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Research article

Screening and identification of prognostic genes associated with eosinophilic features of clear cell renal cell carcinoma☆

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ABSTRACT

Clear cell renal cell carcinoma (ccRCC) is the most common subtype of renal cell carcinoma, which is characterized by transparent cytoplasm. However, some ccRCC also show eosinophilic cytoplasm, and the molecular difference between eosinophilic and clear subtypes is unclear. In this study, we uncovered that under an optical microscope ccRCC with eosinophilic features has a poor prognosis. Eosinophilic ccRCC tends to have a higher histologic grade. Eosinophilic ccRCC has 16 genes significantly up-regulated compared with ccRCC, of which seven genes have multi-cohort validation prognostic value. Immune infiltration analysis suggested a low number of M1 macrophages and NK tissue-resident cells in eosinophilic ccRCC. Enrichment analysis suggests that ccRCC with eosinophilic features may be closely associated with the transport and metabolism of many substances. The findings of this study have important implications for the study of the malignant transformation of ccRCC.

1. Introduction

Renal carcinoma is a solid tumor with an increasing incidence year by year. According to US statistics, it is estimated that there will be 76,080 new cases and 13,780 deaths in 2022 [1]. Renal cell carcinoma (RCC) is the most common form of renal carcinoma, accounting for 85% of cases. It is more common in men than women (1.7: 1), and most people are older, with an average age of 64 [2]. Although patients with early detection and surgical resection have a better prognosis, many patients have progressed when diagnosed as tumors due to atypical symptoms of renal cell carcinoma [3]. The most common subtype of renal cell carcinoma is clear cell renal cell carcinoma (ccRCC), which is also one of the most malignant tumors. As a highly vascularized tumor, the cytoplasm of ccRCC cancer cells is filled with lipid droplets and glycogen, showing a histologically unique transparent cytoplasm [4].

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Due to the heterogeneity within ccRCC tumors, different histological features may be presented in different tissue regions. Some regions of ccRCC exhibit eosinophilic characteristics. These eosinophilic areas were previously considered granular renal cell carcinoma, which is difficult to differentiate from other histologically non-clear renal cell carcinomas. However, the eosinophilic regions of ccRCC have the same genetic characteristics as the clear regions, and they are not currently considered to be separate subtypes [5]. Previous studies have found that eosinophilic cancer cell phenotypes exhibit higher proliferation drive and lower differentiation. Mutations in tumor suppressor p53 (*TP53*) exist only in eosinophilic ccRCC components. The degree of vascularization in the eosinophilic area was low, but there were more infiltrating immune cells. Eosinophilic tissue regions have very little cytoplasmic lipid content, but extremely high mitochondrial content, while clear cell regions do the opposite [6]. It has also been preliminarily confirmed that patients with eosinophilic features in the tumor tissue area of ccRCC have a poor prognosis [7]. However, to date, there is no large-scale clinical cohort to verify the prognostic value of eosinophilic ccRCC. Genetic differences between clear and eosinophilic ccRCC have not been explored and validated by large-scale clinical cohorts.

In this study, we analyzed the differences in prognosis, mutation, and immune infiltration between different subtypes of ccRCC. Key genes of eosinophilic ccRCC were identified and validated, and their prognostic value and potential biological mechanisms were analyzed, which provide new insights into the heterogeneity of ccRCC.

2. Materials and methods

2.1. Patient cohorts and data sources

In this study, we included three clinical cohorts from public databases: The Cancer Genome Atlas (TCGA, https://portal.gdc.cancer.gov/), Clinical Proteomic Tumor Analysis Consortium (CPTAC) [8], and *E*-MTAB-1980 [9], and one of our clinical cohorts was GENERAL. Among them, TCGA, CPTAC, and GENERAL cohorts included hematoxylin-eosin (HE) staining results from patients with confirmed ccRCC. The TCGA, CPTAC, and *E*-MTAB-1980 cohorts included patient survival data and RNA sequencing data. The clinical characteristics, data extraction, and processing procedures of all the patients involved in this study can be found in our previous study [10,11].

2.2. Process of identification of ccRCC subtypes

All patients involved in this study were ccRCC patients, so all patients had been proven to be ccRCC by pathologists through histochemistry before inclusion in this study. According to the characteristics of the cytoplasm under the light microscope of HE staining of ccRCC patients, we defined the completely clear cytoplasm as the clear type, the cytoplasm with clear and eosinophilic components as the middle type, and the completely eosinophilic cytoplasm as the eosinophilic type. Due to the heterogeneity within the tumor, a patient's tumor sample may have multiple types at the same time. When there is an eosinophilic component, it is preferentially defined as an eosinophilic type, and when there is a middle type and a clear type at the same time, it is preferentially defined as middle type. The identification of ccRCC subtypes in all patients was determined by two pathologists from two independent institutions, and for ambiguous samples, it was determined by a third pathologist.

2.3. Hematoxylin-eosin staining

The tumor specimens in the GENERAL cohort were fastened with 4% paraformaldehyde (Sigma–Aldrich), dehydrated with gradient alcohol (70%, 80%, 90%, 95%, 100%), and cleared with xylene (Sigma–Aldrich). The most representative part of the tumor was embedded in paraffin, cut into 4 μm, and treated with HE staining kit (Sigma–Aldrich). The tissues were dyed with hematoxylin, differentiated with hydrochloric acid alcohol and counterstained with eosin. Finally, the tissues were dehydrated with alcohol, cleared with xylene, sealed with neutral gum and dried. The pathological changes of cancer tissues were observed and photographed under a light microscope (Leica Biosystems Inc., Frankfurt, Germany).

2.4. Somatic variant analysis

The somatic mutation data of ccRCC used in this study were obtained from the TCGA cohort. Whole-genome sequencing extracted from consensus coding mutation data was acquired from the cBioPortal [12] for somatic variant analysis. We performed the analysis via maftools package [13] in R.

2.5. Tumor immune microenvironment analysis

We analyzed the landscape of immune cells and differences in immune cell infiltration between different ccRCC subtypes in ccRCC samples from the TCGA cohort by CIBERSORT [14]. The specific implementation methods and parameter setting can be found in our previous research [11].

2.6. Differentially expressed gene analysis

In order to avoid interference caused by the middle type of ccRCC, we selected clear and eosinophilic types of ccRCC as targets for

screening differentially expressed gene (DEGs) analysis. When the | fold change | of DEGs ≥1 and $p < 0.05$, the genes were used for subsequent analysis.

2.7. Enrichment analysis of GO and KEGG by GSEA

Kyoto Encyclopedia of Genes and Genomes (KEGG) pathway and Genetic Ontology (GO) analysis were carried out to explore the potential biological mechanisms in which the DEGs might be involved. Based on the previous DEGs obtained by limma, Gene Set Enrichment Analysis (GSEA) was performed by the R package of clusterProfiler (4.0.5), and the visualization of all results was achieved through ggplot 2 and enrichplot packages.

2.8. Statistical analysis

The proportion of clinical characteristics of the three subtypes was compared by chi-square test. Immune cell proportion and mutation difference were compared with Wilcoxon test. Comparation of continuous variables among more than two groups was carried out through the analysis of variance (ANOVA). Kaplan-Meier curve analysis was used to compare overall survival (OS) and disease-free survival (DFS) with the log-rank test. Part of the survival analysis is done through GEPIA [15]. All statistical analyses and visualization were performed in R (4.0.0) and MedCalc (20.113).

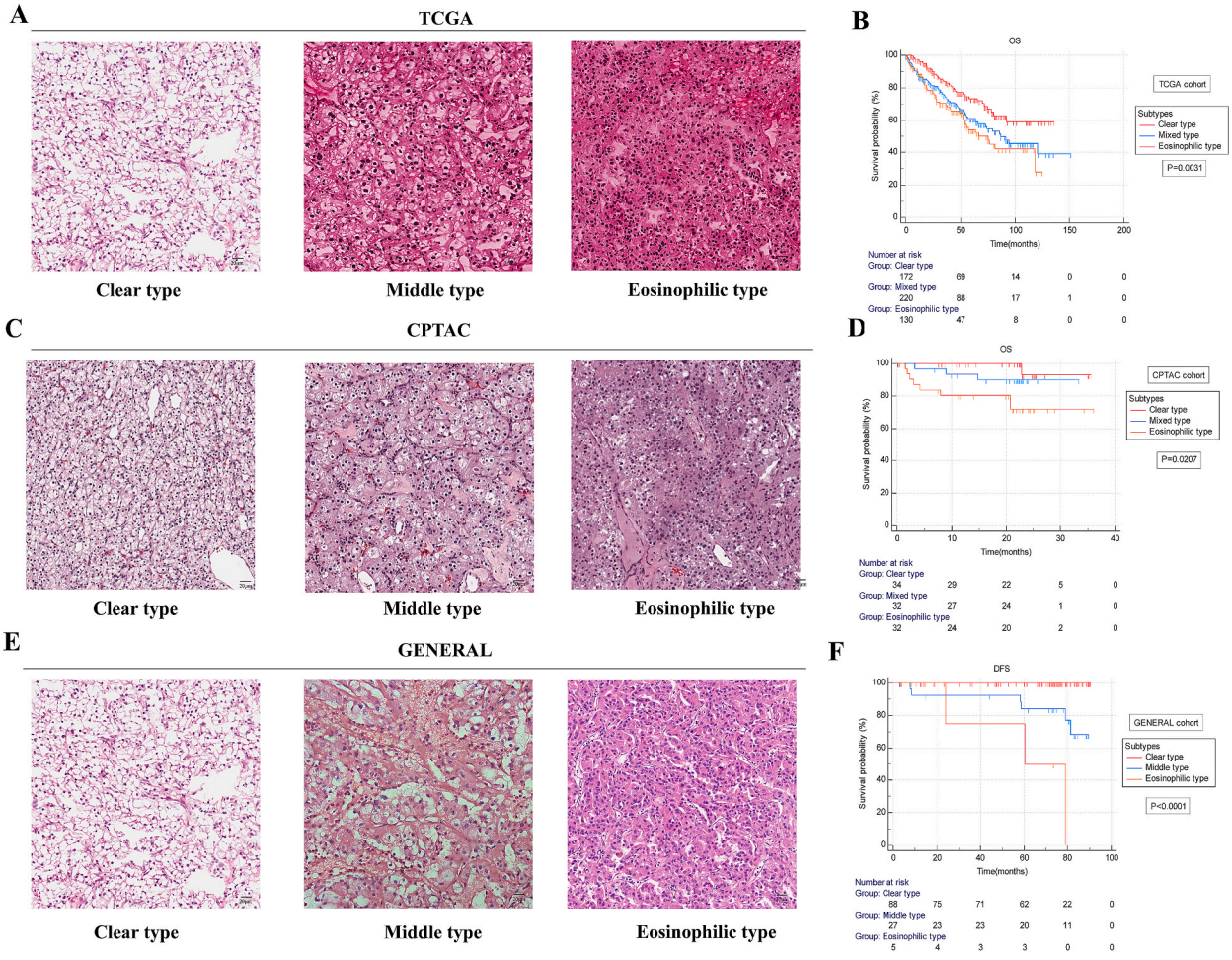

Fig. 1. CcRCC patients with eosinophilic features have a poor prognosis. (A) Examples of classification of tumor samples from TCGA cohort patients under HE staining. (B) Survival analysis of patients with three subtypes in the TCGA cohort. (C) Examples of classification of tumor samples from CPTAC cohort patients under HE staining. (D) Survival analysis of patients with three subtypes in the CPTAC cohort. (E) Examples of classification of tumor samples from GENERAL cohort patients under HE staining. (F) Survival analysis of patients with three subtypes in the GENERAL cohort.

3. Results

3.1. CcRCC patients with eosinophilic features have a poor prognosis

Yoshida et al. preliminarily illustrated that the prognosis of ccRCC patients with eosinophilic features in tumor samples was poor, but there was a lack of large-scale and multi-center clinical cohort validation [6]. In this study, we included multicenter and large-scale cohorts to demonstrate this conclusion, including two public cohorts, TCGA and CPTAC, and one of our GENERAL cohorts. Firstly, in the three cohorts, we divided patients into clear type, middle type, and eosinophilic type according to the degree of eosinophilic staining in the cytoplasm of cancer cells under HE staining (Fig. 1A,C,1E). Then we used the patient's survival data to analyze the survival of the three types of ccRCC. We found that in the TCGA cohort, patients with eosinophilic type had the worst prognosis, followed by middle type, and patients with clear type had the best prognosis (Fig. 1B). Consistent with the results of TCGA cohort, the survival probability of ccRCC patients with eosinophilic features in CPTAC cohort was still the lowest (Fig. 1D). Due to the early detection and early treatment of ccRCC, the survival probability of patients is generally better. Therefore, we analyzed the patient's DFS in the GENERAL cohort. We found that if a patient's first resected tumor had eosinophilic features, the probability of tumor recurrence in that patient was one hundred percent (Fig. 1F). Therefore, ccRCC with eosinophilic features is a sign of malignancy.

3.2. Eosinophilic features of ccRCC are associated with WHO/ISUP grade

Next we wondered whether the eosinophilic features of ccRCC were significantly associated with the patient's stage and WHO/ISUP grade as well as metastasis. We found that ccRCC patients with histologically eosinophilic features tended to have higher tumor stage, although no statistical difference was reached (Fig. 2A). As the histological eosinophilic staining intensified, the patient's grade tended to be elevated, implying that the eosinophilic features may be inextricably linked to the nuclear grade of the tumor, in other words, the two could have occurred concomitantly (Fig. 2B). Similarly, the patient's T stage was elevated with deepening eosinophilic

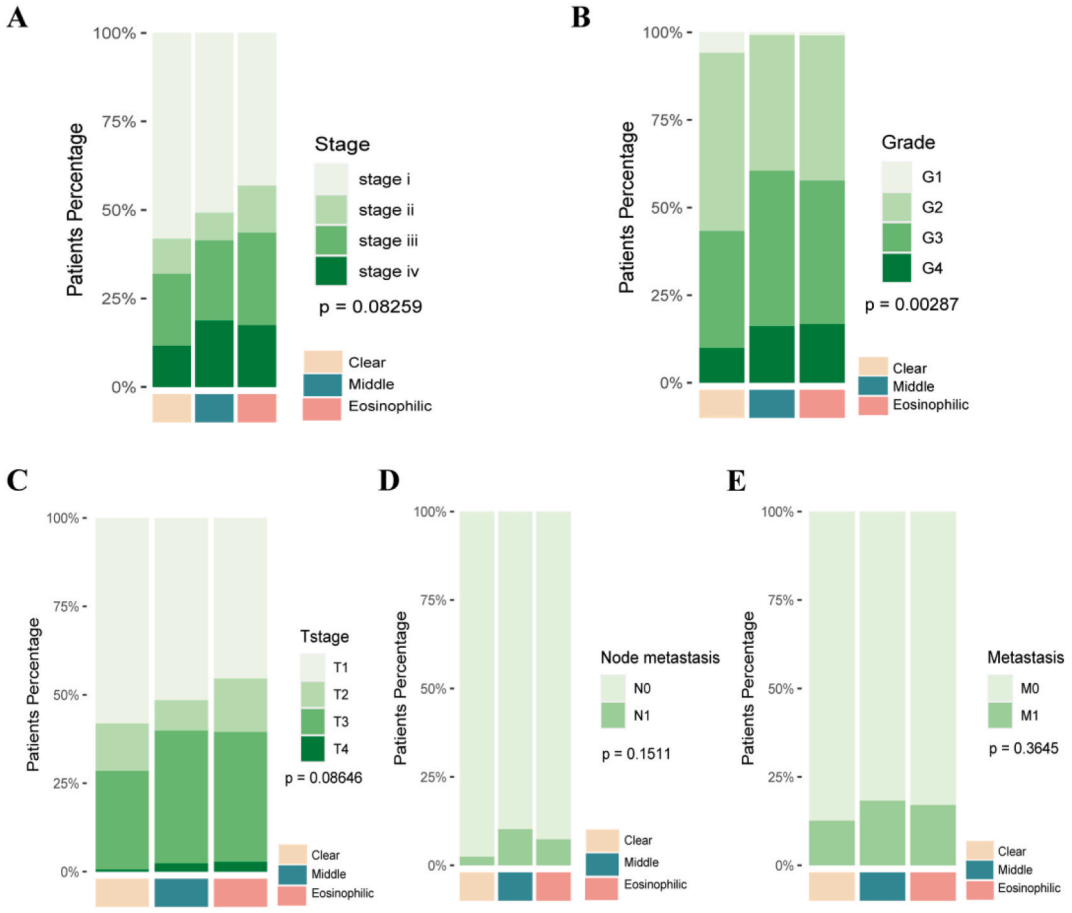

Fig. 2. Eosinophilic features of ccRCC are associated with WHO/ISUP grade. (A) Stage distribution varies of ccRCC among the three subtypes. (B) Grade distribution varies of ccRCC among the three subtypes. (C) T stage distribution varies of ccRCC among the three subtypes. (D) Node metastasis distribution varies of ccRCC among the three subtypes. (E) Distant metastasis distribution varies of ccRCC among the three subtypes.

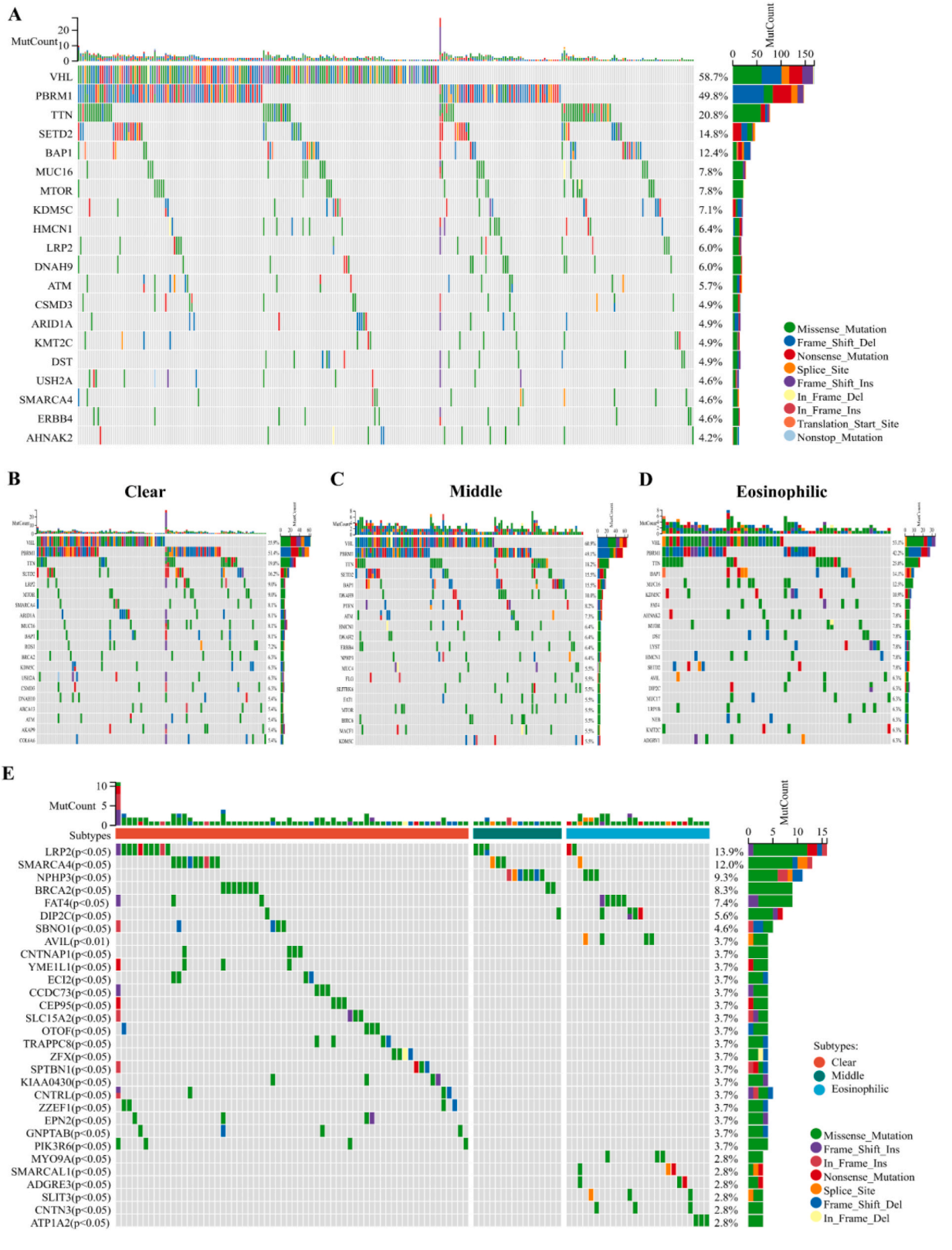

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Fig. 3. Specific mutant genes accompany the eosinophilic features. (A) The top twenty genes with the highest mutation frequency in ccRCC. (B) The top twenty genes with the highest mutation frequency in clear type of ccRCC. (C) The top twenty genes with the highest mutation frequency in middle type of ccRCC. (D) The top twenty genes with the highest mutation frequency in eosinophilic type of ccRCC. (E) The top thirty mutated genes with statistical difference and highest frequency among the three ccRCC subtypes.

staining (Fig. 2C). Although there is no statistical difference, we can still observe that the eosinophilic features of ccRCC are also inseparably linked to the metastasis of cancer cells (Fig. 2D and E). These findings reaffirm that eosinophilic features are associated with tumor progression.

3.3. Genes mutation profile of ccRCC differs between eosinophilic and clear types

Next, we want to know whether there is a difference in the frequency of gene mutations among the three ccRCC subtypes. We first analyzed the top 20 genes with the highest mutation frequency in ccRCC (Fig. 3A). In addition to this, we also show the mutation landscape of each of the three ccRCC subtypes (Fig. 3B–D). We can see that the three genes with the highest mutation frequencies do not differ significantly among the three ccRCC subtypes. We can clearly see that the three genes with the highest mutation frequency have no significant difference in these three ccRCC subtypes. From this point of view, it can also be explained that eosinophilic and clear types of ccRCC are of the same origin and they may be at different stages of tumor progression. To identify genes with different mutation frequencies in the three types of ccRCC, we screened the top thirty mutated genes with statistical differences for display (Fig. 3E). The mutation of FAT4 may have an indelible contribution to the formation of eosinophilic ccRCC.

3.4. Differences in immune cell infiltration among three types of ccRCC

Yoshida et al. found that ccRCC patients with eosinophilic features benefited more from treatment with immune checkpoint inhibitors (ICIs) than with tyrosine kinase inhibitors (TKIs) [7]. However, the infiltration characteristics of immune cells in the tumor microenvironment of patients with eosinophilic stained ccRCC have not been fully defined. In the present study, first, we show the profile of the proportion of different immune cell infiltrations in the tumor microenvironment of ccRCC for each patient using CIBERSORT (Fig. 4A). Subsequently, we showed the percentage abundance of immune cells based on all ccRCC patients in the TCGA cohort (Fig. 4B). This is consistent with our previous study [11]. We then compared the differences in the proportion of immune cell infiltration between the three ccRCC subtypes, and by analysis, we found that five of the immune cells were statistically different between the three subtypes (Fig. 4C). In particular, M1 type macrophages and NK tissue-resident cells with tumor-killing functions are less infiltrated in eosinophilic ccRCC than in clear ccRCC, which may be one of the reasons for the poorer prognosis of eosinophilic type of ccRCC.

3.5. Identification of EDGs between clear and eosinophilic ccRCC

We hypothesized that eosinophilic ccRCC progresses from clear ccRCC, so we wanted to know which genes are involved in this process. We performed EDGs analysis using the gene expression data from the TCGA cohort. First, we performed a visual analysis of the volcano map and we found a total of 16 genes up-regulated and 46 genes down-regulated in eosinophilic ccRCC compared to the clear type (Fig. 5A). Second, the heatmap analysis clearly shows that these 16 genes are significantly highly expressed in eosinophilic ccRCC (Fig. 5B), and the down-regulated genes are not fully shown and can be found in the supplementary S1. We conjecture that if these three ccRCC subtypes are the three stages of tumor progression, then their expression levels would be progressively increasing among these three subtypes. As expected, the mRNA expression of these 16 genes gradually increased with the deepening of eosinophilic staining (Fig. 5C). Therefore, these 16 genes may play an extremely important role in the eosinophilic ccRCC.

3.6. Potential signaling pathways involved in DEGs

Then, we wanted to know which signaling pathways are involved in DEGs, so we performed GO and KEGG gene set enrichment analysis. The results of GO enrichment suggested that genes down-regulated in the eosinophilic ccRCC were associated with transmembrane transport of a variety of substances (Fig. 6A and B). The results of KEGG also suggested that DEGs were correlated with starch and sucrose metabolism (Fig. 6C and D). Studies have found that there are many mitochondria in the cytoplasm of eosinophilic ccRCC [6]. We found that the results of DEGs enrichment were related to the transport and metabolism of multiple substances. Therefore, this may be due to the catabolism of cholesterol and glycogen in the clear type of ccRCC.

3.7. Identification of prognostic value of upregulated genes in eosinophilic ccRCC

Since eosinophilia was a malignant feature, then we hypothesized that the genes upregulated in eosinophilic ccRCC might have certain prognostic value. We divided patients into high-expression and low-expression groups according to the median expression of these 16 genes in the TCGA and *E*-MTAB-1980 cohorts. Subsequently, through survival analysis, we found that SLC6A20, WBSCR17, CKMT1B, CKMT1A, and COCH had no effect on the survival of ccRCC patients in the TCGA cohort (Fig. S1A). KLK4, CCDC78, HAGHL, and FAM40B had survival significance only in the TCGA cohort, but not in the *E*-MTAB-1980 cohort (Fig. S1B). Finally, we found that

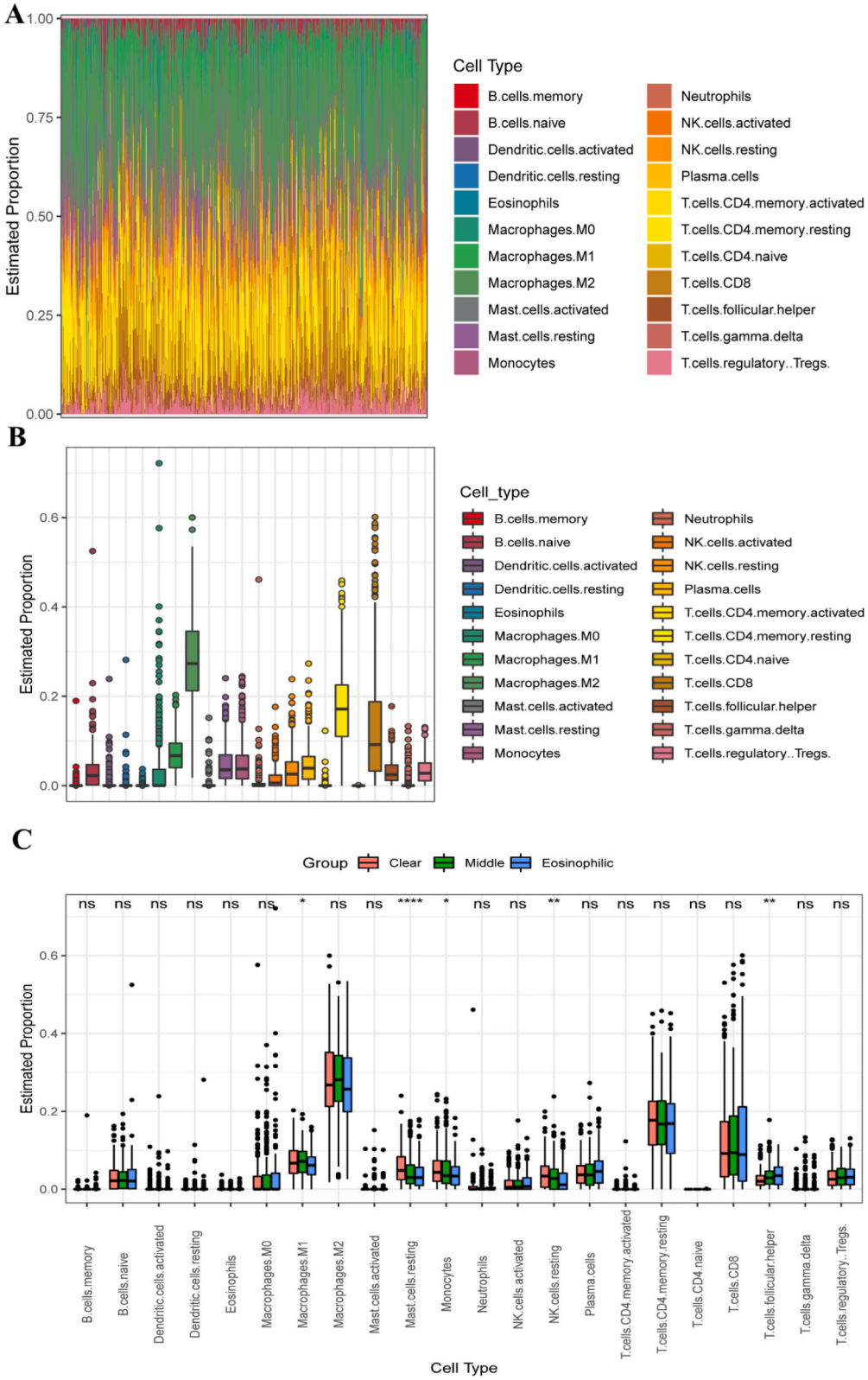

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Fig. 4. Differences in immune cell infiltration among three types of ccRCC. (A) Profile of the proportion of different immune cell infiltrations in the tumor microenvironment of ccRCC for each patient (B) Percentage abundance of immune cells based on all ccRCC patients in the TCGA cohort (C) Comparison of the proportion of immune cell infiltration between three ccRCC subtypes. *: $p < 0.05$; **: $p < 0.01$; ***: $p < 0.001$; ****: $p < 0.0001$; ns: no significance.

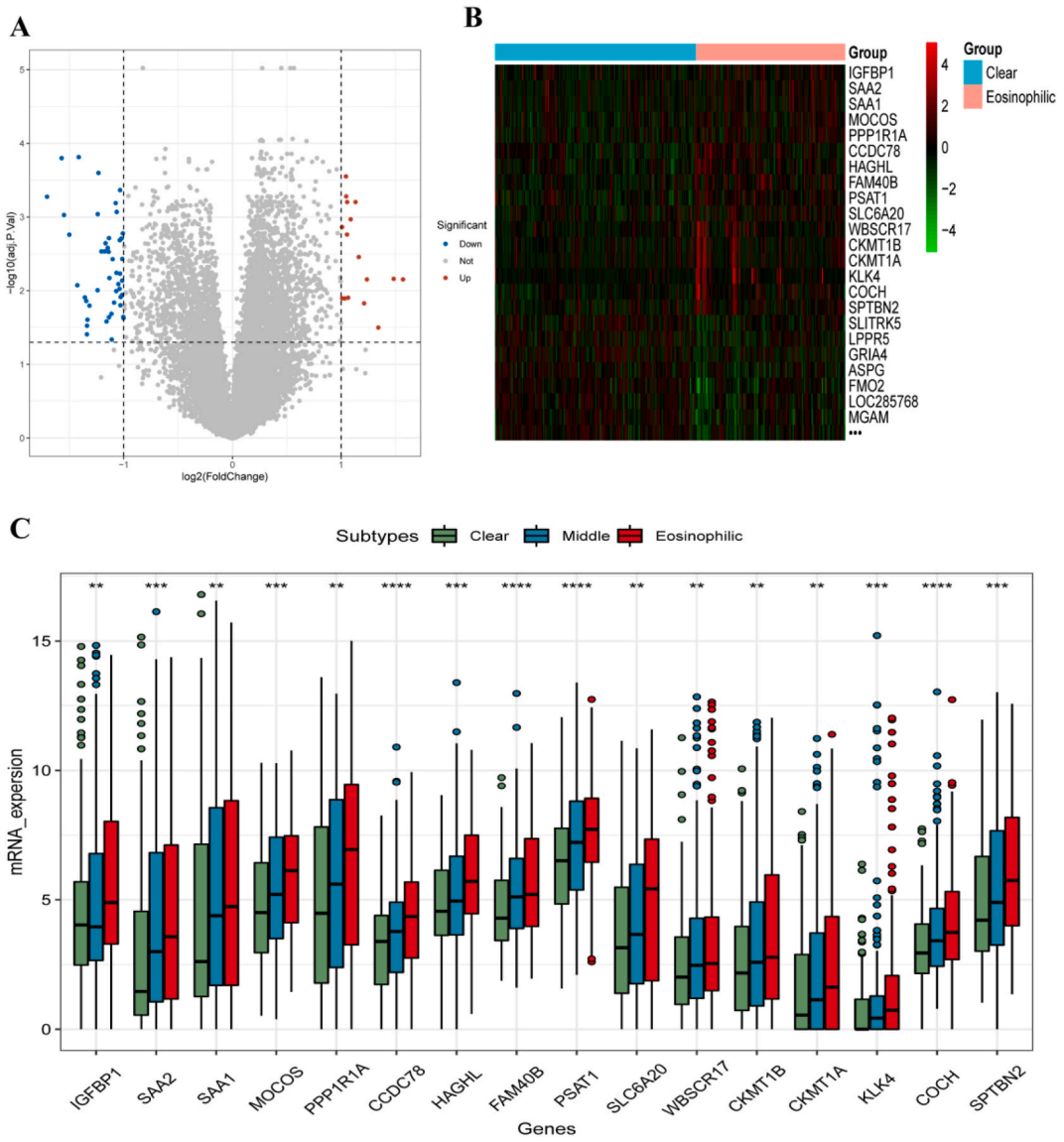

Fig. 5. Identification of EDGs between clear and eosinophilic ccRCC. (A) Visual analysis of volcano map of EDGs. (B) Heatmap visualization analysis of EDGs (Partially displayed). (C) Comparison of the expression of genes upregulated in eosinophilic ccRCC between the three subtypes. *: $p < 0.05$; **: $p < 0.01$; ***: $p < 0.001$; ****: $p < 0.0001$.

the expression of seven genes, MOCOS, PPP1R1 A, PSAT1, IGFBP1, SAA2, SAA1, and SPTBN2, had survival significance in the TCGA and *E*-MTAB-1980 cohorts (Fig. 7A and B). These seven genes were identified to have prognostic significance for ccRCC patients. We speculate that they act as indispensable causes in the formation of eosinophilic ccRCC.

4. Discussion

During the course of tumor progression, cancers often become more heterogeneous. As a result of this heterogeneity, tumors may include collections of cells with different molecular signatures that differ in their sensitivity to therapy [16]. Therefore, assessing the characteristics of tumor heterogeneity is essential for the development of effective therapeutic approaches. After HE staining of tumor

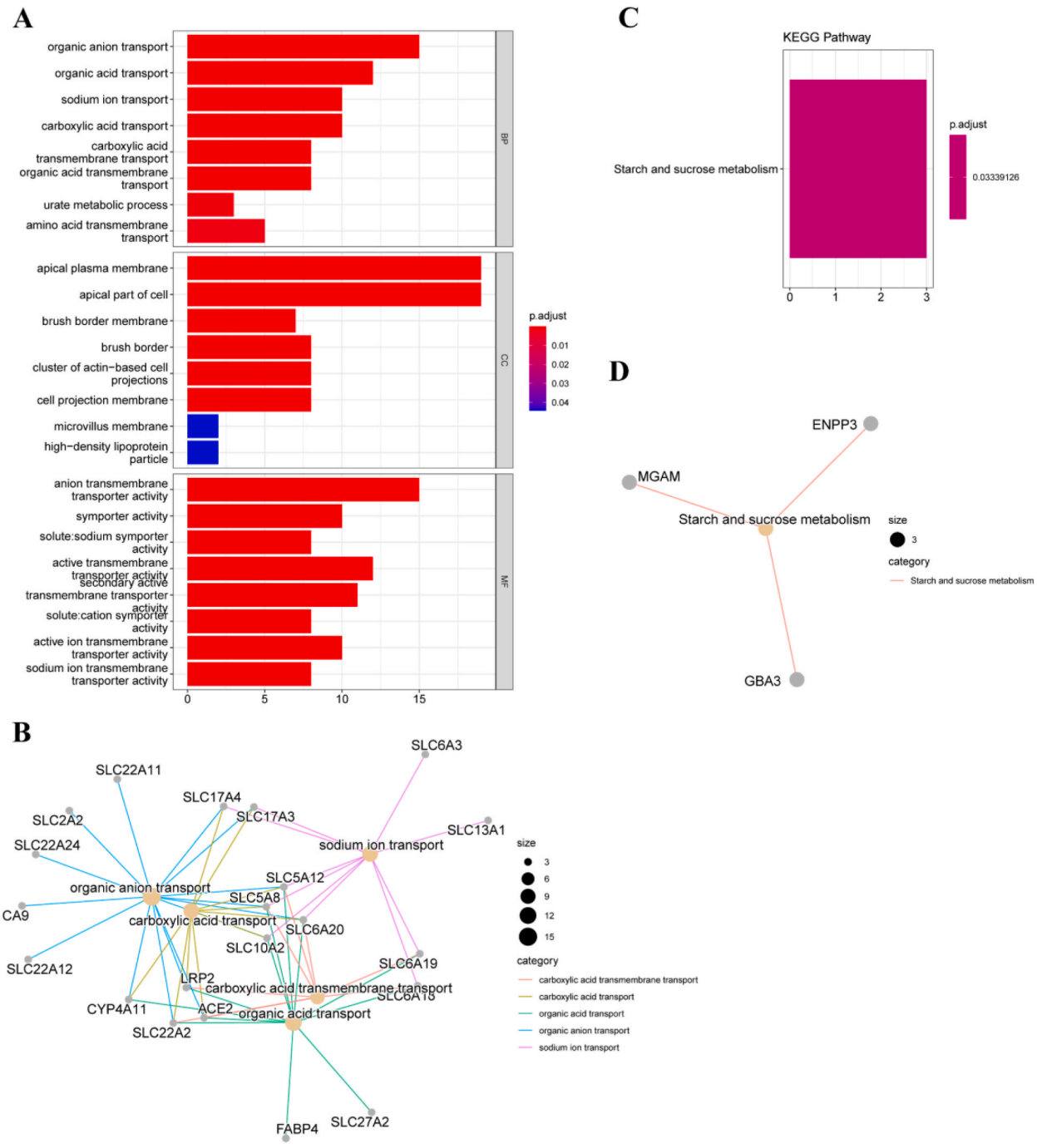

Fig. 6. Potential signaling pathways involved in DEGs. (A) GO enrichment results for DEGs. (B) GO enrichment network. (C) KEGG enrichment results for DEGs. (D) KEGG enrichment network.

samples from ccRCC patients, a subset of patients shows eosinophilic staining in their cancer cells. This is considered to be a malignant feature and heterogeneity within the tumor [6]. However, the molecular characteristics, immune infiltration differences, and differences in mutational profiles between eosinophilic type and the clear type of ccRCC have not been investigated.

In the present study, we validated the poor prognosis of ccRCC patients with eosinophilic features using multicenter data. We found that eosinophilic features were associated with cancer progression, especially grade. In addition to that, we also uncovered that ccRCC patients with acidophilic features had a different gene mutation profile and different immune cell infiltration compared to other types of patients. We also discovered by enrichment analysis that DEGs between clear and eosinophilic types were associated with the

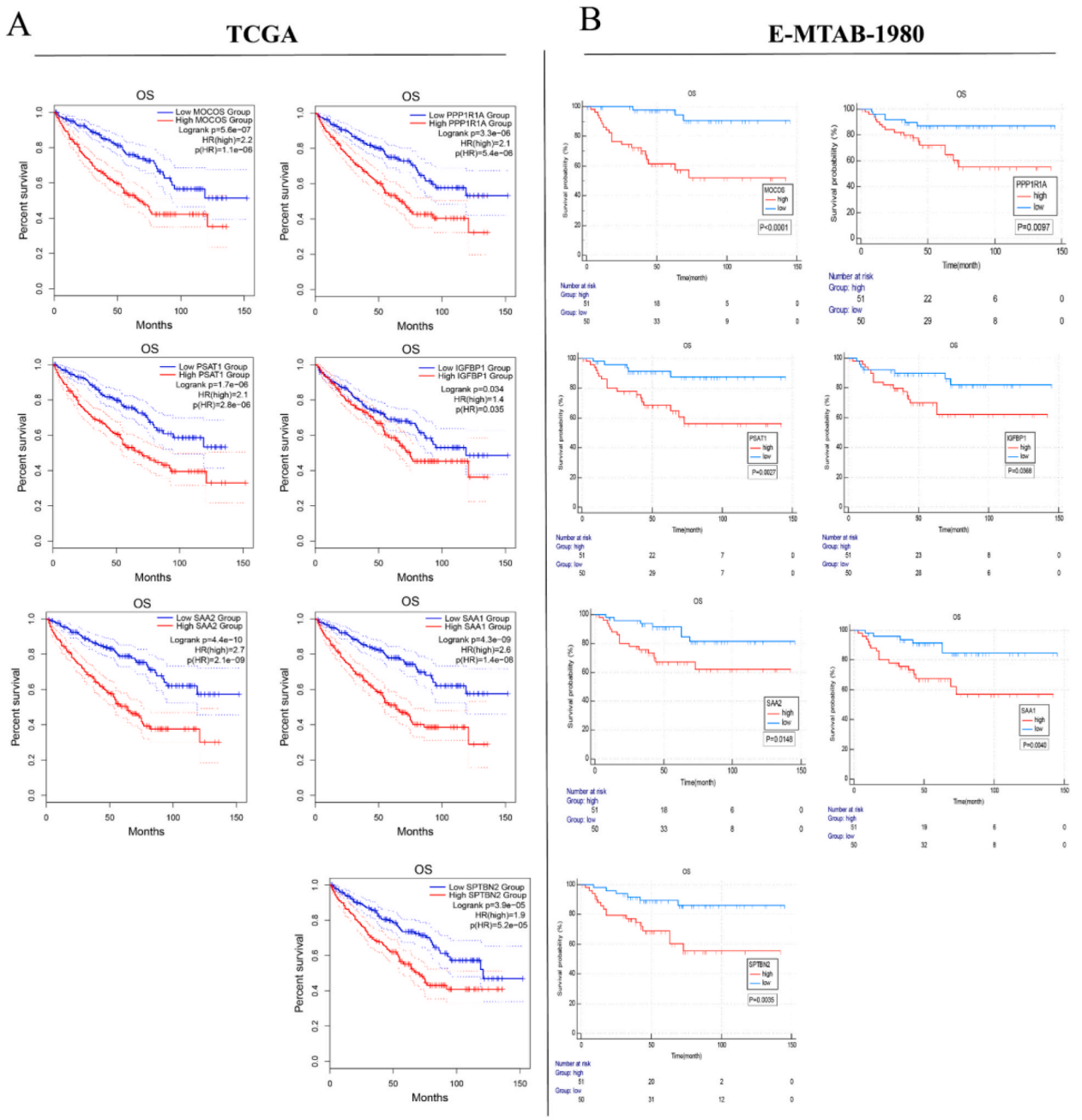

Fig. 7. Potential prognostic genes validated in multiple centers. (A) The prognostic value of eosinophilic features related genes in the TCGA cohort. (B) The prognostic value of eosinophilic features related genes in the *E*-MTAB-1980 cohort.

transport and metabolism of multiple substances. Finally, we identified 7 genes with prognostic value associated with eosinophilic features.

Previous studies have preliminarily found that ccRCC patients with eosinophilic features have a poor prognosis [7], but there is a lack of multicenter data for validation, in this paper we included three cohorts to validate this finding and make the conclusions more persuasive. Nilsson et al. based on small samples of eosinophilic and clear type RNA sequencing also performed analysis of DEGs, but the small sample size and lack of validation by large-scale databases such as TCGA made their study less convincing [6]. Our survival analysis is multicenter-based and the DEGs analysis is based on a large database. Therefore, our conclusions are highly convincing.

The mutation profiles of genes in the three types of ccRCC were different, especially FAT4 had the highest mutation frequency in the eosinophilic ccRCC. Studies have demonstrated that FAT4 is expressed at lower levels in colorectal cancer than in normal colorectal tissue. High expression levels of FAT4 inhibit epithelial-mesenchymal transition (EMT) and induce autophagy in colorectal cancer

[17]. Related reports suggested that the eosinophilic features of ccRCC were associated with EMT [6]. This is consistent with our findings and could indirectly suggest that mutation of FAT4 transforms clear ccRCC to eosinophilic ccRCC. The effect of FAT4 mutation on ccRCC needs to be further investigated urgently. This also implies that targeted EMT therapy in eosinophilic ccRCC may yield better efficacy.

After comparing the results of immune cell infiltration in three types of ccRCC, we found that there were fewer M1 macrophages and NK tissue-resident cells in eosinophilic ccRCC. The role of these two cells is to kill and inhibit tumor progression [18,19]. This may be part of the reason for the poor prognosis of eosinophilic ccRCC.

By analyzing DEGs between clear and eosinophilic types of ccRCC, we identified a total of 62 genes that were differentially expressed. Sixteen genes were up-regulated and 46 genes were down-regulated in eosinophilic ccRCC. We concluded that the prognostic value of genes upregulated in eosinophilic ccRCC was more pronounced. Therefore, we performed survival analysis of these 16 genes, and we identified seven genes with multicenter-validated prognostic value. This suggests that they are important in guiding the prognosis of ccRCC patients. Among them, the prognostic value of IGFBP1 and SAA1 has been reported [20,21]. The remaining five genes have not been reported yet. However, the biological significance of these seven genes in the development and progression of ccRCC has not been investigated.

Through GSEA analysis of DEGs, we found that the enrichment results of GO and KEGG were associated with the transmembrane transport and metabolism of a variety of substances. We speculate that the eosinophilic type of ccRCC is due to the progression through the clear type of ccRCC following alterations in certain genes. The present study identified 16 genes that were significantly upregulated in eosinophilic ccRCC, but how these genes contribute to the transition from the clear type to the eosinophilic type of ccRCC was not explored in depth. We suggest that the eosinophilic transition is associated with the catabolism of cholesterol and glycogen in the cytoplasm of clear type ccRCC. This is also the main content we will study next.

More and more anti-tumor drugs are being developed, depending on the characteristics of different types of ccRCC, the sensitivity to drugs may also vary. Therefore, the development and identification of drugs for different ccRCC subtypes is also a promising direction.

This study inevitably has some drawbacks, such as the screened differentially expressed genes were not experimentally verified. The experimental procedures for HE staining may be different in different centers, so the staining results may be partially biased. In addition to this, there is unavoidably some discrepancy in the assessment of subtypes between different pathologists, which may have potential implications for the study findings.

5. Conclusions

In this study, the prognostic significance of eosinophilic features in ccRCC was determined using multicenter data, and the differences in gene mutation profiles and immune cell infiltration among the three ccRCC subtypes were also described. Finally, seven prognostic genes associated with eosinophilic characteristics that have not been studied were identified. The findings of this study have significant implications for the research on the malignant transformation of ccRCC.

Ethics approval and consent to participate

The ethical approval of this study has been approved by the Research Ethics Committee of Shanghai General Hospital. No further ethical approval was required since all the slice images from the CPTAC cohort and the TCGA cohort were publicly available for research purposes. The patients provided their written informed consent to participate in this study.

Consent for publication

Not applicable.

Authors' contributions

XW (Xiang Wang), SC (Siteng Chen) and NZ (Ning Zhang) conceived and designed the experiments; TG (Tuanjie Guo)and LJ (Liren Jiang) performed the experiments, wrote the paper and analyzed and interpreted the data; YL (Yang Liu), XW (Xuan Wang), TW (Tao Wang) and JZ (Jian Zhang) contributed reagents, materials, analysis tools or data. All authors read and approved the final manuscript.

Funding

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Availability of data and materials

This study included three public datasets, which are the TCGA (https://portal.gdc.cancer.gov/), *E*-MTAB-1980 (https://www.ebi.ac.uk/), CPTAC (https://pdc.cancer.gov/pdc/). The datasets used and analyzed in this study are also available from the corresponding author upon reasonable request.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.heliyon.2023.e16479.

Abbreviations

RCC	renal cell carcinoma
ccRCC	clear cell renal cell carcinoma
TCGA	The Cancer Genome Atlas
CPTAC	Clinical Proteomic Tumor Analysis Consortium
HE	hematoxylin-eosin
DEGs	differentially expressed gene
KEGG	Kyoto Encyclopedia of Genes and Genomes
GO	Genetic Ontology
GSEA	Gene Set Enrichment Analysis
ANOVA	analysis of variance
OS	overall survival
DFS	disease-free survival
ICIs	immune checkpoint inhibitors
TKIs	tyrosine kinase inhibitors
EMT	epithelial mesenchymal transition

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