

Clinical significance of serum melatonin in predicting the severity of oral squamous cell carcinoma

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Abstract. Melatonin, the primary hormone produced by the pineal gland, is intensely assessed for its anticancer properties. This study aimed to reveal the clinical significance of serum melatonin levels in predicting the severity of oral squamous cell carcinoma (OSCC). For this purpose, 40 male patients with OSCC and 30 healthy subjects were enrolled in this study. The serum levels of melatonin were determined by ELISA. The results revealed that the melatonin concentrations

were significantly lower in the patients with OSCC compared with the controls (18.2 vs. 47.6 pg/ml, $P < 0.001$). In addition, the serum melatonin levels had a high predictive accuracy for discriminating patients with OSCC with T-depth of invasion (DOI) II from the healthy controls (89.1%), as well as in discriminating patients with OSCC with nodal metastasis from those without nodal metastasis (83.8%). On the whole, the findings of this study suggest that the serum melatonin concentrations are closely related to the severity of OSCC and may thus be used to assess the different stages of oral cancer objectively and accurately. The present study also supports the conclusion that melatonin may be a potential therapeutic agent for use in the treatment of patients with OSCC.

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Introduction

Oral cancer is one of the most aggressive, invasive and deforming diseases. Therapeutic treatment results in the functional impairment of vital functions of the human body, such as swallowing, chewing and speech. Human papilloma virus (HPV)-associated squamous cell carcinomas represent >90% of oral mucosa neoplasms (1,2). Considering the increased metastatic potential and the relatively unpredictable evolution of oral squamous cell carcinoma (OSCC), even following successful treatment (3), the discovery of novel and precise diagnostic tools with which to identify OSCCs

in the early stages, should be prioritized in order to increase the therapeutic potential and reduce disease progression and/or metastasis. The conventional treatment of OSCC includes surgery (excision of the primary tumor, associated with radical neck dissection for lymph node involvement, based on clinical and surgical findings), radiation therapy (external beam radiotherapy and/or brachytherapy), and coadjuvant therapy (chemotherapy with agents such as cisplatin, carboplatin, 5-fluorouracil, paclitaxel and docetaxel) (4). In oral mucosal lesions, novel therapeutic modalities with natural compounds can be used as a complementary therapy for standard treatment (5-7). Untreated oral cavity tumors develop both locally and distant severe complications, such as fistulas, increasing the risk of aspiration, as well as challenging the treatment of bacterial and fungal infections (8,9).

Previous studies have indicated that melatonin may have oncostatic properties by altering the angiogenic mechanisms or metastatic and proliferative properties of a number of cancer types, including OSCC (10-12). Melatonin (*N*-acetyl-5-methoxytryptamine) is an indolic compound primarily secreted by the pineal gland in response to darkness signals (13-15), although small amounts may be excreted by the retina, the gastrointestinal tract, the skin, the bone marrow (16) and the salivary glands (17). Isola and Lilliu (18) analyzed fragments of salivary glands obtained from male patients undergoing oral tumor resection surgery and demonstrated melatonin subcellular localization in the significant human salivary gland parenchyma (parotid, submandibular and sublingual). There is also evidence to indicate the beneficial and protective effects of melatonin, such as its antioxidant, anti-inflammatory and anti-microbial effects on the oral mucosa (10-12,19-21).

It was thus hypothesized that decreased circulating melatonin levels could be observed in patients with cancer of the oral cavity. The present study was thus carried out in order to identify the clinical significance of serum melatonin concentrations in predicting the severity of OSCC.

Patients and methods

Patients. A total of 40 male subjects with OSCC (aged 57 ± 7 years) undergoing surgery at the Coltea Clinical Hospital, Bucharest, Romania, between November, 2014 and March, 2015, were included in this study. Clinical data were collected from the patients' medical records. All patients had pathological confirmation for diagnostic assessment, according to the 8th Edition of the American Joint Cancer Committee (AJCC) Cancer Staging Manual (22), which provides a much more accurate and reasonable prediction of survival for newly diagnosed subjects with OSCC than the previous edition. This updated classification system has developed a modified staging system that integrates the depth of invasion (DOI) into the tumor size (T) category. Including DOI in the oral cavity better discriminates the higher risk small cancers, as demonstrated by deeply invasive tumors from those with less invasive cancers that have an excellent prognosis (23). For example, a patient with a tumor with a diameter of 2 cm and two positive lymph nodes, diagnosed with tonsil cancer at stage IV according to the 7th Edition, will be diagnosed with stage I disease according to the 8th Edition. New cut-offs for the size and extension of the tumor are 4 cm

for size and 10 mm for depth. According to these boundary lines, patients with OSCC were divided into two subgroups as follows: T-DOI I [small tumors with less invasive lesions ($T \leq 4$ cm; $DOI \leq 10$ mm)] and T-DOI II [large tumors with invasive depth ($T > 4$ cm; $DOI > 10$ mm)]. The histological grading of the tumors was performed according to the WHO Classification of Head and Neck Tumors (24).

In addition, 30 healthy male subjects (aged 56 ± 5 years) with no clinical evidence of ear, nose and throat disorders were randomly selected over the same time period. OSCC is more common among males than females, with a ratio ranging from 2:1 to 4:1 (22). Thus, only male subjects were included in this study (patients and control groups) to avoid inter sex-related variations (25,26).

All the procedures were performed, respecting the principles outlined in the Declaration of Helsinki and were approved by the Coltea Clinical Hospital Ethics Committee. Written informed consent was obtained from each patient and volunteer prior to sample collection.

Blood sample collection and processing. Due to the fact that melatonin is a molecule involved in the circadian and seasonal rhythm regulation of physiological functions, including blood pressure and sleep timing, blood collection was performed under the same conditions for both patients and controls: Cold season (melatonin levels are higher in the autumn and winter, when nights are longer than in the spring and summer) and early in the morning, at 7 a.m. Fasting blood specimens were collected prior to surgery by venipuncture into Vacuette[®] polyethylene terephthalate glycol clot activator tubes (Greiner Bio-One). Serum samples were obtained by clotting (30 min, room temperature) and centrifugation ($3,000 \times g$ for 10 min at 4°C), after which they were aliquoted into labeled cryo-vials and frozen at -80°C for a variable period of a maximum of 12 months.

Melatonin pre-purification by solid-phase extraction. Since melatonin levels recorded in the morning are low, its proper extraction from the blood is essential. In the serum samples, melatonin was extracted through C18 columns from R-Biopharm AG, with a recovery ranging from 87.5 to 94.8% for 10 to 200 pg melatonin/ml. The solid-phase extraction procedure consisted of the following steps: Column conditioning (1 ml of water and 1 ml of pure methanol), sample application [0.5 ml serum sample was passed through a C18 column, which was then washed with 0.5 ml of water and 2 ml of water-methanol (90:10, v/v)], elution of extract (melatonin was eluted from the column with pure methanol), evaporation and reconstitution of extract [the eluate was evaporated to dryness, stored at $2-8^\circ\text{C}$ until the following day (for up to 24 h), and subsequently resuspended in 0.15 ml water for the measurement of melatonin levels]. Melatonin pre-purification from serum samples was essential for a better sensitivity of the analysis method. The procedure described above allowed for the detection of melatonin with high sensitivity and without interference from other components in the sample.

Detection of serum melatonin by enzyme-linked immunosorbent assay (ELISA). Furthermore, the serum concentrations of melatonin were measured using a commercially available

Table I. Clinical and histopathological characteristics of patients with OSCC and healthy controls.

| Patient characteristic | Patients with OSCC | | Healthy controls | | P-value |
|--------------------------------|--------------------|------|------------------|------|---------|
| | n=40 | % | n=30 | % | |
| Mean age (years) | 57±7 | | 56±5 | | 0.130 |
| Risks factors | | | | | |
| Tobacco consumption | 38 | 95.0 | 4 | 13.3 | <0.001 |
| Alcohol consumption | 32 | 80.0 | 0 | 0 | <0.001 |
| Location of tumor | | | | | |
| Base of tongue | 18 | 45.0 | - | - | - |
| Tonsil | 15 | 37.5 | - | - | - |
| Tongue | 5 | 12.5 | - | - | - |
| Lip | 2 | 5.0 | - | - | - |
| Tumor differentiation grading | | | | | |
| G1 | 22 | 55.0 | - | - | - |
| G2 | 8 | 20.0 | - | - | - |
| G3 | 10 | 25.0 | - | - | - |
| Tumor size-depth of invasion | | | | | |
| T-DOI I (T ≤4 cm; DOI ≤10 mm) | 15 | 37.5 | - | - | - |
| T-DOI II (T >4 cm; DOI >10 mm) | 25 | 62.5 | - | - | - |
| Lymph node involvement | | | | | |
| Negative (N0) | 17 | 42.5 | - | - | - |
| Positive (N1 + N2 + N3) | 23 | 57.5 | - | - | - |
| Melatonin (pg/ml) ^a | 18.2 (11.0-39.2) | | 47.6 (37.7-66.4) | | <0.001 |

^aData are expressed as the median values and interquartile ranges (25-75%). OSCC, oral squamous cell carcinoma; T, tumor size; DOI, depth of invasion.

quantitative ELISA kit from DRG International, Inc. The lower limit of detection was 1.6 pg/ml. The sample contamination can cause falsely elevated concentrations. As melatonin is also present in saliva, protective measures were taken to prevent the contamination of the kit reagents while running the tests. The assay was performed in duplicate, following the manufacturer's recommendations.

Statistical analysis. Statistical analysis was performed using Statistica 8.0 software. Quantitative data are presented as the median [interquartile range (IQR) 25-75%], while qualitative data as numbers and percentages (%). Fisher's exact test was used for the nominal data. The distribution of all variables was verified with the Kolmogorov-Smirnov test. The non-parametric Kruskal-Wallis test was used to compare the distribution of continuous variables between different categories for independent samples (subgroups: T-DOI I vs. T-DOI II; N0 vs. N1 + N2 + N3 or G1 vs. G2 vs. G3). Pairwise comparisons with post hoc Bonferroni corrections were used with the Kruskal-Wallis test. The cut-off value of serum melatonin was calculated using the receiver operating characteristic (ROC) curve with its validity parameters (sensitivity and specificity). The area under the ROC curve (AUC) was used to assess the diagnostic performance of melatonin in OSCC. The total AUC is an overall summary of diagnostic accuracy as follows: An AUC >0.9 indicates an excellent diagnostic accuracy, an AUC

between 0.7 and 0.9 indicates a good diagnostic accuracy, an AUC between 0.5 and 0.7 indicates a poor diagnostic accuracy, and an AUC <0.5 indicates the lack of a diagnostic value of the biomarker. For all tests, a value of P<0.05 was considered to indicate a statistically significant difference.

Results

Melatonin concentration is lower in patients with OSCC than in healthy controls. The main clinical and histopathological characteristics of the study subjects are presented in Table I. No significant differences were observed between the ages of the patients and the controls (P=0.13), whereas a significant difference was noted between the risk factors in the two groups (P<0.001). As shown in Table I, 62.5% of patients were classified as T-DOI II, while the remaining patients were classified as T-DOI I. According to the tumor grading system, 55% of the patients had well-differentiated tumors (G1), 20% moderately-differentiated (G2) and 25% poorly-differentiated tumors (G3). The circulating levels of melatonin were significantly lower in the patients with OSCC than in the healthy controls (18.2 vs. 47.6 pg/ml, P<0.001). Furthermore, within the OSCC group (Table II), the melatonin concentrations were significantly lower in the T-DOI II subgroup than in the T-DOI I group (P=0.012) and were also lower in the positive lymph nodes subgroup than in the negative lymph

Table II. Association between melatonin and histopathological features in OSCC patients.

| Variable | T-DOI | | P-value | Nodal involvement | | P-value | Differentiation ^b | | |
|--------------------------------|---------------------|--------------------|---------|---------------------|--------------------|---------|------------------------------|--------------------|--------------------|
| | T-DOI I n=15 | T-DOI II n=25 | | No n=17 | Yes n=23 | | G1 n=22 | G2 n=8 | G3 n=10 |
| Melatonin (pg/ml) ^a | 36.8 (14.5-46.7) | 13.4 (9.4-28.9) | 0.012 | 37.9 (21.5-46.7) | 12.4 (9.4-22.3) | <0.001 | 21.4 (13.1-42.7) | 14.3 (9.0-30.0) | 18.5 (9.4-38.6) |

^aData are expressed as the median values and interquartile ranges (25-75%); ^bno statistical significance. OSCC, oral squamous cell carcinoma; T, tumor size; DOI, depth of invasion.

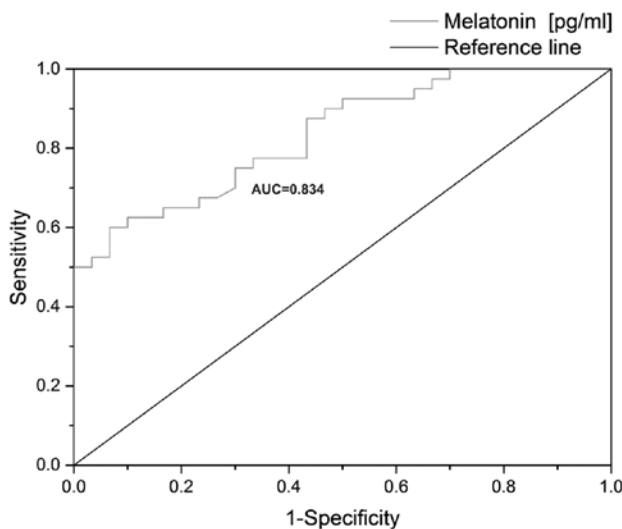


Figure 1. The receiver operating characteristic curve for discriminating patients with OSCC from healthy controls based on the serum melatonin concentrations. OSCC, oral squamous cell carcinoma.

node one ($P < 0.001$). No statistically significant differences were observed in the serum levels of melatonin between the G1, G2 or G3 subgroups of patients.

Melatonin concentration-based AUC may discriminate patients with OSCC from healthy controls. As shown in Fig. 1, the AUC value for discriminating patients with OSCC from healthy controls was high (0.834). The subjects with serum melatonin levels < 38.9 pg/ml had an increased risk of OSCC incidence (sensitivity, 75%; specificity, 76.6%).

Melatonin has the highest predictive accuracy for discriminating patients with OSCC with T-DOI II from healthy controls. The potential utility of melatonin for discriminating patients with OSCC with T-DOI II and T-DOI I from the healthy controls is presented in Fig. 2. It was found that the predictive accuracy of serum melatonin for discriminating subjects with T-DOI II from the healthy controls was higher than that for discriminating subjects with T-DOI I from the healthy controls (AUC = 0.891 vs. AUC = 0.738).

Melatonin concentration-based AUC may discriminate patients with nodal metastasis from those without nodal metastasis. As shown in Fig. 3, the predictive accuracy of serum

melatonin for discriminating subjects with nodal metastasis from those without nodal metastasis was high (AUC = 0.838).

Discussion

The main findings of the current study were the following: i) The serum melatonin concentration was significantly lower in the patients with OSCC than in the healthy controls; ii) the melatonin concentration-based AUC may discriminate patients with OSCC with T-DOI II from healthy controls; iii) the melatonin concentration-based AUC may distinguish patients with nodal metastasis from those without nodal metastasis.

Decreased circulating levels of melatonin have been associated with a high risk of breast (27,28), prostate (29), ovarian (30), or oral cancer (10-12,19,20,31-33). In line with these observations, the findings of this study demonstrated that the serum melatonin concentrations were significantly lower in patients with OSCC compared to the healthy controls (18.2 vs. 47.6 pg/ml, $P < 0.001$). Moreover, the median circulating melatonin concentrations in the T-DOI I (≤ 36.8 pg/ml) or negative lymph node (≤ 37.9 pg/ml) subgroups of patients with OSCC (Table II) were similar to those obtained in the study by Yang *et al* (27) in breast cancer patients (≤ 39.5 pg/ml).

The ROC curve is the most useful tool with which to evaluate the melatonin diagnostic utility in different diseases. The AUC can range from 0.5 to 1, with values close to 1 indicating a high discriminatory ability. In this study, the AUC (0.834) and cut-off level (38.9 pg/ml) for patients with OSCC were similar to those measured in previous studies for systemic lupus erythematosus (AUC = 0.710, cut-off = 18.51 pg/ml) (34) and breast cancer (AUC = 0.72672, cut-off = 39.5 pg/ml) (27).

The results of the present study demonstrated that the under expression of melatonin was related to large invasive tumors (T-DOI II). It was found that serum melatonin had the highest predictive accuracy for discriminating patients with OSCC with T-DOI II from healthy controls (AUC = 0.891) (Fig. 2B). In line with this study, several authors (10-12) have reported an inverse association between melatonin and the clinicopathological features of OSCC.

Local or distant metastases are aggressive hallmarks of OSCC. As regards metastatic dissemination to regional lymph nodes, in this study, melatonin was inversely associated with the presence of lymph node metastases in patients with OSCC (Table II). Therefore, the potential utility of melatonin for predicting the presence or absence of lymph node metastasis was analyzed using the ROC curve. The results

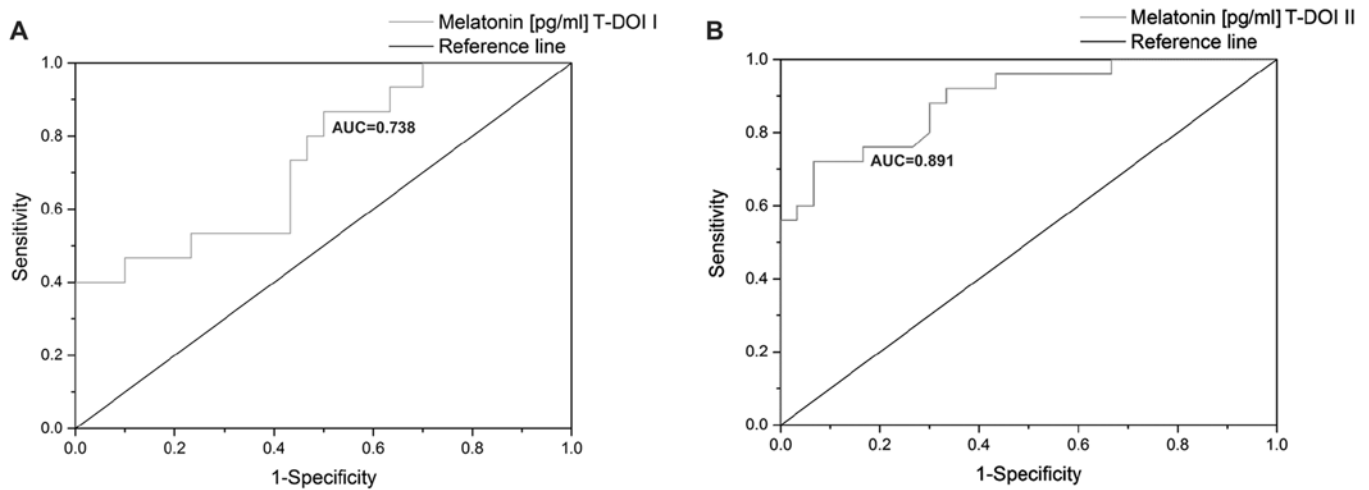


Figure 2. The receiver operating characteristic curve for discriminating patients with OSCC with (A) T-DOI I and (B) T-DOI II from healthy controls based on serum melatonin concentration. OSCC, oral squamous cell carcinoma; T, tumor size; DOI, depth of invasion.

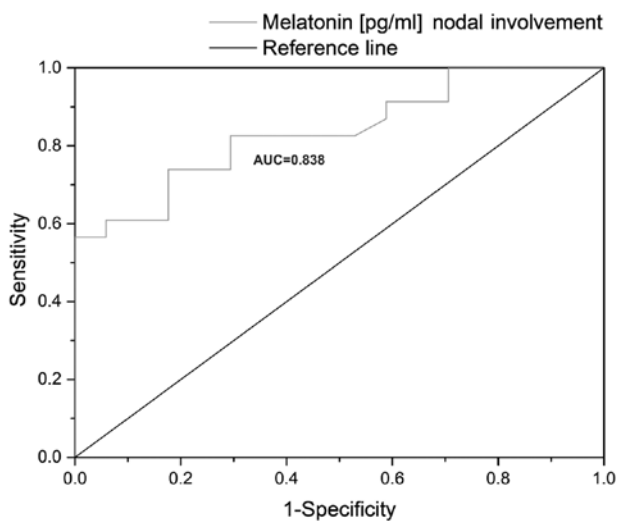


Figure 3. The receiver operating characteristic curve for discriminating patients with OSCC with nodal metastasis from patients without nodal metastasis based on serum melatonin concentration. OSCC, oral squamous cell carcinoma.

revealed that the melatonin concentration-based AUC (0.838) may discriminate patients with nodal metastasis from those without nodal metastasis.

These findings highlight the importance of the human endocrine system in the progression of OSCC (T-DOI II vs. T-DOI I and positive lymph-nodes vs. negative lymph-nodes). In addition, the findings of the present study are in line with those of other studies that depict the high importance of determining the melatonin serum concentration as a robust and reliable biochemical marker in the prevention, diagnosis and evolution assessment of different physiological or pathological conditions (35-38). The results are based on an individual proficient and high-fidelity profile that is accurately modified on a daily circadian base, due to normal activities and light-dark cycles, modulating almost entirely the endocrine human environment. Moreover, melatonin may be a marker of physiological aging, pregnancy, oxidative stress imbalances, or a clinically relevant consequence of a series of diseases (35,39,40).

The main limitation of this study is related to the small number of patients included. However, despite the small sample size, a homogenous population was used, represented only by males in both groups. The samples were collected in the same season (winter), and at the same time in the morning, to discard an artefactual effect.

In conclusion, the findings of this study suggest that serum melatonin concentrations are closely related to the severity of OSCC and may be used to assess different stages of oral cancer progression objectively and accurately. The present study indicates that melatonin may be a predictive biomarker for OSCC metastasis and a potential therapeutic agent for OSCC prevention-progression. The results of the current study however, need to be reinforced by further studies using larger study populations.

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Availability of data and materials

All data generated or analyzed during this study are included within the manuscript.

Authors' contributions

The manuscript was written through contributions of all authors. All authors have given approval to the final version of the manuscript. AES, AZCA and DCG conceived and planned the experiments. AZCA and DCG collected data. Experiments were performed by AES. AES and MMS performed statistical analysis of the results. AES, AZCA, MMS, APS, VJ, CN, AB, TSP, ALA, CMD, RH, ACN, MG, DCG, DAS, AT, MP and ND contributed to the interpretation of the results. AES took the lead in writing the manuscript. AES, AZCA, MMS, APS,

VJ, CN, AB, TSP, ALA, CMD, RH, ACN, MG, DCG, DAS, AT, MP and ND provided critical feedback and helped shape the research, analysis, and manuscript. All authors have read and approved the final manuscript.

Ethics approval and consent to participate

The study conformed to the principles outlined in the Declaration of Helsinki and was approved by the Coltea Clinical Hospital ethics committee. Enrolled patients and volunteers signed an informed consent.

Patient consent for publication

Not applicable.

Competing interests

DAS is the Editor-in-Chief for the journal, but had no personal involvement in the reviewing process, or any influence in terms of adjudicating on the final decision, for this article. All the other authors declare that they have no competing interests.

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