Multiple Omics Analysis of the Rac3 Roles in Different Types of Human Cancer

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\textbf{ABSTRACT} Rac Family Small GTPase 3 (Rac3) is a member of the Rho family of small GTP-binding proteins which play critical roles in the occurrence, progression, and metastasis of various tumors. Nevertheless, previous studies have focused on a single type of human cancer, so that the roles of Rac3 in different cancer types have not been sufficiently clarified. With the progress of biological detection and bioinformatics technology, the emerging cancer genomics databases make it possible to perform a pan-cancer analysis. Therefore, for the first time, we performed multiple omics analysis to investigate the roles of Rac3 in differential expression, survival prognosis, mutative status, DNA methylation, functions, immune infiltration, and immunotherapy across 33 human cancer types. We found that Rac3 expression was abnormal in 17 cancer types and significantly different in both molecular and immune subtypes of 10 cancers ($p < 0.05$). These Rac3 expression dysregulations in cancer tissues significantly affected their corresponding survival prognosis. Our results also indicated that genetic alterations of Rac3 occurred in 27 cancer types and were significantly associated with prognosis. Moreover, methylation of Rac3 was associated with dysfunctional T-cell phenotypes and affected the prognosis of the brain, melanoma, and breast tumors, and Rac3 Copy Number Alterations (CNA) might affect the infiltration levels of different immune cells in nine cancers. Rac3 and Rac3-related genes were enriched in the axon guidance, actin cytoskeleton regulation, and neurotrophin signaling pathway, and involved the regulation of cellular localization and actin cytoskeleton organization, and small GTPase mediated signal transduction. Then, we further explored the correlations between Rac3 expression and 25 immune cells and cancer-associated fibroblasts across 33 human cancers. Furthermore, we found that Rac3 achieved higher predictive power ($AUC > 0.5$) than three standardized biomarkers of tumor immune responses and was associated with cytotoxic T-cell levels (CTLs) and the risk of immunotherapeutic responses in three cancer types. Collectively, our study contributes to a comprehensive and integrative understanding of the oncogenic roles of Rac3 across human cancers and offers a new clue for treatment strategies.

\textbf{INDEX TERMS} Rac3, human cancer, multiple omics, cancer prognosis, immune infiltrating.

\section*{I. INTRODUCTION}

An estimated 19.3 million new cancer cases and almost 10.0 million cancer deaths will occur in 2020 from the Global Cancer Statistics 2020, resulting in cancer being the primary cause of death in the world [1]. Although diagnosis and treatment technology has been continuously developed, cancer is still a major health problem leading to the high cancer burden in China [2], [3]. Therefore, it is crucial and meaningful to identify novel cancer-related genes and investigate their potential molecular mechanisms from the pan-cancer perspective. Meanwhile, with the emerging cancer genomics databases, such as The Cancer Genome Atlas (TCGA), Gene Expression Omnibus (GEO), and Human Protein Atlas (HPA), we could perform pan-cancer analysis of cancer-related genes [4], [5], [6].

Rac3 protein, located at chr17:82,031,678-82,034,204 (GRCh38/hg38), is a member of the Rho family of small
GTP-binding proteins involving in the occurrence, progression, and metastasis of various human tumors [7], [8], [9]. Recent studies revealed that Rac3 was overexpressed in bladder cancer tissues and could promote bladder cancer proliferation, migration, and invasion by activating JAK/STAT signaling [10], [11]. Donnelly et al. found that Rac3 could control the balance of proteolytic and adhesive activity to affect the invasion and metastasis of breast cancer [12]. FBXL19 inhibited rac3 to mediate the E-cadherin expression of esophageal cancer cells [13]. Rac3 regulated the invasion and metastasis of lung adenocarcinoma cells and might serve as a potential therapeutic target [14]. Nevertheless, these studies were limited to a single type of human cancer. To our knowledge, there is still no existing research to explore the potential role of Rac3 from a pan-cancer perspective [15]. However, pan-cancer analysis can provide a novel insight into biomarkers, targeted therapy, and the underlying molecular mechanisms of different human cancers based on the cancer genomics databases [16], [17], [18]. With the progress of biological detection and bioinformatics technology, more and more cancer databases can be easily obtained, making it possible to perform a pan-cancer analysis of Rac3 in different cancer types.

In this study, for the first time, we integrated various multi-omics databases to perform a systematic and comprehensive analysis of Rac3 across human cancers. Our study revealed the oncogenic roles of Rac3 in multiple human cancers, and Rac3 could serve as biomarkers for cancer detection, cancer immune therapy, and prognosis.

II. MATERIALS AND METHODS

As shown in Fig. 1, we first investigated the mRNA and protein expression levels of Rac3 in cancer tissues and adjacent normal tissues. Then, we analyzed the correlation between Rac3 expression and immune and molecular subtypes, survival prognosis, mutative status, DNA methylation, and immune infiltrating. Finally, we explored the Rac3-involved biological process, pathways, and immunotherapeutic responses in various cancers.

A. RAC3 EXPRESSION PROFILE ANALYSIS

We used the ‘Tissue’ (the protein expression data from 44 types of normal tissues and the mRNA expression data from deep sequencing of RNA of 256 different normal tissue types), ‘Single Cell Type’ (the single cell RNA sequencing data from 25 human tissues and peripheral blood mononuclear cells, together with immunohistochemically stained tissue sections), and ‘Pathology’ (the mRNA and protein expression data from 17 different cancer types, together with millions of immunohistochemically stained tissue sections images) parts of the HPA database1 to explore the Rac3 mRNA and protein expression levels in normal and cancer tissues across TCGA human cancers [6]. The Cancer Cell Line Encyclopedia (CCLE) database2 was used to investigate the Rac3 mRNA expression in human cancer cell lines [19]. GEPIA23 contains the RNA data of 8,587 normal samples and 9,736 tumors from the Genotype–Tissue Expression (GTEx) and TCGA databases, and it was used to explore the Rac3 differential expression between human cancer tissues and paired normal tissues [20] (The abbreviations and corresponding full names of human cancers are shown in Table 1). We used UALCAN4 to analyze the difference in Rac3 protein expression between tumor tissues and adjacent normal tissues from the Clinical Proteomic Tumor Analysis Consortium (CPTAC) and International Cancer Genome Consortium (ICPC) datasets [21].

B. RAC3 EXPRESSION IN MOLECULAR SUBTYPES AND IMMUNE SUBTYPES OF DIFFERENT HUMAN CANCERS

Tumor and Immune System Interaction Database (TISIDB)5 is a comprehensive and intuitive web for analyzing tumor and immune system interaction by integrating multiple heterogeneous data types (such as, PubMed database and high throughput screening data) [22]. We used the ‘subtype’ part of TISIDB to assess the distribution of Rac3 expression levels in the immune and molecular subtypes across TCGA cancers.

C. SURVIVAL PROGNOSIS ANALYSIS

Gene Set Cancer Analysis (GSCA)6 is a platform for genomic, pharmacogenomic, and immunogenomic analysis of various cancers by integrating over 10,000 multidimensional genomics data of 33 cancer types from the TCGA dataset. We used the ‘Expression’ module of GSCA to demonstrate the correlations between Rac3 expression levels and overall survival (OS), disease-specific survival (DSS), and progression-free survival (PFS) of patients with different cancers [23]. To further verify our conclusions, PrognoScan7 was also used to explore the relationships between Rac3 expression and patient survival prognosis of patients with BLCA, BRCA, and LGG from the GEO database [24].

D. GENOMIC MUTATION ANALYSIS

We used the ‘Quick select’ part (10967 samples from the ‘TCGA Pan Cancer Atlas Studies’ data) of eBioPortal8 to investigate the mutative status of Rac3 across TCGA cancers [25], [26]. The Overview of Rac3 mutation features among different cancers could be shown in the ‘Cancer Types Summary’ module. The ‘Mutations’ module was used to display Rac3 mutation sites and their 3D structures. We used the ‘Comparison/Survival’ module to study the difference in OS, DSS, and PFS curves between Rac3-altered and Rac3-unaltered groups of pan cancers. The Catalogue Of

1http://www.proteinatlas.org/, accessed on 10 May 2022
2https://portals.broadinstitute.org/ccle, accessed on 10 May 2022
3http://gepia2.cancer-pku.cn/, accessed on 10 May 2022
4http://ualcan.path.uab.edu/, accessed on 10 May 2022
5http://cis.hku.hk/TISIDB/index.php, accessed on 10 May 2022
6https://www.cbioportal.org/, accessed on 10 May 2022
7http://bioinfo.life.hust.cn/GSCA/#, accessed on 10 May 2022
8https://www.prognoscan.org/, accessed on 10 May 2022
Somatic Mutations In Cancer (COSMIC) is the world’s largest database of somatic mutations in human cancer, which includes over 37,000 genomes and 27,000 papers. We further utilized COSMIC\footnote{https://cancer.sanger.ac.uk/cosmic, accessed on 10 May 2022} to explore the distribution of different types of Rac3 mutations for pan cancers \cite{27}. The ‘Mutation’ module of GSCA was used to analyze the association between Rac3 expression and Copy Number Variation (CNV) from human cancers. Additionally, we used the Tumor Immune Estimation Resource (TIMER)\footnote{https://cistrome.shinyapps.io/timer/, accessed on 10 May 2022} to explore the effect of Rac3 copy number alterations (CNA) on immune cell infiltration across TCGA cancer types \cite{28}.

\section*{E. METHYLATION ANALYSIS DATA}
We compared DNA promoter methylation levels of Rac3 between tumor and paired normal tissues in various cancer types by using the UALCAN database, in which the Beta value indicates Rac3 methylation levels ranging from 0 (unmethylated) to 1 (fully methylated) and hypermethylation [Beta value: 0.7-0.5] or hypomethylation [Beta value: 0.3-0.25] is based on different beta value cut-off. We used the ‘Query Gene’ of Tumor Immune Dysfunction and Exclusion (TIDE)\footnote{http://tide.dfci.harvard.edu/, accessed on 10 May 2022} to investigate the effects of Rac3 methylation on CTLs, dysfunctional T-cell phenotypes and risk of various cancers \cite{29}.

\section*{F. PROTEIN–PROTEIN INTERACTION NETWORK CONSTRUCTING}
We searched the query by using protein name: ‘Rac3’ and organism: ‘Homo sapiens’ in the STRING website,\footnote{https://string-db.org/, accessed on 10 May 2022} and
TABLE 1. The abbreviations and corresponding full names of human cancers.

| Abbreviation | Full name                          |
|--------------|-----------------------------------|
| ACC          | Adrenocortical carcinoma          |
| BLCA         | Bladder Urothelial Carcinoma      |
| BRCA         | Breast invasive carcinoma         |
| CESC         | Cervical squamous cell carcinoma and endocervical adenocarcinoma |
| CHOL         | Cholangiocarcinoma                |
| COAD         | Colon adenocarcinoma              |
| DLBC         | Lymphoid Neoplasm Diffuse Large B-cell Lymphoma |
| ESCA         | Esophageal carcinoma              |
| GBM          | Glioblastoma multiforme           |
| HNSC         | Head and Neck squamous cell carcinoma |
| KICH         | Kidney Chromophobe                |
| KIRC         | Kidney renal clear cell carcinoma |
| KIRP         | Kidney renal papillary cell carcinoma |
| LAML         | Acute Myeloid Leukemia            |
| LGG          | Brain Lower Grade Glioma          |
| LIHC         | Liver hepatocellular carcinoma    |
| LUAD         | Lung adenocarcinoma               |
| LUSC         | Lung squamous cell carcinoma      |
| MESO         | Mesothelioma                      |
| OV           | Ovarian serous cystadenocarcinoma |
| PAAD         | Pancreatic adenocarcinoma         |
| PCPG         | Pheochromocytoma and Paraganglioma|
| PRAD         | Prostate adenocarcinoma           |
| READ         | Rectum adenocarcinoma             |
| SARC         | Sarcoma                           |
| SKCM         | Skin Cutaneous Melanoma           |
| STAD         | Stomach adenocarcinoma            |
| TGCT         | Testicular Germ Cell Tumors       |
| THCA         | Thyroid carcinoma                 |
| THYM         | Thymoma                           |
| UCEC         | Uterine Corpus Endometrial Carcinoma |
| UCS          | Uterine Carcinosarcoma            |
| UVM          | Uveal Melanoma                    |

the max number of interactors was set as ‘no more than 100 interactors’ in 1st shell [30]. Finally, the available 100 Rac3-binding proteins were obtained. GeneMANIA\(^{13}\) can predict functions of Rac3 and find other genes that are related to Rac3 from 2830 association networks data [31], which was used to obtain 100 interacted proteins for constructing the Rac3 gene-gene interaction networks. We used Venny\(^{14}\) to find the common proteins between the above two groups.

G. FUNCTIONAL ENRICHMENT ANALYSIS

We first used the ‘Protein Class’ part (209 total terms of PANTHER\(^{15}\) Protein Class) of PANTHER\(^{15}\) by selecting the ‘Homo sapiens’ organism to classify the Rac3 overlapped proteins [32]. Additionally, the function annotation of the Rac3 overlapped proteins was carried out with the ‘Biological Process’ part (30439 biological process terms of Gene Ontology (GO)) of PANTHER. We utilized Metascape\(^{16}\) to perform the biological process of GO and Kyoto Encyclopedia of Genes and Genomes (KEGG) enrichment analysis of these Rac3-related proteins [33], with the ‘Min overlap’ set to 3, ‘P-value Cutoff’ set to 0.05, and ‘Min Enrichment’ set to 1.5. The ‘Expression & Pathway activity’ module of GSCA was used to perform the activity analysis of the cell cycle pathway between high and low Rac3 expression groups in various cancers.

H. IMMUNE INFILTRATION ANALYSIS

We used the ‘Immune’ part of GSCA to investigate the correlation between infiltration levels of different immune cells and Rac3 GSVA scores across TCGA cancers. The ‘Mutation’ module of TIMER 2.0\(^{17}\) was used to analyze and visualize the correlation between Rac3 expression and infiltration of cancer-associated fibroblasts [34]. Furthermore, TISIDB was used to explore the correlation of Rac3 expression with immunomodulators and chemokines (or receptors).

I. IMMUNOTHERAPY ANALYSIS

To analyze the effect of Rac3 on therapeutic responses, the ‘Biomarker Evaluation’ module of TIDE was used to compare the Rac3 predictive power in therapeutic response outcomes with several standardized biomarkers of tumor immune responses. We used TISIDB to evaluate differences of Rac3 between responders and non-responders to Immune Checkpoint Blockade (ICB). In addition, the ‘Query Gene’ module of TIDE was utilized to assess the correlation between Rac3 expression with cytotoxic T-cell levels and survival prognosis of immunotherapeutic responses.

III. RESULTS

A. EXPRESSION LEVELS AND DISTRIBUTION OF RAC3 IN NORMAL HUMAN TISSUES

The Rac3 expression at both gene transcription and translation levels in normal human tissues was shown in Fig. 2 (A), in which the mRNA expression of Rac3 was present in all normal human tissues and was primarily located in the brain, endocrine tissues, proximal digestive tract, gastroin- testinal tract, liver and gallbladder, kidney and urinary bladder, male tissues, and female tissues. In contrast, the protein expression of Rac3 was obviously different. For instance, although the

\(^{13}\)http://www.genemania.org/, accessed on 10 May 2022

\(^{14}\)https://bioinfogp.cnb.csic.es/tools/venny/index.html, accessed on 10 May 2022

\(^{15}\)http://www.pantherdb.org/, accessed on 10 May 2022

\(^{16}\)http://metascape.org/, accessed on 10 May 2022

\(^{17}\)http://timer.cistrome.org/, accessed on 10 May 2022
Rac3 mRNA level of endocrine tissues was the third-highest, the Rac3 protein was not expressed. Except for the eye, connective and soft tissues, pancreas, and endocrine tissues, the Rac3 protein was expressed in all human tissues and organs consistent with the mRNA expression. We further used the GTEx and FANTOM5 datasets to verify the above conclusions. As shown in Fig. 2 (B), the top tissues of Rac3 mRNA level were the testis, pituitary gland, liver, salivary gland, and breast. Moreover, the mRNA levels of Rac3 were primarily expressed in the cerebral cortex, testis, cerebellum, pons, and breast (Fig. 2 (C)). The protein dataset verified that the Rac3 protein was highly expressed in most normal tissues, except for the eye, connective and soft tissues, pancreas, and endocrine tissues (Fig. 2 (D)). From the RNA single-cell-type specificity perspective, Rac3 was highly expressed in glandular epithelial cells, specialized epithelial cells, germ cells, blood and immune cells, and trophoblast cells, especially in the extravillous trophoblasts, spermatogenesis, breast myoepithelial cells (Fig. 2 (E)).

B. DIFFERENTIAL EXPRESSION OF RAC3 BETWEEN HUMAN CANCER AND NORMAL TISSUES

We used the TCGA and CCLE datasets to explore the Rac3 mRNA expression in various cancer tissues and cancer cell lines. It was found that the mRNA levels of Rac3 in prostate cancer, testis cancer, breast cancer, and melanoma were generally higher than in other cancers from the TCGA dataset (Fig. 3 (A)). Also, the Rac3 mRNA levels were highly expressed in prostate cancer, breast cancer, neuroblastoma, liver cancer, and lung cancer from the CCLE dataset (Fig. 3 (B)). Furthermore, we used GEPIA2 to compare the Rac3 mRNA expression between human cancer tissues and paired normal tissues (TCGA normal and GTEx samples). Fig. 3 (C) showed that the Rac3 levels in most cancer types were significantly expressed than adjacent normal tissues. The Rac3 expression in the cancer tissues of BLCA, CESC, DLBC, HNSC, LIHC, LUAD, LUSC, OV, PAAD, PRAD, SKCM, THCA, THYM, UCEC, and UCS was significantly higher than the corresponding normal tissues, while Rac3 showed lower mRNA levels in KIRC and LAML (Fig. 3 (D)). In addition, we found that the protein expression of Rac3 in lung cancer, head and neck cancer, liver cancer, uterine cancer, breast cancer, cervical cancer, ovarian cancer, lymphoma, and skin cancer was higher than in other human cancers (Fig. 3 (E)). Remarkably, compared with normal human tissues, the Rac3 protein expression was significantly expressed in KIRC, GBM, LIHC, and OV tumor tissues (Fig. 3 (F)-(I)).
C. CORRELATIONS BETWEEN RAC3 EXPRESSION AND MOLECULAR SUBTYPES OF DIFFERENT TCGA CANCERS

To further understand Rac3’s mechanism, we used TISDB to explore correlations between differential expression of Rac3 and molecular subtypes of different TCGA cancers. As shown in Fig. 4, we found that Rac3 expression was significantly associated with molecular subtypes of sixteen cancer types, including ACC (P<0.05), BRCA (P<0.001), COAD (P<0.001), GBM (P<0.05), HNSC (P<0.001), KIRC (P<0.05), LGG (P<0.001), LIHC (P<0.001), LUSC (P<0.01), OV (P=0.05), PCPG (P<0.001), PRAD (P<0.001), READ (P<0.01), SKCM (P<0.001), STAD (P<0.001), and UCEC (P<0.001). The highest expression of Rac3 was in the HM-indel subtype of COAD and STAD, and the highest expression of Rac3 was in the G-CIMP-low subtype of LGG and GBM. For ACC, Rac3 had the highest expression in the CIMP-high subtype. Rac3 had the highest expression in the Her2 subtype of BRCA and the classical subtype of HNSC. For KIRC, Rac3 had the highest expression in the C1 subtype. Rac3 had the highest expression in the iCluster:3 subtype of LIHC and the primitive subtype of LUSC. The highest expression of Rac3 was in the proliferative subtype of OV, the ERG subtype of PRAD, and the Kinasesignaling subtype of PCPG. Rac3 was highly expressed in...
expressed in the HM-SNV subtype of READ. Rac3 was highly expressed in the Triple _WT subtype of SKCM and the CN_HIGH subtype of UCEC.

**D. SURVIVAL ANALYSIS OF RAC3 EXPRESSION IN VARIOUS HUMAN CANCERS**

Next, by dividing cancer cases into two groups (Rac3 high and low expression), we analyzed the relationships between Rac3 expression and OS, DSS, and PFS of patients with different cancers from the TCGA and GEO databases. As shown in Fig. 5 (A), we found that Rac3 expression was significantly correlated with OS, DSS, and PFS of five human cancers, including ACC, LGG, KIRC, MESO, and SARC. High expression levels of Rac3 indicated a poor prognosis of OS in ACC ($P=0.0021$), BLCA ($P=0.0026$), KIRC ($P=7.7e^{-05}$), MESO ($P=0.00093$), SARC ($P=0.0072$),
FIGURE 5. Survival analysis of Rac3 expression in human cancers from the TCGA and GEO databases. (A) Overview of the relationships between Rac3 expression levels and OS, DSS, and PFS of different human cancers from the TCGA database. (B) Significant correlations between Rac3 expression and OS of ACC, BLCA, KIRC, LGG, MESO, SARC, and THCA. (C) Significant correlations between Rac3 expression and DSS of ACC, HNSC, KIRC, LIHC, MESO, SARC, and SKCM. (D) Significant correlations between Rac3 expression and PFS of ACC, BLCA, KIRC, LGG, MESO, OV, SARC, and UCEC. (E) Significant correlations between Rac3 expression and OS, DSS of BLCA, OS of BRCA and LGG from the GEO database.

SKCM (P=0.0066), and THCA (P=0.019) (Fig. 5 (B)). DSS analysis results showed that upregulation of Rac3 expression was correlated with poor prognosis in ACC (P=0.0024), HNSC (P=0.015), KIRC (P=2e-04), LIHC (P=0.034), MESO (P=0.0032), SARC (P=0.012), and SKCM (P=0.0077) (Fig. 5 (C)). As for PFS, a high level of Rac3 implied a shorter prognosis in ACC (P=0.0043), BLCA (P=0.01), KIRC (P=0.0033), MESO (P=0.017), OV (P=0.05), SARC (P=0.0012), and UCEC (P=0.014) (Fig. 5 (D)). In contrast, LGG patients with high Rac3 mRNA levels had longer OS (P=1.9e-05), DSS (P=3.3e-05), and PFS (P=2.4e-05). Interestingly, the poor OS in BLCA from the GSE13507 dataset (P=0.010387) and the favorable OS in LGG from the GSE4413 dataset (P=0.000373) were correlated with high Rac3 expression, which further supported our above findings (Fig. 5 (E)). Meanwhile, BLCA patients with Rac3 high mRNA levels indicated poor DSS (P=0.002272, GSE13507), and BRCA patients with Rac3 high mRNA levels demonstrated good OS (P=0.000126, GSE7390).

E. GENOMIC ALTERATION ANALYSIS OF RAC3 ACROSS DIFFERENT TCGA CANCERS

Increasing research has reported that the occurrence and development of human cancers might be correlated with genetic alterations [35], [36], [37]. Thus, we firstly explored the mutative status of Rac3 in different cancers by cBioPortal and COSMIC. The results showed that UCS had the highest alteration frequency of Rac3 (>5%), followed by LIHC, MESO, ACC, and BRCA, and the ‘Amplification’ type was the primary type of Rac3 alterations (mutation, structural variant, amplification, deep deletion, and multiple alterations) across different TCGA cancers (Fig. 6 (A)).
We found that A95V, D65N, P34L/T, and P185L, which were ‘missense’ types, were the most frequent mutation sites in pan-cancers (Fig. 6 (D)). 31 mutations (including 27 missense) were detected and their corresponding 3D structures were shown in Fig. 6 (E). The COSMIC database found that missense substitutions occurred in 48.62% of 109 Rac3 mutation samples (Fig. 6 (B)), which further confirmed our above conclusions. And the substitution mutations mainly occurred in C>T (39.73%) and G>A (24.66%) (Fig. 6 (C)).

To investigate the relationships between alterations of Rac3 and the survival prognosis of various human cancers, we used cBioPortal to find that Rac3-altered patients indicated a statistically poor prognosis in PFS ($P=0.0348$) but not OS ($P=0.741$) and DSS ($P=0.0972$) (Fig. 6 (F)-(H)). Additionally, we analyzed the associations between CNV and Rac3 expression among the 33 human cancers (Fig. 6 (I)). As shown in Fig. 6 (J), positive correlations could be found between CNV and Rac3 expression of 16 human cancers.
(FDR $< 0.05$), including BRCA, LUAD, LUSC, OV, LIHC, CESC, KIRP, HNSC, PAAD, ESCA, UCEC, UCS, STAD, LGG, BLCA, and SKCM. Meanwhile, Rac3 CNV was significantly correlated with poor OS of ACC ($P = 0.046$), COAD ($P = 0.0058$), ESCA ($P = 0.019$), KICH ($P = 0.0019$), LAML ($P = 0.04$), LIHC ($P = 0.046$), PRAD ($P = 0.013$), SARC ($P = 0.044$), and UCEC ($P = 6.8e-07$) (Fig. 6 (K)-(L)).

Furthermore, we performed DNA methylation analysis about Rac3 in different human cancers. Hypermethylation of Rac3 occurred in various cancers, including BLCA, BRCA, CESC, CHOL, COAD, ESCA, KIRC, KIRP, LICH, and READ, while hypomethylation in HNSC, LUAD, LUSC, PRAD, and TGCT (Fig. 7 (A)). Interestingly, Rac3 methylation levels were positively correlated with dysfunctional T cell phenotypes (Fig. 7 (B)), and for patients with brain, melanoma, metastatic melanoma, or breast cancers, hypomethylation of Rac3 induced a poor OS prognosis (Fig. 7 (C)-(F)). These results suggested that methylation of Rac3 was related to dysfunctional T-cell phenotypes and affected the prognosis of the brain, melanoma, metastatic melanoma, and breast cancers.

**F. FUNCTIONAL ANALYSIS OF RAC3-RELATED GENES**

To further explore the Rac3 molecular mechanism in the occurrence and development of human cancers, we found Rac3-related genes to perform serial functional enrichment analysis. Firstly, the 100 Rac3-binding and interacted proteins were obtained from the STRING and GeneMANIA databases, respectively (Fig. 8 (A)-(B)). Cross analysis of the above two groups manifested 18 common proteins: PARDE6A, IQGAP3, RAC1, PAK1, ARHGEF4, DOCK2, WASF3, ARHGDA, RALBP1, RAC2, PHOC, ARHGDI, PARD6G, ABLIM3, WAS, IQGAP2, and ARHDGIB (Fig. 8 (C)). We found that the 18 common proteins were categorized into five protein classes by PANTHER, in which protein-binding activity modulator (57.9%, PC00095) and cell junction protein (15.8%, PC00070) were the two largest classes (Fig. 8 (D)).

Then, we performed the biological process of GO analysis of Rac3 and these overlapped proteins by using both Metascape and PANTHER. The results of Metascape showed that most of these proteins were linked to regulation of cellular localization (GO: 0060341), regulation of actin cytoskeleton organization (GO: 0032956), actin filament polymerization (GO: 0030041), lamellipodium assembly (GO: 0030032), small GTPase mediated signal transduction (GO: 0007264), and positive regulation of protein phosphorylation (GO: 0001934) (Fig. 8 (E), (G)). As shown in Fig. 8 (F), the PANTHER analysis data further suggested that these proteins mostly participated in biological regulation (23.3%, GO: 0065007), cellular process (26.7%, GO: 0000070), and response to stimulus (13.3%, GO: 0050896).
FIGURE 8. Functional analysis of Rac3-related genes. Protein–protein interaction network of Rac3 was constructed by the (A) STRING and (B) GeneMANIA databases. (C) Cross analysis of the Rac3 correlated genes. (D) PANTHER protein class of the Rac3 overlapped proteins. (E) Biological process analysis of the Rac3 overlapped proteins and (G) the corresponding network of enriched terms by Metascape. (H) KEGG pathway analysis of the Rac3 overlapped proteins and (I) the corresponding network of enriched terms by Metascape. (F) Biological process analysis of the Rac3 overlapped proteins by PANTHER. (J) Activity analysis of cell cycle pathway between high and low Rac3 expression groups in various human cancers by GSCA.
The KEGG pathway analysis revealed that these proteins were primarily enriched in axon guidance (hsa04360), regulation of actin cytoskeleton (hsa04810), and neurotrophin signaling pathway (hsa04722) (Fig. 8 (H)-(I)). Furthermore, from the GSCA database, the activity of cell cycle pathway between high and low Rac3 expression groups was found in BLCA, BRCA, KIRC, LUAD, STAD, and UCES (Fig. 8 (J)).

G. CORRELATIONS BETWEEN RAC3 EXPRESSION AND IMMUNE SUBTYPES OF DIFFERENT TCGA CANCERS

We investigated the correlations between Rac3 expression levels and immune subtypes of different TCGA cancers by using the TISDB database. Our results showed that Rac3 expression was significantly associated with six immune subtypes of fifteen human cancers, including BRCA, HNSC, KIRC, KIRP, LGG, LIHC, LUAD, LUSC, MESO, OV, PAAD, PRAD, UCEC, SARC, and STAD (Fig. 9). Rac3 was highly expressed in the wound healing subtype of BRCA, HNSC, KIRC, LGG, MESO, OV, PAAD, PRAD, SARC, and STAD. Moreover, the high expression of Rac3 occurred in the lymphocyte depleted subtype of KIRP, LIHC, LUAD, LUSC, and UCES. In contrast, the low expression of Rac3 was shown in the inflammatory subtype of LIHC, LUAD, LUSC, MESO, PAAD, PRAD, SARC, and STAD. Rac3 was lowly expressed in the lymphocyte depleted subtype of HNSC and OV. In BRCA, KIRC, LGG, and UCEC, Rac3 was lowly expressed in the wound healing subtype.

H. IMMUNE INFILTRATION ANALYSIS OF RAC3 ACROSS TCGA CANCERS

There was a close correlation between immune cells and the occurrence, progression, and metastasis of human tumors, and Rac3 was expressed in immune and blood cells from the single cell type perspective (Fig. 2 (E)). Thus, we firstly used GSCA to explore the association between infiltration levels of different immune cells and Rac3 GSV A scores in various cancers. The results showed that Rac3 had a markedly positive correlation with six types of immune infiltrating cells, including monocyte, Bcell, gamma_delta, CD8_naive, effector_memory, and neutrophil but a markedly negative correlation with central_memory, CD4_T, iTreg, Tfh, Tr1, and Cytotoxic in most human cancers (Fig. 10 (A)). Recent studies have reported that cancer-associated fibroblasts of the tumor microenvironment might participate in regulating functions of infiltrating immune cells. Thus, we applied four algorithms, including EPIC, MCPCOUNTER, XCELL, and TIDE, to analyze the correlation between Rac3 expression and infiltration of cancer-associated fibroblasts among various human cancers. Interestingly, Rac3 expression had a significant positive association with tumor infiltration of cancer-associated fibroblasts in BLCA, CESC, and PAAD but...
Immune Infiltration Analysis of Rac3 in different human cancers. (A) Association between infiltration levels of different immune cells and Rac3 GSVA scores in various cancers (*: P value <= 0.05; #: FDR <= 0.05). (B) Correlation between Rac3 expression and infiltration of cancer-associated fibroblasts by using EPIC, MCPCOUNTER, XCELL, and TIDE algorithms. (C) Bar plot comparing Rac3 with existed biomarkers in immune checkpoint blockade. (D) Differential expression of Rac3 between responders and non-responders to checkpoint blockade. (E) Correlation between Rac3 expression with cytotoxic T-cell levels and the risk of immunotherapeutic response. Correlation analysis between Rac3 expression and (F) immunoinhibitors, (G) immunostimulators, (H) chemokines, and (I) receptors by TISIDB.

a significant negative association in BRCA, GBM, LIHC, and PRAD (Fig. 10 (B)). We used TIDE to compare the biomarker of Rac3 with existing biomarkers in ICB. It was found that Rac3 achieved higher predictive values (AUC>0.5) in 10 of the 25 ICB subcohorts. Compared with the standardized biomarkers, we revealed that Rac3 obtained more counts...
of the predictive score (AUC > 0.5) than tumor mutational burden (TMB), T-cell clonality (T.Clonality), and B-cell clonality (B.Clonality), but fewer counts than microsatellite instability (MSI) score, cluster of differentiation 8 (CD8), and so on (Fig. 10 (C)).

Next, we observed that Rac3 expression had a significant difference between immunotherapy responders and non-responders in urothelial cancer (P = 0.0118) (Fig. 10 (D)). Furthermore, glioblastoma and melanoma patients with Rac3 low expression might obtain the clinical benefits from ICB therapy (PD-1), which was negatively correlated with dysfunctional T cell phenotypes and induced a favorable prognosis. However, bladder patients with Rac3 high expression might have the clinical benefits from ICB therapy (PDL-1) but were negatively associated with dysfunctional T cell phenotypes (Fig. 10 (E)).

Finally, we investigated the correlation between Rac3 expression and various immune-related molecules across TCGA cancers. The results revealed that Rac3 expression was closely associated with different immune cells, including immunoinhibitors, immunostimulators, chemokines, and receptors (Fig. 10 (F)-(I)). The immunoinhibitors showed that Rac3 expression had the highest correlation with PVRL2 in VUM (Spearman: rho = -0.561, P = 1.09e-07) and the lowest correlation with TGFBR1 (Spearman: rho = -0.619, P < 2.2e-16) in THCA, respectively. The immunostimulators showed that Rac3 expression had the highest correlation with CD276 (Spearman: rho = 0.542, P = 3.42e-07) in VUM and the lowest correlation with IL6R (Spearman: rho = -0.539, P = 1.17e-07) in MESO, respectively. We found the most correlations with Rac3 expression for receptors, including CCR10 (PAAD, rho = -0.7850, P = 2.23e-07) and CCR9 (PAAD, rho = 0.78, P = 1.25e-11).

I. EFFECT OF RAC3 COPY NUMBER ALTERATIONS ON IMMUNE INFILTRATION OF DIFFERENT HUMAN CANCERS

We used TIMER to further analyze the correlation of Rac3 CNA with immune cell infiltration across TCGA cancer types. As shown in Fig. 11, the CNA status of Rac3, including arm-level gain and high amplification, significantly affected the infiltration levels of CD4+ T cell, Neutrophil, and Dendritic Cell in BLCA. The ‘arm-level gain’ alteration status of Rac3 could significantly change the infiltration levels of CD8+ T cell, CD4+ T cell, Macrophage, and dendritic cell in BRCA, B cell, CD8+ T cell, neutrophil, and dendritic cell in COAD, CD8+ T cell, neutrophil, and dendritic cell in HNSC, CD8+ T cell, CD4+ T cell, macrophage, neutrophil, and dendritic cell in LUSC, and CD8+ T cell, macrophage, neutrophil, and dendritic cell in SKCM. For LUAD, the ‘arm-level deletion’ alteration status of Rac3 was significantly related to the infiltration levels of B cell, CD8+ T cell, and macrophage. Moreover, for PRAD and UCEC, the ‘high amplification’ of Rac3 significantly changed the infiltration levels of CD8+ T cell, CD4+ T cell, macrophage, neutrophil, and dendritic cell, and CD8+ T cell, respectively.

IV. DISCUSSION

In this study, we first revealed that the mRNA and protein expression of Rac3 were widely expressed in normal human tissues and tumor tissues. From the TCGA and CCLE databases, it was found that the Rac3 mRNA expression in the tumor tissues of prostate cancer, breast cancer, liver cancer,
and lung cancer was generally higher than in other cancers. Compared with normal tissues, Rac3 mRNA levels were significantly overexpressed in BLCA, CESC, DLBC, HNSC, LIHC, LUAD, LUSC, OV, PAAD, PRAD, SKCM, THCA, THYM, UCEC, and UCS, while it was underexpressed in KIRC and LAML. The results were further supported by previous studies involving in bladder cancer [10], [11], lung adenocarcinoma [14], [38], breast cancer [39], [40], prostate carcinomas [41], brain tumors [42], colitis-associated cancer [43], and acute myeloid leukemia [44]. Moreover, the protein expression of Rac3 was highly expressed in lung cancer, head and neck cancer, liver cancer, urothelial cancer, breast cancer, cervical cancer, ovarian cancer, lymphoma, and skin cancer from the HPA database. Remarkably, compared with normal human tissues, we found that the Rac3 protein expression was higher in the primary tumor tissues of KIRC and LIHC but lower in the primary tumor tissues of GBM and OV. These findings indicated that Rac3 might be an oncogenic gene in most human cancers and involve tumorigenesis. Thus, Rac3 might be used as a biomarker for the early detection of these cancers. Furthermore, we found that Rac3 expression was meaningfully correlated with molecular subtypes of sixteen cancers, including ACC, BRCA, COAD, GBM, HNSC, KIRP, LGG, LIHC, LUSC, OV, PCPG, PRAD, READ, SKCM, STAD, and UCEC. There were significant associations between Rac3 expression and six immune subtypes in fifteen cancers. In particular, Rac3 expression was significantly correlated with both molecular subtypes and immune subtypes in ten cancer types, including ACC, BRCA, COAD, GBM, HNSC, KIRP, LGG, LIHC, LUSC, OV, PCPG, PRAD, READ, SKCM, STAD, and UCEC. Somatic mutations might produce new antigens for activating T cells and leading to immune responses [50]. Thus, we further found significant correlations between Rac3 and immune subtypes, which implied that Rac3 might play a critical role in cancer immune therapy. Our study first revealed the significant correlations between Rac3 expression and molecular subtypes or immune subtypes in most cancer types. Thus, to investigate the functions of Rac3 in one of these cancers, we could obtain a new entry point by analyzing the remarkable molecular subtype or immune subtypes.

The results of Rac3 survival analysis indicated distinct differences for various human cancers. High Rac3 expression could cause a worse OS, DSS, or PFS in some cancer types, and genomic alteration of Rac3 could cause a worse OS, DSS, or PFS in some cancer types, indicating a poor recurrence free survival [39]. Therein, we need to point out that Rac3 expression was significantly correlated with OS, DSS, and PFS in five cancer types (ACC, LGG, KIRC, MESO, and SARC), in which high Rac3 expression suggested poor OS, DSS, and PFS of ACC, KIRC, MESO, and SARC except for LGG. Additionally, we found that there was a significant correlation between high Rac3 expression and poor OS (P=0.010387), DSS (P=0.002272) of BLCA from 165 BLCA cases of GSE13507 cohorts, and LGG patients with high Rac3 mRNA levels demonstrated good OS from 74 cases of GSE4412 cohorts (P=0.000373). These results further supported our above findings.

Genomic alteration played an essential role in the occurrence and development of human cancers [36], [47]. Costain et al. showed that the missense variants of Rac3 might cause a novel brain disorder [48]. Rac3 was a hypomethylated gene in colorectal cancer and correlated with a worse prognosis [49]. We found that genetic alterations of Rac3 occurred in most types of human cancer, in which the ‘Amplification’ and ‘mutation’ types were the most types of Rac3 alterations, and the ‘missense’ types of A95V, D65N, P34L/T, and P185L were the most frequent mutation sites. Hwang et al. revealed that mutation of Rac3 occurred in human brain tumors [42], which further confirmed our findings. The specific gene mutation can be used to predict the prognosis of human cancers. Our results showed that Rac3 genetic alterations were statistically associated with poor prognosis in PFS (P=0.0348) but not OS and DSS. Furthermore, Rac3 expression was significantly correlated with CNV in 16 human cancers and significantly related to the prognosis of nine human cancers, including in ACC, COAD, ESCA, KICH, LAML, LIHC, PRAD, SARC, and UCEC. Somatic mutations might produce new antigens for activating T cells and leading to immune responses [50]. Thus, we further found significant correlations between Rac3 CNA and immune cell infiltration in BLCA, BRCA, COAD, HNSC, LUAD, LUSC, PRAD, SKCM, and UCEC. For instance, the ‘arm-level gain’ alteration status of Rac3 significantly changed the immune infiltration levels of B cell, CD8+ T cell, neutrophil, and dendritic cell in COAD. DNA methylation was a critical epigenetic factor of human cancers and used to diagnose human cancers [51], [52]. Compared with normal human tissues, we found that methylation of Rac3 had a statistical difference across TCGA cancers and negatively correlated with mRNA Rac3 expression levels. Hypomethylation of Rac3 was correlated with dysfunctional T cell phenotypes and induced a worse prognosis of the brain, melanoma, metastatic melanoma, and breast cancers. These results suggested that genomic alteration of Rac3 could affect Rac3 expression in TCGA cancers and immuno-invasive phenotypes and the prognosis of various types of cancers. These findings implied that Rac3 might serve as a diagnostic predictor of different cancers and a target of cancer therapies.

Previous research showed that overexpression of RAC3 could activate Janus kinases (JAK)/signal transducers and activators of transcription (STAT) pathway by PYCR1 to promote proliferation and invasion of BLCA cells [11] and regulate the ERK signaling pathway to promote proliferation and migration of LUAD cells [38]. It controlled the proliferation of breast cancer cells by the Rac3–Pak pathway [53]. A single gene may not regulate the entire process of cancer development and progression, and usually conjoins with other related genes [13], [54], [55].
In this study, we firstly obtained 18 Rac3-related proteins intersecting from the STRING and GeneMANIA databases, including PARD6A, IQGAP3, RAC1, PAK1, ARHGEF4, DOCK2, WASF3, ARHGDI1, RALBP1, Rac2, PARD6B, RHOC, ARHGDIG, PARD6G, ABLIM3, WAS, IQGAP2, and ARHGDB, which were mainly categorized into the ‘protein-binding activity modulator’ and ‘cell junction protein’ protein classes. Then we used these intersected genes to perform KEGG and GO enrichment analysis. It was found that they were linked to regulation of cellular localization, regulation of actin cytoskeleton organization, actin filament polymerization, and small GTPase mediated signal transduction. Rho family GTPases could regulate the organization of the actin cytoskeleton [56], which was further supported our findings. The pathways were primarily enriched in axon guidance, regulation of actin cytoskeleton, and neurotrophin signaling pathway. Additionally, the activity of the cell cycle pathway between high and low Rac3 expression groups was found in six types of human cancers, including BLCA, BRCA, KIRC, LUAD, STAD, and UCES. Wang et al. found that Rac3 regulated the proliferation of LUAD cells by cell cycle pathway [46], which was consistent with our results.

Infiltrating immune cells played a crucial role in the occurrence, progression, and metastasis of various human cancers [57], [58], [59]. Cancer-associated fibroblasts were essential for cancer progression and regulated functions of various infiltrating immune cells [60], [61]. We found that Rac3 was expressed in immune and blood cells from the single cell types perspective and closely associated with various immune-related molecules. Rac3 was a markedly positive correlation with six types of immune infiltrating cells but a markedly negative correlation with central_memory, CD4_T, iTreg, Tfh, Tr1, and cytotoxic across most human cancers. Moreover, we found that Rac3 expression was positively associated with tumor infiltration of cancer-associated fibroblasts in BLCA, CESC, and PAAD but had a negative association in BRCA, GBM, LIHC, and OV and paired normal tissues. Costain et al. revealed that the missense variants of Rac3 might cause a novel brain disorder [48]. Our results showed that the missense substitution of Rac3 was the primary mutation type in pan cancers and mainly occurred in C>T and G>A. Moreover, Rac3 genetic alterations significantly shortened the survival time of PFS. Wang et al. found that Rac3 used the cell cycle pathway to regulate the proliferation of LUAD cells [46]. We also revealed that the activity of the cell cycle pathway between high and low Rac3 expression groups was found in the other five cancer types (BLCA, BRCA, KIRC, STAD, and UCES).

V. CONCLUSION

Taken together, we first performed a pan-cancer analysis to reveal the Rac3 roles in different human cancers. Our results showed that the mRNA and protein of Rac3 were widely expressed in various normal tissues and cancer tissues, and Rac3 was remarkably overexpressed in different types of cancers. Rac3 expression was statistically correlated with immune and molecular subtypes, survival prognosis, mutative status, DNA methylation, immune cell infiltration, and immunotherapy in various cancers, which could contribute to understanding the Rac3 roles in the occurrence and development of human cancers, and explored new cancer treatment strategies. However, further studies (e.g., clinical experiment [11], [49], mechanism research [66], and algorithm verification [67], [68]) are required to validate our findings before clinical application in various cancers. Our study workflow can be easily used for multiple omics analysis of other genes, even gene families, from different perspectives, revealing the potential functions of these interesting genes in various cancers.

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