Elevated Expression of *MKRN3* in Squamous Cell Carcinoma of the Head and Neck and Its Clinical Significance

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Abstract

Background: Squamous cell carcinoma of the head and neck (SCCHN) is one of the most common types of cancer and is a relevant cause of cancer-related deaths. Our previous study has revealed that makorin ring finger protein 3 (*MKRN3*) may serve as a key regulator of the SCCHN tumorigenesis, but its specific role in SCCHN progression has never been reported.

Method: TCGA data analysis and qPCR were used to quantify the level of *MKRN3* in SCCHN and its clinical significance was further analyzed. Immunohistochemical staining or immunoblotting analysis was carried out to detect protein expression.

Results: *MKRN3* was ectopically expressed between cancerous and noncancerous SCCHN tissues, and its expression level was tightly associated with high T classifications and advanced clinical stages. qPCR analysis revealed that *MKRN3* was upregulated in the SCCHN cell line. Moreover, Kaplan–Meier and Cox regression analyses indicated that SCCHN patients with high *MKRN3* expression had poorer prognosis and that *MKRN3* was a potential prognostic factor for SCCHN. Using gene ontology and Kyoto Encyclopedia of Genes and Genomes analyses, we determined that *MKRN3* may be involved in the regulation of synthesis and metabolism, cell growth, death and motility, and cancer pathways associated with SCCHN progression. Mechanism investigation further revealed that P53, as a potential target of *MKRN3*, may implicate in the SCCHN tumorigenesis mediated by *MKRN3*.

Conclusions: *MKRN3* is a valuable predictive biomarker and potential therapeutic target in SCCHN.

Background

Squamous cell carcinoma of the head and neck (SCCHN) is a histopathological diagnosis that encompasses epithelial malignancies that arise in the paranasal sinuses, nasal cavity, oral cavity, pharynx, and larynx [1]. Despite advances made in overall oncology treatment strategies, limited improvement in 5-year survival of SCCHN patients was made during the recent decades. The unfavorable outcome has mainly been attributed to late diagnosis, loco-regional recurrences, and cervical lymph node metastasis [2]. Thus, it is important to investigate the pathogenesis of SCCHN and identify molecular biomarkers with prognostic significance in order to enable optimized therapeutic strategies and prolong patient survival when feasible.

In our previous study, we identified *MKRN3* as a candidate regulator of SCCHN tumorigenesis using prediction algorithms [3]. *MKRN3*, an imprinted gene located on the long arm of chromosome 15 (Prader–Willi region), encodes makorin ring finger protein 3, which is involved in gene transcription and ubiquitination [4]. The MKRN3 protein has four zinc finger domains, namely three C3H1 motifs and one C3H4 ring finger with presumed E3 ubiquitin ligase activity [5]. Researchers have initially found that *MKRN3* deficiency causes central precocious puberty in humans [6]. Thus, the biological functions of *MKRN3* were further investigated. At present, *MKRN3* has been determined to be a novel imprinted gene

involved in the progression of osteosarcoma [7]. However, the specific role of *MKRN3* in SCCHN is yet to be determined, particularly with respect to clinical outcomes.

Therefore, our study aimed to investigate the clinical relevance of *MKRN3* expression in SCCHN. In the present work, the expression of *MKRN3* in SCCHN tissue samples and cell lines has been investigated for the first time. Further experiments were performed to assess whether *MKRN3* expression is correlated with clinicopathological parameters and gain insight into the biological pathways and mechanisms regulated by *MKRN3* that are involved in SHNCC pathogenesis.

Materials And Methods

Data Acquisition and Processing

A workflow chart of this study is shown in Figure 1. The expression profiles and clinical information of 522 SCCHN and 44 adjacent noncancerous epithelial samples were obtained from The Cancer Genome Atlas (TCGA) database. The main clinical and pathological parameters of SCCHN patients are summarized in Table 1. $P<0.05$ was set as the significance threshold of differential expression and clinical outcome. The immunohistochemistry (IHC) data was downloaded from Protein Atlas (https://www.proteinatlas.org). IHC evaluation was based on the staining intensity (0–3) and degree (0–4). The expression of the *MKRN3* protein was classified into two groups: high set (score: 4–7) and low set (score: 0–3).

Cell Culture

SCCHN cell lines were kindly provided by professors at a well-known university or purchased from ATCC as previously described [8-10]. Human-derived dysplastic oral keratinocytes, served as a normal cell line, were grown in RPMI 1640 medium. Tu686 cells were cultured in Dulbecco's modified Eagle medium (DMEM)/F12 medium. Fadu and CAL27 cells were maintained in DMEM basic medium, and JHU011 and Tca8113 cells were kept in RPMI 1640 medium. The medium for all cells was supplemented with 10% fetal bovine serum, and all cells were maintained in a humidified incubator at 37°C and 5% CO_2.

SCCHN Patient Samples

A total of 25 SCCHN tissues and 23 noncancerous tissues were collected from patients in our hospital from September 2015 to December 2017（Supplementary Table 5）. The study was approved by Research Ethics Committee of Xiangya Hospital, Central South University, Changsha, China, and all samples were used for analysis under written informed consent from the patients.

Quantitative Reverse Transcriptase Polymerase Chain Reaction

The total RNA was isolated from SCCHN tissues and cell lines using the TRIzol reagent (Invitrogen, Carlsbad, CA, USA), and cDNA was reverse transcribed using the All-in-One™ mRNA cDNA synthesis Kit (GeneCopoeia, Rockville, MD, USA) following the manufacturers' protocols. Relative *MKRN3* expression values were calculated using the $2^{-\triangle\triangle CT}$ method and normalized with GAPDH as a reference [11, 12]. The primers are listed in Supplementary Table 1.

Western blotting assay

Total cell protein was lysed and extracted using RIPA buffer, and separated in 8-12% SDS-PAGE gels and transferred onto PVDF membranes (Millipore, Bedford, MA, USA). Then membranes were blocked with 5% skimmed milk and incubated with primary antibodies at 4°C overnight. GAPDH was used as a loading control. The proteins images were gained by Image Lab 4.1 (Bio-Rad, Hercules, CA, USA) with enhanced chemiluminescence reagents. The relevant antibody information were listed in Supplementary Table S2.

Functional Enrichment Analyses

Protein–protein interaction analysis (PPI), gene ontology (GO) biological process, and Kyoto Encyclopedia of Genes and Genomes (KEGG) pathway enrichment analyses were employed in STRING (http://string-db.org) online database to identify over-represented GO terms in biological processes as well as over-represented KEGG pathway terms. For this analysis, a false discovery rate (FDR) <0.05 and -log FDR >1.301 were considered to indicate statistical significance.

Statistical Analysis

All data were analyzed using SPSS version 22.0 (IBM Corp., Armonk, NY, USA). Results are presented as mean±standard deviation. Statistical comparisons of the two groups were determined using Student's t-test (for equal variances) or Mann–Whitney U test (for unequal variances). Additionally, survival curves were plotted using the Kaplan–Meier method and compared using the log-rank test. *P* values <0.05 were considered statistically significant.

Results

Elevated Expression of *MKRN3* in SCCHN

TCGA sequencing data were used to delineate differential expression scatterplots and paired difference analyses. As a result, *MKRN3* was markedly higher in SCCHN samples than in corresponding adjacent

noncancerous tissues (Figure 2A; *P*<0.01). The same results were recapitulated in 40 paired cases (Figure 2B; *P*<0.01). Next, the relative expression of *MKRN3* was quantified in 25 samples of SCCHN and in 23 samples of adjacent epithelium using qPCR. Our data revealed that the expression level of *MKRN3* in most cases of SCCHN was upregulated compared with that in the normal tissues (Figure 2C; *P*<0.01). Further analysis also showed that the expression level of *MKRN3* was greater in SCCHN than in control cell lines (Figure 2D). Finally, we collected the IHC data for *MKRN3* on SCCHN specimens to investigate whether the *MKRN3* protein level was altered. As shown in Figure 2E-F, the density and intensity of *MKRN3* expression in SCCHN tumor tissues were significantly increased compared with those of para-carcinoma tissues. Together, these results suggest that *MKRN3* expression is elevated in SCCHN.

Correlation between Clinicopathological Features and *MKRN3* Expression in SCCHN

As summarized in Table 2, a high expression of *MKRN3* was closely associated with smoking, advanced clinical stages, and high T classifications in patients with SCCHN (Table 2; all *P*<0.05). These data establish the oncogenic role of *MKRN3* in SCCHN.

A High Expression Level of *MKRN3* Predicts a Worse Prognosis in Patients with SCCHN

The survival analysis indicated that patients with high expression of *MKRN3* had a worse prognosis than those with low expression of *MKRN3* in terms of overall survival including 520 cases (Figure 3A; *P*<0.01) and disease-free survival including 389 cases (Figure 3B; *P*<0.05). Furthermore, the subgroup analysis revealed that high expression of *MKRN3* was an unfavorable factor for the prognosis in SCCHN patients at stages III+IV (Figure 4A; *P*<0.001) and T3+4 (Figure 4B; *P*<0.05), but not in the N (Figure 4C, *P*>0.05) or M classification (Figure 4D, *P*>0.05). Univariate Cox regression analyses revealed that age, sex, clinical stage, lymph node metastasis, T classification, and status of *MKRN3* expression were significantly associated with overall survival (Table 3; all *P*<0.05), whereas clinical stage, metastasis, T classification, and status of *MKRN3* expression were significantly associated with disease-free survival (Table 4; all *P*<0.05). Furthermore, metastasis, T classification, and *MKRN3* expression level were determined to be independent factors with prognostic value for the overall survival (Table 3) and disease-free survival (Table 4) in patients with SCCHN in the multivariate Cox regression analyses. Additionally, the forest plot analysis of overall survival showed statistically significant associations between age, clinical stage, lymph node metastasis, T classification metastasis, and *MKRN3* expression and the outcome in SCCHN patients (Figure 5; all *P*<0.05). Collectively, these findings indicate that *MKRN3* is a valuable biomarker in the surveillance of prognosis in patients with SCCHN.

Functional analysis of *MKRN3*

The functional enrichment clustering of *MKRN3* showed that A total of 63 categories from GO biological process, such as DNA synthesis and repair, cellular response to tumor microenvironment, regulation of cellular cycle, and translation were identified to be important in the development of cancer (Figure 6A; Supplementary Table 3). The KEGG analysis based on these nodes revealed that 17 pathways in cancer, RNA synthesis and metabolism, cell growth, death, and motility that were identified to be significant to SCCHN progression were regulated by *MKRN3* (Figure 6B; Supplementary Table 4). These factors were closely related to the occurrence and development of cancer, suggesting that *MKRN3* expression exhibited a close relationship with the progress of SCCHN. Further PPI analysis of *MKRN3* illustrated that there were mainly 31 nodes based on a combined score ≥0.7 in the STRING analysis, and P53 might be a direct target gene of MKRN3 (Figure 7A). Therefore, we used homologous modeling and molecular docking for *MKRN3* and P53. As shown in Figure 7B, the Ring finger domine of *MKRN3* were supposed to form like a “Goldfish” and tightly bind with the P53. Subsequently, Western blot analysis showed that *MKRN3*cDNA greatly repressed the expression of P53 protein (Figure 7C). Therefore, our data suggest that P53 may involve in *MKRN3* mediated SCCHN tumorigenesis.

Discussion

In this study, we found that elevated expression of *MKRN3* was correlated with the aggressive tumor characteristics in patients with SCCHN, making *MKRN3* an independent prognostic predictor for the survival of patients with SCCHN. Further functional analysis of *MKRN3* provided insight into the biological pathways and mechanisms involved in SHNCC pathogenesis that were regulated by *MKRN3*. Our data provide evidence that *MKRN3* plays an unfavorable role in tumor progression and may serve as a critical promoter of SCCHN.

Most of the previous research on *MKRN3* has focused on its role as a ubiquitin E3 ligase during puberty initiation [4]. In 2013, mutations with loss of function in the *MKRN3* gene were identified from human families with central precocious puberty using whole-exome sequencing [13]. *MKRN3* is a maternally imprinted gene located in the Prader–Willi syndrome critical region (chromosome15q11eq13), and only subjects who inherit the mutation from their father develop central precocious puberty [14]. Although *MKRN3* is postulated to be an inhibitor of gonadotropin-releasing hormone secretion [15]; however, the molecular mechanism of the effect of *MKRN3* on the gonadotropin-releasing hormone network remains unclear. In recent years, therefore, researchers have investigated the correlation between the change in hormonal regulation and cancer. Studies have reported that early age at menarche may act as a cancer promoter in breast carcinomas [16], whereas in other types of cancers, the change in hormonal status can inhibit cancer cells invasion [17]. To our knowledge, there was only one report that has evaluated the prognostic value of sex hormone receptor expression in 50 patients with laryngeal squamous cell carcinoma, whose physiological changes occurred due to rapid development during puberty [18]. However, there are very few studies on the correlation between the expression of *MKRN3* and tumorigenesis of cancer, especially in SCCHN. Thus far, *MKRN3* has only been found as an oncogene in tumorigenesis of gastric cancer [19] and imprinted genes in the process of human

osteosarcoma [7]. Nevertheless, no research has provided more pervasive evidence to strengthen the links between *MKRN3* and tumorigenesis.

With the rapid development of whole-genome sequencing tumor databases, the convenient access to TCGA database allowed large-scale global gene expression profiling and database mining for identifying a potential correlation between gene expression profiles and overall survival in a variety of malignancies, including SCCHN [20-22]. The clinical significance of *MKRN3*, especially its prognostic value in SCCHN, was a key highlight of our current investigation. Herein, we provide evidence that *MKRN3* plays a key role in SCCHN progression. Our findings demonstrated that *MKRN3* expression level was markedly higher in SCCHN samples than in the corresponding adjacent noncancerous tissues, and this result was verified in human SCCHN tissues and cell lines in our laboratory using qRT-PCR. Most importantly, Kaplan–Meyer survival and Cox regression analyses based on the expression level of *MKRN3* strengthened the notion that *MKRN3* was a valuable prognosis biomarker with predictive potential in patients with SCCHN. Further studies are required to test *MKRN3* expression in other solid carcinomas with large patient numbers in order to broaden its clinical significance.

Consistently, through GO and KEGG functional enrichment analyses, we found that a comprehensive molecular mechanism of *MKRN3* action in SCCHN included interferon gamma–mediated signaling pathway, hypoxia-inducible factor 1 signaling pathway, DNA/RNA synthesis and metabolism, and cell cycle regulation. These terms were closely related to the occurrence and development of cancer [23-26], and further mechanism investigation revealed that P53, as a tumor suppressor gene, might be a direct target gene of *MKRN3*, suggesting that *MKRN3* was oncogene and could provide a novel targeted therapeutic strategy for treating SCCHN.

In conclusion, the present study revealed that *MKRN3* was upregulated in SCCHN tissues, and its expression may be a potential marker for prognostic evaluation of SCCHN. However, our study has limitations because the results obtained from bioinformatics analysis are insufficient and need to be confirmed through functional experimental and mechanistic exploration verification. And there had only 1 sample of 522 SCCHN occurred distant metastases. Therefore, further investigation is required to determine whether *MKRN3* may represent an intriguing and novel therapeutic target in SCCHN.

Conclusion

Our data revealed that MKRN3 plays an unfavorable role in tumor progression and may serve as a critical promoter of SCCHN; hence, it can be possibly used as a prognosis biomarker with predictive potential and a novel therapeutic target in patients with SCCHN.

Abbreviations

SCCHN: Squamous cell carcinoma of the head and neck; MKRN3: makorin ring finger protein 3; TCGA: The Cancer Genome Atlas; IHC: immunohistochemistry; DMEM: Dulbecco's modified Eagle medium;

GAPDH: glyceraldehyde-3-phosphate dehydrogenase; SDS-PAGE: sodium dodecyl sulfatepolyacrylamide gel electrophoresis; PPI: Protein–protein interaction analysis; GO: gene ontology; KEGG: Kyoto Encyclopedia of Genes and Genomes.

Declarations

Ethics approval and consent to participate

The study was approved by the Research Ethics Committee of Xiangya Hospital, Central South University, Changsha, China, and all samples were used for analysis under written informed consent from the patients.

Consent for publication

Not applicable.

Availability of data and materials

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

Competing interests

The authors declare that they have no competing interests.

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Authors’ contributions

S. Zhang: Development of methodology, acquisition, analysis and interpretation of data (provided TCGA data sets, acquired HNSCC cells, etc.), and manuscript writing. Y. Qiu: Conception, design, study supervision and editing.

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Tables

Table 1. Clinical characteristics of TCGA SCCHNs patients

Parameters	No. of patients	Percentage (%)
Age		
<59	234	44.8
≥59	287	55.0
NA	1	0.2
Gender		
Female	137	26.2
Male	385	738
Alcohol		
Yes	348	66.7
No	163	31.2
NA	11	2.1
Smoking		
Yes	297	56.9
No	213	40.8
NA	12	2.3
Histological grade		
G1+G2	367	70.3
G3+G4	132	25.3
Gx	18	3.4
NA	5	1.0
Stage		
I+ II	118	22.6
III+ IV	390	74.7
NA	14	2.7
T classification		
T1+T2	186	35.6
T3+T4	274	52.5
Tx	39	7.5

NA	23	4.4
Lymph node metastasis		
N0	176	33.7
N+	246	47.1
N_X	75	14.4
NA	25	4.8
M classification		
M0	188	36.0
M1	1	0.2
MX	62	11.9
NA	271	51.9

NOTE: *. $P < 0.05$ was considered to be statistical significance.

Table 2. Correlations between the expression of *MKRN3* and clinicopathological parameters

Parameters	MKRN3 expression	*t* value	*P*-value*
Age			
<59	66.339±54.931	-0.318	0.751
≥59	67.915±57.543		
Gender			
Female	65.794±60.985	-0.342	0.733
Male	67.712±54.665		
Alcohol consumption			
Yes	65.821±53.437	0.044	0.965
No	66.055±59.506		
Smoking			
Yes	66.190±58.305	-2.311	***0.021***
No	77.953±53.935		
Histological grade			
G1+G2	70.159±58.499	0.963	0.336
G3+G4	64.520±47.820		
Stage			
I+ II	52.571±39.396	-2.928	***0.001***
III+ IV	71.743±62.453		
T classification			
T1+T2	62.429±52.840	-2.348	***0.025***
T3+T4	75.098±60.669		
Lymph node metastasis			
N0	67.450±54.218	-0.989	0.560
N+	72.227±61.835		

NOTE: *. $P < 0.05$ was considered to be statistical significance.

Table 3. Cox model analysis of overall survival

Parameters	Relative risk (95%CI)	*P*-value
Univariate		
Age	1.318(1.005-1.729)	***0.046***
Gender	0.740(0.557-0.983)	***0.042***
Smoking	1.009(0.767-1.327)	0.949
Alcohol	0.877(0.644-1.194)	0.405
Histological grade	0.828(0.643-1.067)	0.144
Stage	1.767(1.212-2.577)	***0.003***
T classification	1.386(1.114-1.723)	***0.003***
Lymph node metastasis	1.346(1.117-1.622)	***0.002***
Distant metastasis	1.050(0.818-1.348)	0.701
MKRN3 expression	1.004(1.002-1.006)	***0.000***
Multivariate		
Lymph node metastasis	1.721(1.358-2.158)	***0.000***
T classification	1.916(1.389-2.643)	***0.000***
MKRN3 expression	1.004(1.002-1.006)	***0.001***

NOTE: All the clinicopathological variables listed in the table were included in the univariate and multivariate analyses.

Abbreviation: 95% CI, 95% confidence interval.

Table 4. Cox model analysis of disease-free survival

Parameters	Relative risk (95%CI)	*P*-value
Univariate		
Age	1.246(0.919-1.691)	0.157
Gender	0.818(0.519-1.137)	0.232
Smoking	0.867(0.643-1.168)	0.348
Alcohol	0.970(0.703-1.337)	0.851
Histological grade	1.036(0.772-1.390)	0.815
Stage	0.541(0.354-0.827)	***0.005***
T classification	2.038(1.439-2.888)	***0.000***
Lymph node metastasis	1.759(1.298-2.483)	***0.000***
Distant metastasis	1.136(1.019-1.266)	***0.022***
MKRN3 expression	1.003(1.001-1.005)	***0.013***
Multivariate		
Lymph node metastasis	1.699(1.215-2.375)	***0.002***
T classification	1.900(1.328-2.718)	***0.000***
Distant metastasis	1.135(1.015-1.269)	***0.027***
MKRN3 expression	1.003(1.001-1.005)	***0.006***

NOTE: All the clinicopathological variables listed in the table were included in the univariate and multivariate analyses.

Abbreviation: 95% CI, 95% confidence interval.

Figures

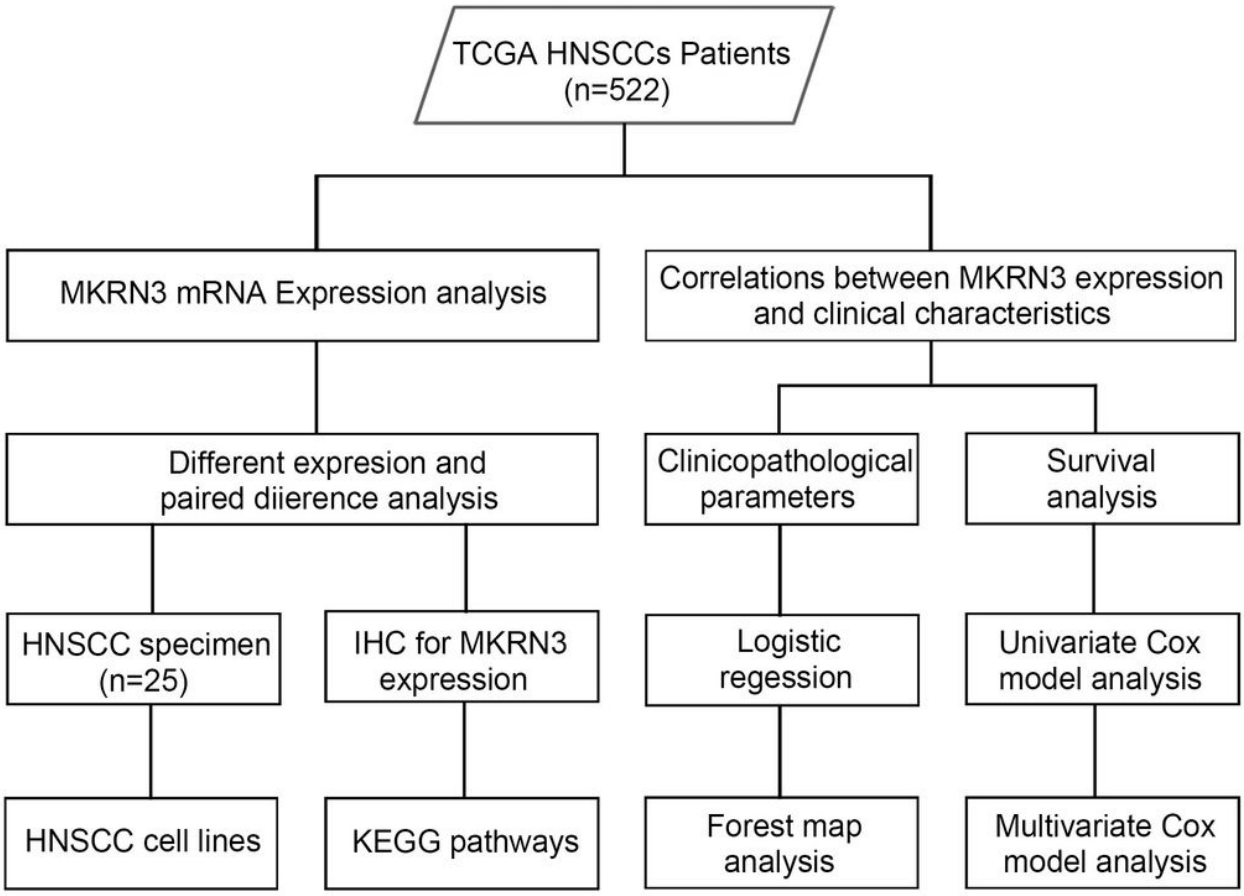

Figure 1

Study flowchart.

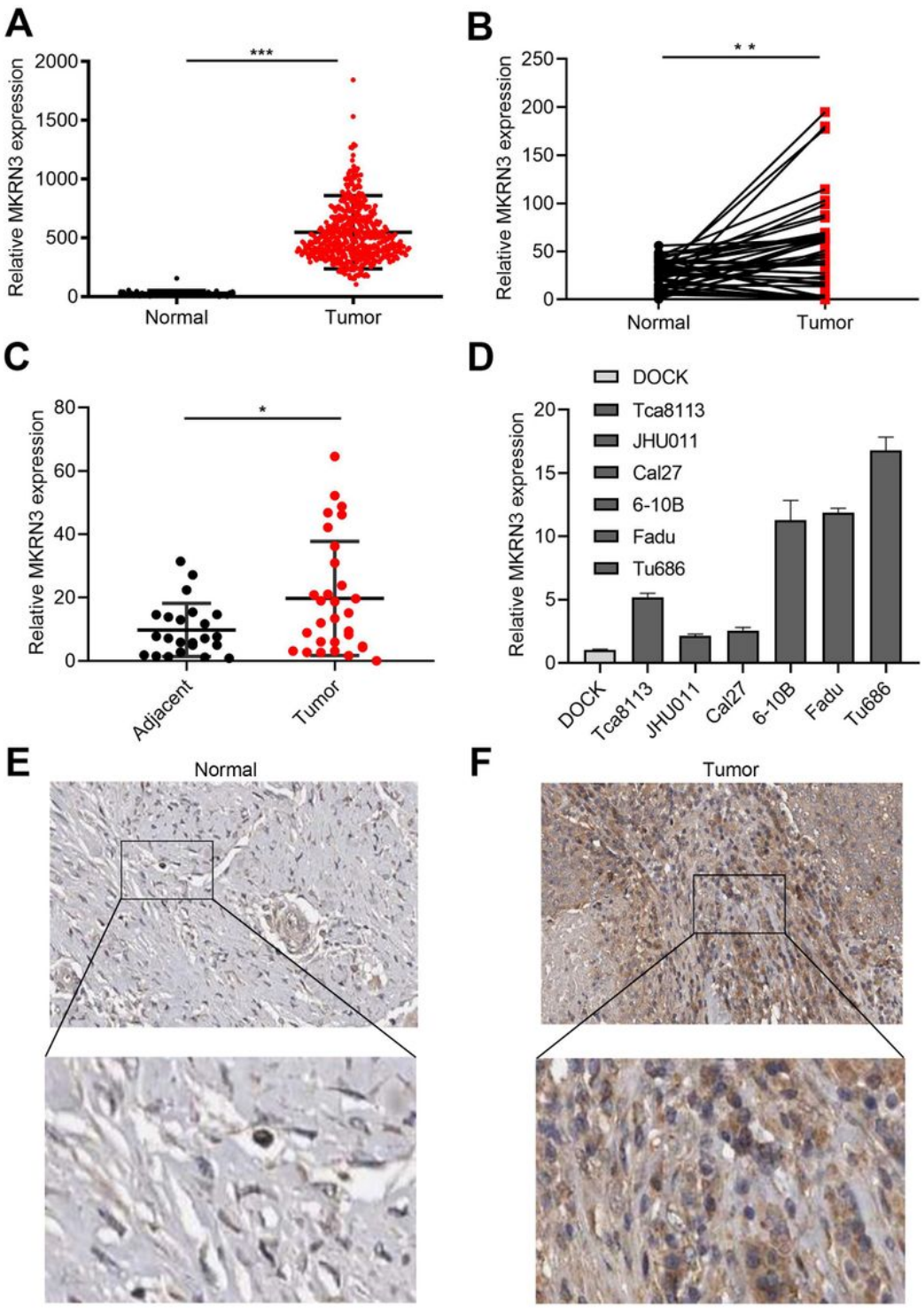

Figure 2.

Figure 2

please see the manuscript file for the full caption

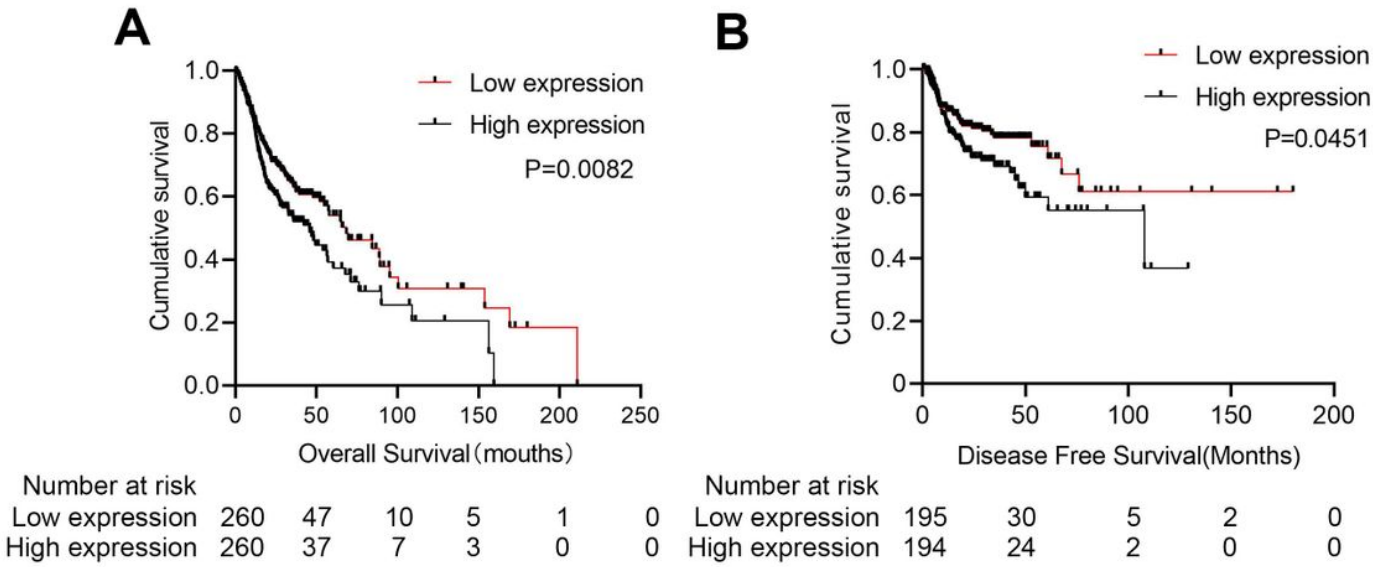

Figure 3

High expression of MKRN3 predicts a worse prognosis in patients with SCCHN. Kaplan–Meier survival analysis of MKRN3 expression in terms of overall survival (A) and disease-free survival (B) in SCCHN.

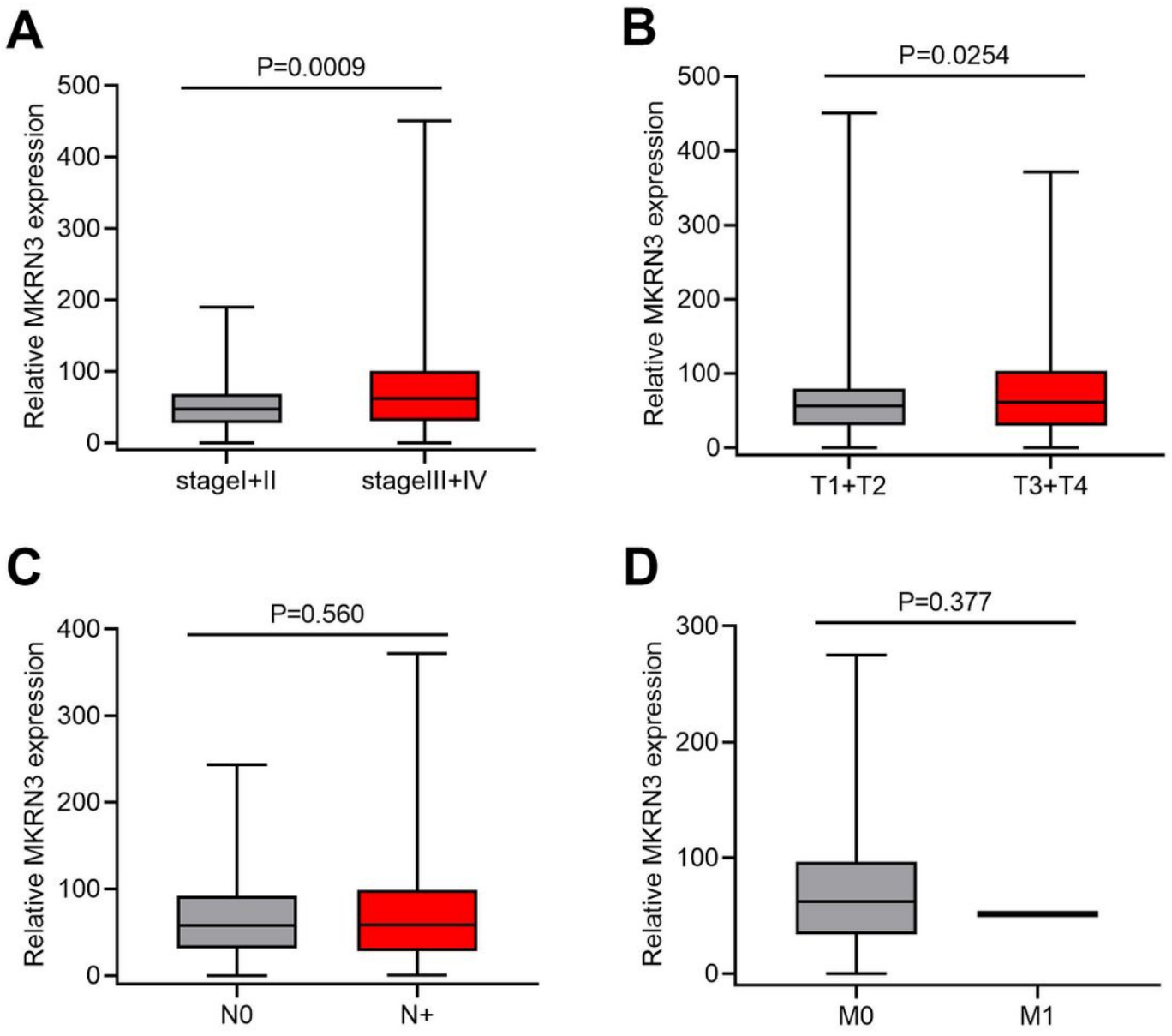

Figure 4

Correlation between MKRN3 expression and clinicopathologic characteristics. (A) Subgroup analysis of clinical stage (stages I+II vs. stages III+IV). (B) T classification (T1+2 vs. T3+T4). (C) N classification (N0 vs. N+). (D) M classification (M0 vs. M1). P-values were calculated using the Mann–Whitney U test.

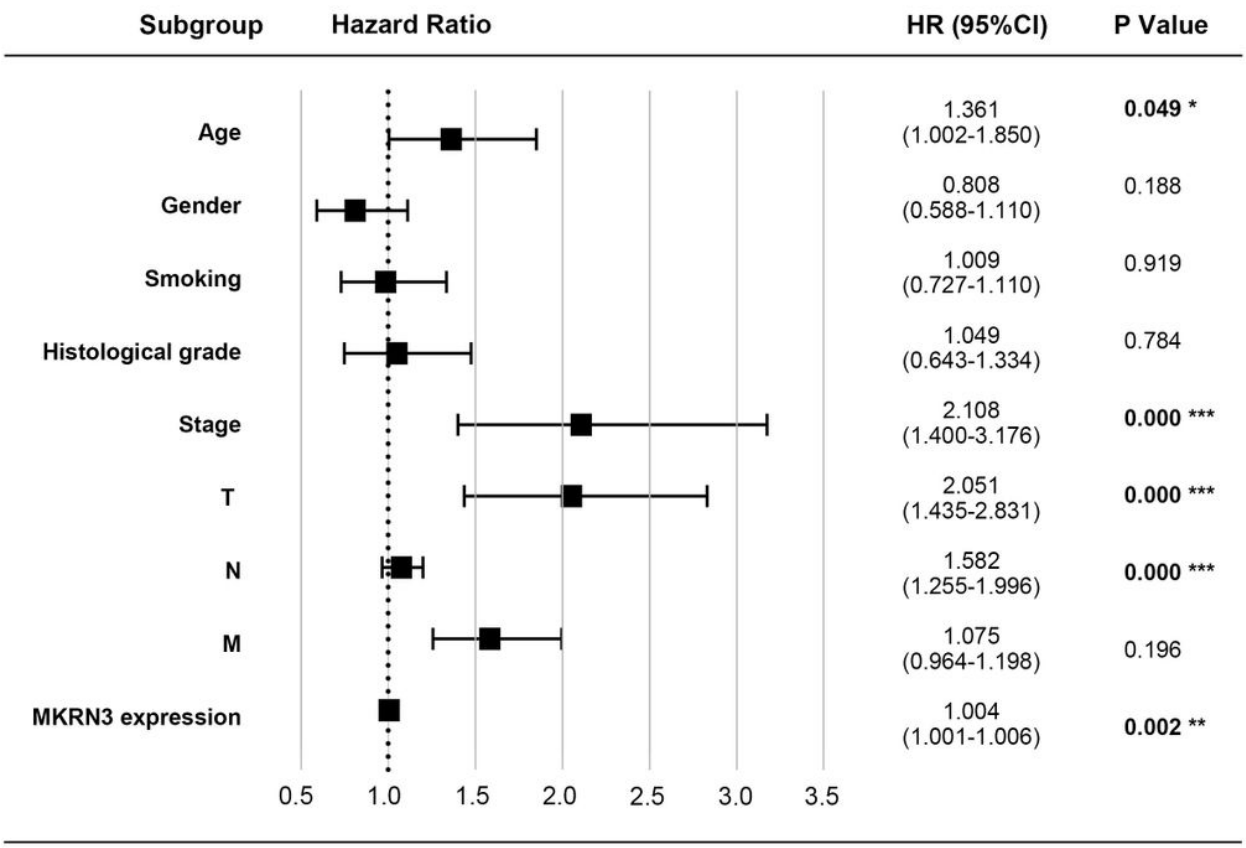

Figure 5

Forest map analysis of MKRN3 expression and clinicopathologic characteristics in SCCHN. P-values were calculated using the Mann–Whitney U test. *P<0.05; **P<0.01; ***P<0.001.

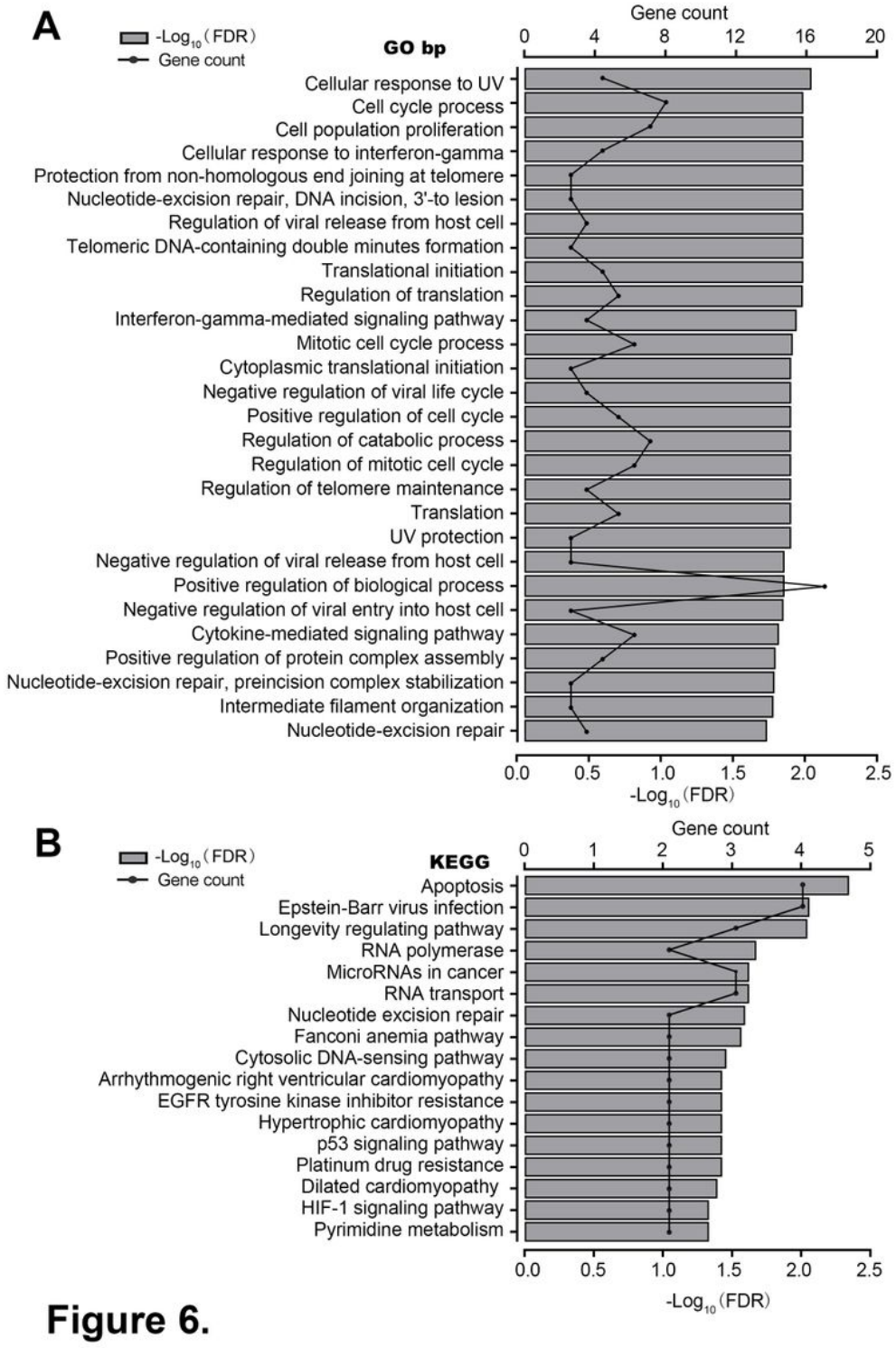

Figure 6.

Figure 6

Functional enrichment analysis of MKRN3 in SCCHN. (A) Enriched KEGG biological pathways. (B) Enriched GO terms in the “biological process” category with FDR <0.02. The columns indicate different significance levels, and the curve indicates the number of genes. FDR, false discovery rate; GO, gene ontology; KEGG, Kyoto Encyclopedia of Genes and Genomes.

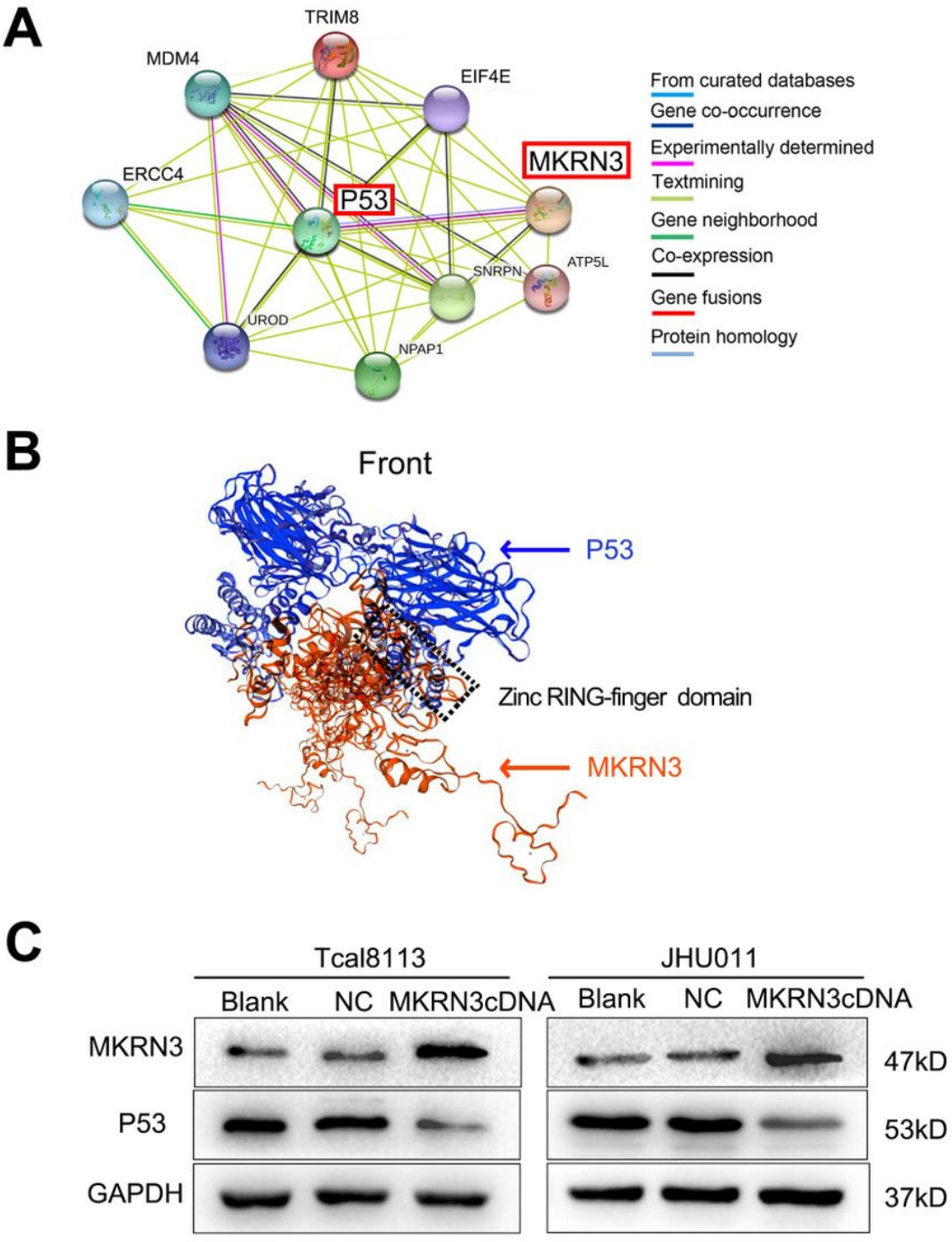

Figure 7

P53 might be a potential target gene of MKRN3. (A) STRING analysis in the protein–protein interaction of MKRN3. Only the proteins with more than one interaction are displayed. (B) The homologous modeling and molecular docking with MKRN3 and P53. Red and Blue cartoon represent MKRN3 and P53 respectively. Rectangle is for highlighting the interacted domain. (C) Relative expression of P53 protein in Tcal8113 and JHU011 cells that transfected with MKRN3cDNA.