Study Protocol

Official Title : Melatonin Effect in Combination with Neoadjuvant Chemotherapy to Clinical Response in Locally Advanced Oral Squamous Cell Carcinoma

Unique ID : MLTOSCC

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1. Objectives

a. General Objectives

- To determine the role of melatonin on locally advanced oral squamous cell carcinoma to neoadjuvant chemotherapy through its oncostatic and antioxidant properties

b. Specific Objectives

- To analyze effect of melatonin administration on locally advanced oral squamous cell carcinoma after neoadjuvant chemotherapy to clinical response compared to placebo
- To analyze effect of melatonin administration on locally advanced oral squamous cell carcinoma after neoadjuvant chemotherapy to HIF-1α expression compared to placebo
- To analyze effect of melatonin administration on locally advanced oral squamous cell carcinoma after neoadjuvant chemotherapy to miR-210 expression compared to placebo
- To analyze effect of melatonin administration on locally advanced oral squamous cell carcinoma after neoadjuvant chemotherapy to CD44 expression compared to placebo
- To analyze effect of melatonin administration on locally advanced oral squamous cell carcinoma after neoadjuvant chemotherapy to CD133 expression compared to placebo

2. Design

This study used a parallel randomized controlled trial design with placebo as comparison. This study is conducted in patients with locally advanced oral squamous cell carcinoma that are receiving neoadjuvant chemotherapy and 20 mg per day of melatonin or placebo.

3. Methods

a. Location and Period of Study

This study is conducted in surgical oncology clinic of dr. Cipto Mangunkusumo National Central General Hospital (RSCM), Dharmais Cancer Hospital (RSKD). Histopathology examination is conducted in Anatomical Pathology Department of FKUI-RSCM. DNA/RNA examination is performed in Integrated Laboratory of FKUI-Jakarta. Period of study is from June 2017 to July 2018

b. Population and Sample

Target population are patients with locally advanced oral squamous cell carcinoma (OSCC) (stage IVA and IVB) and accessible population are patients with locally advanced OSCC on surgical oncology clinic of RSCM and RSKD that are planned to receive neoadjuvant chemotherapy (NC).

Study sample are patients with locally advanced oral squamous cell carcinoma (OSCC) that are planned to receive NC in surgical oncology clinic of RSCM, RSKD, Persahabatan General Hospital (RSP), and Fatmawati General Hospital (RSF) that fulfilled inclusion and exclusion criteria.

c. Study Criteria

c.1. Inclusion Criteria

- Stage IVA and IVB locally advanced OSCC proven by histopathology examination
- Subject planned to receive NC
- Subject had never undergone any definitive surgery or never received NC
- Subject consent to participate by signing informed consent
- Subject with Karnofsky score ≥ 50

c.2. Exclusion Criteria

- Subject not eligible for chemotherapy

c.3. Dropout Criteria

- Subject loss to follow up or passed away
- Subject refused to continue therapy

d. Sample Size

Formula for two unpaired mean hypothesis test with numeric variable

$$\mathbf{n_1 = n_2 = 2\ \{S\ (Z_\alpha + Z_\beta\ )\ /\ (X_1 - X_2)\ \}^2}$$

- Type I error = 5%, unidirectional hypothesis, therefore $Z_\alpha = 1{,}645$
- *Power* of study = 80% and type II error = 20%, therefore $Z_\beta = 0{,}84$

S value is calculated by standard deviation of combination of two group means.

Calculated by formula for standard deviation for two group means:

$$\sigma = \sqrt{\sum(x_1 - \mu)^2/n}$$

$$\sigma = \sqrt{(n_1 - 1)\ S_1^2 + (n_2 - 1)\ S^2/(n_1 - 1) + (n_2 - 1)}$$

Difference in mRNA expression mean to investigator clinical judgement 25%

Combined standard deviation:

$$\sigma = 20.8$$

Inserted into formula:

$$n = 2\ \{20{,}8\ (1{,}645 + 0.84)/(64 - 48)\}^2$$

$$n_1 = n_2 = 21$$

$$n = n_1 + n_2 + 10\% = 46$$

Minimal sample size needed for this study is 42 subjects, added by 10% chance of dropout, therefore sample size becomes 46 subjects.

e. Study Variable

Independent variable is oral melatonin administration while dependent variable is clinical response, HIF-1α expression, miR-210 expression, CD44 and CD133 gene expression.

f. Sampling Method

Sampling method in this study is by consecutive sampling

g. Sample Allocation

Allocation for study subjects are done by block randomization. Concealment is done by giving serial number on drug preparations.

h. Protocol

h.1 OSCC

On all subjects will be performed anamnesis, physical examination, and supporting examination, which is complete blood examination, MRI of oral cavity, chest x-ray, liver ultrasonography, and biopsy

h.2 Supporting Examination

h.2.1 Biopsy

Biopsy will be performed by a surgeon in outpatient clinic by open biopsy (incisional biopsy) or core biopsy with local or general anesthesia

h.2.2 Blood Examination

Blood sample is taken from peripheral blood vessel before receiving treatment, and receiving NC

h.2.3 Radiological Examination

On all subjects will be performed MRI of oral cavity to evaluate organ that is involved and extension of tumor cells. Chest x-ray and liver ultrasonography is performed to evaluate distant metastasis

h.3 Patient selection

Potential subject identification uses list from locally advanced OSCC patients that are planned to receive chemotherapy, done by surgeon on duty in outpatient clinic. The investigator will then explain and hand out informed consent regarding purpose and flow of the study. After understanding and agreeing to participate, study subjects is asked to sign the informed consent. Subjects who wishes to participate will receive a copy of informed consent, while subjects who doesn't participate will undergo procedure according to hospital standard.

h.4 Randomization

If the subjects fulfilled inclusion and exclusion criteria, study is continued by randomization procedure done by a third party so the investigator or researcher doesn't know the drug that were given. Every OSCC patients received chemotherapy and melatonin or placebo with randomization pattern that was computerized, split into two arms which is intervention arm and control arm. Intervention arm will receive capsule containing 20 mg melatonin, while control arm will receive capsule containing placebo. Capsule containing melatonin or placebo is distributed to OSCC patients seven days prior to chemotherapy,

initiated until NC administration is completed (three cycles). If the subjects didn't fulfill inclusion criteria, subjects will be excluded from the study, however will still receive therapy according to hospital protocol.

h.5 Administration of Melatonin and Placebo

1. Intervention arm will receive NC and 20 mg melatonin per day. Melatonin is taken seven days prior to chemotherapy. Then melatonin is taken along with NC until three cycles. Melatonin is taken at night
2. Control arm will receive NC and placebo. Placebo is taken seven days prior to chemotherapy. Then plcebo is taken along with NC until three cycles. Placebo is taken at night

Subjects will receive melatonin on intervention arm and placebo on control arm as much as 74 capsule (a week before NC is initiated and for 1 initial cycle) and then 1 week after first NC patient will be given 50 capsule for second cycle, and then the same goes for third cycle. Patient will be assessed of their adherence by investigator phone call to the subjects twice a week.

h.6 Neoadjuvant Chemotherapy

Neoadjuvant chemotherapy that is given are regimen of docetaxel 75 mg/m^2 on first day; cisplatin 80-100 mg/m^2 or carboplatin on first day, with consideration if the patient has the Karnofsky score of 50-70 will be given cisplatin 80 mg/m^2, however if the score is above 70 will be given cisplatin 100 mg/m^2; and 5-FU 1000mg/m^2 since day 1-5. Docetaxel and cisplatin will be administered simultaneously on day one, while 5FU will be given in day 1-5. Chemotherapy is given every three weeks. Two weeks prior to chemotherapy, the patient is asked for outpatient visit to be examined for blood sample for chemotherapy preparation. If laboratory results didn't fulfill requirements for chemotherapy, the patient will be observed for improvement of condition. Melatonin and placebo will still be administered when the patient is in observation for improvement.

h.7 Evaluation of Neoadjuvant Chemotherapy

Two weeks after third NC, physical examination and supporting examination (Oropharyngeal MRI, Chest X-ray, and Liver USG) is performed. Tumor size will be measured after NC administration. Patients with PR and CR will undergo surgery. Surgery will be performed one week after evaluation of response continued by tissue and blood

examination for qRT-PCR. Patients with SD and PD will be performed secondary biopsy and blood exam for qRT-PCR.

h.8 Gene Expression Examination

h.8.1 Primer Design and gBlocks Gene Fragment Primer Design Synthesis

Primer design for CD44, CD133, miR-210, and HIF-1α gene amplification is performed using Primer Quest Tool IDT software. Sequence information of each gene is obtained from National Centre for Biotechnology Information (NCBI) database.

Creation of gBlocks gene fragments

Measurement of mRNA expression of the 4 gene that is analyzed is performed using qRT-PCR absolute quantification. Therefore, a standard curve for template amplification (gene fragment) result is needed with various known concentration. Gene fragments that is used is obtained by designing gBlocks gene fragment with various concentration, starting from 100 ng/mL that contain HIF-1α, CD44, CD133, miR-210 gene fragments (each sized <150 bp). Sequence selection for each gene and gBlocks synthesis is done by gBlocks Gene Fragments IDT (integrated DNA technologies).

h.8.2 RNA Isolation

Sample used to analyze mRNA expression of CD44, CD133, miR-210, and HIF-1α gene is obtained from incisional biopsy or core biopsy and blood examination. Tissue sample is stored in 1.5 ml microcentrifuge tube containing RNAlater RNA Stabilization Reagent in -80^{0}C temperature until RNA isolation is performed. Total RNA isolation uses QIAamp RNA Blood Mini Kit (Qiagen). Protocol of RNA isolation is according to insert manual kit. Purity and concentration of RNA as result of isolation is measured using NanoDrop 2000 (Thermo Scientific). Result of the measurement is used to determine RNA template volume according to calculation of final concentration that will be used in cDNA synthesis process.

h.8.3 cDNA Synthesis

cDNA synthesis is performed using QuantiTect® SYBR® green PCR kit (Qiagen). Protocol of cDNA synthesis is done according to manual insert kit. Before cDNA synthesis is performed, normalization is done by measuring final concentration of each sample. Final concentration of each blood sample is 1 ng/20 µL while final concentration of tissue sample is 100 ng/20 µL.

h.8.4 Two-Step Quantitative PCR (qPCR)

Gene amplification is done using two-step qRT-PCR absolute quantification. Standard curve creation and mRNA expression quantification of each sample gene is done using QuantiTect® SYBR® green PCR kit (Qiagen) with details as described below. All reagents in the kit is deposited in ice box, and then waited until perfectly thawed. Reagent mix is prepared by adding QuantiTect SYBR Green PCR Master Mix of 12.5 µL, forward primer (10 µM), 0,75 µL, *primer reverse* (10 µM) 0,75 µL, and *RNase-free water* 9 µL until volume reaches 23 µL and then homogenized by up-down pipetting and then continued by adding cDNA of 2 µL, and then the mixture is homogenized.

qPCR Profile Phases

1. PCR initial activation step, in 95^0 C temperature for 15 minutes, once.
2. PCR cycle of 40 times consisting of 15 seconds denaturation at 94^0 C, continued by annealing at 57-63^0 C for 30 seconds, and extension at 72^0C
3. Melt curve, melted in 72-95^0 C, raised by 0.5^0 C in each top phases. Wait for 5 seconds for each top phases.