BBA - Molecular Basis of Disease 1865 (2019) 181–192

Contents lists available at ScienceDirect

BBA - Molecular Basis of Disease

journal homepage: www.elsevier.com/locate/bbadis

KIF14 promotes tumor progression and metastasis and is an independent predictor of poor prognosis in human gastric cancer

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ARTICLE INFO

Keywords:
Gastric cancer
KIF14
Invasion
Migration
Akt

ABSTRACT

The kinesin family member 14 (KIF14) is a potential oncogene and is involved in the metastasis of various cancers. Nevertheless, its function in gastric cancer (GC) remains poorly defined. The expression of KIF14 was examined in GC cell lines and a clinical cohort of GC specimens by qPCR, western blotting and immunohistochemistry (IHC) staining. The relationship between KIF14 expression and the clinicopathological features was analyzed. The effect of KIF14 on cell proliferation, colony formation, invasion and migration were investigated *in vitro* and *in vivo*. The expression of KIF14 was significantly increased in the GC tissues and cell lines. High KIF14 expression was associated with tumor stage, tumor-node-metastasis (TNM) stage and metastasis. KIF14 was an independent prognostic factor for the overall survival of GC, and a higher expression of KIF14 predicted a poorer survival. KIF14 silencing resulted in attenuated proliferation, invasion and migration in human gastric cancer cells, whereas KIF14 ectopic expression facilitated these biological abilities. Notably, the depressed expression of KIF14 inhibited Akt phosphorylation, while overexpressed KIF14 augmented Akt phosphorylation. Additionally, there was a significant correlation between the expression of KIF14 and p-Akt in GC tissues. Importantly, the proliferation, invasion and migration of the GC cells, which was promoted by KIF14 overexpression, was abolished by the Akt inhibitor MK-2206, while Akt overexpression greatly rescued the effects induced by KIF14 knockdown. Our findings are the first to demonstrate that KIF14 is overexpressed in GC, is correlated with poor prognosis and plays a crucial role in the progression and metastasis of GC.

1. Introduction

Gastric cancer (GC) is the fourth most common cancer and the second highest cause of cancer-related death worldwide, especially in East Asia [1,2]. Despite improvements in multidisciplinary treatment strategies, patient prognosis remains poor. Metastasis is the main cause of mortality in GC patients before or after curative surgery, and it severely threatens the survival of the patients [3]. Therefore, it is of great importance to identify new genes that are involved in the progression of GC metastasis and present a predictive value for prognosis.

Recently, we performed a gene profiling analysis of samples from GC patients, and the abnormal expression of the gene KIF14 was identified. Kinesins are ATP-dependent molecular motors that carry cargo along microtubules. They are classified into 14 distinct families, with varying structural and functional characteristics [4]. KIF14 is a member of the kinesin-3 superfamily and is located on chromosome 1q32.1 [5]. The KIF14 protein has a C-terminal motor domain, a citron kinase binding region and an N-terminal extension for the binding of PRC1 [6], and it plays an essential role in the mitotic spindle formation, midbody formation, cell cytokinesis and chromosome segregation. The deletion of KIF14 increases the level of $p27^{Kip1}$ and induces cytokinesis failure in hepatocellular carcinoma cells [7,8]. KIF14 is widely accepted to play a role in tumorigenesis as a chromokinesin. The overexpression of KIF14 may lead to rapid and error-prone mitosis, which induces

Abbreviations: KIF14, Kinesin family member 14; GC, gastric cancer; GSEA, gene set enrichment analysis; FBS, fetal bovine serum; TNM, tumor-lymph node-metastasis; EMT, epithelial-mesenchymal transition; CCK-8, cell counting kit-8; IHC, immunohistochemistry; H&E, hematoxylin and eosin; GO, gene ontology

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https://doi.org/10.1016/j.bbadis.2018.10.039
Received 12 July 2018; Received in revised form 30 October 2018; Accepted 31 October 2018
Available online 04 November 2018

aneuploidy during tumorigenesis [9]. Its high expression is involved in the progression of many types of malignant tumors. The increased expression of KIF14 is identified in retinoblastoma, breast cancer, and lung cancer, which provides evidence that KIF14 may be an oncogene in the progression of multiple cancers [9–11]. Recently, KIF14 was reported to be overexpressed in medulloblastoma and associated with unfavorable progression-free survival and overall survival; moreover, the downregulation of KIF14 suppresses tumor proliferation and induces apoptosis [12]. KIF14 is also reported to induce anchorage-independent growth in the ovarian cancer cell line SKOV3 [13] and indicates a poor prognosis in patients with epithelial ovarian cancer. Moreover, it is strongly correlated with invasion and migration and is reported to be a predictor of grade and outcome of breast cancer [14]. In adult synovial sarcomas, enhanced KIF14 expression is strongly associated with metastasis [15]. Importantly, the deletion of KIF14 in breast cancer cells leads to increased cell spreading, altered focal adhesion dynamics, and the inhibition of cell migration and invasion [5]. In gastric cancer, Tong et al. recently reported that KIF14 was upregulated in a GC dataset [16]. However, the biological role and the underlying molecular mechanism of KIF14 in GC is still not known.

Akt signaling is involved in multiple cellular activities, including cell proliferation and metastasis [17]. The phosphorylation of Akt on serine 473 is involved in the growth and invasion of some cancers, including gastric cancer [18]. However, it is still unknown whether the activation of Akt is involved in the KIF14 dysregulation-mediated tumor growth and metastasis in GC.

In the current study, we identified KIF14 expression and described its clinical significance in GC. We found that KIF14 expression was significantly higher in the advanced stage and correlated with the poor prognosis of GC patients. Furthermore, the phosphorylation of Akt was involved in KIF14 dysregulation, which uncovered an essential role for KIF14 in GC progression. Using this approach, we aimed to elucidate the functional and prognostic implications of KIF14 in GC.

2. Materials and methods

2.1. Cell culture

The GC cell lines SGC-7901 [19,20], AGS, MKN28, and MKN45, as well as the human embryonic kidney cell line, HEK293T and the immortalized gastric mucosal epithelial cell line GES-1, were preserved by our institute [21]. All the cell lines were grown in RPMI-1640 or Dulbecco's Modified Eagle's Medium supplemented with 10% fetal bovine serum (FBS).

2.2. Patients and samples

The GC tissue samples used in the gene profiling analysis were collected over the time period from 2011 to 2013 in Ruijin Hospital Shanghai Jiao Tong University School of Medicine. The samples included 10 GC tissues and their corresponding normal gastric mucosal tissues. Representative sections of the fresh tissue specimens were flash frozen in liquid nitrogen within 15 min of resection and were stored at −80 °C until the RNA isolation step. The information about the patient and disease characteristics was obtained from reviewing the patients' medical records. We used tissue microarrays containing 90 pairs of GC tissue samples and 97 cancer tissues from GC patients who underwent surgery at hospitals that cooperated with Shanghai Outdo Biotech during 2007–2008. The 90-paired microarray included 67 men and 23 women, ranging from 34 to 83 years of age (median: 65 years) with 1–51 months of follow-up information. Paraffin-embedded diagnostic tumor biopsy specimens and their adjacent non-tumor specimens (≥5 cm away from the tumor) were collected before any treatments. All human participants provided informed consent. This study was conducted with the approval of the Ruijin Hospital Ethical Review Board.

2.3. DNA/shRNA transfection and stable cell line generation

Human KIF14 complementary DNA was amplified from the SGC-7901 cells by PCR (forward primer: 5′-ATGTCATTACACAGTA CTC-3′ and reverse primer: 5′-TCACACCCA CTGAATCCTAC-3′) and was subcloned into the pQCXIN retroviral vector (Clontech, Palo Alto, CA) to generate the pQCXIN-KIF14 construct for transfection. Short hairpin RNA (shRNA) sequences, targeting KIF14 (5′-GTTGGCTAGAATTGGG AAA-3′), and a negative control sequence (5′-TGCGTTGCTAGTACC AAC-3′), were synthesized, annealed and ligated into the retroviral pSIREN-RetroQ vector (Clontech). The retrovirus packaging and infection were conducted as described previously using HEK293T cells [22]. The virally infected cells were cultured in medium containing 2 μg/ml puromycin for 7 days, and the stable cells were collected and expanded [23,24]. The GC cells infected with the NC sequence or shRNAs targeting KIF14 were termed NC or shKIF14, respectively.

2.4. Cell proliferation assay and colony formation assay

GC cells were cultured in 96-well plates at a density of 2×10^3/well (200 μl/well). A cell proliferation assay was conducted using a CCK-8 (Cell CountingKit-8) (Dojindo, Kumamoto, Japan) according to the manufacture's protocol. OD 450 was measured by spectrophotometry (BioTek, Vermont, USA) 2 h after being incubated with 10 μl of CCK-8 reagent.

Colony formation assay was performed with equal numbers of cells (1×10^3/well) seeded into 6-well plates and cultured for 2 weeks in RPMI-1640 medium. The colonies were visualized by staining with 3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium-bromide (MTT). Photographs were obtained and the number of colonies was counted.

2.5. Real-time PCR

According to the manufacturer's instructions, the total RNA from the gastric cells was extracted by Trizol (Invitrogen). Then, the cDNA was synthesized using 1 μg of RNA *via* a reverse transcriptional kit (Promega, Madison, WI, USA) abiding by manufacturer's protocol. Quantitative PCR was performed using SYBR green (Thermo Fisher) on a CFX96 Real Time PCR Detection System (Bio-Rad). β-actin, as an internal control, was used to normalize the mRNA expression of each gene. All the reactions were detected in triplicate, at least three independent times. The specific primers for KIF14 were 5′-CAGTGGCAG TACGCGTAAGA-3′ and 5′-TGAGAAGCTAGCAGCACCAC-3′ and for β-actin were 5′-CATCCTCACCCTGAAGTACCC-3′ and 5′-AGCCTGGAT AGCAACGTACATG-3′.

2.6. Migration and invasion assays

The tumor cells (1×10^5 each) were serum-starved and seeded into Boyden chambers with Matrigel-coated inserts (Corning, NY, USA) for the invasion or migration assays. The chambers were then placed in 24-well plates containing medium and 10% FBS. For migration assays, tumor cells were suspended in 500 μl serum-free RPMI 1640 medium (2.5×10^4 cells) and cultured in the upper chamber. Fetal bovine serum-conditioned medium (10%) (750 μl) was added to the lower 24-well plates. For invasion assays, the inserts were coated with Matrigel (50 μl/well) (BD Biosciences) before adding the cells. After 24 h, the cells attached to the undersurface of the membrane were fixed in methanol and stained with crystal violet, and the number of cells that invaded through the Matrigel layer was counted in five random fields with an Olympus BX51 microscope.

2.7. Wound healing assay

The cells were seeded at 80% to 90% confluence in 6-well plates. An area of cells was scratched with a pipette tip drawn across the center of

each well. The cells were washed three times followed by treatment with FBS-free medium. The wound closure was monitored at 12 and 24 h by comparing the wound distance ratio at 0 h. This experiment was independently repeated three times.

2.8. Tumorigenesis and metastasis of xenografts

The *in vivo* experiments were performed using 4-week-old male nude mice in accordance with the guidelines and approval of the Institutional Animal Care and Use Committee of Ruijin Hospital. The SGC-7901, NC, shKIF14, SGC-7901/vector, and SGC-7901/KIF14 cells were harvested, washed, and resuspended in PBS. The mice received 3×10^5 cells in 0.1 ml PBS, which was injected s.c. into the right flank regions or by lateral tail vein injections using 3×10^6 cells. In the peritoneal cavity metastasis model, 2×10^6 cells were intraperitoneally injected into the 5-week-old male nude mice. The two groups of mice were euthanized after 5 or 4 weeks, and the quantity of the planted tumors, the metastatic foci on lung surface or in the abdominal cavity and the volume of ascites were measured. For the tissue morphology evaluation, hematoxylin and eosin staining was performed on sections from the embedded samples. The IHC staining for KIF14 was performed on sections from the xenograft tumors.

2.9. Western blotting

The proteins were separated by sodium dodecyl sulfate–polyacrylamide gel electrophoresis and were transferred to a nitrocellulose membrane (Bio-Rad, Hercules, CA). The primary antibodies recognized KIF14 (A300-233A, Bethyl Laboratories, Inc.), Akt (9272S, Cell Signaling Technology), phospho-Akt (Ser473) (4060S, Cell Signaling Technology), vimentin (5741S, Cell Signaling Technology), snail (3879S, Cell Signaling Technology), e-cadherin (14472, Cell Signaling Technology), fibronectin (610077, BD), and the β-actin antibody (PM053-7, MBL) was used as a loading control. The quantitative changes in the luminescence were estimated with LAS1000 UV mini and Multi Gauge Ver. 3.0 (Fuji Film, Tokyo, Japan).

2.10. IHC staining

IHC staining was performed according to a previous study [25]. Briefly, the IHC staining for KIF14 was performed in paraffin-embedded tissues. The following primary antibodies were used: rabbit anti-KIF14 (1:200) followed by an incubation with a biotinylated secondary antibody (1:100). The IHC staining scores of KIF14 in the gastric tissues were assessed by two pathologists. The percentage of positive cells and the intensity of the immunostaining were quantified and classified into five groups as follows: 0, < 5% positive cells; 1, 5–24% positive cells; 2, 25–49% positive cells; 3, 50–74% positive cells; and 4, ≥75% positive cells. The intensity of the KIF14 protein staining was scored as 1 for yellow staining, 2 for claybank and 3 for sepia staining. The percentage of positive tumor cells and the staining intensity were multiplied to produce a weighted score for each case. The cases with weighted scores of 0–5 were termed negative expression, 6–8 was positive expression, and 9–12 was strong positive expression [25].

2.11. Microarray hybridization

RNA was purified from normal gastric mucosal and GC tissues using RNeasy (QIAGEN, Valencia, CA) according to manufacturer's instructions. Purified mRNA (2 μg) was used to synthesize cDNA with SuperScript II (Invitrogen). cDNAs were purified using RNeasy Mini Kit (QIAGEN), labeled with Cy3, and hybridized at 65 °C for 17 h and samples were hybridized to AffymetrixGenechip® Human Gene 2.0 ST Arrays according to a previous study [26]. The gene expression data discussed in this report have been deposited in the National Center for Biotechnology (NCBI) Gene Expression Omnibus (GEO) database and are accessible through GEO Series accession number GSE118897.

2.12. Bioinformatics analysis

The differentially expressed genes were identified based on RVM *t*-test and FDR (False Discovery Rate) analysis, and genes with at least 2.0-fold change in either direction with p-value < 0.05 were considered to be up- or down-regulated. The hierarchical clustering analysis (Cluster 3.0) and TreeView analysis were performed to generate a dendrogram for each cluster of genes based on their expression profiling similarities. The Gene Ontology (GO) gene sets biological process database from the Molecular Signatures Database–MsigDB was used for the enrichment analysis [27]. To gain further insight into the biological pathways involved in GC pathogenesis through the KIF14 pathway, a GSEA was performed using GSEA version 2.0. The gene sets showing an FDR of 0.25, which was a well-established cut-off for the identification of biologically relevant genes, were considered enriched between the classes under the comparison.

The data sets (the Cancer Genome Atlas Stomach Adenocarcinoma database (TCGA-STAD), GSE27342, and GSE33335) of GC patients from the publicly available TCGA and GEO databases were used as the validation sets. The gene expression data and the corresponding clinical data used in this study were obtained from the GEO database and the related article [28,29]. The TCGA RNA-Seq and the corresponding clinical data were downloaded from the TCGA-STAD website [30]. 443 gastric cancer patient samples copy number variation (CNV) data were isolated from the database TCGA-STAD, and data type was copy number segments. Every patient's cancer and normal control data were contrast with chromosome 1q32 (chr1: 198,700,001-214,400,000) by Bedtools and the CNV data from cancer samples generated in this segment were isolated [31,32]. Then, these extracted data were compared with the gene KIF14 location information (chr1: 200,551,500-200,620,734) and the intersection data were isolated as target CNV data. The number of the CNV segments were calculated and Segment_Mean > 0 were judged as Gain and Segment_Mean < 0 were judged as Loss. Normal sample data were polishing by ′ −1 −1′ and not displayed in the Supplementary Table S4.

2.13. Statistical analysis

All the experiments were performed at least three times, and the results are shown as the mean ± SEM. Student *t*-test was performed when comparing two groups. The significance of the differences among more than two groups was calculated using an ANOVA with Tukey's *post hoc* test. The correlations of the clinical characteristics of the GC patients were analyzed using the chi-squared test and Fisher's exact test. The survival data were used to draw the Kaplan-Meier curves, and the differences among the groups were analyzed by a log-rank assay. The univariate and multivariate Cox proportional hazards modeling was used to evaluate the prognostic significance. The SPSS statistics 20.0 package was used for the other analyses. The correlation between KIF14 and pAkt expression was analyzed by Pearson's correlation. $P < 0.05$ was considered a statistically significant difference.

3. Results

3.1. KIF14 is upregulated in human GC cell lines and tissues

After an informatics analysis of the gene profiling data derived from the 10-pairs GC tissue samples, we identified the most significantly changed genes and enriched GSEA-GOs (Supplementary Fig. S1A–B; Supplementary Table S1), and the gene KIF14 was selected to go on to the next step in the study (Supplementary Fig. S1C and Supplementary Table S2). The expression of KIF14 was verified in GES-1 cells and in another four GC cell lines, including SGC-7901, AGS, MKN28 and MKN45. The gastric cancer cell lines had a relatively high expression

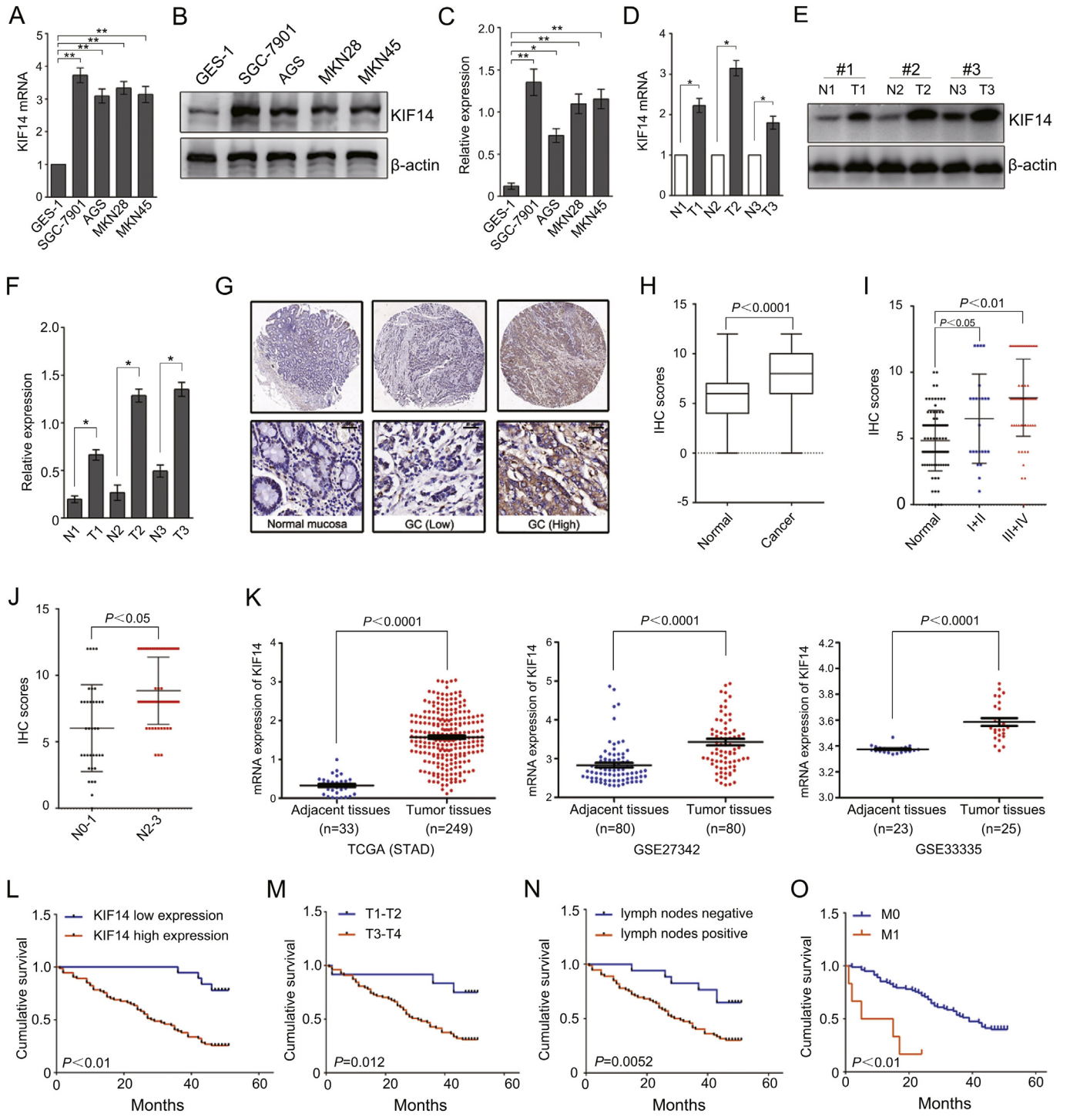

Fig. 1. KIF14 is upregulated in GC cells and tissues and is associated with a poor prognosis in GC. (A) Real-time PCR analyzed the mRNA levels of KIF14 in the GC cell lines SGC-7901, AGS, MKN28, and MKN45 and in the immortal gastric epidermal cell line GES-1 (mean ± SEM, $n = 3$, $^{**}P < 0.01$, an ANOVA with Tukey's *post hoc* test, GES-1 mRNA expression normalized to 1). (B) KIF14 expression was examined by western blotting. (C) The relative expression of the KIF14 protein levels in the cell lines. The histogram indicates the signal intensity of the proteins against β-actin (mean ± SEM, $n = 3$, $^{*}P < 0.05$, $^{**}P < 0.01$, an ANOVA with Tukey's *post hoc* test). (D–E) Real-time PCR and western blotting analysis for the KIF14 mRNA and protein levels in 3 pairs of human gastric samples (mean ± SEM, $n = 3$, $^{*}P < 0.05$, Student *t*-test). N, normal tissue; T, GC tissue. (F) The relative expression of KIF14 protein levels in GC tissues. The histogram indicates the signal intensity of the proteins against β-actin (mean ± SEM, $n = 3$, $^{*}P < 0.05$, Student *t*-test). (G) Representative IHC staining of KIF14 in the normal and cancer tissues (up: ×40; below: ×400). (H) Statistical analysis of KIF14 expression in the normal mucosa and GC tissues. ($n = 90$, $P < 0.0001$, Student *t*-test). (I–J) The expression of KIF14 was associated with pathological stage and lymph nodes metastasis. A scatter plot of the KIF14 IHC scores according to the tumor pathological stages (Normal *vs* I + II; Normal *vs* III + IV) and lymph nodes metastasis (N_{0-1} *vs.* N_{2-3}). (K) An analysis of KIF14 expression in GC and the matched mucosa in three independent datasets (TCGA, GSE27342, and GSE33335) ($P < 0.0001$, Student *t*-test). (L) Kaplan-Meier curves of the cumulative survival of the GC patients with high and low KIF14 expression ($P < 0.01$). (M–O) Kaplan-Meier curves of the cumulative survival of the GC patients with different tumor stages (T1-T2 *vs* T3-T4), lymph node states (lymph nodes negative *vs* lymph nodes positive) and metastasis (M0 *vs* M1) ($P < 0.01$).

level of KIF14 compared with the GES-1 cells, both at the mRNA and protein levels (Fig. 1A–C). These findings were consistent with the results in 3 pairs of GC tissue samples (Fig. 1D–F).

To further illuminate the expression pattern of KIF14, we performed IHC for KIF14 using the tissue microarrays that contained 90 pairs of GC tissue samples. The GC tissues showed a relatively higher expression of KIF14 compared with the normal tissues [24] (Fig. 1G–H; Supplementary Table S3). Additionally, the KIF14 expression was examined in the GC metastasis tissues, such as the abdominal wall, greater omentum and ovary. As expected, these tissues demonstrated a high expression of KIF14 (Supplementary Fig. S2).

The high expression of KIF14 was positively associated with pathological stage ($P < 0.01$), lymph nodes metastasis ($P < 0.05$) (Fig. 1I–J), the TNM stage and metastasis (Table 1). However, there were no significant correlations between the expression levels of KIF14 and other clinicopathologic variables, such as gender and age (Table 1). Furthermore, to testify our results, we set out to determine the expression pattern of KIF14 in a large panel of samples from the TCGA, GSE27342, and GSE33335 datasets. KIF14 expression was significantly increased in the GC tissues when compared with the adjacent tissues of GC patients in the TCGA independent dataset and the GSE27342 and GSE33335 cohorts (Fig. 1K). Additionally, using gastric cancer copy number variation (CNV) data from 443 samples in TCGA-STAD database we found that 27.3% KIF14 copy number gain occurred in the chromosome 1q32 (Supplementary Tables S4 and S5).

3.2. High KIF14 expression correlates with poor prognosis of GC patients

Due to the fact that KIF14 is highly expressed in the GC, we hypothesized that high levels of this protein predicted poor survival in GC patients. To test this hypothesis, the indicated 90 patients were analyzed for overall survival. GC Patients with a high KIF14 expression had a significantly detrimental prognosis compared to those with a low KIF14 expression ($P < 0.01$; Fig. 1L). Additionally, the overall survival of the patients was also significantly different between the groups of patients with different tumor stages (T1-T2 *vs* T3-T4), lymph nodes metastasis states (lymph nodes negative *vs* lymph nodes positive) and metastasis (M0 *vs* M1) ($P < 0.01$; Fig. 1M–O). A further multivariate Cox analysis showed that KIF14 high expression was an independent prognostic factor for poor survival in GC patients (HR, 4.90; 95%CI, 1.73–13.86; $P = 0.003$; Table 2). Thus, it appears that high expression of KIF14 is a poor prognostic indicator for GC patients.

Table 1
Expression of KIF14 in gastric cancers and its correlation with clinicopathologic variables.

Variables	Total	KIF14			*P*
		Negative	Positive	Strong	
Gender					
Male	67	13(14.4%)	31(34.4%)	23(25.6%)	0.507
Female	23	6(6.7%)	12(13.3%)	5(5.6%)	
Age					
< 60	29	7(7.8%)	14(15.6%)	8(8.9%)	0.836
≥60	61	12(13.3%)	29(32.2%)	20(22.2%)	
Tumor size					
≤5 cm	43	12(13.3%)	23(25.6%)	8(8.9%)	0.214
> 5 cm	47	7(7.8%)	20(22.2%)	20(22.2%)	
T stage					
$T_{1–2}$	12	6(6.7%)	5(5.6%)	1(1.1%)	0.001
T_3	60	12(13.3%)	33(36.7%)	15(16.7%)	
T_4	18	1(1.1%)	5(5.6%)	12(13.3%)	
Lymph nodes					
$N_{0–1}$	36	16(17.8%)	13(14.4%)	7(7.8%)	0.003
$N_{2–3}$	54	3(3.3%)	30(33.3%)	21(23.3%)	
Metastasis					
Absent	84	19(21.1%)	42(46.7%)	23(25.6%)	0.030[a]
Present	6	0(0)	1(1.1%)	5(5.6%)	
TNM stage					
I	6	4(4.4%)	1(1.1%)	1(1.1%)	< 0.001
II	29	13(14.4%)	12(13.3%)	4(4.4%)	
III	49	2(2.2%)	29(32.2%)	18(20.0%)	
IV	6	0(0%)	1(1.1%)	5(5.6%)	

[a] Fisher's exact test.

3.3. KIF14 down-regulation attenuates GC cell proliferation, colony formation and xenograft tumor formation

For an in-depth understanding of the biological functions of KIF14 and its role in carcinogenesis, we performed a GSEA analysis of the RNA-sequencing data of the GC cohort from TCGA-STAD, GSE27342 and GSE33335, which showed that the gene signatures of cell proliferation, metastasis and epithelial-mesenchymal transition (EMT) correlated with the patients with KIF14-higher expression (Supplementary Fig. S3). Interfering shRNAs were designed and infected into SGC-7901 cells. The expression of KIF14 was validated by real-time PCR and western blotting (Fig. 2A–B). KIF14 knockdown significantly suppressed SGC-7901 cell growth (Fig. 2C) and colony formation (0.96 ± 0.12, 1 and 0.39 ± 0.99 in SGC-7901, NC, and shKIF14 cells) (Fig. 2D).

Furthermore, to examine whether the down-regulation of KIF14 expression inhibited the tumorigenicity *in vivo*, the growth of the xenograft tumors from the SGC-7901, NC and shKIF14 cells were compared. Tumor formation and the volume of the tumor in each mouse were examined, measured and recorded for 5 weeks, and the tumor growth curves were determined. Our results demonstrated that after the same amount of the three groups of cells were injected into mice, tumors appeared in all of the animals from the two control groups at day 10. However, mice that received the injection of the shKIF14 cells developed relatively small tumors at a later date (day 19) (Fig. 2E), and the tumor weight was much smaller in the shKIF14 group (Fig. 2F, G). Additionally, the protein expression of KIF14 and Ki67 protein was low in tumors derived from the shKIF14 cells by IHC (Fig. 2H).

3.4. Inhibition of KIF14 suppresses the migration and invasion of GC cells in vitro and in vivo

We next examined cell invasion and migration to understand the molecular basis by which KIF14 regulates cancer metastasis. Typical images from the cell invasion, migration and wound healing assay are shown in Fig. 3A–B, which show a significant reduction in cell invasion and migration of shKIF14 cells. To investigate whether KIF14 affects tumor metastasis *in vivo*, the shKIF14 and control cells were intravenously and intraperitoneally injected into nude mice. The average number and volume of the metastatic nodules in the lungs of the mice injected with the shKIF14 cells were reduced compared to those injected with parental and NC cells (Fig. 3C). Metastatic lung nodules were confirmed by hematoxylin and eosin (H&E) staining (Fig. 3C). In the peritoneal metastasis models, the ascites volume and peritoneal nodules were decreased in the shKIF14 group (Fig. 3D–E). The expression of vimentin, snail, fibronectin, the EMT marker, was suppressed *via* the downregulation of KIF14 in the metastatic tissues (Fig. 3F–G). Collectively, these data suggest that the downregulation of KIF14 suppresses the metastasis of GC cells *in vitro* and *in vivo*.

3.5. Forced expression of KIF14 promotes the proliferation, colony formation, invasion and migration of GES-1 cells

To determine the effect of KIF14 overexpression on normal gastric mucosal epithelial cells, we constitutively expressed KIF14 in GES-1 cells, which have a relatively low KIF14 expression. The ectopically expressed KIF14 was confirmed by real-time PCR and western blotting (Fig. 4A). The overexpression of KIF14 promoted proliferation and colony formation in the GES-1 cells (Fig. 4B–C). Additionally, the KIF14 overexpressing cells had significantly greater invasive and migratory

Table 2
Univariate and multivariate Cox regression analysis of different prognosis factors in gastric cancer patients.

Factors	Univariate analysis			Multivariate analysis		
	HR	95%CI	*P*	HR	95%CI	*P*
KIF14 expression (low *vs* high)	4.71	1.56–14.28	0.0004	4.90	1.73–13.86	0.003
Gender (male *vs* female)	1.10	0.60–2.01	0.7538			
Age (< 60 y *vs* ≥ 60 y)	1.26	0.72–2.23	0.4231			
Tumor size (≤5 *vs* > 5 cm)	2.86	1.64–4.99	0.0002	2.07	1.17–3.66	0.013
Lymph node status (N_{0-1} *vs* N_{2-3})	1.85	1.06–3.22	0.0296	0.83	0.27–2.58	0.376
Metastasis (absent *vs* present)	4.58	1.86–11.29	0.0009	3.07	1.24–7.63	0.015
T stage (early *vs advanced stage*)	4.18	1.30–13.41	0.0162	1.82	0.51–6.44	0.204
TNM stage (stage I–II *vs* stage III–IV)	2.44	1.36–4.37	0.0027	1.64	0.46–5.86	0.152

HR Hazard ratio, CI Confidence interval.

capacities compared to the control groups (Fig. 4D–E). To further confirm these results, KIF14 was knocked down in the KIF14 overexpressing cells (Fig. 4F). As seen in Fig. 4G and H, the depletion of KIF14 markedly suppresses the invasiveness and migration of GES-1 cells, thus confirming that the enhancement of invasion and migration is mediated by KIF14. However, increased KIF14 expression in GES-1 cells is not sufficient to increase tumorigenicity of GES-1 cells *in vivo* (data not shown). These observations were also extended to human gastric cancer SGC-7901 cells, when KIF14 overexpressing GC cells were injected into the mice, the xenograft tumors appeared much larger and heavier (Fig. 4I). Taken together, these results indicate that KIF14 expression is responsible for the proliferation, invasion and migration promotion of gastric mucosal epithelial cells *in vitro*.

3.6. KIF14 activates p-Akt to promote GC cells proliferation, invasion and migration

Akt activation is known to correlate with tumor cell proliferation, metastasis as well as EMT [33–35], and thus, we evaluated the expression of p-Akt in the SGC-7901, NC and shKIF14 cells by an immunoblot analysis. The phosphorylation of Akt was significantly inhibited following KIF14 knockdown (Fig. 5A). Consistently, when KIF14 was re-expressed, p-Akt expression was rescued (Fig. 5B). In addition, there was a significant positive correlation between the expression of KIF14 and p-Akt, and this was evaluated in the same tissue microarray containing 97 human GC tissues (Fig. 5C–D). p-Akt activation correlated with high KIF14 expression in the same samples (Fig. 5C case 2). Accordingly, the GC samples with a low KIF14 expression demonstrated reduced p-Akt activation (Fig. 5C case 1; Supplementary Table S6). These results suggested that the involvement of p-Akt activation in the effect of KIF14 on GC. Furthermore, to confirm the effect of Akt, a novel allosteric kinase inhibitor of Akt, MK-2206, was used [36]. The NC and shKIF14 cells transfected with the vector and KIF14 were treated with or without 0.5 μmol/L MK-2206, and Akt phosphorylation was inhibited both in the control and KIF14 overexpressed cells (Fig. 5E). Simultaneously, the proliferation, invasion and migration abilities of cancer cells were largely repressed in the MK-2206-treated cells (Fig. 5F–H). However, when Akt was overexpressed in shKIF14 cells (I) the effects mediated by KIF14 knocked down was greatly rescued (J–M). Taken together, these data suggest that the activation of p-Akt is involved in KIF14 stimulated cell growth and metastasis.

4. Discussion

KIF14 is reported to play a role in various tumors [9,10,13]. However, this study provided the first demonstration of its crucial functions in GC by combining high-throughput data analysis and functional assays. In the current study, KIF14 was significantly overexpressed in GC tissues by a gene profiling analysis. The *in vitro* experiments demonstrated that KIF14 was overexpressed both in human GC cell lines and tissues. Additionally, KIF14 was positively correlated with tumor stage, lymph nodes metastasis and poor prognosis. The inhibition of KIF14 expression greatly repressed the proliferation, invasion and migration of the GC cells. Conversely, the ectopic overexpression of KIF14 in the GES-1 cells greatly enhanced proliferation, invasion and migration. Notably, in the xenograft models, KIF14 was required for tumor formation and for the efficient dissemination of the GC cells to lung and peritoneal cavity.

Mounting evidence suggests that KIF14 may indeed be an oncogenic potential gene. As reported, the identification of KIF14 genomic gain in benign retinoma lesions and its overexpression in premalignant breast tissue demonstrate that the gain of KIF14 is an early event in tumorigenesis [37]. In breast cancer, Singel et al. presented an oncogenic role of KIF14 with its ectopically expression while knockdown correlated with decreased Akt phosphorylation and activity. And inhibition of KIF14 with siRNA or an experimental small-molecule inhibitor showed a chemosensitizing effect and correlated with decreasing activation of Akt. Their results demonstrated that therapeutic targeting of KIF14 was feasible and targeting KIF14 may increase chemosensitivity in “triple-negative” breast cancers [38]. Moreover, Corson et al. reported that KIF14 expression was an independent prognostic factor for disease-free survival and knockdown decreased tumorigenicity *in vitro* in lung cancer, showing that it was a clinically relevant oncogene and an exciting therapeutic target [39]. Besides, KIF14 plays an oncogenic role in glioma/glioblastoma through a mechanism involving the activation of Akt and function as a candidate prognostic marker and a promising molecular target for human gliomas [40,41]. Recently, Zhang et al. reveals that KIF14 acts as a potential oncogene that contributes to tumor progression and poor prognosis in prostate cancer, which may represent a novel and useful prognostic biomarker for prostate cancer [42]. Apart from KIF14, many other kinesin family members are also reported as tumor promoting genes, including KIF3A, KIF5B, KIF1B, KIF4A, KIF7, and KIF2a [43–48]. However, there are also contrary opinions about the role of KIF14 in human cancers. Hung et al. demonstrated that expression of KIF14 was negatively correlated with clinical outcomes in the lung adenocarcinoma patients, modulating the expression of KIF14 inhibited cell migration, invasion and adhesion by controlling the recruitment of adhesion molecules cadherin 11 to the cell membrane [49]. Moreover, Abiatari et al. found a significant decrease in KIF14 expression in highly invasive clones of pancreatic cell lines using a rat vagal nerve model by an expression profiling analysis [50]. Ectopic expression of cadherin 11 was reported to induce tumor cell apoptosis, inhibit Akt signaling and EMT, thus further inhibiting tumor cell migration and invasion [51]. So, one explanation for the discrepancy of KIF14 function may due to that in certain type of cancers KIF14 recruits excessive cadherin 11, which induces apoptosis and inhibits Akt activity and EMT, as a result contributing to KIF14 suppressive role in tumorigenicity. Other reason may stem from the use of different cell models, diverse experimental conditions and inter-cohort variation. Therefore, validation of these findings is crucial for expanding the understanding of the role of KIF14 in cancer. In addition,

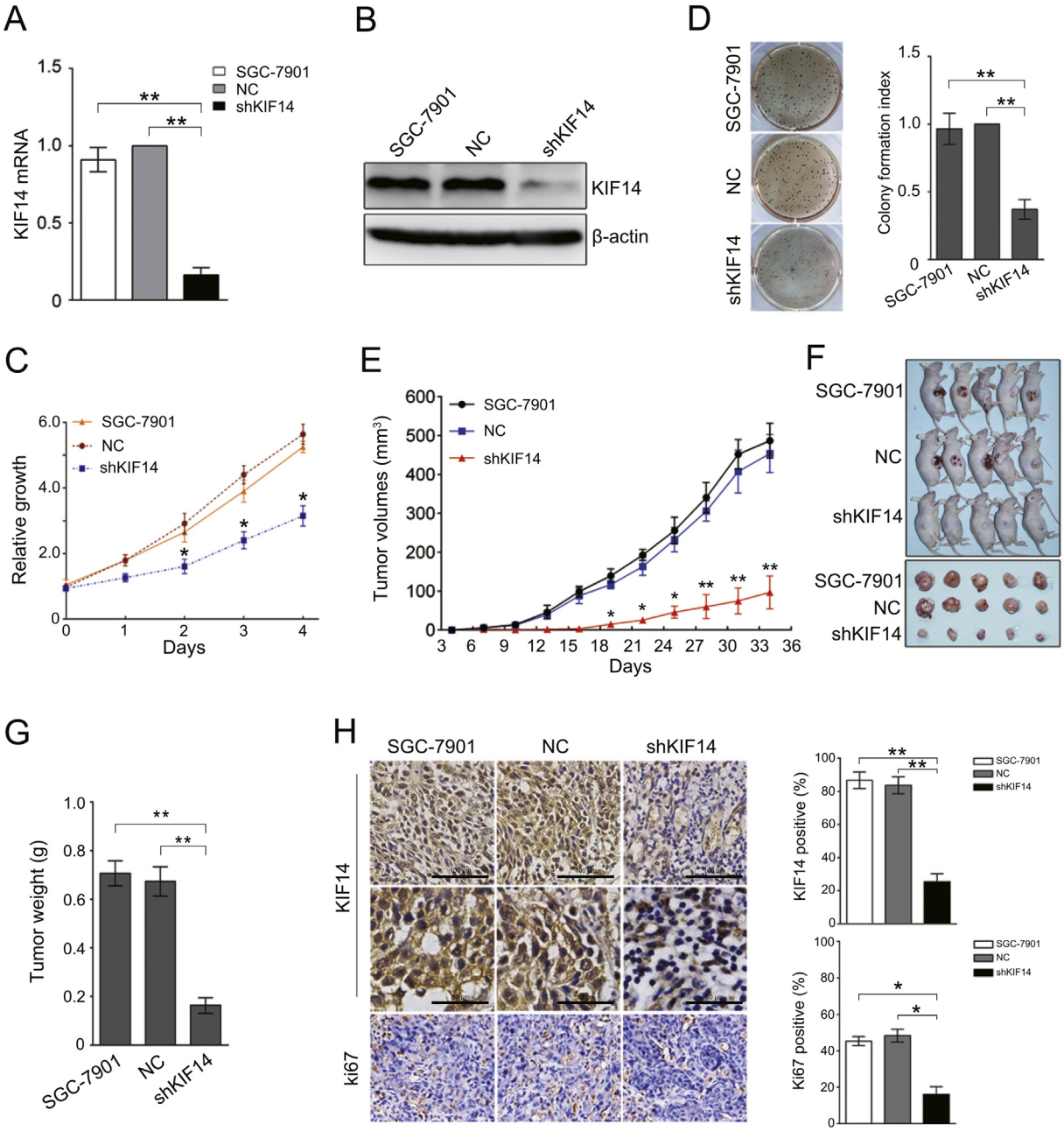

Fig. 2. Down-regulation of KIF14 inhibits the proliferation, colony formation, and xenograft tumor formation of SGC-7901 cells. (A-B) The expression of KIF14 was examined by real-time PCR and western blotting (mean ± SEM, $n = 3$, $^{**}P < 0.01$, an ANOVA with Tukey's *post hoc* test). (C) The growth of the SGC-7901, NC and shKIF14 cells was measured within 4 days by a CCK-8 assay (mean ± SEM, $n = 3$, $^{*}P < 0.05$, an ANOVA with Tukey's *post hoc* test). (D) A colony formation assay was performed to examine colony formation in the SGC-7901, NC and shKIF14 cells (mean ± SEM, $n = 3$, $^{**}P < 0.01$, an ANOVA with Tukey's *post hoc* test). (E) The SGC-7901, NC and shKIF14 cells were inoculated into nude mice. The tumor diameters were measured every 3 days. (mean ± SEM, $n = 5$, $^{*}P < 0.05$, $^{**}P < 0.01$, an ANOVA with Tukey's *post hoc* test). (F) Image of the excised xenografted tumors that originated from the SGC-7901, NC and shKIF14 cells. (G) Histogram depicting the tumor weight of the xenografted tumors (mean ± SEM, $n = 3$, $^{**}P < 0.01$, an ANOVA with Tukey's *post hoc* test). (H) KIF14 and ki67 expression was determined by IHC staining in the xenografted tumors derived from the SGC-7901, NC and shKIF14 cells (×200, top; ×400, bottom) (mean ± SEM, $n = 3$, $^{*}P < 0.05$, $^{**}P < 0.01$, an ANOVA with Tukey's *post hoc* test).

the conflicting reports promote an urgent need to explore the biological function of KIF14 in human cancers.

Furthermore, we demonstrated that the prognosis was poor for GC patients with a high KIF14 expression ($P < 0.01$). These findings suggest that KIF14 is a promising target for GC therapy, although it requires further verification. To date, many of the reported kinesin inhibitors have been tested, but not against KIF14 [52,53]. It is possible that some existing molecules already target this protein. The progression of other mitotic kinesin inhibitors into clinical trials and the fact that the motor domain is selectively targetable with inhibitors also offer promise for KIF14 as a potential therapeutic target.

Although the exact mechanism of how KIF14 is involved in the tumorigenesis of GC remains to be clarified, our GSEA analysis showed that a higher expression of KIF14 was associated with proliferation, metastasis and EMT pathways. These pathways all contribute to the tumorigenesis of GC [54], and the bioinformatics analyses were in agreement with the aforementioned *in vitro* and *in vivo* results. Moreover, we showed that Akt phosphorylation was inhibited by KIF14

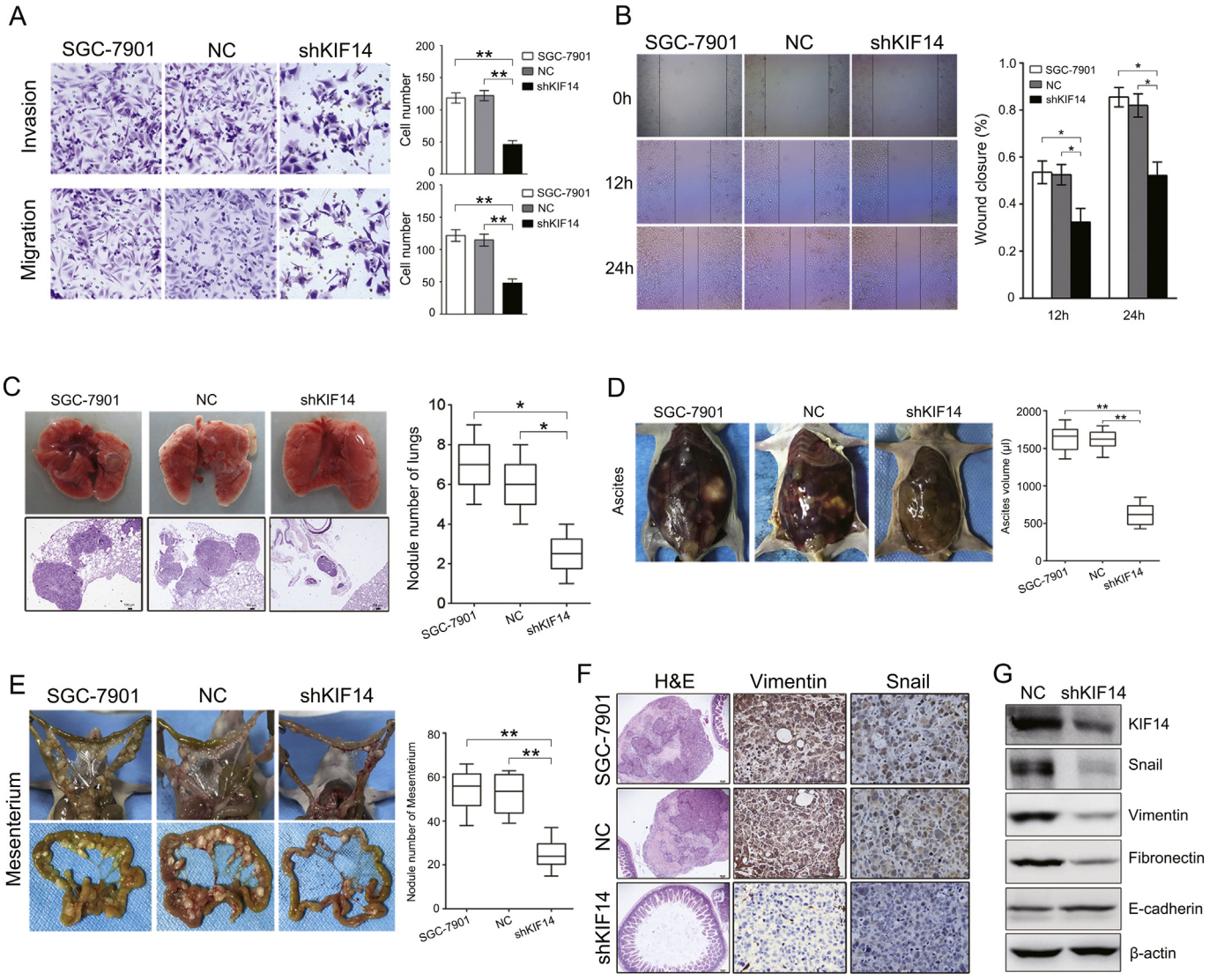

Fig. 3. KIF14 down-regulation suppresses the migration and invasion of GC cells *in vitro* and *in vivo*. (A) A Matrigel invasion and migration assay and a scratch-wound assay (B) were performed. The wound closure (%) was calculated (mean ± SEM, $n = 3$, $^{*}P < 0.05$, $^{**}P < 0.01$, an ANOVA with Tukey's *post hoc* test). (C) Representative photographs of lungs with metastatic nodules. Micrographs of the lung tissues with metastatic cells are shown by H&E staining at a magnification of ×40 (bottom). The number of metastatic nodules per lung in the SGC-7901, NC and shKIF14 cell-injected mice (mean ± SEM, $n = 10$, $^{*}P < 0.05$, an ANOVA with Tukey's *post hoc* test) (right). (D) Representative photos of the ascites in the SGC-7901, NC and shKIF14 cell intraperitoneally injected mice. Box plot of the ascites volumes collected from the control and KIF14 knockdown cells (mean ± SEM, $n = 10$, $^{**}P < 0.01$, an ANOVA with Tukey's *post hoc* test). (E) Effect of KIF14 knockdown on peritoneal metastasis (mean ± SEM, $n = 10$, $^{**}P < 0.01$, an ANOVA with Tukey's *post hoc* test) (right). (F) H&E staining of the metastatic tumors (left); IHC staining of the Vimentin and Snail in the metastatic tumors from the mesentery of the small intestine of the mice (right). (G) Expression of KIF14, Vimentin, Snail, Fibronectin and E-cadherin in the metastatic tumors was examined by western blotting.

downregulation, which suggested a significant role for KIF14 in the activation of the Akt in GC. By using the Akt inhibitor MK-2206 and Akt re-expression, we demonstrated that the activation of p-Akt was involved in KIF14 stimulated cell growth and metastasis, but the underlying mechanism still needs a deeper exploration.

In conclusion, our findings showed, for the first time, that GC patients expressing high level of KIF14 had a poorer survival compared with those with a low KIF14 expression, and KIF14 expression was an independent prognostic factor for gastric cancer. The dysregulation of KIF14 mediated the proliferation, invasion and migration of GC, and the Akt pathway was involved in this process. Therefore, KIF14 expression could be used for the prediction of cancer progression, metastasis and prognosis in GC patients.

Supplementary data to this article can be found online at https://doi.org/10.1016/j.bbadis.2018.10.039.

Transparency document

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Acknowledgements

This work was supported by National Natural Science Foundation of China No. 81772518, 81201915, 81772509, 91529302; Multicenter Clinical Trial of Shanghai Jiao Tong University School of Medicine NO. DLY201602; and Foundation for Shanghai Jiao Tong University School

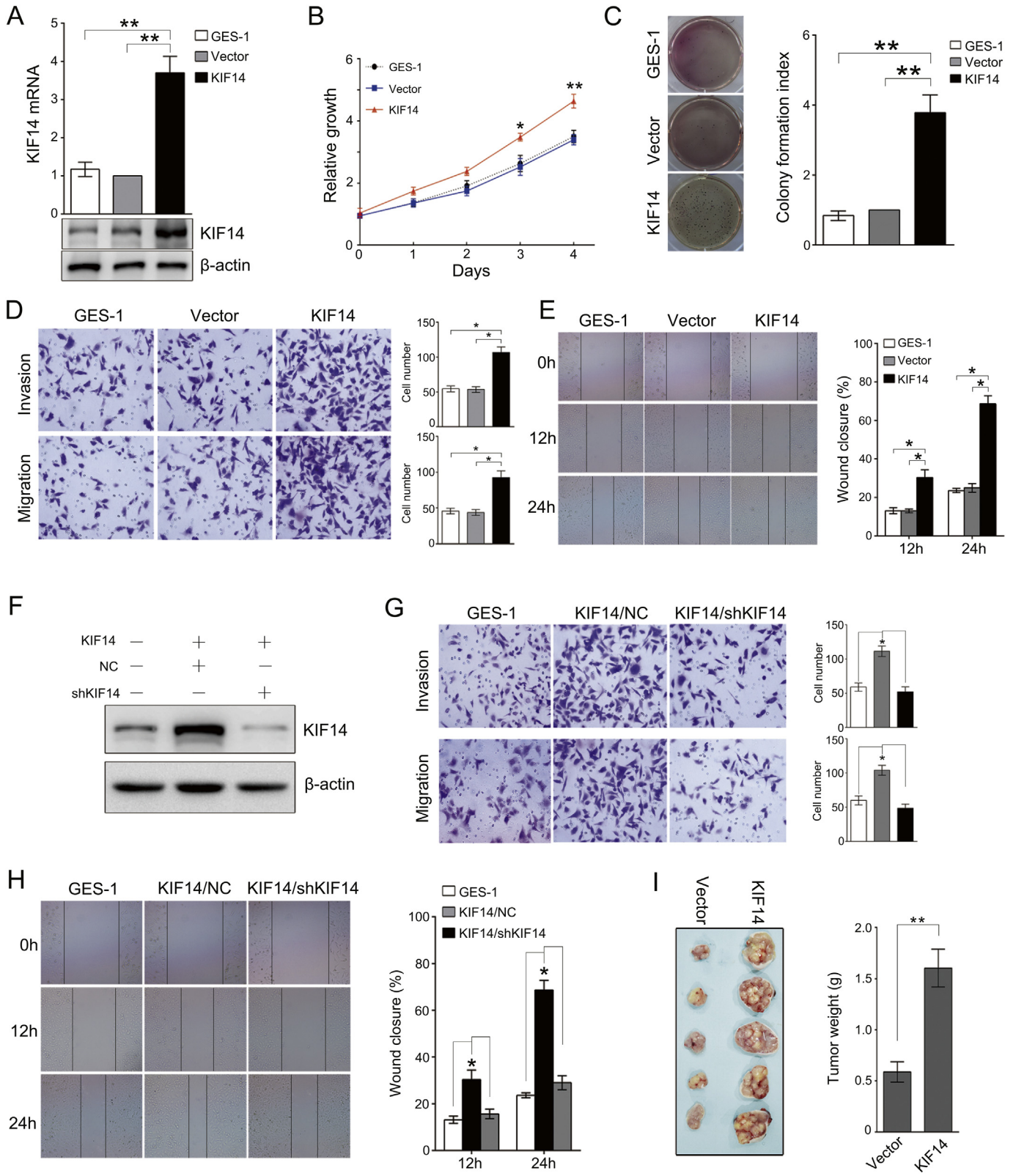

Fig. 4. Forced expression of KIF14 promotes the proliferation, invasion and migration of GES-1 cells. (A) Real-time PCR and western blotting analysis were used to examine the expression of KIF14 in the GES-1, GES-1 transfected with vector (Vector) and pQCXIN-KIF14 (KIF14) cells (mean ± SEM, $n = 3$, $^{**}P < 0.01$, an ANOVA with Tukey's *post hoc* test). (B) The growth of the GES-1, Vector and KIF14 cells was measured within 4 days by a CCK-8 assay (mean ± SEM, $n = 3$, $^{*}P < 0.05$, $^{**}P < 0.01$, an ANOVA with Tukey's *post hoc* test). (C) A colony formation assay was performed to examine colony formation in the GES-1, Vector and KIF14 cells (mean ± SEM, $n = 3$, $^{**}P < 0.01$, an ANOVA with Tukey's *post hoc* test). (D) Matrigel invasion, migration and scratch-wound assays (E) were performed (mean ± SEM, $n = 3$, $^{*}P < 0.05$, an ANOVA with Tukey's *post hoc* test). (F) The knockdown of KIF14 in the KIF14 overexpression cells was confirmed by western blotting. The cell counting in the invasion, migration (G) and wound closure (%) (H) assays were calculated (mean ± SEM, $n = 3$, $^{*}P < 0.05$, an ANOVA with Tukey's *post hoc* test). (I) Image of the excised xenografted tumors that originated from the Vector and KIF14-overexpression cells, and the tumor weight was calculated (mean ± SEM, $n = 3$, $^{**}P < 0.01$, Student *t*-test).

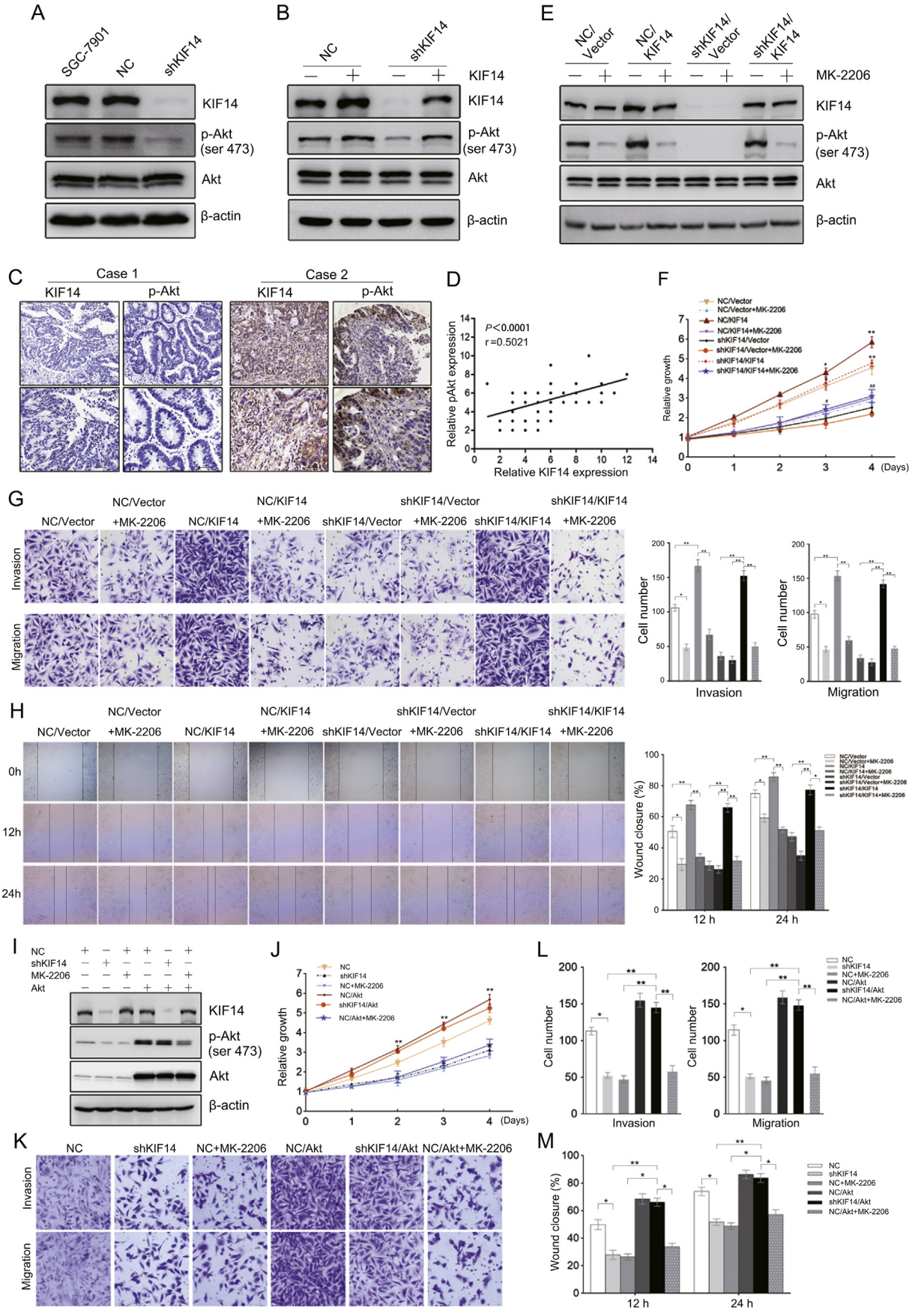

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Fig. 5. Activation of p-Akt is involved in KIF14-stimulated cell growth and metastasis. (A) Western blotting was used to examine the expression of KIF14, Akt and p-Akt in the SGC-7901, NC and shKIF14 cells. (B) KIF14 overexpression NC and shKIF14 cells were examined by western blotting. (C) Representative IHC staining for KIF14 and p-Akt in serial sections of GC patients. (D) There was a positive correlation between the expression level of KIF14 and p-Akt in GC tissues. (E) KIF14 overexpression NC and shKIF14 cells were treated with or without 0.5 μmol/L MK-2206, and the expression of KIF14, Akt and p-Akt were examined by western blotting. (F) The growth of the above 8 groups of cells were measured within 4 days (mean ± SEM, $n = 3$, $^{*,\#}P < 0.05$, $^{**,\#\#}P < 0.01$, an ANOVA with Tukey's *post hoc* test). Matrigel invasion, migration (G) and scratch-wound assays (H) were performed. The cell counting in the invasion and migration, and wound closure (%) assays were calculated (mean ± SEM, $n = 3$, $^{*}P < 0.05$, $^{**}P < 0.01$, an ANOVA with Tukey's *post hoc* test). (I) Akt was overexpressed in NC or shKIF14 cells, the expression of KIF14, Akt and p-Akt were examined by western blotting. (J) The growth of the above 6 groups of cells were measured within 4 days (mean ± SEM, $n = 3$, $^{**}P < 0.01$, an ANOVA with Tukey's *post hoc* test). (K–L) Matrigel invasion, migration and scratch-wound assays (M) were performed with Akt overexpressed cells. The cell counting in the invasion and migration, and wound closure (%) assays were calculated (mean ± SEM, $n = 3$, $^{*}P < 0.05$, $^{**}P < 0.01$, an ANOVA with Tukey's *post hoc* test).

of Medicine NO. 13XJ10011. And we would like to thank the Genminix Informatics Ltd., Co. for assisting us with data analysis.

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