



# Integration of transcriptomics and metabolomics reveals a novel gene signature guided by FN1 associated with immune response in oral squamous cell carcinoma tumorigenesis

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## Abstract

**Purpose** Oral squamous cell carcinomas (OSCCs) are primary head and neck malignant tumours with a high incidence and mortality. However, the molecular mechanisms involved in OSCC tumorigenesis are not fully understood.

**Methods** OSCC and paired para-carcinoma samples were collected and used to perform multi-omics study. Transcriptomic analysis was used to reveal significant alterations in inflammatory and immune processes in OSCC. Ingenuity Pathway Analysis (IPA) combined with the LASSO Cox algorithm was used to identify and optimize a crucial gene signature. Metabolomics analysis was performed to identify the important metabolites which linked to the crucial gene signature. The public data TCGA-HNSCC cohort was used to perform the multiple bioinformatic analysis.

**Results** These findings identified a FN1-mediated crucial network that was composed of immune-relevant genes (FN1, ACP5, CCL5, COL1A1, THBS1, BCAT1, PLAU, IGF2BP3, TNF, CSF2, CXCL1 and CXCL5) associated with immune infiltration and influences the tumour microenvironment, which may contribute to OSCC tumorigenesis and progression. Moreover, we integrated the relevant genes with altered metabolites identified by metabolic profiling and identified 7 crucial metabolites (Glu-Glu-Lys, Ser-Ala, Ser-Ala, N-(octadecanoyl) sphing-4-enine-1-phosphocholine, N-methylnicotinamide, pyrrhocanthinol and xanthine) as potential downstream targets of the FN1-associated gene signature in OSCC. Importantly, FN1 expression is positively correlated with immune infiltration levels in HNSCC, which was confirmed at the single-cell level.

**Conclusions** Overall, these results revealed the differential genetic and metabolic patterns associated with OSCC tumorigenesis and identified an essential molecular network that plays an oncogenic role in OSCC by affecting amino acid and purine metabolism. These genes and metabolites might, therefore, serve as predictive biomarkers of survival outcomes and potential targets for therapeutic intervention in OSCC.

**Keywords** Oral squamous cell carcinomas · Transcriptomics · Metabolomics · IPA analysis · FN1

## Introduction

Oral squamous cell carcinoma (OSCC) is a common type of oral cancer that affects the front two-thirds of the tongue, the gingiva (gums), the floor of the mouth, the lips, the alveolar ridge, the buccal mucosa, the hard palate, the retromolar trigone and other sites (Chai et al. 2020). OSCC is one of the most aggressive head and neck cancers and is characterized by aggressiveness and frequent metastasis within the oral cavity, accounting for ~370 000 new cases and ~170 000 deaths in 2020 (Chaturvedi et al. 2013; Sung et al. 2021). Although advanced treatments such as surgery, radiotherapy, and chemotherapy have improved over time, OSCC patients still have poor prognoses, and the 5-year survival rate has not obviously

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increased (Wu et al. 2018). Thus, it is imperative to clarify the molecular mechanisms underlying OSCC tumorigenesis to improve patient outcomes and develop effective targeted therapeutics.

Novel omics technologies and bioinformatics hold enormous promise for investigating the molecular mechanisms underlying OSCC tumorigenesis (Rahman and Rahman 2018). Regarding the mechanisms of action that underlie cancer progression, transcriptomics can identify broad-scale changes in the expression levels of genes, which may provide valuable information for researchers to elucidate the specific molecular responses to the disease (Dong et al. 2018). As tumours initiate and progress, metabolic alterations in cancer cells must accumulate, which allows them to increase bioenergetic and biosynthetic demand for its proliferation and survival (Martínez-Reyes and Chandel 2021). In the context of tumorigenesis, metabolomics can provide information about system-wide alterations in metabolism and facilitate the identification of early biomarkers (Xiao and Zhou 2017). In cancer tissues, OSCC triggers specific biological events and disrupts global metabolism, which merits investigation using a combination of transcriptomic and metabolomic approaches.

Fibronectin 1 (FN1), a member of the FN family, plays a crucial role in the progression of multiple cancers, such as breast cancer, (Zhang et al. 2020a) colorectal carcinogenesis (Cai et al. 2018), gastric cancer (Zhang et al. 2017), ovarian cancer (Kujawa et al. 2020) and head and neck squamous cell carcinoma (Liu et al. 2020). In HNSCC, FN1 was identified as a potential biomarker that was obviously highly expressed in cancer tissues and positively associated with poor prognosis (Jin and Qin 2020; Qadir et al. 2019). Likewise, Surui Sheng et al. revealed that FN1 played an oncogene role in HNSCC in a manner dependent on hypermethylation and an aberrant immune microenvironment (Sheng et al. 2021). In general, FN1 has been reported as a diagnostic and prognostic marker of HNSCC, but the mechanism by which FN1 drives OSCC carcinogenesis is unknown to date.

In this study, a multi-omics study including transcriptomics and metabolomics analysis was performed to investigate the crucial genetic and metabolic alterations in OSCC tissue (tumour tissue) compared to adjacent noncancerous tissue (Ntumour tissue). Our study sheds new light on the FN1-mediated immune response, which plays a crucial role in cancer progression, and these findings could be beneficial for understanding the underlying regulatory mechanisms of OSCC tumorigenesis.

## Materials and methods

### Chemical reagents

Ammonium acetate (NH4AC) was purchased from Sigma-Aldrich, acetonitrile was purchased from Merck, and ammonium hydroxide (NH4OH) and methanol were purchased from Fisher.

### Sample collection

OSCC and paired para-carcinoma samples were collected from the Department of Oral and Maxillofacial Surgery, the Second Xiangya Hospital of Central South University. Detailed information is shown in Table S1.

### Sample preparation and transcriptomics analysis

Sample preparation and transcriptomics analysis were performed by Shanghai Applied Protein Technology Co., Ltd., as described in a previous study (Chen et al. 2020). Detailed information is shown in Supplementary method 1.

### Sample preparation and metabolomics analysis

The patient tissues (10 pairs of OSCC tissues and para-carcinoma tissues) were quickly frozen in liquid nitrogen immediately after dissection and were sent to Shanghai Applied Protein Technology Co., Ltd., for metabolomics analysis as described in a previous study (Hong et al. 2022). Detailed information is shown in Supplementary method 2.

### Transcriptomic data mining by Ingenuity Pathway Analysis (IPA)

To provide deep insights into the underlying mechanisms of OSCC progression, the differentially expressed genes and metabolites identified by transcriptomics and metabolomics were analysed by IPA (Qiagen) as described in a previous study (Liu et al. 2021). The DEGs with  $P\text{-adj} < 0.05$  and  $\log_2\text{FC} > 2$  or  $\log_2\text{FC} < -2$  and the changed metabolites with  $P\text{ value} < 0.05$  and VIP  $> 1$  were selected for integrated analysis by IPA.

### Cell culture and FN1-siRNA transfection in OSCC cell line

The OSCC cell lines (SCC9 and Cal27) were purchased from the American Type Culture Collection (ATCC, USA). All these cells were cultured in DMEM (Thermo Fisher, 11,995,040) supplemented with 10% FBS (Thermo

Fisher, 10,091,155) and 1% (v:v) penicillin/streptomycin (Sigma–Aldrich, P4333) at 37 °C in a humidified 5% CO<sup>2</sup> atmosphere. FN1 knockdown was performed by small interfering RNAs (siRNAs) synthesized from RiboBio Co., Ltd. (RiboBio, China) using Lipofectamine RNAiMAX (Thermo Fisher, 13,778,075) in SCC9 and Cal27 cells which were performed following the recommendations of the manufacturers. A scramble sequence as negative control (RiboBio, siN0000001-1–5) was used to the control group. The siRNA sequence of the *FN1* is listed in Table S2.

### **Cellular proliferation, migration and invasion assays**

Cell counting kit-8 (Dojindo, CK04) was used to investigate the change of cell proliferation ability after FN1 knockdown in the SCC9 and Cal27 cell lines. CCK-8, cell migration and invasion assays were performed as our previous studies (Fan et al. 2018, 2022).

### **Quantitative real-time PCR detection**

The extraction of total RNA, the synthesis of cDNA, the mRNA expression levels quantified and calculated were completed as our previous report (Fan et al. 2022). Actb was performed as endogenous controls to normalize mRNA expression. The gene primers are listed in Table S3.

### **Analysis of the clinical characteristics of FN1**

Gene Expression Profiling Interactive Analysis (GEPIA) and UALCAN Database Analysis based on the TCGA database were performed to determine the clinical characteristics of FN1 in HNSCC. According to the median FN1 gene expression, a low cut-off (50%) and high cut-off (50%) were used as the expression thresholds for dividing the patients into two groups, as described in a previous study (Chandrashekhar et al. 2017; Tang et al. 2017).

### **Overall survival and progression-free survival analysis using the GEPIA database**

Overall survival (OS) and progression-free survival (RFS) of FN1 in HNSCC were analysed with the GEPIA online database (Tang et al. 2017).

### **Immune infiltration analysis**

To analyse the association between the FN1 expression level and immune infiltration on the basis of TCGA tumour data, we used the Tumour Immune Estimation Resource 2.0 (TIMER2.0) (<http://cistrome.org/TIMER/>) webserver and the TISIDB database (<http://cis.Hku.hk/TISIDB/>) as

described in a previous study (Li et al. 2020; Wang et al. 2022).

### **Tumour Immune Single-Cell Hub (TISCH) database analysis**

To verify the correlation between FN1 expression and immune cell infiltration at the single-cell level, the TISCH database was used as described in a previous study (Sun et al. 2021). An important component of the TISCH database is that it provides detailed cell-type annotations at the single-cell level, which makes it possible to explore the tumour microenvironment (TME) across a variety of cancer types (Sun et al. 2021).

### **LASSO algorithm analysis**

LASSO algorithm analysis was performed as described in a previous study (Wu et al. 2021). The TCGA dataset provides RNA-sequencing expression (level 3) profiles and clinical information for HNSCC. Conversion of counts to TPM was performed, and the data were normalized by log2 (TPM + 1) while keeping samples with intact clinical information ( $n=503$ ). In the analysis, features were selected using the LASSO regression algorithm, tenfold cross-validation was performed, and glmnet was used in the R package. The formula used to calculate the risk value was as follows: risk score =  $\sum_{i=1}^n (\text{coef} \times \exp)$ . The optimal model was selected as the final model. The log-rank test was used to compare differences in survival between groups. Time-ROC (tROC) analysis was used to compare the predictive accuracy of genes and risk scores. First, multifactor Cox regression was used to analyse the data, and then the step function was used for iteration. Using the log-rank test and univariate Cox proportional hazards regression, *p* values and hazard ratios (HRs) with 95% confidence intervals (CIs) were calculated for Kaplan–Meier curves. The analysis methods and R packages were all implemented using R version 3.6.3. A *p* value  $<0.05$  was considered statistically significant.

### **Statistical analysis**

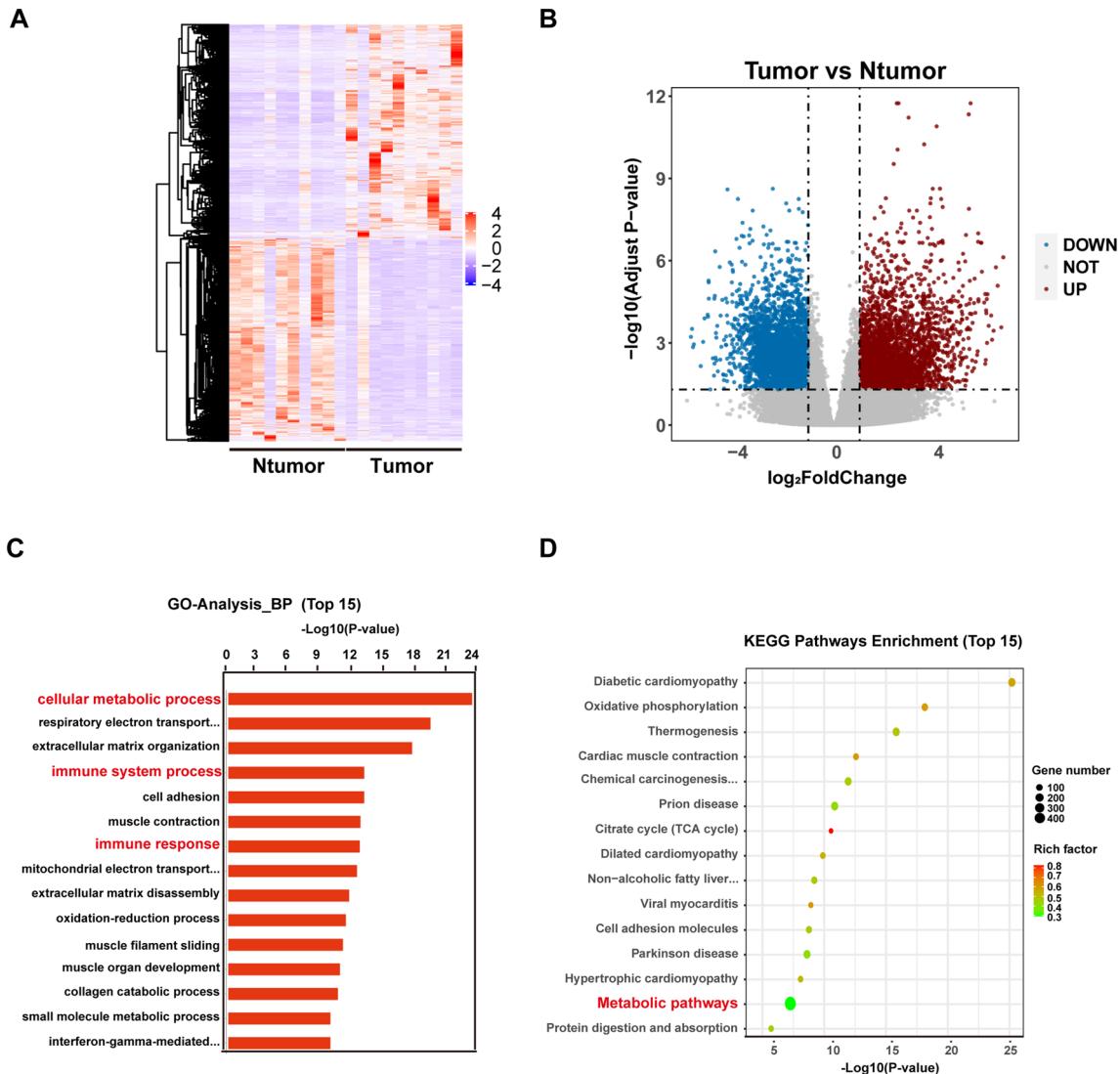
All statistical tests were bilateral and were performed using R version 3.6.3 and Bioconductor. Metabolic pathway analysis was conducted on the MetaboAnalyst website (<http://www.metaboanalyst.ca>). The pathway plots were based on the Kyoto Encyclopedia of Genes and Genomes (KEGG) database. The correlation analysis was performed by Spearman's test.  $P < 0.05$  was considered statistically significant (\* $P < 0.05$ , \*\* $P < 0.01$ ).

## Results

### Transcriptomic profile analysis of OSCC and para-carcinoma tissues

We collected 10 pairs of primary OSCC and para-carcinoma tissues from patients with a mean age of 50.0 years (range from 41 to 64 years, male). The detailed clinical characteristics of the OSCC patients in our study are summarized in Table S1. To explore the genetic alterations that occurred during OSCC tumorigenesis, RNA-seq-based transcriptome analysis was performed to identify the DEGs between the OSCC tumour and Ntumour groups. Of the 42,641 genes profiled, 6151 exhibited

obvious alterations in OSCC with the cut-off criteria  $\log_2\text{FC} > 1$  and  $P\text{-adj} < 0.05$  (Fig. 1A, B). Notably, a total of 3103 obviously upregulated genes and 3048 downregulated genes were filtered and subjected to GO and KEGG enrichment analyses. The results of GO-biological process (BP) enrichment analysis revealed that the DEGs were significantly enriched in specific terms, including cellular metabolic process, respiratory electron transport chain, extracellular matrix organization, immune system process, cell adhesion, and the immune response (Fig. 1C). These results indicated that compared with the adjacent noncancerous tissue, the DEGs of OSCC tissues significantly altered cellular metabolism and immune processes, which may influence cellular energetic metabolism and the tumour microenvironment. Furthermore, the KEGG



**Fig. 1** Transcriptomics profiling of OSCC tissues compared with para-carcinoma tissues. **A** Heatmap of differentially expressed genes between the Ntumour and Tumour groups. **B** Volcano plot of differ-

entially expressed genes between the Ntumour and Tumour groups. **C** GO-BP enrichment analysis results for the DEGs. **D** KEGG pathway enrichment analysis results for the DEGs

enrichment results were similar to the results of GO-BP analysis, which revealed significant clusters in the citrate cycle and metabolic pathways (Fig. 1D).

### Transcriptomic data mining by IPA

IPA is a web-based bioinformatics tool that enables researchers to upload data and integrative analysis results from high-throughput experiments such as microarray, next-generation sequencing, metabolomics and proteomics data (Orlando et al. 2020). The significantly altered genes in OSCC tissues were subjected to IPA. The top modules identified in disease and biological function analysis indicated significantly activated disease or biological processes involving cancer, humoral immune response, inflammatory response, immune cell trafficking, and cellular movement (Fig. 2A, B). Likewise, canonical pathway analysis revealed that the tumour microenvironment (TME) pathway was obviously activated, which indicated that TME changes also played a crucial role in OSCC progression (Fig. 2C). Regulator effect network (REN) analysis is able to predict master upstream regulators that operate through other regulators that regulate disease progression (Krämer et al. 2014). Using REN in IPA with the transcriptomics data as input, we revealed that FN1 was found obviously upregulated and positively regulated a crucial network involved in cell migration and movement (Fig. 2D, E). This network consisted of ACP5, BCAT1, CCL5, COL1A1, CSF2, CXCL1, CXCL5, CXCL8, IFNG, IGF2BP3, MMP1, MMP13, MMP14, MMP3, MMP9, PLAUR, SERPINE1, SLC2A1, SOX9, THBS1, and TNF, which are involved in regulating the progression of inflammatory and immune responses. These findings indicated that the altered genes significantly disturbed the inflammatory and immune response processes and the FN1-mediated network was predicted to be a crucial regulator network for OSCC progression.

### Optimizing and evaluating the clinical value of the crucial FN1-mediated network in HNSCC

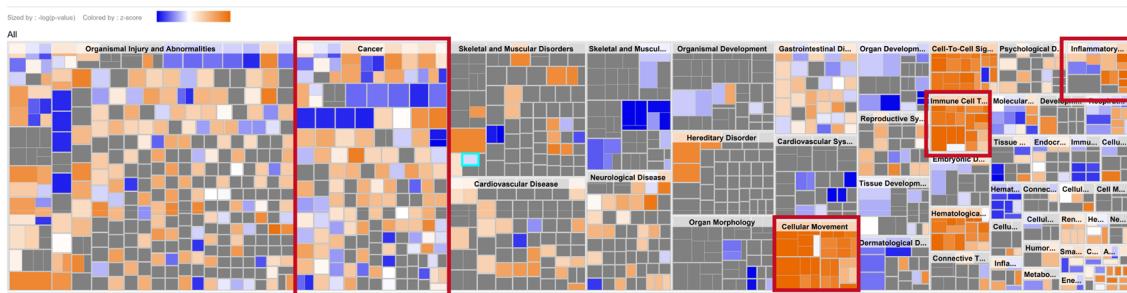
The genes originating from the FN1-mediated network were subjected to the LASSO regression algorithm to identify the optimal gene network associated with the prognosis of HNSCC based on the TCGA database, following a previous study (Zhang et al. 2020b). As a result of LASSO regression analysis, the coefficients of these genes related to the prognosis of patients were calculated: Risk score =  $(-0.0181) \times CXCL5 + (0.0375) \times CXCL1 + (0.0178) \times CSF2 + (0.1034) \times TNF + (0.0037) \times IGF2BP3 + (0.1678) \times PLAUR + (0.0536) \times BCAT1 + (0.0809) \times THBS1 + (-0.0689) \times COL1A1 + (-0.0727) \times CCL5 + (-0.0073) \times ACP5 + (0.0132) \times FN1$ . Figure 3A–C indicates the top associated genes (FN1, ACP5, CCL5,

COL1A1, THBS1, BCAT1, PLAUR, IGF2BP3, TNF, CSF2, CXCL1 and CXCL5) and the analysis results for the risk score, survival time and survival status of the selected dataset. As shown in Fig. 3D, KM survival curves showed that the high-expression group had worse OS than the low-expression group, and the median survival time (LT50) was 2.3 and 5.7 years, respectively. HR (High exp) represents the hazard ratio of the low-expression sample relative to the high-expression sample, and HR (2.26) > 1 indicates a risk factor for these genes (Fig. 3D). Likewise, the AUC value of multiple genes (1 year: 0.645, 3 years: 0.694, 5 years: 0.655) by tROC analysis within a 95% confidence interval indicated good predictive performance in HNSCC (Fig. 3E). In brief, these findings revealed that a twelve-gene signature modulated by FN1 demonstrated good prognostic value and merited further investigation.

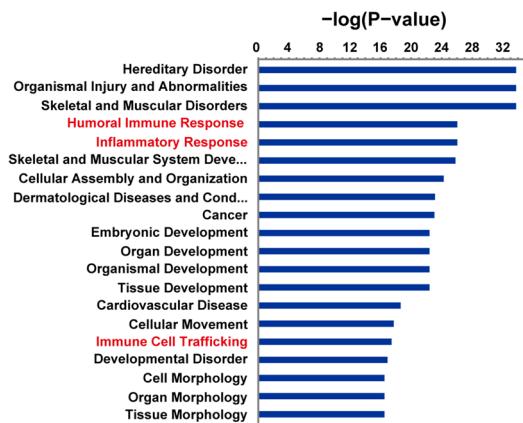
### The crucial metabolites linked to the FN1-mediated crucial network in OSCC

It has become evident that metabolic remodelling facilitates tumorigenesis and metastasis (Bose et al. 2020). We speculated that metabolites linked to the FN1-associated genes signature which may play a crucial role in OSCC initiation and progression. Hence, untargeted metabolomics analysis was performed by high-resolution LC-MS/MS with positive and negative ionization modes. The PCA score plots and permutation test indicated satisfactory system stability and robustness of these analyses (Figs. S1–2). A total of 2044 metabolites were identified in positive (1368 metabolites) and negative (676 metabolites) ionization modes (Figs. S1–2). The VIP value of each variable in the OPLS-DA model represented its contribution to the classification, and a total of 205 metabolites were significantly altered with the following criteria: VIP > 1 and  $p$  value < 0.05 in positive and negative ionization modes (Fig. 4A, B, Figs. S1–2). In addition, these altered metabolites mainly belonged to the organic acid and derivative superclass and were significantly enriched in multiple important pathways, such as central carbon metabolism in cancer, aminoacyl-tRNA biosynthesis, biosynthesis of amino acids, purine metabolism, and alanine, aspartate and glutamate metabolism (Fig. 4C, D). Furthermore, integrated transcriptomics and metabolomics analysis was performed to identify the crucial metabolites that may be regulated by FN1-mediated immune-related genes. A total of 209 differentially expressed metabolites and FN1-mediated genes were subjected to integrated analysis with  $p$  < 0.05, as determined by the Pearson correlation value. As shown in Fig. 5A, we found that the FN1-mediated network was positively linked to 7 metabolites (Table S4), including 3 types of organic acids and derivatives (Glu-Glu-Lys, Ser-Ala, Ser-Ala, N-(octadecanoyl) sphing-4-enine-1-phosphocholine, N-methylnicotinamide, pyrroloanthinol

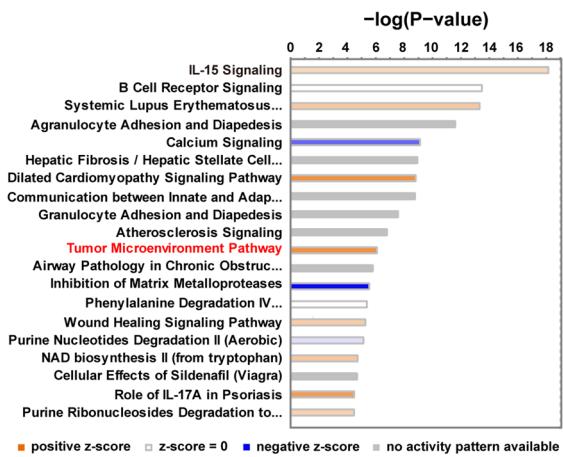
## A Diseases and functions



## **B Diseases and Bio Functions (Top 20)**



## C Canonical Pathway (Top 20)

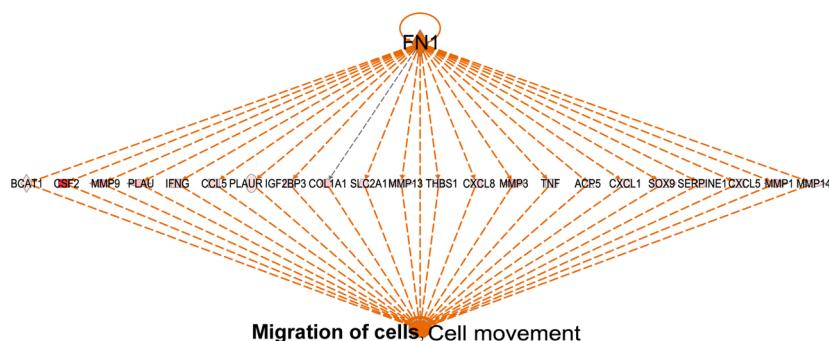


D

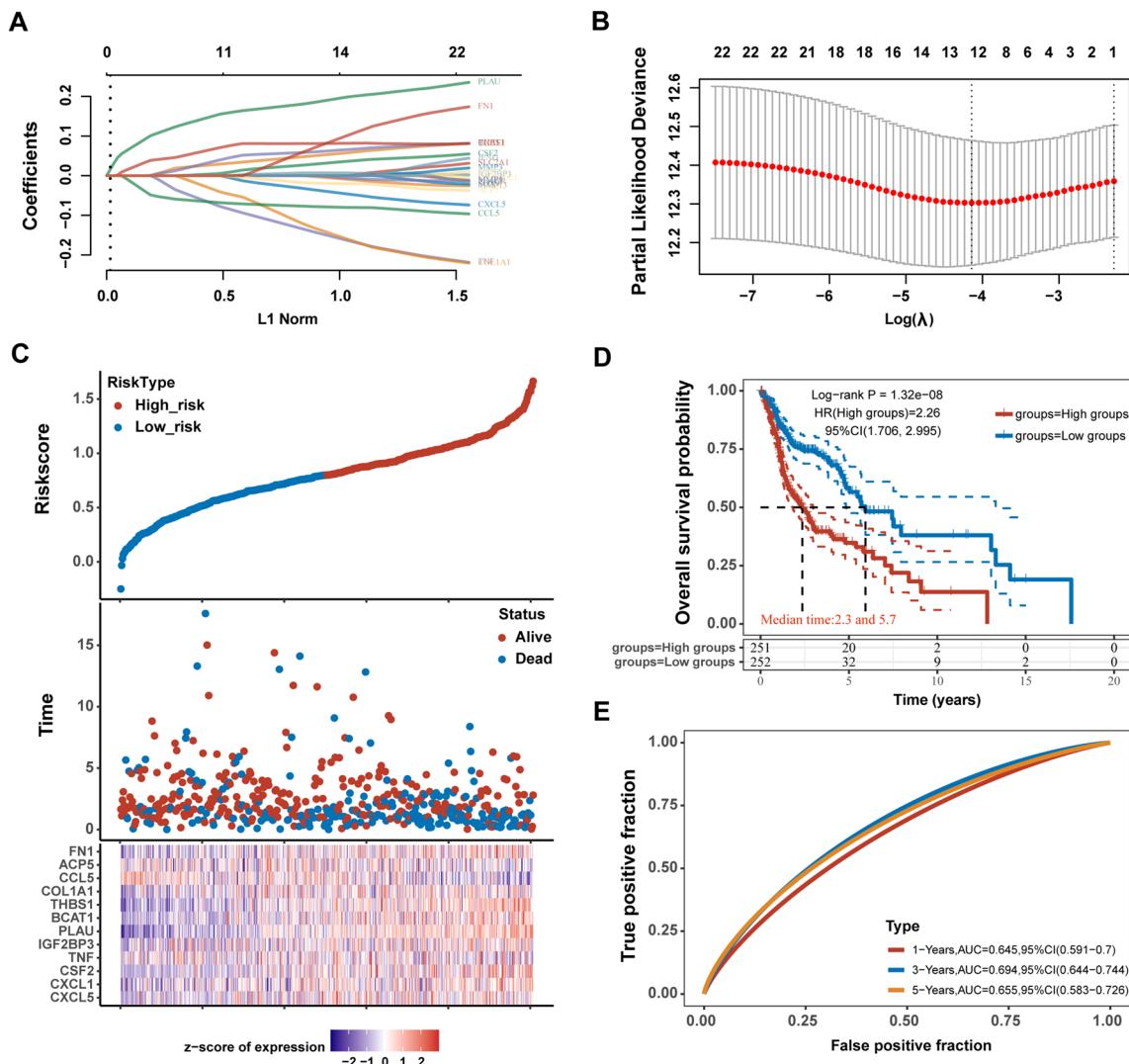
## Regulator Effect Network (TOP5)

Regulators	Target Total	Target Molecules in Dataset	Diseases and Functions
FN1	22	ACP5, BCAT1, CCL5, COL1A1, CSF2, CXCL1, CXCL5, CXCL8, IFNG, IFGBP3, MMP1, MMP13, MMP14, MMP3, MMP9, PLAU, PLAU, SERPINE1, SLC2A1, SOX9, THBS1, TNF	Migration of cells
		ACP5, BCAT1, CCL5, COL1A1, CSF2, CXCL1, CXCL5, CXCL8, IFNG, IFGBP3, MMP1, MMP13, MMP14, MMP3, MMP9, PLAU, PLAU, SERPINE1, SLC2A1, SOX9, THBS1, TNF	Cell movement
FN1	22	CCL17, CCL6, CD80, CD86, CSF2, CXCL1, CXCL10, CXCL6, CXCL8, DEFBA4/DEFB4B, GZMB, IFNG, IGHG1, IL23A, INHBA, IRF1, MMP14, MMP9, MSR1, PTGS2, SERPINE1, SLC7A2, TNF	Activation of cells
		CCL11, CCL17, CCL20, CCL5, CCL7, CSF2, CXCL1, CXCL10, CXCL11, CXCL13, CXCL5, CXCL6, CXCL8, CXCL9, DEFBA4/DEFB4B, MMP14, TNF, VEGFC	Attraction of cells
IL1B	18	CCL11, CCL17, CCL20, CCL5, CCL7, CSF2, CXCL1, CXCL10, CXCL11, CXCL13, CXCL5, CXCL6, CXCL8, CXCL9, DEFBA4/DEFB4B, TNF	Attraction of leukocytes
		CCL11, CCL17, CCL20, CCL5, CCL7, CSF2, CXCL1, CXCL10, CXCL11, CXCL13, CXCL5, CXCL6, CXCL8, CXCL9, DEFBA4/DEFB4B, TNF	Attraction of leukocytes

E



**Fig. 2** IPA analysis of transcriptomic data. **A–B** The results of the top disease and biological function analysis of the DEGs. **C** Top 20 canonical pathways identified. **(D–E)** The results of regulator effect network (REN) analysis with the transcriptomics data as input



**Fig. 3** The clinical characteristics and diagnostic value of the FN1-mediated crucial network. (A–C) The optimized analysis of the candidate genes using the LASSO regression algorithm and the results for risk score, survival time and survival status in the TCGA-HNSCC cohort ( $n=503$ ). (D) KM curves showed that the FN1-mediated

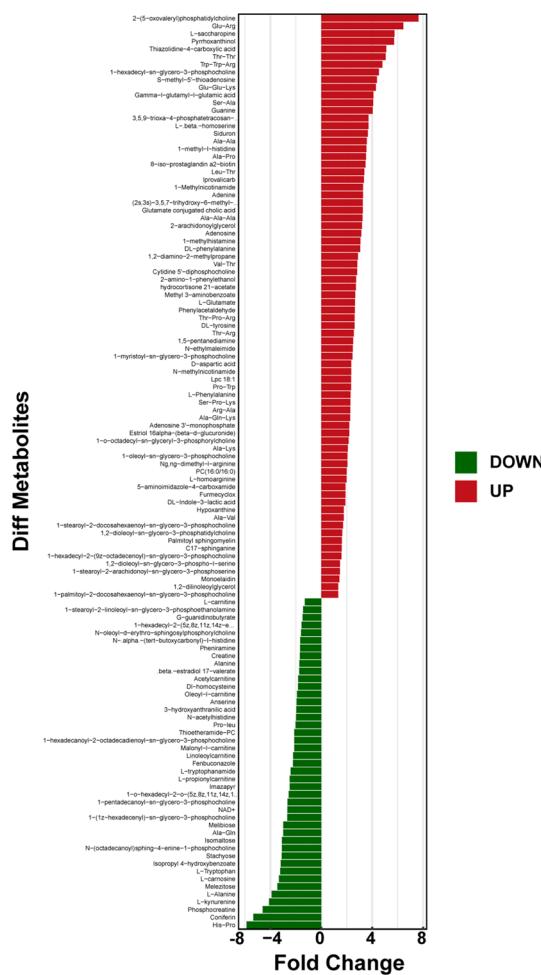
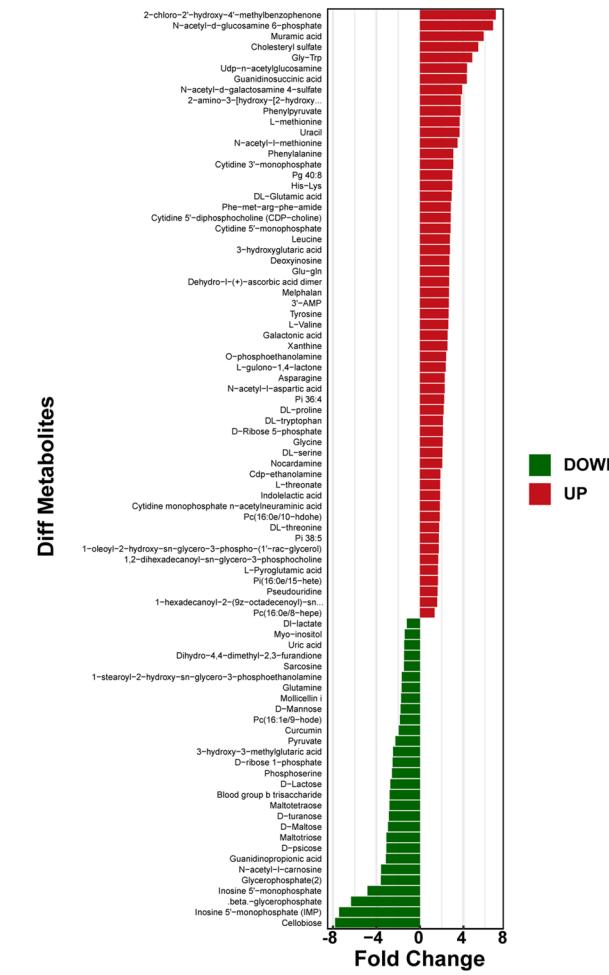
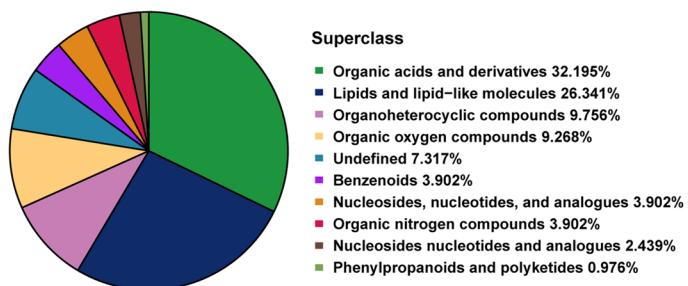
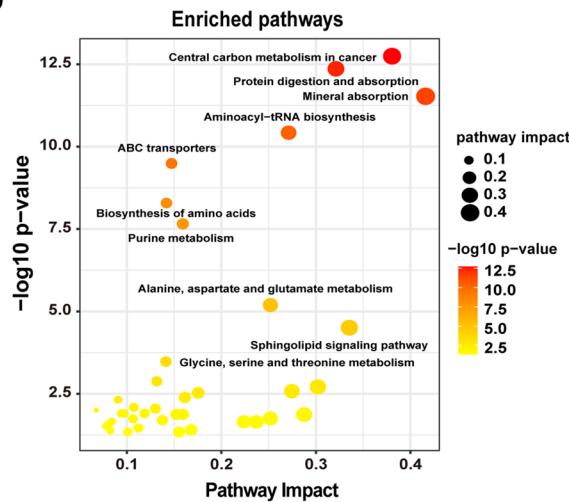
genes were positively associated with worse overall survival in HNSCC ( $n=503$ ). (E) tROC curves showed the predictive potential of the FN1-mediated genes for HNSCC patients based on TCGA cohorts

and xanthine). Except for N-(octadecanoyl) sphing-4-enine-1-phosphocholine, the contents of the other metabolites were obviously increased in OSCC tissues compared to para-carcinoma tissues, as identified by metabolomic profiling (Fig. 5B)P. Above all, these results provide new insight into 7 potential metabolites that are correlated with the FN1-mediated network and play a vital role in OSCC initiation and progression.

#### Analysis of the clinical characteristics of FN1 in HNSCC

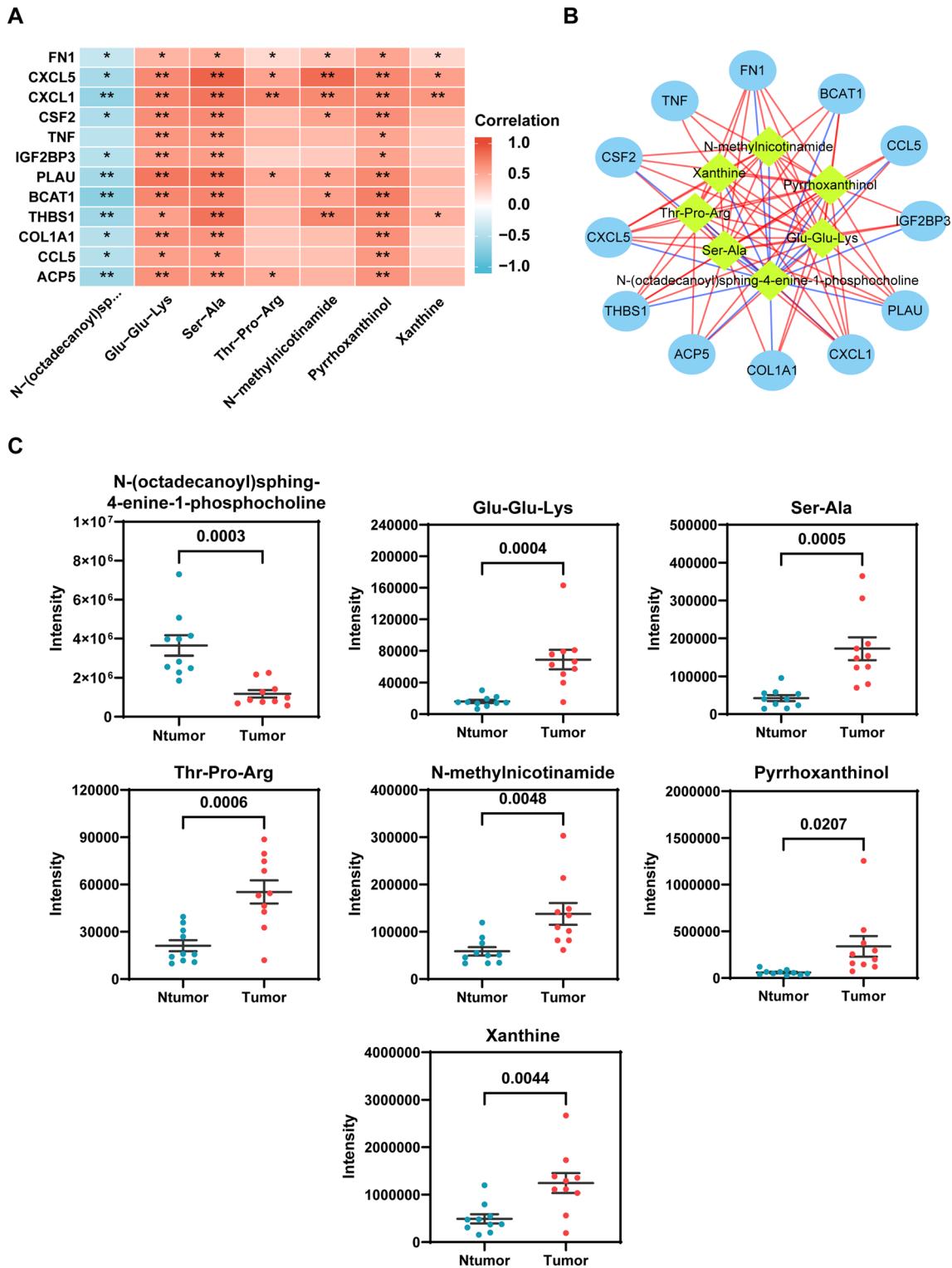
Furthermore, we knocked down FN1 in SCC9 and Cal27 cells to confirm its crucial role in OSCC progression. As

Figure S3A shown, FN1 siRNA-1 was used to perform further study because its excellent inhibition efficiency. FN1 knockdown significantly inhibited the ability of SCC9 and Cal 27 cells in proliferation, migration and invasion (Fig. S3B-D). We further verified the clinical characteristics of FN1 in HNSCC using the TCGA database. As illustrated in Fig. 6A, the transcript levels of FN1 were obviously increased in 15 of the 33 cancer tissues compared to adjacent normal tissue (ACC, BRCA, DLBC, GBM, HNSC, KIRC, LGG, LIHC, PAAD, PCPG, STAD, TGCT, THCA, THYM, and THCA), especially in HNSCC. Furthermore, we investigated the correlation between FN1 expression levels and multiple clinical parameters in HNSCC (age, sex, tumour stage, N stage, M stage and survival outcomes). The results

**A****B****C****D**

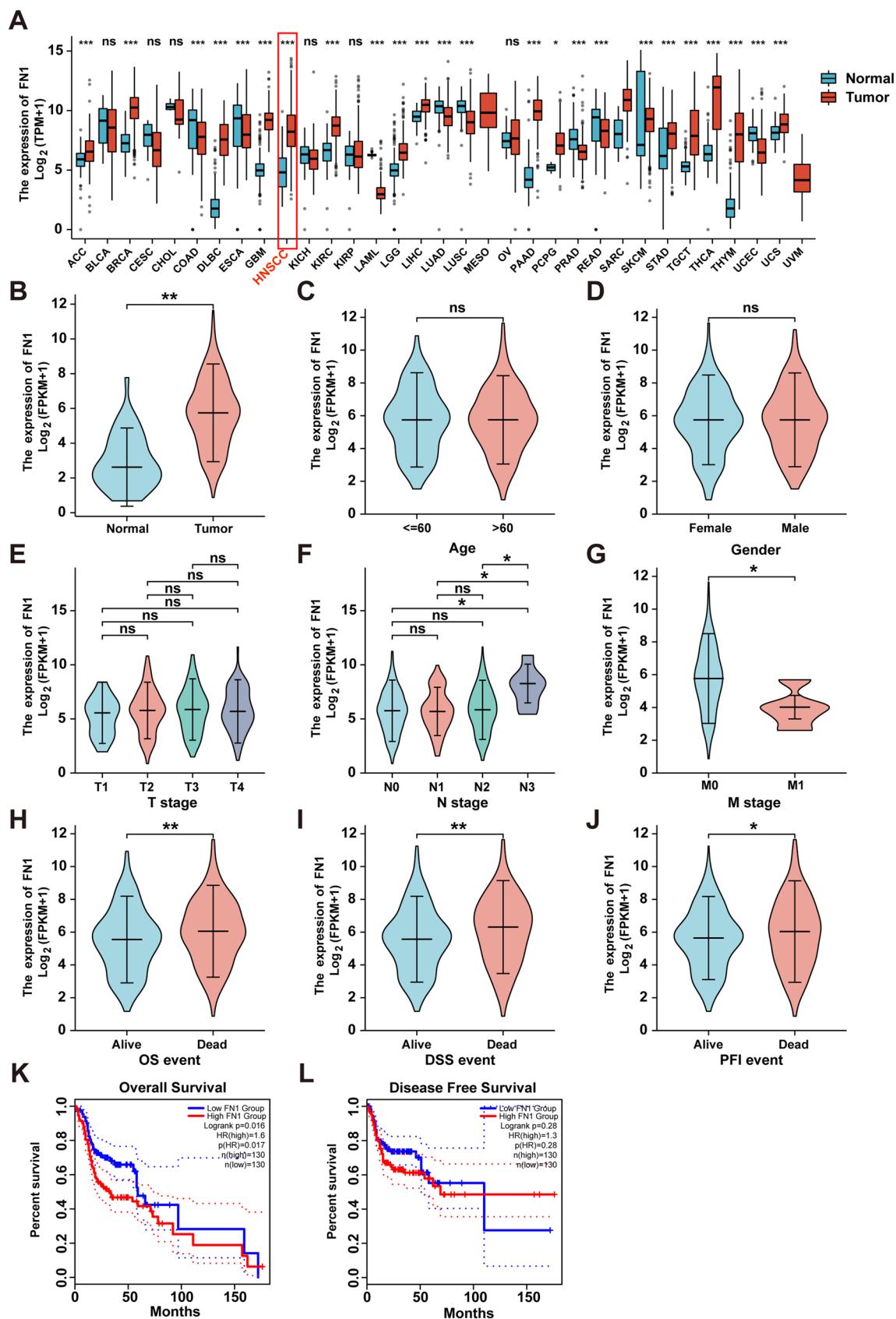
**Fig. 4** The crucial metabolites positively related to the FN1-mediated network in OSCC. The bar plot shows the fold changes of altered metabolites in positive ionization **A** or negative ionization mode

**(B).** **C** The classification of total altered metabolites in OSCC. **D** The most important metabolic pathways of the altered metabolites enriched in OSCC



**Fig. 5** Integrated metabolomics and transcriptomics analysis identified crucial metabolites that were positively linked to the FN1-mediated crucial network. **A** FN1 associated gene signature positively

linked to 7 metabolites. **B** Network diagram of the FN1-mediated network and relevant metabolites in OSCC. **C** The contents of the 7 metabolites in OSCC tissues or para-carcinoma tissues



**◀Fig. 6** The clinical characteristics of FN1 in cancers. **A** FN1 mRNA expression in various cancers, including HNSCC. **B** FN1 expression levels in HNSCC tissue and normal tissue from the TCGA dataset. **C–G** FN1 expression level in HNSCC according to age, sex, different T stages, different N stages and different M stages. **(H–J)** FN1 expression level in HNSCC according to OS events, DSS events, and PFI events. \* $P < 0.05$ ; \*\* $P < 0.01$  were considered statistically significant. OS, overall survival; DSS, disease-specific survival; PFI, progression-free interval; ns, no significance. Kaplan–Meier estimates of overall survival (OS) (**K**) and recurrence-free survival (RFS) (**L**) according to GEPIA,  $n=260$  containing atypical subtypes, basal subtypes, classical subtypes, mesenchymal subtypes

indicated that a higher FN1 mRNA expression level was observed in patients in the lymph node stage (N stage) and pathologic metastasis stage (M stage) related to OS, DSS and PFI events, but no notable difference was found in the associations of FN1 mRNA expression levels with age, sex and advanced tumour stage (T stage) (Fig. 6B–J). Kaplan–Meier plotter analysis indicated that a high expression level of FN1 was positively associated with poor prognosis related to OS but not RFS (Fig. 6K, L). Taken together, these results demonstrated that the augmentation of FN1 was positively related to HNSCC progression and could be considered a potential therapeutic target for OSCC.

### FN1 expression is positively correlated with immune infiltration levels in HNSCC

Several studies have shown that immune cell infiltration is a significant component of the tumour microenvironment, promoting cancer initiation, development, and metastasis (Sokratos et al. 2017). Hence, we investigated whether FN1 expression was correlated with immune infiltration levels in HNSCC. We first quantified the infiltration levels of 24 immune cell types in each of the HNSCC samples ( $n=546$ ) using single-sample gene set enrichment analysis (ssGSEA) with the R package gsva in TCGA. The results indicated that the expression level of FN1 was significantly correlated with the abundance of multiple infiltrating immune cells, with the strongest association observed for infiltrating macrophages (Fig. 7).

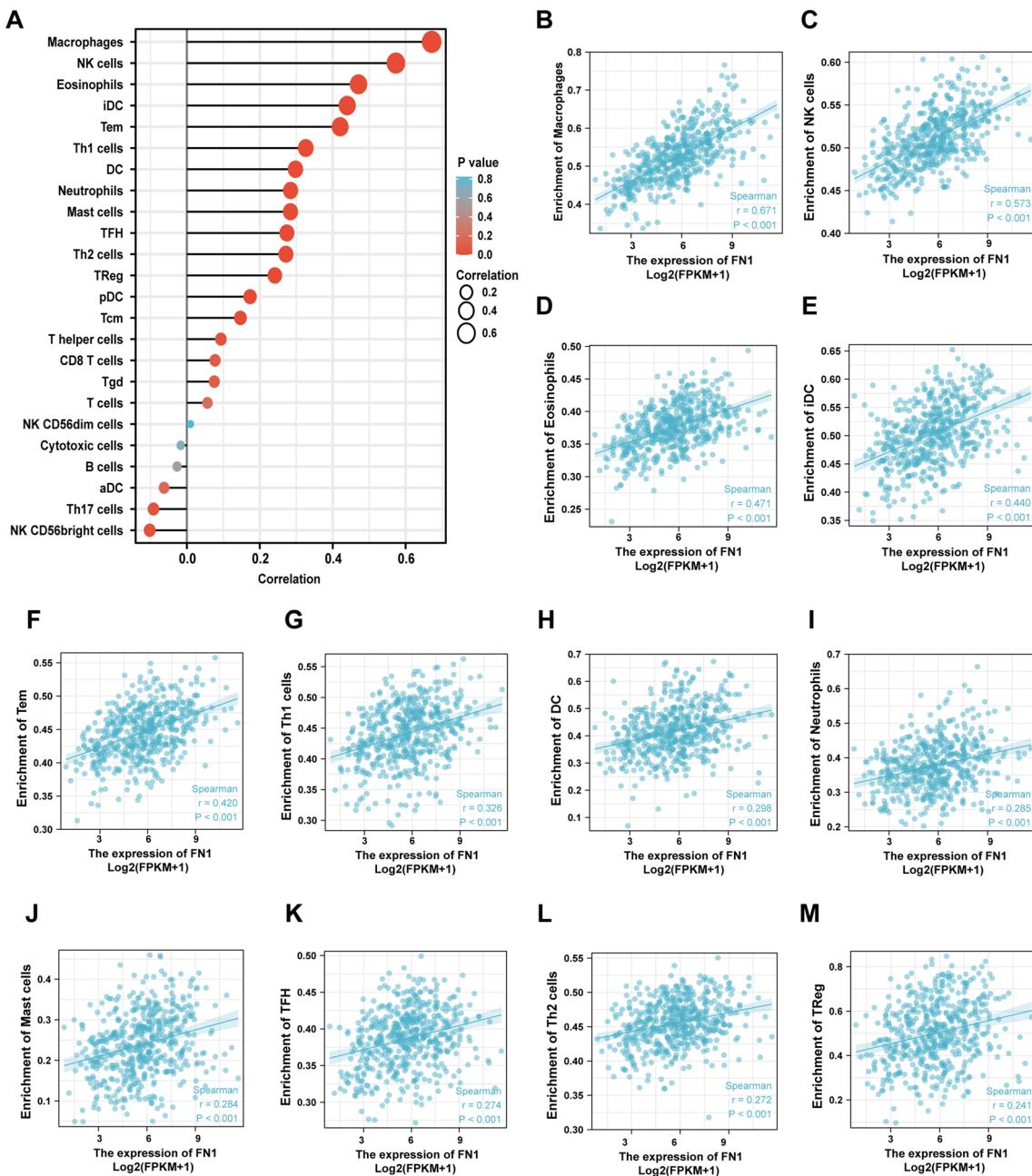
Given the important role of macrophage infiltration in HNSC malignancy, we further validated and analysed the correlation between FN1 expression levels and immune infiltration at single-cell resolution with the HNSC-GSE139324 dataset in the Tumour Immune Single-Cell Hub (TISCH) database. UMAP and bar charts revealed that FN1 expression is positively associated with multiple immune cell types, which was in line with the above results (Fig. 8A, B). As we predicted, monocytes/macrophages were the second most abundant immune cells associated with the FN1 expression level, behind only mast cells (Fig. 8C). Notably, FN1 expression was strongly associated with the infiltration of cluster 17 macrophages and monocytes, which indicated

the heterogeneity of tumour-associated macrophages (TAMs) in HNSCC (Fig. 8D, E).

## Discussion

Among malignant tumours of the head and neck, OSCC is the most common, and overall survival rates remain below 50% after 5 years (Chamoli et al. 2021). Searching for the underlying mechanisms and essential biomarkers of OSCC provides a crucial approach to identify therapeutic options for its treatment. In this study, transcriptomic analysis integrated with IPA revealed the significantly altered inflammatory and immune process pathways and identified a FN1-mediated crucial network. Similarly, global nontargeted metabolomics analysis was performed to investigate the crucial changes in metabolic phenotypes associated with OSCC and screen the important metabolites that may be regulated by the FN1-mediated network in OSCC tumorigenesis and progression. This study not only explored crucial genetic and metabolic pathways but also identified an essential FN1 associated gene signature that showed significant value for predicting prognosis and may serve as a potential target for OSCC treatment.

In recent years, research using omics approaches has been spurred by advances in technology that have allowed systematic, quantitative characterization of the molecular machinery involved in disease progression (Advani et al. 2017; Reel et al. 2021). In our study, GO-BP and KEGG enrichment analyses revealed that the DEGs were significantly enriched in immune-related processes (immune system process, immune response), pathways related to energy metabolism (respiratory electron transport chain, oxidative phosphorylation, mitochondrial electron transport), and extracellular matrix organization and disassembly, which was similar to the previous report (Serafini et al. 2020). In particular, we used IPA, a powerful bioinformatic tool for the resolution of omics data analysis, and verified the critical pathway signatures associated with OSCC tumorigenesis, which were enriched in inflammatory and immune processes, the TME and metabolic pathways (Fig. 2). These signatures have been reported in various types of carcinomas, including oral cancer, which verified the reliability of our study (Fraga et al. 2021; Greten and Grivennikov 2019). Wenchao Gu et al. reported the regulatory mechanism of ferroptosis-related immune activation in the TME during OSCC progression (Gu et al. 2021). Consistent with Wenchao Gu's findings, we also found an essential role of the immune response and TME activation in OSCC but showed a different mechanism. We identified a critical network modulated by FN1 that ranked first in Regulator Effect Networks in IPA analysis and involved members of the MMP family, cytokines, chemokines, and other inflammation-related genes (Fig. 2).



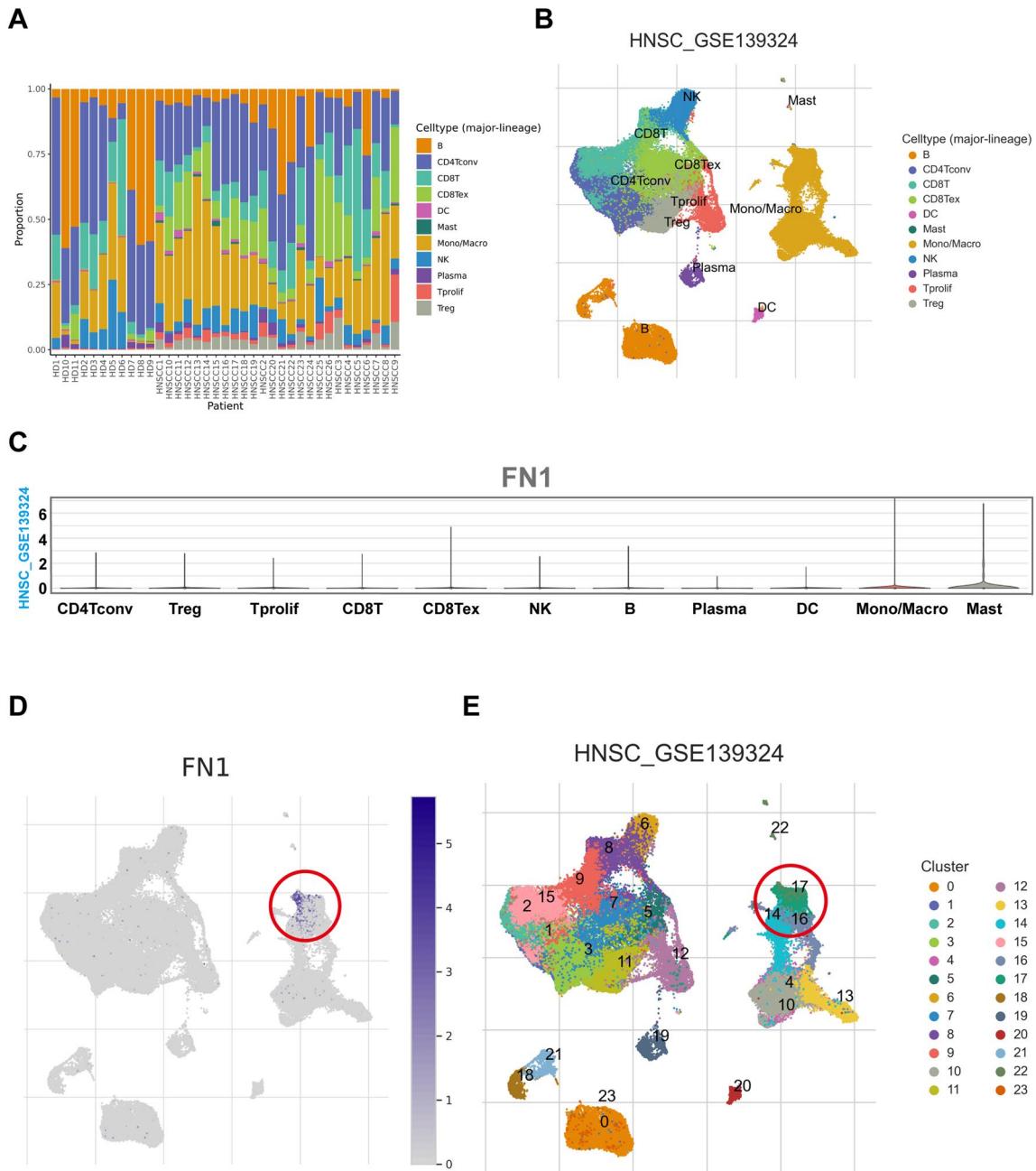
**Fig. 7** Correlation between FN1 expression and immune infiltration levels in HNSCC. **A** Correlation between FN1 expression and the 24 infiltrating immune cell types in the TCGA-HNSCC cohort. **B–M**

Correlations between FN1 expression and the infiltration level of different immune cells were analysed by Spearman correlation test

To the best of our knowledge, this is the first report of an FN1-mediated immune response network that plays an oncogenic role and deserves further investigation.

The glycoprotein fibronectin 1 (FN1) is found on the surface of cells and in the extracellular matrix in both dimeric and multimeric forms (Zollinger and Smith 2017). In previous studies, FN1 was reported to play an oncogenic role and facilitate tumour development, drive extracellular matrix remodelling, and promote epithelial-to-mesenchymal

transition (EMT) and metastasis in various cancers (Li et al. 2019; Liu et al. 2020; Song et al. 2021). In HNSCC, we found that FN1 expression levels were obviously upregulated and positively related to poor prognosis and immune infiltration levels, which was consistent with a previous report on HNSCC (Sheng et al. 2021). Those authors also reported that FN1 expression was closely related to multiple immune cell types, but we further verified that FN1 expression was not only positively associated with macrophage infiltration



**Fig. 8** FN1-associated immune cell type distribution using the TISCH database. **A–B** Bar charts and UMAP plot showing the alterations of cell type distribution in the TME based on the HNSC-GSE139324 dataset. **C** Violin plot showing the distribution of FN1 expression in different cell types in the HNSC-GSE139324 dataset.

**D** Distribution of FN1 in different cells in the HNSC-GSE139324 dataset. **E** The cluster classifications of the TME associated with FN1 expression and immune infiltration of cluster 17 in the HNSC-GSE139324 dataset

but also significantly related to cluster 17 macrophages using the TISCH-HNSCC cohort (Fig. 8). These findings confirmed the relationship between FN1 and immune cell infiltration at the single-cell level and further revealed the heterogeneity of TAMs affected by FN1, which identified a favourable target for the precise treatment of HNSCC. Similarly, Linfeng Wu reported that FN1 was significantly

positively associated with M1 macrophages in thyroid cancer (Wu et al. 2021), which indicated that FN1 might be widely linked to macrophage polarization during the progression of multiple tumours.

Furthermore, we chose LASSO regression analysis to further optimize the FN1-mediated network and confirmed its prognostic value for OS using the TCGA-HNSCC cohort.

A prognostic twelve-gene signature was ultimately obtained through LASSO and served as an independent prognostic factor in HNSCC patients (Fig. 3). The genes signature consisted of FN1, ACP5, CCL5, COL1A1, THBS1, BCAT1, PLAU, IGF2BP3, TNF, CSF2, CXCL1 and CXCL5 (Fig. 3). C–C motif chemokine ligand 5 (CCL5) was reported to activate AKT signalling to recruit and repolarize TAMs, which promoted the development of malignant PTs (Nie et al. 2019). Meng Xiao et al. found that THBS1 derived from OSCC exosomes polarized macrophages into an M1-like state, which facilitated malignant migration in OSCC (Xiao et al. 2018). TNF- $\alpha$  is a major proinflammatory cytokine found in the TME of breast cancer patients that is primarily produced by tumour-associated macrophages and is involved in epithelial-to-mesenchymal transition (EMT) and metastasis of breast cancer (Cruceriu et al. 2020). Colony-stimulating factor 2 (CSF-2) was found to promote macrophage-derived CXCL8 secretion, which determines immune evasion in gastric cancer (Lin et al. 2019). Several studies have indicated that the expression of the proinflammatory chemokine CXCL5 is promoted by TAM activation, which induces an immunosuppressive tumour microenvironment in nonmelanoma skin cancers (Fujimura and Aiba 2020). These reports substantiated the relationship between these genes and TAMs, which indicated that the twelve-gene signature not only has good diagnostic value but also represents a potential target for treatment strategies for OSCC patients.

Metabolic remodelling is an important characteristic of tumour cells, and metabolite alterations usually play a fundamental role in tumour initiation and progression (Lyssiotis and Kimmelman 2017; Yang et al. 2020). Hence, we performed metabolomics analysis and investigated the perturbations of metabolism in OSCC tissues. Our study revealed that the altered metabolites were obviously enriched in several pathways, such as central carbon metabolism in cancer, aminoacyl-tRNA biosynthesis, biosynthesis of amino acids, purine metabolism, and alanine, aspartate and glutamate metabolism (Fig. 4). Interestingly, Lihua Zuo et al. reported similar alterations in metabolic pathways from the plasma of 73 patients with primary OSCC, which were enriched in citrate cycle metabolism, purine metabolism, alanine, aspartate and glutamate metabolism, pyrimidine metabolism, and sphingolipid metabolism (Zuo et al. 2021). These results indicated that the perturbation of purine metabolism and alanine, aspartate and glutamate metabolism might be hallmarks of OSCC initiation and progression, and the differences in metabolite alterations in tissues and plasma indicated differential characteristics and functions in OSCC patients. Similarly, Chia-Wei Hsu et al. revealed disturbance of the polyamine pathway and identified potential metabolic biomarkers of OSCC using integrated metabolomics and transcriptomics analysis (Hsu et al. 2019). Unlike these previous studies, we speculated that metabolite changes

and perturbations of metabolic pathways mediated by gene alterations could also be considered crucial mechanisms for tumour occurrence and development. Hence, we integrated the altered metabolites with the FN1-mediated gene network and revealed 7 crucial metabolites positively linked to the twelve-gene signature, which may be involved in OSCC tumorigenesis and metastasis (Fig. 8). Xanthine is involved in purine metabolism, which has been identified in multiple cancers and deemed a potential target for cancer therapy (Hsu et al. 2019). N-Methylnicotinamide is generated by the methylation of nicotinamide catalysed by nicotinamide N-methyltransferase (NNMT), which is a master metabolic regulator of cancer-associated fibroblasts for tumour initiation and progression (Eckert et al. 2019; Gao et al. 2019). Hence, we concluded that the 7 metabolites might be positively related to OSCC initiation and development, which is regulated by the FN1-associated twelve-gene network.

However, it must be acknowledged that several limitations should be taken into consideration when interpreting the results. The number of cases was small, and 7 crucial metabolites linked to the FN1-mediated molecular network need to be verified in further studies.

## Conclusions

In brief, integrated transcriptomics and metabolomics analysis of OSCC tissues and para-carcinoma tissues revealed that the oncogenic role of the FN1-mediated immune response may contribute to the biosynthesis of amino acids and purine metabolism during OSCC tumorigenesis, which provides new insights for the identification of new prognostic markers and potential therapeutic targets for OSCC.

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**Author contributions** TFF: conceptualization; YCP: data curation, formal analysis, methodology, validation, visualization, and writing—original draft; DHY and XXL: data curation, formal analysis, methodology, validation, and visualization; KW, WL, YXH, XYL, ZHR, and XY: data curation and investigation; TFF, ZYZ, and SZ: formal analysis, funding acquisition, project administration, supervision, validation, visualization, writing—review and editing. All authors have read and approved the final version of this manuscript.

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**Availability of data and materials** The datasets presented in this study can be found in online repositories. The names of the repository/repositories and accession number(s) can be found below: <https://www.ncbi.nlm.nih.gov/>, PRJNA876085 for transcriptomics raw data.

## Declarations

**Conflict of interest** None.

**Informed consent** Informed consent was obtained from all subjects involved in the study.

**Institutional review board statement** OSCC and paired para-carcinoma samples were collected from the Department of Oral and Maxillofacial Surgery, the Second Xiangya Hospital of Central South University. Informed consent was obtained from all participants in writing. The protocols were approved by the Clinical Research Ethics Committee of the Second Xiangya Hospital of Central South University (NO. JBWKQA001), and the provisions of the Helsinki Declaration were followed when conducting the research.

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