Tumor antigens and immune subtypes guided mRNA vaccine development for kidney renal clear cell carcinoma

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Abstract
Current treatment strategy for kidney renal clear cell carcinoma (KIRC) is limited. Tumor-associated antigens, especially neoantigen-based personalized mRNA vaccines represent new strategies and manifest clinical benefits in solid tumors, but only a small proportion of patients could benefit from them, which prompts us to identify effective antigens and suitable populations to facilitate mRNA vaccines application in cancer therapy. Through performing expression, mutation, survival and correlation analyses in TCGA-KIRC dataset, we identified four genes including DNA topoisomerase II alpha (TOP2A), neutrophil cytosol factor 4 (NCF4), formin-like protein 1 (FMNL1) and docking protein 3 (DOK3) as potential KIRC-specific neoantigen candidates. These four genes were upregulated, mutated and positively associated with survival and antigen-presenting cells in TCGA-KIRC. Furthermore, we identified two immune subtypes, named renal cell carcinoma immune subtype 1 (RIS1) and RIS2, of KIRC. Distinct clinical, molecular and immune-related signatures were observed between RIS1 and RIS2. Patients of RIS2 had better survival outcomes than those of RIS1. Further comprehensive immune-related analyses indicated that RIS1 is immunologically “hot” and represent an immunosuppressive phenotype, whereas RIS2 represents an immunologically “cold” phenotype. RIS1 and RIS2 also showed differential features with regard to tumor infiltrating immune cells and immune checkpoint-related genes. Moreover, the immune landscape construction identified the immune cell components of each KIRC patient, predicted their survival outcomes, and assisted the development of personalized mRNA vaccines. In summary, our study identified TOP2A, NCF4, FMNL1 and DOK3 as potential effective neoantigens for KIRC mRNA vaccine development, and patients with RIS2 tumor might benefit more from mRNA vaccination.

Keywords: mRNA vaccine, Kidney renal clear cell carcinoma, Immunotherapy, Tumor antigens, Immune subtypes, Immune landscape

Background
Kidney renal clear cell carcinoma (KIRC) occupies about 85% of renal cell carcinoma (RCC) [1]. The prognosis of KIRC, compared to other histological subtypes, was generally worse before adjustment of tumor stage and grade [2, 3]. Therefore, the need of new strategies treating KIRC has become necessary and urgent. The use of mRNA-based vaccine has been proposed as a promising approach of combatting tumors two decades ago, and has again become a hotspot under the background of coronavirus disease-2019 (COVID-19) pandemic [4–7]. However, the application of mRNA vaccine in KIRC is somehow lagged. Although a certain degree of immune response was observed, only a
limited number of studies have investigated mRNA vaccines in KIRC [8, 9] and their results were still far from satisfactory. Hence, the present study aims to explore novel candidate tumor antigens for KIRC mRNA vaccine development. Additionally, immune subtypes of KIRC will be classified and patients suitable for mRNA vaccination will be identified. Taken together, this study may pave an avenue for the development of KIRC mRNA vaccine and the identification of KIRC patients suitable for mRNA vaccination.

Results and discussion
Screening of candidate antigens in KIRC
The workflow of our study was presented in Fig. S1. A total of 1098 aberrantly expressed genes were identified (Fig. S2a-b) and the chromosomes distribution of these genes were showed in Fig. 1a. Next, we identified 11,162 genes that mutated in KIRC (Fig. S2c) and the top 30 mutated genes were showed in Fig. 1b. The altered genome fraction and mutation counts in individual samples were demonstrated in Fig. 1c-d. Of note, PBRM1, TTN and VHL were also the most frequently mutated genes considering both altered genome fraction and mutation counts (Fig. 1e-f). Combining the expression and mutation data of KIRC, 572 genes that were highly expressed and mutated in KIRC were identified as potential candidate antigens (Fig. 1g). The Gene Ontology (GO) analysis demonstrated that these 572 genes were involved in immune response-related pathways (Fig. 1h, Fig. S2d-f). The results indicated that these genes were aberrantly highly expressed and mutated in KIRC, which might stimulate tumor-specific immune response. Thus, the 572 genes were the potential candidates for mRNA vaccine development.

To explore the key genes that functioned as best candidates for mRNA vaccine targets, we further identified 37 genes which were associated both OS and RFS from the 572 genes (Fig. 1i-j). Given the pivotal role of antigen-presenting cells (APCs) in the function of mRNA vaccines, we analyzed the association of these 37 genes with APCs using single sample gene set enrichment analysis (ssGSEA) [10] (Fig. 1k). Finally, 4 genes including TOP2A, FCN4, FMNL1 and DOK3 that were closely associated with APCs were identified (spearman correlation coefficient > 0.3; Fig. 1l). All of these four genes were positively associated with APCs and could serve as potential tumor antigens that can be recognized and processed by the APCs to T cells, finally triggering strong immune response against tumor cells. More importantly, the survival analysis demonstrated that the high expression of these four genes were associated with decreased survival in KIRC (Fig. 1m), suggesting the four genes were of importance in KIRC development and progression. Taken together, TOP2A, NCF4, FMNL1 and DOK3 were significantly upregulated, mutated and positively associated with APCs infiltration in KIRC. Therefore, mRNA vaccines encoding these 4 genes might induce anti-tumor immune response and eliminate malignant cells.

Immune subtypes identification
Tumor immune microenvironment might impact the efficacy of immunotherapy and immune subtypes might be helpful to identify patients who can respond to mRNA vaccination. We analyzed the expression profiles of 1621 immune-related genes in KIRC samples to construct consistent clusters. We selected k = 2 (Fig. S3a-b) for stable clustering of immune-related genes and obtained two immune subtypes, named kidney renal cell carcinoma immune subtype 1 (RIS1) and RIS2, respectively (Fig. 2a). The principal component analysis (PCA) validated that these two subtypes could be well distinguished (Fig. S3c). Survival analysis revealed that RIS1 had significant worse prognosis than RIS2 (Fig. 2b) and RIS1 had significant higher pathological T, N and M stages than RIS2 (Fig. 2c). Overall, our immune subtyping was well distinguished and could be applied to identify KIRC patients with better pathological and survival outcomes.

Immune subtypes with tumor mutation burden (TMB)
As TMB was associated with immunotherapy response [11], we assessed the TMB, mutation counts and copy number alteration (CNA) status between RIS1 and RIS2. Our results showed that there was no difference between groups regarding TMB (Fig. 2d), while RIS1 had significantly higher mutation number than RIS2 (Fig. 2e). The waterfall diagrams of different subtypes were showed in

(See figure on next page.)

Fig. 1 Identification of potential antigens in KIRC. a Chromosomal distribution of up- and down-regulated genes in KIRC; b Waterfall diagram of the top 30 mutant genes; c Distribution of mutation frequency; d Distribution of mutation number; e Distribution of mutation number of the top 10 genes; f Distribution of mutation frequency of the top 10 genes; g Overlapped genes identified through intersection; h GO enrichment analysis of 572 genes after intersection of overexpressed and mutated genes; i Univariate Cox regression analysis of the 37 potential antigens for OS (i) and RFS (j); k Correlation analysis of 37 genes with immune infiltrating cells, red box indicates genes closely related to APCs (threshold: spearman correlation coefficient > 0.3); l Association of TOP2A, MCF4, FMNL1 and DOK3 with B cell, macrophage, and dendritic cells, m Kaplan-Meier curves of the association of TOP2A, MCF4, FMNL1 and DOK3 with OS and DSS. GO, Gene Ontology; KEGG, Kyoto Encyclopedia of Genes and Genomes; OS, overall survival; RFS, recurrence-free survival; DSS, disease specific survival. * p < 0.05 and ** p < 0.01
Fig. 1 (See legend on previous page.)
RIS1 is immunologically “hot” and represents an immunomarkedly higher in RIS1 than RIS2 (Fig. S4). Therefore, that plasma cells, CD8 T cells, memory CD4 T cells were immune response. The mRNA vaccine targeting RIS2 might reinforce its “cold” tumor to “hot”. It might stimulate the immune response, namely turning proportion of C4 than RIS1. This result further validated the mRNA vaccines might function better in RIS2 for its relatively low ICPs expressions. Moreover, we showed that RIS1 had markedly higher immune score, higher stromal score and lower tumor purity (Fig. 2i) than RIS2. In addition, we also conducted cytolytic activity (CYT) analysis and the results showed that RIS1 had higher CYT than RIS2 (Fig. 2i). Furthermore, we analyzed the differences of 28 immune infiltrating cells between groups (Fig. 2i). The results showed that plasma cells, CD8 T cells, memory CD4 T cells were markedly higher in RIS1 than RIS2 (Fig. S4). Therefore, RIS1 is immunologically “hot” and represents an immunosuppressive phenotype, whereas RIS2 represents an immunologically “cold” phenotype. Previous study [12] had identified six pan-cancer subtypes, including C1 (Wound Healing), C2 (IFN-γ Dominant), C3 (Inflammatory), C4 (Lymphocyte Depleted), C5 (Immunologically Quiet) and C6 (TGF-β Dominant). The distributions of these six types in RIS1 and RIS2 were analyzed as well. As exhibited in Fig. 2k, RIS1 had significantly higher proportion of C2 than RIS2, while RIS2 had significantly higher proportion of C4 than RIS1. This result further validated the “hot” phenotype of RIS1 and the “cold” phenotype of RIS2. Therefore, mRNA vaccine administration in RIS2 might stimulate the immune response, namely turning “cold” tumor to “hot”.

Immune landscape of KIRC
The expression profile of immune-related genes in each KIRC sample was selected to construct the immune landscape of KIRC. We found that the point distribution in RIS1 and RIS2 is relatively discrete (Fig. 2i). Principal component 1 (PC1, horizontal axis) was most negatively correlated with plasma cells, CD8 T cells and T follicular helper cells, and most positively correlated with resting NK cells and resting mast cells. On the contrary, principal component 2 (PC2, vertical axis) was most negatively correlated with resting memory T cells and most positively correlated with Tregs (Fig. 2m). The correlation of different immune cells between PC1 and PC2 further indicated the accuracy of our classification. Moreover, heterogeneities in each cluster can be observed from Fig. 2i that even RIS1 and RIS2 also exhibited opposing distribution. Therefore, we further divided RIS1 and RIS2 into two subgroups according to the distribution location of immune cell groups (Fig. 2n). RIS1B and RIS2A had significantly lower CD8 T cells compared with their counterparts, suggesting mRNA vaccines might be viable in RIS1B and RIS2A (Fig. S5a-b). In addition, by comparing the prognosis of samples with extreme distribution in the immune landscape, we found that patients in group 8 had best survival outcomes and patients in group 7 had worst survival outcomes (Fig. 2o-p). Taken together, construction of immune landscape of KIRC enabled us to accurately identify the immune cell components of each KIRC patients and predict their survival outcomes, finally assist the development of personalized mRNA vaccines.

Identification of immune gene co-expression modules and immune hub genes of KIRC
The immune gene co-expression module clustered the KIRC samples through weighted gene coexpression network analysis (WGCNA, Fig. S6a-d). We subsequently analyzed the distribution of characteristic genes of the RIS1 and RIS2 in these 9 modules (Fig. S6e). RIS1 showed the higher eigengenes in black, brown, magenta, pink, red and turquoise modules than...
Fig. 2 (See legend on previous page.)
Identification of differential expression genes (DEGs) between immune subtypes
To explore our immunotyping more deeply, 107 genes were identified as the DEGs between RIS1 and RIS2 (Fig. S9a). Pathway enrichment analyses were showed in Fig. S9b-e and they were enriched in immune response pathways such as “active immune response”, “lymphocyte mediated immunity” and “hemal immune response”, further indicating that our immunotyping could reflect the antitumor immune response in KIRC. We next performed univariate Cox regression analysis on these 107 DEGs and 31 genes were significantly correlated with decreased survival outcomes (threshold \( P < 0.01 \), Fig. S10a). Then we performed lasso regression analysis on these 31 genes and finally obtained 7 high-risk genes (CCL19, CCL5, IGLV9-19, IGLV3-27, IGLV3-21, IGLC2 and IGHG3, Fig. S10b-d). Of note, two of these 7 genes (IGHG3 and IGIC2) were highly expressed and mutated genes in KIRC. A risk model was built based on the 7 genes according to the median of risk score (Fig. S10e-f) and the survival curves indicated the risk model had prognostic efficacy (Fig. S10g).

The expression heatmap of these 7 genes was showed in Fig. S10h. It can be found that RIS1 had significantly higher scores and greater percentage of high-risk samples than RIS2 (Fig. S10i-j). The expression of the above 7 genes in TCGA-SKCM were picked up and calculated the risk score of each sample to build a model. The results showed that high risk group had significantly higher anti-CTLA-4 and anti-PD-1 response than low risk group (Fig. S10k), indicating that RIS2 had lower immune checkpoint efficacy than RIS1. Thus, immune checkpoint blockade might not suitable for KIRC patients with RIS2 and mRNA vaccines might be effective for RIS2 populations.

Conclusions
In summary, our study identified TOP2A, NCF4, FMNL1 and DOK3 as potential effective neoantigens for KIRC mRNA vaccine development, and patients with RIS2 tumor might benefit more from mRNA vaccination. Our study paved a way for future mRNA vaccine development and define the suitable population for vaccination.

Methods and availability of supporting data
Methods and materials used in our study are attached as supplementary information. All data are freely available from the public databases and the other necessary and reasonable information could be obtained from the corresponding author.

Abbreviations
ccRCC: Clear cell renal cell carcinoma; KIRC: Kidney renal cell carcinoma; mRNA: Messenger ribonucleic acid; TCGA: The Cancer Genome Atlas; GO: Gene ontology; KEGG: Kyoto encyclopedia of genes and genomes; PCA: Principal component analysis; ssGSEA: Single sample gene set enrichment analysis; CYT: Cytolytic activity; RIS: KIRC immune subtype; CNA: Copy number alteration; TMB: Tumor mutation burden; ICP: Immune-checkpoint; WGCNA: Weighted gene coexpression network analysis; DEG: Differential expression gene; PFS: Progression-free survival; OS: Overall survival; APCs: Antigen-presenting cells; ICB: Immune checkpoint blockade; SKCM: Skin cutaneous melanoma.

Supplementary Information
The online version contains supplementary material available at https://doi.org/10.1186/s12943-021-01466-w.
of genes in the risk model, l; risk score distribution between immune subtypes; J; Distribution of high and low risk group samples among subtypes; k; immune response inference based on SKCM, RIS, renal cancer immune subtype; SKCM, human skin cutaneous melanoma; CTLA-4, Cytotoxic T-Lymphocyte Associated Protein 4; PD-1, programmed cell death protein 1; nonR, non-responder; R, responder.

Acknowledgements
The authors would like to thank TCGA (http://cancergenome.nih.gov) for data collection, as well as GEPIA2 (http://geopia2.cancer-pku.cn) for the data processing and customizable functions.

Authors’ contributions
H.X., X.Z., S.Z. and X.Y. performed the literature search and bioinformatics analysis, and prepared the figures; S.Z., X.Y. and T.Z. helped with data collection, analysis, and interpretation; Q.W. and H.L. revised and polished the manuscript; J.A. conceived the study and revised the manuscript. The authors read and approved the final version of manuscript.

Funding
This study was supported by a grant from National Natural Science Foundation of China (82070784, 81702536) to J.A., a grant from 1.3.5 project for disciplines of excellence, West China Hospital, Sichuan University (ZYGD18011) to H.L., grants from China Postdoctoral Science Foundation (No. 2021 M692306) and Post-Doctor Research Project of West China Hospital of Sichuan University (No. 2021HX8H026) to X.Z.

Declarations

Ethics approval and consent to participate
Not applicable.

Consent for publication
Not applicable.

Competing interests
None.

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Received: 26 August 2021  Accepted: 16 November 2021
Published online: 06 December 2021

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