JCOMS eISSN:2783-3127 https://journalofcoms.com

Journal of

Current Oncology and Medical Sciences

Vol. 5, No. 2

Original Free Access

Exploring neoantigens and genetic profiles in renal cell carcinoma: a study of Iranian patients

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Abstract

Introduction: Kidney cancer accounts for 3.7% of all newly diagnosed cancer cases and represents a considerable global health challenge. Although there have been advancements in treatment, renal cell carcinoma (RCC) continues to show resistance to conventional cytotoxic chemotherapy, highlighting the need for innovative therapeutic strategies. Current research is focusing on vaccine approaches that target tumor neoantigens, utilizing next-generation sequencing to pinpoint tumor-specific mutations. A deeper understanding of the molecular characteristics of RCC, particularly gene mutations such as BAP1, PBRM1, SETD2, and VHL, is essential for the development of personalized treatment modalities. This study aimed to investigate potential tumor neoantigens in samples from Iranian patients diagnosed with RCC, with an emphasis on peptide sequences that exhibit a strong binding affinity for Iranian Human Leukocyte Antigen (HLA).

Materials and methods: Databases and relevant literature were employed to identify neoantigens with the highest prevalence. Tumor samples were obtained from patients with RCC, and primary cells were isolated and cultured in RPMI complete medium. Total DNA was extracted, followed by polymerase chain reaction (PCR) using specifically designed primers, and the resulting PCR products were sequenced using Sanger sequencing.

Results: Our examination did not identify the specified nucleotide changes in the DNA sequencing chromatogram of the cell samples, indicating that the anticipated mutations were absent. Nevertheless, other mutations were observed in the analyzed regions of the genes.

Conclusion: Although certain mutations were not identified in the sequenced samples, this research highlights the necessity for further investigation. Comprehensive studies are vital to gain a complete understanding of the genetic mutation profile of RCC in Iranian patients. Mapping the gene mutation landscape among RCC patients in Iran presents significant opportunities for the development of effective cancer vaccines and tailored treatment strategies.

Keywords: Human Leukocyte Antigen (HLA), Renal cell carcinoma, Tumor, Mutation, Vaccines

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Received: 2025.4.1, Accepted: 2025.6.28

Introduction

Kidney cancer represents 3.7% of newly diagnosed cancer cases and ranks among the ten most prevalent cancers. The predominant type, RCC, is found in 85% of kidney cancer instances. This condition is more prevalent in men than in women, with a ratio of 1.7:1, and it generally affects older adults, with an average age of 64 at diagnosis (1). The rate of metastasis is notably high, as 30% of patients are diagnosed with metastatic disease, and an additional 30% develop metastases during subsequent follow-ups (2). Renal cell carcinomas can be categorized into clear cell (CCRCC), papillary (PRCC), and chromophobe (ChRCC) subtypes, which account for 65-70%, 15-20%, and 5-7% of all RCC cases, respectively (3). Recent research has identified genetic factors, including mutations in the VHL, PBRM1, BAP1, and SETD2 genes, as significant contributors to the risk of developing RCC (4).

Among the innovative strategies for treating RCC, antigen-directed approaches such as monoclonal antibodies, adoptive cell therapies, and therapeutic vaccines show promise in enhancing the effectiveness of existing immunotherapies (5). Various neoantigen vaccine platforms, including synthetic long peptide (SLP), RNA, dendritic cell (DC), and DNA vaccines, are currently undergoing evaluation in early-phase clinical trials (6). The SLP vaccine platform has emerged as the most extensively studied neoantigen vaccine in preclinical and early-phase clinical settings, offering notable advantages such as a well-established safety profile, a thoroughly characterized GMP manufacturing process, excellent stability, and ease of administration in human trials (7). The advent of next-generation sequencing (NGS) technologies has further expedited the discovery of tumor-specific mutations, facilitating the development of more targeted and personalized treatment strategies (8).

Recent studies have validated the therapeutic promise of SLP neoantigen vaccines across various human cancers, including melanoma, glioblastoma, non-small cell lung cancer (NSCLC), colorectal cancer, and urothelial cancer (9-13). A clinical trial involving the peptide-based neoantigen vaccine iNeo-Vac-P01 in patients with advanced solid tumors revealed a favorable safety profile and encouraging efficacy, achieving a 71.4% disease control rate and robust immune responses in nearly 80% of participants (9). In this context, Ott et al. have undertaken multiple investigations. In one study involving six melanoma patients, clinicians administered up to 20 neoantigens per individual. Remarkably, four patients with stage IIIB/C disease experienced no recurrence over a 25-month period, while two patients with previously untreated stage IVM1b disease (lung metastases) achieved complete regression following vaccination and subsequent anti-PD-1 therapy (12). Similar findings were reported in glioblastoma patients (11). Furthermore, Ott et al. introduced a neoantigen-based vaccine, NEO-PV-01, in conjunction with PD-1 blockade for patients with melanoma, NSCLC, or bladder cancer. This vaccine elicited CD4+ and CD8+ T cell responses post-vaccination, characterized by a cytotoxic phenotype capable of infiltrating tumors and inducing tumor cell destruction. The treatment was deemed safe, with no adverse events reported (NCT02897765) (13).

In tumor cells, mutations can lead to the formation of novel self-antigen epitopes, referred to as neoantigens. The advent of NGS has created opportunities for the efficient and cost-effective identification of tumor-specific mutations in individual patients, facilitating the exploration of therapies targeting these mutated proteins in clinical trials studies (14). This process encompasses several stages, including the collection of tumor samples, sequencing, bioinformatic analysis, and peptide synthesis, all of which demand considerable time and resources. Despite advancements in sequencing technology facilitating rapid progress, the identification and validation of neoantigens remain labor-intensive and costly, with the preparation of vaccines from tissue samples typically requiring 3 to 5 months (15, 16).

In RCC, the scenario is different because RCC is a cancer with low mutational burden (17). Neoantigens can be divided into two categories: shared neoantigens (public neoantigens) and personalized neoantigens. Off-the-shelf vaccines are designed to target shared neoantigens, which are anticipated to have a high expression frequency and provoke strong anti-tumor immune responses, thus making them suitable for a

wider array of cancer specially RCC patients (18). The molecular profiles of RCC have been examined through NGS in various research initiatives, including The Cancer Genome Atlas (TCGA) and other studies conducted in France, Japan, and the European Union (19). Wang et al. analysis of individual gene mutations was shown that the most prevalent mutations include PBRM1 (74.1%), BAP1 (74.1%), SETD2 (74.1%), and VHL (74.91%) (20). In our forthcoming research aimed at developing an off-the-shelf peptide vaccine, we explored potential tumor neoantigens in RCC samples collected from Iranian patients, focusing on peptide sequences with a strong affinity for the Iranian HLA.

Materials and methods

3.1. Data sources

A genomic analysis of RCC data was performed using the TCGA database (https://portal.gdc.cancer.gov/). Literature reviews and original articles were also investigated to find RCC mutations. The potential neoantigens were explored using cBioPortal (http://www.cbioportal.org, v3.2.11). The Human Gene Mutation Database (HGMD, www.hgmd.com) was used to select peptide sequences considering a moderate to strong affinity for Iranian HLA binding. The bioinformatics analysis included the prediction of MHC I-binding epitopes using tools such as NetMHCpan (v4.0) to identify peptides with a moderate to high affinity for HLA molecules.

3.2. Patients and samples

Cancerous tissues were obtained from patients diagnosed with RCC following surgical procedures at the Department of Urology at Imam Khomeini Hospital. Pathologists verified the pathological features of these samples through histopathological analysis. The section on Ethical Considerations outlines the ethical approval for utilizing the primary cells of patients, with written informed consent secured from all participants (Ethical Approval Code: IR.TUMS.IAARI.REC.1399.010). The inclusion criteria specified that participants must be diagnosed with RCC and have not received any prior treatment. Patients with different histological subtypes of RCC or those who had previously undergone treatment were excluded from the study.

3.3. Primary Cell Culture

RCC solid tumor tissue was first rinsed with phosphate-buffered saline (PBS) and then cut into pieces measuring approximately 1-2 mm. The tissue was subsequently filtered through a 70 µm cell strainer without the use of enzymatic digestion. The resulting filtered cells were washed twice with PBS and cultured in RPMI 1640 medium supplemented with 15% fetal bovine serum (FBS) and 1X Penicillin-Streptomycin (Pen/Strep) in a 37°C incubator with 5% CO2 and 95% humidity for a duration of 72 hours. Following this incubation, the suspended cells were removed by washing with sterile PBS. The adherent cells were then maintained in fresh RPMI medium containing 10% FBS and 1X Pen/Strep for one week, allowing them to achieve 90% confluency. The cells were subsequently passaged using Trypsin-EDTA (0.25%) and seeded at a density of $1x10^6$ cells per T-75 flask for further experimentation.

3.4. DNA Extraction and Polymerase Chain Reaction

To validate the potential neoantigen peptides in cultured cells, adherent cells were harvested. Total DNA was extracted utilizing the SanPrep Column DNA Miniprep Kit (Bio Basic, Canada) in accordance with the manufacturer's guidelines. The PCR was performed using Taq DNA Polymerase 2x Master Mix and 1.5 mM MgCl2 (Ampliqon, Denmark), following the standard protocol with an annealing temperature of 58°C for the primers. Specific primers for the peptides were designed using Primer3. To ensure the specificity and accuracy of the designed primers, their sequences were subjected to a BLAST search in NCBI and Gene Runner. The sequences of the primers are provided in Table 1.

Table 1. The details of oligonucleotide primers utilized for PCR.

Gene	Forward Primer	Reverse Primer	Product Size
VHL (1)	GCGTTCCATCCTC TACCGAG	GCTTCAGACCG TGCTATCGT	525 bp
VHL (2)	ACCGGTGTGGCTC TTTAACA	ACGTACAAAT ACATCACTTCC ATTT	334 bp
VHL (3)	GGCAAAGCCTCTT GTTCGTT	CGATATGCTGC AATTCCCACT	579 bp
PBRM1	TGATGCACATATC CTGGAGAAGTTA	CCATGCTGGAG TACAGTGAGTT	217 bp
BAP1	GCTGCTCTCTGAA GCTTTGC	AGCAGTTGAG CCAGGGAATC	555 bp
SETD2	AAACTTTGAAGCT GGTAGTCAGGA	TTAATGGTCAG AACAGCAATC GTG	310 bp
TP53	CTCCTAGGTTGGC TCTG	GAGGCTGGGG CACAGCAGGC CAGTG	167 bp
FLCN	GCTGGGGAGGTTT CATGGAG	CCCCTGAGAA GCAGTCTGTG	451 bp
PIK3CA (1)	GGGAAAAATATG ACAAAGAAAGC	CTGAGATCAGC CAAATTCAGTT	250bp
PIK3CA (2)	CTCAATGATGCTT GGCTCTG	TGGAATCCAG AGTGAGCTTTC	241bp

3.5. Analysis of PCR Product

The PCR products underwent sequencing at Pishgam Co. in Iran, utilizing the Sanger sequencing technique. Following the amplification of the selected mutant regions with the specified primer sets (refer to Table 1), a total of 10 PCR product samples were dispatched for sequencing. The sequencing chromatograms were first analyzed using Chromas Lite v.2.5 software, followed by alignement and comparison to reference sequences using CLC Sequence Viewer 7.7.1 software to investigate the potential presence of neoantigen mutations in the cultured primary tumor cells.

Ethical consideration

The Vice Chancellor for Research Affairs at Tehran University of Medical Sciences in Tehran, Iran, granted approval for the acquisition of the primary cell culture utilized in this study (Ethical Approval Code: IR.TUMS.IAARI.REC.1399.010). All procedures conducted adhered to the ethical standards set forth by both the institutional and national research committees, as well as the 1964 Helsinki Declaration and its subsequent amendments or equivalent ethical guidelines.

Data Analysis

Statistical methods were not applied in the analysis of sequencing data. Instead, descriptive statistics were utilized to summarize the frequency and distribution of the identified neoantigens. Additionally, bioinformatic tools, including NetMHCpan 4.1, were employed to predict the binding affinity of the identified peptides to HLA molecules.

Results

4.1. Bioinformatic Data

The potential tumor antigens associated with RCC were identified through the utilization of cBioPortal, The Cancer Genome Atlas (TCGA), the International Cancer Genome Consortium (ICGC) cohorts, and comprehensive literature reviews. Following the identification of the most prevalent antigens exhibiting mutations, involve of 17 mutations from 7 genes (VHL, PBRM1, BAP1, SETD2, TP53, FLCN, and PIK3CA). By advantage of bioinformatics tools, including NetMHCpan (v4.1), MHC-I binding epitopes predication, common Iranian population MHC-I are HLA-B*35:01, HLA-A*02:01, HLA-A*24:02, HLA-B*51:01, HLA-A*03:01, and HLA-A*01:01, in both wild-type and mutant of 1105 nine-mer peptides were done. Mutant peptides with moderate to high binding affinities in compare to wild type were selected based on their predicted binding capabilities. Finally, based on Table 2, twenty nine peptides were selected (15,21).

A summary of the RCC potential tumor antigens data, along with the common MHC-I alleles in the Iranian population, is presented in Figure 1 and Table 2.

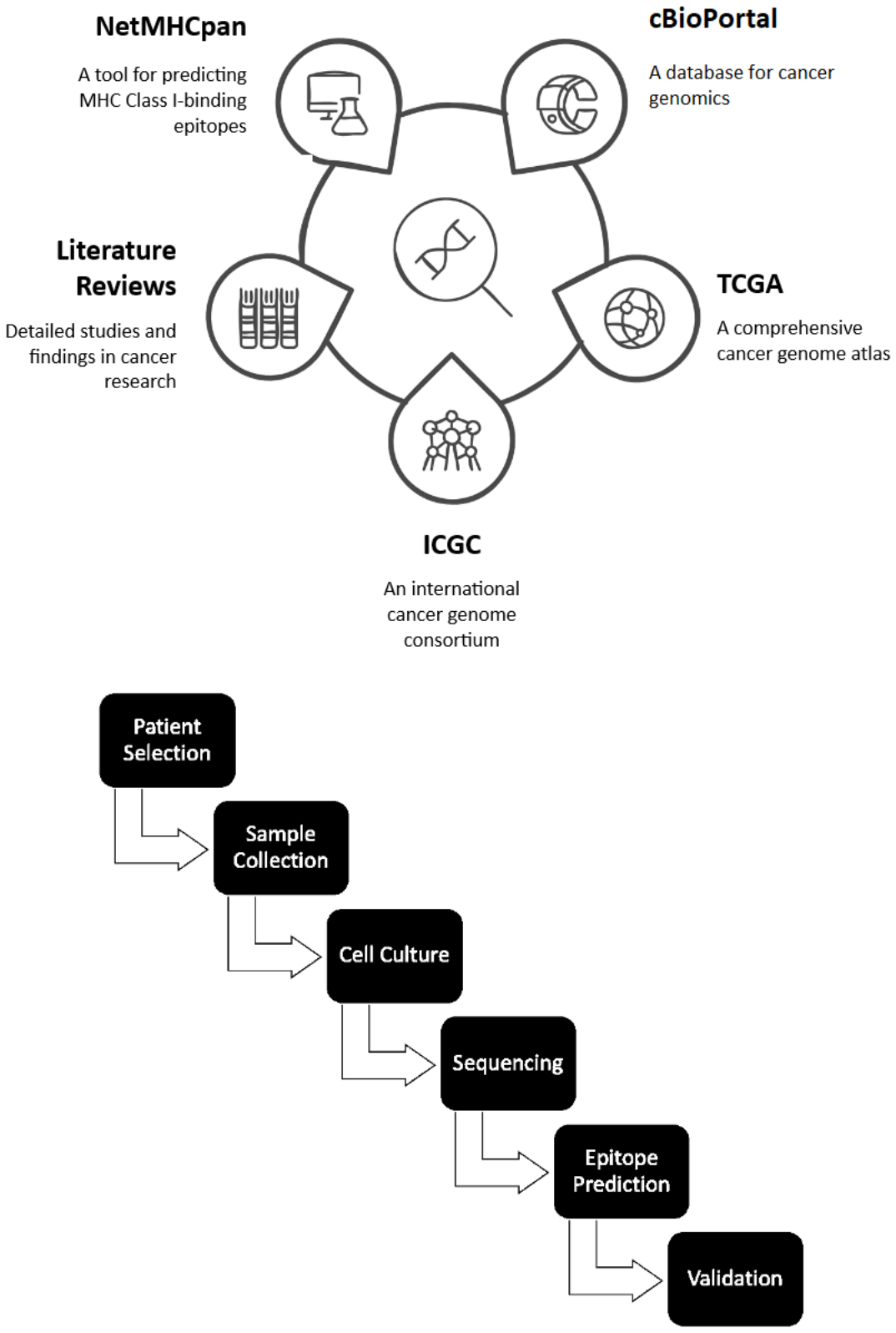

Figure 1. Schematic of selected potential tumor antigens of renal cell carcinoma.

Table 2. The details of selected potential tumor antigens of Renal Cell Carcinoma. The peptides with strong binding affinity to HLA were shown with green color. peptides exhibiting moderate to strong HLA-binding affinity (IC50 < 150 nmol/l) being more likely to activate CD8+ T cells.

Gene	Mutation	Immunizing peptide	HLA	Binding Affinity (nM)	
				Wild	Mutant
VHL	W88L	SPRVVLPVL	HLA-B*35:01	3839.92	1613.19
		VVLPVLLNF	HLA-A*24:02	376.32	1420.44
	H115Y	SYRGYLWLF	HLA-A*24:02	14.56	12.14
	D121Y	RYAGTHDGL	HLA-A*24:02	26237.97	387.69
	V130F	GLLFNQTEL	HLA-A*02:01	176.25	108.44
		LLFNQTELF	HLA-A*24:02	4051.01	2081.94
		LLFNQTELF	HLA-B*35:01	1934.56	1456.79
	N131K	GLLVKQTEL	HLA-A*02:01	176.25	197.59
		GTHDGLLVK	HLA-A*03:01	22426.29	342.46
		LLVKQTELF	HLA-A*24:02	4051.01	2578.81
		LLVKQTELF	HLA-B*35:01	1934.56	2834.24
	L135F	LVNQTEFFV	HLA-A*02:01	736.1	140.49
		LLVNQTEFF	HLA-B*35:01	1934.56	1702.89
	I151T	FANTTLPVY	HLA-A*01:01	2066.66	1207.22
		TTLPVYTLK	HLA-A*03:01	31.53	39.98
		FANTTLPVY	HLA-B*35:01	4.85	3.96
	L169P	LQVVRSPVK	HLA-A*03:01	807.91	1784.2
PBRM1	G626V	VPLPDDDDM	HLA-B*35:01	416.12	58.65

BAP1	F170V	RTMEAFHVV	HLA-A*02:01	7.22	29.91
		RTMEAFHVV	HLA-A*24:02	588.39	703.75
		MEAFHVVSY	HLA-B*35:01	59.51	62.01
		EAFHVVSYV	HLA-B*51:01	2045.28	1749.79
SETD2	H1629Y	NMYSCEPNC	HLA-A*02:01	12006.73	3790.55
TP53	R248L	NLRPILTII	HLA-A*02:01	26600.99	3128.28
		MNLRPILTI	HLA-A*24:02	9934.93	3516.92
FLCN	R18H	ELHGPHTLF	HLA-A*24:02	16730.88	8074.98
		ELHGPHTLF	HLA-B*35:01	13223.15	9173.56
PIK3CA	E542K	ISTRDPLSK	HLA-A*03:01	35089.89	930.82
	H1047L	ALHGGWTTK	HLA-A*03:01	5388.83	47.14

4.2. Pathological Characteristics of RCC

The identification of renal cell tumor was validated through Hematoxylin and eosin (H&E) staining (Fig.1). This staining technique allowed for a comprehensive visualization of cellular morphology and tissue structure, thereby affirming the presence of distinctive characteristics associated with RCC. Following a one-week culture period, RCC cells were analyzed using an inverted microscope to assess their morphology and growth patterns (Fig.2). The cells displayed the typical morphological traits of RCC, characterized by clear cytoplasm and well-defined cell boundaries. Finally, 10 RCC patient cells were selected for DNA sequencing.

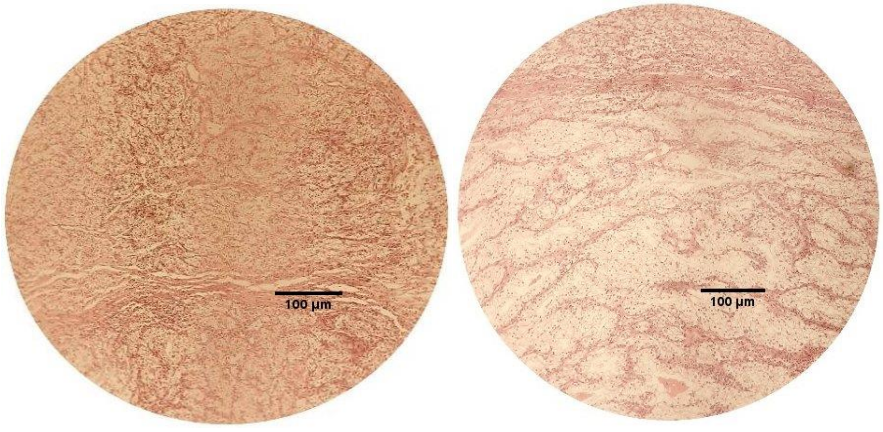

Figure 2. Two different RCC tissues from the studied patient #008 were stained with the hematoxylin-eosin staining method.

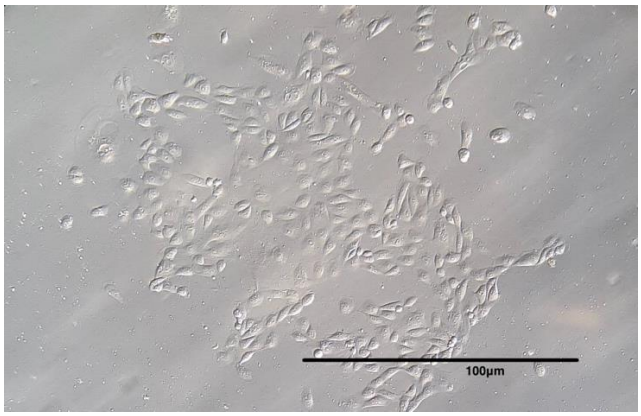

Figure 3. Primary RCC cell obtained from patient #004 (20X magnification).

Table 3. The patient demographics of those with RCC who used their tumor samples for the study.

Patient No.	Sex	Age (Years)	Diagnosis
01	Female	46	Clear cell
02	Male	53	Chromophobe
03	Male	41	Clear cell
04	Female	48	Clear cell
05	Male	39	Papillary
06	Female	46	Clear cell
07	Male	71	Clear cell
08	Male	45	Clear cell
09	Male	66	Papillary
10	Female	74	Chromophobe

4.3 Sanger Sequencing Analysis

The results from Sanger sequencing revealed that the expected mutations were absent in the analyzed samples when compared to the reference sequences. However, additional mutations in the analyzed genes, which are not the chosen mutations, were observed. Below are some of those mutations found in the sequenced regions that include: PIK3CA p.Leu1067del, VHL L135I, VHL c.491delA (p.Gln164ff), TP53 D259E, …

This discrepancy indicates that the identified neoantigens may not be common among Iranian RCC patients. To validate these findings, additional sequencing with a larger sample size may be required.

Discussion

The global incidence of renal cell carcinoma (RCC) is on the rise, with an estimated mortality rate of approximately 20% (22). Neoantigen-based vaccines may prove effective for patients suffering from metastatic RCC, potentially leading to improved overall survival rates, diminished tumor burden, and a reduction in cancer progression (23). It is crucial to identify tumor-associated antigens that are specific to RCC and to develop strategies to counteract tumor-induced immunosuppression, thereby making vaccine therapy a feasible treatment option (24). The ideal antigen should be exclusive to tumor cells, vital for tumor initiation and progression, identifiable by the immune system, and capable of inducing cytotoxic effects on tumor cells (25). Although numerous tumor antigens show promise, only a limited number meet the criteria for tumor selectivity and robust immune response activation, underscoring the necessity for ongoing research and development in this field (26). Previous investigations have confirmed the safety and feasibility of employing mutated VHL peptides as a vaccine for metastatic RCC (27). In the majority of ccRCC cases, mutations in genes such as SETD2, BAP1, MTOR, PTEN, KDM5C, and PBRM1 are frequently observed in the chromosome 3p regions, alongside VHL gene mutations (28). Recent research has emphasized the potential of these mutations as targets for innovative therapeutic strategies, indicating that a multi-targeted vaccine approach may enhance the efficacy of RCC immunotherapy. In a study conducted by Razafinjatovo et al. in 2017, DNA samples from 30 ccRCC patients were analyzed through NGS, focusing on a specific panel of 18 genes, including VHL, BAP1, HIF1a, PDGFRA, PDGF(R)B, TP53, CARD11, NFkB, TSC1, MTOR, EGFR, PBRM1, SETD2, KDM5C, KDM6A, PTEN, and PIK3CA, all of which are known to harbor mutations in ccRCC. Frequent mutations in these genes were detected, and their mutational status,

particularly in genes such as PBRM1, BAP1, CARD11, and HIF1α, was correlated with responses to targeted therapies, potentially serving as predictors of therapeutic outcomes in ccRCC patients (29).

These findings have established a basis for the identification of potential tumor antigens. The advent of advanced sequencing technologies has facilitated more accurate and thorough analyses of these mutations, thereby deepening our comprehension of their significance in RCC. Such insights are vital for the creation of targeted vaccines and personalized therapies that can effectively address the genetic diversity present in RCC tumors. Recent investigations have indicated that neoantigen-based vaccines administered to RCC patients have shown encouraging outcomes. Nevertheless, two major challenges persist in this area. The first challenge is the impracticality of a peptide-based approach due to prohibitive costs and time demands (30). For example, while a particular study reported positive outcomes, the process was impeded by these financial and temporal constraints (12). The application of NGS techniques for personalized treatment and the identification of neoantigens may prove to be highly effective. Research conducted by Bles and Ott (2021) has illustrated that personalized neoantigen-based vaccines, supported by rapid sequencing technologies and bioinformatics, exhibit strong immunogenic profiles and preliminary indications of anti-tumor efficacy in melanoma patients. Furthermore, findings from Ott et al. (2017) highlight that these vaccines can provoke robust anti-tumor immune responses and contribute to tumor size reduction in certain melanoma patients (31, 32).

In this context, the exploration of universal peptides as an alternative strategy is particularly compelling. Universal peptides are present across a wide range of cancer types and have the unique ability to stimulate immune responses against multiple tumors simultaneously. This capacity enables them to activate various subsets of T cells, positioning universal peptides as promising candidates for broad-spectrum vaccines in cancer therapy. The NeoVax brand is focused on identifying and utilizing these universal peptides to cater to different cancer types. By adopting this approach, we can significantly reduce the time and costs associated with the collection of individual tumor samples while ensuring a consistent method of application. This could provide a hopeful pathway for addressing the challenges faced in the treatment of RCC and other cancers.

However, there remains a substantial gap in research specifically addressing the application of these vaccines for renal cell carcinoma. This shortfall may be attributed to the distinct genetic characteristics of RCC, which exhibit unique mutational patterns different from those seen in melanoma. Moreover, the tumor microenvironment in RCC can significantly impact the immune system's ability to recognize and respond to neoantigens.

In our research, we carefully examined a variety of potential tumor antigens from samples collected from Iranian patients, as outlined in Table 2. While we started with optimistic expectations, we found that we could not validate the mutations in the sequenced samples from Iran. This lack of confirmation might be influenced by several factors, including sample heterogeneity and varying mutation frequencies among patients in this population. Future investigations should prioritize the use of higher coverage sequencing methods and an expanded sample size to address these challenges. Furthermore, incorporating additional techniques like whole exome sequencing (WES) or RNA sequencing (RNA-seq) could yield a more thorough understanding of the genetic landscape of RCC across diverse populations(33, 34). The genetic profile of renal tumors exhibits spatial variation within individual tumors as well as differences among patients. There is a notable deficiency in data concerning the intratumor heterogeneity present across various racial groups. Preliminary evidence indicates that genetic variations may exist among different racial demographics affected by RCC, warranting further investigation (35). For instance, analysis of the TCGA database reveals that black patients demonstrate elevated expression of BAP1 at the genetic level (36). Future studies should focus on clarifying the degree of genetic heterogeneity within RCC tumors and its potential implications for personalized treatment strategies. Gaining insight into these variations is crucial for the development of targeted therapies that are more effective for distinct racial and ethnic populations.

Conclusion

As time consuming and cost of personalized neoantigen based vaccine and also low mutation burden in RCC case we designed this study. In other hands, to develop an optimal universal neoantigen-based vaccine, it is essential to first identify the predominant mutations associated with RCC within a specific population. Following the identification of relevant mutations, the vaccine can be designed accordingly. But, based of racial variety we did not find mentioned mutations in studied samples and we think there is necessary comprehensive investigations to map the genetic mutation landscape of RCC patients in Iran in future. Moreover, similar studies should be conducted across Asia to gain a broader understanding of the genetic variations and mutations associated with RCC in diverse populations throughout the continent.

Author contribution

ZM, **GAK**, formal analysis, investigation, data curation, visualization, and writing original draft preparation. **GAK**, conceptualization, methodology, validation, and project administration. **GAK,** writing review and editing, supervision and funding acquisition. **AF** made significant contributions to the preparation of the primary cells. **MDA** contributed to the study and problem-solving related to the paper, writing review and editing. **SMS** contributed to writing, reviewing and editing. All authors read and approved the final manuscript.

Ethics approval

The author declares no conflict of interest.

Funding

The ethical committee of Tehran University of Medical Science confirmed this study [Ethic no. IR.TUMS.IAARI.REC.1399.010].

Consent for publication

All authors have reviewed and approved the final manuscript for publication. All participants provided informed consent for their data and images to be published.

Funding

Tehran University of Medical Science (Grant No. 48430) supported this work. The authors declare that no funds, grants, or other support were received during the preparation of this manuscript.

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