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ORIGINAL RESEARCH

TRIM Expression in HNSCC: Exploring the Link Between Ubiquitination, Immune Infiltration, and Signaling Pathways Through Bioinformatics

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Objective: Ubiquitination is an important post-translational modification. However, the significance of the TRIM family of E3 ubiquitin ligases in head and neck squamous cell carcinoma (HNSCC) has not been determined. In this study, the roles of TRIM E3 ubiquitin ligases in lymphovascular invasion in head and neck squamous cell carcinoma (HNSCC) were evaluated.

Materials and Methods: TRIM expression and related parameters were obtained from UbiBrowser$^{2.0}$, UALCAN, TIMER, TISIDB, LinkedOmics, STRING, and GeneMANIA databases. Immunohistochemistry was used to confirm their expression.

Results: *TRIM2, TRIM11, TRIM28*, and *TRIM56* were upregulated in HNSCC with lymphovascular invasion. TRIM expression was strongly associated with immune infiltration, including key treatment targets, like *PD-1* and *CTL4*. Co-expressed genes and possible ubiquitination substrates included tumor-related factors. The TRIMs had predicted roles in ubiquitination-related pathways and vital signaling pathways, eg, *MAPK, PI3K-Akt*, and *JAK-STAT* signaling pathways.

Conclusion: Ubiquitination mediated by four TRIMs might be involved in the regulation of tumor immunity, laying the foundation for future studies of the roles of the TRIM family on the prediction and personalized medicine in HNSCC. The four TRIMs might exert oncogenic effects by promoting lymphovascular invasion in HNSCC.

Keywords: ubiquitination, TRIM, head and neck squamous cell carcinoma, bioinformatic analysis, lymphovascular invasion

Introduction

Head and neck tumors are among the most prevalent cancers in the USA and around the world.[1] Head and neck squamous cell carcinoma (HNSCC) is the main pathological type, accounting for 90% of malignant head and neck tumors.[2] Cervical lymph node metastasis is the most common form of tumor metastasis. About 50% of patients with HNSCC already have hidden or obvious cervical lymph node involvement at the time of diagnosis. The invasion of tumor cells into the space between lymphatic endothelial cells is an indispensable step in metastasis.[3] Accordingly, a clear understanding of lymphovascular invasion is critical for the treatment and prognostic assessment of HNSCC. Moreover, head and neck tumors have entered the era of immunotherapy.[4,5] Therefore, analyses of the relationships between lymphovascular invasion-related indicators and immunity will provide a reference for precise diagnosis and treatments.

The occurrence and development of HNSCC involve multiple factors and multi-step processes, such as oncogenes, tumor suppressor genes, epigenetic modifications, and post-translational regulation as well as the tumor immune microenvironment.[6–10] Among these factors, the ubiquitination-mediated degradation of target proteins with high selectivity is a key pathway for post-translational modification, with a significant role in oncogenesis and pathological processes, for example, *Parkin* targeted *HIF-1α* for ubiquitination and degradation, which served to inhibit the progression of breast tumors.[11] Ubiquitination, an important post-translational modification, refers to the specific modification of target proteins by ubiquitin molecules under the action of a series of special enzymes, such as ubiquitin activating

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enzyme E1, binding enzyme E2, ligase E3, and degrading enzyme. After ubiquitination, the substrate can enter the proteasome for degradation or can exhibit structural alterations, affecting its properties and functions. Ubiquitination plays an important role in protein localization, metabolism, function, regulation, degradation and many biological processes.[12,13] Studies have shown that ubiquitination is closely related to the pathogenesis of tumors and other diseases.[14,15] It has become an important area of research on tumor-related mechanisms.

E3 ubiquitin ligase is a vital component of the ubiquitination cascade; it binds directly to substrates by controlling mutual specificity and is regarded as a promising anticancer target, with roles in important cancer signaling pathways including ERK signaling pathway.[16,17] The tripartite motif (TRIM) protein family is a new kind of RING finger ubiquitin ligase family with a conserved protein structure and rapid evolution. The Most members of this family function as E3 ubiquitin ligases.[18] The TRIM family includes more than 70 members, with roles in various biological processes and tumorigenesis.[19–21] This family has gradually become an important area of cancer research. An increasing number of studies have implicated TRIM family members in various processes, including apoptosis, cell cycle regulation, viral response, autophagy, and the epithelial–mesenchymal transition,[19,22–27] as well as multiple important signaling pathways, like NF-κB, PI3K/AKT, and JAK signaling pathways, in a variety of tumors such as osteosarcoma, breast cancer and lung cancer.[26,28–31] Clinically, some TRIM family members are closely linked to prognosis of gastric cancer and lung adenocarcinoma.[32,33] However, the significance of E3 ubiquitin ligase TRIM family members in HNSCC is still unclear.

In total, 62 ubiquitin ligases (E3) in the TRIM family have been identified based on a query of UbiBrowser$^{2.0}$. In this study, we focused on four TRIM family members with newly identified associations with lymphovascular invasion of HNSCC research, including *TRIM11, TRIM28, TRIM56*, and *TRIM2* (designated the four TRIMs). We used a data mining approach with various public databases to evaluate the relationship between the expression of TRIMs and HNSCC, including analyses of lymphovascular invasion, functional networks, immune cell infiltration, protein interactions, and ubiquitination substrates as well as functional and pathway enrichment analyses of co-expressed genes. Our results support the important role of TRIM-related ubiquitination in HNSCC.

Materials and Methods

UbiBrowser2.0

UbiBrowser2.0 (http://ubibrowser.bio-it.cn/ubibrowser_v3/) is a comprehensive resource for known and predicted ubiquitin ligase (E3)/deubiquitinase (DUB)-substrate interactions in eukaryotic species. These E3/DUB-substrate interactions are derived from five data sources: manual curation, GO annotation, protein domains, protein motifs, and network topology. To evaluate each biological evidence, the golden standard positive and negative data sets of UbiBrowser2.0 are constructed. 1322 E3-substrate interactions and 495 DUB-substrate interactions were manually extracted from the literature downloaded from PubMed. These data were used as golden standard positive (GSP) dataset. The GSN (golden standard negative) dataset was defined as the randomly combination of human E3s/DUBs and other human proteins, removing the overlap with GSP. Human E3 data was collected from UUCD (version 1.0). In this study, the E3 ligases of the TRIM family and predicted ubiquitination substrates were explored using UbiBrower2.0. We explored the TRIM family by selected the query protein as Ubiquitin ligase (E3), species as H. sapiens and submit the protein as TRIM. Then we chose the four TRIMs separately for prediction and analysis of the top 20 ubiquitination substrates of four TRIMs.

UALCAN Analysis

UALCAN (http://ualcan.path.uab.edu) is an interactive web portal for in-depth analyses of The Cancer Genome Atlas (TCGA) gene expression data (primary tumor n=520 and normal n=44) and protein expression analyses of data from Clinical Proteomic Tumor Analysis Consortium (CPTAC) and the International Cancer Proteogenome Consortium (ICPC) (primary tumor n=108 and normal n=71). In this study, expression data and clinical parameters for TRIM family members in HNSCC tissues and normal tissues were investigated. TCGA (https://portal.gdc.cancer.gov/) was used to analyze lymphatic vascular invasion in HNSCC based on HTSeq and RNAseq data in FPKM format and clinical data. Please refer to the Results section for details.

Relationships Between Immune Cell Infiltration and Four TRIMs

TIMER (http://cistrome.org/TIMER/) is a user-friendly web server for the systematic and comprehensive analysis of immune cell infiltration across various tumor types based on TCGA database. Levels of immune cell infiltration for the four TRIMs in HNSCC were estimated using the TIMER database (Spearman correlation). Correlations between TRIM expression and the abundance of six types of infiltrating immune cells ($CD8^+$ T cells, $CD4^+$ T cells, B cells, dendritic cells, macrophages, and neutrophils) were evaluated. TIMER was also used to analyze the associations between immune-related genes and TRIMs. In addition, it was used to investigate the correlations between tumor immune infiltration levels and copy number variation in TRIM family members.

Furthermore, the TISIDB database (http://cis.hku.hk/TISIDB) is a public portal for tumor and immune system interactions based on multiple heterogeneous data types. It integrates multiple heterogeneous data types include: Literature mining results from PubMed database, High throughput screening data (eg CRISPR-Cas9, shRNA and RNAi) for detecting resistance and sensitivity of tumor cells to T cell-mediated killing, Exome and RNA sequencing data set of patient cohorts with immunotherapy (responders and non-responders), Genomics, transcriptomics and clinical data of 30 cancer types from TCGA and Public databases, including UniProt, GO, DrugBank etc. TISIDB was used to evaluate associations between expression levels of four TRIMs and immune cell infiltration, immune modulators, and chemokines in HNSCC. First, we created the landscape of correlation between four TRIMs expression and 28 TILs across multiple cancer types via "lymphocyte" module separately. Specifically, we searched and screened the appropriate candidates with the statistical significance of Spearman correlation. In addition, Spearman correlation analysis was also performed to evaluate the correlation of four TRIMs expression with immune checkpoint gene levels and chemokines/chemokine receptors via "Immunomodulator" and "chemokine" module.

LinkedOmics Database Analysis

The LinkedOmics database (http://www.linkedomics.org/login.php) was utilized to analyze 32 TCGA cancer-associated multi-dimensional data sets. The differentially expressed genes related to four TRIMs were screened from the TCGA HNSCC cohort through the LinkFinder module in the database. The first step was to selected cancer cohort HNSCC (TCGA_HNSC). Then we selected search dataset (TCGA_HNSC, RNAseq, HiSeq RNA, Firhose_RSEM_log2), with Step-2b for histological_type head and neck squamous cell carcinoma [N:517]. Step-3 was to select four TRIMs separately and then to select target dataset as step-2. Correlations were evaluated by the Spearman correlation coefficient and are presented in a volcano plot and heat maps. Enriched Gene Ontology biological processes (GO_BP) and Kyoto Encyclopedia of Genes and Genomes (KEGG) pathways were evaluated by a gene set enrichment analysis (GSEA) using the LinkInterpreter module.

Gene and Protein Network Analysis

STRING (https://string-preview.org/) and GeneMANIA (http://genemania.org/) integrating multiple heterogeneous data types were used to construct and analyze gene and protein networks based on the four TRIMs. STRING showed the result with the main settings such as meaning of network edges ("evidence"), minimum required interaction score ["Medium confidence (0.400)"], and max number of interactors to show ("no more than 10 interactors") operated.

Immunohistochemistry

An independent set of formalin-fixed, paraffin-embedded archival tissue specimens including HNSCC tissues obtained at Xiangya Hospital of Central South University (the number of the samples was 48), China, between January 2021 and December 2023 were analyzed. Among lymphovascular invasion group, 24 were male, ranging from 40 to 82 years old with an average age of 59.88 ± 9.18 years old, while among the control group, 24 were male, ranging from 38 to 79 years old with an average age of 57.90 ± 11.00 years old. Tissue samples were cut into 4 μm sections that were deparaffinized, rehydrated, and treated with an antigen retrieval solution (10 mmol/l sodium citrate buffer, pH 6.0). The sections were incubated with antibodies against *TRIM2* (1:150; Proteintech), *TRIM11* (1:800; Proteintech), *TRIM28* (1:200; Abcam) and *TRIM56* (1:800; Abcam). A total score (ranging from 0–6) was obtained by adding the extent and intensity scores for each case: 0–3, low staining; 4–6, high staining.

Statistical Analysis

Data are presented as median. The non parametric test was used the compare the quantitative data of two groups; Spearman correlation analysis was also performed to evaluate the correlation. A two-tailed *P* value < 0.05 was considered significant.

Results

Members of the TRIM Family of E3 Ligases and Aberrant Expression in Patients with HNSCC

Members of the TRIM family of E3 ligases are listed in Table S1 (N = 62), as determined by using UbiBrowser$^{2.0}$. To investigate the expression profiles of TRIM family members in HNSCC, TCGA datasets were used to compare mRNA expression levels of TRIMs in HNSCC tissues (N = 502) and normal tissues (N = 44). A total of 45 aberrantly expressed genes were identified within the human HNSCC tissues, including 39 genes that were up-regulated in HNSCC tissues and 6 genes that were down-regulated in HNSCC tissues (Figure 1a and b). Next, CPTAC databases were used to compare the protein expression levels of TRIM family members in HNSCC tissues versus normal tissues. Both the mRNA and protein expression levels of *TRIM5, TRIM11*, TRIM21, *TRIM22, TRIM25, TRIM28*, and *TRIM56* were strongly up-regulated, while *TRIM2, TRIM7, TRIM13, TRIM55* and *TRIM63* were down-regulated in patients with HNSCC in different datasets. Figure 1c summarizes the protein expression levels of these TRIMs, showing members who with similar differences in expression at the protein and mRNA levels between HNSCC and control groups ($P < 0.05$).

Then, we evaluated the lymphovascular invasion features of twelve TRIMs in HNSCC by using TCGA datasets (219 without invasion vs 122 with invasion). Four TRIMs were associated with lymphovascular invasion, namely *TRIM11, TRIM28, TRIM56*, and *TRIM2*, as shown in Figure 2a.

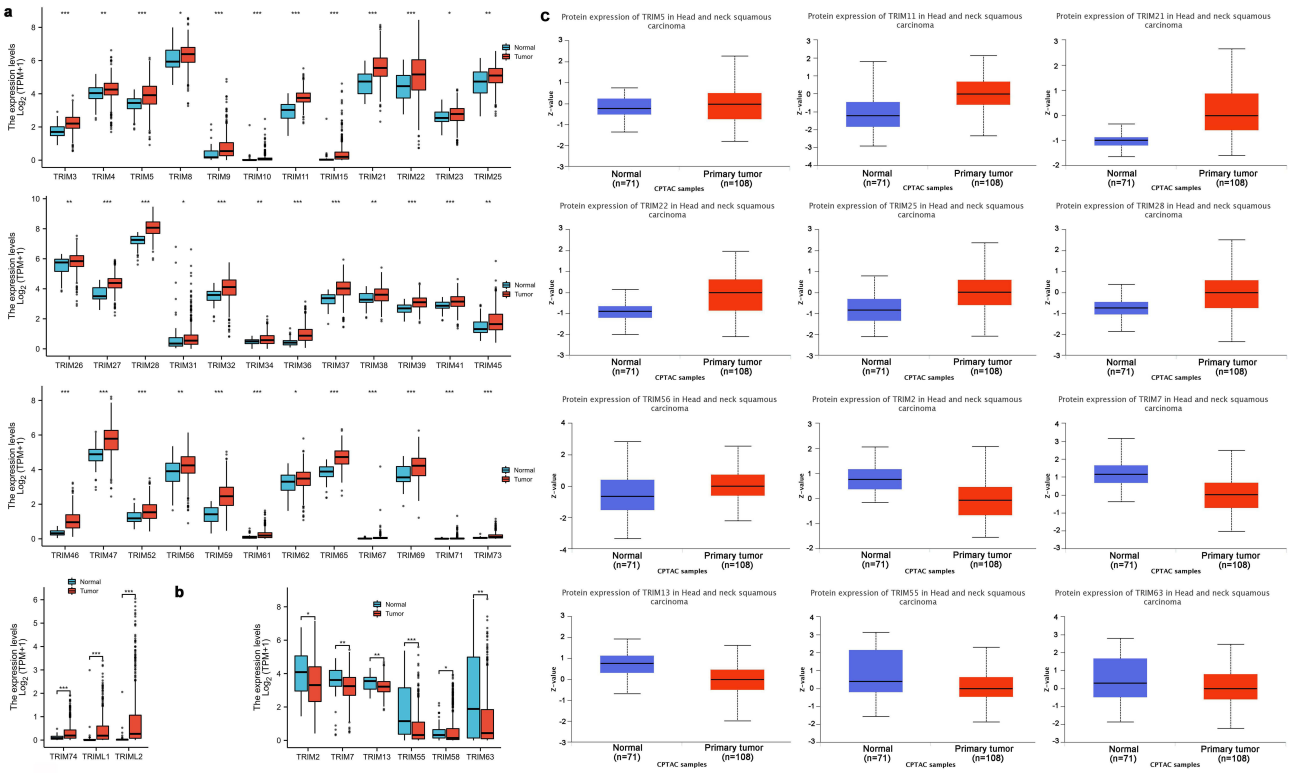

Figure 1 Members of the TRIM family of E3 ligases and their aberrant transcriptional levels and protein level in patients with HNSCC. (**a**) Using TCGA database, TRIM family members that are aberrantly expressed in head and neck tumors tissues (n = 502) versus normal tissues (n = 44). TRIM family members that are highly expressed in HNSCC tissues. **P*< 0.05; ***P*<0.01; ****P*<0.001. (**b**) TRIM family members that are lowly expressed in HNSCC tissues. **P*< 0.05; ***P*<0.01; ****P*<0.001. (**c**) Using CPTAC database, TRIM family members that are aberrantly expressed in head and neck tumors tissues (n = 108) versus normal tissues (n = 71). *P*<0.05. Z-values represent standard deviations from the median across samples for the given cancer type. Log2 Spectral count ratio values from CPTAC were first normalized within each sample profile, then normalized across samples.

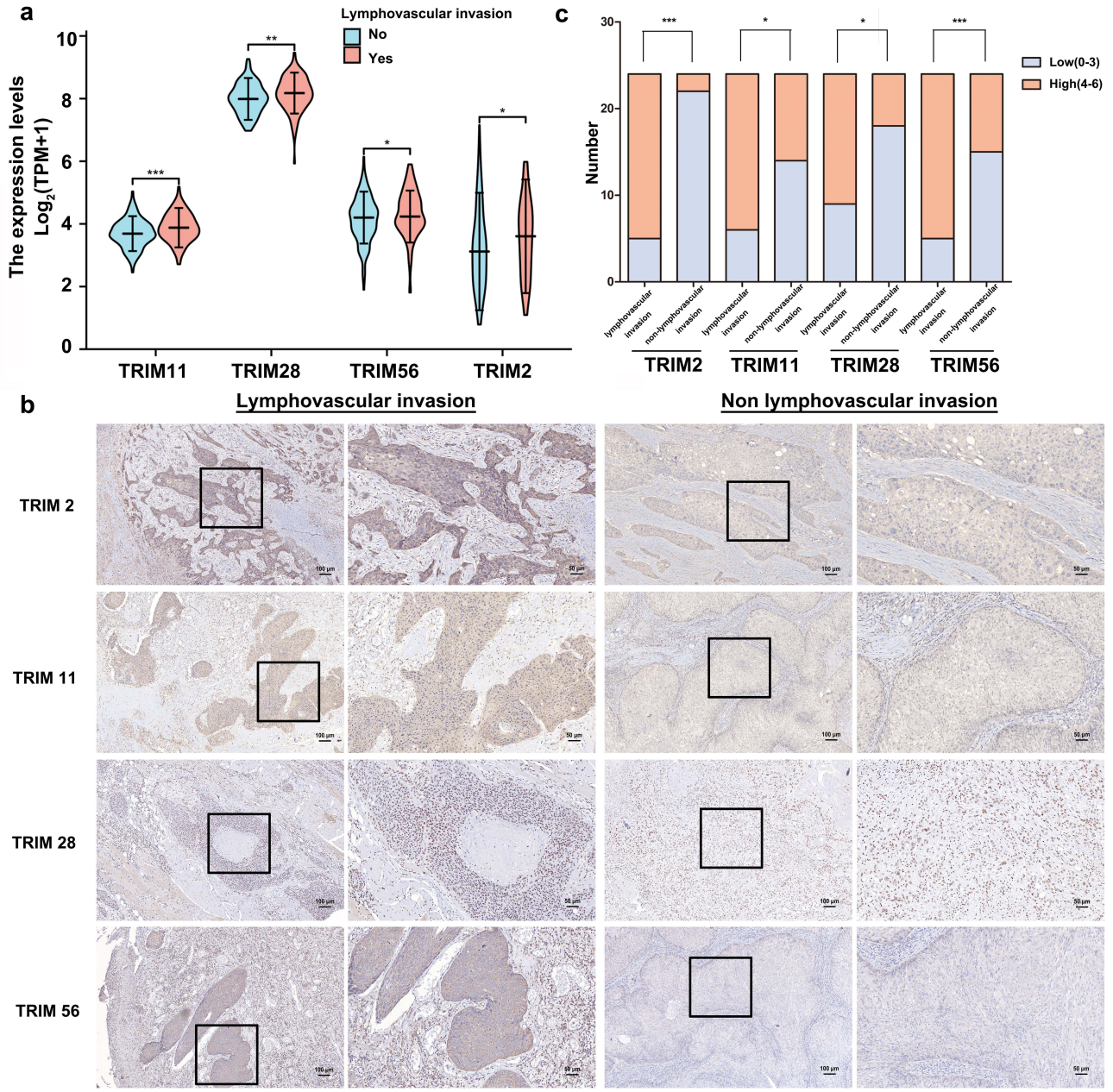

Figure 2 Four-TRIMs expression between lymphovascular invasion and non lymphovascular invasion in HNSCC. (**a**) The lymphovascular invasion features of four-TRIMs using TCGA database. **P*< 0.05; ***P*<0.01; ****P*<0.001. (**b**) Representative immune-histochemistry results showing four-TRIMs expression in lymphovascular invasion and non lymphovascular invasion HNSCC tissues. (**c**) The bar chart of immunohistochemical expression levels and quantities of four-TRIMs. Mann–Whitney *U*-test **P*< 0.05; ***P*<0.01; ****P*<0.001.

We also confirmed and quantified the protein expression by immunohistochemistry. The results showed that the four TRIMs were expressed at a higher level in lymphovascular invasion tissues as compared to non lymphovascular invasion ones (Figure 2b and c), which were consistent with the results of the bioinformatics analysis.

Expression Levels of Four TRIMs and Immune Cell Infiltration in HNSCC

The occurrence and development of tumors are inseparable from the immune system, and the rise of immunotherapy is changing the paradigm of HNSCC treatment. Therefore, to examine whether the four TRIMs are immune targets and to determine relationships between the expression levels of four TRIMs and immune cell infiltration in HNSCC, we used the TIMER online tool. As shown in Figure 3a, the expression levels of the four TRIMs were positively associated with $CD4^+$T and macrophage infiltration; *TRIM11, TRIM28*, and *TRIM2* were related to tumor purity and B cell infiltration.

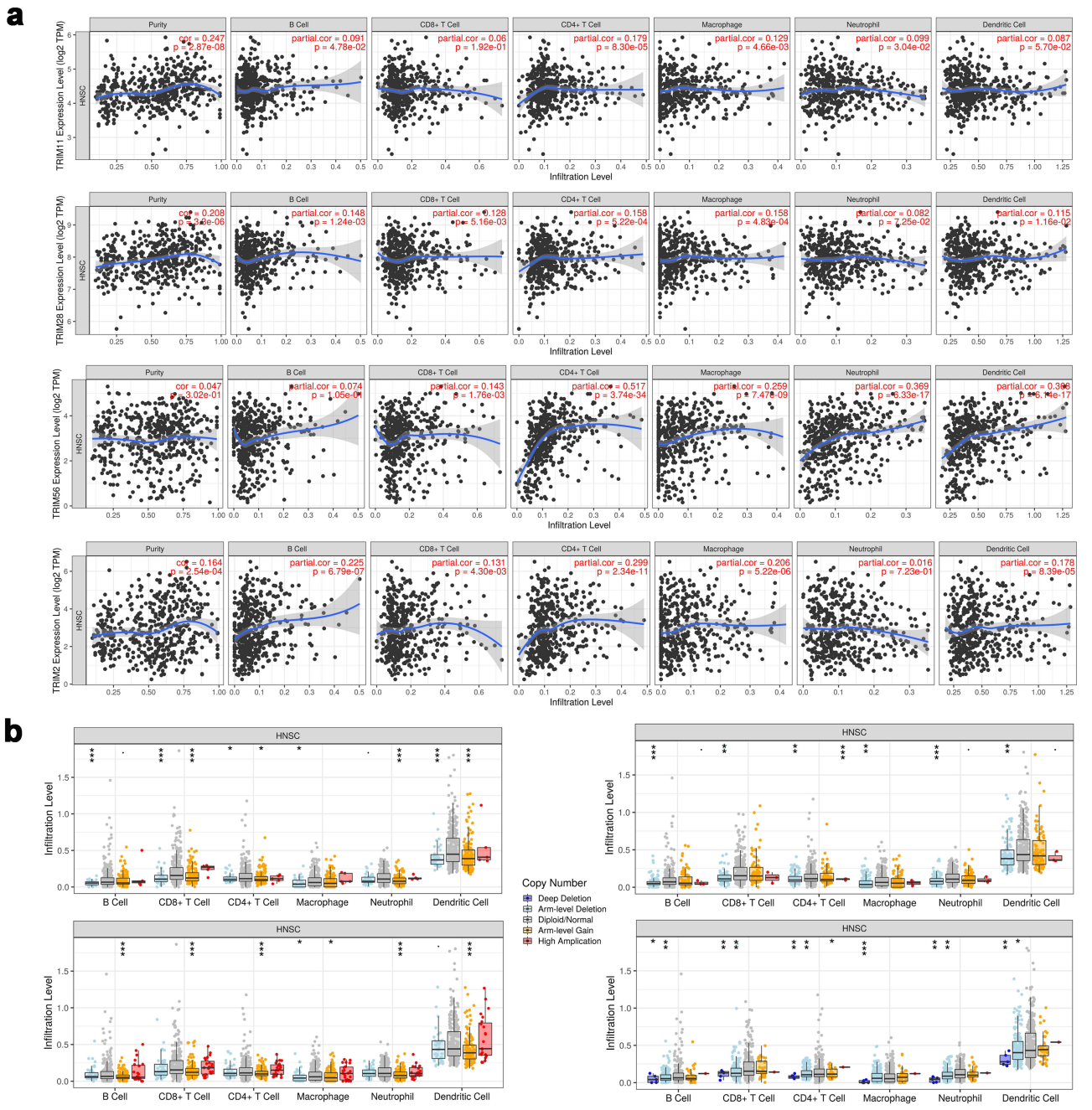

Figure 3 Correlations of four-TRIMs expression with immune infiltration level in HNSCC (TIMER). (**a**) The correlation between the abundance of immune cells and the expression of four-TRIMs in HNSCC. (**b**) four-TRIMs copy number variation affects the infiltrating levels of immune cells in HNSCC. **P*< 0.05; ***P*<0.01; ****P*<0.001.

TRIM28, TRIM56, and *TRIM2* were correlated with $CD8^+$T cell and dendritic cell infiltration. *TRIM11* and *TRIM56* were linked to neutrophil cell infiltration. Furthermore, copy number variation in the four TRIMs was related to infiltrating levels of B cells, $CD8^+$T cells, $CD4^+$T cells, macrophages, neutrophils, and dendritic cells in HNSCC (Figure 3b). Thus, the four TRIMs may affect immunity in HNSCC via distinct mechanisms.

To explore the detailed roles of the four TRIMs in the infiltration of various immune cells in HNSCC, TIMER was also used to explore the relationships between expression levels and well-established marker sets for different immunocytes, such as B cells, $CD8^+$ T cells, Th cells, macrophages, NK, and dendritic cells (DCs), in HNSCC (Table S2). In addition, various functional T cells, including Th1, Th2, Th17, Tfh, and Tregs, were examined. The levels of most immune sets (eg, T cells, tumor-associated macrophages, M1/M2 macrophages, monocytes, and DCs) were associated with the expression

levels of the four TRIMs, especially *TRIM56*, in HNSCC. For T cell exhaustion, the results were consistent with the analysis of four TRIMs, which were significantly associated with *TIM-3* and partly related to *CD274* (*PD-L1), LAG3*, and *PD*-1. It is important to emphasize that *CD274* (*PD-L1*), a biomarker of the response to immune-checkpoint inhibitors, was significantly correlated with *TRIM56* expression in HNSCC.

Therefore, the TISIDB database was utilized for a more detailed immune infiltration analysis of the four TRIMs in HNSCC among subgroups, such as Act_B, Act_CD8, and Act_DC, with different functions. *TRIM11* and *TRIM28* were negatively related to the infiltration of various immune cells, especially activated B cells, NK cells, and macrophages, indicating that the expression of these proteins might be associated with a suppressive tumor immune microenvironment in HNSCC. *TRIM56* expression was related to activated B cells, activated $CD8^+$ T cells, NK cells, and macrophages (Figure 4a and b). Subsequently, the correlations between the expression levels of four TRIMs and levels of 50 common

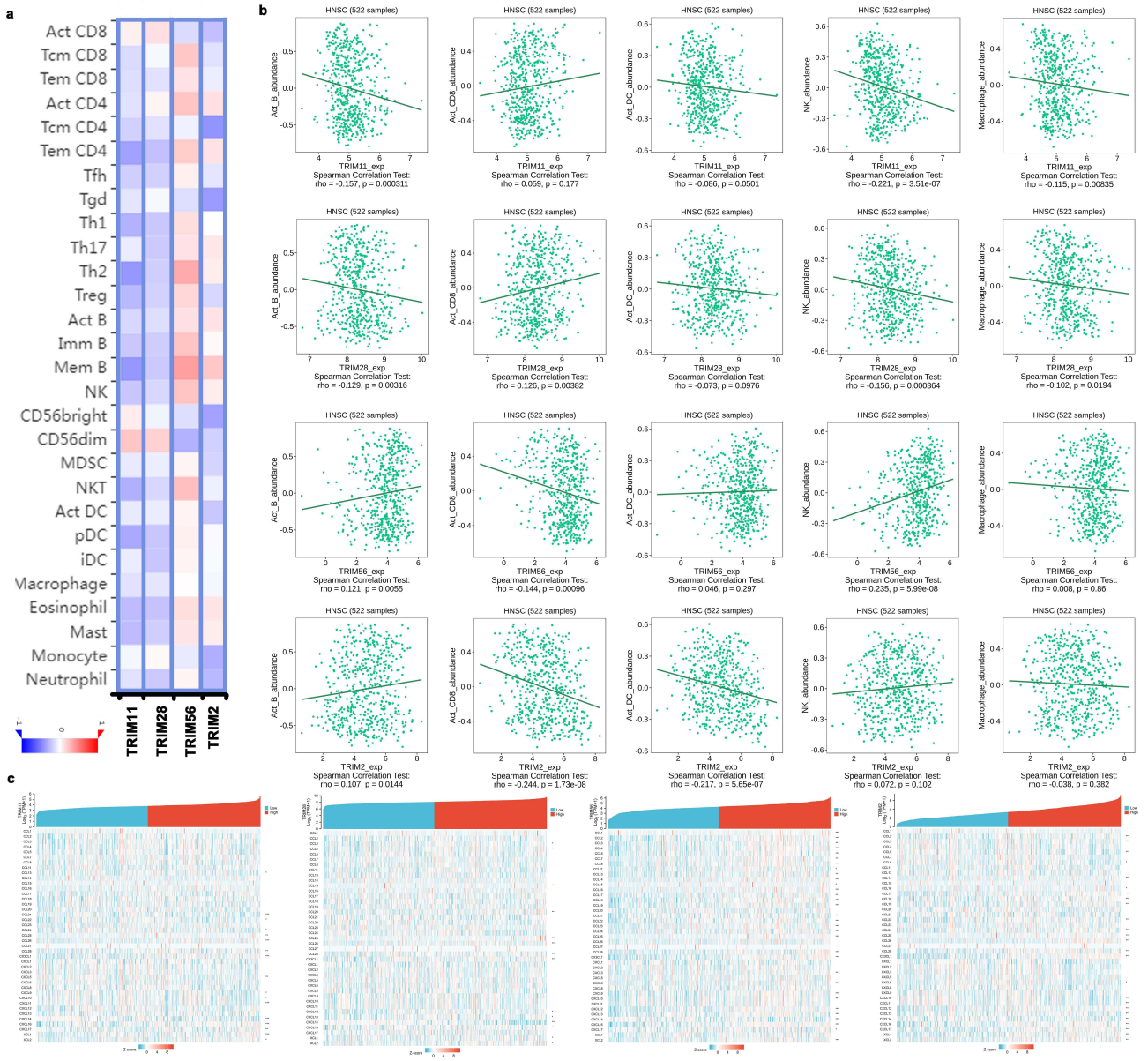

Figure 4 Association of four-TRIMs expression with immune infiltration level, Immunomodulator and chemokines in HNSCC (TISIDB). (**a**) Heat map of immune infiltrate correlation for four-TRIMs. (**b**) Scatter plot of the correlation between the expression level of four-TRIMs and immune cell infiltration, including Act_B cells, Act_$CD8^+$T cells, Act_DC, NK cells and macrophages (Spearman correlation, N = 522). (**c**) Chemokines heatmap associated with four-TRIMs expression levels in HNSCC.
Abbreviations: Act, activated; NK, natural killer cells; DC, dendritic cells.

immune control genes were analyzed (Table 1, Immunomodulator genes). Interestingly, in HNSCC, *TRIM56* expression was associated with levels of almost all immune checkpoint markers, such as *CD276, CD27, CD244, IL-10*, and *CD274*. These results strongly demonstrated that TRIM genes and ubiquitination may play a crucial role in tumor immunity. To

Table 1 Correlation Analysis Between Four-TRIMs Expression and Immunomodulator Genes

Immunomodulator	TRIM2		TRIM11		TRIM28		TRIM56	
	Cor	*P*	Cor	*P*	Cor	*P*	Cor	*P*
ADORA2A	0.345	<0.001	0.149	<0.001	0.253	<0.001	0.441	<0.001
BTLA	0.228	<0.001	−0.026	0.565	0.079	0.079	0.329	<0.001
CD160	0.198	<0.001	0.093	0.036	0.130	0.003	0.421	<0.001
CD244	0.060	0.176	−0.092	0.039	−0.095	0.033	0.159	<0.001
CD27	0.167	<0.001	0.009	0.841	0.101	0.024	0.260	<0.001
CD274	−0.007	0.875	−0.051	0.253	0.016	0.716	0.251	<0.001
CD276	−0.030	0.499	−0.005	0.910	0.171	<0.001	0.172	<0.001
CD28	0.236	<0.001	−0.090	0.044	0.038	0.390	0.342	<0.001
CD40	0.178	<0.001	0.112	0.012	0.248	<0.001	0.377	<0.001
CD48	0.133	0.003	−0.029	0.516	0.059	0.188	0.273	<0.001
CD70	0.016	0.713	0.083	0.063	0.180	<0.001	0.097	0.029
CD80	0.095	0.033	−0.070	0.115	0.053	0.232	0.296	<0.001
CD86	0.069	0.122	−0.047	0.297	0.063	0.157	0.301	<0.001
CD96	0.209	<0.001	0.018	0.696	0.103	0.021	0.359	<0.001
CTLA4	0.036	0.421	0.014	0.750	0.116	0.009	0.276	<0.001
ENTPD1	0.305	<0.001	0.023	0.609	0.205	<0.001	0.398	<0.001
HAVCR2	0.109	0.015	0.037	0.410	0.134	0.003	0.309	<0.001
HHLA2	0.351	<0.001	0.240	<0.001	0.153	<0.001	0.250	<0.001
ICOS	0.031	0.484	−0.098	0.028	0.059	0.189	0.281	<0.001
ICOSLG	0.293	<0.001	0.143	0.001	0.165	<0.001	0.270	<0.001
IDO1	−0.027	0.541	0.028	0.528	0.106	0.018	0.275	<0.001
IL10	0.022	0.626	−0.170	<0.001	−0.027	0.542	0.241	<0.001
IL6	0.054	0.223	−0.065	0.143	−0.029	0.519	0.078	0.082
KIR2DL1	−0.061	0.169	0.038	0.395	0.041	0.356	0.149	<0.001
LAG3	−0.079	0.079	0.093	0.038	0.177	<0.001	0.225	<0.001
LGALS9	0.308	<0.001	0.224	<0.001	0.289	<0.001	0.408	<0.001
LTA	0.191	<0.001	0.071	0.113	0.163	<0.001	0.370	<0.001
MICB	0.068	0.127	0.112	0.012	0.236	<0.001	0.390	<0.001
NT5E	−0.157	<0.001	−0.195	<0.001	−0.118	0.008	0.083	0.064
PDCD1	0.055	0.218	0.066	0.138	0.143	0.001	0.293	<0.001
PVR	−0.106	0.018	0.061	0.172	0.188	<0.001	0.143	0.001
RAET1E	−0.281	<0.001	−0.266	<0.001	−0.385	<0.001	−0.299	<0.001
TGFB1	−0.041	0.355	−0.040	0.368	0.064	0.153	0.107	0.016
TIGIT	0.136	0.002	0.004	0.920	0.092	0.040	0.326	<0.001
TMIGD2	0.034	0.445	0.076	0.090	0.104	0.020	0.249	<0.001
TNFRSF14	0.154	<0.001	0.259	<0.001	0.240	<0.001	0.374	<0.001
TNFRSF17	0.183	<0.001	−0.019	0.663	0.068	0.131	0.196	<0.001
TNFRSF18	0.336	<0.001	0.411	<0.001	0.245	<0.001	0.117	0.009
TNFRSF25	−0.026	0.561	0.334	<0.001	0.150	<0.001	0.124	0.005
TNFRSF4	0.071	0.111	0.136	0.002	0.160	<0.001	0.198	<0.001
TNFRSF8	0.056	0.207	0.049	0.276	0.157	<0.001	0.210	<0.001
TNFRSF9	0.205	<0.001	−0.042	0.342	0.156	<0.001	0.366	<0.001
TNFSF13	0.404	<0.001	0.153	<0.001	0.143	0.001	0.492	<0.001
TNFSF14	0.137	0.002	−0.072	0.105	−0.016	0.725	0.322	<0.001

(*Continued*)

Table 1 (Continued).

Immunomodulator	TRIM2		TRIM11		TRIM28		TRIM56	
	Cor	*P*	Cor	*P*	Cor	*P*	Cor	*P*
TNFSF15	0.349	<0.001	0.108	0.015	0.261	<0.001	0.432	<0.001
TNFSF18	0.361	<0.001	0.017	0.711	0.014	0.762	0.261	<0.001
TNFSF4	0.367	<0.001	0.027	0.544	0.207	<0.001	0.355	<0.001
TNFSF9	−0.037	0.407	0.024	0.591	−0.105	0.019	−0.222	<0.001
ULBP1	0.518	<0.001	0.257	<0.001	0.257	<0.001	0.194	<0.001
VTCN1	0.608	<0.001	0.313	<0.001	0.125	0.005	0.262	<0.001

Abbreviation: Cor, R value of Spearman correlation.

further elucidate the association between TRIM expression and immune cell migration, we comprehensively analyzed the connections between expression levels and chemokine levels (Figure 4c and Table S3). The results proved that the expression levels of the four TRIMs, especially *TRIM56*, were positively correlated with levels of immune cell-associated chemokines, such as *CX3CL1* (r = 0.412, $P < 0.001$), *CXCL12* (r = 0.309, $P < 0.001$), *CXCL16* (r = 0.405, $P < 0.001$), and *CCL2* (r = 0.267, $P < 0.001$). Since levels of these chemokines increased as the *TRIM56* expression level increased, high *TRIM56* expression may promote the migration of immune cells to the tumor microenvironment (TME). Therefore, the four TRIMs were widely involved in the regulation of various immune molecules in HNSCC lymphovascular invasion, affecting immune cell infiltration in the TME.

Genes Co-Expressed with Four TRIMs in HNSCC

To unveil the functions and mechanism of action of the four TRIMs in HNSCC, we first used LinkedOmics to identify correlated expression patterns based on mRNA sequencing data for 517 patients with HNSCC. For example, as shown in Figure 5a, *TRIM56* expression was positively correlated with levels of 5991 genes (dark red dots) and negatively correlated with levels of 4930 genes (dark green dots). Figure 5b and c show heat maps of the top 50 genes that were positively and negatively associated with *TRIM56* expression, respectively. We next evaluated GO term annotations by a GSEA of genes that were co-expressed with *TRIM56*. GO term annotation showed that co-expressed genes were mainly involved in the regulation of small GTPase-mediated signal transduction, protein autophosphorylation, protein localization to endoplasmic reticulum, receptor complex, transcriptional repressor complex, ribosome, *NADH* dehydrogenase complex, protein serine/threonine kinase activity, protein kinase A binding, antioxidant activity, and structural constituent of ribosome (Figure 5d–f). A KEGG pathway analysis indicated enrichment in the JAK-STAT signaling pathway, signaling pathways regulating the pluripotency of stem cells, Inositol phosphate metabolism, cGMP-PKG signaling pathway, Ribosome, oxidative phosphorylation, proteasome, and metabolic pathways (Figure 5g). Analyses of co-expressed genes for *TRIM2, TRIM11*, and *TRIM28* are summarized in Figure S1 and Table S4.

Gene and Protein Networks of Four TRIMs

Gene–gene and protein–protein interaction networks were generated by using GeneMANIA and STRING for the four TRIMs (Figure 6). As determined using GeneMANIA, *TRIM11* potentially interacted with 20 proteins, including *TRIM35, PHOX2B, MED15, UBE2N*, and *RNF126. TRIM28* was likely to have a connection with *ZNF* family members, *TRIM33*, and *POGK*. *TRIM2* interacted with *ERN2, ERN1, BCL2L11, TRIM3, PDHA1*, and so on. Interestingly, *TRIM56* was related to *STING1*, which was closely related to *p53*.[34,35] These results may guide future studies of the functional interactions of the four TRIMs in HNSCC lymphovascular invasion. We further used the STRING online tool to analyze the protein–protein interaction network of the four TRIMs to determine interactions in the lymphovascular invasion of HNSCC. The top proteins are listed in Figure 6f–i. These proteins included *MPHOSPH8* (*MPP8*), which is essential for sustaining the self-renewal of ground-state pluripotent stem cells and mediates the down-regulation of *CDH1* expression,[36,37] *MDM2*, which interacts with *p53, TMEM173* (*STING1*), and *PUM1*, which promotes cancer cell proliferation and migration.[38,39]

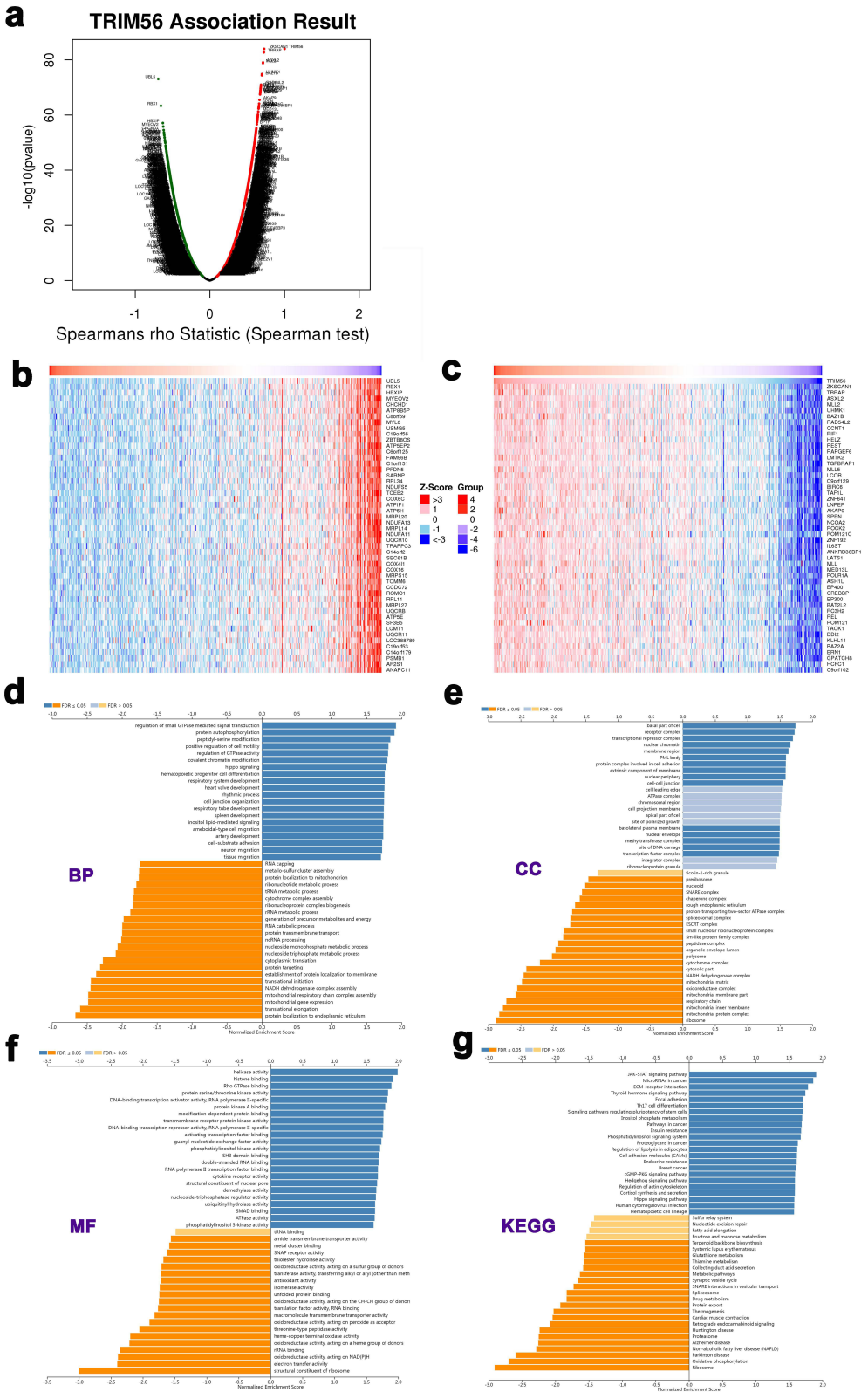

Figure 5 Enrichment analysis of *TRIM56* functional networks in HNSCC (LinkedOmics). (**a**) The genes strongly correlating with *TRIM56* as identified by Spearman's test in HNSCC. (**b** and **c**) Heat maps showing genes positively and negatively correlating with *TRIM56* in HNSCC (TOP 50). (**d–g**) Significantly enriched Gene Ontology annotations and Kyoto Encyclopedia of Genes and Genomes pathways of *TRIM56* in HNSCC.
Abbreviations: CC, Cellular component; BP, Biological process; MF, Molecular function.

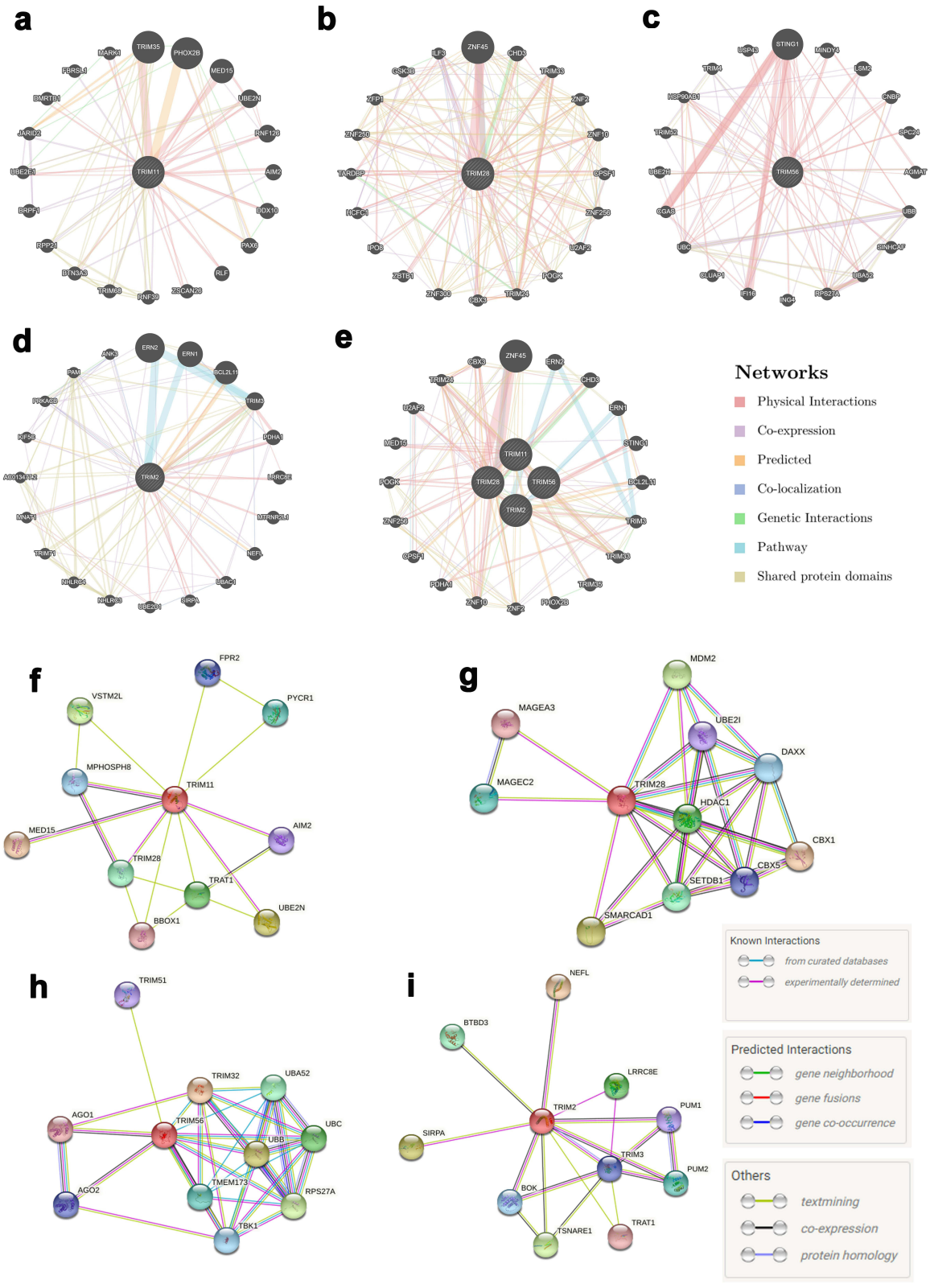

Figure 6 Gene–gene and protein–protein interaction network of genes of the Four-TRIMs. (**a–d**) A network diagram of interactions between proteins encoded by genes of the four-TRIMs, drawn by using STRING. (**e–i**) The gene network associated with the four-TRIMs gene, drawn by using GeneMANIA.

Prediction and Analysis of Ubiquitination Substrates of Four TRIMs

UbiBrowser was used to predict and analyze the interaction relationships between the four TRIMs and possible substrates. Both known substrates reported in the literature and the top 20 predicted substrates are presented in Figure 7a–d. *AXIN1, DUSP6, ESR1, PHLPP1, IRF7, PRKAA1, TP53, STING1, VIM, BCL2L11*, and *NEFL* were substrates of all four TRIMs. The substrates of the four TRIMs included *TP53, TP63, TP73, CCND1, LMNA*, and other members of the TRIM family. Then, GO term annotation was performed to explore functions of the ubiquitin substrates of the four TRIMs. The proteins were mainly involved in the pattern specification process, regionalization, signal transduction by *p53* class mediator, binding such as DNA-binding transcription activator activity, enhancer binding, and *p53* binding, transcription coactivator activity, transcription factor complex, transcriptional repressor complex, nuclear matrix, and nuclear membrane (Figure 7e). A KEGG pathway analysis indicated enrichment for various pathways, including the MAPK signaling pathway, PI3K-Akt signaling pathway, Wnt signaling pathway, p53 signaling pathway, JAK-STAT signaling pathway, Toll-like receptor signaling pathway, TNF signaling pathway, NF-kappa B signaling pathway, IL-17 signaling pathway, PD-L1 expression, and PD-1 checkpoint pathway in cancer (Figure 7f). The predicted substrates of the four-TRIMs are listed in Supplementary Table S5.

Discussion

The TRIM family is a three-domain protein family, the majority of which function as E3 ubiquitin ligases, queried by UbiBrowser[2.0]. How to identify the E3 ubiquitin ligases related to lymphovascular invasion in HNSCC among numerous TRIM family members to explore the potential relationship between ubiquitination and lymphovascular invasion, as well as inflammation? We summarized the contribution of each bioinformatics analysis to the final understanding of the role played by the four TRIMs associated with lymphovascular invasion (Figure 8).

Through analysis of the TCGA database, we discovered that most TRIM family members had a cancer-promoting effect in HNSCC and were more highly expressed in tumor tissues than in normal tissues. In the process of lymphovascular invasion, the four TRIM members were highly expressed, suggesting that further experiments should explore their roles in lymphovascular invasion. We analyzed the expression levels of E3 ligases in the TRIM family in HNSCC, with verification using different data types from CPTAC. We found that four-TRIMs were closely related to lymphovascular invasion in HNSCC. Therefore, we focused on the significance of these four ligase E3 proteins. Next, using various databases, we evaluated relationships with immune infiltration, co-expressed genes, gene and protein networks, and ubiquitination substrates in HNSCC. Finally, we evaluated related functions and pathways based on co-expressed genes and predicted substrates by GO and KEGG enrichment analyses. Although some TRIM family members have known roles in oncogenesis and tumor progression like *TRIM72* in non-small cell lung cancer and *TRIM22* in glioblastoma,[40,41] comprehensive bioinformatics analyses of these loci in HNSCC are lacking. We performed the first analyses of the transcription levels, immune cell infiltration, co-expressed genes, and predicted substrates of four TRIM family members in HNSCC. These findings establish the link between the TRIM family and HNSCC, improve our understanding of the relationship between ubiquitination and lymphovascular invasion, and provide potential targets for diagnosis and treatment.

Owing to the complex TME and the great progress in tumor immunotherapy, studies of factors involved in immune cell regulation are critical. We focused on the molecular mechanism underlying ubiquitination mediated by E3 ligase family members in lymphovascular invasion in HNSCC and tumor immunity. Numerous studies have confirmed that tumor immune cell infiltration could influence the efficacies of chemotherapy, radiotherapy, or immunotherapy as well as metastasis, such as ovarian tumor, neuroblastoma, and so on.[42–45] Our results suggested that the four TRIMs are positively correlated with $CD4^+$ T cells and macrophages. $CD4^+$ T cells and tumor-associated macrophages could participate in tumor development via many signaling pathways, such as the Wnt and NF-κB signaling pathways.[46–48] For example, the factor in TME and tumor-associated macrophages promotes glioma progression. These findings indicated that tumor immune cell infiltration (eg, via the regulation of $CD4^+$ T cell and macrophage-related proteins) might account, in part, for TRIM-mediated lymphovascular invasion in HNSCC.

Further analyses of infiltrating lymphocyte markers showed that many lymphocyte markers were related to the expression of these four TRIMs. Interestingly, almost all of the four TRIMs were strongly correlated with *TIM-3* (*HAVCR2*), an important

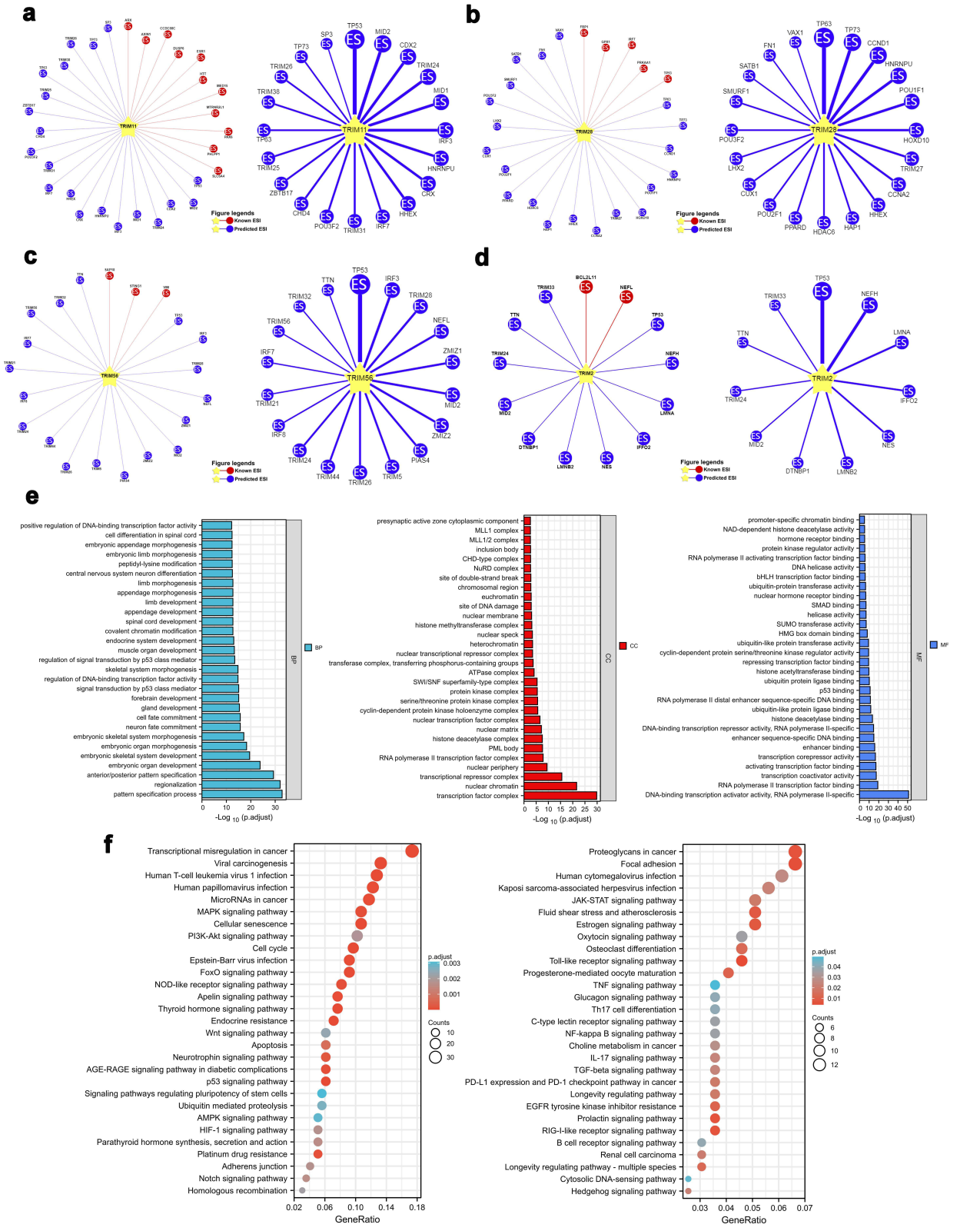

Figure 7 Predicted ubiquitination substrates of the four-TRIMs and functional analysis (UbiBrowser$^{2.0}$). (**a–d**) Ubiquitination substrates of the four-TRIMs by UbiBrowser$^{2.0}$. The left one on each figure was all of the ubiquitination substrates, the right one was the predicted ubiquitination substrates. The thickness of blue line represented the score of each protein. (**e**) Significantly enriched Gene Ontology annotations of predicted ubiquitination substrates. (**f**) Kyoto Encyclopedia of Genes and Genomes pathways of predicted ubiquitination substrates.

Abbreviations: CC, Cellular component; BP, Biological process; MF, Molecular function.

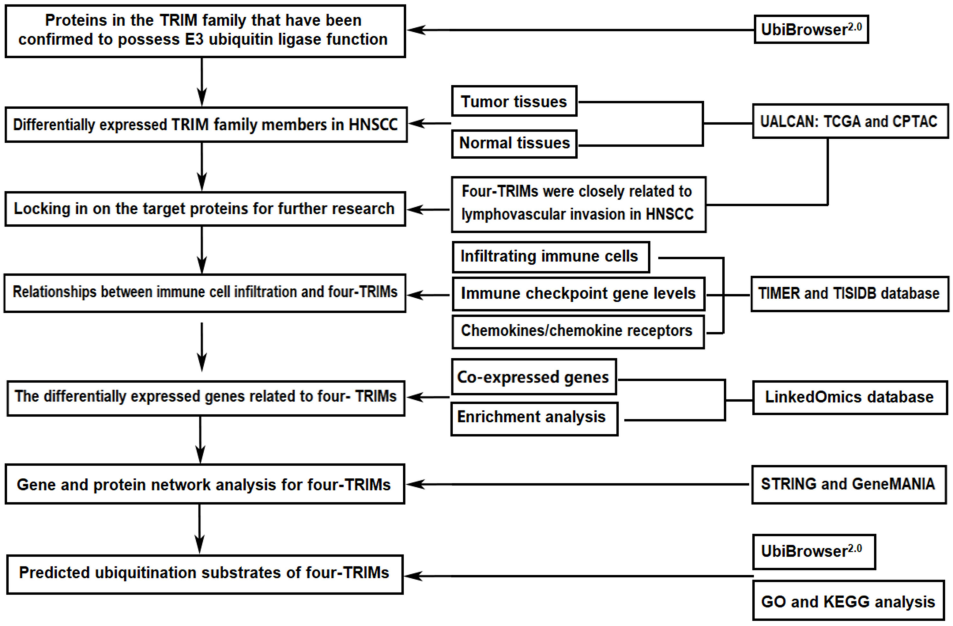

Figure 8 Summarized the contribution of bioinformatics analysis to understanding of the role played by the four TRIMs associated with lymphovascular invasion.

gene for T cell exhaustion, which could inhibit anti-tumor immunity.[49] Treg cells create an immunosuppressive tumor microenvironment and Treg cell infiltration in tumors is associated with a poor prognosis.[50] The four-TRIMs were all related to *STAT5B*, indicating that ubiquitination mediated by the TRIM family might play an important role in the TME.

Immune checkpoints have resulted in significant improvements in the treatment of HNSCC. The expression levels of the four TRIMs were positively associated with levels of *CD160, HHLA2, ICOSLG, TNFRSF14, ULBP1*, and *VTCN1* and were negatively correlated with levels of *RAET1E*. *TRIM56* was related to most immunomodulators, such as *CTLA4, LAG3*, and *PDCD1*. Programmed cell death protein-1 (*PD*-1, encoded by *PDCD1*) is expressed in activated T cells and suppresses the activation of lymphocytes and cytokine production by interacting with its ligand, *PD*-L1. The blockade of *PD*-1, *LAG3*, and *CTL4* signaling using corresponding antibodies has promising therapeutic effects in a variety of cancers, such as melanoma and non-small-cell lung cancer.[51–53] In addition, *CD160* is expressed in NK, NKT, $CD8^+$ T cells, intra-epithelial T cells, and $CD4^+$ T cells in humans.[54,55] *CD160* inhibits T cells and stimulates NK cells. Impaired NK cell function is related to a poor clinical prognosis in cancer.[56,57] Because the expression levels of four TRIMs were positively corrected with levels of *CD160*, we speculated that these proteins promoted lymphovascular invasion in HNSCC by inhibiting the function of NK cells.

According to TISIDB, the expression levels of the four TRIMs were associated with levels of some chemokines, such as *CX3CL1, CXCL16* and *CXCL14*. *CX3CL1* and its receptor *CX3CR1* play a complex role in the genesis and development of various tumors, especially in the process of tumor metastasis. Accordingly, *CX3CL1* and *CX3CR1* have become targets for tumor therapy. *CXCL16* is expressed in many tumors and can promote tumor invasion and metastasis. It can also regulate tumor immunity.[58] *CXCL16* is highly expressed by activated dendritic cells occupying the vascular microenvironment. Moreover, the receptor of this chemokine, *CXCR6*, promotes cytotoxic T cell accumulation in this microenvironment, which is crucial for their survival in tumors. *CXCL14* can promote the enrichment of M2 macrophages, promote differentiation into M2 macrophages, and inhibit differentiation into M1 macrophages.[59] Levels of the four TRIMs were related to levels of these chemokines, indicating that ubiquitination might contribute to the regulation of chemokines and thereby play a role in lymphovascular invasion in HNSCC. These results clearly showed that the four TRIMs have a wide range of effects on immune cells in the TME of HNSCC and are candidates for further research in the field of tumor immunotherapy.

A LinkedOmics database analysis revealed that in addition to the four TRIMs, genes that were co-expressed with these TRIMs in HNSCC (whether positively or negatively) might also be closely related to the lymphovascular invasion of HNSCC. GO and KEGG signaling pathway analyses of these co-expressed genes revealed roles in important signaling pathways, providing targets for future research on ubiquitination. We constructed protein/gene networks for the four TRIMs and found that related proteins included some important molecules, such as the cGAS-STING1 pathway, a recent focus of immunity research, as well as *ERN1*.[60] The factors related to TRIM-mediated ubiquitination provide a basis for follow-up studies of the mechanism underlying lymphovascular invasion.

Despite achieving some innovative results from the research, there are still certain limitations in this study. For instance, we did not further explore the relationships between TRIM family members and some factors such as tumor staging and HPV infection. However, these will be investigated and supplemented in subsequent studies.

Conclusions

We found that four TRIMs are highly expressed in HNSCC with lymphovascular invasion and are potential unfavorable prognostic factors for HNSCC. They were strongly associated with immune cell infiltration. Co-expressed genes and ubiquitination substrates also included molecules that were closely related to tumors. GO and KEGG pathway analyses showed that they were involved in many key signaling pathways. Therefore, the specific roles and mechanisms of action of the four TRIMs in HNSCC should be investigated further by both in vitro and in vivo assays. To sum up, the four TRIMs evaluated in this study might play an oncogenic role to promote lymphovascular invasion in HNSCC. The mechanism of action and role of ubiquitination and the TME in lymphovascular invasion deserve further study.

Abbreviations

HNSCC, head and neck squamous cell carcinoma; TRIM, tripartite motif; PHLPP2, pleckstrin homology domain leucine-rich repeat protein phosphatase 2; DUB, deubiquitinase; TCGA, The Cancer Genome Atlas; CPTAC, Clinical Proteomic Tumor Analysis Consortium; GO, Gene Ontology; KEGG, Kyoto Encyclopedia of Genes and Genomes; GSEA, gene set enrichment analysis; DCs, dendritic cells; TME, tumor microenvironment; PD-1, Programmed cell death protein-1.

Ethics Approval and Consent to Participate

The study was reviewed and approved by Ethics Committee of Xiangya Hospital of Central South University, which conformed to the ethical guidelines of the 1975 Declaration of Helsinki. The patients have signed their informed consent to participate in this study.

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Disclosure

The authors declare that they have no competing interests in this work.

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