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Melatonin Exerts Anticancer Effects in Human Tongue Squamous Cell Carcinoma Cells by Promoting Autophagy

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Abstract. *Background/Aim: The global prevalence of head and neck squamous cell carcinoma (HNSCC) remains high, and its prognosis poor. We investigated the anticancer effects of melatonin in human tongue squamous cell carcinoma cells (SCC-25) and its mechanisms of action. Materials and Methods: MTT assay was used to determine cell viability. To assess the effects of melatonin on SCC-25 cell metastasis, we conducted cell formation, wound healing, transwell migration and invasion assay. Western blot analysis was performed to measure the levels of autophage marker proteins. Results: We found that melatonin treatment significantly reduced the viability and colony formation ability of SCC-25 cells, impairing cell migration and invasion. Western blotting assay revealed that melatonin increased the levels of autophagy markers, such as LC-3B and Beclin-1. Consequently, melatonin induces autophage in SCC-25 cells. Conclusion: Melatonin may be a promising anticancer agent for the treatment of human tongue squamous cell carcinoma.*

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Head and neck squamous cell carcinomas (HNSCC) are tumors of the oral cavity, nasal cavity, pharynx, and larynx and are among the 10 most prevalent malignancies worldwide (1). Oral squamous cell carcinoma accounts for almost 20% of all head and neck cancers, and its incidence is increasing rapidly, especially among young and middle-aged individuals. Approximately 600,000 individuals are diagnosed with HNSCC per year globally, making HNSCC the sixth most frequent cancer in the world (2). Among patients with oral squamous cell carcinoma, 25-50% are diagnosed with tongue carcinoma (3).

Alcohol consumption and tobacco exposure are significant risk factors for developing oral carcinoma. Infection with human papillomavirus is another potential risk factor for oropharyngeal squamous cell carcinoma independent of smoking status (4). Over 50% of patients with tongue carcinoma are diagnosed at a locally advanced stage, and current treatment for these patients includes a combination of surgery, radiotherapy, and chemotherapy. Despite treatment, more than 50% of patients with tongue carcinoma die within 5 years of diagnosis (5). The poor treatment outcomes and high toxicity of current treatments call for development of improved and less toxic therapeutic approaches for patients with oral squamous cell carcinoma (6).

Melatonin (N-acetyl-5-methoxytryptamine) is an indoleamine hormone secreted by the pineal gland (7, 8) that plays a key role in many biological processes, including sleep regulation, redox balance, and immune system regulation (8, 9). Recently, melatonin has been shown to exert anticancer effects by modulating oxidative stress pathways, inhibiting cancer cell migration, and inducing apoptosis and autophagy (10-14). Consistently, melatonin has shown strong suppression of the growth and migration of various cancer cell types *in*

vitro (13-20). However, melatonin's safety as an anticancer agent (21) and its efficacy against tongue squamous cell carcinoma remain unclear.

In this study, we investigated the anticancer effects of melatonin in human tongue squamous cell carcinoma cells and its mechanisms of action.

Materials and Methods

Cell culture. The human tongue squamous cell carcinoma cell line SCC-25 was kindly provided by Prof. Chul-Ho Kim (Ajou University, Suwon, Republic of Korea). Cells were cultured in minimal essential medium (Gibco, Carlsbad, CA, USA) supplemented with 10% fetal bovine serum (FBS; Gibco) and penicillin/streptomycin (100 U/ml each; Gibco). Cells were maintained at 37˚C and 5% CO_2 in a humidified atmosphere.

Reagents and antibodies. MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide) and melatonin were purchased from Sigma-Aldrich (St. Louis, MO, USA) and dissolved in phosphate-buffered saline (PBS) and ethanol, respectively. To prepare the stock solution, 400 mM melatonin was dissolved in ethanol with minimal air and light exposure. For all experiments, cells were treated with the indicated concentrations of melatonin or equal volumes of ethanol (as a control). Antibodies against Beclin-1 and LC3 were purchased from Cell Signaling Technologies (Beverly, MA, USA). The anti-β-actin antibody was purchased from Sigma-Aldrich.

Cell proliferation assays. Cell proliferation was assessed by MTT assay. Briefly, cells were seeded in 12-well plates at a density of 5×10^4/well and allowed to grow overnight. Subsequently, the cells were treated with melatonin (1, 2, or 4 mM) for 24, 48, or 72 h and then incubated with MTT reagent (0.5 mg/ml) for 4 h. The purple-colored formazan produced by viable cells was dissolved in dimethyl sulfoxide, and the optical absorbance at 570 nm was measured using an ELISA reader (Multiskan EX, Thermo Fisher Scientific, Vantaa, Finland). Cell viability was reported as a percentage relative to the untreated controls. Representative images of the melatonin-treated and control cells were obtained under a phase-contrast microscope (micros Austria, Veit An Der Glan, Austria).

Colony formation assays. Cells were seeded in 6-well plates (500 cells/well) and allowed to grow for 48 h before adding the indicated chemicals. After 14 days, cells were fixed in methanol and stained with 0.5% crystal violet (Sigma-Aldrich) for 15 min at room temperature. Colonies in 10 random fields of view in each dish were counted under a microscope (Austria micros) using Image J. All experiments were performed in triplicate.

Wound healing assays. Wound healing assays were performed to assess cell migration ability. Cells were seeded in 6-well plates and treated with melatonin, after which artificial wounds were created using 200 μl pipette tips. Wound closure was monitored by imaging the cells at the time of scratching, as well as 24 and 48 h after scratching. The migration rate was reported as the fraction of cell coverage across the scratch line.

Transwell migration and invasion assays. Transwell chambers with or without Matrigel (Corning, Bedford, MA, USA) were used to test the effects of melatonin on the migration and invasion abilities of SCC-25 cells. For the migration assay, 200 μl cell culture medium without FBS was added to the cell-containing upper chamber, and 600 μl medium containing 10% FBS was added to the lower chamber as a chemoattractant. For the invasion assay, cells suspended in 200 μl medium without FBS were added to the upper chamber with Matrigel, and 600 μl medium containing 10% FBS was added to the lower chamber. Melatonin (2 or 4 mM) was added to the upper chambers, and after 24 h, the cells in the lower chambers were fixed in 4% paraformaldehyde for 30 min and stained with 0.5% crystal violet for 15 min. The cells that remained in the upper chambers were carefully removed using cotton swabs. The fixed and stained cells in the lower chambers were dried at room temperature and observed under a microscope (Austria micros) at a magnification of 100×. All assays were performed in triplicate.

Flow cytometry. Cell apoptosis was assessed using the Annexin V fluorescein isothiocyanate (FITC) apoptosis detection kit (BD Biosciences, San Jose CA, USA) according to the manufacturer's protocol. Briefly, cells were treated with melatonin (2 or 4 mM) for 24 h, washed with cold PBS, and stained with Annexin V-FITC and 7-amino-actinomycin D (7-AAD) at room temperature for 15 min. Stained cells were analyzed by flow cytometry (FACSCanto II; BD Biosciences), and flow cytometric data were analyzed using FACSDiva software (BD Biosciences).

Western blotting assay. Following the different treatments, cells were washed twice with cold PBS and lysed in PRO-PREP protein extraction solution (Intron Biotechnology, Sungnam, Republic of Korea). Equal amounts of protein were resolved by 8-12% sodium dodecyl sulfate–polyacrylamide gel electrophoresis and then transferred onto polyvinylidene difluoride membranes (Millipore, Billerica, MA, USA). Membranes were blocked with 4% nonfat milk in Tris-buffered saline containing 0.1% Tween 20 at room temperature. Subsequently, the membranes were incubated with primary antibodies and a horseradish peroxidase-conjugated secondary antibody (Jackson ImmunoResearch, West Grove, PA, USA). Protein signals were detected using an enhanced chemiluminescence detection system and Pierce ECL western blotting substrate (Pierce, Rockford, IL, USA) following the manufacturer's instructions. Each blot was also probed for β-actin as an internal control. Signal intensities were analyzed using ImageJ and normalized to the respective β-actin signal intensities.

Statistical analysis. All data are presented as means±standard deviation based on at least three independent experiments. Comparisons between two groups were performed using Student's *t*-test. *p*-Values<0.05 were considered statistically significant.

Results

Melatonin affects the viability of human tongue squamous cell carcinoma cells. To assess the effects of melatonin on the viability of human tongue squamous cell carcinoma cells, we performed MTT assays of SCC-25 cells treated with different concentrations of melatonin (1-4 mM) for 24, 48, or 72 h. Cell viability was decreased by more than 80% in SCC-25 cells exposed to melatonin for 72 h (Figure 1). Importantly, the effect of melatonin on SCC-25 cell survival was time-dependent (Figure 1).

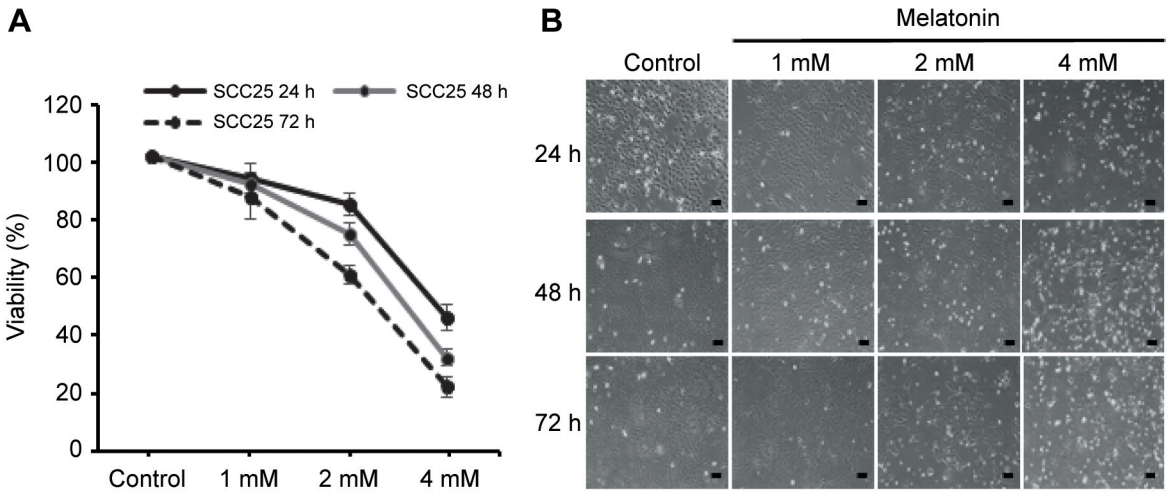

Figure 1. *Effects of melatonin on the viability and morphology of SCC-25. The cells were treated for 0, 24, 48, or 72 h with various concentrations of melatonin (control, vehicle 1%, 0.5, 1, 2, 4, and 5 mM). Viability was assessed using the MTT assay. Representative images from three independent experiments are shown. *p<0.05; **p<0.01 vs. control cells.*

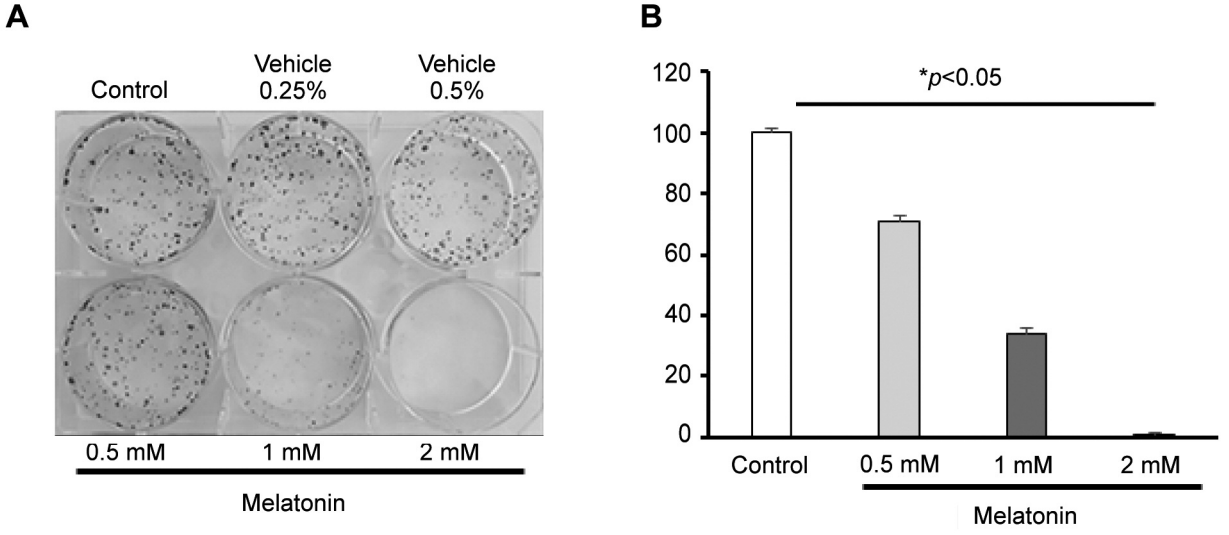

Figure 2. *Effects of melatonin on colony formation of SCC-25 cells. The cells were incubated without (cell control) or with 0.5, 1, and 2 mM of melatonin for 14 days; melatonin inhibits colony formation in SCC-25 cells. The results are represented as the means of three independent experiments. *p<0.05 vs. control cells. Mel: Melatonin.*

Effects of melatonin on colony formation, migration, and invasion in SCC-25 cells. Since metastasis is the primary cause of death in patients with tongue squamous cell carcinoma, we assessed the effects of melatonin on the migration and invasion abilities of SCC-25 cells. We found that treatment with 1 or 2 mM melatonin significantly decreased the number of colonies formed by the cells. Notably, no colonies formed in SCC-25 cells treated with 2 mM melatonin (Figure 2). Next, we performed wound healing assays of SCC-25 cells treated with different concentrations of melatonin for 24 or 48 h. We found that 4 mM melatonin significantly decreased the migration ability of SCC-25 cells (Figure 3). Consistently, Transwell migration and invasion assays revealed significant inhibition of cell migration after melatonin treatment (Figure 4). These findings suggest that melatonin inhibits the migration and

invasion of tongue squamous cell carcinoma cells in a concentration-dependent manner.

Effects of melatonin on SCC-25 cell survival. To assess the effects of melatonin on the survival of tongue squamous cell carcinoma cells, we performed flow cytometric analysis of cells treated with melatonin (2 or 4 mM for 24 h) using Annexin V-FITC and 7-AAD staining. Since 7-AAD does not penetrate intact cells, it does not stain early apoptotic or viable cells. In contrast, the increased membrane permeability of necrotic and late apoptotic cells allows 7-AAD to enter the cells and bind to nucleic acids, emitting red fluorescence. The viability of control cells grown in nutrient-deprived medium was greater than 90% (Figure 5), whereas that of melatonin-treated cells were 77.6% (2 mM) and 69.9% (4 mM), with a significant increase in the percentage of late apoptotic/necrotic cells (15% and 20%, respectively).

Melatonin induces autophagy in SCC-25 cells. Next, we assessed the effects of melatonin on autophagy induction in SCC-25 cells. To this end, we investigated the expression levels of the autophagic markers Beclin-1, LC3, cytoplasmic LC-3A (16 kDa), and pre-autophagosomal and autophagosomal membrane-bound LC-3B (14 kDa) in SCC-25 cells treated with different concentrations of melatonin. Treatment of SCC-25 cells with 4 or 6 mM melatonin significantly increased the levels of LC-3A, LC-3B, and Beclin-1 (Figure 6A).

Janus kinase (JAK)/signal transducer and activator of transcription (STAT), phosphatidylinositol-4,5-bisphosphate 3-kinase (PI3K)/protein kinase B (Akt), mitogen-activated protein kinase (MAPK)/extracellular signal-regulated kinases (ERK), and glycogen synthase kinase 3-beta (GSK3β) play critical roles in autophagy. Interestingly, we found that 6 mM melatonin treatment significantly decreased the phosphorylation levels of JAK/STAT, PI3K/Akt, MAPK/ERK, and GSK3β in SCC-25 cells. Similarly, melatonin treatment decreased the total levels of STAT3, JAK-2, and GSK3 (Figure 6B). These findings suggest that melatonin induces autophagy in tongue squamous cell carcinoma cells.

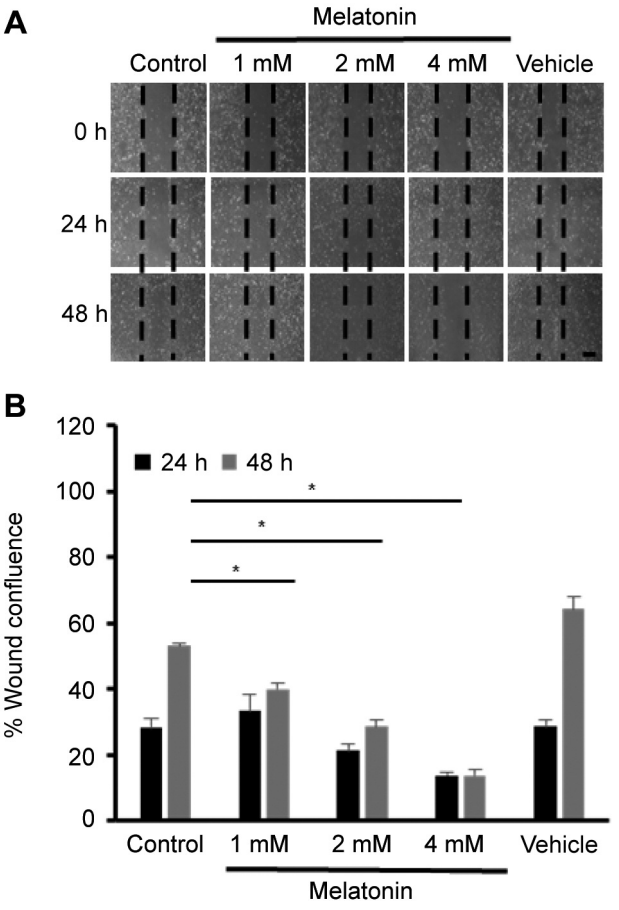

Figure 3. *Melatonin inhibits wound confluence in SCC-25 cells. The cells were treated with 0 (control) to 4 mM melatonin for 0, 24, and 48 h (the dotted lines indicate the width of wound closure in the 0 h group and are used as a comparison for the other groups); analysis of level of wound closure by cells following treatment with 0 (control), 1, 2, and 4 mM of melatonin at 24 and 48 h compared to that at 0 h in the control group. Representative images from three independent experiments are shown. *p<0.05 vs. control cells.*

Discussion

HNSCC is characterized by high rates of early recurrence and high malignancy. Despite recent advances in cancer diagnosis and treatment, the survival rates of patients with HNSCC remain low. In addition to the low efficacy and high toxicity of chemotherapy, the development of resistance to chemotherapeutic agents further limits the clinical success of chemotherapy. Therefore, the development of novel drugs with potent anticancer properties and reduced toxicity is urgently required to improve the prognosis of HNSCC.

Melatonin, an indoleamine hormone synthesized in the pineal gland, has recently been shown to exert anticancer effects with minimal side-effects (11-21). Yeh *et al*. recently reported the anticancer effects of melatonin in oral cancer cells (HSC-3, OECM-1) (22). Since metastatic disease is the leading cause of death in patients with HNSCC, we examined the effects of melatonin on the migration and invasion of human tongue squamous cell carcinoma cells. Notably, we found that melatonin significantly reduced the migration and invasion abilities of SCC-25 cells.

As apoptosis induction is the primary mechanism of action of most anticancer agents, we also assessed the effects of melatonin on apoptosis levels in SCC-25 cells. Although melatonin has been previously shown to induce apoptosis in colorectal cancer cells (23), we found no significant differences in apoptosis levels after melatonin treatment in

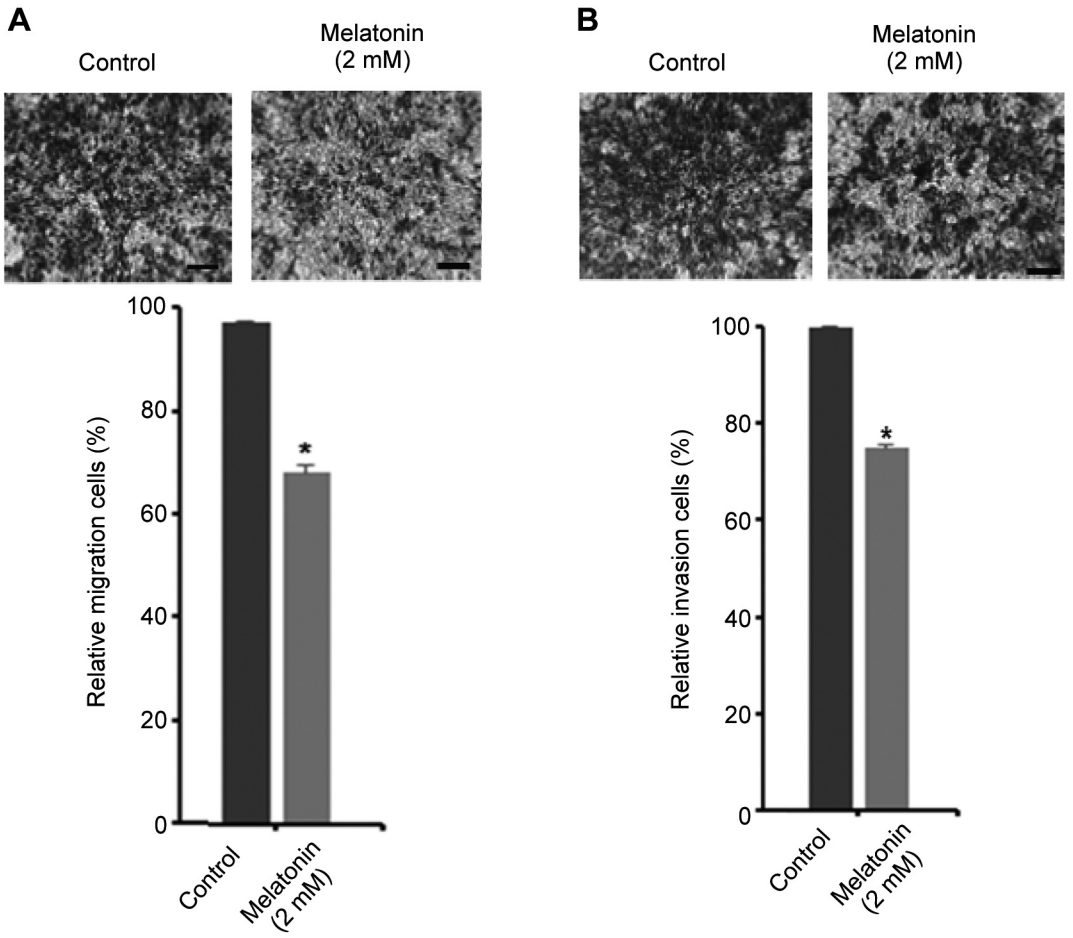

Figure 4. *Melatonin inhibits the migration and invasion of SCC-25. Analysis of the relative migration of cells after 24 h following treatment with 0 (control), 2, and 4 mM of melatonin, compared to those at 0 h in the untreated control group. (A) The migration of SCC-25 cells. (B) The invasion of SCC-25 cells. Representative images from three independent experiments are shown. *p<0.05 vs. control cells.*

SCC-25 cells, suggesting that melatonin may cause cell death via alternative mechanisms, such as autophagy or necroptosis.

Autophagy is an evolutionarily conserved cellular process involving the engulfment of cellular proteins and organelles by autophagosomes, followed by their degradation in lysosomes to maintain cellular homeostasis (24, 25). Autophagy is activated in response to different stresses in the tumor microenvironment (26). Autophagy has been demonstrated to act as an alternative programmed cell death mechanism that ultimately leads to tumor suppression (27, 28). Additionally, temozolomide has been shown to exert cytotoxic effects in apoptosis-resistant tumors by modulating autophagy (29). Hence, targeting autophagy may represent an attractive approach to eliminate apoptosis-resistant cancer cells. In this study, although melatonin did not significantly affect apoptosis of SCC-25 cells, it strongly induced autophagy, indicated by the increased levels of various autophagic markers.

We also found that melatonin treatment in SCC-25 cells suppressed pro-survival cascades by inhibiting the JAK2/STAT3, ERK/MAPK, and Akt pathways. ERK signaling is a critical pro-survival mechanism involved in malignant transformation and tumor progression (30). In HNSCC, the ERK pathway has been linked to lymph node metastasis, increased cancer cell proliferation, and tumor recurrence (31). The data presented here suggest that melatonin strongly suppresses ERK signaling activation. High levels of phospho-Akt have been linked to local recurrence in HNSCC (32), and Akt pathway activation has been associated with a poor prognosis in various cancer types (33). Upon phosphorylation by PI3K, Akt phosphorylates numerous adaptor proteins, transcription factors, cell-cycle regulators, and oncoproteins (34). Aberrant PI3K/Akt pathway activation has been reported in several cancer types, including HNSCC (35); thus, numerous PI3K/Akt pathway inhibitors have been tested as anticancer

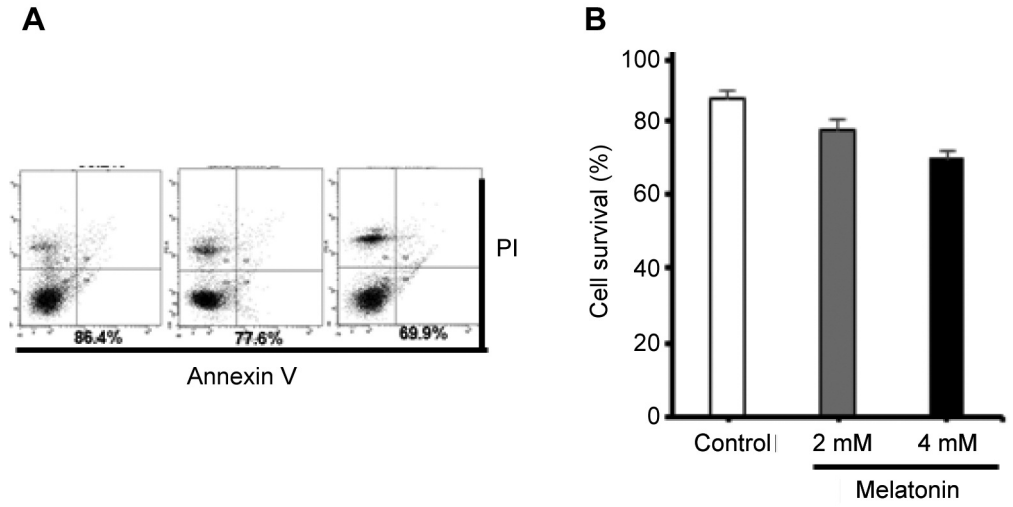

Figure 5. *Melatonin induces apoptosis in SCC-25 cells. (A) Representative images of Annexin V FITC and PI dual staining assays and quantification of dual negative cells in SCC-25 cells treated with melatonin (control, 2 mM, 4 mM) for 24 h. (B) Analysis of cell survival percentages following treatment for 24 h with 0, 2, and 4 mM of melatonin. Data are represented as mean±SD of at least three independent experiments. Representative images from three independent experiments are shown. *p<0.05 vs. control cells.*

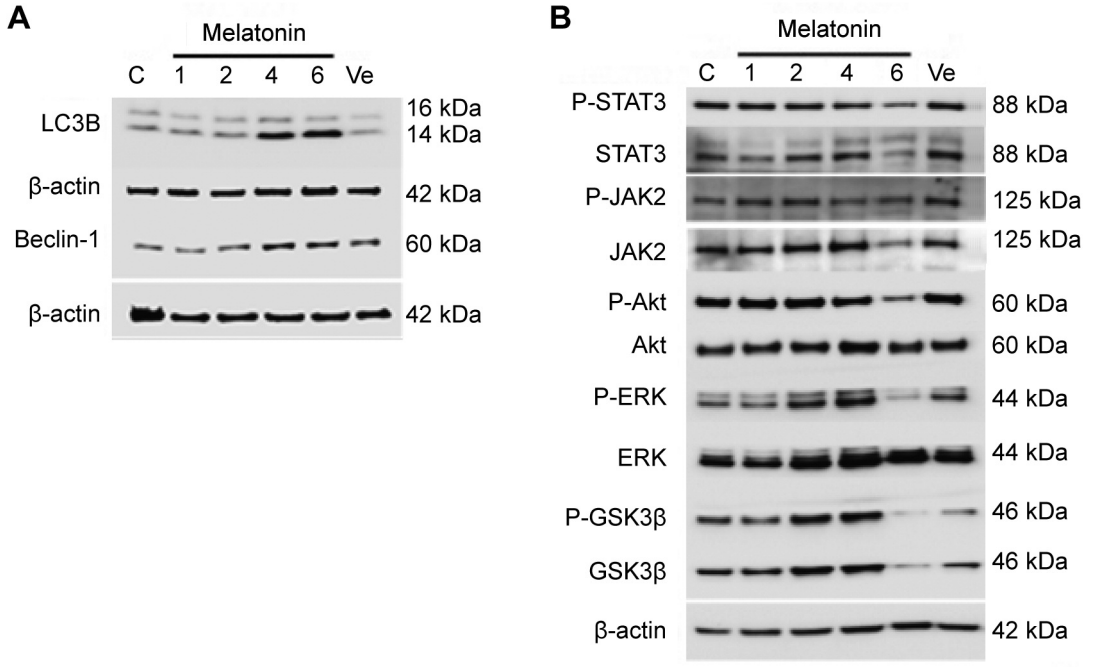

Figure 6. *Immunoblots of cell extracts from SCC-25 cells after treatment with indicated concentrations of melatonin for 24 h; β-actin was used as a loading control. (A) LC3B, Beclin-1 (B) p-JAK2, p-STAT3, p-Akt, p-ERK and p-GSK3β. Data are represented as mean±S.E.M of at least three independent experiments. p-Stat3: Phosphor-Stat3; p-JAK2: phospho-JAK2; p-Akt: phospho-Akt; p-ERK; phosphor-ERK; p-GSK3β: phospho-GSK3β.*

drugs in clinical trials (34). Notably, PI3K targeting has been shown to inhibit both Akt and Rac1/ERK pathways (36). GSK3β is a downstream effector of the Akt pathway (37), and melatonin has been recently demonstrated to inhibit GSK3β in breast cancer cells in a dose-dependent manner (38). JAK/STAT, PI3K/Akt, MAPK/ERK, and GSK3 pathways usually inhibit autophagy induction and cause cell survival in several cancers (39). However, Xu *et al.* published ERK/p-

ERK pathway activation of autophagy in colon cancer cells (40). In addition, we showed that Beclin 1, an important initiator of autophagy, and LC3 B, a central component of autophagosome formation demonstrated the effect of melatonin on autophagy induction in SCC-25 cells. There was a significant up-regulation of LC3 B as well as Beclin 1 in SCC-25 (Figure 6A). Since autophagy and Beclin 1 regulate stemness of breast cancer stem cells (CSCs) (41), Beclin1 is likely to be involved in the CSCs of oral squamous cell carcinoma. Because CSCs are responsible for the failure of anticancer therapy, regulating CSCs is crucial to promote the efficiency of anticancer therapy (42). Therefore, further investigation of the autophagic relationship between melatonin treatment and CSCs of oral squamous cell carcinoma should be necessary. Taken together, our findings suggest that melatonin affects the activation status of all of these pathways, thereby regulating the survival and autophagy of SCC-25 cells.

In this study, we confirmed the strong anticancer effects of melatonin in human tongue squamous cell carcinoma cells. Additionally, we identified JAK/STAT, Akt, ERK, and GSK3β signaling pathways mediating the effects of melatonin on the viability of tongue squamous cell carcinoma cells. Therefore, melatonin may represent a promising anticancer agent in tongue squamous cell carcinoma. Future studies are required to further dissect the molecular mechanisms underlying the anticancer effects of melatonin and identify novel therapeutic targets for human tongue squamous cell carcinoma.

In conclusion, we found that melatonin inhibited cell proliferation, colony formation, migration, and invasion in human tongue squamous cell carcinoma cells. We also found that melatonin affected cell viability by promoting autophagy. Melatonin may represent a promising therapeutic approach for tongue squamous cell carcinoma, exerting potent anticancer effects by inhibiting pro-survival signaling pathways.

Conflicts of Interest

The Authors declare that they have no competing interests.

Authors' Contributions

ESS, JCL, and WGA were responsible for the concept and design of this study. ESS, JUK, IWL, and SWC performed the experiments and procedures. ESS, JCL and WGA wrote the article. YTA and HBJ commented on the manuscript and interpreted data. JCL acquired funding for conducting the entire study. All Authors read and approved the final manuscript.

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