Research Article

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Low expression of TRIM74 predicted poor prognosis and promoted the proliferation and migration of liver cancer cells

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Abstract

The aim of this study was to investigate the expression and clinical significance of the Tripartite motif-containing 74 gene in liver cancer.

Methods : Bioinformatics methods were used to analyze the gene expression of TRIM74 in liver cancer and normal liver tissues by using TCGA and GTEx databases.Wilcoxon test was used to analyze the differences in the expression of TRIM74 and to investigate the relationship between clinicopathological features.Kaplan-Meier method was used to examine the clinicopathological features of the correlation between TRIM74 expression and overall survival (OS).The expression of TRIM74 protein and mRNA in liver cancer cell lines were detected by Western blotting and reverse transcription quantitative polymerase chain reaction (RT-qPCR), respectively.The overexpression of exogenous TRIM74 in HCC cells was used to observe the effects on the proliferation,migration and invasion of HCC cells.
Results : Compared with the non-cancerous liver tissues,the expression of TRIM74 in liver cancer tissues was significantly decreased, and the decreased expression was significantly correlated with the disease progression.Compared with HCC patients with high expression of TRIM74,the survival time of HCC patients with low expression of TRIM74 was significantly shortened.The protein and mRNA expression levels of TRIM74 in hepatoma cell lines were significantly decreased compared with normal liver cell lines.Functional studies showed that overexpression of TRIM74 significantly reduced the proliferation,invasion and migration of HepG2 and Huh-7 cells.

Keywords : *TRIM74,hepatocellular carcinoma,prognosis,cell proliferation*

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INTRODUCTION

Liver cancer is a common malignant tumor,accounting for 8.3% of global cancer deaths and the third leading cause of cancer-related deaths [1].Due to the limited number of clinical studies and accurate biomarkers,the early diagnosis of liver cancer is difficult [2].In addition,without any intervention,the survival time is only 6-20 months [3].Therefore,the search for more effective and novel tumor biomarkers is of great importance.

The TRIMs protein family is a large protein family with extensive members and complex functions,including more than 80 highly conserved proteins,which are usually composed of a RING (R) domain, one or two B boxes (B) and a coiled-coil region (CC)[4][5].In recent years,the role of TRIMs protein family in cancer development has aroused much research interest,many novel tumor promoters and tumor inhibitors have been demonstrated in members of the TRIMs family[6][7],suggesting that they may have potential application value as new therapeutic targets or prognostic markers.

TRIM74 is a new member of the TRIMs family.So far,there have been very limited reports on the function of TRIM74,and its biological function is far from clarified.

Material and Methods

Data collection and preprocessing

The Cancer Genome Atlas (TCGA) database (https://portal.gdc.cancer.gov/) is used to obtain the HCC patients' RNA sequencing (RNA-seq) transcriptome data and clinical data. The RNA sequence data sets of normal liver tissues were obtained from the Genotypic Tissue Expression (GTEx) (www.gtexportal.org).mRNA profiles were included from 374 liver cancer tissues and 110 normal tissues,then normalized using the "limma" package in R (version 4.2.1).The cexpression difference between normal tissues and liver cancer tissues was analyzed,and the block diagram was used for visualization according to the expression difference of discrete variables [8]. Patients with incomplete clinical data were excluded to further analyze the RNASeq gene expression HTSeq count data and clinical data of HCC patients.The expression differences of discrete variables were visualized by the beeswarm package in R.Survival packs were used to make Kaplan-Meier curves to compare the overall survival rates of the high-expression and low-expression groups.

Table 1.TCGA liver cancer patient characteristics

Characteristics	**Number of sample,n (%)**	
	n	**Percentage (%)**
Age (years)		
≤ 65	157	71.1
> 61	64	28.9
Gender		
Male	69	31.3
Female	152	68.7
T stage		
T1	109	49.3
T2	47	21.2
T3	55	24.9
T4	10	4.5
N stage		
N0	218	98.7
N1	3	1.3
M stage		
M0	218	98.6
M1	3	1.4
Stage		
I	108	48.9
II	46	20.8
III	61	27.6
IV	6	2.7
Vital status		
Living	152	68.8
Deceased	69	31.2
Total	221	100

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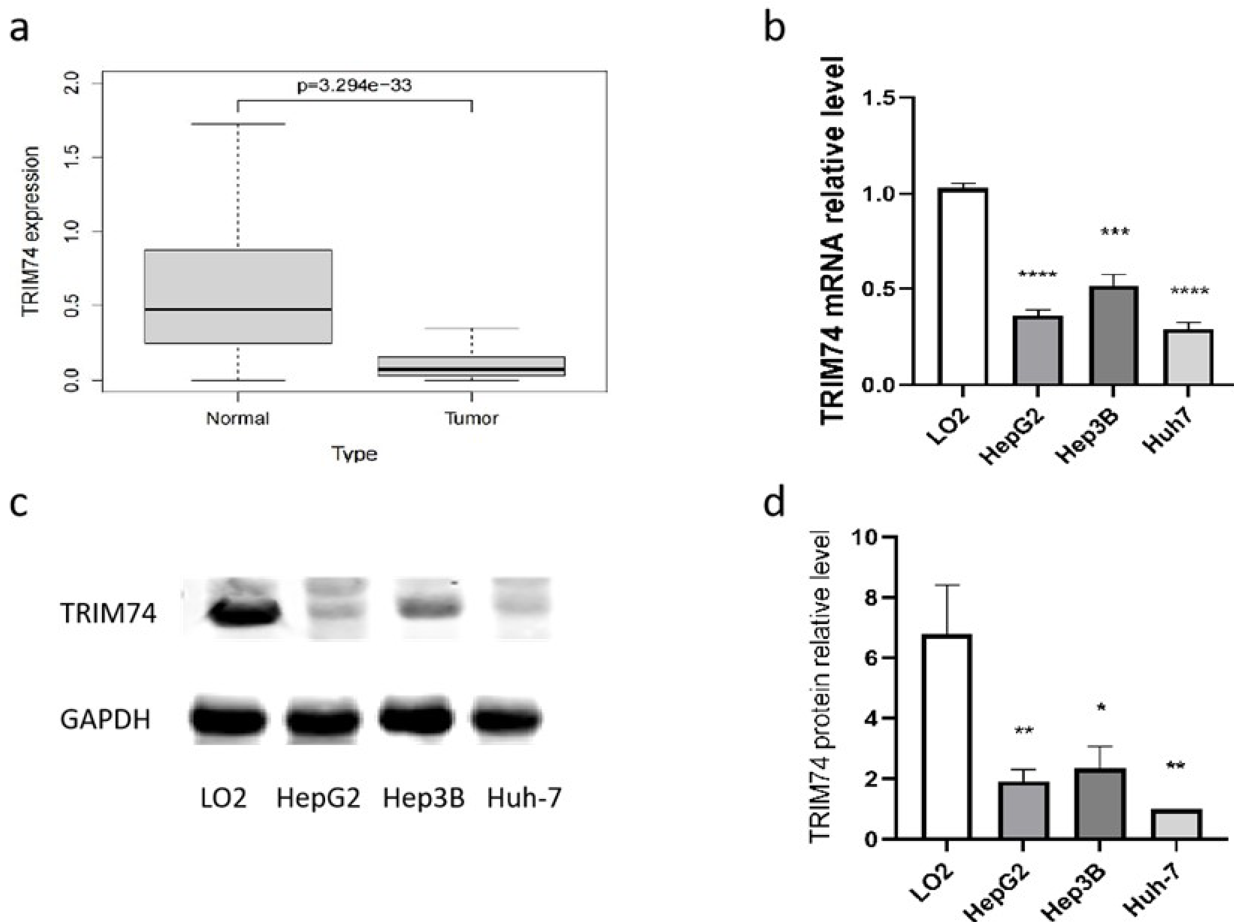
Quantitative real-time PCR (qRT-PCR)

Total RNA was extracted from cells with Trizol reagent(Invitrogen,Carlsbad,CA,USA).cDNA was generated by reverse transcription of total RNA as template using the RT kit (Takara Biotechnology Co.,Ltd.,Dalian, China).Expression levels were quantitatively analyzed by SYBR Green qPCR Master Mixes (Takara Biotechnology Co.,Ltd.,Dalian,China).The primer sequence is as follows: TRIM74 forward:5'-CCACCATGAGTTCATCTGGAA GT-3';TRIM74 reverse:5' -AACCAGTGGAGCTGAGATGCC-3';GAPDH forward: 5'-AATCCACACCTCTC-3'; GAPDH reverse:5'-AGGCTGTTGTCATACTTC-3'. ΔCt evaluated the changes of mRNA expression .

Western blot assay

Total protein was extracted using RIPA lysis buffer.After quantification,protein in cell lysates was added to SDS sample buffer and denatured the protein at 95^{0}C.Then,protein was isolated by 10% sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and transferred to a polyvinylidene fluoride (PVDF) membrane (Millipore) and blocked in 5% skim milk .Then,the membranes were incubated with the primary antibodies (TRIM74,Bioss,Beijing,China) overnight at 4^{0}C.The next day,the membrane was washed with TBST and then conjugated at 37^{0}Cwith secondary antibodies.Detected using an enhanced chemiluminescence (ECL) detection system (Beyotime, Shanghai, China).

Figure 1 : The expression of TRIM74 in liver cancer tissues and hepatoma cell lineswas significantly lower than that in normal tissues and liver cancer cell lines.a.The expression of a TRIM74 in normal tissues was significantly higher than that in cancer tissues (P=3.294e−33).b .RT-qPCR was used to detect TRIM74 mRNA levels in LO2,HepG2,Hep3B and Huh-7 cells.c,d . Western blot was used to detect TRIM74 protein levels in LO2,HepG2,Hep3B and Huh-7 cells.

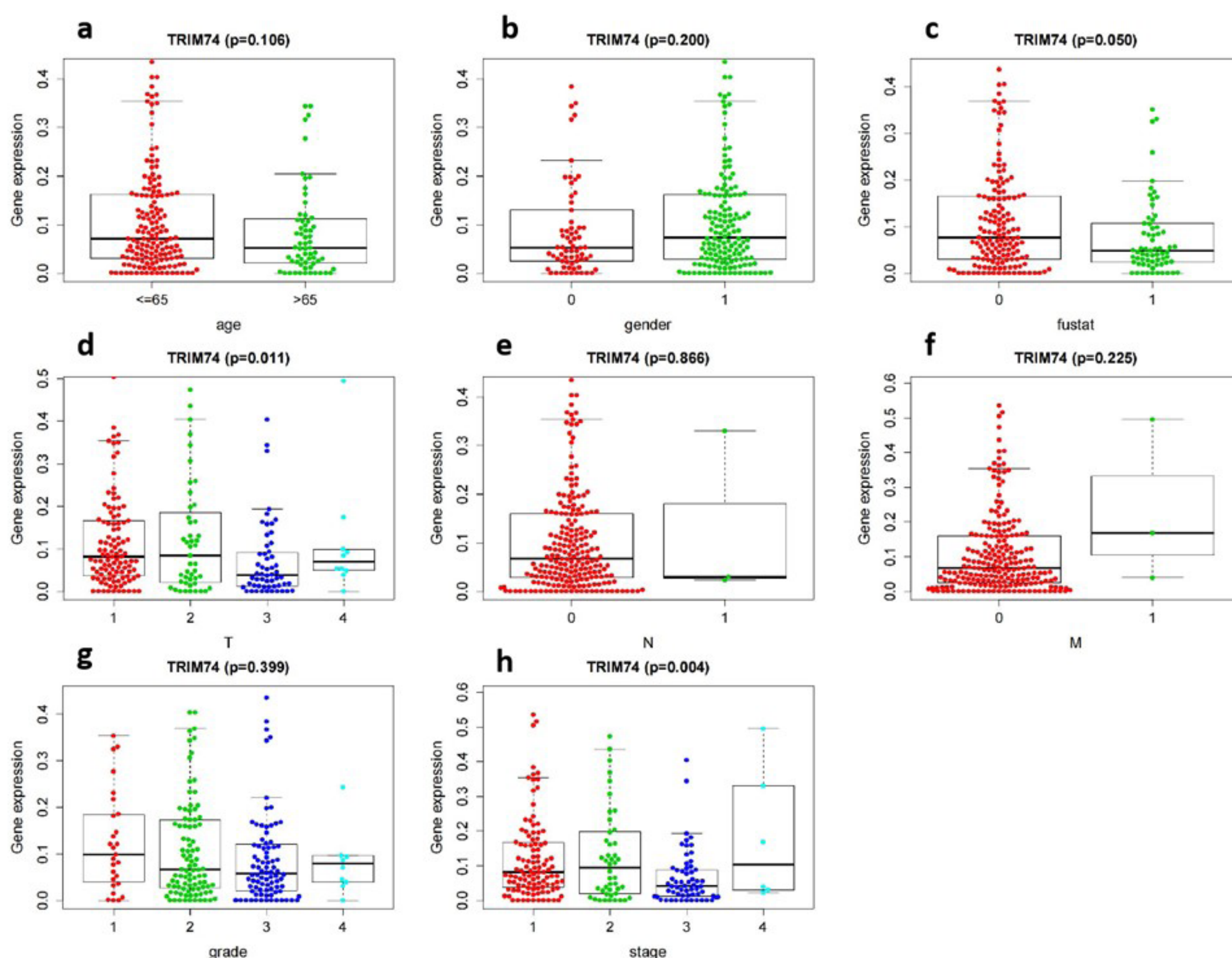


Cell culture and transfection

Human HCC cell lines HepG2,Huh-7,and Hep3B,and immortalized normal human liver cell lines LO2 were obtained from ATCC (Manassas,Virginia,USA) and cultured in high-glucose Dulbecco Modified Eagle's Medium (DMEM,Gibco, Waltham,MA,USA; Invitrogen, Waltham, MA, USA) containing 10% fetal bovine serum (FBS, ExCell Bio, Shanghai, China),incubated at 37^{0}C,5%CO2 and 95% air.

The expression plasmid pcDNA3.1-TRIM74 was constructed using pcDNA3.1 vector (Miaoling Bio, Wuhan, China.The coding sequence of human TRIM74 was amplified by PCR.A blank Vector was used as the negative control.According to the manufacturer's instructions,the cells were seeded in a 6-well plate and transfected with pcDNA3.1-TRIM74 and blank vector groups using Lipofectamine 2000 reagent (Invitrogen,Carlsbad, CA,USA) .TRIM74 group, Vector group and Control group,respectively.

Figure 2 : Relationship between TRIM74 expression and clinicopathological features in TCGA patients with liver cancer.a.age,b. sex,c.living status,d-f .TNM stage,f.grade grade,g.stage.



CCK8 assay

After transfection,HepG2 and Huh-7 cells (5×10^3 cells/well) were placed in 96-well plates and cultured for 0,24,48 and 72h,respectively. 100μL 10%CCK-8 solution (Signalway Antibody,College Park,Maryland,USA) was added in each wells and then incubated in 5%CO2 at 37°Cfor 2 h.Using a microplate spectro-photometer to obtained an optical density at 450 nm.

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Transwell assay

Transwell chamber(8μm pore size; Corning Costar) coated with Matrigel gel was used to detect the invasiveness.2×10^4 transfected HepG2 and Huh-7 cells and control groups were placed in the upper chamber with 300μL serum-free DMEM. DMEM (700μL) containing 10%FBS was added to the lower chamber.After incubation for 24h,the cells migrated to the bottom surface of the membrane,were washed with PBS,fixed,stained with 0.5% crystal violet.Then under the microscope(×200; Olympus Tokyo,Japan) photographed and counted the aggressive cells found at the bottom of each chamber.The migration capacity of HepG2 and Huh-7 cells was measured using Transwell without Matrigel gel.

Cell scratch test

5.0×10^5 cells per well were inoculated into Six well plates. After culture at 37°C for 24h,single-layer linear scratches were made to record the wound healing status at 0 h and 24h.Use ImageJ software for analysis.

Data analysis and statistics

Statistical analysis was performed using R(v.4.2.1) and GraphPad Prism software(version 9.0).Wilcoxon rank sum test was used to compare TRIM74 expression between liver cancer group and normal group.Subjects were divided into two groups based on median gene expression,excluding patients with incomplete clinical data.Wilcoxon test was used to evaluate the relationship between TRIM74 and clinicopathologic variables.

Kaplan-Meier analysis was performed to compare OS between high and low TRIM74 expression groups.Quantitative variables were analyzed using the T-test or one-way analysis of variance (ANOVA).Data are shown as mean±standard deviation.P<0.05 was considered statistically significant.

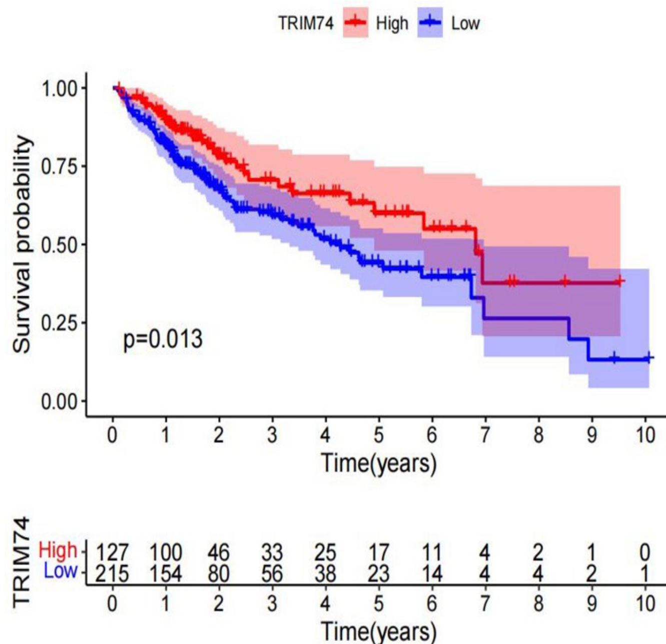
Results

TRIM74 was down-regulated in liver cancer tissues and cells.

Wilcoxon rank sum test was used to analyze the relationship between the expression of TRIM74 in different tissues,and the results showed that the expression of TRIM74 was significantly higher in normal tissues than in liver cancer tissues (P=1.724×−32)(Fig.1a).

Compared with normal liver cell lines LO2,RT-qPCR and Western blot assay showed that TRIM74 was low expressed in HCC cell lines,including HepG2 ,Hep3B and Huh-7 cells (Fig.1b-d).

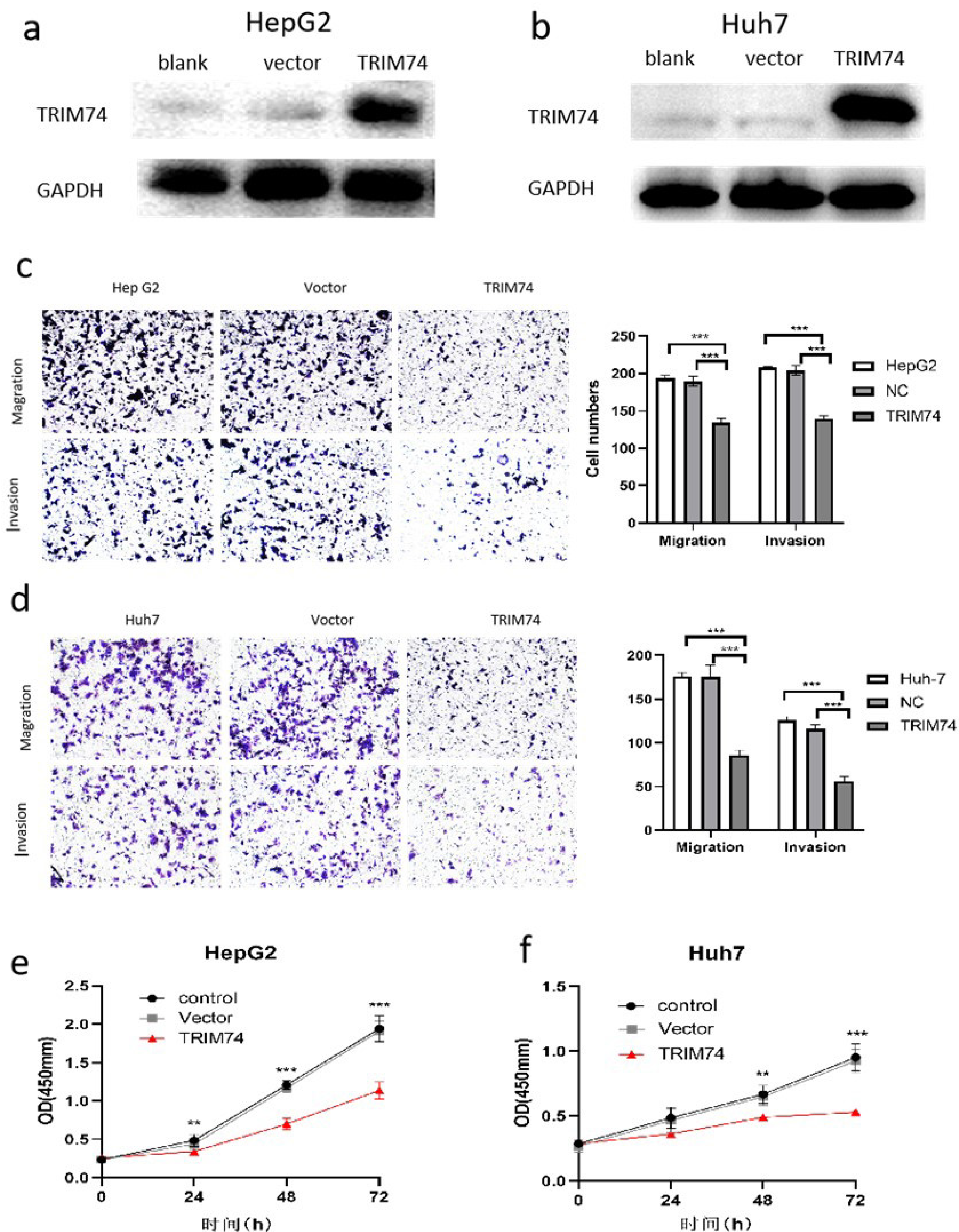
Figure 3 : Patients were divided into high expression group and low expression group to study the effect of TRIM74 expression on the overall survival rate of liver cancer patients in the TCGA cohort.



Low expression of TRIM74 was associated with clinicopathology and predicted a poor prognosis

Characteristics of 221 patients with HCC,including sex,age,TNM stage,clinical stage,histological grade,and survival status,were analyzed based on the patients of HCC in TCGA (Table 1).Wilcoxon rank sum test showed that up-regulation of TRIM74 was significantly correlated with T stage (P=0.011) and clinical stage (P=0.004) (Fig.2).Kaplan-Meier survival analysis was used to detect the role of TRIM74 expression in predicting the prognosis of patients with liver cancer.The results showed that patients with high expression of TRIM74 had a higher overall survival than those with low expression of TRIM74 (P=0.013) (Fig.3).

Figure 4 : a ,b Transfected HepG2 and Huh7 cells with TIRM74 expression plasmid or empty load plasmid,and western blot analysis was performed to determine the successful exogenous overexpression of TRIM74 in HCC cells.c,d.HepG2 and Huh7 cells were transfected with TRIM74 plasmid or no-load plasmid,and transwell assay was performed to study the migration and invasion ability of HCC cells.e,f.HepG2 and Huh7 cells were transfected with TRIM74 plasmid or no-load plasmid,and the proliferation status of transfected HCC cells was detected by CCK8 assay at 0 h,24 h,36 h,48 h and 72 h.


a
HepG2
blank
vector
TRIM74
TRIM74
GAPDH
b
Huh7
blank
vector
TRIM74
TRIM74
GAPDH
c
Hep G2
Voctor
TRIM74
Magration
Invasion
Cell numbers
Migration
Invasion
HepG2
NC
TRIM74
d
Huh7
Voctor
TRIM74
Magration
Invasion
Migration
Invasion
Huh-7
NC
TRIM74
e
HepG2
control
Vector
TRIM74
OD(450mm)
时间(h)
f
Huh7
control
Vector
TRIM74
OD(450mm)
时间(h)

The up-regulation of TRIM74 suppressed the proliferation of HCC cells

Blank vector (pcDNA3.1) was used as negative control. As shown in Fig.4(a-b),up-regulation of TRIM74 significantly increased TRIM74 protein expression in HepG2 and Huh-7 cells compared with blank pcDNA3.1 (vector) transfection.In addition,CCK-8 analysis showed that,compared with vector transfection,up-regulation of TRIM74 significantly decreased the proliferation of HepG2 and Huh-7 cells at 24,48 and 72 h,respectively (Fig.4 e-f).These results suggest that TRIM74 up-regulation suppressed HCC cell proliferation.

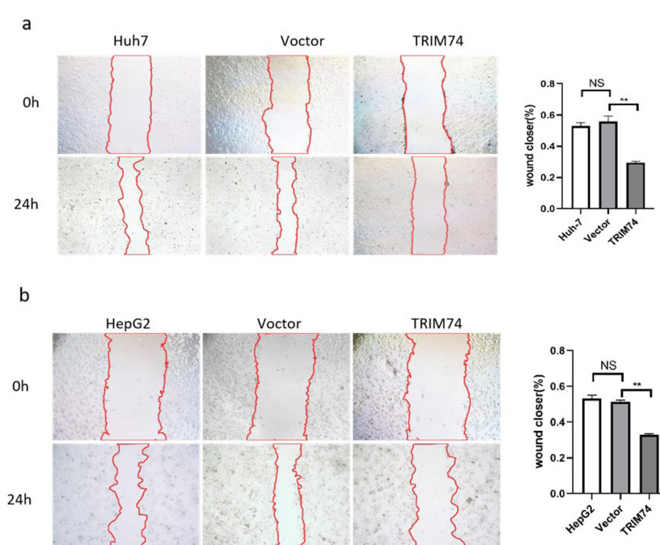
Up-regulation of TRIM74 suppressed cell migration and invasion of HCC cells.

Transwell assay and scratch assay further investigate the role of TRIM74 in the migration and invasion of HCC cell lines.Transwell assay showed that up-regulation of TRIM74 inhibited the invasion ability of Huh7 and HepG2 cells by 52.1% and 31.7%, respectively.Meanwhile, the migration ability of Huh7 and HepG2 cells was inhibited by 51.0% and 29.3%(Fig.4 c-d).Scratch experiments showed that,compared with NC transfection, up-regulation of TRIM74 significantly inhibited the scratch area of Huh7 and HepG2 cells by 23.6% and 18.5%,respectively (Fig.5).These results indicated that up-regulation of TRIM74 suppressed the migration and invasion of HCC cells.

Discussion

TRIMs family proteins are involved in many biological processes,suchascellproliferation,differentiation,cytoskeletal remodeling,membrane repair,carcinogenesis and apoptosis [9][10].Meanwhile,mutations of TRIMs protein genes will lead to various pathological changes,such as neurological diseases,viral infections and carcinogenesis [11].Recent studies have shown that TRIMs family proteins influence many aspects of cancer.Previous studies have shown that many studies have found that TRIM family proteins are closely related to tumor proliferation,invasion and migration,ubiquitination,p53 signaling pathway,NF-κB signaling pathway, autophagy,chromosomal ectopia, transcriptionand many other aspects [12].However, the role of TRIM74 in the occurrence and development of liver cancer is rarely reported.

Figure 5 : Huh7 (a) and HepG (b) cells were transfected with TIRM74 expression plasmid or no-load plasmid,and the wound healing status was recorded at 24h.



Therefore,in order to study the effect of TRIM74 on liver cancer,we studied the expression level and biological behavior of this gene.The expression of TRIM74 gene in liver cancer and its related clinical information were extracted from the public database.We found that TRIM74 gene was highly expressed in normal tissues compared to liver cancer tissues. Meanwhile,through the analysis of clinical data,it was found that the expression level of TRIM74 was negatively correlated with tumor stage,which also suggested that TRIM74 might be a protective factor in liver cancer.Kaplan-meier method was used to analyze the relationship between TRIM74 and survival time,and the results showed that HCC patients with high expression of TRIM74 had longer survival time.This is consistent with previous studies [13].Several recent studies show that downregulation of TRIM50,TRIM3 and TRIM26 leads to poor prognosis in HCC patients, suggesting the presence of tumor inhibition [6][14][15]. However,TRIM52 and TRIM11 can promote the proliferation and invasion of HCC cells[7][16].RT-qPCR and Western blotting assay were used to detect the expression of TRIM74 mRNA and protein in different cell lines.The results showed that TRIM74 was underexpressed in HCC cell lines.Meanwhile, the up-regulation of TRIM74 inhibited the invasion, migration and proliferation of HCC cells.

Therefore,TRIM74 up-regulates and influences the proliferation and migration of liver cancer cells,and has potential value as a prognostic biomarker for liver cancer. However, our research still needs a lot of improvement. In order to fully elucidate the heterogeneity of TRIM74

in liver cancer,it is necessary to conduct further studies combined with clinical samples.Only the effects on cell function were observed without elucidating the related molecular mechanisms. We will continue to explore the deep mechanism of the occurrence and development of liver cancer and provide new ideas for the prevention and treatment.

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