is a crucial factor in the development of anti-cancer vaccines. The *KRAS* neoepitopes identified in this study exhibit a reduced likelihood of causing allergic reactions, toxicity, or adverse effects due to cross-reactivity with human proteomes, highlighting their potential as safe components for future vaccines and immunotherapy.

Computational immunology offers a cost-effective and efficient method for discovering and developing immunotherapeutic agents. However, its application requires caution due to inherent limitations and assumptions in immunoinformatic tools. The study utilized a limited set of experimentally validated neoepitopes and considered factors affecting neoepitope immunogenicity, further research is necessary to establish benchmarks for identifying effective neoepitopes.

The integrative approach in the study successfully identified and characterized 21 potential CD8+ neoepitopes linked to shared oncogenic *KRAS* mutations in pancreatic, lung, and colorectal cancers. A workflow for neoepitope identification from neoantigen sequences with known oncogenic mutations was also developed. Among these, 10 candidate neoepitopes exhibited high immunogenic potential, featuring TCR-facing mutated residues and significant differential agretopicity indices. Safety assessments confirmed that all 21 neoepitopes could be incorporated into immunotherapeutic anti-cancer agents. Future efforts should focus on in vitro, animal, and clinical evaluations of the neoepitopes before their inclusion in vaccine formulations for patients with oncogenic *KRAS* mutations. Investigating the use of these neoepitopes in individual or combination therapies is also crucial.

Author Contribution Statement

The conceptualization, data collection, data analysis and paper write-up were all conducted by the sole author.

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Ethics Approval

This study did not conduct methods that involve human participants, samples and data. Thus, ethics review was not deemed necessary. Thus, ethics review was not deemed necessary.

Conflict of Interest

The author declares no conflict of interest.

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