

Original Article

KLF4 suppresses the migration of hepatocellular carcinoma by transcriptionally upregulating monoglyceride lipase

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Abstract: The dysregulation of cellular metabolism, particularly lipid metabolism, is essential for cancer progress. Monoglyceride lipase (MGLL) is an important fatty acid metabolism enzyme, which converts monoacylglycerides to free fatty acids and glycerol. Despite the expression level of MGLL was reported to be downregulated in Hepatocellular carcinoma (HCC), the clinical significances and molecular mechanism of MGLL downregulation remains unknown. In the current study, the clinical significances of MGLL expression were investigated in 95 patients with HCC and the transcription factors of MGLL were identified in HCC cells. We found that MGLL was frequently downregulated in HCC samples, especially in metastatic tumor tissues. Patients with low MGLL expression owned remarkably lower 5 year-overall survival (5-OS). Functionally, we found that MGLL played an important role in HCC cell migration. Overexpression of MGLL suppressed cell migration and depletion of MGLL by shRNA promoted cell migration. Further studies indicated that KLF4 directly bound to the promoter of MGLL and accelerated MGLL expression, which then led to HCC cell migration decrease. Additionally, the expression levels of KLF4 were positive association with MGLL expression in HCC tissues. Collectively, our data suggest that KLF4 is a key regulator of MGLL. The KLF4-MGLL axis plays an essential role in suppressing HCC cell migration.

Keywords: KLF4, hepatocellular carcinoma, MGLL, migration, lipid metabolism

Introduction

Hepatocellular carcinoma is the most common type of liver cancer, which accounts for 75-85% of the primary malignancies in the liver. Approximately 50% of cases occur in China [1, 2]. Although several advanced therapeutic strategies have been used, the 5-year survival rate of HCC patients remains poor. Therefore, an in-depth study of the molecular mechanisms underlying hepatic carcinogenesis is important for improving the diagnosis and management of human HCC.

Monoglyceride lipase (MGLL), also designated monoacylglycerol lipase (MAGL), is a major enzyme catalysing the hydrolysis of monoacylglycerol (MGs) into glycerol and fatty acids. MGLL was indicated to play critical roles in many physiological and pathological processes,

such as pain, inflammation, neuroprotection and cancer [3, 4]. In tumours, because MGLL could convert MGs into glycerol and free fatty acids that might be utilized by cells as an energy source, one would anticipate that MGLL might be an oncogene and promote tumorigenesis. However, there are conflicting reports about the role of MGLL in carcinogenesis. MGLL has been shown to be overexpressed in multiple tumour types, such as melanoma, ovarian, breast, and prostate cancers, positively regulating tumorigenesis [5]. Conversely, studies in colon cancer show that MGLL is downregulated and might function as a tumour suppressor [6, 7]. A recent study reported that the mRNA and protein levels of MGLL were downregulated in HCC tissues compared with normal liver tissues. The decreased MGLL enhanced HCC cell proliferation [8]. However, the molecular mechanism of the MGLL decrease remains unclear.

KLF4 transcriptionally upregulates MGLL expression

In this study, we found that MGLL overexpression suppressed HCC cell migration. Conversely, the depletion of MGLL by shRNA promoted cell migration. Downregulation of MGLL was indicated in HCC tissues, which was associated with HCC metastasis and the poor prognosis of patients. Further studies indicated that KLF4 directly bound to the promoter of MGLL leading to MGLL upregulation in HCC cells. Additionally, KLF4 suppressed HCC cell migration by regulating MGLL expression. Thus, our data suggest that KLF4 is a key transcription factor of MGLL, and the KLF4-MGLL pathway plays an essential role in suppressing HCC cell migration.

Materials and methods

Cell culture and reagents

The human HCC cell lines SNU449 and BEL-7402 were obtained from the Cell Bank of the Chinese Academy of Sciences (Shanghai, China). BEL7402 cells were grown in DMEM supplemented with 10% foetal bovine serum, 1% penicillin, streptomycin and 0.1% Savelt™. SNU449 cells were grown in 1640 supplemented with 10% foetal bovine serum, 1% penicillin, streptomycin and 0.1% Savelt™. The cells were maintained in a humidified atmosphere with 5% CO₂ at 37°C. The following antibodies and reagents were used: MGLL (1:100, sigma, HPA011348), beta-actin (1:1000, Proteintech Group, #60008-1-Ig), KLF4 (1:500, Cell Signaling Technology, #12173), and Savelt™ (Hanbio Co., LTD 1:1000).

Transwell assays

The transwell system was used for the determination of cell migration. Cells were seeded in serum-free medium in chambers (8.00 mm pores, BD, Biosciences), and then, they were allowed to migrate across uncoated inserts using serum-containing medium for 24 h. Cells on the apical surface of the insert were scraped off, and the membranes invaded with cells were fixed with 1% paraformaldehyde and stained with crystal violet. Cell counts were expressed as the average number of cells per field of view. Three independent experiments were performed.

RNA interference and virus infection

RNA interference was performed as previously described [9, 10]. The shRNA was purchased

from Sigma. The various targeting sequences were as follows: MGLL-1, 5-CCAATCCTGAATCTGCAACAA-3; MGLL-2, 5-CAACTCCGTCTTCCATGAAAT-3; and KLF4-1, 5-ATCGGTCATCAGCGTCAGCAA-3; KLF4-2, 5-AAGTCATCTTGTGAGTGGA-TAA-3.

Quantitative real-time polymerase chain reaction assay (Q-PCR)

Total RNA was isolated using Trizol (Invitrogen). One microgram of total RNA was used to synthesize cDNA using the PrimeScript™ RT reagent kit (Takara, RR047A) according to the manufacturer's instructions. The primers were as follows: MGLL up: 5-ACAACCTTCAAGGTCCTT-3, dn: 5-CGAGAGAGCAGCTGGAG-3; and ACTIN up: 5-GACCTGACTGACTACCTCATGAAGAT-3, dn: 5-GTCACACTTCATGATGGAGTTGAAGG-3.

Promoter reporters and dual-luciferase assay

The promoter of MGLL was constructed into the pGL3-basic vector. Luciferase activity was measured in a 1.5-ml Eppendorf tube with the Promega Dual-Luciferases Reporter Assay kit (Promega E1980) according to manufacturer's protocols after transfection. Relative Renilla luciferase activity was normalized to firefly luciferase activity. The assay was performed as previously described [11, 12].

Tissue microarray, immunohistochemistry (IHC) and histological studies

The HCC tissue microarrays containing 95 HCC tissues and the adjacent normal tissues were purchased from Shanghai Outdo Biotech (Shanghai, China). Sections were stained with Masson's trichrome and H&E (haematoxylin and eosin) for histopathological examination. For immunohistochemistry, sections were subjected to antigen retrieval using microwave heating at 95°C in citrate buffer (pH=6.0, for MGLL). The indicated antibodies were specific for MGLL (1:300).

Statistical analysis

All results are shown as the mean \pm s.d. of multiple independent experiments, not technical replicates. Detailed *P* values for each panel in the figures are stated in the corresponding legends. A Student's *t*-test, a Mann-Whitney test (for two group comparisons) or a Kruskal-Wallis one-way ANOVA followed by Dunn's multiple comparison tests (for more than two group

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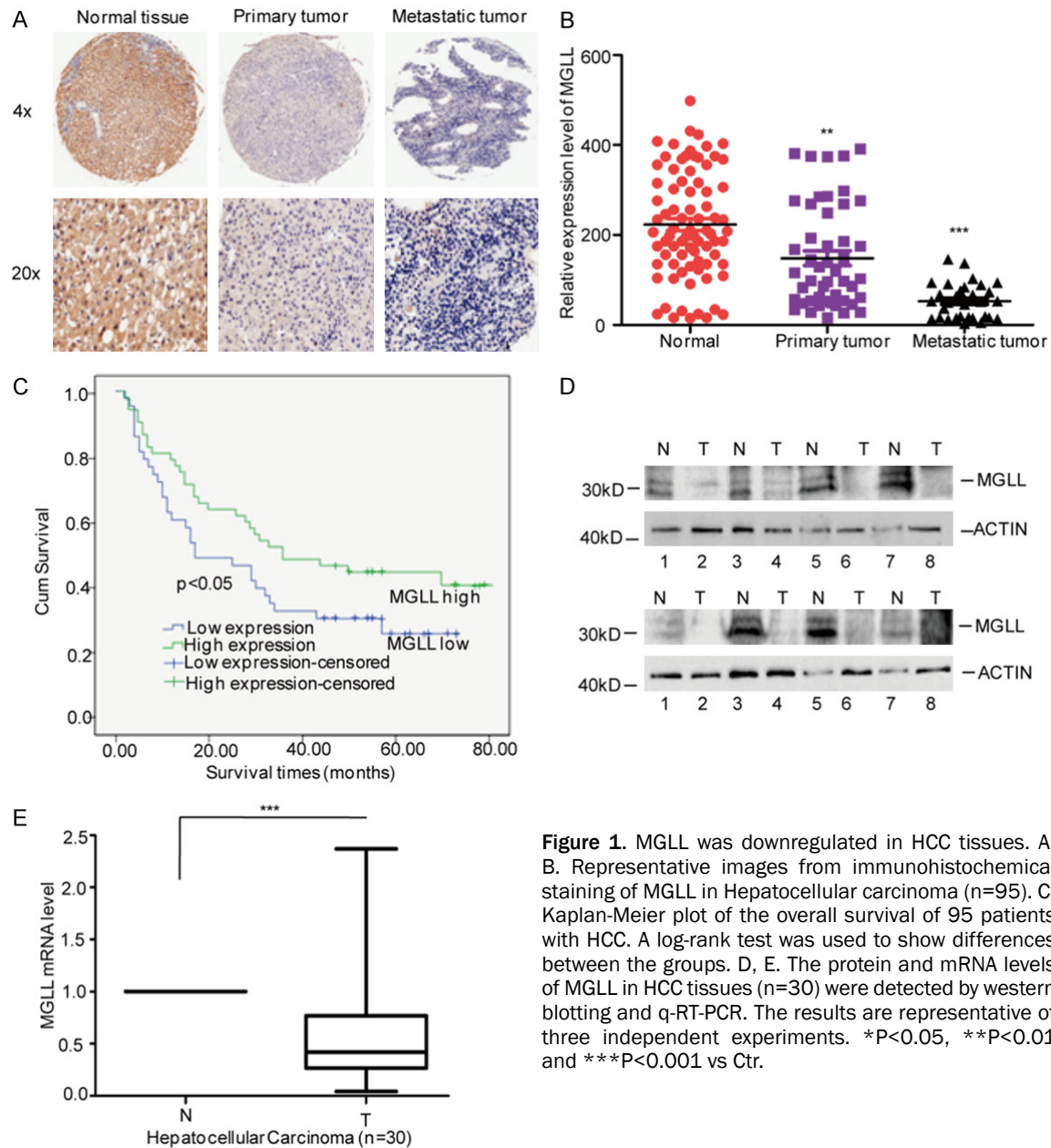


Figure 1. MGLL was downregulated in HCC tissues. A, B. Representative images from immunohistochemical staining of MGLL in Hepatocellular carcinoma (n=95). C. Kaplan-Meier plot of the overall survival of 95 patients with HCC. A log-rank test was used to show differences between the groups. D, E. The protein and mRNA levels of MGLL in HCC tissues (n=30) were detected by western blotting and q-RT-PCR. The results are representative of three independent experiments. * $P < 0.05$, ** $P < 0.01$ and *** $P < 0.001$ vs Ctr.

comparisons) was used for statistical analyses. All statistical analyses were performed with GraphPad Prism 5 and SPSS 19.0 software. All statistical tests were two-sided, and P values < 0.05 were considered to be statistically significant.

Results

Downregulation of MGLL expression is associated with high metastasis and poor prognosis of patients with HCC

To reveal the clinical relevance of MGLL expression in HCC, we collected 95 HCC tissues and

their adjacent normal tissues. Through immunohistochemistry assays, we observed that MGLL was decreased in tumour tissues and the decrease was further amplified in metastatic tumours (**Figure 1A** and **1B**). Subsequently, the correlations between MGLL expression and age, tumour size, gender, distant metastasis, TNM of the patients were obtained. As shown in **Table 1**, the expression levels of MGLL were significantly associated with tumour size ($P = 0.025$), distant metastasis ($P = 0.012$), and TNM ($P = 0.035$). Additionally, the relationship between MGLL expression and the prognosis of patients with HCC was also analysed. The

Table 1. MGLL protein expression and tumor index correlation analysis

	Variables	N	MGLL expression			P value
			Low	High	%	
Age	≥65	52	43	9	54.7	0.576
	<65	41	32	9	43.1	
Gender	Male	85	69	16	89.4	0.025
	Female	9	7	2	9.4	
Size	≤5 cm	19	7	12	20	0.806
	>5 cm	74	69	5	77.9	
Metastasis	M0	19	2	17	20	0.012
	M1	69	68	1	72.6	
TNM	TNM ₁ -TNM ₂	25	11	14	26.3	0.035
	TNM ₃ -TNM ₄	62	58	4	65.3	

Table 2. Cox-regression analysis for prognosis of 95 HCC patients

Variables	HR	95.0% CI	P value
Tumor size	1.087	0.539~2.191	0.0309
Metastasis	2.294	0.435~3.851	0.0422
TNM (I~II):(III~IV)	2.949	0.468~8.121	0.0.039

result showed that patients with low MGLL expression owned notably lower 5 year-overall survival (5-OS) (**Figure 1C** and **Table 2**). To further investigate whether the decrease of MGLL in HCC relied on its mRNA downregulation, we collected 30 fresh HCC tissues and adjacent normal tissues. The protein and mRNA levels of MGLL were analysed by western blotting and q-RT-PCR. As shown in **Figure 1D** and **1E**, the MGLL mRNA levels were decreased in HCC tissues and the downregulation of MGLL mRNA levels were positively correlated with the protein levels.

MGLL inhibits the migration of HCC cells

To investigate the effect of MGLL on the migration of HCC cells, we knocked down MGLL expression with 2 independent shRNAs in BEL-7402 and SNU449 cell lines. As shown in **Figure 2A**, stable cell lines expressing these shRNAs showed significantly reduced MGLL levels. In subsequent transwell assays, the numbers

of migrated cells in BEL7402 and SNU449 cells with MGLL knockdown were remarkably increased compared to the control cells (**Figure 2B** and **2C**). Furthermore, in the wound healing assays, the migratory capabilities of BEL7402 and SNU449 cells with MGLL depletion were also elevated (**Figure 2D** and **2E**). After that, we used the opposite approach to investigate the effect of MGLL overexpression on HCC cells and generated BEL7402 and SUN449 cells that stably overexpressing MGLL (**Figure 2F**). Similarly, the overexpression of MGLL decreased cell metastasis (**Figure 2G-J**).

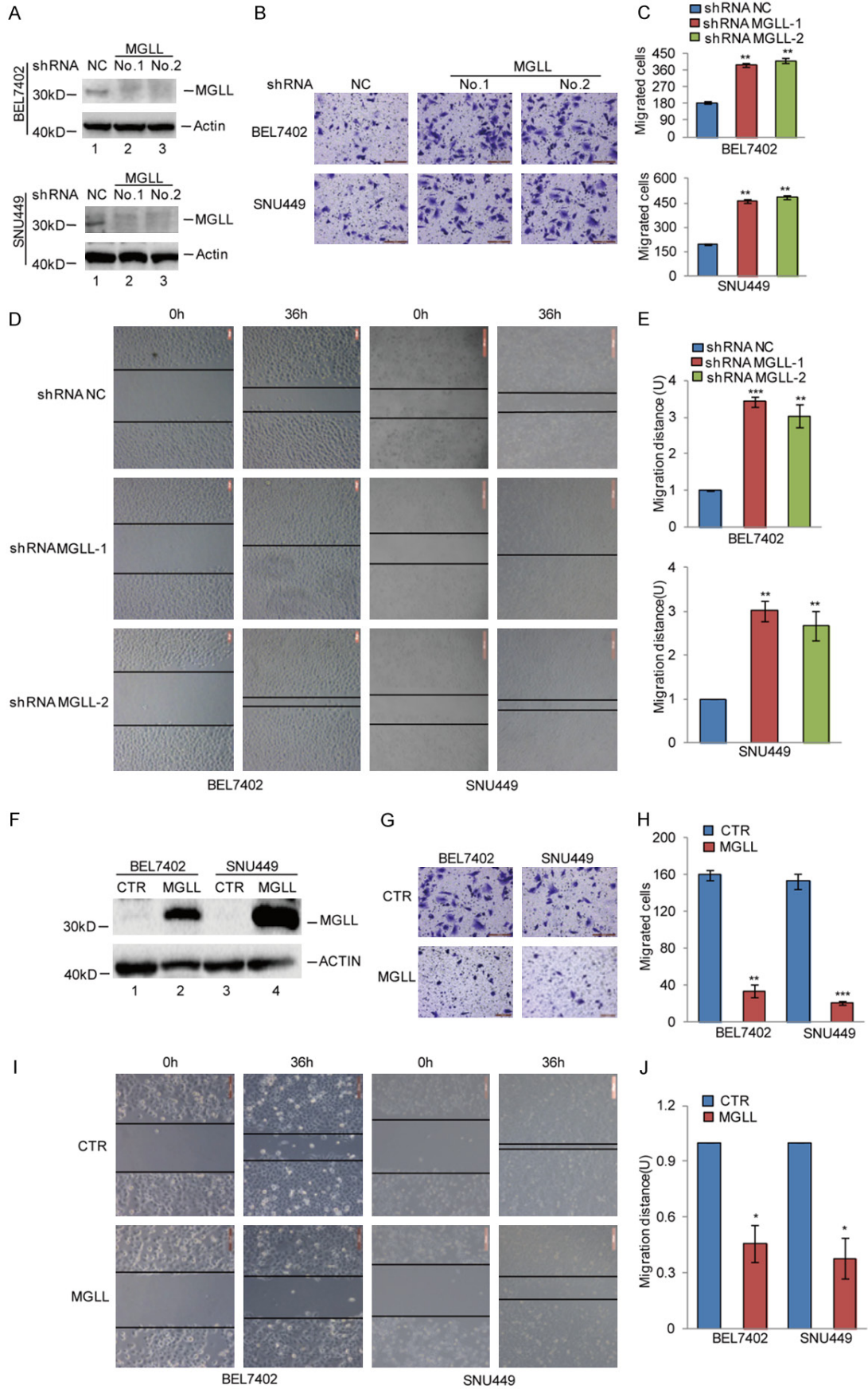
KLF4 binds to the promoter of MGLL in HCC cells

To uncover the molecular mechanism of MGLL downregulation in HCC, we designed and obtained a 1000 bp biotin-labelled double-stranded DNA of the MGLL promoter to pull down transcription factors which bind to the promoter of MGLL. After mass spectrum analysis, we found that the transcription factor KLF4 was a potential binding protein of the MGLL promoter (**Figure 3A** and **3B**). To confirm it, the chromatin immunoprecipitation (ChIP) assay was used. As shown in **Figure 3C**, the chromatin fragments of MGLL promoter were specifically present in anti-KLF4 immunoprecipitates and the fragments were increase in KLF4 overexpressing cells. After that, the interaction between KLF4 and MGLL promoter was further investigated in KLF4 depleted HCC cells. Similar with the previous result, deficiency of KLF4 inhibited the binding of KLF4 on MGLL promoter (**Figure 3D**). Thus, these data indicated KLF4 could bind to the promoter of MGLL.

KLF4 upregulates MGLL expression by binding to the MGLL promoter region (nt -594 to -413)

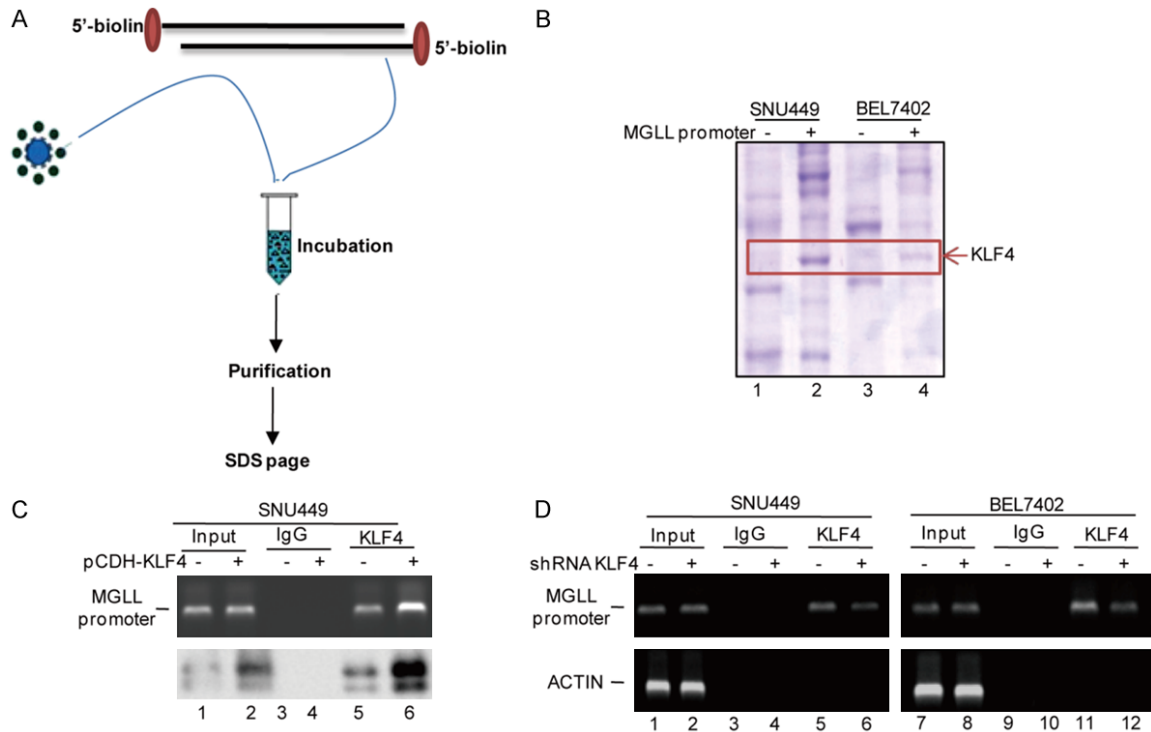
KLF4 is a zinc finger-type transcription factor which usually binds to the GC rich element of the promoters [13]. To seek the potential KLF4 binding sites, we inspected the sequence of the MGLL promoter by JASPAR software and found three putative KLF4 binding sites on the MGLL promoter. To verify that these potential KLF4-binding sites are indeed responsive to KLF4, a series of pGL3-based luciferase reporter plasmids named P1, P2, P3, and P4 were generated (**Figure 4A**). These plasmids were individually transfected into SNU449 and BEL7402

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Figure 2. MGLL inhibits HCC cells migration. A. MGLL was knocked down in BEL7402 and SNU449 cells. The protein levels of MGLL were detected by western blotting. B-E. Effects of MGLL on migration were examined by transwell and wound healing assays. The results are representative of three independent experiments. * $P < 0.05$, ** $P < 0.01$ and *** $P < 0.001$ vs Ctr. F. MGLL was overexpressed in BEL7402 and SNU449 cells. The expression levels of MGLL were detected by western blotting. G-J. Effects of MGLL on migration were examined by transwell and wound healing assays. The results are representative of three independent experiments. * $P < 0.05$, ** $P < 0.01$ and *** $P < 0.001$ vs Ctr.



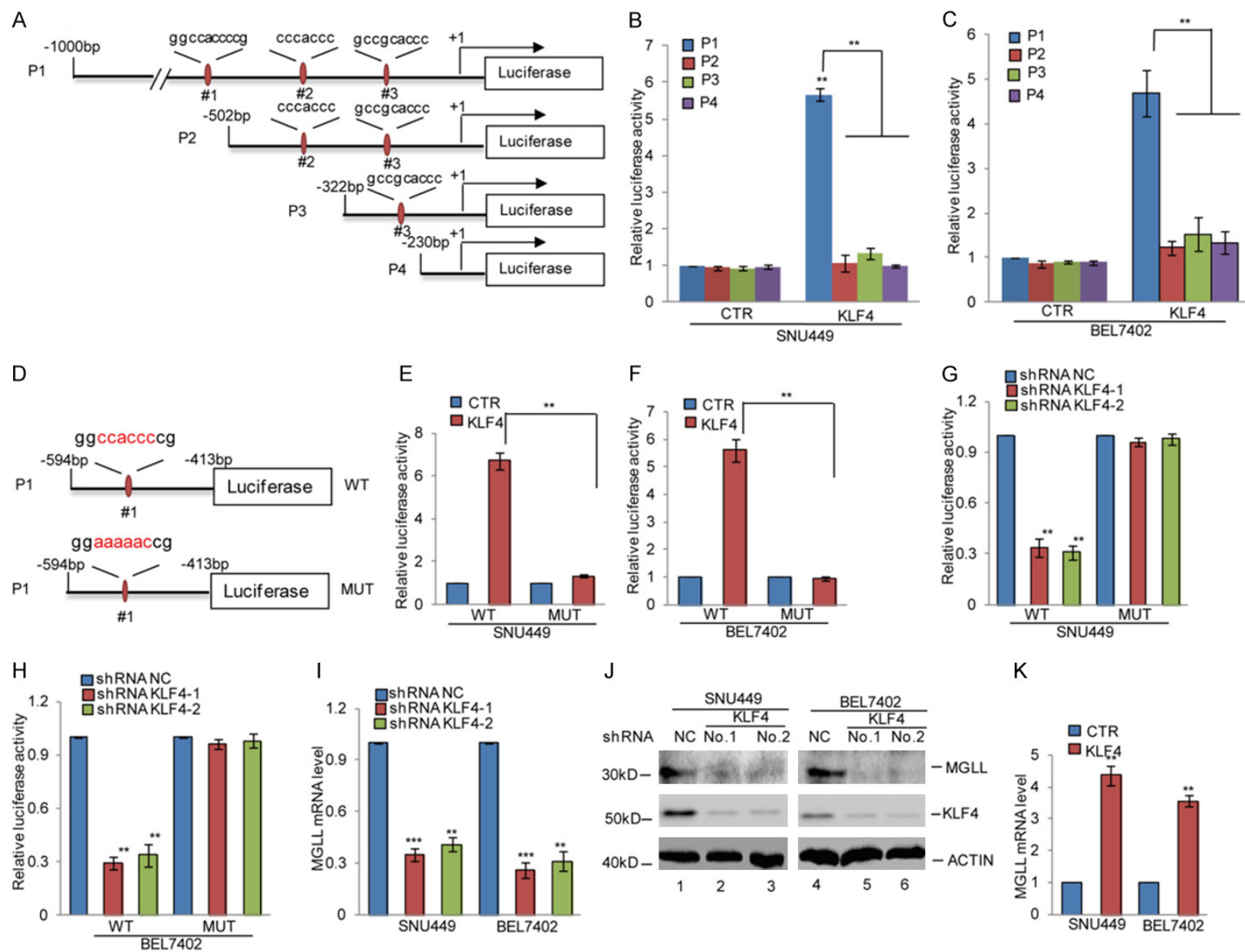
cells with or without KLF4 overexpression. As shown in **Figure 4B** and **4C**, the luciferase activity of P1, but not P2, P3, and P4, was significantly increased in KLF4-overexpressing cells indicating that the first binding site was a positive KLF4 binding site on the MGLL promoter. To further confirm this, two pGL3-based luciferase reporter plasmids containing the wild type KLF4 binding region (WT) and a mutant KLF4 binding region (MUT) were generated (**Figure 4D**). As shown in **Figure 4E** and **4F**, the luciferase activity of WT was dramatically increased in KLF4 overexpressing BEL7402 and SNU449 cells. However, the increase was disappeared when the binding site was mutated. Similar results were obtained in KLF4 depleted

SNU449 and BEL7402 cells (**Figure 4G, 4H**). Subsequently, the mRNA and protein levels of MGLL were analysed in KLF4-depleted SNU449 and BEL7402 cells. As shown in **Figure 4I** and **4J**, we found that the inhibition of KLF4 expression significantly decreased MGLL expression and vice versa (**Figure 4K, 4L**).

KLF4 inhibits HCC cell migration by regulating MGLL expression

Next, we want to prove whether the suppression of KLF4 on HCC cell migration was relied on MGLL. To investigate it, we stably overexpressed KLF4 in SNU449 and BEL7402 cells with or without MGLL knockdown. As shown in

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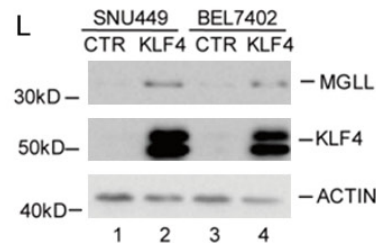


Figure 4. KLF4 enhanced MGLL expression. A. Schematic illustration of pGL3-based reported constructs were used in luciferase assays to examine the transcriptional activity of MGLL. B, C. The promoters of MGLL named P1, P2, P3, and P4 were individually transfected into SNU449 and BEL7402 cells with or without KLF4 overexpression. Luciferase activity was measured. The results are representative of three independent experiments. * $P < 0.05$, ** $P < 0.01$ and *** $P < 0.001$ vs Ctr. D. Schematic illustration of the MGLL promoter (WT) or the binding site mutant (MUT) used in luciferase assays. E-H. The WT and MUT of the MGLL promoter was individually transfected into SNU449 and BEL7402 cells with or without KLF4 overexpression or knockdown. Luciferase activity was measured. The results are representative of three independent experiments. * $P < 0.05$, ** $P < 0.01$ and *** $P < 0.001$ vs Ctr. I, J. KLF4 was knocked down in SNU449 and BEL7402 cells. The mRNA and protein levels of MGLL were detected by q-RT-PCR and western blotting. The results are representative of three independent experiments. * $P < 0.05$, ** $P < 0.01$ and *** $P < 0.001$ vs Ctr. K, L. KLF4 was overexpressed in SNU449 and BEL7402 cells. The mRNA and protein levels of MGLL were detected by q-RT-PCR and western blotting. The results are representative of three independent experiments. * $P < 0.05$, ** $P < 0.01$ and *** $P < 0.001$ vs Ctr.

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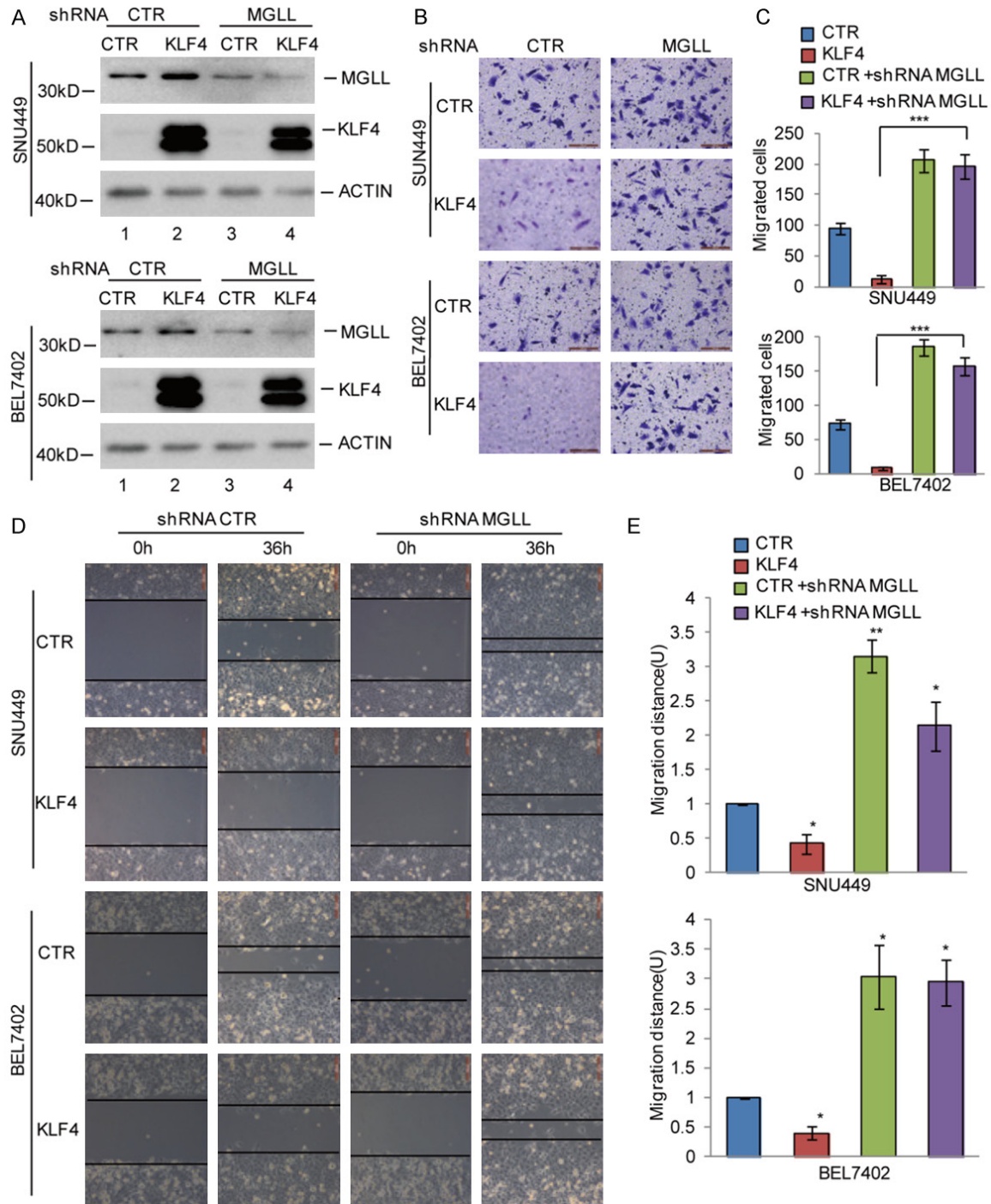


Figure 5. KLF4 suppressed HCC migration via regulation of MGLL. A. MGLL was knocked down in SNU449 and BEL7402 cells with or without KLF4 overexpression. Cell lysates were then subjected to western blotting analysis using the indicated antibodies. B-E. Effects of MGLL on migration were examined by transwell and wound healing assays. The results are representative of three independent experiments. * $P < 0.05$, ** $P < 0.01$ and *** $P < 0.001$ vs Ctr.

Figure 5A, exogenous overexpression elevated the KLF4 levels in both the control HCC cells and those depleted of MGLL cells. Transwell assays were performed using these cells. The

results showed that KLF4 overexpression inhibited cell migration. However, the suppression was reversed by MGLL depletion (**Figure 5B**, **5C**). Additionally, in the wound healing assay,

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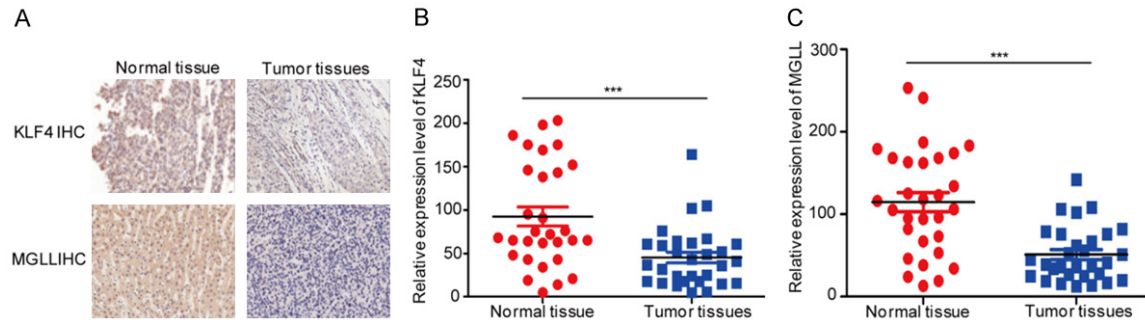


Figure 6. Decrease of MGLL in HCC was associated with KLF4 protein expression. A-C. The expression levels of KLF4 and MGLL were examined by immunohistochemistry. Data represent the mean \pm SD of three independent experiments. *** $P < 0.001$ vs control.

Table 3. The correlation between KLF4 and MGLL in 30 HCC tissues

KLF4	MGLL expression		P value
	High	Low	
High	5	1	0.016
Low	3	21	

MGLL depletion also rescued the defect of cell migration caused by KLF4 overexpression (**Figure 5D, 5E**). Thus, these data indicated that the suppression of KLF4 on HCC cell migration was relied on the regulation of MGLL.

The expression of KLF4 is decreased in HCC tissues and is associated with MGLL expression

To further confirm the regulation of MGLL by KLF4 in HCC, the protein levels of KLF4 and MGLL in HCC tissues and normal tissues were analysed by immunohistochemical staining (**Figure 6A**). We found that the relative expression levels of KLF4 were significantly decreased in HCC tissues and it was positive correlation with MGLL expression (**Figure 6B, 6C** and **Table 3**).

Discussion

In the study, we describe a novel role of MGLL in the suppression of HCC cell migration. MGLL has been shown to decrease in the HCC tissues. However, the molecular mechanism is still unknown. This is the first documentation that KLF4 could directly bind to the promoter of MGLL and promote MGLL expression. Thus, our study uncovers the molecular mechanism of MGLL downregulation in HCC.

The conversion of the cell from the normal to the cancerous state is accompanied by repro-

gramming the metabolic pathway [14]. Among the dysregulated metabolic pathways, heightened de novo lipid biosynthesis, or the development of a “lipogenic” phenotype, has been posited to play a major role in cancer. Therefore, many enzymes responsible for fatty acid biosynthesis are correlated with poor prognosis in cancer patients [15]. MGLL is a major enzyme catalysing the hydrolysis of monoacylglycerol (MGs) into glycerol and fatty acids that may be utilized by cancer cells as an energy source. MGLL was anticipated to promote tumourigenesis. However, the conflicting roles of MGLL in cancers were reported. In melanoma, ovarian, breast, and prostate cancers, MGLL was indicated to be overexpressed and promote carcinogenesis [5]. In colon cancer, MGLL was shown to decrease and inhibit tumour progress [6, 7]. Similar to colon cancer, MGLL was also downregulated in HCC and suppressed cell proliferation [8]. Here, we also obtained the same result that MGLL was decreased in HCC tissues. Our data first showed that the overexpression of MGLL inhibited HCC cell migration. HCC patients with low MGLL owned notably lower 5 year-overall survival.

The subsequent study indicated that a significant decrease in MGLL mRNA levels was detected in human HCC tissues. Thus, apart from post-translational regulation by SND1, there is a regulation of MGLL at a transcriptional level, which leads to its downregulation in HCC patients. Here, we found that KLF4 could directly bind to the promoter of MGLL and enhance MGLL expression. The expression level of KLF4 was shown to be downregulated in HCC patients, which might be correlated with MGLL expression. KLF4 was also indicated to inhibit HCC cell migration [16]. Consistently, our data also showed that KLF4 could suppress HCC cell

migration via regulating MGLL expression. Thus, our study suggests that the KLF4-MGLL pathway plays an essential role in suppressing HCC cell migration and MGLL is an important tumour suppressor in HCC.

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Disclosure of conflict of interest

None.

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