Comprehensive analysis of the expression, prognostic value and biological importance of OVO-like proteins in clear cell renal cell carcinoma

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Abstract. This study examined the expression levels of OVO-like proteins (OVOLs) in clear cell renal cell carcinoma (ccRCC) tissues and their value in predicting disease prognosis. The transcript levels, genetic alterations, and biological functions of OVOLs and their correlation with tumor immune cell infiltration and drug sensitivity and survival outcomes, as well as their prognostic values, in patients with ccRCC were analyzed based on data obtained from The Cancer Genome Atlas, Gene Expression Profiling Interactive Analysis, cBioPortal, and GSCALite databases. Gene Ontology and Kyoto Encyclopedia of Genes and Genomes analyses were performed using R software (Bioconductor, clusterProfiler packages). A protein-protein interaction network was established and visualized using the R software with the ggplots package. The ggstatsplot package was used to plot the correlation between gene expression and immune cell infiltration. The mRNA expression levels of *OVOL1* and *OVOL2* were significantly downregulated in patients with ccRCC, whereas those of *OVOL3* were upregulated. *OVOL1* expression was correlated with tumor stage and histological grades. The *OVOL1*, *OVOL2*, and *OVOL3* levels were significantly correlated with the prognosis of patients with ccRCC, the infiltration of immune cells, and drug sensitivity. Multivariate and univariate analyses showed that the expression of *OVOL1* was an independent prognostic factor for the overall survival (OS) of patients with ccRCC. The OVOL proteins were associated with various pathways, including tight junction, cell adhesion molecules, and ether lipid metabolism. Additionally, *OVOL3* upregulation, and *OVOL1* and *OVOL2* downregulation in clinical ccRCC samples were experimentally verified. Thus, OVOL1 and OVOL2 are potential therapeutic targets and prognostic markers for ccRCC. Additionally, OVOL1 can serve as an independent prognostic factor for OS in patients with ccRCC.

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Introduction

Renal cell carcinoma (RCC) accounts for 2-3% of all malignant tumors in adults, as well as 90% of all malignant kidney tumors (1,2). The incidence and mortality rates of kidney cancer have been increasing worldwide. According to the 2021 GLOBOCAN data, 431,288 cases of kidney cancer were diagnosed in 2020 worldwide, accounting for 2.2% of all new cancer cases and 1.8% (179,368) of all new cancer-related deaths (3). Clear cell RCC (ccRCC), which is the most common subtype of renal cancer (4), is characterized by increased malignancy and has the highest mortality rate among genitourinary system cancers (5). Radiation therapy and chemotherapy are ineffective against ccRCC. Currently, surgical intervention is the primary treatment for ccRCC. Although the incidence of kidney cancer is increasing annually, the development of early detection techniques has markedly decreased kidney cancer-related mortality rates in recent decades (6). The majority of patients with ccRCC are asymptomatic and are diagnosed during imaging examinations, such as computed tomography scan or ultrasound (7). The lack of effective diagnostic methods prevents early diagnosis and treatment of patients with ccRCC, contributing to poor prognosis and poor survival rates. The survival rate of patients with kidney cancer who have distant metastases is only 12%, while that of patients with localized cancer is 67% (8). Surgical resection of locally advanced RCC is the only curative treatment (5). Approximately 20-30% of patients with kidney cancer exhibit relapse after nephrectomy (9,10). Recently, several treatment strategies, such as immunotherapy, radiotherapy, and molecular targeted drugs, have been developed, which have markedly improved the clinical outcomes of

advanced diseases (11,12). However, the clinical outcomes of kidney cancer are poor owing to the low objective response rates, local recurrences, or distant metastases. Therefore, there is an urgent need to identify novel molecular prognostic markers for ccRCC.

Evaluation of the prognostic value of OVO-like proteins (OVOLs) in patients with ccRCC can potentially improve the prediction of clinical outcomes and aid in the development of effective treatments. In mammals, OVOLs encode C2H2 zinc finger transcription factors (13). OVOLs, which are members of the zinc finger protein family, function as transcription factors to regulate gene expression during differentiation (14). The three members of the OVOL family are OVOL1, OVOL2, and OVOL3. Molecular profiling of human tumors revealed that OVOL deregulation is associated with adverse outcomes in various carcinomas and is directly related to metastasis (15-17). The activity of OVOL can stabilize a hybrid phenotype between epithelial and mesenchymal states, resulting in several benefits for both tumors and healthy stem cells (18,19). Epithelial-to-mesenchymal transition (EMT) plays a key role in the stromal invasion of tumor cells (20). OVOL1 and OVOL2 are reported to be key regulators of EMT and its mirror process. mesenchymal-to-epithelial transition (MET) (16). Previous studies have reported that OVOLs are associated with the clinical stage, EMT, and tumor metastasis and that they can modulate cancer cell stemness. Additionally, OVOLs are potential prognostic prediction factors (17,21-23). The distinct expression/mutation pattern and prognostic significance of OVOLs have not been evaluated in ccRCC.

This study evaluated the potential of OVOLs as predictors of the prognosis of ccRCC using experimental and bioinformatics approaches.

Materials and methods

Ethics statement. The research protocol used in the present study was approved by the Ethics Committee of the First Affiliated Hospital of Nanchang University (approval no. 12-110). The datasets were retrieved from public databases. All data were collected after obtaining written consent.

Patient and tumor samples. In total, 20 pairs of kidney renal clear cell carcinoma (KIRC) and adjacent non-tumorous tissues obtained via radical nephrectomy were collected from patients who had been pathologically confirmed to have cancer by two independent pathologists. The samples collected at the First Affiliated Hospital of Nanchang between January 2021 and December 2022 were immediately stored in liquid nitrogen.

Cell lines and cell culture. The KIRC, HK-2, 786-O, Caki-1, and ACHN cell lines were purchased from the American Type Cell Collection. The cell lines were maintained as monolayers in minimal essential medium, RMPI-1640 medium, and Dulbecco's modified essential medium (Gibco; Thermo Fisher Scientific, Inc.) supplemented with 10% (v/v) fetal bovine serum at 37˚C in a humidified incubator supplied with 5% CO_2.

RNA isolation and reverse transcription-quantitative (RT-q) PCR. Total RNA was isolated from ccRCC tissues and adjacent non-tumorous tissues, as well as from ccRCC cell lines, using TRIzol® reagent (Invitrogen; Thermo Fisher Scientific, Inc.). The extracted RNA was reverse-transcribed to cDNA using the First-Strand cDNA synthesis kit (Qiagen GmbH) according to the manufacturer's protocol. qPCR was performed using the SYBR Real-Time PCR kit (Qiagen GmbH). The PCR thermocycling conditions were as follows: 95˚C for 2 min, followed by 40 cycles of 95˚C for 5 sec and 60˚C for 10 sec. The relative gene expression levels were calculated using the $2^{-\Delta\Delta Cq}$ method (24). Each analysis was performed in triplicate. *ACTB* served as an internal reference gene. The sequences of the primers used for qPCR were: GAPDH forward, 5'-GCCACATCGCTCAGACACCAT-3' and reverse, 5'-CCCATACGACTGCAAAGACCC-3'; human OVOL1 forward, 5'-AGACACGTCCGAACTCACAC-3' and reverse, 5'-TGCTGCACACCATGGATCTT-3'; human OVOL2 forward, 5'-CAACGACACCTTCGACCTGA-3' and reverse, 5'-TCAGGTGGGACTCCAGAGAG-3'; human OVOL3 forward, 5'-TTCGATCTCAAGCGCCACAT-3' and reverse, 5'-GCTGTCCATGCACCTTAGCA-3'.

Gene Expression Profiling Interactive Analysis (GEPIA) dataset. GEPIA (http://gepia.cancer-pku.cn/) was used to comparatively examine the tumor and non-tumor tissue datasets from The Cancer Genome Atlas (TCGA; https://portal.gdc.com) and the non-tumor datasets from the Genotype-Tissue Expression database (25). The differential expression levels of *OVOL1*, *OVOL2*, and *OVOL3* between ccRCC tissues and adjacent non-tumor tissues are represented using box plots. The correlation between gene expression and cancer stage was examined using the GEPIA software package.

cBioPortal analysis. cBioPortal is a free, open-access online resource integrating data from large-scale genomic projects, including but not limited to TCGA and the International Cancer Genome Consortium (26). In the present study, 512 ccRCC samples (TCGA, provisional) with known mutations, putative copy-number alterations (identified using the GISTIC module) (27), and z-scores (RNA Seq V2 RSEM) for mRNA expression were analyzed. Additionally, the correlation between genetic mutations in OVOL-encoding genes with the overall survival (OS) of patients with ccRCC was examined. A log-rank test was used to compare the difference in survival between the altered and unaltered groups.

STRING analysis. STRING (http://string-db.org) was used to construct protein-protein interaction networks, including both physical binding and functional associations, between immune checkpoints and tumor immune microenvironment-related factors (28). The networks were visualized using R software (version 4.2.1; https://www.r.project.org).

GSCALite. GSCALite (http://bioinfo.life.hust.edu.cn/web/GSCALite/) provides a platform for the analysis of gene sets in cancer (29). In the present study, the correlation between microRNA (miRNA) and corresponding OVOLs was analyzed using GSCALite. Drug sensitivity data and gene expression profiles of cancer cell lines were retrieved from the Genomics of Drug Sensitivity in Cancer (GDSC) database and the Therapeutics Response Portal (CTRP).

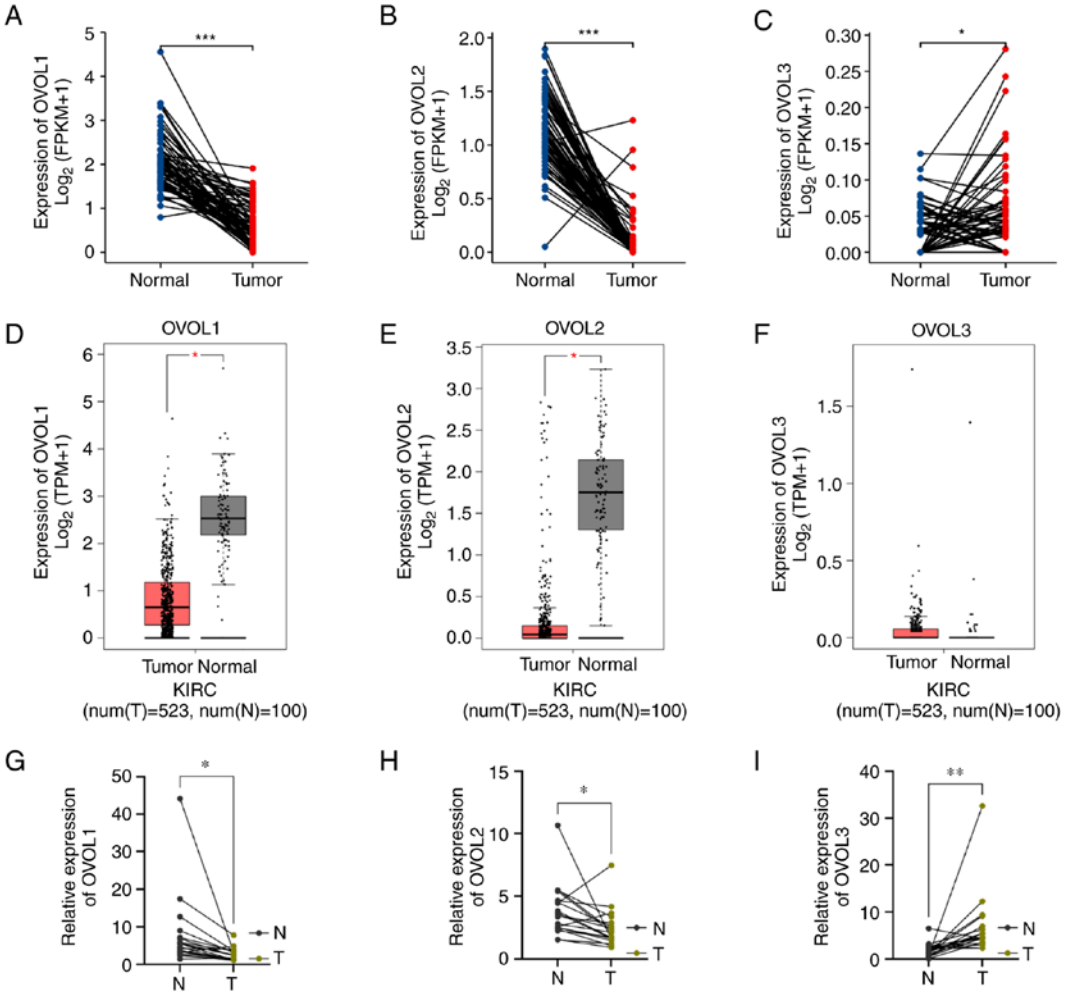

Figure 1. The expression of distinct OVOL1, OVOL2 and OVOL3 in KIRC tissues and adjacent normal kidney tissues. (A-C) The differential mRNA expression levels of *OVOL1*, *OVOL2*, and *OVOL3* between 72 pairs of kidney cancer tissues and adjacent non-cancerous kidney tissues, which were obtained from The Cancer Genome Atlas and Genotype-Tissue Expression databases. (D-F) The expression levels of *OVOL1*, *OVOL2*, and *OVOL3* in clear cell renal cell carcinoma and healthy kidney tissues were analyzed using the Gene Expression Profiling Analysis datasets. (G-I) The mRNA levels of *OVOL1*, *OVOL2*, and *OVOL3* in ccRCC tissues and paired adjacent non-tumor kidney tissues were analyzed using reverse transcription-quantitative PCR. *P<0.05, **P<0.01, ***P<0.001. ns, not significant; OVOL, OVO-like protein; KIRC, kidney renal clear cell carcinoma; FPKM, fragments per kilobase million; TPM, transcripts per million.

Statistical analysis. All statistical analyses were performed using R software (version 3.6.2). The differential expression levels of OVOLs in ccRCC were analyzed using the 'limma' R package and a Wilcox test. The prognostic significance of OVOLs was evaluated using Kaplan-Meier survival analysis and Cox proportional hazards regression analysis. The effect of clinicopathological parameters and mRNA levels of OVOLs on the survival of patients with ccRCC was determined using univariate Cox regression analysis. Further analyses were performed using a P<0.1 threshold. All statistical analyses were performed using two-sided tests. P<0.05 was considered to indicate a statistically significant difference. The RNA-sequencing expression (level 3) profiles and corresponding clinical information for ccRCC were downloaded from TCGA. The R software ggstatsplot packagehttps://CRAN.R-project.org/package=ggstatsplot) was used to plot the correlation between gene expression and immune score, while the pheatmap package (https://CRAN.R-project.org/package=pheatmap) was used to plot multi-gene correlation. Gene Ontology (GO) (30,31) and Kyoto Encyclopedia of Genes and Genomes (KEGG) pathway (32) enrichment analyses were performed using R. To predict the functional roles of target host genes, GO enrichment analysis was performed based on biological process (BP), cellular component (CC) and molecular function (MF) using the R package ggplot2 v3.3.2 (33).

Results

Differential mRNA levels of OVOLs in patients with ccRCC. A TCGA-KIRC dataset was analyzed to comparatively evaluate the expression levels of OVOL family members between ccRCC samples and 72 paired non-tumor tissue samples. As shown in Fig. 1A-C, the *OVOL1* and *OVOL2* mRNA levels in non-tumor samples were significantly higher than those in ccRCC tissues. In contrast, the *OVOL3* mRNA levels in tumor tissues were upregulated when compared with those in the non-tumor tissues. Next, the *OVOL1*, *OVOL2*, and *OVOL3* expression levels in ccRCC datasets obtained from the GEPIA database were analyzed. As shown in Fig. 1D-F, the *OVOL1* and *OVOL2* mRNA levels in non-tumor tissues were significantly upregulated when compared with those in

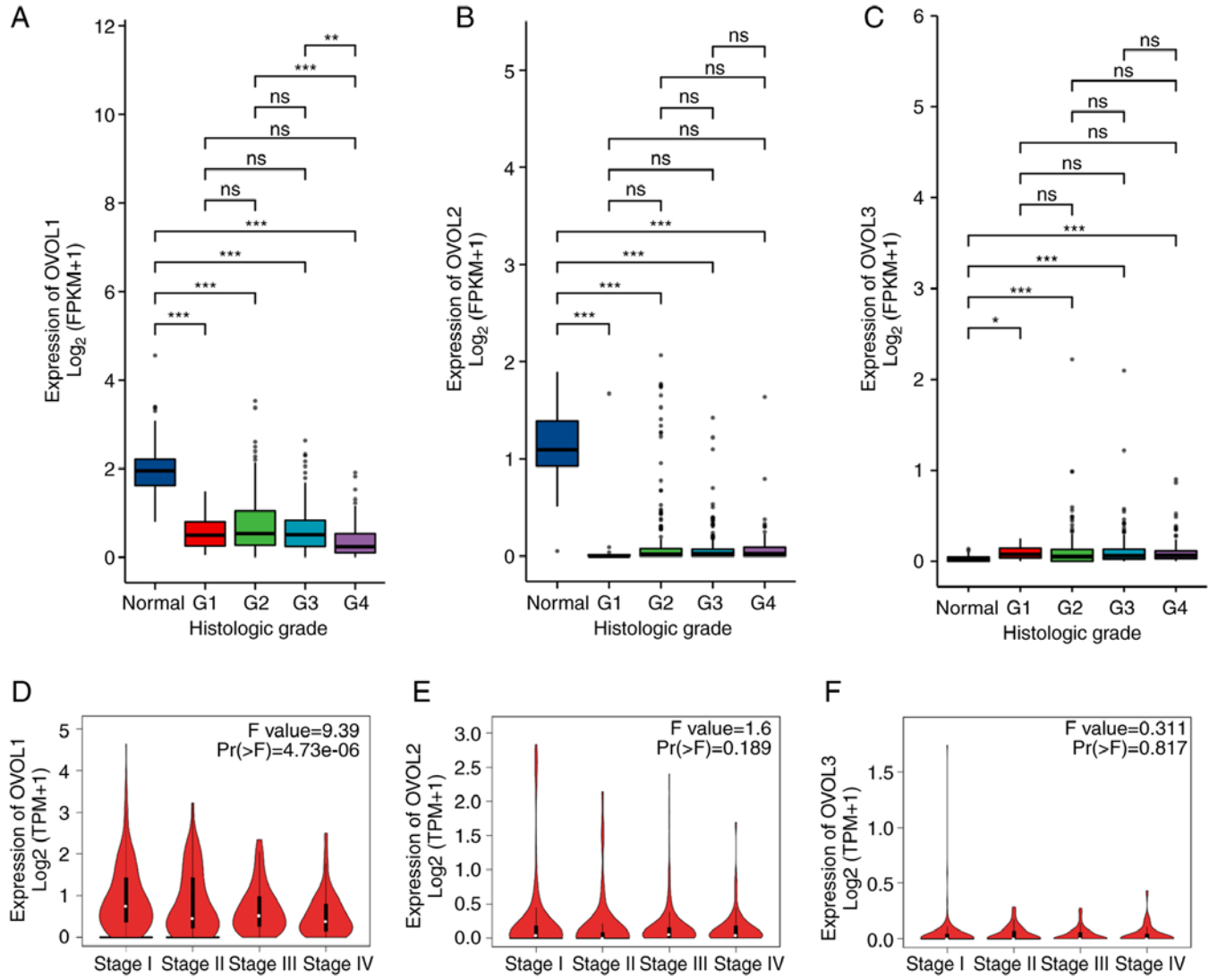

Figure 2. Relationship between the micro RNA levels of OVOL1/2/3 and the clinicopathological parameters of patients with ccRCC. (A-C) Association between the expression levels of the OVOL family members and the histological grade of ccRCC. (D-F) A violin plot was used to analyze the correlation between OVOL-encoding mRNA levels and clinical stages of patients with ccRCC using the Gene Expression Profiling Interactive Analysis dataset. *P<0.05, **P<0.01, ***P<0.001. OVOL, OVO-like protein; ccRCC, clear cell renal cell carcinoma; ns, not significant; FPKM, fragments per kilobase million; TPM, transcripts per million.

ccRCC tissues. However, the mRNA levels of *OVOL3* were not significantly different between ccRCC and non-tumor tissues. To further validate this conclusion, 20 pairs of cancerous and adjacent non-cancerous tissues were subjected to RT-qPCR analysis. As shown in Fig. 1G-I, the *OVOL3* mRNA levels in tumor tissues were higher than those in adjacent non-tumor tissues. Meanwhile, the *OVOL1* and *OVOL2* mRNA levels in tumor tissues were downregulated when compared with those in adjacent non-tumor tissues. These results indicate that the mRNA levels of *OVOL1* and *OVOL2* in paired non-tumor tissues were significantly lower than those in ccRCC tissues, while those of *OVOL3* exhibited contrasting expression patterns. Healthy kidney and kidney cancer cell lines were subjected to RT-qPCR analysis to determine the mRNA levels of *OVOL1*, *OVOL2*, and *OVOL3* (Fig. S1). The mRNA levels of *OVOL1* and *OVOL2* in kidney cancer cell lines were lower than those in healthy cell lines. In contrast, the *OVOL3* mRNA levels in kidney cancer cell lines were upregulated when compared with those in healthy cell lines.

Correlation between OVOLs and the clinicopathological parameters of patients with ccRCC. Next, the correlation between the *OVOL1*, *OVOL2*, and *OVOL3* mRNA levels and clinicopathological parameters (including histological grade and pathological stage obtained from TCGA and GEPIA datasets) was evaluated. As shown in Fig. 2A-C, the *OVOL1* mRNA expression levels were positively correlated with tumor histological grade. However, the *OVOL2* and *OVOL3* mRNA levels were not correlated with the histological grade. As shown in Fig. 2D-F, the *OVOL1* mRNA levels were correlated with the clinical stage of ccRCC, whereas the *OVOL2* and *OVOL3* mRNA levels were not correlated. Patients at advanced pathological stages exhibited downregulated *OVOL1* levels. Thus, the mRNA expression levels of the OVOL family members were significantly correlated with

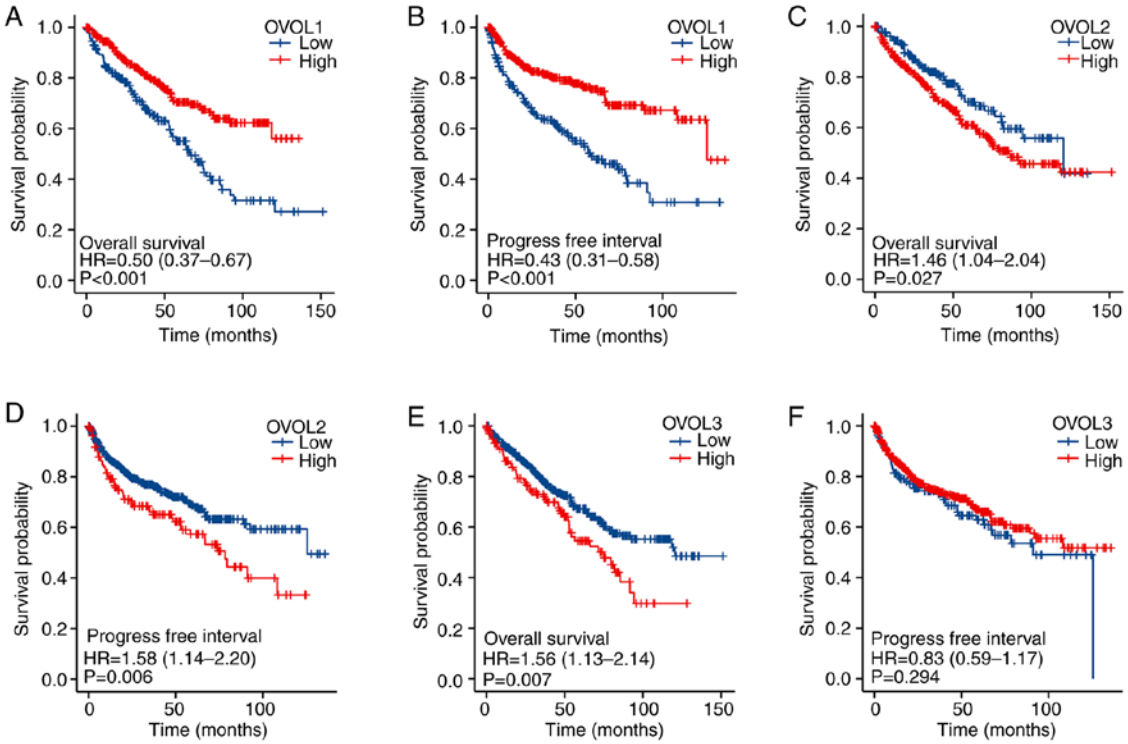

Figure 3. Prognostic value of OVOL-encoding mRNA expression levels in patients with ccRCC. (A, C, and E) The association between OVOL-encoding mRNA expression levels and the overall survival of patients with ccRCC was investigated using The Cancer Genome Atlas datasets. (B, D, and F) The correlation between OVOL-encoding mRNA expression levels and progression-free interval of patients with ccRCC was analyzed using R software. OVOL, OVO-like protein; HR, hazard ratio.

several clinical and pathological parameters in patients with ccRCC.

Prognostic value of OVOLs mRNA levels in patients with ccRCC. The prognostic value of the expression of OVOL-encoding mRNAs in patients with ccRCC was analyzed using Kaplan-Meier survival curves using TCGA datasets. As shown in Fig. 3, the mRNA levels of OVOL family members were significantly associated with the prognosis of patients with ccRCC. The downregulated *OVOL1* mRNA levels [(hazard ratio (HR)=0.50, 95% confidence interval (CI)=0.37-0.67, and P<0.001] were associated with a poorer OS in patients with ccRCC. Meanwhile, the upregulated *OVOL2* (HR=1.46, 95% CI=1.04-2.04, P=0.027) and *OVOL3* (HR=1.56, 95% CI=1.13-2.14, P=0.007) mRNA levels were associated with a poorer OS in patients with ccRCC. The upregulated mRNA levels of *OVOL1* (HR=0.43, 95% CI=0.31-0.58, P<0.001) were associated with a favorable progression-free interval (PFI). However, the *OVOL3* mRNA levels were not associated with the PFI of patients with ccRCC. The *OVOL2* mRNA levels (HR=1.58, 95% CI=1.14-2.20, P=0.006) were negatively correlated with PFI. Thus, the mRNA expression levels of OVOLs were significantly correlated with the prognosis of patients with ccRCC. Additionally, the mRNA levels may be used as indicators for predicting clinical outcomes, including the OS, of patients with ccRCC.

Independent prognostic value of OVOL mRNA levels for predicting OS in patients with ccRCC. The mRNA levels of OVOLs were significantly associated with the OS of patients with ccRCC. Further, the independent prognostic value of the mRNA expression levels of OVOL family members in patients with ccRCC was examined using a TCGA dataset and Cox survival regression analysis (34). Univariate Cox regression analysis revealed that the upregulated expression of *OVOL1* (HR=0.492, 95% CI=0.347-0.700, P<0.001) was significantly associated with a favorable OS. Multivariate analysis revealed that the upregulated mRNA levels of *OVOL1* (HR=0.645, 95% CI=0.446-0.933, P=0.020) were independently correlated with a favorable OS in patients with ccRCC. Thus, the mRNA levels of *OVOL1* are an independent prognostic factor in patients with ccRCC (Table SI).

Genetic mutations in the OVOL family and their association with the OS of patients with ccRCC. To further evaluate the expression patterns of the OVOL family members, genetic alterations in OVOL-encoding genes and their association with the OS of patients with ccRCC were examined using the cBioPortal online tool. Genetic alterations in OVOL-encoding genes are shown in Fig. 4A. The frequency of genetic alterations according to the cBioPortal database is shown in Fig. 4B. The ccRCC dataset analysis indicated that the percentages of DNA alterations in *OVOL1*, *OVOL2*, and *OVOL3* were 2.3, 3, and 0%, respectively. Next, the association between OVOL-encoding gene alterations and survival outcomes was examined. Mutations in the OVOL-encoding gene family were not associated with the OS (Fig. 4C). These results indicated that DNA alterations were not the primary cause for the dysregulation of OVOL family members. Multiple non-coding RNAs, including hsa-miR-9-5p and hsa-miR-30a-5p, regulated

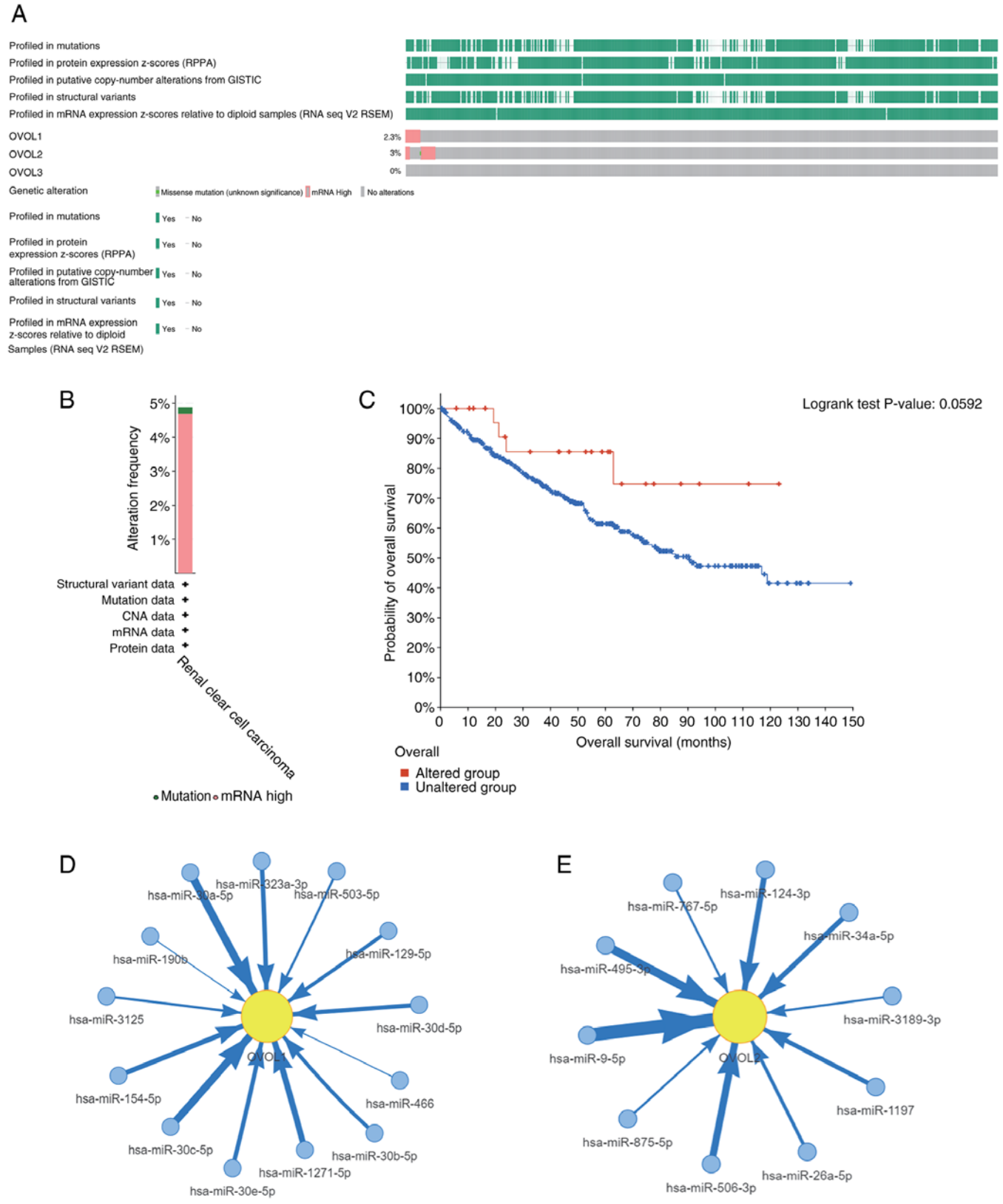

Figure 4. OVOL1/2/3 gene expression and mutation analysis in ccRCC and interaction network between OVOL1/2/3 gene and mRNAs (A) Analysis of the genetic alterations of OVOL-encoding genes in ccRCC. (B) Alteration frequency of OVOL-encoding genes according to the cBioPortal database. (C) Kaplan-Meier plots and log-rank tests revealed the overall survival of patients with ccRCC with or without OVOL alterations. (D and E) Interaction network of *OVOL1*, *OVOL2*, and the associated microRNAs. OVOL, OVO-like protein; clear cell renal cell carcinoma.

OVOL family mRNAs, which suggested that non-coding RNA-mediated regulation may play a key role in OVOL alterations (Fig. 4D-E).

Predicted functions and pathways of altered OVOLs and the 100 most frequently altered neighboring genes in patients with ccRCC. An integrated network was constructed by

analyzing 100 genes related to the OVOL mutants. The top 100 genes that were co-expressed and associated with OVOLs were retrieved from the cBioPortal database. A protein-protein interaction network was constructed using R. As shown in Fig. 5A, the cell-cell junction assembly-related genes, including *CDH1*, *UGT8*, *GRHL2*, *MARVELD3*, *CRB3*, *MARVELD2*, and *OCLN*, were significantly associated with OVOL mutations. BPs, such as GO:0007043 (cell-cell junction assembly), GO:0034329 (cell junction assembly), GO:0070830 (bicellular tight junction assembly), GO:0120192 (tight junction assembly), and GO:0009913 (epidermal cell differentiation) were significantly associated with OVOL alterations in ccRCC. CCs, including GO:0070160 (tight junction), GO:0016324 (apical plasma membrane), GO:0043296 (apical junction complex), GO:0045177 (apical part of the cell), and GO:0005923 (bicellular tight junction), were significantly associated with OVOL alterations. MFs, such as GO:0015605 (organophosphate ester transmembrane transporter activity) were significantly associated with OVOL alterations. KEGG analysis revealed that OVOL mutations were enriched in the following five pathways in ccRCC: has04530 (Tight junction), has04514 (Cell adhesion molecules), has04966 (Collecting duct acid secretion), has05130 (Pathogenic *Escherichia coli* infection), and ha00565 (Ether lipid metabolism) (Fig. 5B and Table SII).

Association between OVOLs and immune infiltration in ccRCC. The correlation between genes and immune infiltration was evaluated using the R software pheatmap package. The abundance of CD8+ T cells was negatively associated with the expression levels of OVOL1 and OVOL2. Additionally, the abundance of CD4+ T cells was negatively associated with the expression of OVOL3. The abundance of neutrophil cells was positively associated with the expression of OVOL3 but negatively associated with the expression of OVOL2. Furthermore, the abundance of macrophages and dendritic cells was negatively associated with the expression of OVOL2. The abundance of B cells was positively associated with OVOL1 expression (Fig. 6A). These data suggested that immune infiltration was closely associated with the OVOL family members in patients with ccRCC.

Verification of the role of OVOLs in drug sensitivity. Analysis of the GDSC revealed that the expression levels of *OVOL1* and *OVOL2* were positively associated with certain drugs (Fig. 6B). Additionally, analysis of CTRP revealed that the mRNA levels of *OVOL1* and *OVOL2* were negatively associated with drugs or small molecules and positively associated with small molecules (Fig. 6C). Thus, OVOL1 and OVOL2 expression levels were associated with drug resistance, which indicated that they may be used to determine drug sensitivity.

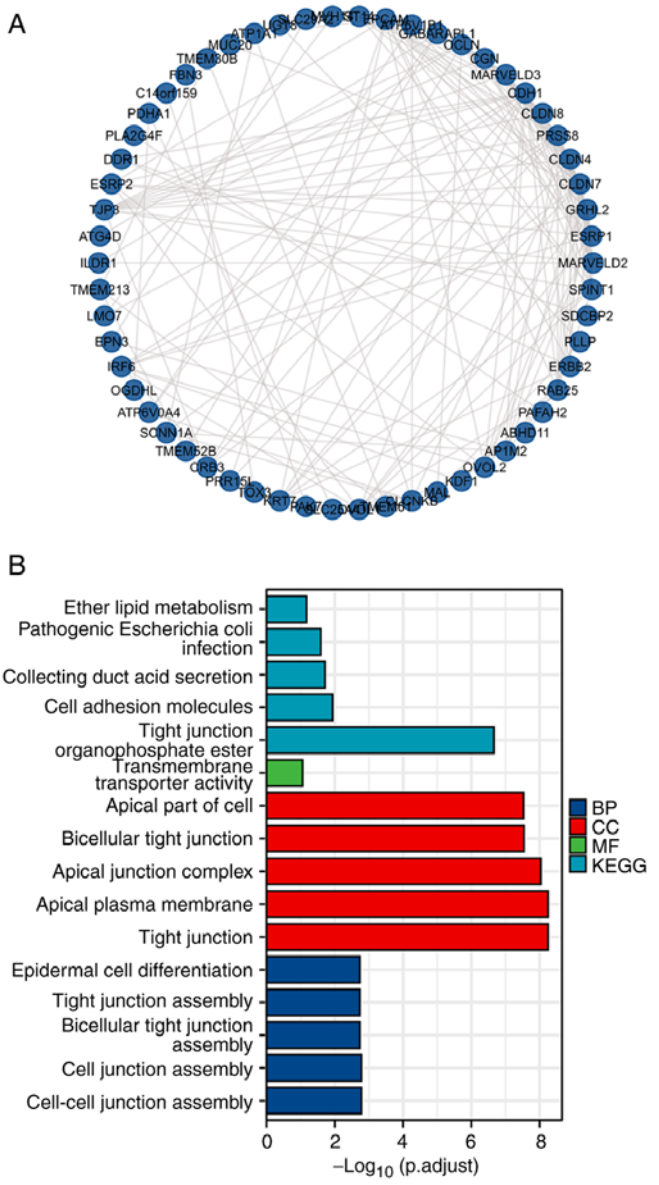

Figure 5. Predicted functions and pathways of OVOL1/2/3 and their neighbor genes in ccRCC by the analysis of GO and KEGG. (A) Analysis of the protein-protein interaction network involving the OVOL family members, and the 100 neighboring genes related to OVOL mutants in ccRCC. (B) Analysis of the functional enrichment of the OVOL family members and 100 neighboring genes related to the OVOL mutants in ccRCC using Gene Ontology KEGG. OVOL, OVO-like protein; ccRCC, clear cell renal cell carcinoma; GO, Gene Ontology; KEGG, Kyoto Encyclopedia of Genes and Genomes; BP, biological process; CC, cellular component; MF, molecular function.

Discussion

Several studies have reported that OVOLs function as transcription factors to regulate gene expression during various differentiation processes (14) and induce MET in several types of cancer (16). The dysregulation of OVOLs has been reported in several types of cancer (22,23,35). In the present study, bioinformatics and experimental studies were used to investigate the correlation between OVOLs and the prognosis of ccRCC. A comprehensive analysis of OVOLs in ccRCC has not been previously performed to the best of our knowledge. The mRNA levels, protein interactions, and functional enrichment of OVOLs and their correlation with immune infiltration and prognosis were investigated in the present study. The results of this study indicated that OVOLs are potential therapeutic targets and prognostic markers for ccRCC.

OVOL1 is a key regulator of epithelial lineage determination and MET. Studies have demonstrated that OVOL1 inhibits breast cancer cell invasion by promoting the degradation of TGF-β type I receptor (36). OVOL1 overexpression promoted

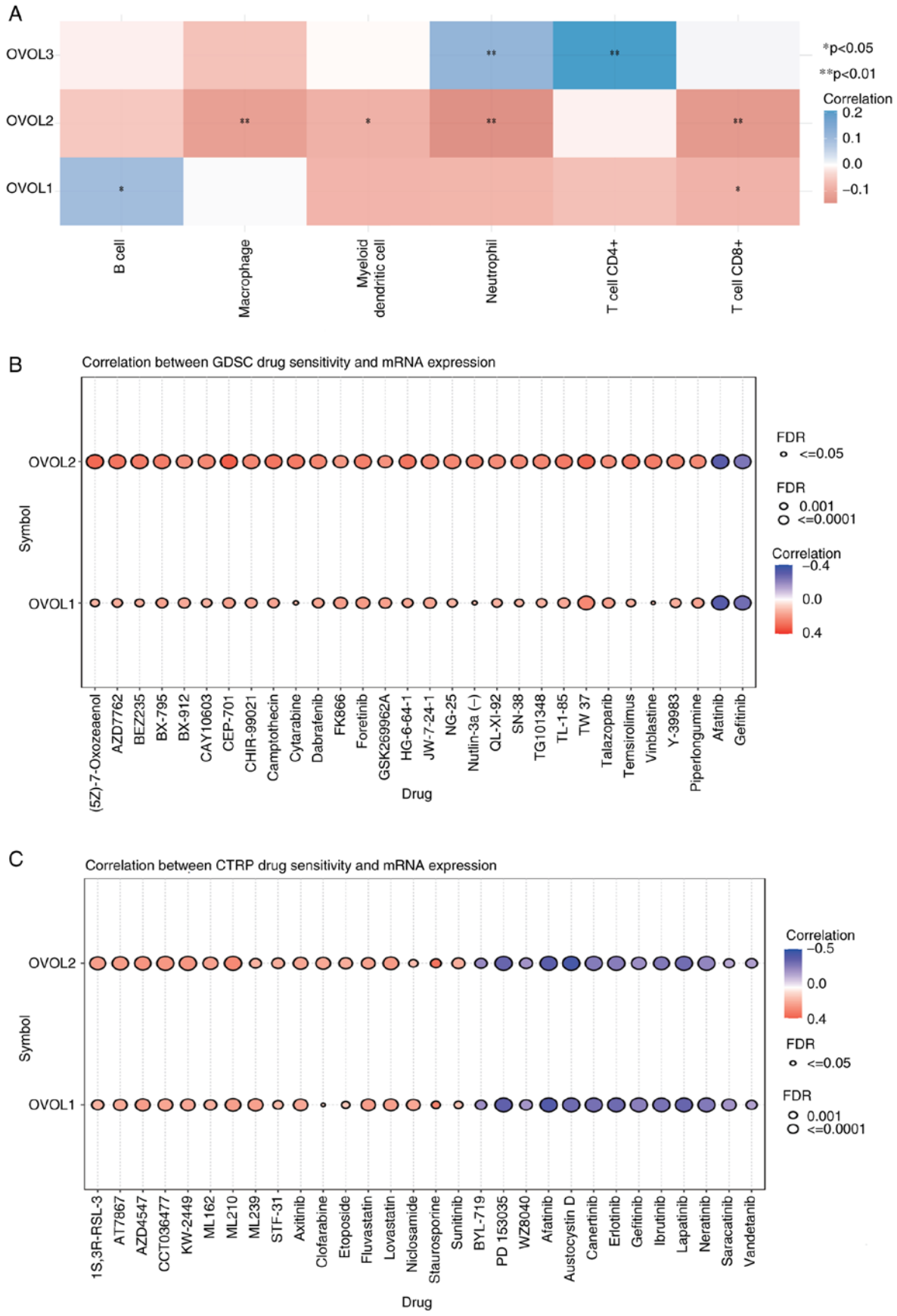

Figure 6. Analysis of the correlation between OVOL1/2/3 and immune cells and drug sensitivity analysis of OVOL1/2/3 (A) Heatmap of the correlation between multiple genes or models and immune score. The abscissa and ordinate represent genes, while different colors represent different correlation coefficients (blue and red represent positive and negative correlations, respectively). The darker the color, the stronger the correlation. (B and C) Drug sensitivity of *OVOL1* and *OVOL2* according to the GSCA Lite database. The bubble plot shows the correlation between gene expression and Food and Drug Administration-approved drugs. A positive Spearman correlation coefficient indicates that upregulated gene expression is associated with drug resistance according to GDSC and CTRP. **P<0.01, *P<0.05. GDSC, Genomics of Drug Sensitivity in Cancer; CTRP, Cancer Therapeutics Response Portal.

oral squamous cell carcinoma (OSCC) progression by inhibiting ZEB1. Thus, OVOL1 is a potential prognostic marker for OSCC (22). Additionally, OVOL1 is significantly downregulated in cutaneous squamous cell carcinoma. Mechanistically, OVOL1 functions as an upstream suppressor of c-Myc and OVOL2. The OVOL1-OVOL2 axis and a modulator of c-Myc regulate the invasiveness of cutaneous squamous cell carcinoma. OVOL1 expression was upregulated in eccrine poroma and hidradenoma and may play an important role in human skin morphogenesis and tumorigenesis (37). In the present study, the expression of OVOL1 in ccRCC tissues was downregulated when compared with that in adjacent non-tumor tissues. The miRNA network revealed the mRNA expression of *OVOL1* was regulated by multiple miRNAs with the coordination between several miRNAs regulating the expression of a single mRNA (38). The miRNAs bind to their target mRNAs to form RNA-induced silencing complexes that degrade or inhibit the translation of mRNA (39). The upregulated mRNA expression of *OVOL1* was significantly correlated with prolonged OS and PFI in ccRCC, suggesting that OVOL1 can function as a tumor suppressor. Additionally, multivariate Cox regression analyses showed that the downregulated *OVOL1* expression independently predicted poor outcomes in ccRCC. OVOL1 was also correlated with the infiltration of immune cells in ccRCC, which suggested that OVOL1 may regulate cancer immunity. These findings suggest that OVOL1 is a promising prognostic and therapeutic target for patients with ccRCC.

OVOL2, which functions as a transcription factor to regulate gene expression by directly binding to the promoter regions, plays an important role in tumor development and metastasis. A previous study reported that OVOL2 is closely associated with EMT during tumor invasion. The expression of OVOL2 is downregulated in non-small cell lung cancer (NSCLC). Consistently, OVOL2 overexpression inhibited the survival of NSCLC cells (40). Additionally, a previous study reported that OVOL2 inhibited EMT in breast cancer by suppressing the direct transcription of *ZEB1*. Patients with nasopharyngeal carcinoma (NPC) exhibiting downregulated OVOL2 levels were associated with poor OS. Thus, OVOL2 is a potential prognostic indicator for NPC (23). OVOL2 also inhibits EMT and metastasis in colorectal cancer by suppressing Wnt signaling (41). Similarly, OVOL2 inhibits EMT in liver cancer by indirectly promoting the expression of miR-200 (35). In the present study, the expression of OVOL2 in non-tumor tissues was higher than that in kidney tumor tissues. However, the upregulated OVOL2 expression was significantly correlated with a poorer OS and PFI, suggesting an oncogenic role of OVOL2. miRNA network analysis revealed that the oncogenic role of OVOL2 can be attributed to the non-coding RNA-mediated regulation of *OVOL2* mRNA. As the gene expression levels varied in each cancer cell, tumor heterogeneity may significantly contribute to differential mRNA expression. It has been demonstrated that OVOL2 overexpression in macrophages significantly inhibited M2 polarization and consequently inhibited breast cancer metastasis by regulating *IL10* transcription and modulating the tumor microenvironment (42). In the present study, OVOL2 expression was negatively correlated with the infiltration of immune cells. Thus, these results indicate that OVOL2 is a potential prognostic biomarker and a therapeutic target for ccRCC and that *OVOL2* is an oncogene.

OVOL3 has not been previously studied as it is expressed only in early embryos. Thus, the correlation between OVOL3 and cancer is unclear. In the present study, the mRNA expression levels of *OVOL3* in ccRCC tissues were significantly higher than those in healthy kidney tissues. OVOL3 expression was significantly correlated with poor OS but not with PFI. The expression of OVOL3 was positively correlated with the infiltration of immune cells, including neutrophils and CD4+ T cells. However, there are no studies examining the role of OVOL3 in different subtypes of ccRCC to the best of our knowledge.

The present study investigated the expression levels and prognostic value of OVOLs in ccRCC. The findings of this study improved our understanding of the molecular heterogeneity and complexity of ccRCC. Additionally, experimental evidence was generated for the expression of OVOLs in ccRCC tissues. However, this study has some limitations. Although the mRNA levels of *OVOL1* were demonstrated to be an independent prognostic factor associated with a short OS in patients with ccRCC, further studies are needed to validate the findings of this study and explore the clinical application of OVOLs in ccRCC. Second, the mechanisms of the different types of OVOLs were not elucidated. Future studies should explore the potential mechanisms of OVOLs in ccRCC. Finally, this study was based on retrospective data.

In conclusion, the present study evaluated the expression levels and prognostic value of OVOLs in ccRCC. The upregulated mRNA expression levels of *OVOL2* and *OVOL3* were significantly correlated with the OS in patients with ccRCC. Additionally, the upregulated mRNA expression of *OVOL1* was associated with a favorable OS. The *OVOL1* mRNA levels were significantly associated with clinical cancer stage and histological grade in patients with ccRCC. Multivariate analysis revealed that *OVOL1* mRNA expression was independently associated with a short OS in patients with ccRCC. The mRNA expression levels of OVOLs were closely associated with immune infiltration in patients with ccRCC and drug sensitivity. Therefore, these results indicate that OVOL1 and OVOL2 are potential therapeutic targets for ccRCC and that OVOL1 is a novel prognostic factor for ccRCC.

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Availability of data and materials

The datasets used and/or analyzed during the current study are available from the corresponding author on reasonable request.

Authors' contributions

PZ designed and supervised the project. RC and MJ performed the bioinformatics analysis and conducted the experiments. JL and GC were responsible for data interpretation, literature search, critical revision of the manuscript for scientific and factual content and confirmed the authenticity of all the raw data. All the authors have seen and confirmed the authenticity of the raw data generated during the study. All authors have read and approved the final manuscript.

Ethics approval and consent to participate

All subjects provided written informed consent. The study was conducted according to the ethical principles of the Declaration of Helsinki and was approved by the Institutional Ethics Committee of First Affiliated Hospital of Nanchang University. (approval no. 202012-110).

Patient consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

References

1. Ferlay J, Soerjomataram I, Dikshit R, Eser S, Mathers C, Rebelo M, Parkin DM, Forman D and Bray F: Cancer incidence and mortality worldwide: Sources, methods and major patterns in GLOBOCAN 2012. Int J Cancer 136: E359-E386, 2015.
2. Siegel RL, Miller KD, Fuchs HE and Jemal A: Cancer statistics, 2022. CA Cancer J Clin 72: 7-33, 2022.
3. Sung H, Ferlay J, Siegel RL, Laversanne M, Soerjomataram I, Jemal A and Bray F: Global cancer statistics 2020: GLOBOCAN estimates of incidence and mortality worldwide for 36 cancers in 185 countries. CA Cancer J Clin 71: 209-249, 2021.
4. Moch H, Cubilla AL, Humphrey PA, Reuter VE and Ulbright TM: The 2016 WHO classification of tumours of the urinary system and male genital organs-part A: Renal, penile, and testicular tumours. Eur Urol 70: 93-105, 2016.
5. Ljungberg B, Albiges L, Abu-Ghanem Y, Bensalah K, Dabestani S, Fernández-Pello S, Giles RH, Hofmann F, Hora M, Kuczyk MA, *et al*: European association of urology guidelines on renal cell carcinoma: The 2019 update. Eur Urol 75: 799-810, 2019.
6. Wong MCS, Goggins WB, Yip BHK, Fung FDH, Leung C, Fang Y, Wong SYS and Ng CF: Incidence and mortality of kidney cancer: Temporal patterns and global trends in 39 countries. Sci Rep 7: 15698, 2017.
7. Padala SA, Barsouk A, Thandra KC, Saginala K, Mohammed A, Vakiti A, Rawla P and Barsouk A: Epidemiology of renal cell carcinoma. World J Oncol 11: 79-87, 2020.
8. Siegel RL, Miller KD and Jemal A: Cancer statistics, 2020. CA Cancer J Clin 70: 7-30, 2020.
9. Williamson TJ, Pearson JR, Ischia J, Bolton DM and Lawrentschuk N: Guideline of guidelines: Follow-up after nephrectomy for renal cell carcinoma. BJU Int 117: 555-562, 2016.
10. Jamil ML, Keeley J, Sood A, Dalela D, Arora S, Peabody JO, Trinh QD, Menon M, Rogers CG and Abdollah F: Long-term risk of recurrence in surgically treated renal cell carcinoma: A post hoc analysis of the eastern cooperative oncology group-American college of radiology imaging network E2805 trial cohort. Eur Urol 77: 277-281, 2020.
11. Unverzagt S, Moldenhauer I, Nothacker M, Roßmeißl D, Hadjinicolaou AV, Peinemann F, Greco F and Seliger B: Immunotherapy for metastatic renal cell carcinoma. Cochrane Database Syst Rev 5: CD011673, 2017.
12. Meyer E, Pasquier D, Bernadou G, Calais G, Maroun P, Bossi A, Theodore C, Albiges L, Stefan D, de Crevoisier R, *et al*: Stereotactic radiation therapy in the strategy of treatment of metastatic renal cell carcinoma: A study of the getug group. Eur J Cancer 98: 38-47, 2018.
13. Tsuji G, Ito T, Chiba T, Mitoma C, Nakahara T, Uchi H and Furue M: The role of the OVOL1-OVOL2 axis in normal and diseased human skin. J Dermatol Sci 90: 227-231, 2018.
14. Kumar A, Bhandari A, Sinha R, Sardar P, Sushma M, Goyal P, Goswami C and Grapputo A: Molecular phylogeny of OVOL genes illustrates a conserved C2H2 zinc finger domain coupled by hypervariable unstructured regions. PLoS One 7: e39399, 2012.
15. Watanabe K, Villarreal-Ponce A, Sun P, Salmans ML, Fallahi M, Andersen B and Dai X: Mammary morphogenesis and regeneration require the inhibition of EMT at terminal end buds by Ovol2 transcriptional repressor. Dev Cell 29: 59-74, 2014.
16. Roca H, Hernandez J, Weidner S, McEachin RC, Fuller D, Sud S, Schumann T, Wilkinson JE, Zaslavsky A, Li H, *et al*: Transcription factors OVOL1 and OVOL2 induce the mesenchymal to epithelial transition in human cancer. PLoS One 8: e76773, 2013.
17. Wang ZH, Li Z, Hu M, Yang QJ, Yan S, Wu RS, Li BA and Guo M: Ovol2 gene inhibits the epithelial-to-mesenchymal transition in lung adenocarcinoma by transcriptionally repressing Twist1. Gene 600: 1-8, 2017.
18. Jia D, Jolly MK, Boareto M, Parsana P, Mooney SM, Pienta KJ, Levine H and Ben-Jacob E: OVOL guides the epithelial-hybrid-mesenchymal transition. Oncotarget 6: 15436-15448, 2015.
19. Jolly MK, Jia D, Boareto M, Mani SA, Pienta KJ, Ben-Jacob E and Levine H: Coupling the modules of EMT and stemness: A tunable 'stemness window' model. Oncotarget 6: 25161-25174, 2015.
20. Nieto MA: The ins and outs of the epithelial to mesenchymal transition in health and disease. Annu Rev Cell Dev Biol 27: 347-376, 2011.
21. Liu J, Wu Q, Wang Y, Wei Y, Wu H, Duan L, Zhang Q and Wu Y: Ovol2 induces mesenchymal-epithelial transition via targeting ZEB1 in osteosarcoma. Onco Targets Ther 11: 2963-2973, 2018.
22. Xu C, Yan T and Yang J: OVOL1 inhibits oral squamous cell carcinoma growth and metastasis by suppressing zinc finger E-box binding homeobox 1. Int J Clin Exp Pathol 12: 2801-2808, 2019.
23. Qi XK, Han HQ, Zhang HJ, Xu M, Li L, Chen L, Xiang T, Feng QS, Kang T, Qian CN, *et al*: OVOL2 links stemness and metastasis via fine-tuning epithelial-mesenchymal transition in nasopharyngeal carcinoma. Theranostics 8: 2202-2216, 2018.
24. Livak KJ and Schmittgen TD: Analysis of relative gene expression data using real-time quantitative PCR and the 2(-Delta Delta C(T)) method. Methods 25: 402-408, 2001.
25. Tang Z, Kang B, Li C, Chen T and Zhang Z: GEPIA2: An enhanced web server for large-scale expression profiling and interactive analysis. Nucleic Acids Res 47: W556-W560, 2019.
26. Cerami E, Gao J, Dogrusoz U, Gross BE, Sumer SO, Aksoy BA, Jacobsen A, Byrne CJ, Heuer ML, Larsson E, *et al*: The cBio cancer genomics portal: An open platform for exploring multidimensional cancer genomics data. Cancer Discov 2: 401-404, 2012.
27. Mermel CH, Schumacher SE, Hill B, Meyerson ML, Beroukhim R and Getz G: GISTIC2.0 facilitates sensitive and confident localization of the targets of focal somatic copy-number alteration in human cancers. Genome Biol 12: R41, 2011.
28. Szklarczyk D, Gable AL, Lyon D, Junge A, Wyder S, Huerta-Cepas J, Simonovic M, Doncheva NT, Morris JH, Bork P, *et al*: STRING v11: Protein-protein association networks with increased coverage, supporting functional discovery in genome-wide experimental datasets. Nucleic Acids Res 47: D607-D613, 2019.
29. Liu CJ, Hu FF, Xia MX, Han L, Zhang Q and Guo AY: GSCALite: A web server for gene set cancer analysis. Bioinformatics 34: 3771-3772, 2018.
30. Ashburner M, Ball CA, Blake JA, Botstein D, Butler H, Cherry JM, Davis AP, Dolinski K, Dwight SS, Eppig JT, *et al*: Gene ontology: Tool for the unification of biology. The gene ontology consortium. Nat Genet 25: 25-29, 2000.
31. The Gene Ontology Consortium: The gene ontology resource: 20 years and still GOing strong. Nucleic Acids Res 47: D330-D338, 2019.
32. Kanehisa M and Goto S: KEGG: Kyoto encyclopedia of genes and genomes. Nucleic Acids Res 28: 27-30, 2000.

33. Yu G, Wang LG, Han Y and He QY: clusterProfiler: An R package for comparing biological themes among gene clusters. OMICS 16: 284-287, 2012.
34. Liu J, Lichtenberg T, Hoadley KA, Poisson LM, Lazar AJ, Cherniack AD, Kovatich AJ, Benz CC, Levine DA, Lee AV, *et al*: An integrated TCGA pan-cancer clinical data resource to drive high-quality survival outcome analytics. Cell 173: 400-416.e11, 2018.
35. Fu H, Qi L, Chen L, He Y, Zhang N and Guo H: Expression of ovol2 is related to epithelial characteristics and shows a favorable clinical outcome in hepatocellular carcinoma. Onco Targets Ther 9: 5963-5973, 2016.
36. Fan C, Wang Q, van der Zon G, Ren J, Agaser C, Slieker RC, Iyengar PV, Mei H and Ten Dijke P: OVOL1 inhibits breast cancer cell invasion by enhancing the degradation of TGF-beta type I receptor. Signal Transduct Target Ther 7: 126, 2022.
37. Mitoma C, Nakahara T, Uchi H, Ito T, Inatomi Y, Ide T, Jinnai S, Jinnai N, Iwasaki N, Sakamoto K, *et al*: Preferential expression of OVOL1 in inner root sheath of hair, sebaceous gland, eccrine duct and their neoplasms in human skin. Fukuoka Igaku Zasshi 105: 166-173, 2014.
38. Li J, Yu T, Cao J, Liu L, Liu Y, Kong HW, Zhu MX, Lin HC, Chu DD, Yao M and Yan MX: MicroRNA-148a suppresses invasion and metastasis of human non-small-cell lung cancer. Cell Physiol Biochem 37: 1847-1856, 2015.
39. Bartel DP: MicroRNAs: Genomics, biogenesis, mechanism, and function. Cell 116: 281-297, 2004.
40. Zhang R, Geng GJ, Guo JG, Mi YJ, Zhu XL, Li N, Liu HM, Lin JF, Wang JW, Zhao G, *et al*: An NF-κB/OVOL2 circuit regulates glucose import and cell survival in non-small cell lung cancer. Cell Commun Signal 20: 40, 2022.
41. Ye GD, Sun GB, Jiao P, Chen C, Liu QF, Huang XL, Zhang R, Cai WY, Li SN, Wu JF, *et al*: OVOL2, an inhibitor of WNT signaling, reduces invasive activities of human and mouse cancer cells and is down-regulated in human colorectal tumors. Gastroenterology 150: 659-671.e16, 2016.
42. Wu RS, Lin J, Xing YM, Gao WL, Jiang YX, Chen LX, Zhang XP and Dai ZL: OVOL2 inhibits macrophage M2 polarization by regulating IL-10 transcription, and thus inhibits the tumor metastasis by modulating the tumor microenvironment. Immunol Lett 242: 17-26, 2022.