



# Melatonin effect on hypoxia inducible factor-1 $\alpha$ and clinical response in patients with oral squamous cell carcinoma receiving neoadjuvant chemotherapy: A randomized controlled trial

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Submitted: 15-Aug-2020

Revised: 21-Dec-2020

Accepted: 06-Jan-2021

Published: 07-May-2021

## Abstract:

**CONTEXT:** Chemoresistance is a major issue in patients with locally advanced oral squamous cell carcinoma (OSCC). In this study, we evaluated the effectiveness of melatonin in conjunction with neoadjuvant chemotherapy (NC) on hypoxia-inducible factor-1 $\alpha$  (HIF-1 $\alpha$ ) expression and clinical response in locally advanced OSCC patients.

**AIMS:** To study the effects of melatonin on HIF-1 $\alpha$  expression and its effect on the clinical response of patients with locally advanced OSCC.

**SETTINGS AND DESIGN:** A randomized controlled trial was conducted, wherein patients were recruited from several hospitals in Jakarta, Indonesia. Patients were randomized into two groups using computerized block randomization.

**SUBJECTS AND METHODS:** Both groups were given NC, with treatment group receiving melatonin. Outcomes measured in this study were HIF-1 $\alpha$  expression from tissue samples and clinical response based on the RECIST 1.1 criteria. Twenty-five patients completed the study protocol and were included in the data analysis.

**STATISTICAL ANALYSIS USED:** Shapiro–Wilk test was used to test the data normality. For data with normal distribution, we conducted an independent *t*-test to compare between the two groups. Data with abnormal distribution were analyzed using Mann–Whitney U-test. The mean difference between the two groups was analyzed using Shapiro–Wilk normality test.

**RESULTS:** Our study showed a significant decrease in HIF-1 $\alpha$  expression in the melatonin group compared to the placebo group ( $P < 0.05$ , relative risk 3.08). However, the degree of reduction of HIF-1 $\alpha$  expression in the melatonin group did not differ significantly ( $P = 0.301$ ).

**CONCLUSIONS:** Our study showed that melatonin administered at 20 mg/day could reduce the expression of HIF-1 $\alpha$  and residual tumor percentage, but did not affect the clinical response in OSCC patients.

## Keywords:

Chemoresistance, hypoxia-inducible factor-1 $\alpha$ , melatonin, oral squamous cell carcinoma

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**How to cite this article:** Kartini D, Taher A, Panigoro SS, Setiabudy R, Jusman SW, Haryana SM, *et al.* Melatonin effect on hypoxia inducible factor-1 $\alpha$  and clinical response in patients with oral squamous cell carcinoma receiving neoadjuvant chemotherapy: A randomized controlled trial. J Carcinog 2021;20:5.

## Introduction

Head-and-neck squamous cell carcinoma (HN-SCC) is currently the sixth most common cancer in the world.<sup>[1]</sup> Recent data have shown that there are 500,000 cases of HN-SCC with 40,000 new cases each year in the United States alone.<sup>[2]</sup> In Japan, cases of oral SCC (OSCC), which is a part of HN-SCC, have increased from 2100 cases in 1975 to 6900 cases in 2000.<sup>[3]</sup>

Management of HN-SCC, especially OSCC, presents specific challenges due to the requirement of protecting important anatomical structures that are prone to be damaged by tumor infiltration and cancer therapy.<sup>[4,5]</sup>

Platinum-based neoadjuvant chemotherapy (NC) has been proven to cause tumor shrinkage and to improve tumor resectability.<sup>[6]</sup> A retrospective study by Patil *et al.* in 2013 showed that 68% of the patients with locally advanced oral cavity cancer who received three NC regimens of NC (platinum + 5-FU [PF] + docetaxel)/TPF achieved significant resectability, compared to 37% in the group treated with two regimens (platinum + taxane) ( $P=0.029$ ).<sup>[7]</sup> The use of the TPF regimen as NC has been also supported by a meta-analysis by Blanchard *et al.* in 2013, which showed that the TPF regimen was not only better in increasing local control but also in increasing overall survival compared to using PF.<sup>[8]</sup>

In addition to the chemotherapy regimen, age, tumor location, and stage, chemoresistance is also an issue in locally advanced OSCC. Chemoresistance is a complex process with three possible causes: drug-target interaction, intrinsic tumor cell defense, and conditions affecting tumor cell survival.<sup>[9]</sup> Hypoxic stress is a microenvironment condition that causes chemoresistance. In hypoxic tissues, a poor vascular system causes an inefficient drug delivery that further causes decrement of drug concentration in the target tissue.<sup>[10]</sup>

Hypoxia-inducible factor (HIF), especially HIF-1 $\alpha$ , is a transcription factor that plays an important role in cancer development during hypoxic conditions and whose activity is highly influenced by oxygen. Under normoxic conditions, HIF-1 $\alpha$  is degraded by the enzyme prolyl hydroxylase. In contrast, if hypoxia occurs, HIF-1 $\alpha$  will be dimerized with HIF-1  $\beta$  to form HIF-1. HIF-1 will then activate several genes, some of which encode the vascular endothelial growth factor (VEGF), and a few other enzymes that metabolize glucose, iron, and nucleosides.<sup>[11]</sup> Therefore, increased HIF-1 $\alpha$  activity will affect angiogenesis, maintenance of cancer stem cells (CSCs), tumor invasion, metastasis, and resistance to radiotherapy and chemotherapy.<sup>[12]</sup> The connection between HIF-1 $\alpha$  and hypoxia has prompted this study,

which investigated whether melatonin supplementation, considered a potent antioxidant, was able to decrease gene expression of HIF-1 $\alpha$ .

Melatonin (5-methoxy-N-acetyltryptamine) is naturally produced and secreted by the pineal gland. This hormone is different from other hormones due to its ability to influence the activity of almost every cell type without having a specific target organ.<sup>[13,14]</sup> Its mechanism of action begins with the activation of its receptor proteins, namely MT1 and MT2. In general, activation of these two receptors will stimulate the nervous system, causes vasoconstriction of arteries, inhibition of cancer cell proliferation, stimulation of the immune system, as well as assistance in metabolic and reproductive functions. One of the effects of melatonin is the inhibition of cancer cell proliferation through the MT1 receptor. Therefore, melatonin possesses an oncostatic property.<sup>[15]</sup>

In a previous study, the administration of melatonin resulted in reduced proliferation, state of stemness, and invasiveness in human ovarian CSCs.<sup>[16]</sup> Melatonin also demonstrated its anti-metastatic property by halting cancer cell migration and invasion of triple-negative breast cancer cell, which is an aggressive subtype of breast cancer that is associated with high metastatic rates and poorer prognosis.<sup>[17]</sup> Besides its anti-metastatic effect, an *in vitro* study by Cheng *et al.*, showed that melatonin also served as an anti-angiogenic agent through the downregulation of the hypoxia/HIF-1 $\alpha$ /ROS/VEGF pathway.<sup>[18]</sup> In addition, melatonin administration was shown to significantly reduce tumor volume by almost 70%.<sup>[19]</sup>

Considering the effectiveness of melatonin in cancer, it has been deemed worthwhile to evaluate the effects of melatonin on advanced-stage cancer where chemotherapy is the treatment of choice and chemoresistance is a challenge. Previous studies related to melatonin supplementation mostly involved patients with solid cancer.<sup>[20-24]</sup> Therefore, this study formulated a well design method with a specific population to obtain a solid result as a pilot study. Furthermore, we tried to evaluate the effect of melatonin in hypoxic condition not only in the clinical level but also in the molecular level to assess causality. With these considerations, we decided to evaluate melatonin's effects in the reduction of tissue HIF-1 $\alpha$  expression and improvement of the clinical response to chemotherapy.

## Subjects and Methods

### Study design

We conducted a double-blind, parallel randomized, and placebo-controlled trial. The trial was registered as a clinical trial study in [www.clinicaltrial.gov](http://www.clinicaltrial.gov) on

October 24, 2019, under registration identification number NCT04137627. This study was reported in accordance with the CONSORT guidelines for clinical trials.

## Subjects

The study was performed from June 2017 to July 2018 in the Surgical Oncology Clinic of CMN General Hospital, PSH General Hospital, and DCA Hospital, Jakarta, Indonesia. Subjects of this study were patients with stage IVA and IVB OSCC diagnosed by using histopathological examination and with no history of definitive surgery or chemotherapy. To be eligible for inclusion in this study, patients must have been scheduled to receive NC and a Karnofsky score of  $\geq 50$ . The exclusion criteria of this study are those patients who did not meet the criteria for receiving chemotherapy (including poor general conditions and treatment refusal) and any metastasis. Informed consent was obtained from each patient included in this study. At the beginning of the study, 62 subjects were assessed for eligibility. A total of twelve patients were excluded due to poor general conditions,<sup>[7]</sup> treatment refusals,<sup>[3]</sup> and metastasis.<sup>[2]</sup> The remaining 50 patients were randomized into intervention and control groups. Hence, each group contained of 25 patients. In the intervention group, 13 patients were lost to follow-up, whereas 12 were lost to follow-up from the control group. Ultimately, 25 patients completed the study protocol and were included in the data analysis. The recruitment flow of study participants is shown in Figure 1 of the referenced study.<sup>[25]</sup>

## Intervention

The intervention group received capsules containing 20 mg of melatonin + NC, while the control group received capsules containing placebo + NC.<sup>[26]</sup> The NC regimen that was administered consisted of docetaxel of 75 mg/m<sup>2</sup> + cisplatin 80–100 mg/m<sup>2</sup> or carboplatin + 5-FU 1000 mg/m<sup>2</sup>. Docetaxel was given on the 1<sup>st</sup> day of the chemotherapy session with cisplatin/carboplatin, whereas 5-FU was also given on the 1<sup>st</sup> day and continued until the fifth day. We administered a dose of 80 mg/m<sup>2</sup> of cisplatin if the subject had a Karnofsky score of 50–70 and 100 mg/m<sup>2</sup> for Karnofsky scores above 70. Chemotherapy was administered every 3 weeks. We performed blood tests soon after every session of chemotherapy and 3–7 days before the next cycle to assess the patient's eligibility for chemotherapy. If the results from the blood tests did not meet the criteria for chemotherapy, the patient was admitted to improve clinical conditions. Melatonin or placebo capsules were consumed at night, 7 days before NC initiation, until the third cycle of chemotherapy was completed. Melatonin or placebo was administered even if the patient was admitted for improvement in the clinical conditions.

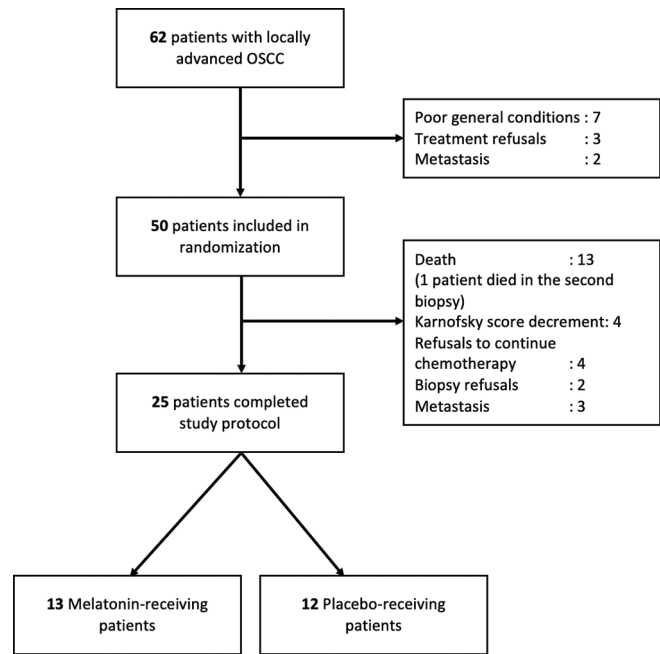


Figure 1: The recruitment flow diagram of subjects

A research assistant assessed the patient's compliance by contacting the patient twice per week.

## Outcomes

The three outcomes evaluated in this study were HIF-1 $\alpha$  expression, clinical response, and residual tumor percentage. All outcomes were compared before and after the treatment. For tissue examination, we performed incisional biopsy for pretreatment and either incisional biopsy or surgery for posttreatment. HIF-1 $\alpha$  expression was measured in tissue samples in the form of messenger RNA (mRNA) concentration. First, we made a primer for HIF-1 $\alpha$  using the Primer Quest Tool IDT (Integrated DNA Technologies in Singapore) with genetic sequence information provided by the National Centre for Biotechnology Information (HIF-1 $\alpha$  with accession number NM\_001530.3). Then, we measured HIF-1 $\alpha$  gene expression using quantitative real-time polymerase chain reaction absolute quantification based on the standard curve of the template (gene fragment) amplification result with known various concentrations. Gene fragment was obtained by designing gBlocks gene fragments (from IDT in Coralville, Iowa, USA) with various concentrations, starting from 100 ng/mL, which contains the gene fragment sequence of HIF-1 $\alpha$  (sized <150 bp). The selection of sequence and gBlock synthesis was performed using the gBlocks Gene Fragments IDT. Clinical response outcomes from chemotherapy were evaluated using the RECIST 1.1 criteria. These outcomes, namely complete response (CR), partial response (PR), stable disease (SD), and progressive disease (PD), were categorized into positive (CR and PR) and negative (SD and PD) responses for the purpose of our analysis. We

assessed the response and residual tumor percentage by comparing tumor diameter and lymph node involvement from magnetic resonance imaging results obtained before and after treatment. Besides, the adverse events of melatonin administration were recorded in this study.

### Sample size

We calculated sample size using the formula for two independent variables with the continuous outcome for superiority trial design. With this formula, we required a minimum of 42 subjects to fulfil the objectives of this study, assuming a 5% level of significance and 80% power. Assuming a 10% dropout rate, a total of 46 patients denoted the minimum sample size required for this study. Consecutive sampling with randomization was used for the sampling method.

### Randomization and blinding

Research subject allocation was randomized by a third party using computerized block randomization with concealment by ascribing serial numbers to drug preparations. The authors, who also acted as care providers and outcome assessors, as well as the subjects, were blinded.

### Statistical method

Statistical analysis was performed using IBM SPSS Statistics for Windows, Version 20.0. Armonk, NY: IBM Corp. We used the Shapiro–Wilk test to test data normality. Data with normal distribution is presented in the form of mean  $\pm$  standard deviation, while data with abnormal distribution is presented as median, minimum, and maximum values. For data with normal distribution, we used an independent *t*-test to compare between two groups. Data with abnormal distribution were analyzed using the Mann–Whitney U test. The mean difference between the two groups was analyzed using the Shapiro–Wilk normality test. A *P* < 0.05 was considered as statistically significant.

### Ethics

The authors declare that all procedures in this study have been approved by The Ethics Committee of the Faculty of Medicine, UNI under registration number 1071/UN2.F1/ETIK/2018. Informed consents from patients to participate in the study have been obtained in written forms.

## Results

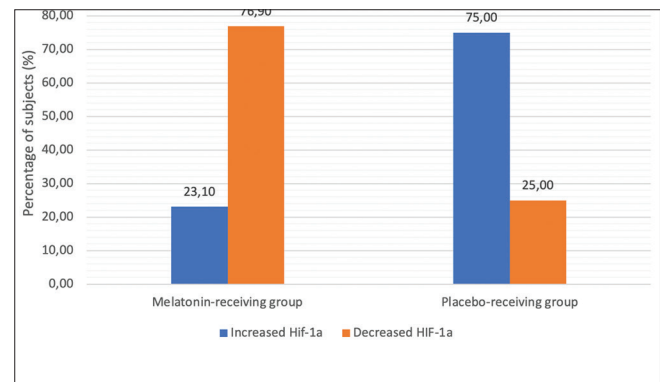
### Baseline characteristics of study subjects

From the 50 patients enrolled at the beginning of the study, only 25 patients successfully completed the study protocol. This was due to: (i) Decrement of Karnofsky scores (*n* = 4), refusal of patients to continue the chemotherapy session or biopsy (*n* = 6), events of

metastasis (*n* = 3), and deaths of the patients (*n* = 12). Characteristics of the 25 patients who completed the study are described in Table 1.

### Effect of melatonin on hypoxia-inducible factor-1 $\alpha$ expression

Melatonin administration decreased gene expression of HIF-1 $\alpha$  in 10 out of 13 patients in the intervention group compared to 3 out of 12 in the control group. This finding was statistically significant



**Figure 2:** Percentage of subjects that showed change in hypoxia inducible factor-1 $\alpha$  expression in melatonin-receiving group (13 subjects) and placebo-receiving group (12 subjects)

**Table 1: Characteristics of patients who completed the study protocols for melatonin-receiving group and placebo-receiving group**

Characteristics	Melatonin, <i>n</i> (%)	Placebo, <i>n</i> (%)
Age (years)		
Range	32-69	33-68
Mean $\pm$ SD	51.38 $\pm$ 13.01	48.92 $\pm$ 9.99
Sex		
Male	8 (61.5)	7 (58.3)
Female	5 (38.5)	5 (41.7)
Tumor location		
Tongue	10 (76.9)	9 (75.0)
Buccal	1 (7.7)	1 (8.3)
Palatum	0 (0)	1 (8.3)
Mandibular	1 (7.7)	1 (8.3)
Gingiva	1 (7.7)	0 (0)
Stage		
IV A	12 (92.3)	10 (83.3)
IV B	1 (7.7)	2 (16.7)
Keratin/nonkeratin		
Keratinized	12 (92.3)	6 (50.0)
Nonkeratinized	1 (7.7)	6 (50.0)
Differentiation		
Well differentiated	5 (23.1)	4 (33.3)
Moderately differentiated	5 (38.5)	3 (25.0)
Poorly differentiated	3 (38.5)	5 (41.7)
Grade		
High	6 (46.2)	11 (91.7)
Low	7 (53.8)	1 (8.3)

SD: Standard deviation



( $P = 0.009$ ; relative risk = 3.08) [Figure 2]. However, there was no significant difference in the degree of reduction or delta reduction of HIF-1 $\alpha$  gene expressions between the two groups before and after the administration of melatonin and placebo [Table 2].

### Relationship between hypoxia-inducible factor-1 $\alpha$ gene expression and residual tumor percentage

Table 3 shows the decrements of HIF-1 $\alpha$  gene expressions found in the intervention group; the decrement was not directly proportional to the reduction of residual tumor percentage after NC. As shown in Table 3, the majority of patients receiving melatonin showed HIF-1 $\alpha$  decrement (in 10 out of 13 patients).

### Effect of melatonin on clinical response to neoadjuvant chemotherapy

The results from our analysis that evaluated the effect of melatonin on the positive clinical response after receiving NC showed that there was no significant difference between the two groups [Table 4]. However, the evaluated residual tumor percentage was 21.4% lower in the melatonin-receiving group compared to that in the placebo-receiving group [Figure 3].

**Table 2: Expression of hypoxia inducible factor -1 $\alpha$  before and after melatonin and placebo administration**

Tissue HIF-1 $\alpha$	Median (range)		<i>P</i>
	Melatonin ( <i>n</i> =13)	Placebo ( <i>n</i> =12)	
Before	0.018 (0.0030-0.142)	0.0048 (0.0005-0.254)	0.301
After	0.012 (0.0014-0.139)	0.0087 (0.0004-0.060)	
Change	-0.008	0.0027	
After-before	-0.131 $\rightarrow$ +0.085	-0.238 $\rightarrow$ +0.053	

HIF: Hypoxia-inducible factor

**Table 3: Effect of melatonin on hypoxia-inducible factor -1 $\alpha$  gene expression and residual tumor percentage**

Variable Tissue HIF-1 $\alpha$	Melatonin ( <i>n</i> =13)	Percentage of residual tumor	<i>P</i>
Decreased	10	94.77	0.128
Increased	3	41.47	

HIF: Hypoxia-inducible factor

**Table 4: Comparison of chemotherapy response in melatonin-receiving group and placebo-receiving group**

Group	Response		Total, <i>n</i> (%)	RR (95% CI)
	Positive, <i>n</i> (%)	Negative, <i>n</i> (%)		
Melatonin-receiving group	5 (50)	8 (53.3)	13 (52)	0.923 (0.35-2.41)
Placebo-receiving group	5 (50)	7 (46.7)	12 (48)	
Total	10 (100)	15 (100)	25 (100)	

RR: Risk ratio, CI: Confidence interval

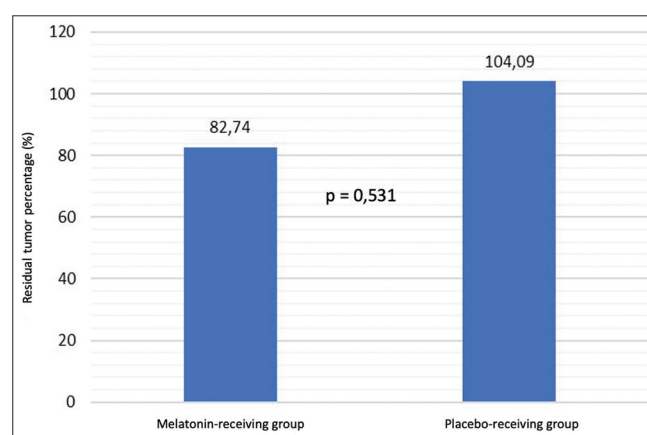
### Adverse events of melatonin

Adverse events observed in this study included sleep disorders, fatigue, headache, etc. [Table 5]. The most common adverse event is drowsiness in 4 out of 13 patients. Patients with adverse events were administered with symptomatic therapy without any effects on chemotherapy administration.

### Discussion

Although there was no significant difference in the general baseline characteristics between the two groups, none could influence the results of the study. Clavel *et al.* described pathological parameters, including tumor differentiation, as prognostic factors of chemotherapeutic response. Their study showed that chemotherapeutic response and 2-year survival were better in nonkeratinizing tumors. It was also shown that in rapidly proliferating tumors, recurrence occurred in 76% of the cases compared to slow-proliferating tumors where only 36.4% recurred.<sup>[27]</sup> In the present study, we found no significant relationship between SCC grading and chemotherapeutic response.

Our results show that melatonin administration reduces HIF-1 $\alpha$  gene expression compared to that in the control group. This finding is in line with the findings of the study conducted by Goncalves *et al.* on cell lines in 2014. The study evaluated the effect of melatonin administration on two different cell lines that originated from SCC of the tongue in patients aged 25 years old (SCC9) and 75 years old (SCC 25). The study showed that in SCC9,



**Figure 3: Comparison of clinical response based on the residual tumor percentage**

**Table 5: Adverse events in melatonin-receiving group and placebo-receiving group**

Adverse events	Melatonin (n)	Placebo (n)
Sleep pattern		
No	11/13	10/12
Yes	2/13	2/12
Fatigue		
No	11/13	9/12
Yes	2/13	3/12
Headache		
No	10/13	11/12
Yes	3/13	1/12
Decreased alertness		
No	11/13	12/12
Yes	2/13	0
Drowsiness		
No	9/13	9/12
Yes	4/13	3/12
Emotional changes		
No	11/13	9/12
Yes	2/13	3/12
Others		
No	12/13	12/12
Yes	1/13	0

administration of 1 mM melatonin in hypoxic tissues decreased HIF-1 $\alpha$  gene expression compared to that in the control group ( $P < 0.001$ ).<sup>[28]</sup> Moreover, other studies conducted in prostate cancer, colon cancer, and glioblastoma at the cellular levels showed similar results.<sup>[29-31]</sup>

The current study revealed that the decrement degree in HIF-1 $\alpha$  gene expression was not significantly different between the two groups and the decrease in HIF-1 $\alpha$  expression was not directly proportional to the decrease in residual tumor percentage after chemotherapy. This might have been caused by the presence of confounding factors due to the *in vivo* nature of this study. Other studies were conducted *in vitro*, where the heterogeneity of the cell culture could be tightly controlled. However, *in vitro* studies also have limitations where the translation to a clinical phenotype is often difficult.<sup>[32]</sup> Moreover, a study by Park in 2009, which evaluated the melatonin effect on several different phases of HIF-1 $\alpha$  formation, showed a significant decrease in HIF-1 $\alpha$  expression at the protein level. However, HIF-1 $\alpha$  expression did not change at the mRNA level. This may indicate that melatonin affects HIF-1 $\alpha$  gene expression in posttranslational regulation.<sup>[33]</sup> In the present study, HIF-1 $\alpha$  expression was only evaluated at the mRNA level, therefore further studies are necessary to validate the action of melatonin in reducing HIF-1 $\alpha$  expression, especially at the protein levels.

Unlike previously published studies on various types of solid cancer, the results from our study showed no

significant difference in chemotherapeutic response. A systematic review by Seely *et al.* evaluating the melatonin effect on the chemotherapeutic response found positive results whereby melatonin administration could increase CR and PR up to 2.33 (95% confidence interval [CI] = 1.29–4.20) and 1.90 (95% CI = 1.43–2.51), respectively.<sup>[34]</sup> Similar results were also found in a systematic review reported by Block *et al.* in 2007. Block also reported limitations in the studies, such as the difference in the proportion of the patients with PD in the two trial groups which could affect the results.<sup>[35]</sup> Moreover, the review included studies of various types of solid cancer and several included papers were published by the same group, hence could lead to biased conclusions.<sup>[34,35]</sup>

The differences seen in the results of our study compared to the two aforementioned systematic reviews could be due to the heterogeneity of the tumors. Tumor heterogeneity is caused by different molecular properties of the various cell types that constitute a tumor, which may lead to varying sensitivity to treatment. This heterogeneity could be caused by the variability in cell properties in one or various locations of the tumor (spatial heterogeneity) or time (temporal heterogeneity) where the molecular makeup of a single lesion may vary over time. Results from these evaluations would shift the paradigm of cancer therapy to one that could become more personalized and genotype-guided.<sup>[36]</sup>

Despite the results showing no significant difference in the chemotherapeutic response between the two groups, our study revealed that the percentage of residual tumor reduction after chemotherapy was 21.4% greater in the melatonin-receiving group compared to that in the control. This finding may assist clinicians in progressing to the next therapeutic phase, where clinicians might consider to add melatonin as a supplementation therapy to obtain their effects such as the reduction in residual tumor percentage. Hence, it is hoped that operating field of the tumor becomes easier to handle.

There were adverse events of melatonin administration shown in Table 5, where drowsiness is the most common adverse event (4/13). Melatonin is able to decrease the pain threshold, being an antidepressant, anxiolytic, and regulate locomotor activity.<sup>[37]</sup> Furthermore, the dosage used in this study, 20 mg/day, was in line with a previous study stating that there was not any significant toxicity effect.<sup>[38]</sup>

Nevertheless, some of the limitations of this study must be stated. Owing to intratumoral heterogeneity, incisional biopsy is unlikely to provide an accurate result that represents the entire lesion. This constitutes a limitation of this study. In this study, we minimized bias by documenting pretreatment biopsy location as a guide

for posttreatment biopsy location so that the pre- and post-treatment tissue examined were from similar locations. In addition, we acknowledge that only half of the recruited samples were used in the analysis. This high dropout rate might lead to a significantly smaller sample size and results in a lower statistical power.

## Conclusion

Melatonin administration of 20 mg/day can lower HIF-1 $\alpha$  expression ( $P < 0.05$ , RR 3.08). However, there was no significant difference in the degree of reduction of HIF-1 $\alpha$  gene expression between the two groups. Patients who received melatonins showed residual tumor reductions 21.4% greater than that of the placebo-receiving group. This should be taken into consideration by clinicians when deciding on supplemental therapeutic options for their patients.

## Financial support and sponsorship

Nil.

## Conflicts of interest

There are no conflicts of interest.

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