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Melatonin inhibits lung metastasis of gastric cancer *in vivo*

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Highlights

- Melatonin inhibits IL-1β-induced lung metastasis of GC.
- Melatonin downregulates the expression and activation of MMP-2, MMP-9, and NF-κB in GC cells *in vivo*.
- Melatonin would be as a supplementary therapy for patients with advanced GC.

Abstract

Aim

Melatonin shows therapeutic benefits in gastric cancer, but the mechanism underlying its anticancer effects remains elusive. The aim of this study was to determine whether melatonin inhibits lung metastasis in gastric cancer.

Main methods

A lung metastasis model of gastric cancer was established in nude mice injected with human gastric adenocarcinoma MGC80-3 cells. Mice were divided into control, IL-1β-treated, melatonin-treated, and IL-1β plus melatonin-treated groups and analyzed for the formation of lung metastatic nodules by flow cytometry and hematoxylin and eosin staining. The mRNA expression of epithelial-mesenchymal transition (EMT) markers was assessed by RT-qPCR. The activities of matrix metalloproteinase (MMP)-2 and MMP-9 were determined by gelatin zymography and their protein expression by western blotting and immunohistochemistry. The levels of NF-κB p65 and phosphorylated (p)-p65 were detected by immunohistochemistry.

Key findings

The number of lung metastases in the IL-1β plus melatonin group was significantly lower and the sizes of nodules were smaller than those in the IL-1β group. Furthermore, melatonin reversed changes in the expression of EMT markers induced by IL-1β by increasing mRNA levels of β-catenin and E-cadherin and decreasing those of fibronectin, vimentin, and Snail compared to IL-1β. Treatment with IL-1β upregulated the expression and activities of MMP-2 and MMP-9 and expression of NF-κB p65 and phospho-p65 (p-p65), but melatonin alleviated these effects.

Significance

Melatonin inhibited IL-1β-induced lung metastasis of gastric cancer through downregulation of MMP-2, MMP-9, and NF-κB p65 expression and activities. These findings provide a basis for potential use of melatonin as a supplementary therapy for patients with advanced gastric cancer.

Previous Next

Abbreviations

GC, gastric cancer; EMT, epithelial-to-mesenchymal transition; GA, gastric adenocarcinoma

Keywords

Gastric cancer; Metastasis; IL-1β; Melatonin; Matrix metalloproteinase

1. Introduction

Gastric cancer (GC) is the fourth most common malignancy in the world with the second highest death rate among all cancers [1]. Although the incidence and mortality of GC have been significantly reduced over the recent years owing to increasing use of gastroscopy, its 5-year survival rate remains poor because GC is often diagnosed when it is already in an advanced stage [2]. Surgery is the most effective treatment for primary GC tumors, but the advanced tumors are prone to invasion and metastasis, which complicates the surgery and reduces its effectiveness. Tumor metastasis is a dynamic multi-step process occurring through epithelial-to-mesenchymal transition (EMT), which is considered an important mechanism triggering tumor invasion and migration[3]. Experimental evidence indicates that the development, invasion, and metastasis of GC are associated with the activation of EMT-related transcription factors and signaling pathways [[4], [5], [6]]; therefore, inhibition of EMT may be a strategy to prevent tumor invasion and metastasis in GC. Although GC patients with advanced tumors may be treated with adjuvant radiotherapy and/or chemotherapy, the prognosis remains unsatisfactory as these therapies have serious side effects and their success varies because of individual differences [7]. Therefore, comprehensive treatment modes for advanced GC should be developed to increase the survival rate.

Melatonin, a hormone produced by the pineal gland, has pleiotropic effects and can regulate diverse biological processes. Accumulating evidence indicates that melatonin has not only hormonal functions but also protects cells against oxidative stress and xenobiotics [8] by regulating anti-oxidant response, hematopoiesis, and immune reactivity [9,10]. Furthermore, several studies have reported that melatonin exerts significant antitumor effects in several cancers [[11], [12], [13]]. Thus, melatonin was shown to reduce the metastatic potential of cultured breast cancer cells by regulating the expression of EMT markers, thus decreasing cell viability and invasiveness [14]. Melatonin also exhibited anti-angiogenic properties by upregulating miRNA3195 and miRNA374b and downregulating the expression of angiogenesis-related factors HIF-1α, HIF-2α and VEGF in prostate cancer cells under hypoxia [15] and was shown to decrease proliferation of prostate cancer cells by inactivating NF-κB [16]. Furthermore, melatonin has been found to reduce tumor size and weight and to inhibit cell proliferation and angiogenesis in a mouse model of GC [17]. Retrospective analysis indicated that serum melatonin levels in women with ovarian cancer were significantly lower compared with control groups ($p < 0.05$), suggesting that the reduction in circulating melatonin concentrations might contribute to the pathogenesis of ovarian cancer [18]. Our *in vitro* study indicated that melatonin could significantly suppress IL-1β-induced EMT and reduce the invasion and metastatic potential of GC cell lines MGC80-3 and SGC-7901 possibly by inhibiting the activities of NF-κB and matrix metalloproteinases MMP-2 and MMP-9 [19]. The purpose of this study was to further clarify the effects of melatonin on GC cell invasion and metastasis into the lung *in vivo* and determine the underlying signaling mechanisms, which have not been previously investigated.

2. Materials and methods

2.1. Cell culture

Human gastric adenocarcinoma (GA) cell line MGC80-3 was obtained from the Cell Bank of the Chinese Academy of Sciences (Shanghai, China) and cultured in RPMI-1640 medium (Hyclone, Logan, UT, USA) with 10% fetal bovine serum (HyClone) at 37 °C in an incubator containing 5% CO_2.

2.2. Animals

Six-week-old male BALB/c nude mice were purchased from Beijing Vital River Laboratory Animal Technology Co., Ltd (Beijing, China) and housed in the Animal Laboratory Center of Fuzhou General Hospital in specific pathogen-free conditions. All experiments were approved by the Laboratory Animal Care Committee of Fuzhou General Hospital, complied with the ARRIVE guidelines, and carried out in accordance with the U.K. Animals (Scientific Procedures) Act.

2.3. Invasion assay in mice

The effects of melatonin (Sigma-Aldrich Co., St Louis, MO, USA) on lung metastasis of GC *in vivo* was examined as previously described [20] with minor modifications. Twenty-four nude mice were injected with 200 μl RPMI-1640 medium containing 2×10^6 MGC80-3 cells into the tail vein, and then randomly divided into four groups (n = 6/group). The day following GA cell injection, groups 1 (control) and 3(melatonin treatment)were intraperitoneally injected with 200 μl PBS and groups 2 (IL-1β treatment) and 4 (IL-1β plus melatonin treatment) injected with IL-1β (Prospec, Rehovot, Israel) at a concentration of 20 μg/kg/day in 200 μl PBS; the injections were then performed every two days for 14 days. Starting from day 15, groups 3 and 4 received intraperitoneal injections of melatonin (100 mg/kg/day in 200 μl PBS) for one week, whereas groups 1 and 2 received PBS under the same conditions. In addition, three normal mice were set as a negative control group (n = 3). Mice were observed daily for food intake, weight, and activities. The used dose of melatonin had no significant effect on food consumption, activities, mental state, weight, body temperature, heart rate, or blood pressure of mice. After 45 days, mice were sacrificed and the lungs were dissected. One third of each lung tissue was used to prepare paraffin sections, which were stained with hematoxylin and eosin and observed under a microscope (Olympus, Tokyo, Japan) to confirm the presence of metastasis. The total number of metastatic nodules in each lung was counted in corresponding lung sections.

2.4. Flow cytometry analysis

Lung metastatic nodules from mice of the control group and normal lung tissues were used to prepare single-cell suspensions. The expression of cyclooxygenase-2 (COX-2) in metastatic nodules and normal lung tissue were detected by staining with APC-labeled anti-human COX-2 antibodies according to the manufacturer's instructions (Becton Dickinson, San Diego, CA, USA).

2.5. Immunohistochemistry (IHC) analysis

Lung tissues of nude mice were fixed with 4% paraformaldehyde, embedded in paraffin, and sectioned. The sections were dewaxed in xylene, and rehydrated in ethanol series and then in water; the detailed IHC protocol is described in the manufacturer's instructions (MXB Biotechnologies Inc., Fuzhou, China). To determine the expression of metastasis-related factors, primary antibodies against the following human proteins were used: COX-2 (1:100; Proteintech, Chicago, IL, USA), MMP-2 and MMP-9 (1:100; ZSGB, Beijing, China), β-catenin (1:500; Abcam, Cambridge, MA, USA), NF-κB p65 and phospho-p65 (1:100; Abcam). Briefly, the sections were incubated with the primary antibodies overnight at 4 °C, rinsed three times with PBS, and incubated with biotin-labeled secondary antibodies for 10 min at room temperature. After treatment with *Streptomyces* sp. peroxidase, the sections were incubated with DAB solution, stained with hematoxylin for 20 s, and observed under a microscope (Olympus, Tokyo, Japan). The results were evaluated as previously described [21].

2.6. Reverse transcription-quantitative polymerase chain reaction (RT-qPCR) analysis

Total RNA was extracted from lung metastatic nodules of nude mice with TRIzol reagent (Invitrogen; Thermo Fisher Scientific, Inc., Carlsbad, CA, USA). RT-qPCR was performed as previously described using GAPDH as an internal control [19,22]. Primer sequences are given in Table 1. Relative expression of the target genes was calculated using the $2^{-\Delta\Delta Ct}$ method. All experiments were repeated three times independently.

Table 1. The primers used for RT-qPCR.

Gene	Squence	
	Sence(5′-3′)	Antisence(5′-3′)
β-catenin	TGCCAAGTGGGTGGTATAGAG	CGCTGGGTATCCTGATGTGC
Fibronectin	CGAGCTTCCCCAACTGGTAACCC	GGTGGCACCTCTGGTGAGGC
E-cadherin	TTGCTCACATTTCCCAACTCCTC	CACCTTCAGCCATCCTGTTTCTC
Vimentin	GCTGAATGACCGCTTCGCCAACT	AGCTCCCGCATCTCCTCCTCGTA
Snail	TCTAGGCCCTGGCTGCTACAA	ACATCTGAGTGGGTCTGGAGGTG
MMP-2	CCTGATGTCCAGCGAGTG	AGCAGCCTAGCCAGTCG
MMP-9	CAGTCCACCCTTGTGCTCTTC	TGCCACCCGAGTGTAACCAT
GAPDH	AGAAGGCTGGGGCTCATTTG	AGGGGCCATCCACAGTCTTC

2.7. Western blotting analysis

Total protein was extracted from lung metastatic nodules on ice with radioimmunoprecipitation assay lysis buffer containing 1% phenylmethyl sulfonyl fluoride (Beyotime Institute of Biotechnology, Nantong, China) and the protein was then subjected to western blotting analysis as previously described [19,22] using rabbit polyclonal anti-human MMP-2 and MMP-9 antibodies (1:1,000; Cell Signaling Technology, Danvers, MA, USA). Anti-GAPDH antibody (1:2,000; Cell Signaling Technology) was used to ensure equal loading.

2.8. Gelatin zymography

Proteolytic activities of MMP-2 and MMP-9 in sera of nude mice were detected by gelatin zymography. Briefly, total serum proteins were separated by sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) in 8% acrylamide gels containing 1 mg/ml gelatin (Xinfan Technologies Inc., Shanghai, China) at a constant current of 40 mA; renaturing and development were performed in accordance with the manufacturer's instructions. Gels were stained with Coomassie blue, rinsed with 7% acetic acid solution for destaining, and evaluated using a gel scanner (Epson, Nagano, Japan) equipped by image analysis software.

2.9. Statistical analysis

Data analysis was performed using SPSS version 22.0 (SPSS Inc., Chicago, IL, USA). The results were presented as the mean ± SD and the significance of differences between groups was assessed by one-way analysis of variance (ANOVA); $p < 0.05$ was considered statistically significant.

3. Results

3.1. Human COX-2 expression is increased in lung metastatic nodules of mice injected with GA cells

In normal tissue, COX-2 is mostly absent or expressed at low levels, whereas during inflammation or carcinogenesis its expression is significantly induced and has been found to be abnormally high in most GC tissues and cell lines [23,24]. Therefore, we selected COX-2 as a marker to verify the formation of lung metastatic nodules in nude mice injected with human GA cells. Flow cytometry and IHC analyses showed that COX-2 was highly expressed in the metastatic nodules compared with normal lung tissue of nude mice (Fig. 1), confirming the invasion of lung tissue by GA cells.

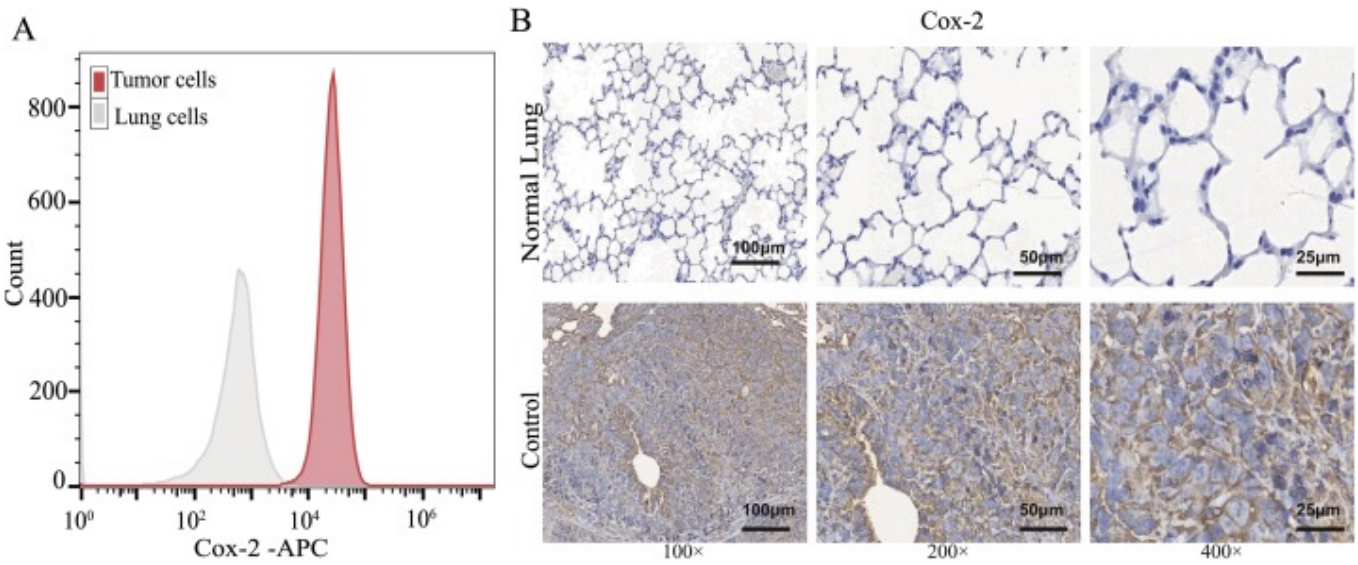

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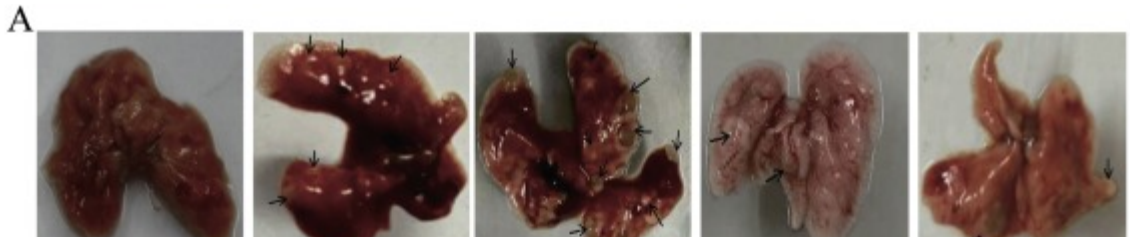
Fig. 1. Overexpression of COX-2 in lung metastatic nodules of nude mice. (A) Flow cytometry analysis showed that lung metastatic nodules had increased levels of COX-2 expression compared with normal mouse lung tissues. (B) Immunohistochemical analysis of COX-2 expression in lung metastatic nodules and normal lung tissue. COX-2 was overexpressed in metastatic nodules (control group), but not in normal lung tissue.

3.2. Melatonin inhibits IL-1β-induced metastatic potential of GA cells *in vivo*

Our previous research indicated that melatonin decreased IL-1β-induced invasion of GA cells *in vitro* [19]. To determine whether melatonin also inhibits GC metastasis *in vivo*, we performed an invasion assay in nude mice injected with MGC80-3 cells and then treated or not with IL-1β or IL-1β with melatonin. After 45 days, 50% of control PBS-treated mice (3/6) developed lung metastases, whereas all IL-1β-treated mice (100%, 6/6) had lung metastases. However, only two mice in the melatonin-treated group (33%, 2/6) and IL-β plus melatonin-treated group (33%, 2/6) had metastatic nodules in the lung (Fig. 2A). Moreover, the average number of lung metastatic nodules in mice treated with melatonin (2 nodules) or IL-1β plus melatonin (2 nodules) was significantly lower compared with those treated with IL-1β (9 nodules) and even lower than in the control group (5 nodules) (Fig. 2B). In addition, the size of nodules was significantly smaller in the IL-1β plus melatonin group compared to the IL-1β group (Fig. 2C).



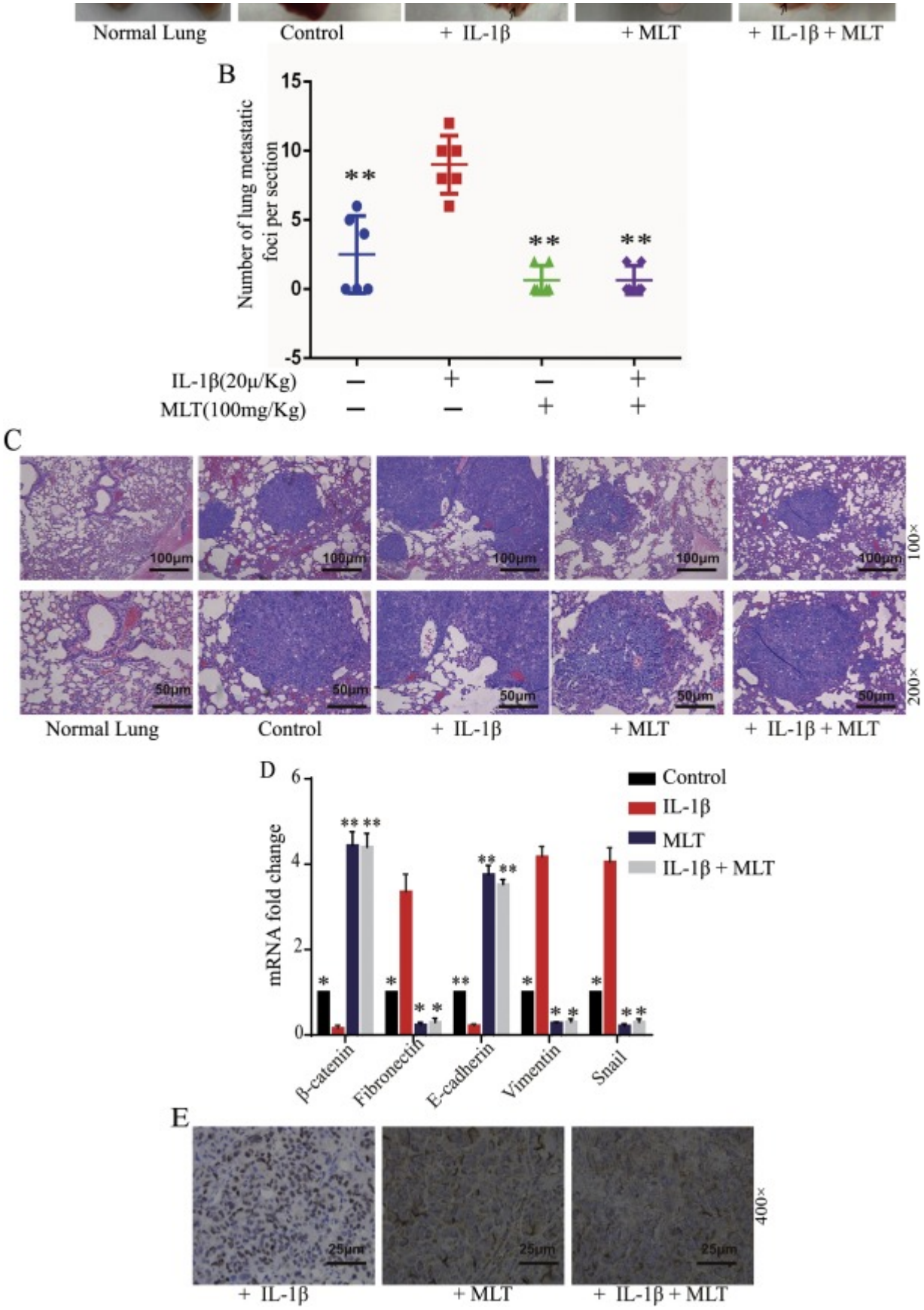


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Fig. 2. Melatonin decreased the metastatic potential of GA cells in IL-1β-treated nude mice. Mice were injected GA cells and treated with IL-1β, melatonin, and IL-1β + melatonin, or left untreated (control) (n = 6 mice/group). (A) Representative images of mouse lungs with metastatic nodules. (B) The number of lung metastatic nodules per section in different groups of mice that developed lung metastases; $^{**}p \leq 0.001$ vs. the IL-1β-treated group. (C) Representative images of HE-stained mouse lungs with metastases (Scale bars, up panels, 100μm; down panels, 50μm). (D) The mRNA expression of EMT-related markers in lung metastatic nodules analyzed by RT-qPCR. The data were presented as the mean ± SD from at least three independent experiments; $^{*}p < 0.05$ and $^{**}p \leq 0.001$ vs. the IL-1β-treated group. (E) The expression location of β-catenin in different groups was identified by IHC. Representative images were shown; magnification, ×400. MLT, melatonin.

To further confirm the inhibitory effect of melatonin on the metastatic process of GC in nude mice, we examined the expression of genes encoding EMT markers β-catenin, E-cadherin, fibronectin, vimentin, and Snail implicated in tumor invasion by RT-qPCR. The mRNA levels of β-catenin and E-cadherin in lung metastatic nodules were decreased, whereas those of fibronectin, vimentin, and Snail were increased in response to IL-1β, but melatonin reversed the effects of IL-1β on gene expression (Fig. 2D). Besides, we performed IHC to examine the expression of β-catenin and found that in the IL-1β treatment group, the positive staining was in nuclei; while in the melatonin treatment group and IL-1β+melatonin treatment group that was on membrane (Fig. 2E). These results suggest that melatonin inhibited IL-1β-induced metastasis of MGC80-3 cells *in vivo* through regulation of EMT.

3.3. Melatonin downregulates the expression and activities of MMP-2 and MMP-9 induced by IL-1β

A previous study reported that a strong correlation between MMP-2 and MMP-9 expression and cancer invasion and metastasis [25]. Therefore, we investigated whether melatonin inhibited the invasion of GA cells into the lung through downregulation of MMP-2 and MMP-9. The results of RT-qPCR, western blotting, and IHC analyses indicated that IL-1β upregulated the expression of MMP-2 and MMP-9 in lung metastatic nodules (Fig. 3A–C), and gelatin zymography revealed that it also induced their enzymatic activities (Fig. 3D). However, in mice treated with IL-1β plus melatonin, such effects of IL-1β were reduced (Fig. 3).

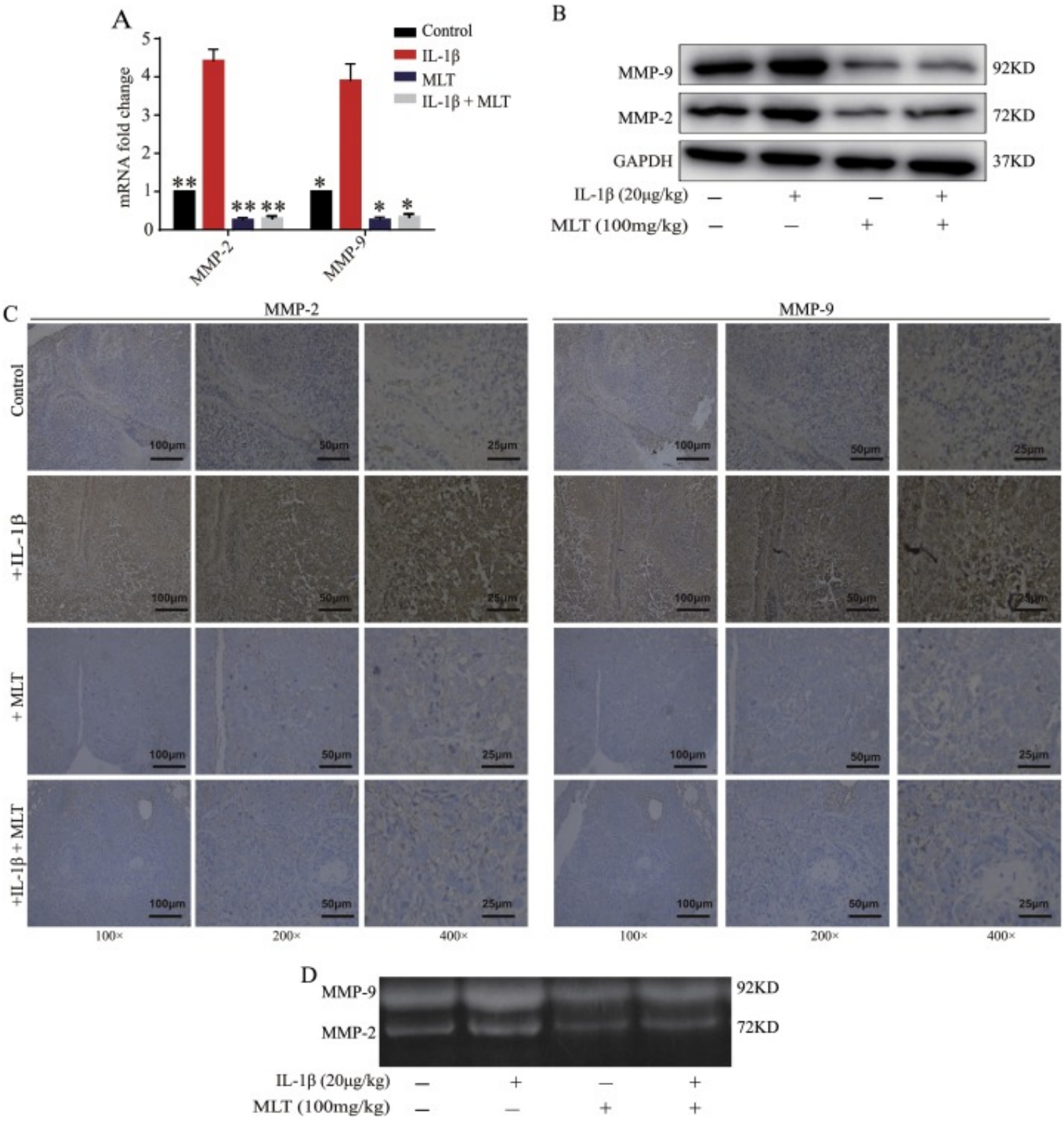

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Fig. 3. Melatonin downregulates the expression and activities of MMP-2 and MMP-9 upregulated by IL-1β in mouse lung metastatic nodules. Mice were injected GA cells and treated with IL-1β, melatonin, and IL-1β + melatonin, or left untreated (control) (n = 6 mice/group). (A) The mRNA expression of MMP-2 and MMP-9 in lung metastatic nodules was analyzed by RT-qPCR. The data were presented as the mean ± SD from at least three independent experiments; *$p < 0.05$ and **$p \leq 0.001$ vs. the IL-1β-treated group. (B, C) MMP-2 and MMP-9 protein expression in lung metastatic nodules was analyzed by western blotting (B) and IHC (C). Representative IHC images

were shown; magnification, ×100, ×200, and ×400. (D) Enzymatic activities of MMP-2 and MMP-9 in mouse serum was evaluated by gelatin zymography. MLT, melatonin.

3.4. Melatonin decreases IL-1β-induced expression of NF-κB

NF-κB is a transcription factor known to be involved in the regulation of cancer development, especially tumor invasion and metastasis [26]. Our previous results revealed that melatonin inhibited the activity of NF-κB in GC cell lines [19]; therefore, we examined the expression of NF-κB in our mouse model of GC metastasis. IHC analysis revealed that treatment with IL-1β increased the expression and phosphorylation of NF-κB p65 in mouse lung metastatic nodules compared with the control group, whereas melatonin mitigated these effects (Fig. 4).

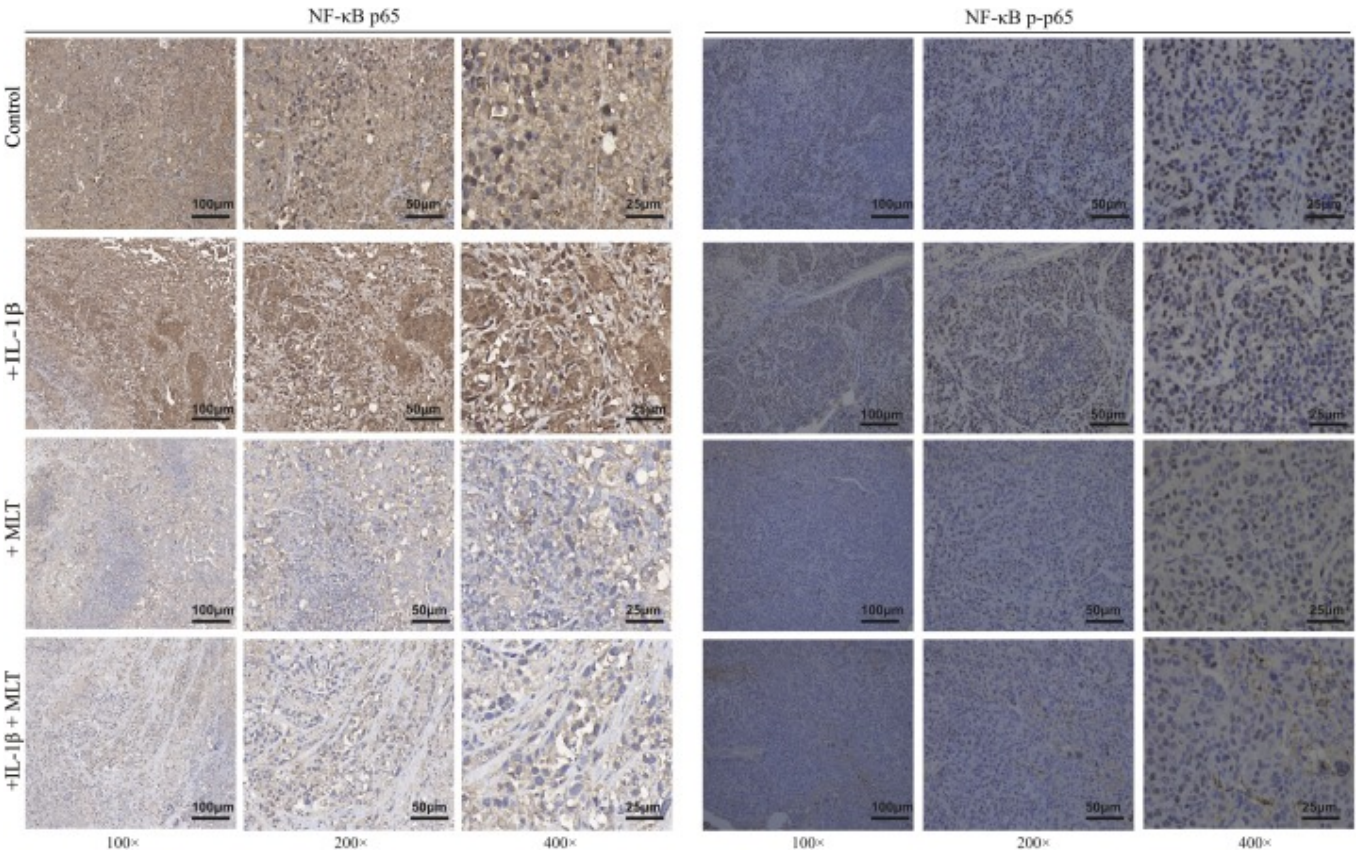

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Fig. 4. Melatonin decreases IL-1β-induced expression of NF-κB in mice. NF-κB p65 and phospho (p)-p65 expression in lungs metastatic nodules of nude mice subjected to the different treatments was analyzed by IHC. Representative images were shown; magnification, ×100, ×200 and ×400 (n = 6 mice/group). MLT, melatonin.

4. Discussion

GC is the most common gastrointestinal malignancy originating from the epithelium and presenting a severe threat to human health. Most GC patients are diagnosed at an advanced stage; therefore, despite the combined treatment, including surgery, chemotherapy, radiotherapy, and targeted therapy, their 5-year survival remains poor, which is largely due to the development of metastases [2]. Metastasis is not only a sign of disease deterioration but also a major cause of treatment failure and death [27]. Accordingly, effective therapeutic agents targeting GC metastasis may significantly improve patient's outcome and quality of life. EMT is a complex process of reprogramming epithelial cells, which plays an essential role in tumor metastasis in several cancers, especially in GC [28]. During EMT, cells lose their epithelial characteristics, including cell-cell adhesion proteins such as E-cadherin and β-catenin and acquire mesenchymal features, including expression of pro-migratory cytoskeletal proteins such as vimentin and fibronectin [29].

Melatonin may exert its anticancer activity by inhibiting tumor cell proliferation and metastasis through stimulation of anti-tumor immunity and induction of cancer cell apoptosis [[30], [31], [32], [33], [34]]. It was revealed that melatonin was able to reduce proliferation, clone formation, migration, and invasion, and induce apoptosis of a GC cell line AGS by activating JNK and p38 MAPK and inhibiting NF-κB [35]. In addition, melatonin significantly enhanced the anti-tumor effect of cisplatin and showed low systemic toxicity [35]. In cancer clinical trials, melatonin was mainly used with other chemotherapeutic drugs as an adjuvant. Thus, Lissoni et al. [36] evaluated the effect of a concomitant administration of melatonin (20 mg/day orally) in patients with metastatic non-small cell lung cancer receiving a chemotherapeutic regimen consisting of cisplatin and etoposide. In their study, melatonin-treated patients had significantly higher rates of overall tumor regression and 5-year survival, and better tolerance to chemotherapy. Similarly, co-administration of melatonin with irinotecan resulted in a higher percent of disease control in patients with metastatic colorectal cancer compared to irinotecan alone, as indicated by increased number of patients with partial response and stable disease [37].

In a previous study, we found that melatonin inhibited the IL-1β-induced EMT process and reduced the invasion and migration of MGC80-3 and SGC-7901 cells *in vitro* [19]. To further clarify the relationship between the effects of IL-1β and melatonin on metastatic processes in GC *in vivo*, in this study we used an animal model to determine if melatonin could reduce GC metastasis to the lung induced by IL-1β and to define the underlying signaling mechanism.

Our results indicate that IL-1β stimulated the formation of lung metastatic nodules by GA cells in the lungs of nude mice, as evidenced by increased number and size of nodules compared to the control group. However, treatment with melatonin significantly inhibited the process of metastatic nodule formation induced by IL-1β. The difference in GA cell invasion of the lung corresponded to changes in the expression of EMT-related factors. Thus, the levels of β-catenin

and E-cadherin in lung metastatic nodules were decreased and those of fibronectin, vimentin, and Snail increased by IL-1β, but these effects were attenuated by melatonin.

MMPs are zinc-dependent endopeptidases, which play a significant role in the degradation of basement membrane and extracellular matrix. MMP-2 and MMP-9 are important members of the MMP family involved in the development and progression of cancer, especially in tumor invasion and metastasis [25]. Therefore, we analyzed the proteolytic activities of MMP-2 and MMP-9 in mouse serum and their expression in lung metastatic nodules. Our results showed that the activities and expression of MMP-2 and MMP-9 increased following treatment with IL-1β, but melatonin inhibited the IL-1β-induced overactivation of MMP-2 and MMP-9 during GA cell metastasis, which is consistent with our previous results *in vitro*.

It has been demonstrated that tumor cells with constitutively active NF-κB are highly metastatic and that the reduction of NF-κB activity in these cells significantly decreases their invasiveness [38]. In this study, IHC analysis indicated that IL-1β treatment increased NF-κB levels in mouse lung metastatic nodules, but administration of melatonin could inhibit IL-1β-induced upregulation of NF-κB, indicating a decrease in its functional activity.

5. Conclusions

Taken together, these results suggested that melatonin inhibits IL-1β-induced lung metastasis of GC by regulating EMT-related genes and preventing activation of MMP-2, MMP-9, and NF-κB. Our findings provide a basis for the application of melatonin alone or in combination with other therapeutic methods to reduce metastasis in patients with advanced GC.

Availability of data and materials

The analyzed data are available from the corresponding author upon reasonable request.

Conflict of interest

The authors declare that they have no conflict of interest.

Acknowledgements

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