Melatonin inhibits gallbladder cancer cell migration and invasion via ERK-mediated induction of epithelial-to-mesenchymal transition

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Abstract. Melatonin is a naturally occurring molecule secreted by the pineal gland that exhibits antitumor properties and prevents the development of human cancer. However, little is known regarding the effects of melatonin on gallbladder cancer (GBC) cells. The present study aimed to investigate the role of melatonin on the prevention of GBC cell invasion. The GBC cell line, GBC-SD, was treated with different concentrations of melatonin for different time periods, and the data indicated that melatonin markedly inhibited the invasion of GBC cells. Following treatment of GBC cells with melatonin, the protein levels of the epithelial marker, E-cadherin, significantly increased, while the expression levels of the mesenchymal markers, N-cadherin, Snail and vimentin, notably decreased. In addition, melatonin inhibited the phosphorylation of ERK1/2. Following treatment of the cells with the ERK activator, tert-Butylhydroquinone, the anti-invasive effects of melatonin were reversed by rescuing epithelial-to-mesenchymal transition in GBC cells. Taken together, these results suggest that melatonin inhibits GBC invasiveness by blocking the ERK signaling pathway. Thus, melatonin may be used as a potential novel cancer therapeutic drug for the treatment of GBC.

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Introduction

Gallbladder cancer (GBC) is the most common malignancy of the biliary tract originating from epithelial cells of the gallbladder with a particularly high incidence (up to 7.5 per 100,000 for men and 23 per 100,000 for women) in native American and South American populations (1). It ranks sixth among malignancies of the alimentary tract and its prevalence is higher in women than in men (2). The global incidence rate of GBC has recently increased, notably in India, Eastern Europe and China (3). GBC is closely associated with genetic and environmental factors, such as chronic infection of the gallbladder, exposure to toxic compounds and heavy metals and dietary habits (1,4). GBC is highly heterogeneous and invasive, which develops liver metastases due to its adjacency to the liver and lack of serosa layer (2). The diagnosis of early-stage GBC is considerably difficult and the disease develops lymphatic metastasis (5). Patients with GBC have a high mortality rate, with an overall 5-year survival rate of ~5% (6). Notably, the metastatic spread of GBC is the main reason for its high morbidity (2). Although radical surgery is a preferred method for the treatment of GBC, its resection rate is low (1). Current treatment methods for GBC are ineffective, with very poor treatment efficacy (2). Thus, this cancer type is considered a serious threat to public health. Based on this evidence, it is important to identify specific and efficient drugs for the treatment of GBC.

Melatonin or N-acetyl-5-methoxytrypamine is a neurohormone predominantly synthesized and secreted by the pineal gland, with potent antioxidant, immunomodulatory and anticancer effects (7). *In vivo* and *in vitro* data have indicated that melatonin can inhibit tumor growth (8-10). However, the function and mechanism of action of melatonin vary in different types of cancer. For example, melatonin induces the inhibition of leiomyosarcoma cell proliferation and invasion, which is mediated by aerobic glycolysis (known as the Warburg effect) (11). However, in lung cancer cells, melatonin suppresses cancer metastasis by inhibiting epithelial-to-mesenchymal transition (EMT) by targeting Twist (12). In renal cell carcinoma, melatonin inhibits matrix

metalloproteinase-9 by suppressing the AKT/MAPK pathway and the NF-κB DNA-binding activity (13). In human epidermal growth factor receptor 2-positive human breast cancer cells, melatonin represses metastasis by suppressing the expression of ribosomal protein S6 kinase 2 (14). Despite its extensive antimetastatic function, the effects of melatonin on GBC remain unknown.

In the present study, the antimetastatic effects of melatonin on GBC were investigated. In addition, the molecular mechanism of melatonin involved in gallbladder cancer invasion and metastasis was investigated. This hormone may be considered as a potential candidate drug in the treatment of metastatic GBC.

Materials and methods

Cell culture. The human GBC cell line, GBC-SD, was purchased from the Cell Bank of Type Culture Collection of the Chinese Academy of Sciences. The NOZ cell line was gifted by Dr Jianwen Ye, Department of Hepatobiliary and Pancreatic Surgery, The First Affiliated Hospital of Zhengzhou University (Zhengzhou, China). Cells were maintained in RPMI-1640 medium (Beijing Solarbio Science & Technology Co., Ltd.) supplemented with 10% fetal bovine serum (FBS, Gibco; Thermo Fisher Scientific, Inc.) and 100 μg/ml penicillin/streptomycin, at 37˚C with 5% CO_2 (Thermo Fisher Scientific, Inc.).

Drug treatment. Melatonin (>99%) was purchased from MedChemExpress and dissolved in DMSO (Sigma-Aldrich; Merck KGAa). GBC-SD and NOZ cells were treated with melatonin (0.00, 0.25, 0.50, 1.00, 2.00 and 3.00 mM) for 24 or 48 h, and DMSO was used as the control. Tert-Butylhydroquinone (tBHQ) was purchased from MedChemExpress and dissolved in DMSO. For detection of the ERK pathway, GBC-SD cells were treated with 2 mM melatonin and 50 μM tBHQ for 3, 6 and 12 h. Cell morphology was assessed using an inverted fluorescence microscope (IX71; Olympus Corporation) (magnification x100).

Cell Counting Kit-8 (CCK-8) assay. Cell viability was assessed via the CCK-8 assay (Suzhou Yuheng Biological Technology Co., Ltd.). Briefly, GBC-SD cells were seeded into 96-well plates at a density of $5x10^3$ cells/well. Cells were treated with different concentrations of melatonin (0.25, 0.5, 1, 2 and 3 mM) for 24 and 48 h. Melatonin was diluted in DMSO (0.01%). The control group contained DMSO, which was adjusted according to the corresponding concentration of the highest melatonin dosage (3 mM) to exclude the possibility of the solvent causing effects on the cells. Subsequently, 10 μl CCK-8 solution was added to each well and the plates were incubated at 37˚C for 1 h. The absorbance was measured at a wavelength of 450 nm, using the Multiskan Spectrum (Varioskan LUX; Thermo Fisher Scientific, Inc.).

Wound healing assay. GBC-SD and NOZ cells were seeded into 6-well plates at a density of $1x10^6$ cells/well and cultured at 37˚C with 5% CO_2. Once cells reached 100% confluence, the monolayers were scratched to create a linear wound, using a 100 μl pipette tip. Cells were washed three times with PBS, and RPMI-1640 medium (1% FBS) with 3 mM melatonin or DMSO were added to each well. Following the indicated treatment periods (24 or 48 h), cells were observed under an inverted fluorescence microscope (IX71; Olympus Corporation) (magnification x100) and the images were captured. The proportion of wound closure was calculated according to the migration over the denuded area.

Cell invasion assay. The invasion assay was performed using Transwell chambers (Corning, Inc.). Matrigel (BD Biosciences) was diluted at a ratio of 1:10. A total of 100 μl diluted Matrigel was added vertically in the upper chambers and cultured at 37˚C with 5% CO_2 overnight. Once the cell monolayer reached 90% confluence, GBC-SD and NOZ cells were digested with trypsin. Cells were subsequently suspended in serum-free medium with or without melatonin (2 mM) and the concentration was adjusted to $2x10^5$/ml. Subsequently 100 μl cell suspension was added to the upper chamber. A total of 600 μl RPMI-1640 medium with 10% FBS was plated in the lower chambers, and cells were incubated at 37˚C for 24 h. Non-invasive cells were removed, while invasive cells were fixed in 4% formalin at room temperature for 20 min and subsequently stained with 0.1% crystal violet at room temperature for 15 min. Stained cells were counted in five randomly selected fields using a light microscope (magnification x200).

Western blotting. GBC-SD and NOZ cells were lysed with RIPA lysis buffer (Beijing Solarbio Science & Technology Co., Ltd.) on ice for 20 min. The cell lysate was collected and centrifuged at 13,000 x g for 15 min. Total protein was quantified using Bicinchoninic acid (Beijing Solarbio Science & Technology Co., Ltd.). Proteins (50 μg/lane) were separated by 10% SDS-PAGE. The separated proteins were subsequently transferred onto polyvinylidene fluoride membranes and blocked with non-fat milk for 3 h at room temperature. The membranes were incubated with primary antibodies against E-cadherin (1:5,000; cat. no. 20874-1-AP); N-cadherin (1:5,000; cat. no. 22018-1-AP); vimentin (1:5,000; cat. no. 10366-1-AP); Snail (1:800; cat. no. 13099-1-AP); ERK (1:1,000; cat. no. AB3373); phosphorylated (p)-ERK (1:1,000; cat. no. AB3355); AKT (1:1,000; cat. no. AY0420); p-AKT (1:1,000; cat. no. AY0421); NF-κB-p65 (1:1,000; cat. no. AY5034), p-NF-κB-p65 (1:1,000; cat. no. CY6372); PI3K (1:1,000; cat. no. CY5355) and p-PI3K (1:1,000; cat. no. CY6427), overnight at 4˚C. E-cadherin, N-cadherin, Vimentin and Snail polyclonal antibodies were purchased from ProteinTech Group, Inc. ERK, p-ERK, AKT, p-AKT, NF-κB-p65, p-NF-κB-p65, PI3K and p-PI3K antibodies were purchased from Shanghai Abways Biotechnology Co., Ltd. Membranes were washed three times with TBS and Polysorbate 20, and subsequently incubated with Rabbit Anti-Goat IgG (H+L) horseradish peroxidase (HRP) (1:5,000; cat. no. AB0103; Shanghai Abways Biotechnology Co., Ltd.) for 1 h at room temperature. Protein bands were visualized using an ECL kit (Suzhou Xinsaimei Biotechnology Co., Ltd.). The gray levels of the protein bands were analyzed using ImageJ 1.8.0 software (National Institutes of Health) and the relative protein levels were normalized to GAPDH.

Reverse transcription-quantitative (RT-q)PCR. Total RNA was extracted from GBC-SD cells using TRIzol® reagent

Table I. Primer sequences used for quantitative PCR.

Number	Name	Primer sequences
Primer 1	h-GAPDH	F: GGAGCGAGATCCCTCCAAAAT R: GGCTGTTGTCATACTTCTCATGG
Primer 2	h-E-Cadherin	F: ATTTTTCCCTCGACACCCGAT R: TCCCAGGCGTAGACCAAGA
Primer 3	h-N-Cadherin	F: AGCCAACCTTAACTGAGGAGT R: GGCAAGTTGATTGGAGGGATG
Primer 4	h-Vimentin	F: TGCCGTTGAAGCTGCTAACTA R: CCAGAGGGAGTGAATCCAGATTA

F, forward; R, reverse.

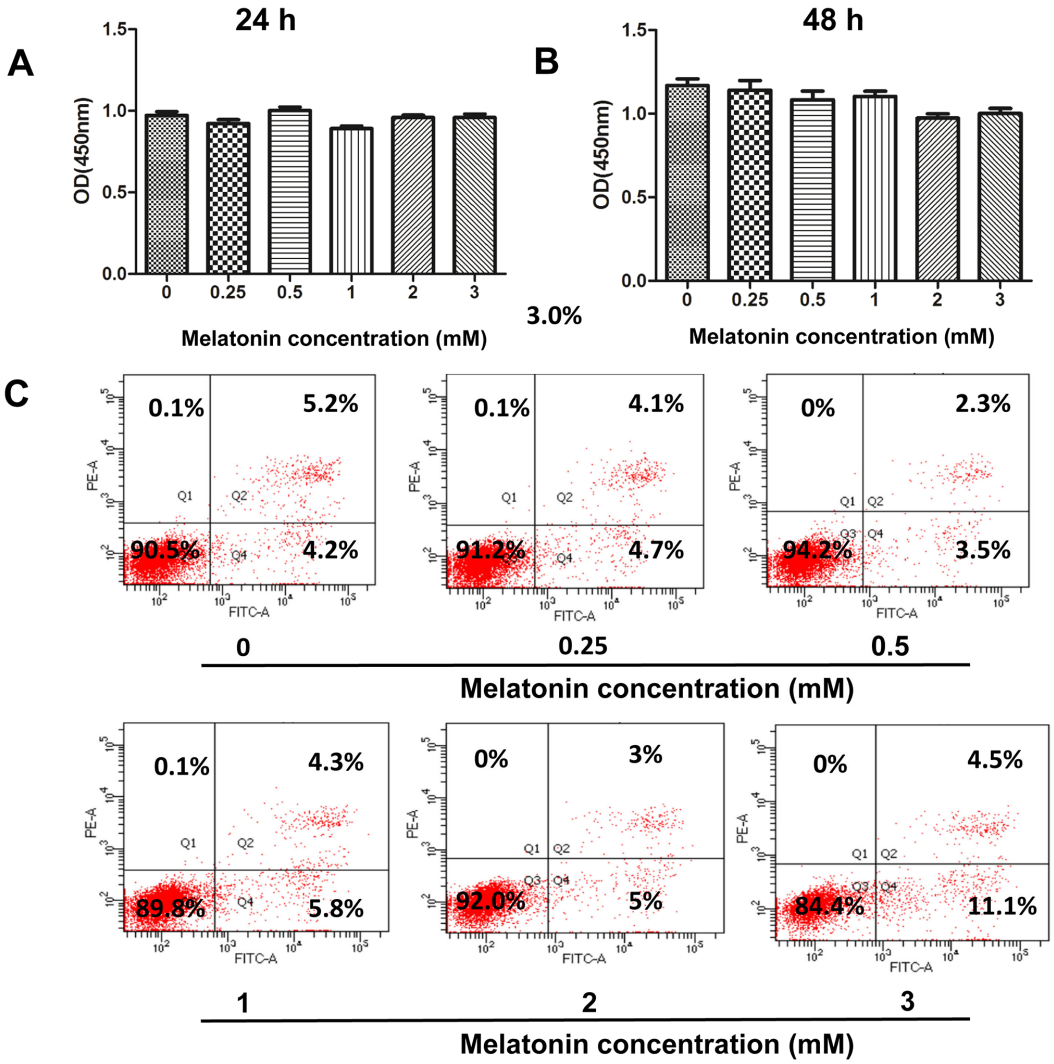

Figure 1. Effect of melatonin on the viability of human GBC cells. The Cell Counting Kit-8 assay was performed to assess the effect of melatonin on the viability of GBC cells treated with melatonin (0-3 mM) for (A) 24 or (B) 48 h. (C) GBC cells were treated with melatonin (0-3 mM) for 48 h and subsequently subjected to flow cytometry to detect apoptosis. GBC, gallbladder cancer; OD, optical density.

(Gibco; Thermo Fisher Scientific, Inc.), according to the manufacturer's instruction. cDNA was synthesized using the HiScript II Reverse Transcriptase kit (Vazyme Biotech Co., Ltd.) according to the manufacturer's protocol. The reverse transcription conditions used were as follows: 25˚C for 5 min, 50˚C for 15 min and 85˚C for 2 min. qPCR was subsequently performed using the PowerUp™ SYBR-Green Master mix (Applied Biosystems; Thermo Fisher Scientific, Inc.), with

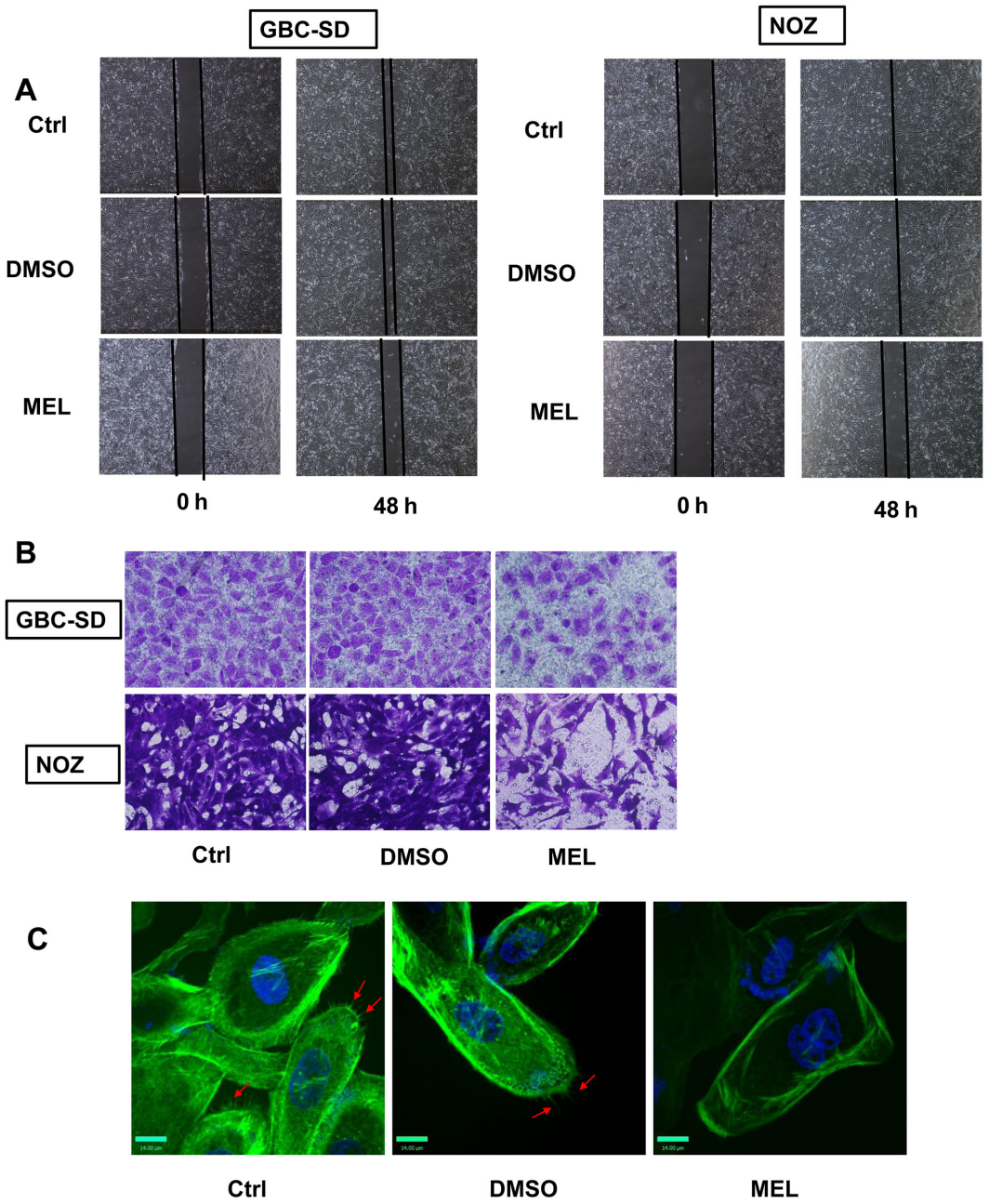

Figure 2. Antimetastatic effect of melatonin in GBC cells. (A) The wound healing assay was performed to assess cell migration (x100) following treatment with melatonin (2 mM) for 48 h. (B) The Transwell assay was performed to assess cell invasion (x200) following treating with melatonin (2 mM) for 48 h. (C) Fluorescence images (x400) of GBC cells stained with YF Dye Phalloidin Conjugates (F-actin) and DAPI (nuclei) following treatment with melatonin (2 mM) for 48 h. Red arrow indicate microfilament. GBC, gallbladder cancer; Ctrl, control; MEL, melatonin.

specific primer sequences. The thermocycling conditions used were as follows: Pre-denaturation at 95˚C for 30 sec, denaturation at 95˚C for 5 sec and annealing at 59.5˚C for 30 sec for a total of 40 cycles. The fold change in relative mRNA levels was expressed as the relative quantification calculated by the average of the standardized gene expression levels $2^{-\Delta\Delta Cq}$ (15). GAPDH was used as the housekeeping gene. The primer sequences used for qPCR are listed in Table I.

Phalloidin staining. GBC-SD cells were seeded into 24-well plates with carry sheet glass and treated with melatonin (2 mM) for 6 h. Cells were fixed with 4% paraformaldehyde for 15 min at room temperature. Cells were re-washed 3 times with PBS and YE Dye phalloidin conjugates (US Everbright) staining was performed according to the manufacturer's instructions. DAPI was used for nuclear staining at room temperature for 5 min. The images were acquired using a laser scanning confocal microscope (Nikon A1R/A1; Nikon Corporation) (magnification x400).

Flow cytometry. The Annexin-V assay was performed to assess the detection of exposed phosphatidylserine, which provides a highly sensitive method for detecting apoptosis (16). A total of $3x10^5$ GBC-SD cells were seeded into 6-well plates and treated with different concentrations of melatonin (0, 0.25, 0.5, 1, 2 and 3 mM) for 48 h. The Annexin-V/PI double staining kit (BD

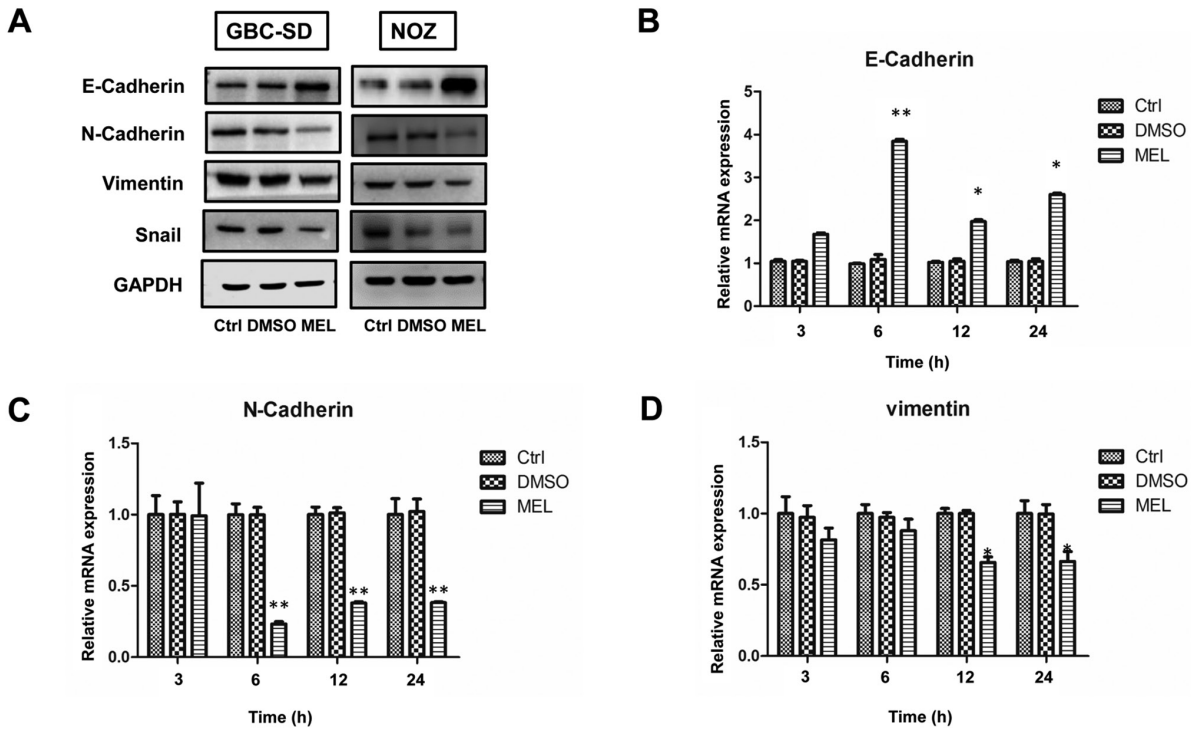

Figure 3. Melatonin regulates the epithelial-to-mesenchymal transition phenotype of GBC cells. (A) Western blot analysis was performed to detect the protein expression levels of E-cadherin, N-cadherin, Snail and vimentin in GBC and NOZ cells following treatment with 2 mM melatonin for 48 h. (B-D) The mRNA expression levels were detected via reverse transcription-quantitative PCR analysis. Data are presented as the mean ± SD. *P<0.05; **P<0.01 vs. the control group. GBC, gallbladder cancer; Ctrl, control; MEL, melatonin.

Biosciences) was used to detect apoptotic cells, according to the manufacturer's instructions. Apoptosis cells were detected by flow cytometry (BD Canto II; BD Biosciences). Cell apoptosis was analyzed by BD FACSDiva Software v.8.0.1 (BD Biosciences). Cells that were positive for Annexin-V and negative for PI were considered viable cells, while cells that were in the early apoptotic phase were Annexin-V positive and PI negative. Cells that were non-viable or late apoptotic were Annexin-V and PI-positive.

Statistical analysis. Statistical analysis was performed using SPSS 17.0 software (SPSS Inc.). All experiments were performed in triplicate and data are presented as the mean ± SD. Paired Student's t-test followed by the Shapiro-Wilk W test were used to compare differences between two groups, while one-way ANOVA followed by Bonferroni's test were used to compare differences between multiple groups. P<0.05 was considered to indicate a statistically significant difference.

Results

Cytotoxicity of melatonin in GBC cells. Melatonin induces apoptosis in various human cancer cells, such as hepatocellular carcinoma, lung cancer, cholangiocarcinoma and pancreatic cancer cells (11-14). Thus, the present study initially investigated whether melatonin promotes cellular apoptosis in human GBC cells. Treatment of GBC-SD cells with melatonin (0.25, 0.5, 1, 2 and 3 mM) for 24 or 48 h did not significantly affect cell viability, according to the CCK-8 assay (Fig. 1A and B). To further confirm the cytotoxicity of melatonin, Annexin V-FITC/PI double staining was performed to determine the induction of apoptosis via flow cytometry. The number of early and late apoptotic cells did not significantly increase following treatment with melatonin (0-2 mM) for 48 h (Fig. 1C). However, the apoptotic rate of GBC-SD cells increased to 15.6% (4.5+11.1%) following an increase in the concentration of melatonin to 3 mM. Thus, the concentration range of melatonin (0-2 mM) was adjusted in subsequent experiments to investigate its antimetastatic properties.

Melatonin inhibits GBC cell migration and invasion. Metastasis plays a significant role in the malignant status of GBC (2). Thus, the effects of melatonin on GBC cell invasion and migration were examined in the present study. The wound healing assay was performed to assess cell migratory, and the results demonstrated that melatonin (2 mM) significantly inhibited the rate of wound closure compared with the control group (Fig. 2A). The effects of melatonin were also verified using Matrigel chambers. Melatonin significantly decreased the number of cells that passed through the membrane (Fig. 2B). To assess the effects of melatonin on cell movement, YF dye phalloidin conjugates were used to stain the actin filaments of GBC cells. Phalloidin is found in amanita phalloides, and can specificity bind to polymerized microfilaments (17). By binding to the polymerized microfilaments, the cyclic peptides inhibit the disintegration of the microfilaments, and destroy the dynamic balance of polymerization and depolymerization of the microfilaments (17). Treatment of GBC cells with melatonin (2 mM) decreased the number of microfilaments compared with the control group (Fig. 2C). Taken together, these results suggest that melatonin mitigates the invasive and migratory abilities of GBC cells.

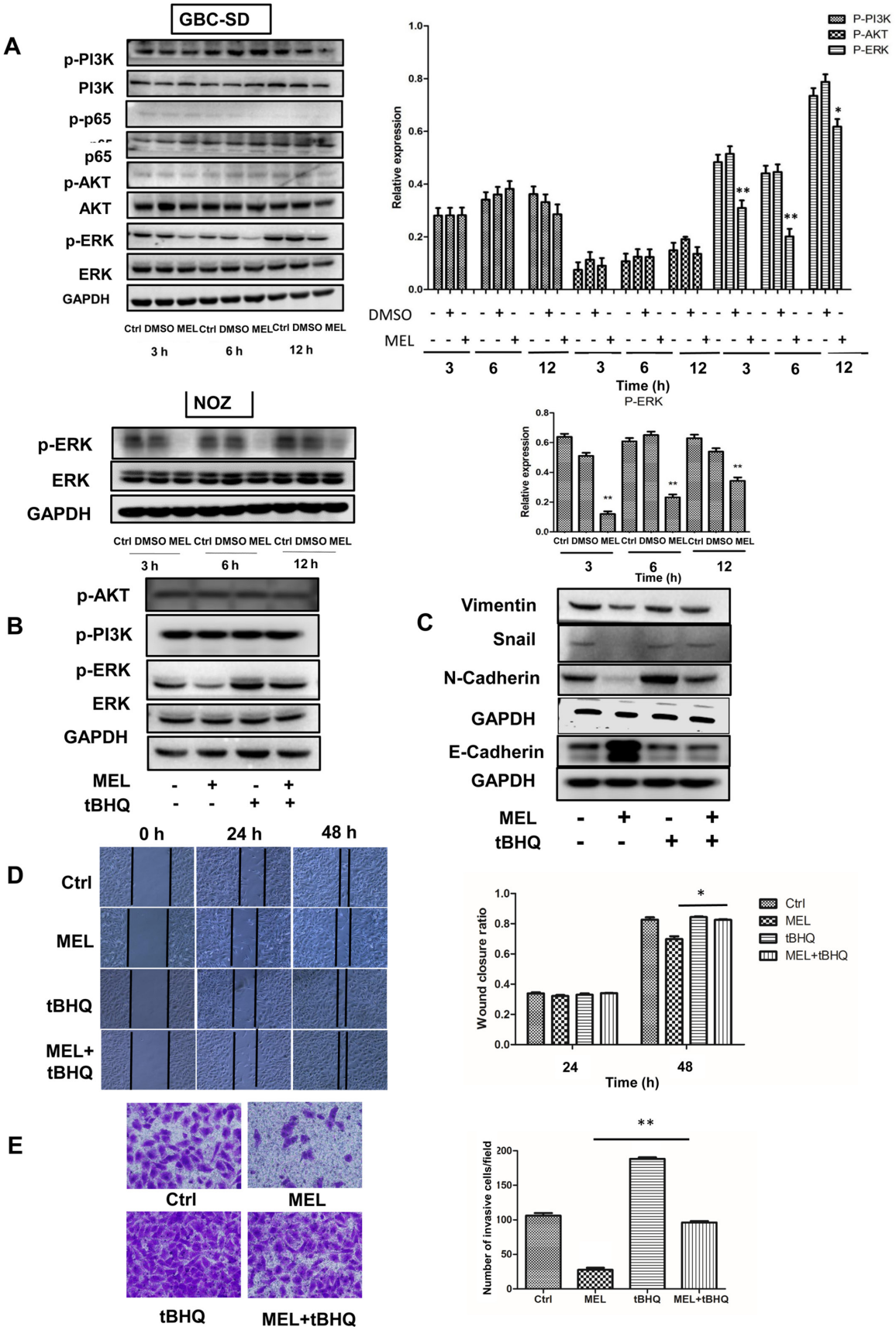

Figure 4. Effects of the ERK signaling pathway on the invasion and epithelial-to-mesenchymal transition of GBC cells. (A) Western blot analysis was performed to detect the protein expression levels of AKT, p-AKT, p65, p-p65, PI3K, p-PI3K, ERK and p-ERK in GBC and NOZ cells treated with melatonin (2 mM) for different time points. (B and C) Western blot analysis was performed to detect the protein expression levels of ERK, p-ERK, N-cadherin, E-cadherin, snail and vimentin in GBC cells following treatment with melatonin and/or tBHQ (50 μM) for 48 h. GAPDH was used as the control. (D) The wound healing assay was performed to assess cell migration (magnification x200) following treatment with melatonin and/or tBHQ (50 μM) for 48 h. The wound closure rate was analyzed. (E) The Transwell assay was performed to assess cell invasion (magnification x200). Data are presented as the mean ± SD. *P<0.05; **P<0.01 vs. the MEL group. GBC, gallbladder cancer; p, phosphorylated; tBHQ, tert-Butylhydroquinone; MEL, melatonin; Ctrl, control.

Melatonin inhibits EMT in GBC cells. EMT promotes migration and invasion of human cancer cells (9). Studies have demonstrated that melatonin can downregulate EMT (9,12). Thus, it was speculated that melatonin exerts its inhibitory effect by blocking EMT in gallbladder carcinoma cells. To assess the underlying molecular mechanisms of the antimetastatic effects of melatonin in GBC, cells were treated with 2 mM melatonin for specific time periods. The expression levels of the epithelial marker, E-cadherin, in the melatonin-treated group significantly increased, whereas the expression levels of the mesenchymal phenotype markers, N-cadherin and vimentin, significantly decreased (Fig. 3A). These results were further confirmed via RT-qPCR analysis (Fig. 3B-D). However, the PCR result of Snail was not shown as the gene expression level was much lower compared with the other markers assessed (data not shown). Collectively, these results suggest that melatonin exerts its inhibitory effect by blocking EMT.

Melatonin attenuates EMT and exerts antimetastatic effects by inhibiting the ERK1/2 pathway in GBC cells. Increasing evidence suggest that the AKT, NF-κB, PI3K, ERK and Wnt/β-catenin signaling pathways are involved in the regulation of metastasis caused by melatonin (9). Thus, the present study investigated whether these signaling pathways are involved in melatonin-induced inhibition of GBC-SD cell motility. The results demonstrated that cells treated with melatonin (2 mM) diminished activation of ERK, whereas the phosphorylation levels of AKT, PI3K and NF-κB were unaffected. To confirm the role of melatonin in downregulating the ERK signaling pathway, NOZ cells were treated with melatonin, and the results demonstrated that the expression of phosphorylated ERK decreased (Fig. 4A). These findings suggest that the ERK signaling pathway may be involved in melatonin-mediated inhibition of GBC cells.

To verify the role of the ERK signaling pathway in regulating the migratory potential and EMT, tBHQ (an activator of ERK1/2) was added to GBC cells (18-20). Notably, tBHQ (50 μM) reversed the effects of melatonin in inhibiting phosphorylation of ERK (Fig. 4B). In addition, the expression levels of E-cadherin significantly decreased following treatment with tBHQ, whereas the expression levels of Snail, vimentin and N-cadherin significantly increased compared with cells treated with melatonin (Fig. 4C). Accordingly, the inhibitory effects of melatonin on migration (Fig. 4D) and invasion (Fig. 4E) of GBC cells was partially reversed following treatment with tBHQ. Taken together, these results suggest that melatonin downregulates activity of the ERK signaling pathway, which in turn inhibits EMT and attenuates the metastatic effect observed in GBC cells (Fig. S1).

Discussion

GBC is the most common malignancy of the biliary tract that exhibits poor prognosis (3). Metastasis remains an important factor that affects the prognosis of patients with GBC (1). Currently, the therapeutic outcome of GBC treatment is extremely poor (21). Melatonin is a naturally occurring hormone secreted by the pineal gland that exhibits anticancer effects on different types of cancer, including glioma, uterine neck, ovarian, colon, liver and lung cancers (12,22-26). Previous studies have demonstrated reduced cytotoxicity of melatonin over a range of doses (9,26-28). The oral administration of 0.5 mg or more of melatonin instantaneously makes itself available in blood plasma, but it does not mimic the endogenous profile (29). However, the effects of melatonin on migration and invasion of GBC cells have not yet been investigated. The results of the present study demonstrated that melatonin inhibited the invasion of GBC cells by blocking activity of the ERK signaling pathway.

To achieve the desired therapeutic effects, endogenous substances are usually administered at considerably high concentrations compared with those corresponding to the physiological levels (30). Melatonin concentrations at >1 μM are described as pharmacological, while the physiological concentrations of this hormone include those <1 nM (31,32). In the present study, only high concentrations (2 and 3 mM) of melatonin were effective in decreasing GBC cell metastasis. However, treatment of cells with melatonin (3 mM) induced partial apoptosis. Thus, subsequent experiments were performed using 2 mM melatonin. The results of the present study demonstrated that melatonin decreased the invasive and migratory abilities of GBC cells.

EMT is a process during which epithelial cells shift towards the mesenchymal state, which is considered a principal process in tumor metastasis (33,34). The molecular and cellular changes observed in EMT are characterized by upregulation of specific proteins, such as N-cadherin, Snail and vimentin and by downregulation of E-cadherin (35). Thus, these proteins are used as EMT markers. It has been reported that EMT may be associated with the prognosis and clinicopathological characteristics of patients with GBC (36-38). Loss of epithelial markers and dysfunction of EMT-associated transcription factors promotes metastasis of GBC cells (39,40). To further investigate the mechanism of action of melatonin on GBC cells, the expression levels of the EMT-associated markers were assessed in the present study. The results demonstrated that melatonin promoted the expression levels of E-cadherin, while attenuating the levels of N-cadherin, Snail and vimentin. Thus, melatonin may inhibit GBC cell migration and/or invasion by influencing EMT.

Signaling pathways, such as the ERK, NF-κB, Wnt, Notch, Hedgehog, activator protein 1 and the growth factor signaling pathways, induce or modulate the EMT process (9,41). Evaluation of the phosphorylated levels of ERK, AKT, p65 and PI3K in the present study demonstrated that only the ERK signaling pathway was inhibited by melatonin. Overactivation of the ERK signaling pathway has been reported in both GBC tissues and cells (42). Melatonin inhibits cancer migration by blocking activation of ERK in different types of human cancer (11-14,42,43). Thus, the present study investigated whether the induction of EMT by melatonin was mediated by regulating the ERK signaling pathway in GBC cells. tBHQ is an ERK activator that induces the phosphorylation of ERK protein (43). The results of the present study demonstrated that the anti-invasive effects of melatonin were reversed by tBHQ, suggesting that this hormone can inhibit EMT via the ERK signaling pathway in GBC cells.

In conclusion, the results of the present study demonstrated that melatonin inhibited the invasion of GBC cells by blocking

the ERK signaling pathway. This study lays a foundation for future studies investigating the mechanisms of melatonin in GBC and may provide insights into a new therapeutic agent for GBC. However, the lack of further validation *in vivo* is a limitation of the present study. Further *in vivo* studies will help to elucidate the role of melatonin in the antitumor therapy of gallbladder cancer.

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Availability of data and materials

All data generated or analyzed during this study are included in this published article.

Authors' contributions

HWT, XYS and PFZ performed the experiments. WZG, SJZ and JL designed the study. HWT and BY analyzed and interpreted the data. HWT and SJZ wrote the article. HWT, SJZ and XYS confirm the authenticity of all the raw data. All authors have 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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