Melatonin downregulates nuclear receptor RZR/RORγ expression causing growth-inhibitory and anti-angiogenesis activity in human gastric cancer cells *in vitro* and *in vivo*

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Abstract. An adequate supply of oxygen and nutrients, derived from the formation of novel blood vessels, is critical for the growth and expansion of tumor cells. It has been demonstrated that melatonin (MLT) exhibits marked *in vitro* and *in vivo* oncostatic activities. The primary purpose of the present study was to evaluate the *in vitro* and *in vivo* antitumor activity of MLT on the growth and angiogenesis of gastric cancer cells, and explore the underlying molecular mechanisms. The present results revealed that MLT inhibited the growth of gastric cancer SGC-7901 cells in a dose- and time-dependent manner. In addition, the present study demonstrated that low concentrations (0.01, 0.1 and 1 mM) of MLT had no clear effect on vascular endothelial growth factor (VEGF) secretion, whereas a high concentration (3 mM) of MLT suppressed VEGF secretion in SGC-7901 cells. Notably, administration of MLT caused suppression of gastric cancer growth and blockade of tumor angiogenesis in tumor-bearing nude mice. Furthermore, MLT treatment reduced the expression of the MLT nuclear receptor RZR/RORγ, SUMO-specific protease 1, hypoxia-inducible factor-1α and VEGF at transcriptional and translational levels within gastric cancer cells during tumorigenesis. In conclusion, MLT nuclear receptor RZR/RORγ may be of great importance in the MLT mediated anti-angiogenesis and growth-inhibitory effect in gastric cancer cells. Since RZR/RORγ is overexpressed in multiple human cancers, MLT may be a promising agent for the treatment of cancers.

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Introduction

Gastric cancer is characterized by a rapid progression and decreased survival time, poor response to conventional chemotherapy and poor prognosis of patients, and is one of the most common malignancies across the world (1,2). Worldwide, 952,000 cases of gastric cancer were newly diagnosed, and 723,000 associated mortalities occurred in 2012 (3). Improving the survival rate of patients with gastric cancer is currently a major clinical challenge. Tumor growth, metastasis and prognosis are associated with angiogenesis (4,5), which is a multifactorial and multi-step process (6). Inhibition of tumor cell growth, invasion, migration and metastasis may prevent tumor angiogenesis. It is therefore accepted that angiogenesis blockade is a novel treatment for cancer (7,8).

Melatonin (MLT), an indoleamine synthesized in the pineal gland and other organs, is known to have a wide variety of physiological functions, including inhibiting inflammation, regulating circadian rhythms and increasing the activity of antioxidant enzymes (9,10). Recently, numerous studies have demonstrated anti-tumor effects of MLT (11-13). Furthermore, an increased expression of MLT nuclear receptors RZR/ROR have been demonstrated to contribute to the antitumor activity of MLT (14,15).

The present study was performed using *in vivo* and *in vitro* experiments and aimed to evaluate the effect of MLT on gastric cancer growth. The present results demonstrated that MLT inhibited angiogenesis in gastric cancer, and MLT nuclear receptor was involved in the inhibition of gastric cancer cell growth caused by MLT. The data support the theory that MLT may regulate gastric cancer angiogenesis, which provides novel insights into the treatment of gastric cancer.

Materials and methods

Human gastric cancer SGC-7901 cell culture. SGC-7901 cells were purchased from the Shanghai Institutes for Biological Sciences, Chinese Academy of Sciences (Shanghai, China). The cells were cultured in RPMI-1640 medium supplemented with 10% fetal bovine serum (Invitrogen™; Thermo Fisher Scientific, Inc., Waltham, MA, USA) at 37˚C in a humidified incubator

containing 5% CO_2. Hypoxia was induced by adding $CoCl_2$ (final concentration, 100 μM) in the culture medium. Cells with satisfactory growth were selected for subsequent experiments.

Microscopic observation of cell morphology. SGC-7901 cells were seeded onto 6-well plates (Corning Inc., Corning, NY, USA) at a density of $1x10^6$ cells/well, and incubated in a 37˚C incubator in a humidified atmosphere containing 5% CO_2. One day later, the cells were treated with 1 and 3 mM MLT (Sigma-Aldrich, St. Louis, MO, USA), and cell morphology was visualized 24 h post-treatment with a TE2000 inverted fluorescence microscope (Nikon Corporation, Tokyo, Japan).

Cell proliferation and viability assays. The SGC-7901 cells were seeded onto 96-well plates (Corning Inc.) at a density of 5,000 cells/well), and treated with 0, 0.01, 0.1, 1 and 3 mM MLT one day later. Following a further culture for 0.2, 2, 16 and 24 h, the viability and proliferation of the SGC-7901 cells was detected using a cell counting kit-8 (CCK-8) assay (Dojindo Molecular Technologies, Inc., Kumamoto, Japan), according to the manufacturer's protocol. Cell density was determined by measuring the absorbance value (A value) at 450 nm with a Varioskan™ Flash Multimode Reader (Thermo Fisher Scientific, Inc.).

In vivo xenograft tumor growth. A total of 60 4-week-old male nude mice of the BALB/c strain [BALB/cA-nu (nu/nu)] were purchased from Shanghai Laboratory Animal Center (Shanghai, China), and were maintained in a specific pathogen-free facility at 20-25°C in a relatively humidity of 40-60% under a 12 h-light/12 h-dark cycle, with free access to food and water. The mice were subcutaneously injected in the flank with SGC-7901 cells at a density of $3x10^6$ cells per mouse, and were randomly assigned into four groups by concentration of MLT that the mice were treated with: Control (0 mg/kg), low (50 mg/kg), medium (100 mg/kg) and high (150 mg/kg). The indicated amounts of MLT, which were intraperitoneally injected into the mice, were not administered until the diameter of the xenografts reached 2 mm. The long (L) and short (W) axes of the tumors were measured with a caliper, and the tumor size (V) was calculated using the following formula: $V = 4/3\pi \times l/2 \times (W/2)^2$.

Immunohistochemical staining of the tumors. Mice were sacrificed using the cervical dislocation method, and tumors were excised 7 days following MLT treatment. The excised tumors were cut into 5-μm sections, and transferred to gelatin-coated slides (Sigma-Aldrich). Following a 30-min incubation in phosphate-buffered saline (PBS) containing 0.3% Triton X-100 (Sigma-Aldrich), the sections were incubated with a primary antibody against RZR/ROR receptor [rabbit anti-RZR/RORα polyclonal antibody (1:500; catalog number ab60134; Abcam, Cambridge, MA, USA), rabbit anti-RZR/RORβ polyclonal antibody (1:500; catalog number ab78007; Abcam) or rabbit anti-RZR/RORγ polyclonal antibody (1:500; catalog number ab188756; Abcam) or vascular endothelial growth factor (VEGF) (rabbit anti-VEGF polyclonal antibody (1:200; catalog number ab46154; Abcam) at 37˚C overnight. After washing with 1X-PBS three times, the sections were incubated with a polyclonal goat anti-rabbit biotinylated antibody (1:100; catalog number BA1003; Boster Biological Technology Co., Pleasanton, CA, USA) for 1 h, and subsequently incubated in VECTASTAIN® Elite ABC Reagent (Vector Laboratories, Inc., Burlingame, CA, USA). Subsequently, the sections were stained with 3,3'-diaminobenzidine (DAB; Sigma-Aldrich). Images were taken with a Leica DM4000B photo microscope (Leica Microsystems GmbH, Wetzlar, Germany; magnification, x400).

Enzyme-linked immunosorbent (ELISA) assay. SGC-7901 cells were seeded onto dishes (60 mm diameter) and incubated at 37˚C for 24 h. Subsequently, the cells were incubated in darkness with MLT at various concentrations (0, 50, 100 and 150 mM) and 100 μM $CoCl_2$ (Sigma-Aldrich) at 37˚C for 24 h. The cell cultures were transferred to centrifuge tubes and centrifuged at 4˚C, 18,000 x g for 15 min on a 5417 R refrigerated microcentrifuge (Eppendorf, Hamburg, Germany). The supernatants were collected and the VEGF level in the supernatant was determined using a Human VEGF Quantikine ELISA kit (R & D Systems, Inc., Minneapolis, MN, USA) by measuring the A value at 450 nm. The cell count was estimated with a hemocytometer at least three times, and the standard curve was plotted.

Western blotting assay. Total protein was isolated from the tumor tissues using a Total Protein Extraction kit (Beijing Transgen Biotech Co., Ltd., Beijing, China), and the protein concentration was quantified with a Bicinchoninic Acid Protein Assay kit (Sigma-Aldrich). The protein samples were separated by 12% sodium dodecyl sulfate polyacrylamide gel electrophoresis (110 V; 1.5 h), and the proteins were transferred to polyvinylidene difluoride (PVDF) membranes (EMD Millipore; Billerica, MA, USA) at 4˚C (110 V; 1.5 h) using a wet transfer technique. Subsequently, the membranes were blocked in 5% skimmed milk in PBS, incubated with a primary antibody against SUMO-specific protease 1 (SENP1) (rabbit anti-SENP1 polyclonal antibody; 1:1,000; catalog number SAB2102107; Sigma-Aldrich) or hypoxia-inducible factor-1α (HIF-1α) (rabbit anti-HIF-1α monoclonal antibody; 1:1,000; catalog number ab51608; Abcam) at 4˚C overnight, washed in Tris-buffered saline-Tween 20 (TBST), and incubated with a monoclonal goat anti-mouse IgG secondary antibody (1:4,000; catalog number, A3562; Sigma-Aldrich) at room temperature for 1.5 h. Subsequently, the PVDF membranes were washed in TBST and the immunoreactive bands were visualized through exposure to X-ray film for 1-15 min. The protein purity was evaluated by densitometric analysis using the Quantity One software version 4.4.2 (Bio-Rad Laboratories, Inc., Hercules, CA, USA). The quantity of target protein was calibrated with respect to β-actin, and control values and relative intensities were obtained.

Reverse transcription-quantitative polymerase chain reaction (qPCR). Total RNA was extracted from the tumor tissues using TRIzol reagent (Invitrogen™; Thermo Fisher Scientific, Inc.), according to the manufacturer's protocol. The RNA was reverse-transcribed into first-strand cDNA using the GoScript™ Reverse Transcription System (Promega Corporation, Madison, WI, USA), which contained M-MLV reverse transcriptase. The primers used for RT were designed using the Primer Express

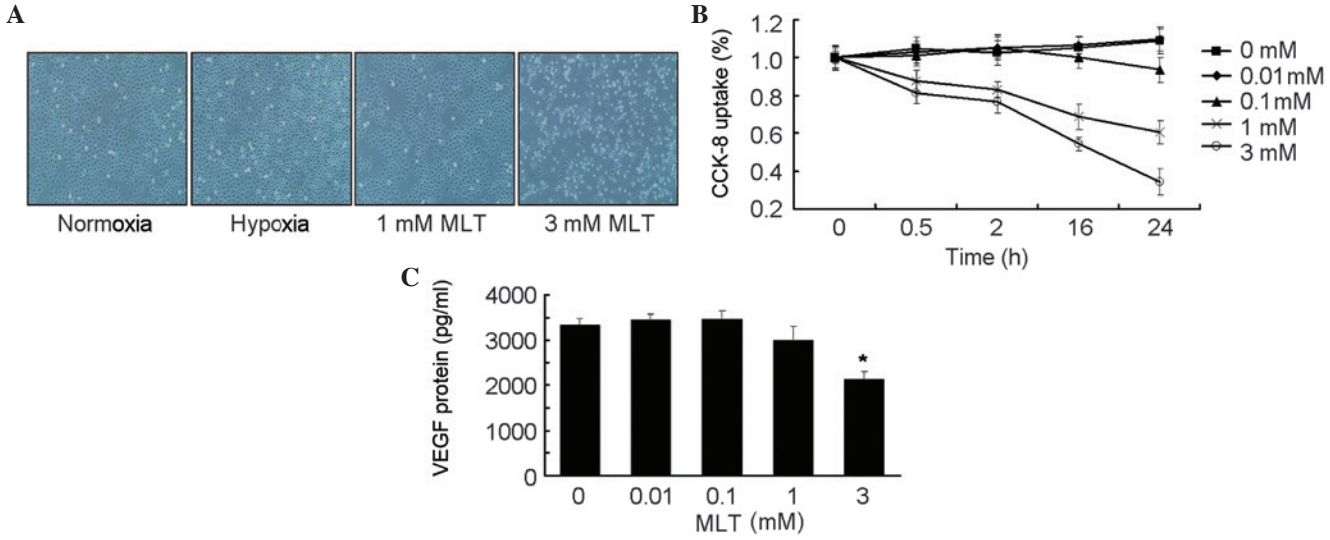

Figure 1. Effects of MLT on human gastric cancer SGC-7901 cell morphology, viability and VEGF secretion. Cells were cultured in RPMI-1640 containing MLT [0 (control), 0.01, 0.1, 1 and 3 mM]. (A) Cellular morphology was observed by optical microscopy in the indicated conditions (magnification, x200). (B) Cell viability was assayed using a CCK-8 assay. (C) VEGF protein content in the supernatant was determined by enzyme-linked immunosorbent assay. Data are expressed as the mean ± standard error of the mean of three independent experiments. *P<0.05 vs. control group. MLT, melatonin; VEGF, vascular endothelial growth factor; CCK-8, cell counting kit-8.

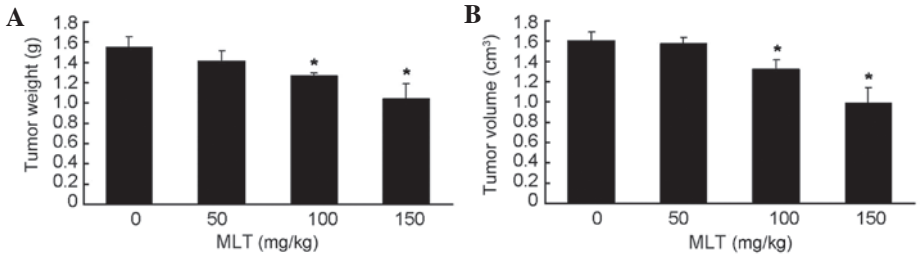

Figure 2. Comparison of tumor (A) weight and (B) volume between tumor-bearing control nude mice and tumor-bearing MLT-treated nude mice. Data are presented as the mean ± standard error of the mean. *P<0.05 vs. control group. MLT, melatonin.

version 5.0 software (Applied Biosystems®; Thermo Fisher Scientific, Inc.), and were synthesized by BioSune Biotechnology Co. (Shanghai, China). The cycling conditions were as follows: 95°C for 30 sec, followed by 40 cycles of 95°C for 5 sec and 60°C for 34 sec. The reaction was conducted on a ABI PRISM 7500 Real-time PCR System (Applied Biosystems®; Thermo Fisher Scientific, Inc.). The relative expression of RZR/ROR receptor, SENP1, HIF-1α, VEGF and glyceraldehyde-3-phosphate dehydrogenase (GAPDH) genes was determined using qPCR with Brilliant III SYBR Green QRT-PCR Master Mix (Agilent Technologies, Inc., Santa Clara, CA, USA) on a StepOnePlus® Real-Time PCR system (Applied Biosystems®; Thermo Fisher Scientific, Inc.). qPCR was performed using the following primers: RZR/RORγ, forward 5'-GAGGCCATTCAGTACGTGGT-3' and reverse 5'-GCAATCTCATCCTCGGAAAA-3'; SENP1, forward 5'-GAGGATGGATGCTGGAGAAG-3' and reverse 5'-TGTCTGAGGAAGGATTATCTGAG-3'; HIF-1α, forward 5'-ACTCAGGACACAGATTTAGACTTG-3' and reverse 5'-ATCAGTGGTGGCAGTGGTAG-3'; VEGF, forward 5'-CTTGCCTTGCTGCTCTAC-3' and reverse 5'-ACCACTTCGTGATGATTCTG-3'; GAPDH, forward 5'-CCGAGAATGGGAAGCTTGTC-3' and reverse 5'-TTCTCGTGGTTCACACCCATC-3'. The cycling conditions were as follows: 40 cycles of 95˚C for 30 sec, and 60˚C for 1 min. Relative mRNA expression levels were calculated using the $2^{-\Delta\Delta Cq}$ method (16). Melting curve analysis was employed to check the specificity of the amplification reaction.

Statistical analysis. All experiments were repeated at least three times. All data are expressed as the mean ± standard error of the mean, and all statistical analyses were performed with SPSS version 16.0 software (SPSS, Inc., Chicago, IL, USA). Data were analyzed by one-way analysis of variance, followed by Fisher's least significant difference test. P<0.05 was considered to indicate a statistically significant difference.

Results

MLT suppresses gastric cancer cell growth and VEGF secretion. To examine the effects of MLT treatment on SGC-7901 cells, cell morphology and cellular viability in response to MLT stimuli was examined. Microscopy revealed a significantly reduced number of SGC-7901 cells in the MLT treatment groups compared to the control cells. In addition, MLT treatment inhibited the growth of SGC-7901 cells in a time- and dose-dependent manner (Fig. 1A and B). Subsequently, whether angiogenesis was impaired following MLT treatment was assessed by measuring VEGF protein content in the supernatant

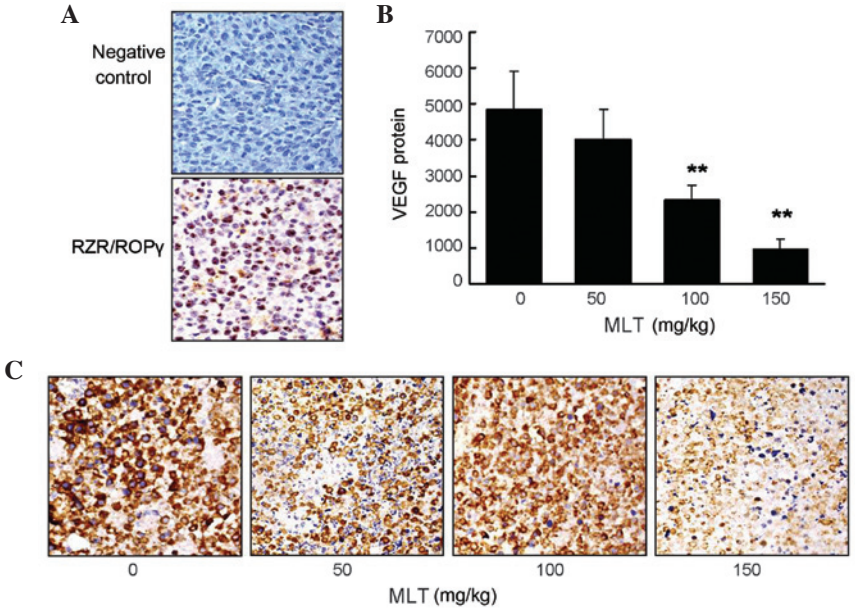

Figure 3. MLT nuclear receptor RZR/RORγ expression is increased and VEGF expression is decreased in gastric cancer tissue by MLT treatment. (A) Immunohistochemistry staining of RZR/RORγ protein in tumor tissues (magnification, x200). (B and C) VEGF protein expression in the tumor tissues of control mice and tumor-bearing MLT-treated mice (magnification, x200). Data are presented as the mean ± standard error of the mean. **P<0.05 vs. control mice. MLT, melatonin; VEGF, vascular endothelial growth factor.

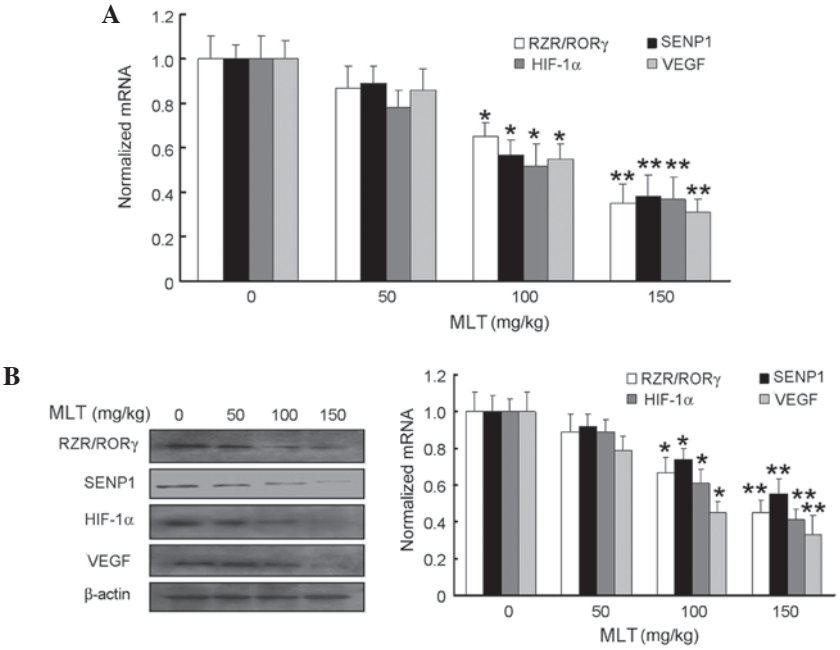

Figure 4. (A) mRNA and (B) protein expression of RZR/RORγ, SENP1, HIF-1α and VEGF in tumor tissues treated with various concentrations of MLT. Data are presented as the mean ± standard error of the mean. *P<0.05; **P<0.01 vs. control mice. MLT, melatonin; VEGF, vascular endothelial growth factor; SENP1, SUMO-specific protease 1; HIF-1α, hypoxia-inducible factor-1α.

of the cells. The present findings demonstrated that treatment with low concentrations of MLT had no remarkable effect on VEGF secretion, while 3 mM MLT inhibited VEGF production in SGC-7901 cells (P=0.023), which was in agreement with the growth inhibitory effects (Fig. 1C).

MLT inhibits tumorigenesis of gastric cancer cells in vivo. Since the antitumor activity of MLT has been extensively described previously, the present study sought to determine the effective concentration of MLT that was required to inhibit the *in vivo* xenograft tumor growth of gastric cancer in nude mice. Tumor weight and volume (Fig. 2A and B, respectively) were decreased in tumor-bearing mice treated with low (50 mg/kg) doses of MLT, and significantly decreased in tumor-bearing mice treated with medium (100 mg/kg; P=0.043 and 0.041 in Fig. 2A and B, respectively) and high (150 mg/kg; P=0.039 and 0.031 in Fig. 2A and B, respectively) doses of MLT compared with untreated mice. These

results additionally confirmed the antitumor activity of MLT against gastric cancer cells.

Nuclear receptor RZR/RORγ is expressed and MLT decreases VEGF expression in gastric cancer tissue. Immunohistochemistry staining results revealed that RZR/RORγ was expressed in gastric cancer tissues (Fig. 3A), while the expression of RZR/RORα or RZR/RORβ was not detected (data not shown). To investigate the effect of effective concentrations of MLT on VEGF expression in gastric cancer tissue, immunohistochemical staining was performed to detect VEGF protein expression. The present findings demonstrated that there was a significantly reduced VEGF protein expression in the tumor tissues collected from MLT-treated tumor-bearing mice compared with control mice (P=0.009 and 0.003 for 100 and 150 mg/kg MLT, respectively; Fig. 3B and C).

Effects of MLT on RZR/RORγ, SENP1, HIF-1α and VEGF expression at mRNA and protein levels in gastric cancer tissue. To explore the underlying mechanisms of the anti-tumor activity of MLT in gastric cancer tissue, the present study sought to determine the expression of RZR/RORγ, SENP1, HIF-1α and VEGF following treatment with MLT. The transcriptional and translational expression of these molecules was determined using qPCR and western blot analysis, respectively. The present findings revelaed significantly reduced mRNA and protein levels of RZR/ROR receptor, SENP1, HIF-1α and VEGF in the tumor tissues derived from the MLT-treated tumor-bearing mice compared with untreated mice (Fig. 4A and B). The P-values obtained for 100 mg/kg MLT in regards to mRNA expression were as follows: P=0.038 for RZR/RORγ, P=0.035 for SENP1, P=0.033 for HIF-1α and P=0.034 for VEGF, while for 150 mg/kg MLT, the results revealed P=0.005 for RZR/RORγ, P=0.007 for SENP1, P=0.006 for HIF-1α and P=0.004 for VEGF (Fig. 4A). In terms of protein expression, 100 mg/kg MLT resulted in P=0.040 for RZR/RORγ, P=0.042 for SENP1, P=0.038 for HIF-1α and P=0.028 for VEGF, while 150 mg/kg MLT led to P=0.006 for RZR/RORγ, P=0.009 for SENP1, P=0.005 for HIF-1α and P 0.004 for VEGF (Fig. 4B).

Discussion

The physiological adjustment in response to alterations in oxygen tension is reported to be critical for normal embryonic development and the pathophysiology of ischemic vascular disorders (17-19). Low oxygen induces the stabilization and activity of HIF-1α, a critical transcription factor for the adaptation to the hypoxic tumor microenvironment. Indeed, it has been demonstrated that HIF-1α is involved in the progression of solid tumors through the stimulation of VEGF transcription under a hypoxic microenvironment (19-21). Antibodies and soluble proteins targeting VEGF and its receptors have therefore been developed and tested for their anti-angiogenic efficacy against solid tumors, and satisfactory anti-angiogenic activity was achieved (22-24).

MLT, which was firstly identified as a major secretory product from the pineal gland of bovines, is a critical regulator in the coordination of circadian rhythms and seasonal reproduction, and exhibits antioxidant, oncostatic and anti-proliferative actions (12,25-27). It has been recently demonstrated that MLT markedly reduces the risk of mortality and adverse events of multiple malignancies, notably in breast cancer, colon cancer, melanoma, lung cancer and malignant glioblastoma (28-30). Previous studies performed by the present authors demonstrated that MLT inhibited the growth of murine foregastric carcinoma cells *in vivo* and *in vitro* (31,32). The findings of the current study reveal that MLT inhibits the proliferation of SGC-7901 cells in a dose- and time-dependent manner, and treatment with 3 mM MLT suppresses VEGF protein secretion in SGC-7901 cells exposed to $CoCl_2$. As expected, MLT treatment caused an inhibition on tumor growth and formation of novel blood vessels, and suppressed tumor angiogenesis in nude mice, as shown by a decrease in VEGF expression. The results suggest that MLT functions as an antitumor agent due to its anti-angiogenesis activity. Therefore, it is hypothesized that MLT may alter tumor adaptation to hypoxia and inhibit angiogenesis of solid tumors to exhibit anticancer activity.

It has been proposed that the putative nuclear MLT receptor belongs to a novel subclass of orphan nuclear receptors, and it was also suggested that the immunomodulatory and antitumor effects of MLT was dependent on its intracellular action in response to nuclear signaling (14,15). In addition, the actions of MLT had been reported to be mediated by mechanisms that are either receptor-dependent or independent. The present study investigated whether MLT inhibited HIF-1α protein expression in a receptor-dependent manner. Previously, MLT nuclear receptors were cloned and termed retinoid Z receptor (RZR) and retinoid acid receptor-related orphan receptor (ROR). The RZR/ROR family consists of three subtypes (α, β and γ) with four splicing variants of the α-subtype (33). The present study revealed that RZR/RORγ receptors were highly expressed in gastric cancer tissue from tumor-bearing nude mice. HIF-1α is reported to be critical for hypoxia-dependent angiogenesis during tumor progression, and its expression is associated with a poor prognosis in patients with solid tumors (34). Therefore, it appears that MLT may act as a HIF-1α inhibitor in tumor microenvironments, including hypoxia. Consequently, it may be assumed that MLT effectively decreases HIF-1α expression through the removal of MLT nuclear receptors during tumor progression.

Hypoxia induces nuclear translocation and SUMOylation of HIF-1α, which binds to von Hippel-Lindau molecule in a hydroxyl proline-independent manner, which finally leads to its ubiquitination and proteasomal degradation (35). SENP1, which is predominately a nuclear protein, is well-positioned to regulate the activity and stability of HIF-1α in the nucleus by removing SUMO (36-38). Thus, SENP1 is critical in maintaining HIF-1α stability during hypoxia and is involved in hypoxia-dependent angiogenesis during tumor progression. Several inhibitors and oncolytic adenoviruses, which function during HIF-1α modification or in the downstream signaling pathways, have been previously developed (39,40). MLT has been reported to suppress tumor angiogenesis by inhibiting HIF-1α stabilization under hypoxic conditions (41,42). The current study demonstrated that MLT effectively decreased the levels of RZR/RORγ, SENP1, HIF-1α and VEGF at mRNA and protein levels in developing gastric cancer. It is hypothesized

that MLT reduces HIF-1α expression by removing RZR/ROR receptors and SENP1 during tumor progression. In addition, MLT has been identified to lower HIF-1α expression by inhibiting its stability and accumulation (43). The present findings demonstrate that MLT suppressed HIF-1α accumulation via RZR/ROR receptor and SENP1 inactivation, and MLT may act as a promising agent for treatment of gastric cancer.

In summary, the results of the current study demonstrate the anti-angiogenesis and antitumor activity of MLT against gastric cancer by targeting HIF-1α, which stabilizes the tumor microenvironment and stimulates tumor angiogenesis. In addition, MLT effectively decreased the expression of RZR/RORγ, SENP1, HIF-1α and VEGF at mRNA and protein levels in developing gastric cancer. These findings suggest that MLT may be vital in tumor suppression by acting through the RZR/RORγ, SENP1, HIF-1α, VEGF signaling pathway, and reduces angiogenesis in the developing tumor. Therefore, MLT nuclear receptor RZR/RORγ may be important in inhibiting gastric cancer angiogenesis and tumor growth. The present findings provide a novel option to support the treatment of gastric cancer. Additional clinical trials to evaluate the efficacy and safety of MLT against gastric cancer appear to be justified.

Acknowledgements

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