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Web Server issue

TIMER3: an enhanced resource for tumor immune analysis

Hao Cui[1,†], Guile Zhao[1,†], Yiwen Lu[1,†], Siying Zuo[1], Dingyu Duan[1], Xiaobo Luo[1], Hang Zhao[1], Jing Li[1], Zexian Zeng[2], Qianming Chen[1,*], Taiwen Li[1,*]

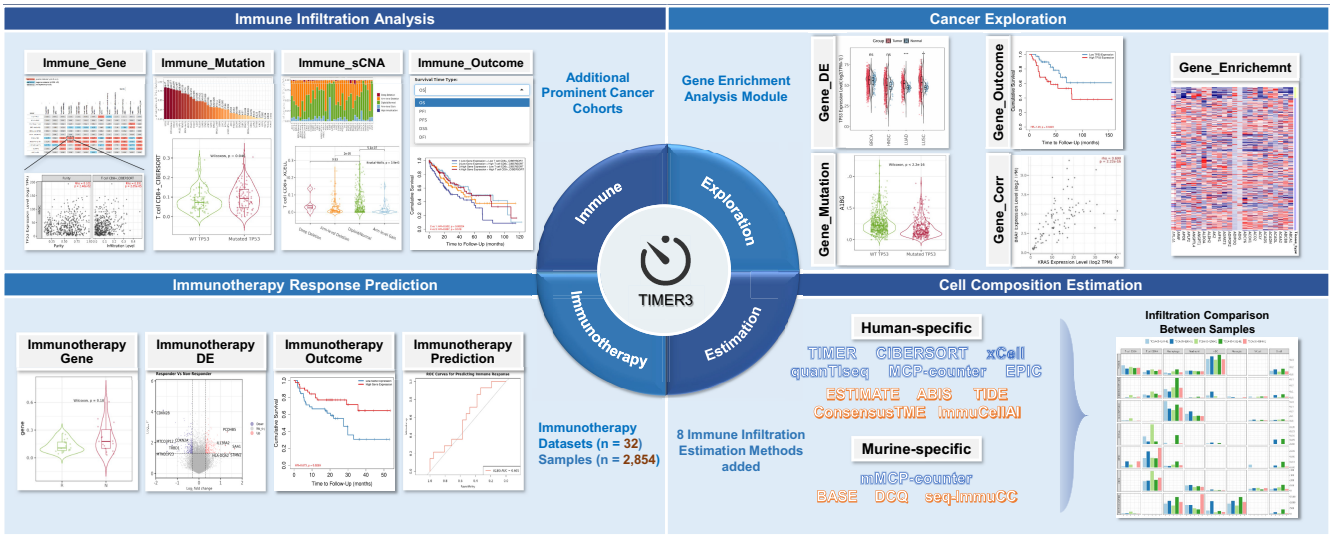
[1]State Key Laboratory of Oral Diseases, National Center for Stomatology, National Clinical Research Center for Oral Diseases, Research Unit of Oral Carcinogenesis and Management, Chinese Academy of Medical Sciences, West China Hospital of Stomatology, Sichuan University, Chengdu 610041, Sichuan, China
[2]Peking–Tsinghua Center for Life Sciences, Center for Quantitative Biology, Academy for Advanced Interdisciplinary Studies, Peking University, Beijing 100084, China

[*]To whom correspondence should be addressed. Email: litaiwen@scu.edu.cn
Correspondence may also be addressed to Qianming Chen. Email: qmchen@scu.edu.cn
[†]The first three authors should be regarded as Joint First Authors.

Abstract

The tumor immune microenvironment plays a critical role in tumor progression and immunotherapy response, with the abundance and composition of infiltrating immune cells serving as key determinants of therapeutic outcomes. Given the limitations of direct experimental methods, computational deconvolution algorithms are widely applied to infer immune cell infiltration from bulk RNA-seq data. While such estimates have proven valuable for studying tumor–immune interactions, large-scale, systematic analyses across multiple cancer types and treatment contexts remain limited. To address this need, we developed TIMER3 (https://compbio.cn/timer3/), an upgraded web server that substantially extends the functionality and scope of its predecessors. TIMER3 integrates 15 state-of-the-art immune deconvolution algorithms, including both human-specific and mouse-specific methods, to improve the robustness and interpretability of immune cell estimation. It incorporates an expanded collection of public RNA-seq datasets of immunotherapy-related cohorts, enabling large-scale analyses of immune dynamics across pre-treatment and post-treatment conditions. TIMER3 introduces new analytical modules for immunotherapy response, signature-based functional profiling, and interactive visualization of cell composition, gene expression, and survival associations. Collectively, TIMER3 provides a comprehensive, user-friendly platform for dissecting the TIME across diverse datasets, facilitating translational research and precision immuno-oncology.

Graphical abstract



Introduction

The tumor immune microenvironment plays a pivotal role in tumor initiation, progression, and treatment response, with the infiltration patterns and functional states of immune cells directly influencing antitumor immunity and therapeutic efficacy [1–5]. Accurately characterizing and quantitatively

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Table 1. Comparative overview of TIMER3 versus TIMER2.0: key advancements in data, methodology, and usability

Feature	TIMER2.0	TIMER3	Advancement
Datasets	TCGA	Including TCGA, 10 large cancer cohorts, and 32 cancer cohorts with immunotherapy	Broader coverage of cancer types and immunotherapy contexts
Survival analysis	OS only	Including OS, PFS, PFI, DFS, and DSS	Expanded survival endpoints support more nuanced prognostic evaluations across multiple clinical outcomes
Deconvolution methods	Six algorithms: TIMER, CIBERSORT, quanTIseq, xCELL, MCP-counter, EPIC	Updated 15 state-of-the-art algorithms (11 human-specific, 4 mouse-specific), including ABIS, ConsensusTME, ESTIMATE, ImmuCellAI, TIDE, seq-ImmuCC, DCQ, and BASE	Improved robustness and comprehensiveness of immune cell estimation; supports cross-species analysis (human/mouse)
Immunotherapy module	Not available	Including gene expression analysis, DEG analysis, survival analysis, and ROC-based prediction	Enabled immunotherapy biomarker discovery and response prediction
Gene enrichment analysis	Not available	Incorporating enrichment analysis with GO, KEGG, and HALLMARK gene sets	Facilitated systematic exploration of enrichment pathways in cancer
User interface	Basic gene search and static visualizations	Enhanced interactivity: selectable cancer types, gene alias support, and clickable heatmaps for detailed exploration	Improved usability and customization; streamlined workflow for diverse research questions

OS: overall survival; PFS: progression-free survival; PFI: progression-free interval; DFS: disease-free survival; and DSS: disease-specific survival.

assessing the immune microenvironment is essential for understanding tumor biology and optimizing treatment strategies [6, 7]. The advent of high-throughput sequencing has led to the accumulation of vast amounts of tumor transcriptomic data. This surge in data has created an urgent need for quantitative analytical tools that can transform gene expression profiles into reliable estimates of immune cell composition and functional states.

To address this need, TIMER was developed as a computational tool for immune microenvironment analysis [8, 9]. Previous versions of TIMER, initially based on the TCGA database, provided precomputed estimates of major immune cell infiltration levels across various cancer types by leveraging bulk RNA-seq data, enabling researchers to infer the relative abundance of immune cell populations within tumors. Later iterations introduced significant improvements, incorporating multiple state-of-the-art immune cell infiltration estimation algorithms to enhance the robustness and accuracy of infiltration quantification. Additionally, these versions expanded functionality by enabling users to analyze their own transcriptomic data, thereby significantly enhancing the ability to dissect the tumor immune landscape.

In recent years, the clinical adoption of cancer immunotherapy, such as immune checkpoint inhibitors, has expanded rapidly [10, 11]. Concurrently, large-scale RNA-seq datasets from patients receiving immunotherapy have been accumulated, offering valuable resources for investigating how the immune microenvironment influences therapeutic responses [12, 13]. However, effectively leveraging these vast datasets requires more advanced computational tools and analytical platforms. Resources such as CAMOIP, CancerImmunityQTL, GSCA, GEPIA, TIGER, and TIMEDB each offer unique strengths in areas including immune infiltration analysis, genetic variation, and immunotherapy [14–19]. Collectively, these platforms have significantly enhanced our understanding of the tumor immune microenvironment from a human perspective. Nevertheless, these databases based on bulk RNA-seq data generally lack systematic quantification of immune cell infiltration and do not incorporate a comprehensive framework of immune estimation algorithms. Single-cell databases such as TISCH and scTIME compile scRNA-seq datasets across diverse cancer types, enabling high-resolution characterization of the tumor immune microenvironment at the cellular level [20–22]. However, the relatively small sample sizes in single-cell studies pose challenges for large-scale, statistically robust analyses. To address these limitations, TIMER3 introduces targeted enhancements by integrating multiple bulk RNA-seq cohorts across different cancer types to enable large-sample analyses. In particular, TIMER3 incorporates a broad collection of publicly available immunotherapy-related bulk RNA-seq datasets. By integrating multiple immune infiltration estimation algorithms and supporting cross-cancer, cross-cohort analyses, TIMER3 provides a more comprehensive and systematic analytical framework for large-scale studies of the tumor immune microenvironment.

New development

Compared to TIMER2.0, TIMER3 has introduced the following key updates (Table 1), reflecting major advancements in data coverage, analytical capabilities, algorithm integration, and user interface design. These improvements collectively aim to provide a more intuitive, flexible, and personalized analytical experience for users.

Cohort data expansion

The TIMER is dedicated to comprehensive tumor immune microenvironment profiling based on large-scale cancer cohort RNA-seq data. By integrating quantitative immune cell infiltration analysis with transcriptomic features, it systematically elucidates tumor–immune interaction mechanisms. TIMER3 significantly advances its previous versions through systematic data updates and resource expansion, incorporating key

Table 2. TIMER3 module overview: inputs, submodule, functions, and outputs

Module	Submodule	Functions	Outputs
Immune	Immune_Gene Immune_Mutation Immune_sCNA Immune_Outcome	Enables correlation analysis between gene expression and immune infiltration across cancer types. Supports purity-adjusted association testing, mutation/sCNA-based comparisons, and survival modeling	Correlation heatmaps and scatter plots; heatmaps and violin plots of infiltration differences (mutation/sCNA); prognostic heatmaps and Kaplan–Meier survival curves
Exploration	Gene_DE Gene_Outcome Gene_Mutation Gene_Corr Gene_Enrichment	Supports differential expression analysis (tumor versus normal), gene–gene correlation, mutation-associated expression comparison, survival modeling, and pathway-level signature exploration	Box plots for tumor versus normal gene expression; violin plots for mutation-related expression differences; correlation heatmaps; Kaplan–Meier curves and Cox model heatmaps; pathway heatmaps with hierarchical clustering
Immunotherapy	Immunotherapy_Gene Immunotherapy_DE Immunotherapy_Outcome Immunotherapy_Prediction	Enables comparative analysis of gene expression under immunotherapy versus non-immunotherapy, as well as between responders and non-responders and pre- versus post-treatment. Supports survival and predictive modeling	Violin plots of gene expression under different treatment conditions; volcano plots for differential gene expression; heatmaps of Cox regression coefficients; Kaplan–Meier survival curves; ROC curves with AUC values
Estimation	Estimation for human Estimation for mouse	Allows users to upload human or mouse RNA-seq data and estimate immune cell composition using 15 deconvolution algorithms	Table of estimated immune cell fractions; bar plots of immune composition across samples; pie charts of cell composition per sample

cohorts from the field, including TCGA datasets, additional prominent cancer cohorts, and immunotherapy-related RNA-seq datasets. These enhancements not only improve data quality and scale but also expand the platform's dimensionality and clinical utility, with plans for continuous real-time updates to ensure that the latest and most relevant data are available for research.

Immunotherapy response analysis

This module focuses on in-depth analysis and visualization of immunotherapy transcriptomic data, employing multidimensional analytical approaches to elucidate molecular characteristics associated with immunotherapy. Key functions include single-gene expression profiling before and after immunotherapy, differential gene expression analysis, multivariate Cox regression analysis survival analysis, and immunotherapy prediction analysis. In this update, we have incorporated 2854 samples from 32 datasets, encompassing dynamic changes in gene expression before and after treatment as well as long-term prognostic outcomes. The module supports comparative analyses between responders and non-responders as well as pre-treatment and post-treatment conditions. To facilitate the exploration of differential responses to immunotherapy, the interactive visualization system integrates heatmaps, violin plots, volcano plots for differentially expressed genes, and Kaplan–Meier survival curves. Notably, the heatmap feature supports interactive click-based exploration, enabling users to delve into specific gene expression patterns with ease.

Gene enrichment analysis

TIMER3 has introduced an added gene enrichment analysis module, enabling standardized gene enrichment analysis across cohorts [23]. This module allows users to systematically evaluate gene enrichment scores for specific cancer types and visualize gene expression heatmap of the gene sets, providing an intuitive representation of expression patterns across different samples. The module incorporates 6852 fundamental functional annotation sets including GO, KEGG, and HALLMARK. Through enrichment analysis, it facilitates the prediction of tumor microenvironment immune responses in cancer patients, providing a comprehensive resource for researchers to explore and characterize immune functional responses in tumors.

Cell composition estimation

TIMER3 has significantly enhanced the accuracy and biological interpretability of immune infiltration quantification by integrating more multimodal deconvolution algorithms. This update introduces eight newly integrated immune infiltration algorithms, including five human-specific methods (ABIS, ConsensusTME, ESTIMATE, ImmuCellAI, and TIDE) and three mouse-specific methods (seq-ImmuCC, DCQ, and BASE) [24–31]. ABIS performs deconvolution of RNA-seq data to estimate the absolute proportions of 29 immune cell types. ConsensusTME decomposes gene expression data into weighted contributions of various immune and stromal cell types, quantifying the abundance of 18 cell types. ESTIMATE computes stromal and immune scores based on gene expression profiles and ultimately estimates tumor purity. ImmuCellAI utilizing the single-sample gene set enrichment analysis method quantifies immune infiltration levels and assesses 24 immune cell types, including 18 T-cell subpopulations and 6 other immune cell types (e.g. B cells and NK cells). TIDE evaluates immune exclusion signatures, particularly for cancer-associated fibroblasts and myeloid-derived suppressor cells. TIMER3 integrates multiple immune infiltration algorithms, significantly enhancing analytical accuracy.

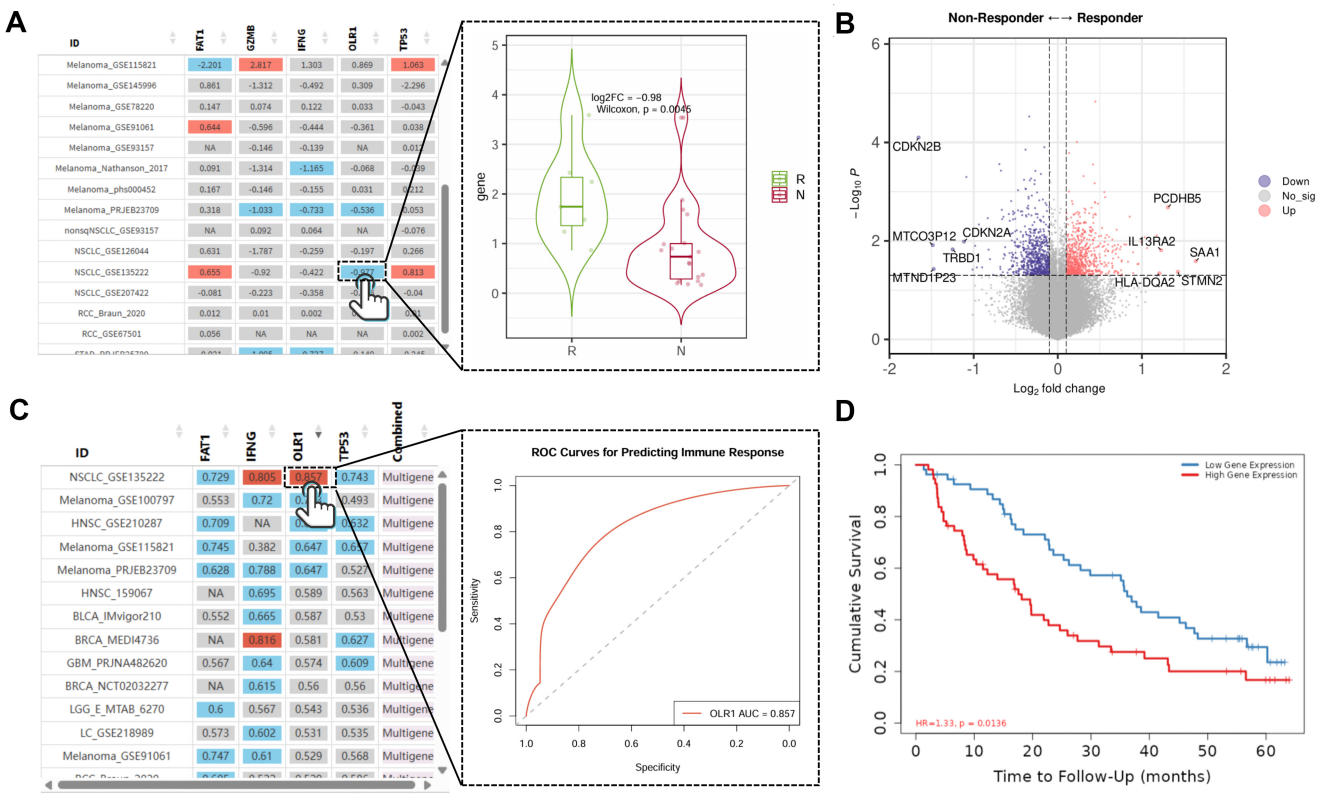

Figure 1. An illustration of how TIMER3 enables interactive, quantitative, and biologically interpretable analysis of immunotherapy-associated gene features. TIMER3 provides a suite of visualization tools to explore the relationship between gene expression and immunotherapy response across multiple datasets. (**A**) An interactive heatmap displays the differential expression (log_2FC) of selected genes (e.g. *OLR1*, *FAT1*, *TP53*) across immunotherapy cohorts. The accompanying violin plot shows that *OLR1* expression is significantly downregulated in non-responders compared to responders (log_2FC = –0.98, Wilcoxon $P = .0045$), suggesting its potential as a positive predictive marker for immunotherapy efficacy. (**B**) The volcano plot highlights genes that are differentially expressed between responders and non-responders. Notably, *IL13RA2*, *PCDHB5*, and *SAA1* are significantly upregulated in responders, suggesting a potential role in enhancing antitumor immunity or serving as response biomarkers. In contrast, genes such as *CDKN2A/B*, *MTCO3P12*, and *TRBD1* are downregulated in responders, possibly reflecting the suppression of cell cycle or mitochondrial pathways in non-responders. (**C**) ROC analysis indicates that *OLR1* expression predicts immunotherapy response in the lung squamous cell carcinoma cohort, with an AUC of 0.857, reflecting strong predictive performance. (**D**) Kaplan–Meier analysis shows that high *OLR1* expression is associated with worse overall survival (HR = 1.33, P = .0136), suggesting its potential prognostic value in patients receiving immunotherapy. Collectively, these results highlight *OLR1* as a candidate biomarker associated with treatment resistance and poor prognosis in certain cancers, demonstrating how TIMER3 facilitates data-driven hypothesis generation.

Interactive features update

In response to user feedback and to enhance usability and personalized interactive experiences, TIMER3 has introduced several new updates, including selectable cancer types, gene alias search functionality, and additional prognostic analysis indicators. We have added an input box for optional cancer types to enhance the flexibility of user-customized analysis for multiple cancer types. To enhance the user experience during gene searches, we have integrated a comprehensive gene alias list, ensuring that inputs such as *TP53* or *P53* will accurately return the analysis results for *TP53*. Furthermore, within the immune and exploration modules, we have introduced clinical indicators for survival analysis, such as progression-free survival (PFS), allowing users to directly obtain PFS survival prognosis results.

Results

Overview

TIMER3 is a freely accessible web server for the research community, implemented using the Shiny web framework in R. It primarily consists of four key components: immune, exploration, estimation, and immunotherapy. The immune component consists of four modules, enabling users to analyze the relationship between estimated immune infiltrates and gene expression, somatic mutations, somatic copy number alterations (sCNAs), and clinical outcomes across tumor cohorts. The exploration enables users to identify key associations within tumors. It supports comparisons of gene expression differences between tumor and normal tissues. It also allows for the analysis of correlations between gene expression and patient survival rates. Furthermore, it facilitates the investigation of relationships between gene mutation status and expression levels. Additionally, users can systematically evaluate gene enrichment scores for specific cancer types. The immunotherapy component comprises four modules, enabling users to evaluate immunotherapy biomarkers, analyze differential gene expression, predict treatment efficacy, and conduct survival analysis, thereby providing comprehensive insights into immunotherapy response mechanisms and patient outcomes. The estimation component can infer immune cell infiltration of user-provided expression profiles using the 15 estimation algorithms (Table 2).

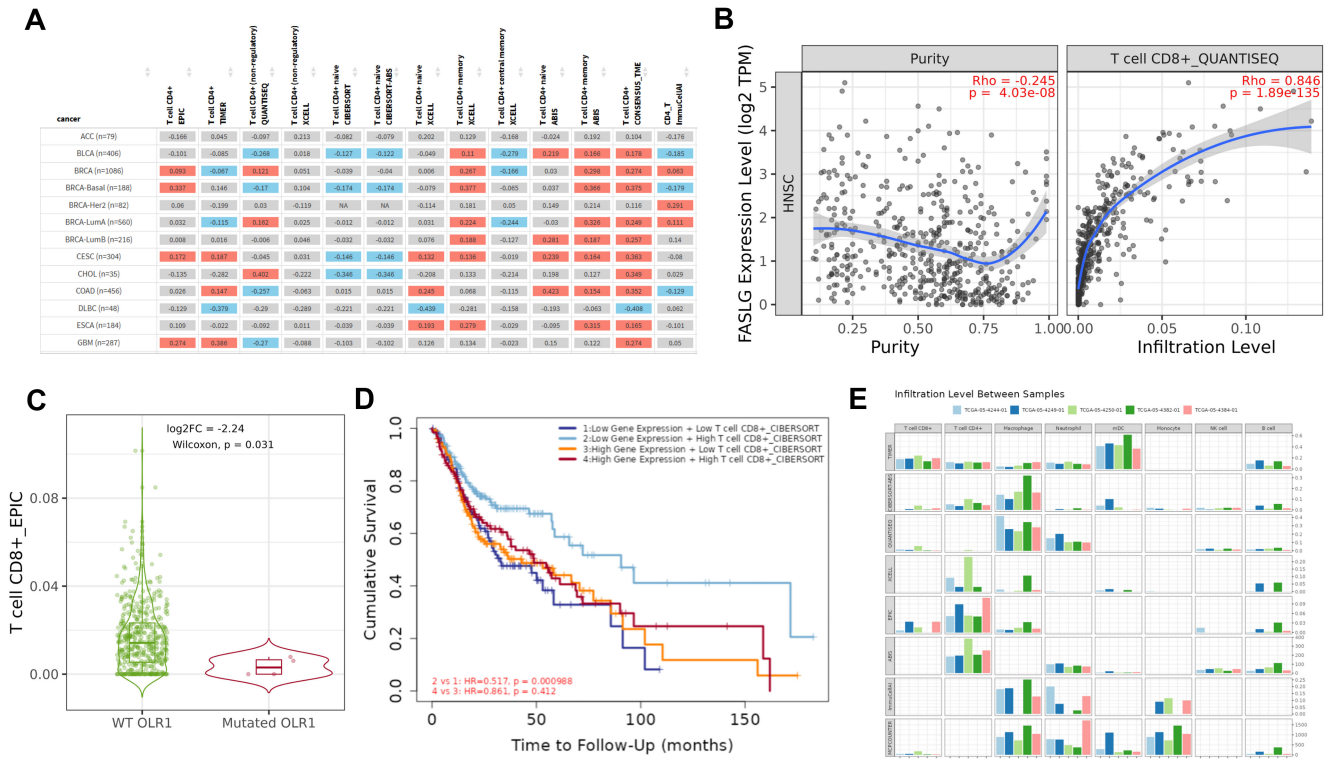

Figure 2. An illustration of how TIMER3 enables comprehensive analysis of immune infiltration patterns and their clinical relevance across cancer types. TIMER3 provides multidimensional tools to examine the abundance of immune cell types, their association with gene expression, mutation status, and clinical outcomes. (**A**) Heatmap of immune cell infiltration scores across cancer types. Each row represents a cancer type and each column represents an immune cell subset estimated by one of the integrated deconvolution algorithms. Notably, $CD8^+$ T cells show relatively high infiltration in BRCA, ESCA, and GBM, whereas certain memory subsets show cancer type-specific suppression, revealing inter-tumoral immune heterogeneity. (**B**) Scatter plots show that the expression of *FASLG* is negatively correlated with tumor purity ($\rho = –0.245$, $P = 4.03e–08$) and positively correlated with $CD8^+$ T-cell infiltration ($\rho = 0.846$, $P = 1.89e–135$) in HNSC, reinforcing its role as an effector T-cell-associated immune marker. (**C**) Violin plot of $CD8^+$ T-cell infiltration levels (EPIC score) by *OLR1* mutation status. Tumors with *OLR1* mutations exhibit significantly reduced $CD8^+$ T cell infiltration compared to wild-type tumors ($\log_2FC = –2.24$, Wilcoxon $P = .031$), suggesting a potential role of *OLR1* mutations in impairing cytotoxic T-cell recruitment. (**D**) Kaplan–Meier analysis stratified by both *OLR1* expression and $CD8^+$ T cell infiltration levels reveals that patients with high T cell infiltration and low *OLR1* expression have the best prognosis, while those with low T-cell infiltration and high *OLR1* expression have the worst survival outcomes (HR = 0.517, $P = .000988$), highlighting the combined prognostic significance of gene expression and immune contexture. (**E**) A bar plot displays the estimated infiltration levels of major immune cell types across multiple TCGA samples, demonstrating inter-tumoral heterogeneity and enabling cross-sample immune profiling. Collectively, these analyses underscore how TIMER3 can integrate immune deconvolution with genomic and clinical variables to uncover immune escape mechanisms and inform immunotherapy strategies.

Immunotherapy response component

Immunotherapy_Gene

To evaluate whether a gene plays a key role in the immunotherapy process, its expression is typically analyzed for specificity or high levels in tissues from immunotherapy recipients or responders. This module provides an interactive table and heatmap to visualize differential gene expression between pre-treatment and post-treatment conditions or between responders and non-responders, enabling users to explore immunotherapy-related biomarkers and resistance mechanisms. By clicking on any cell within the heatmap, users can access detailed expression information of the selected gene within the dataset. A violin plot is then used to visually depict its differential expression patterns, facilitating a thorough analysis of the gene's expression characteristics across different conditions (Fig. 1A).

Immunotherapy_DE

To evaluate the therapeutic targeting potential and response to immunotherapy, differential gene expression analysis is typically performed on samples before and after treatment to identify key molecules with significant changes during the therapeutic process. This module allows users to select specific immunotherapy datasets to analyze differential gene expression between pre-immunotherapy and post-immunotherapy conditions, as well as between responders and non-responders (Fig. 1B). After selecting the immunotherapy condition and dataset, TIMER3 will generate the corresponding volcano plot for differential analysis and provide a detailed results table.

Immnotherapy_Outcome

By analyzing gene expression profiles, computational methods can now predict patient survival and treatment efficacy, offering valuable insights for precision oncology. The survival analysis tab also provides an interactive heatmap to display survival analysis results, enabling users to evaluate biomarkers for immune therapy response. When users click on a cell in the interactive heatmap, detailed information is displayed, including Cox analysis models and Kaplan–Meier curve plots

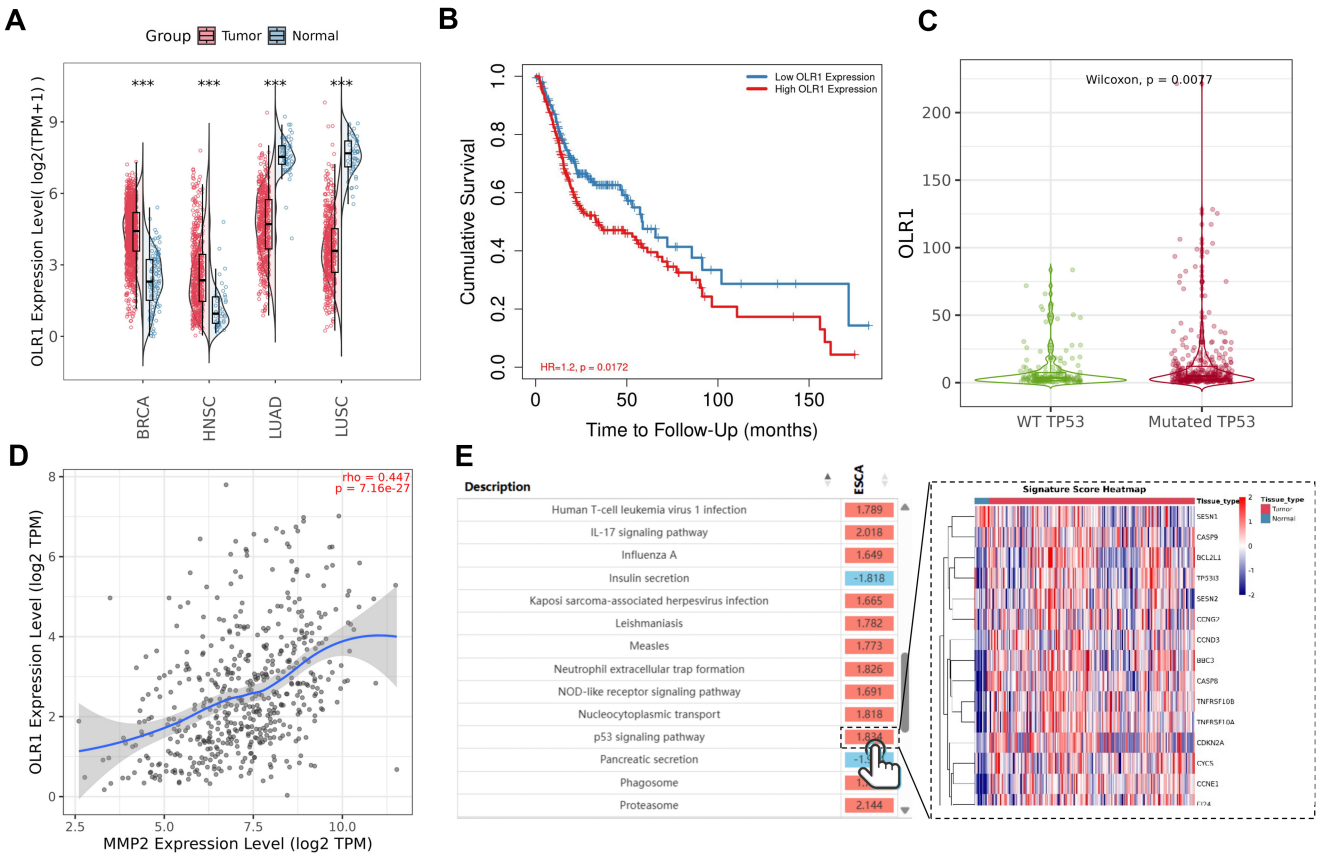

Figure 3. An illustration of TIMER3's capacity to integrate expression analysis, mutation association, pathway activity, and survival outcomes to interpret the biological role of *OLR1*. TIMER3 enables multifaceted interrogation of gene expression patterns, mutation associations, pathway activities, and clinical relevance across cancer types. (**A**) Violin plots comparing *OLR1* expression between tumor and adjacent normal tissues across multiple cancer types (e.g. BRCA, HNSC, LUAD, LUSC). OLR1 is significantly upregulated in tumors in all four cancer types (Wilcoxon test, $^{**}P < .001$), suggesting its potential role as a tumor-associated gene. (**B**) Kaplan–Meier survival analysis indicates that high *OLR1* expression is associated with significantly worse overall survival compared to low expression (HR = 1.2, $P = .0172$), suggesting that *OLR1* may serve as a prognostic marker in cancer. (**C**) Violin plot showing that *OLR1* expression is significantly higher in tumors harboring *TP53* mutations compared to *TP53* wild-type tumors (Wilcoxon P = .0077), implying a potential regulatory link between *TP53* mutation status and *OLR1* upregulation. (**D**) Scatter plot demonstrating a strong positive correlation between *OLR1* and *MMP2* expression levels ($\rho = 0.447$, P = 7.16e–27), indicating that *OLR1* may participate in extracellular matrix remodeling or invasion-related pathways. (**E**) Heatmap of gene signature set expression levels reveals differential activity across multiple hallmark pathways between tumor and adjacent nontumor tissues. Normalized enrichment score highlight elevated activity of the p53 signaling pathway in tumor tissues relative to adjacent controls, supporting the association between dysregulation of the p53 signaling pathway and tumorigenesis. Collectively, these results suggest that *OLR1* is not only overexpressed in tumors and associated with poor prognosis, but also potentially regulated by *TP53* mutation and involved in tumor-promoting pathways such as ECM remodeling signaling, demonstrating the utility of TIMER3 for integrative cancer transcriptomics.

(Fig. 1C). Additionally, users can adjust survival analysis parameters to optimize visualization results.

Immunotherapy_Prediction

The application of ROC curves in immunotherapy prediction is of significant importance. It can evaluate the diagnostic performance of predictive models, helping to identify patients who are likely to respond to treatment or not. Additionally, by comparing AUC values, it assesses the effectiveness of immunotherapy. This module predicts the efficacy of immunotherapy before and after treatment at the gene level, presenting the AUC values in an interactive heatmap (Fig. 1D). The heatmap includes four categories of prediction results: AUC < 0.6 represents weak predictive performance; $0.6 \leq$ AUC < 0.8 represents moderate predictive performance; and AUC ≥ 0.8 represents strong predictive performance. Clicking on a cell in the heatmap displays the corresponding detailed ROC curve, further illustrating the diagnostic performance of the model.

Cell composition component

TIMER3 integrates 15 immune infiltration algorithms, comprising 11 human-specific methods and 4 mouse-specific methods, to improve computational accuracy and biological relevance in tumor immune microenvironment analysis [8, 24–28, 32–36]. TIMER3 has comprehensively updated the immune cell type estimation results, allowing users to explore associations between specific immune cell infiltration types and genomic alterations or clinical outcomes through interactive heatmap visualization (Fig. 2A–D). Concurrently, the Estimation module enables users to submit RNA-seq transcriptomic data derived from human or mouse samples to conduct a comprehensive evaluation of immune cell infiltration characteristics (Fig. 2E).

Exploration component

We have implemented a series of optimizations to the visualizations in the Gene_DE module, upgrading the original gene differential bar plots to half-violin plots to more intuitively

display differences in gene expression levels (Fig. 3A). Additionally, users can now customize the selection of specific cancer types for generating differential expression plots, significantly enhancing the module's flexibility and user-friendliness. We have incorporated multiple clinical prognostic indicators into the survival analysis, including PFS, progression-free interval (PFI), disease-free survival (DFS), and disease-specific survival (DSS), significantly expanding the analytical scope (Fig. 3B). Simultaneously, we have integrated a comprehensive gene alias list to enhance user interaction during gene searches (Fig. 3C and D).

The Gene_Enrichment module computes normalized enrichment scores using GSEA methodology, providing an interactive heatmap data table, allowing users to select specific cancer types and gene signature names, and by clicking on heatmap cells, intuitively visualize the expression level heatmap of all genes in the gene signature for the selected cancer type through feature analysis (Fig. 3E).

Discussion

TIMER3 represents a significant advancement in tumor immune microenvironment analysis, integrating comprehensive updates across data resources, analytical algorithms, and interactive features. By expanding cohort data to include immunotherapy-related RNA-seq datasets and enhancing cell composition estimation through multimodal deconvolution algorithms, TIMER3 provides researchers with a more robust and versatile platform for exploring immune infiltration dynamics. The introduction of gene enrichment analysis and improved interactive features, such as selectable cancer types and gene alias search, further enhances usability and facilitates in-depth investigations into tumor–immune interactions. These updates not only improve analytical accuracy but also empower users to derive clinically relevant insights, ultimately advancing cancer immunotherapy research.

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Conflict of interest

None declared.

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Data availability

TIMER3 is freely available at https://compbio.cn/timer3/. This website is free and open to all users and there is no login requirement.

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