TMEM100 negatively regulated by microRNA-106b facilitates cellular apoptosis by suppressing survivin expression in NSCLC

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Abstract. Non-small cell lung cancer (NSCLC) is a common malignant tumour. Nevertheless, the 5-year survival rate of NSCLC patients remains poor. Thus, identifying critical factors involved in regulating the progression of NSCLC is important for providing potential treatment targets. In the present study, it was observed that transmembrane protein 100 (TMEM100) was significantly downregulated in NSCLC tissues compared with paired peritumoral tissues. Decreased TMEM100 expression was associated with poor clinical outcomes in NSCLC patients. Moreover, TMEM100 overexpression inhibited colony formation and facilitated apoptosis by suppressing survivin expression in NSCLC cells, whereas TMEM100 knockdown had the opposite effect. In addition, microRNA (miR)-106b, a miR with controversial roles in different human cancers, was upregulated in NSCLC and directly downregulated TMEM100 expression. The roles of miR-106b in cell survival were mitigated by the restoration of TMEM100. The aforementioned results indicated that TMEM100 induced cell apoptosis and inhibited cell survival by serving as a tumour suppressor and that miR-106b-mitigatedTMEM100 expression defined a potentially oncogenic pathway in NSCLC.

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Introduction

Lung cancer is a common malignant tumour (approximately 11.4% of all cancers diagnosed), and its cancer-related mortality (18.0% of the total cancer-related deaths) ranks first among various types of tumours (1). There are two major pathological types of lung cancer: Non-small cell lung cancer (NSCLC) and small-cell lung cancer. NSCLC, as the main subtype, accounts for more than 75% of cases (2). The standard treatment option for NSCLC is surgical resection combined with adjuvant radiotherapy/chemotherapy and molecular-targeted agents. However, numerous patients with NSCLC are diagnosed at a late stage and are not suitable for surgical treatment (3). Currently, the molecular mechanisms of NSCLC progression remain incompletely elucidated, and the treatment efficiency is far from what individuals anticipate. The 5-year survival rate is <15% in individuals with NSCLC. Accordingly, it is necessary to determine the oncogenic factors and/or tumour suppressors in the progression of NSCLC to provide potential treatment targets.

An important matter for tumour occurrence and development is the overgrowth of cancer cells without limitation, which is derived from the dysfunction of cell death. Thus, regulators of cell apoptosis are regarded as tumour-related molecules and inducing apoptosis in cancer cells is considered an important method for mitigating tumour progression (4). Accumulating evidence has indicated that a few transmembrane proteins (TMEMs) are up- or downregulated in some types of tumours and participate in regulating tumour progression and tumorigenesis (5-7). The upregulation of TMEM16A has been reported to promote cancer metastasis and be correlated with a poor prognosis in human gastric cancer (8). Cytosolic TMEM88has been revealed to increase the expression of Snail and promote the metastasis of cancer cells in triple-negative breast and lung cancer (9,10). TMEM100, belonging to the family of transmembrane proteins, is well conserved in vertebrates. There are two putative transmembrane domains in TMEM100 (11). The known cellular functions of TMEM100 mainly include arterial endothelial differentiation and vascular morphogenesis. In mouse embryos, TMEM100 knockdown leads to cardiovascular developmental disorders, heart defects,

and a failure of vascular remodelling (12). During atrioventricular canal cushion formation, endothelial-mesenchymal transformation is impaired by TMEM100 deficiency (13). Moreover, TMEM100 mitigates the metastasis and proliferation of cancer cells in hepatocellular carcinoma (14). However, the pathophysiologic roles of TMEM100 in the survival of NSCLC cells remain to be explored.

The present research aimed to determine the regulatory roles of TMEM100 in the apoptosis of NSCLC cells and the corresponding mechanisms involved to provide potential treatment targets for this fatal disease.

Materials and methods

Cell culture. H358, H1650, H1299, A549, H1975, and H460 cells were obtained from the American Type Culture Collection (ATCC). Cells were cultured in cell culture flasks in DMEM (Gibco; Thermo Fisher Scientific, Inc.) supplemented with 10% fetal bovine serum (FBS; Gibco; Thermo Fisher Scientific, Inc.). Cells were cultured in an atmosphere containing 5% CO_2 at 37˚C. Serum deprivation (SD) was utilized as an apoptotic model to induce cell apoptosis in the appropriate experiments.

Cell transfection. Cells at the logarithmic growth phase were trypsinized and plated in 6-well plates at a density of ~1x10^5 cells/well. After 24 h of incubation, Lipofectamine 2000 (Invitrogen; Thermo Fisher Scientific, Inc.) was used to transfect negative control (NC; 50 nM) and miR-106b mimics (miR-106b; 50 nM) or miR-106b inhibitor (anti-miR-106b; 50 nM) and anti-NC (50 nM) or empty vector and recombinant survivin plasmid (1 μg), diluted with Opti-MEM according to the manufacturer's instructions. After 6 h of transfection at 37˚C, the culture medium was renewed and then the cells were collected for subsequent experiments. The sequences were as follows: NC forward, 5'-UCACAACCUCCUAGA AAGAGUAGA-3' and reverse, 5'-UCUACUCUUUCUAGG AGGUUGUGA-3'; miR-106b mimics forward, 5'-UAAAGU GCUGACAGUGCAGAU-3' and reverse, 5'-AUCUGCACU GUCAGCACUUUA-3'; anti-miR-106b 5'-AUCUGCACU GUCAGCACUUUA-3'; and anti-NC 5'-UCUACUCUUUCU AGGAGGUUGUGA-3'. The recombinant survivin plasmid was obtained from Addgene, Inc. Both miR mimics and inhibitors were purchased from Shanghai Biotend Biological Technology, Co., Ltd. The lentiviruses (the lentiviral expression vector pLKO.1) of TMEM100 overexpression and knockdown were purchased from Shanghai GenePharma Co., Ltd. Briefly, pLKO.1/puro was used as the lentiviral plasmid and lentiviruses were generated by the 2nd generation system. Lentiviruses were produced by co-transfection of 293T cells (ATCC) with the lentiviral vector (1.5 μg) and packaging vectors (0.5 μg psPAX2 and 0.5 μg pMD2.G). After 48 h of transfection, the lentiviral supernatant was collected. The lentiviruses were utilized to infect the target cells (1x10^5) at a multiplicity of infection (MOI) of 20, including H358, H460, H1299, and H1975, in the presence of 5 μg/ml polybrene (Sigma-Aldrich; Merck KGaA). After 48 h of incubation, the cells were cultured in DMEM with 10% FBS at 37˚C in the presence of 3 μg/ml puromycin. After a week, cells were then used for the subsequent experimentations.

Western blot analysis. The proteins were extracted from cell lines (H358, H460, H1299, and H1975) by using RIPA (P0013B; Beyotime Institute of Biotechnology) and protein concentrations were determined by bicinchoninic acid (BCA). Cell lysates in equal amounts (50 μg protein) were loaded on sodium dodecyl sulfate-polyacrylamide gels (12%), and then the gels were transferred to polyvinylidene difluoride membranes (Roche Diagnostics). After 2 h of transfer, the membranes were incubated with 5% skimmed milk for 1 h at 25˚C for blocking, and then the primary antibodies were added to the membranes overnight at 4˚C. Primary antibodies included TMEM100, survivin and cleaved caspase-3 (1:500; product codes ab117973, ab76424, and ab2302, respectively; all from Abcam), Bim and cytochrome *c* (both 1:500; product nos. 2933 and 11940, respectively), or β-actin (1:3,000; product no. 3700; all from Cell Signaling Technology, Inc.). After extensive washing with TBST (0.1% Tween) for ~40 min, the corresponding horseradish peroxidase-conjugated secondary antibodies (1:5,000; product nos. 7074 and 7076; Cell Signaling Technology, Inc.) were added to the membranes for 1 h at room temperature. After extensive washing with TBST for ~40 min, the signal was next visualized with chemiluminescence substrate (Pierce Biotechnology; Thermo Fisher Scientific, Inc.) by chemiluminescence detection. Densitometric analysis was performed using Image Lab software (version 4.1; Bio-Rad Laboratories, Inc.).

Reverse transcription-quantitative (RT-q)PCR. RT-qPCR was performed as previously described (15). TRIzol reagent (Invitrogen; Thermo Fisher Scientific, Inc.) and a miRNA isolation kit (Ambion; Thermo Fisher Scientific, Inc.) were used to extract total RNA according to the protocol provided by the supplier. For miRNA analysis, RT-qPCR TaqMan probes for miR-106b were obtained from Applied Biosystems; Thermo Fisher Scientific, Inc., and TaqMan premix (Takara Bio, Inc.) was used for RT-qPCR. For mRNA analysis, a SYBR Green PCR system (Applied Biosystems; Thermo Fisher Scientific, Inc.) was applied after reverse transcription. The primers were designed and were as follows: TMEM100 forward, 5'-TGC TGTGGTTGTCTTCATCG-3' and reverse, 5'-CTCTCCCGT CTCTTGGCTTTC-3'; and β-actin forward, TTGTTACAG GAAGTCCCTTGCC and reverse, 5'-ATGCTATCACCT CCCCTGTGTG-3'. Actin and snRNA U6 (cat. no. 4427975; Thermo Fisher Scientific, Inc.) were utilized as normalization controls to quantify mRNA and miRNA expression levels, respectively.

Cell viability assay. Cell Counting Kit-8 (CCK-8; Dojindo Molecular Technologies, Inc.) was used to determine cell viability. Cells were cultured in a 96-well plate at 3x10^3 cells/well at 37˚C. After adding 10 μl of CCK-8 solution to each well for 2 h at 37˚C, the absorbance was measured with a microplate reader at 450 nm.

Colony formation assay. Cells were cultured at a density of 0.8x10^3 cells (H358 cells) per well or at a density of 3x10^3 cells (H460 cells) per well in 6-cm Petri dishes. A total of 15 days later, 500 μl of 4% paraformaldehyde was added to each well for 10 min at 25˚C. Then, 1% crystal violet (Sigma-Aldrich; Merck KGaA) was utilized to stain the cells for 10 min at 25˚C.

Colonies (approximately >60 cells) could then be observed directly with the unaided eye, and only clearly visible colonies (megascopic cell colonies) observed with the unaided eye were counted and analysed.

Caspase-3 activity assay. A caspase-3 assay kit (Invitrogen; Thermo Fisher Scientific, Inc.) was utilized to detect the activity of caspase-3, according to the manufacturer's instructions. Briefly, 50 μl of cell lysis buffer from the kit was added to the harvested cells on ice for 30 min. Then, the lysed cells were centrifuged at 2,795 x g for 5 min at 4˚C. After adding 50 μl of 2X substrate working solution, the mixture was incubated at room temperature for 30 min. The fluorescence was then measured using a microplate reader (excitation, 342 nm and emission, 441 nm).

Analyses of GEO datasets. To determine the expression of mRNA or miRNA in NSCLC tissues, GEO datasets (the public datasets, www.ncbi.nlm.nih.gov/gds) were analysed in our study. GSE19804 and GSE27262 were used to examine the expression of TMEM100 in NSCLC tissues and paired peritumoral tissues (16,17). GSE3141 was utilised to investigate the clinical significance of TMEM100 in NSCLC tissues (18). MiRNA expression profiling of NSCLC tissues and non-tumor adjacent tissues was acquired from the datasets (GSE63805 and GSE36681) (19,20). Oncomine (a public database; www.oncomine.org) was utilized to examine the expression of TMEM100 in different cancers.

Luciferase experiments. TargetScan (version 7.2; www.targetscan.org) and miRanda (www.microRNA.org) were utilized to identify the potential miRNAs that target TMEM100 based on the sequence of TMEM100 3'untranslated region (UTR) (21,22). As previously described, a dual luciferase reporter gene assay was conducted (15). Briefly, the full-length human TMEM100 3'UTRs containing the wild-type (WT) sites or mutant-type (MT) sites were cloned into the pGL3-Promoter vector (Promega Corporation). By using Lipofectamine 2000 (Invitrogen; Thermo Fisher Scientific, Inc.), control vectors, pGL3-TMEM100 3'UTR and miRNA mimics (Life Technologies; Thermo Fisher Scientific, Inc.) were transfected into 293 cells. According to the protocol provided by the supplier (Promega Corporation), the dual luciferase reporter assay was performed, and firefly luciferase luminescence was recorded by a VICTOR multilabel counter (Berthold Technologies).

TUNEL assay. A One Step TUNEL apoptosis assay kit was purchased from Beyotime Institute of Biotechnology. TUNEL-positive cells were determined according to the manufacturer's instructions. Briefly, cells were cultured in a 6-well plate at $5x10^4$ cells/well at 37˚C for 12 h. Then, the plate was washed with PBS for 5 min and fixed in 4% paraformaldehyde for 30 min at 25˚C. After washing with PBS for 5 min, PBS containing 0.3% Triton X-100 was added to the cells for 5 min at room temperature. Then, the cells were washed twice with PBS for 5 min and treated with 100 μl TUNEL test solution (10 μl TdT enzyme and 90 μl fluorescent labelling solution) per sample at 37˚C for 60 min. After adding 200 μl mounting medium (P0126; Beyotime Institute of Biotechnology), the stained samples (without the nuclear stain) were observed under a fluorescence microscope (magnification, x200; BX61; Olympus Corporation). A total of 5 visual fields were selected randomly for each group.

Statistical analysis. All values are expressed as the mean ± standard error of the mean (SEM) from at least three independent experiments. Paired and unpaired Student's t-tests or one-way ANOVA followed by Dunnett's post hoc test were performed to evaluate the statistical significance. Survival analysis was carried out by using Kaplan-Meier analysis and log-rank testing was selected to compare patient survival between subgroups. Correlations were calculated by Pearson's rank correlation coefficients. $P<0.05$ was considered to indicate a statistically significant difference. GraphPad Prism version 5.0 (GraphPad Software, Inc.) was utilized to perform the statistical analyses.

Results

Downregulation of TMEM100 is associated with poor clinical outcomes in NSCLC patients. The expression of TMEM100 using Oncomine was analysed, and it was revealed that TMEM100 expression was downregulated in some types of cancer tissues, including breast, colorectal and lung cancer (Fig. 1A). To determine the role of TMEM100 in the progression of NSCLC, TMEM100 expression in NSCLC tissues and paired peritumoral tissues was firstly examined via two public data sets (GSE19804 and GSE27262). As revealed in Fig. 1B and C, the mRNA levels of TMEM100 were markedly decreased in 60 cases of tumor tissues compared with paired peritumoral tissues. Consistent results were acquired in the other data set. The expression of TMEM100 in tumour tissues was downregulated compared with paired peritumoral tissues in 25 patients (Fig. 1D and E).

The clinical significance of TMEM100 in NSCLC tissues (GSE3141) was then investigated. The patients were divided into two groups based on the mRNA levels of TMEM100 (the high-TMEM100 group and the low-TMEM100 group). As revealed in Fig. 1F, our results indicated that patients with low TMEM100 expression were associated with poorer overall survival (median overall survival time, 28.4 months; $P<0.01$) than patients with high TMEM100 expression (median overall survival time, 55.4 months). Thus, these results indicated that TMEM100 could be considered as a potential molecule for predicting the prognosis in individuals with NSCLC.

Overexpression of TMEM100 suppresses cell growth and elicits apoptosis in NSCLC cells. To investigate the cellular function of TMEM100 in the progression of NSCLC, the endogenous expression of TMEM100 was then detected in six NSCLC cell lines (Fig. 2A). Based on the endogenous expression of TMEM100 in these cells, TMEM100 was stably overexpressed in H358 cells (low expression levels of endogenous TMEM100) via lentiviral infection. RT-qPCR and western blot analyses were utilized to confirm the overexpression efficiency (Fig. 2B and C). As revealed in Fig. 2D and E, the overexpression of TMEM100 decreased cell viability and inhibited colony formation in H358 cells. Moreover, the expression of key proapoptotic proteins

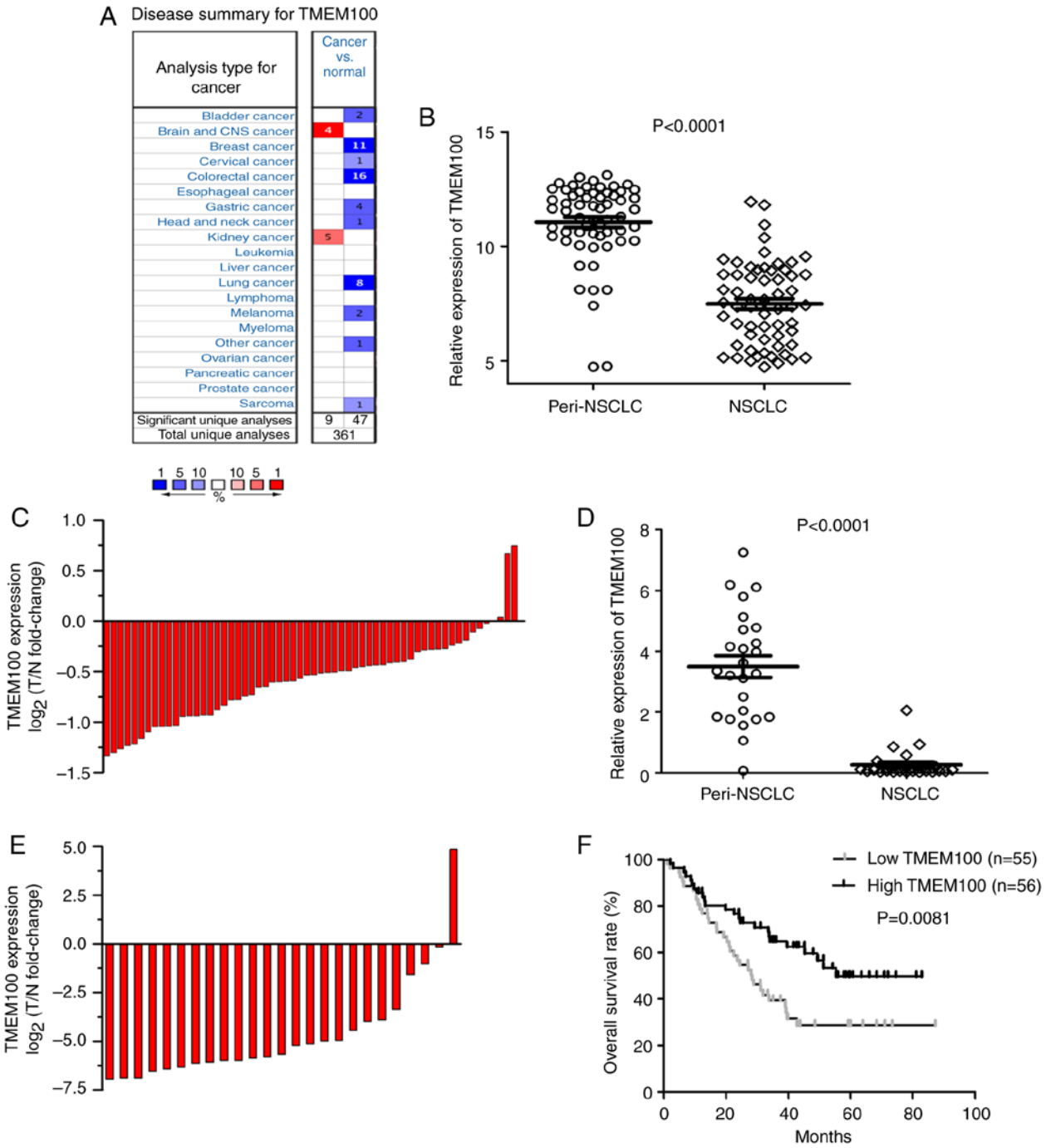

Figure 1. TMEM100 is frequently downregulated in human NSCLC. (A) Expression of TMEM100 in different types of cancer tissues in Oncomine. (B) mRNA levels of TMEM100 were significantly decreased in 60 cases of NSCLC tissues compared with paired peritumoral tissues. (C) Expression data of TMEM100 in the paired samples of NSCLC are presented as the fold change [$\log_2$ (T/N)]. (D) Expression of TMEM100 was significantly downregulated in 25 cases of NSCLC tissues. (E) TMEM100 expression in the paired samples of NSCLC is presented as the fold change [log2 (T/N)]. (F) Kaplan-Meier analysis of overall survival in NSCLC patients based on TMEM100 expression. The results revealed that low expression, rather than high expression, of TMEM100 was associated with poorer overall survival. TMEM100, transmembrane protein 100; NSCLC, non-small cell lung cancer; T, tumour tissues; N, paired normal tissues.

(Bim and cytochrome *c*) in mitochondrial apoptosis, the activity and protein expression of cleaved caspase-3 and TUNEL assays were examined to validate apoptosis in NSCLC cells. The results revealed that the overexpression of TMEM100 induced the expression of Bim and enhanced cytochrome *c* release into the cytoplasm (Fig. 2F and G). Consistent with these results, overexpression of TMEM100 significantly increased the activity and protein expression of cleaved caspase-3 and the number of TUNEL-positive cells (Fig. 2H-J). These results indicated that TMEM100 induced apoptosis in NSCLC cells.

TMEM100 knockdown facilitates cell survival in NSCLC. To represent the physiological functions of TMEM100 on cell survival in NSCLC, TMEM100 expression was knocked down with a shRNA. As revealed in Fig. 3A and B, the efficiency of knockdown was confirmed by RT-qPCR and western blot analyses in H460 cells (high expression levels of endogenous

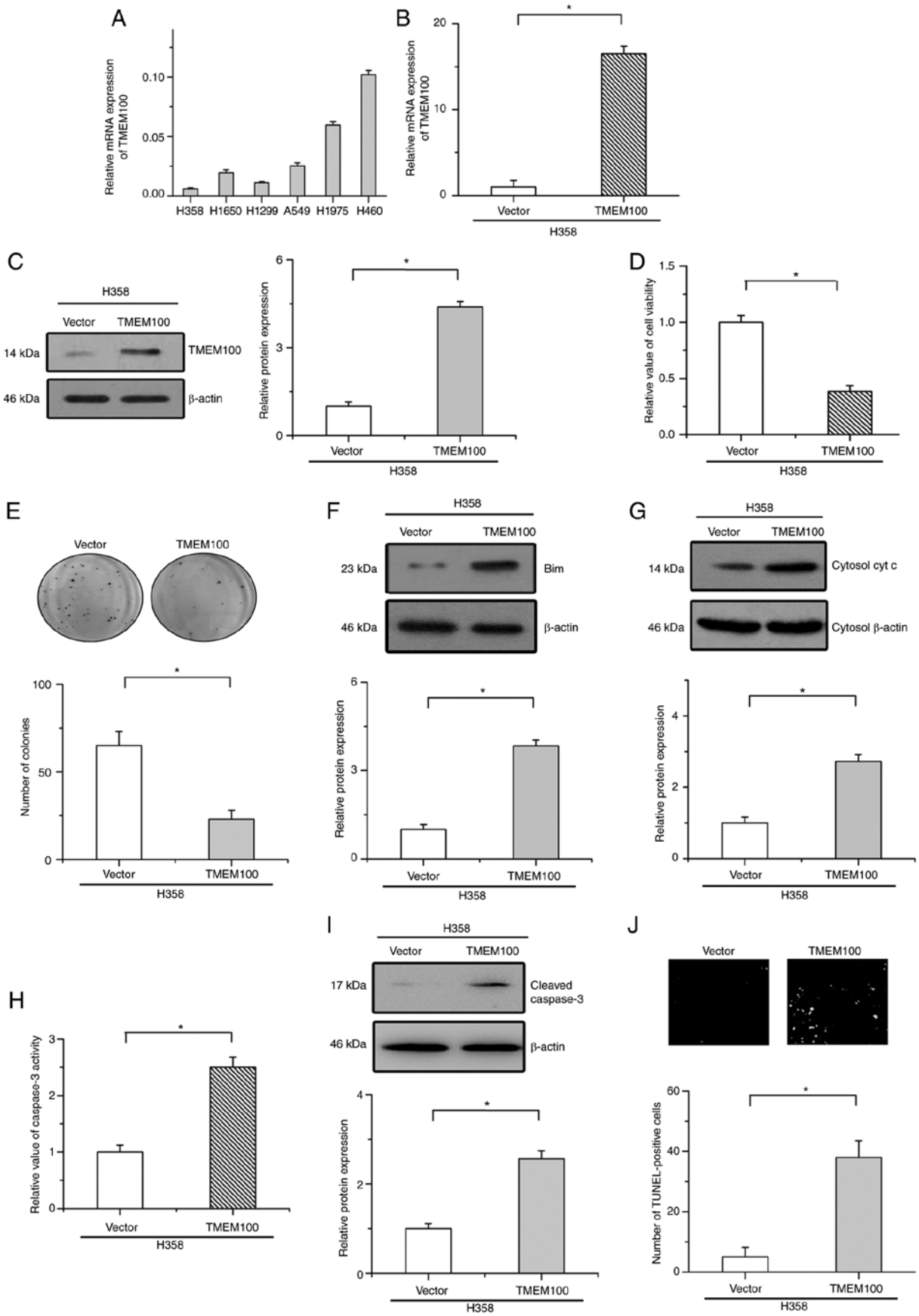

Figure 2. Overexpression of TMEM100 mitigates cell growth and induces cell apoptosis. (A) Endogenous expression of TMEM100 in six NSCLC cell lines. (B and C) Overexpression efficiency was examined by (B) reverse transcription-quantitative PCR and (C) western blot analyses. (D) TMEM100 overexpression suppressed cell viability and inhibited growth in H358 cells. (E) Colony formation was mitigated by the overexpression of TMEM100. (F and G) Overexpression of TMEM100 (F) facilitated Bim expression and (G) induced the release of cytochrome c into the cytoplasm. (H) Caspase-3 activity and (I) cleaved caspase-3 expression were facilitated by TMEM100 overexpression in H358 cells. (J) Overexpression of TMEM100 significantly increased the number of TUNEL-positive cells. *P<0.05. TMEM100, transmembrane protein 100; NSCLC, non-small cell lung cancer.

TMEM100). SD was then utilized as an apoptotic model to examine the effects of TMEM100 on cell apoptosis. It was established that the knockdown of TMEM100 significantly increased cell viability and enhanced colony formation after treatment with SD in H460 cells (Fig. 3C and D). Moreover, the protein levels of Bim and cytochrome *c* in the cytoplasm were both attenuated by TMEM100 knockdown in starved H460 cells (Fig. 3E and F). The knockdown of TMEM100 suppressed the activation and expression of cleaved caspase-3 and decreased the number of TUNEL-positive cells in starved H460 cells (Fig. 3G-I). These results indicated that TMEM100 knockdown promoted cell survival and inhibited cell apoptosis in NSCLC.

TMEM100 negatively regulates cell survival in NSCLC cells. Furthermore, to determine whether the regulatory effects of TMEM100 on cell apoptosis are general in other NSCLC cells, H1299 and H1975 cells were then selected to establish TMEM100 overexpression and TMEM100-knockdown cells, respectively. The overexpression and knockdown efficiencies were verified by RT-qPCR (Fig. 4A and B). As demonstrated in Fig. 4C, overexpression of TMEM100 significantly suppressed H1299 cell viability. The expression of cleaved caspase-3 and the number of TUNEL-positive cells were increased by TMEM100 overexpression (Fig. 4D and E). Conversely, knockdown of TMEM100 significantly increased cell viability and mitigated the expression of cleaved caspase-3 and the number of TUNEL-positive cells in starved H1975 cells (Fig. 4F-H). These results indicated that TMEM100 facilitated cell apoptosis in NSCLC cells.

MiRNA-106b acts as an upstream regulator of TMEM100 in NSCLC. MiRNAs, which can block the translation or initiate the transcript degradation of target mRNAs, play a critical role in reducing protein expression. To search for miRNAs that potentially target TMEM100 and to identify the upstream regulator of TMEM100, TargetScan and miRanda were utilized to determine miRNAs that potentially target the 3'UTR of TMEM100 mRNA (21,22). Based on the analysis results, 30 potential upstream miRNAs (including miR-520e, miR-106b, miR-195, miR-497, miR-16 andmiR-15a) were identified. The expression of these miRNAs in NSCLC tissues and peritumoral tissues was then examined via two public datasets (GSE63805 and GSE36681). Among these miRNAs, it was revealed that only miR-106b was significantly upregulated in NSCLC tissues in both datasets (as revealed in Fig. 5A and B), indicating that miR-106b likely functions as an upstream regulator of TMEM100 in NSCLC.

Then, it was studied whether TMEM100 was regulated by miR-106b. As revealed in Fig. 5C, there was a specific sequence conserved in the 3'UTR of TMEM100 mRNA, which was predicted to integrate with miR-106b. Results of RT-qPCR revealed that expression of miR-106b was significantly increased by miR-106b mimics (miR-106b) and decreased by a miR-106b-specific inhibitor (anti-miR-106b) (Fig. 5D). A luciferase reporter assay was utilized to demonstrate the predicted combination of miR-106b and the 3'UTR of TMEM100 mRNA. Our results revealed that the luciferase activity of the WT plasmid was significantly suppressed by miR-106b, while the luciferase activity of the MT plasmid was not affected by miR-106b (Fig. 5E). Moreover, it was also demonstrated that the mRNA and protein expression levels of TMEM100 were significantly downregulated by miR-106b (Fig. 5F and G), whereas anti-miR-106b led to the increased expression of TMEM100 (Fig. 5H and I). These results indicated that miR-106b served as an upstream regulator of TMEM100 in NSCLC.

miR-106b facilitates cell survival by serving as an oncogenic factor. The regulatory effects of miR-106b on cell survival in NSCLC were then examined. Our results revealed that the inhibition of miR-106b mitigated cell growth and suppressed colony formation (Fig. 6A and B). The protein levels of Bim and caspase-3 activity were significantly increased by treatment with anti-miR-106b in H358 cells (Fig. 6C and D). Conversely, the efficiency of TMEM100 overexpression was verified by RT-qPCR in H460 cells (Fig. 6E). It was revealed that miR-106b increased cell viability and promoted colony formation after treatment with SD in H460 cells, which was attenuated by the reintroduction of TMEM100 (Fig. 6F and G). Additionally, miR-106b-inhibited Bim expression and caspase-3 activation were eliminated by the restoration of TMEM100 in starved H460 cells (Fig. 6H and I). These results indicated that miR-106b inhibited apoptosis by reducing TMEM100 expression in NSCLC cells.

Effects of TMEM100 on cell apoptosis are attenuated by the reintroduction of survivin. Survivin, a critical regulator of cell apoptosis, has been reported to play important roles in the progression of NSCLC (23). As revealed in Fig. 7A, the expression of survivin was significantly upregulated in 60 NSCLC tissues (GSE19804) compared with paired peritumoral tissues. There were highly negative correlations between TMEM100 expression and survivin expression in the same NSCLC tissues ($r=-0.46$, $P<0.001$) (Fig. 7B). It was then examined whether survivin was involved in the inhibitory effects of TMEM100 on cell survival. Our results revealed that the overexpression of TMEM100 mitigated the protein levels of survivin, while the expression of survivin was increased by TMEM100 knockdown (Fig. 7C and D). Furthermore, to determine the roles of survivin in TMEM100-regulated cell apoptosis, survivin expression was increased by transfection with a recombinant plasmid (Fig. 7E). It was revealed that the inhibitory effects of TMEM100 on cell viability were mitigated by the increased expression of survivin (Fig. 7F). In addition, TMEM100-enhanced Bim expression and caspase-3 activation were antagonized by the restoration of survivin (Fig. 7G and H). These results indicated that the roles of TMEM100 in cell survival were, at least in part, mediated by negatively regulating survivin in NSCLC.

Discussion

Mounting evidence has indicated that transmembrane proteins play important roles in the progression and development of some malignancies (8,10,24). Cytosolic TMEM88 has been revealed to stimulate the invasion and metastasis of cancer cells. Higher expression of cytosolic TMEM88 was revealed to be closely associated with poorer differentiation, higher TNM stage, and worse overall survival (10). Increased expression

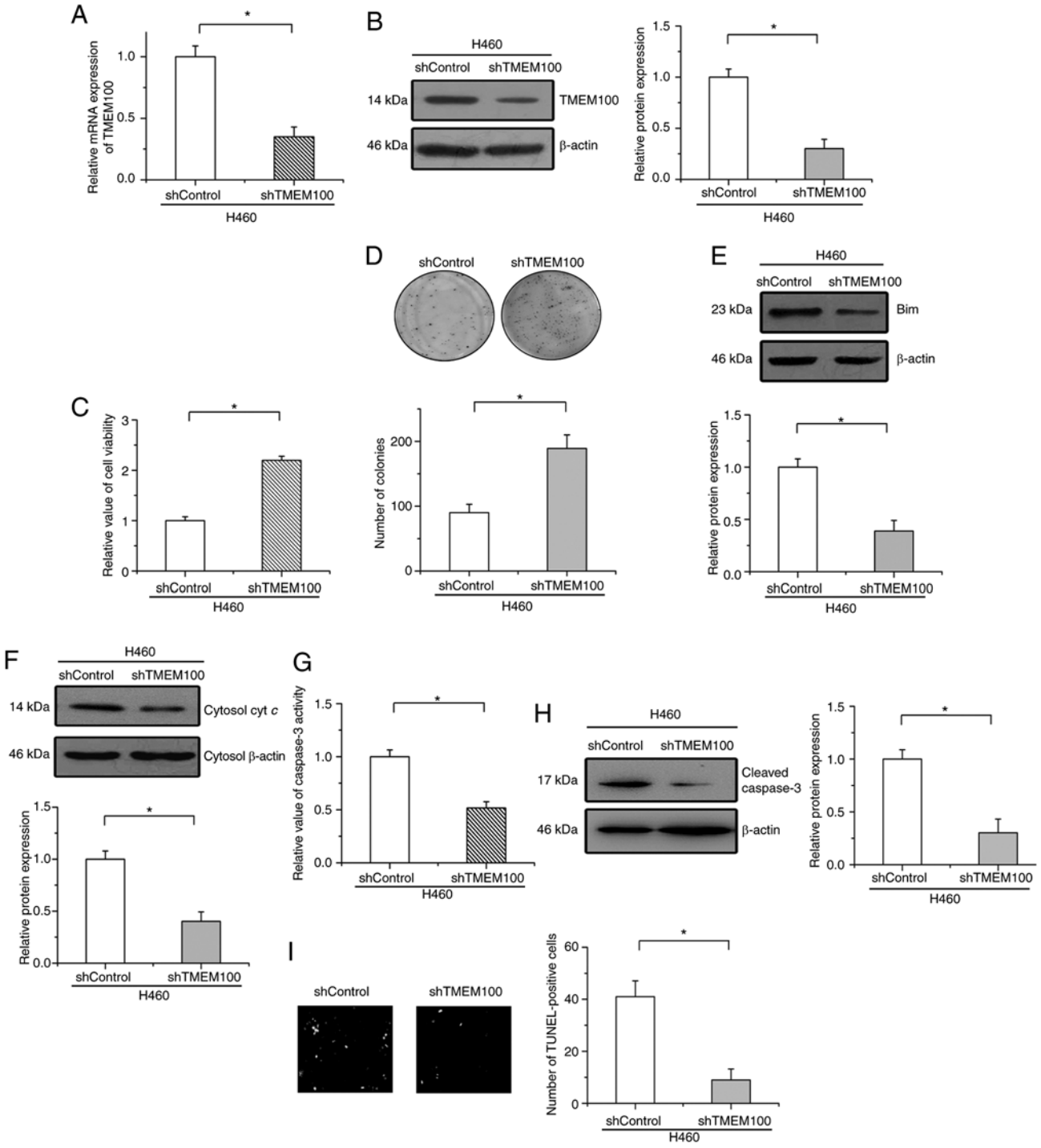

Figure 3. Knockdown of TMEM100 inhibits cell apoptosis. (A) Reverse transcription-quantitative PCR and (B) western blot analyses were used to verify the knockdown efficiency. (C) Cell viability and (D) colony formation were facilitated by the knockdown of TMEM100 after treatment with SD in H460 cells. (E and F) TMEM100 knockdown suppressed (E) the protein levels of Bim and (F) the release of cyt *c* into the cytoplasm under conditions of SD. (G) The activation and (H) expression of cleaved caspase-3 were mitigated by the knockdown of TMEM100 in starved H460 cells. (I) TMEM100 knockdown significantly decreased the number of TUNEL-positive cells. *P<0.05. TMEM100, transmembrane protein 100; SD, serum deprivation; sh-, short hairpin.

of TMEM16A has been revealed in some tumours, including gastric cancer (8), prostate carcinoma (24), as well as head and neck squamous cell carcinoma (25). Additionally, the increased expression of TMEM16A was revealed to be positively associated to tumour stage and negatively associated with the overall survival of patients (8). TMEM100, a member of the transmembrane protein family, has also been revealed to participate in regulating the progression of some cancers. A previous study revealed that TMEM100 inhibited metastasis and proliferation, and the levels of TMEM100 were correlated with tumour size, TNM stage, overall survival and disease-free survival in hepatocellular carcinoma (14). Han *et al* revealed that overexpression of TMEM100 suppressed cell proliferation and mitigated the migration and invasion of NSCLC cells, whereas TMEM100 knockdown promoted cell proliferation and migration (26). However, the proliferation of cancer cells

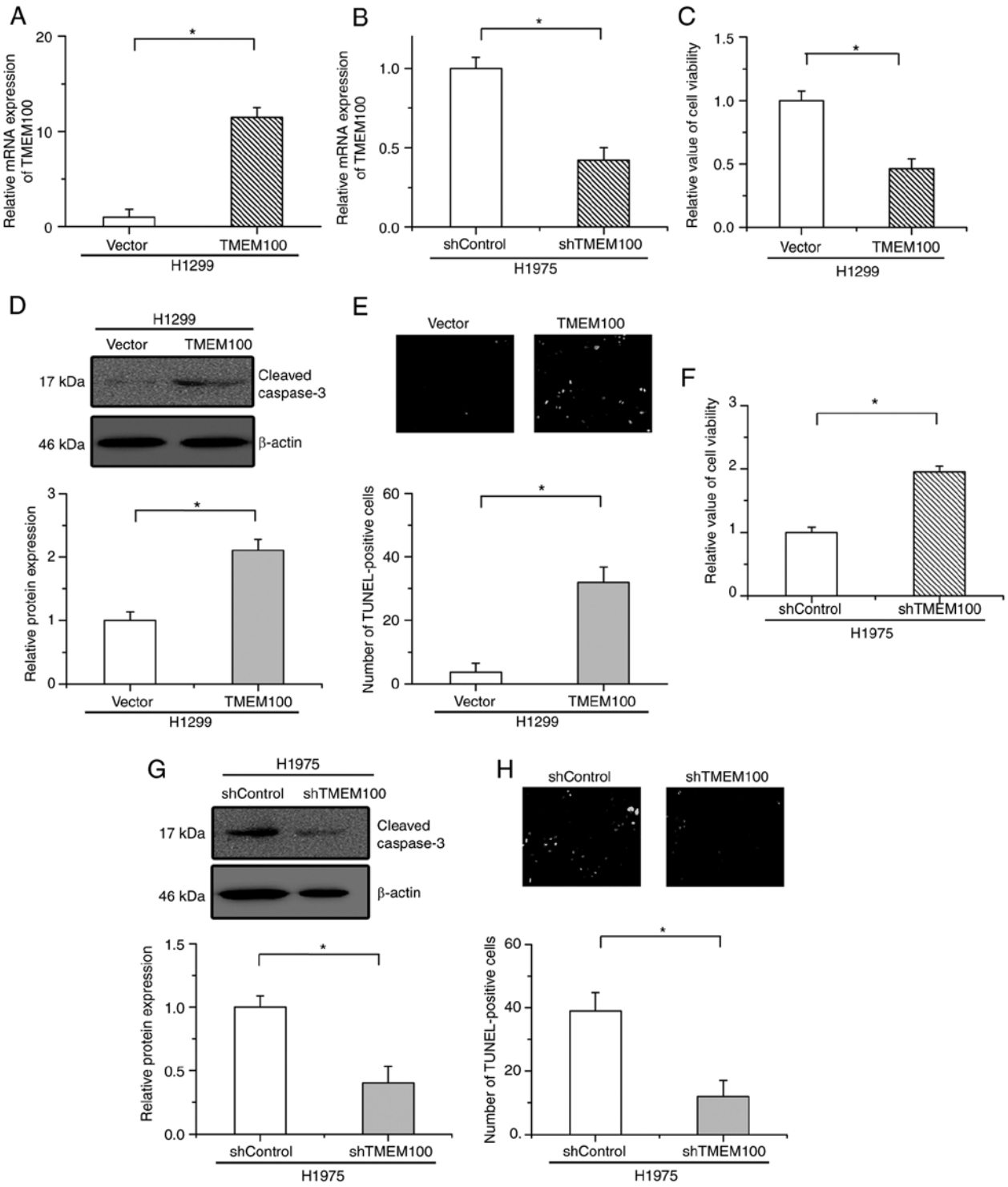

Figure 4. TMEM100 facilitates cell apoptosis in NSCLC cells. (A and B) Reverse transcription-quantitative PCR was utilized to verify the (A) overexpression and (B) knockdown efficiencies. (C) Cell viability was significantly suppressed by overexpression of TMEM100 in H1299 cells. (D and E) Overexpression of TMEM100 increased (D) the expression of cleaved caspase-3 and (E) the number of TUNEL-positive cells. (F) Knockdown of TMEM100 increased cell viability in starved H1975 cells. (G) The expression of cleaved caspase-3 and (H) the number of TUNEL-positive cells were attenuated by TMEM100 knockdown in starved H1975 cells. *P<0.05. TMEM100, transmembrane protein 100; NSCLC, non-small cell lung cancer; sh-, short hairpin.

is determined not only by cell proliferation but also by cell death. Apoptosis, an important form of regulated cell death, also plays a critical regulatory role in the progression of cancer (4). At present, whether the regulation of cell apoptosis is also involved in the inhibitory effects of TMEM100 on NSCLC progression remains uncertain. In the present study, new evidence was provided to indicate that TMEM100, negatively regulated by miR-106b, induced cell apoptosis and inhibited the survival of NSCLC cells by suppressing survivin expression, consistent with a previous study revealing that TMEM100 served as a tumour suppressor in NSCLC (26).

Apoptosis acts as a negative regulator of cell growth. Accumulating evidence has indicated that the inhibition of cell apoptosis is commonly observed in some cancer tissues, and

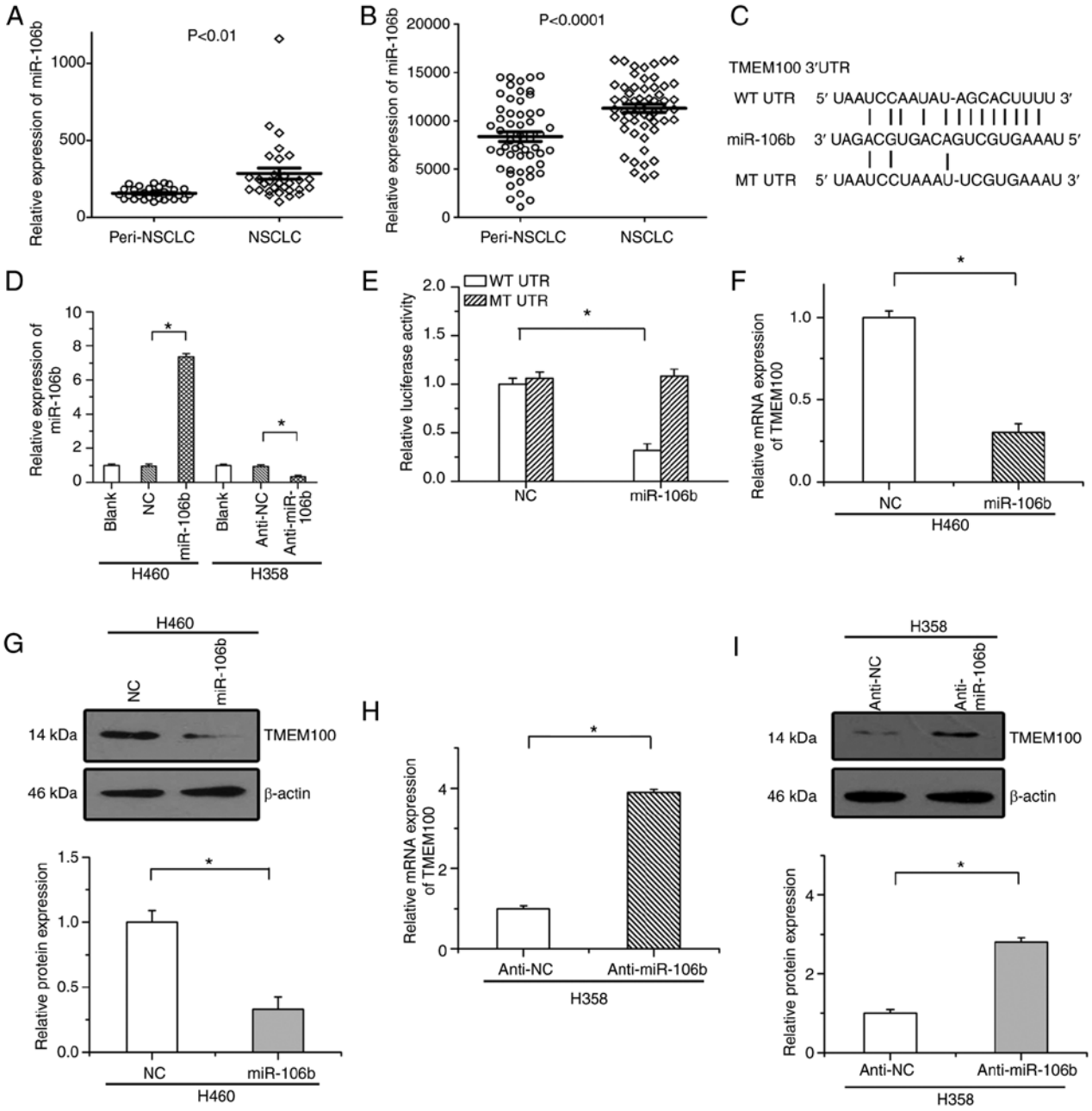

Figure 5. miR-106b downregulates TMEM100 expression in NSCLC. (A and B) miR-106b was significantly upregulated in NSCLC tissues compared with paired peritumoral tissues. (C) miR-106b was predicted to integrate with the sequence conserved in the 3'UTR of TMEM100 mRNA. (D) Expression of miR-106b was significantly increased by miR-106b mimics and decreased by anti-miR-106b. (E) Luciferase activity of the WT plasmid was significantly suppressed by miR-106b, while the luciferase activity of the MT plasmid was not affected by miR-106b. (F) The mRNA and (G) the protein expression of TMEM100 were significantly downregulated by miR-106b. (H and I) Inhibition of miR-106b led to the increased expression of TMEM100 at (H) the mRNA and (I) protein levels. *P<0.05. miR, microRNA; TMEM100, transmembrane protein 100; NSCLC, non-small-cell lung cancer; UTR, untranslated region; WT, wild-type; MT, mutant-type.

genes involved in modulating cell apoptosis are regarded as a new class of tumour-related genes (27,28). Inducing cellular apoptosis is one of the most effective strategies for relieving the progression of tumours. A previous study revealed that mitochondrial dysfunction was an early-stage event that triggered the intrinsic apoptotic pathway (29). Bim is a critical mediator of the mitochondrial apoptotic pathway and participates in regulating the function of mitochondria. Bim can cause a decrease in the mitochondrial membrane potential and induce the opening of the mitochondrial permeability transition pore either by directly activating proapoptotic Bax/Bak or by antagonizing antiapoptotic Bcl-2 (30). A disruption in the mitochondrial membrane potential leads to the release of cytochrome *c* from the mitochondria into the cytoplasm, which in turn triggers the activation of caspase cascades and elicits cell apoptosis (31). To study the role of TMEM100 in the survival of NSCLC cells, the expression of Bim, cytochrome *c* release and caspase-3 activation were examined under conditions of TMEM100 overexpression or knockdown. Our results revealed that the overexpression of TMEM100 induced the expression of Bim, promoted the release of cytochrome *c* into the cytoplasm, and increased the activity of caspase-3. Conversely, TMEM100 knockdown had the opposite effects. These results indicated that TMEM100 elicited cell apoptosis

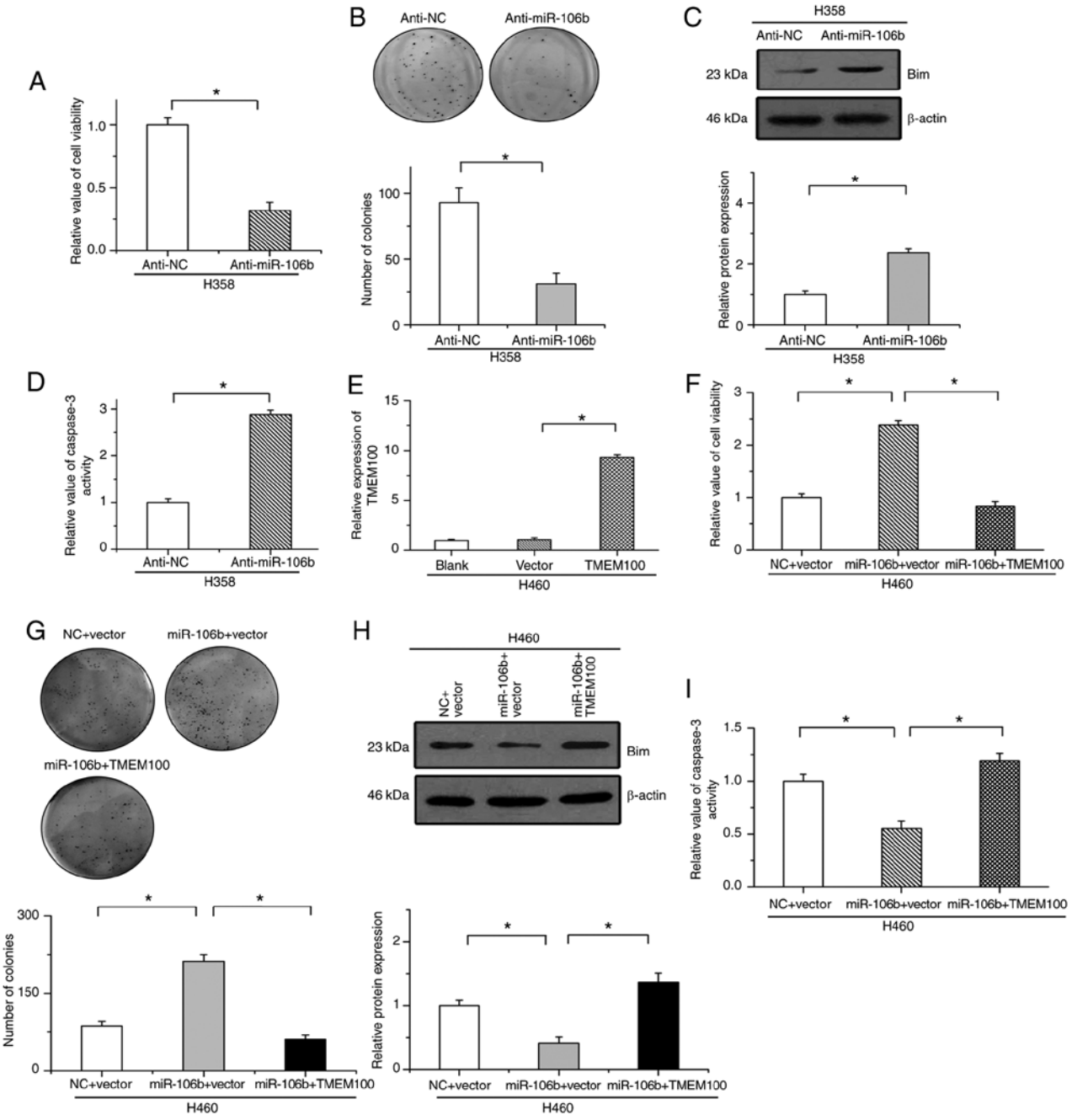

Figure 6. miR-106b inhibits apoptosis by suppressing TMEM100 expression in NSCLC cells. (A) Cell viability and (B) colony formation were attenuated by miR-106b inhibition in H358 cells. (C and D) Treatment with anti-miR-106b (C) induced the protein expression of Bim and (D) enhanced the activation of caspase-3. (E) The efficiency of TMEM100 overexpression was verified by reverse transcription-quantitative PCR in H460 cells. (F) Cell viability and (G) colony formation increased by miR-106b were antagonized by the reintroduction of TMEM100 after treatment with serum deprivation in H460 cells. (H and I) miR-106b-mitigated (H) Bim expression and (I) caspase-3 activity were eliminated by the restoration of TMEM100 in starved H460 cells. *P<0.05. miR, microRNA; TMEM100, transmembrane protein 100; NSCLC, non-small cell lung cancer; NC, negative control.

and inhibited the progression of NSCLC by acting as a tumour suppressor.

An important finding of the present study was that TMEM100 facilitated cell apoptosis by suppressing survivin expression in NSCLC. Survivin belongs to the inhibitor of apoptosis (IAP) gene family and plays important roles in the progression of some human malignancies, including NSCLC (23,32). Its roles in cell survival and apoptosis have been widely studied in NSCLC. The inhibitory effects of nicotine on chemotherapeutic drug-induced cell apoptosis have been reported to be mediated by upregulating XIAP and survivin (33). Ezponda *et al* reported that SF2/ASF promotes the stability of survivin mRNA and that the downregulation of SF2/ASF induces apoptosis by reducing the expression of survivin in NSCLC cells (23). In LKB1-deficient lung adenocarcinomas, survivin was responsible for promoting malignant progression by acting as the downstream mediator of YAP (34). In the present study, our results revealed that the expression of survivin was negatively correlated with TMEM100 expression in NSCLC tissues. Moreover, the roles of TMEM100 in cell apoptosis were attenuated by the reintroduction of survivin. These results indicated that TMEM100

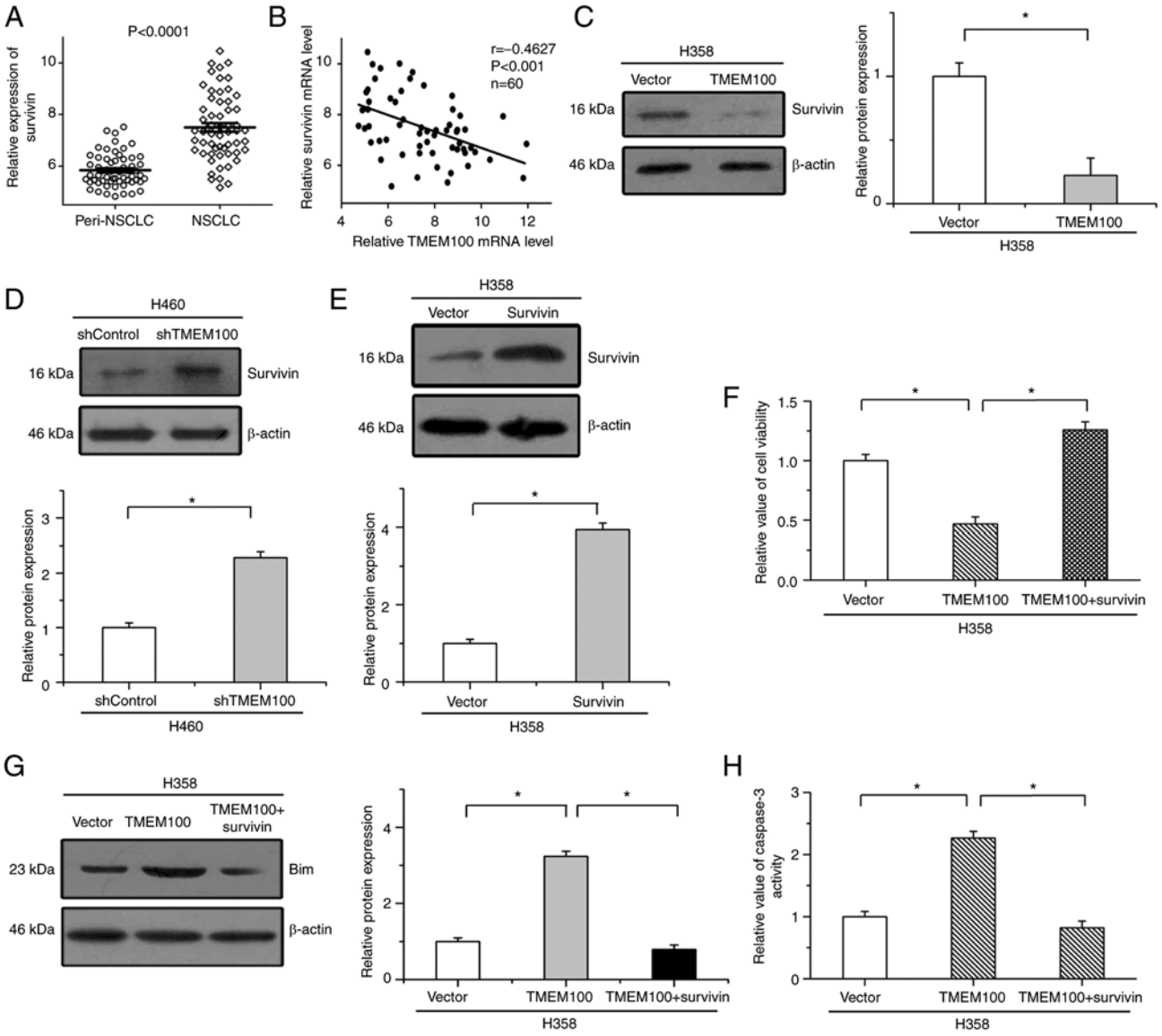

Figure 7. Effects of TMEM100 on cell apoptosis are attenuated by the reintroduction of survivin. (A) Expression of survivin was significantly upregulated in 60 cases of tumor tissues compared with paired peritumoral tissues. (B) TMEM100 expression was negatively associated with the expression of survivin in the same NSCLC tissues. (C) Overexpression of TMEM100 suppressed the protein levels of survivin, (D) while the expression of survivin was facilitated by TMEM100 knockdown. (E) Expression of survivin was significantly increased by the transfection of a recombinant plasmid in H358 cells. (F) TMEM100 overexpression-inhibited cell growth was mitigated by the increased expression of survivin. (G) The increased expression of Bim and (H) activation of caspase-3 induced by TMEM100 overexpression were antagonized by the restoration of survivin protein. *P<0.05. TMEM100, transmembrane protein 100; NSCLC, non-small cell lung cancer.

facilitated cell apoptosis, at least in part, by inhibiting survivin expression in NSCLC.

Another notable finding of the present study was that TMEM100 was negatively regulated by miR-106b in NSCLC cells. MiRNAs, a large family of short, noncoding endogenous RNAs, can bind to a specific sequence conserved in the 3'UTR of their target genes and post-transcriptionally regulate their expression (35). Currently, a number of microRNAs have been indicated to regulate various types of pathological processes, including the initiation and development of cancers (36-38). However, the effects of miR-106b on different human cancers are not consistent. Previous studies have demonstrated that miR-106b enhanced the metastasis and proliferation of cancer cells and boosted tumorigenesis in colorectal cancer (39), hepatocellular carcinoma (40), and glioma (41).Conversely, miR-106b, as a tumour suppressor, has been reported to be significantly downregulated in clinical samples of giant bone cell tumours and inhibited metastasis and tumorigenesis in thyroid (42) and breast cancer (43). To date, the precise roles of miR-106b in NSCLC have been inconclusive. In the present research, our results revealed that miR-106b, which was markedly upregulated in NSCLC tissues, interacted with the 3'UTR of TMEM100 mRNA and suppressed its expression. Moreover, the inhibitory roles of miR-106b in cell apoptosis were eliminated by the restoration of TMEM100 in NSCLC. These results indicated that miR-106b-mitigated TMEM100 expression promoted the progression of NSCLC. However, further studies are still needed to validate the roles of TMEM100 in NSCLC *in vivo*. Although it was demonstrated that TMEM100 regulates

cell survival by survivin in NSCLC, other parallel pathways involved should be investigated in future studies.

In conclusion, our results revealed that TMEM100 induced cell apoptosis in NSCLC by acting as a tumour suppressor. Moreover, miR-106b was responsible for the decreased expression of TMEM100 in NSCLC. These results indicated an important underlying regulatory mechanism and provided potential treatment targets for NSCLC.

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Availability of data and materials

All data generated and/or analyzed during the present study are included in this published article.

Authors' contributions

JM and LZ conceived and designed the experiments. JM, CM and TY performed the experiments. YB, MY and XM analyzed the results. XM, CM and LZ wrote the manuscript. All authors read and approved the final manuscript.

Ethics approval and consent to participate

Not applicable.

Patient consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

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