The Difference in the Cyclin D1 Expression in Advanced Stage Nasopharyngeal Cancer Based on Treatment Response: A Retrospective Cohort Study

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ABSTRAK

Latar belakang: *kanker nasofaring (KNF) merupakan kanker leher kepala terbanyak di Indonesia (28,4%). Sebagian kasus (18,9%) terdiagnosis pada stadium lanjut. Kemoterapi memainkan peranan penting pada kasus stadium lanjut. Akan tetapi, pasien dengan stadium yang sama dapat memiliki respon pengobatan yang berbeda, karena adanya perbedaan karakteristik biologi molekular. Cyclin D1 adalah suatu protein yang berperan dalam siklus sel yang akan mempercepat proliferasi. Penelitian ini menilai ekspresi cyclin D1 serta hubungannya dengan respon pengobatan.* ***Metode:*** *penelitian ini menggunakan disain kohort retrospektif pada pasien KNF stadium lanjut yang mendapatkan kemoterapi di RSCM pada kurun waktu 2015-2018. Pewarnaan imunohistokimia cyclin D1 menggunakan antibodi monoklonal cyclin D1 Novocastra™ dengan teknik pengambilan antigen suhu tinggi. Penilaian ekspresi dilakukan dengan menggunakan h-skor. Respons pengobatan ditinjau ulang berdasarkan kriteria RECIST 1.1.* ***Hasil:*** *terdapat 15 subjek (48,4%) dengan ekspresi cyclin D1 positif. Peneliti menemukan ekspresi cyclin D1 positif lebih banyak pada subjek yang respon terhadap kemoterapi (66,7% vs. 33,3%, p = 0,032). Rerata h-skor antara kelompok respons dan tidak respons juga memiliki perbedaan bermakna (116,24 SD57,80 vs. 77,97 SD45,27, p = 0,048).* ***Kesimpulan:*** *penelitian ini menunjukkan ekspresi cyclin D1 berhubungan dengan respon pengobatan yang baik pada pasien KNF.*

Kata kunci: *Cyclin D1, kanker nasofaring, respon pengobatan.*

ABSTRACT

Background: *nasopharyngeal cancer (NPC) is the most common type of head and neck cancer in Indonesia (28.4%). Reports showed that 18.9% of cases came with advanced stage. Chemotherapy play important role in advanced stage. However, patients with the same stage of the disease may have different treatment response, likely due to the different tumor biological characteristics. Cyclin D1 is a protein involved in the cell cycle, which will stimulate proliferation. This study aimed to examine the proportion of cyclin D1 in NPC and its association with treatment response.* ***Methods:*** *a retrospective cohort study was conducted on advanced NPC patients that underwent chemotherapy at Cipto Mangunkusumo Hospital from 2015 until 2018. Cyclin D1*

immunohistochemistry staining was done by antigen retrieval methods using the cyclin D1 Novocastra™ monoclonal antibody. The cyclin D1 expression was evaluated with h-score. Treatment response was reviewed based on the RECIST 1.1 criteria. ***Results:*** *fifteen subjects (48.4%) had a positive expression of cyclin D1. Higher proportion of cyclin D1 positive was found in responsive group compare with non-responsive group (66.7% vs. 33.3%, p = 0.032). Statistically significant difference in mean h-score was observed between the subjects who responded and those who did not respond (116.24 SD57.80 vs. 77.97 SD45.27, p = 0.048).* ***Conclusion:*** *this study suggests that a higher expression of cyclin D1 is associated with a good treatment response in NPC patients.*

Keywords: *cyclin D1, nasopharyngeal cancer, treatment response.*

INTRODUCTION

Nasopharyngeal cancer (NPC) is the most common type of head and neck cancer in Indonesia (28.4% cases). In 2012–2015, there were 878 NPC patients at Cipto Mangunkusumo General Hospital.[1] The incidence of NPC is about 12,000 cases/year. NPC is mostly diagnosed in the locally advanced and advanced stages of the disease (30.1% and 18.9%).[1]

Treatment of this stage is chemotherapy and radiotherapy.[2] The stage of the disease and the lymph nodes' involvement are some factors proven associated with patient survival.[1,3] However, patients with the same stage of the disease exhibited different results in terms of disease progression, treatment response, and recurrence rate, likely due to the different characteristics of molecular biology that are not included in stage determination.[4]

Cyclin D1 is a protein involved in cell cycle progression from the G1 to the S phase. It is encoded by the CCND1 gene located in chromosome 11q13.[5] It binds to CDK 4/6, activating Rb protein. The transcription factor E2F will be released from Rb protein, obliterating its repressive trait against cyclin E transcription. The upregulation of cyclin E will be followed by binding between cyclin E and CDK 2, initiating the S phase.[6] Therefore, the overexpression of cyclin D1 will shorten phase G1 of the cell cycle.[4]

The overexpression of cyclin D1 has been found in many cancers, but studies regarding the association between the expression level of cyclin D1 and chemotherapy response, until now, have shown a different results. Some studies reported that NPC[7] and bladder cancer[8,9] with an overexpression of cyclin D1 have a better response to induction chemotherapy with cisplatin. Meanwhile, other studies of germ cell tumors,[10] pancreatic cancer,[11] and head and neck cancer[10] give an opposite results. In Indonesia, data concerning the cyclin D1 expression in NPC do not yet exist. Therefore, in this study, we examine the proportion of cyclin D1 in NPC and its association with treatment response.

METHODS

This retrospective cohort study included all NPC patients at Cipto Mangunkusumo General Hospital from 2015 until 2018. Patient data were collected based on medical records. The inclusion criteria were: an age greater than 18 years, histopathological results showing NPC, diagnosis as stage IV B NPC, completion of six series of cisplatin–fluorouracil (5FU) chemotherapy or discontinuation after three cycles due to progressive disease in treatment. Patients with incomplete medical records, with incomplete treatment, and whose chemotherapy schedule was delayed more than 21 days or their imaging evaluation was not done in eight weeks post-chemotherapy were excluded from this study.

The response to treatment was reviewed based on the Response Evaluation Criteria in Solid Tumors (RECIST) 1.1 criteria by staff of the Radiology Department of the Faculty of Medicine, Universitas Indonesia/ Cipto Mangunkusumo General Hospital. The immunohistochemistry examination was conducted in the Department of Pathological Anatomy of the Faculty of Medicine, Universitas Indonesia/Cipto Mangunkusumo General Hospital.

A paraffin block of advanced NPC patients that matched the inclusion criteria and not

the exclusion criteria were reviewed using hematoxylin and eosin (H&E) staining to ensure the results of histopathological examination showed NPC. Then, cyclin D1 immunohistochemistry staining was carried out at room temperature (25°C) by antigen retrieval methods using the cyclin D1 Novocastra™ monoclonal antibody. The staining was read together with a supervisor using an Olympus microscope.

Each preparation was chosen for 10 locations that were considered representations of the preparation (**Figure 1**). Each was assessed using the h-score method by using the ImageJ 1.50i application.

The sample size was calculated using a comparison of the two means test formula, and we obtained a minimum sample size of 16 subjects in each group. The data will be recorded in the case record form and then entered into the SPSS program version 23. The unpaired t-test or Mann–Whitney analysis was performed to determine the mean difference between the two groups, depending on the data distribution. Then, a cut-off score was determined to divide the expression of cyclin D1 into positive or negative; thus, the mean difference of each group could be determined.

The study was conducted under ethical clearance no. 1224/UN2.F1/ETIK/2018 from ethical commission Faculty of Medicine University of Indonesia.

RESULTS

Out of 84 registered subjects with NPC, 34 subjects' data were incomplete and they did not undergo complete treatment, having an incomplete initial imaging examination, delayed chemotherapy for more than three weeks, or not come back for treatment. Nineteen of the rest 50 subjects were excluded because their paraffin block could not be found. Finally, 31 subjects were included in the study.

Clinical Characteristics of Subjects

The clinical characteristics of the subjects, including demographic data, clinical profiles, and treatment responses, are shown in **Table 1**. The majority of subjects were men and they lived outside Jakarta. The chief complaint of the subjects was mostly a mass in the neck (58.1%). The mean age of the subjects when diagnosed was 44.87 years.

In this study, majority of the subjects had complained the symptoms for >6 months before definitive diagnosis was established (64.5%). WHO-III was the most common histopathological finding (93.6%). More than half of the subjects had T4 and N3 disease (64.5 and 54.8%, respectively).

Bone is the most common organ affected by metastasis. Most subjects only have one meta-location. Six subjects had metastases in more than one organ, three subjects had metastases in the bone and lung, and two subjects had metastases in the bone and liver. As well, one subject had metastasis locations in the bones, liver, and lungs. In addition, there was one subject with a meta-location on the skin.

All study subjects received a chemotherapy regimen using cisplatin–5FU at the first administration. Patients who experienced

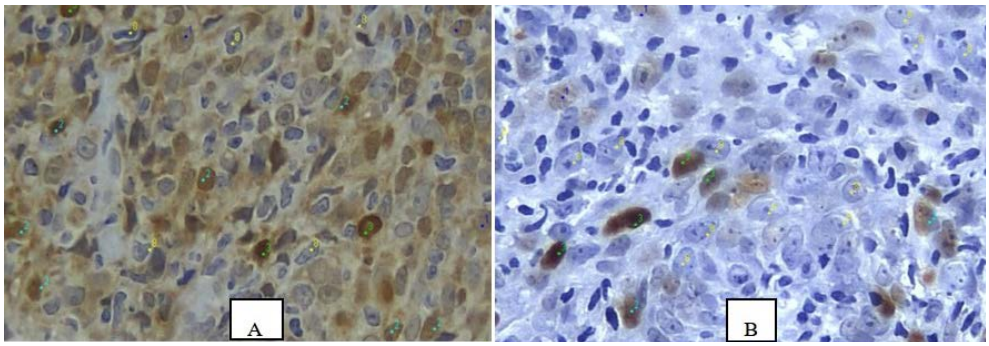

Figure 1. Results of cyclin D1 immunohistochemical examination.
(A) Immunohistochemical features of subjects with positive cyclin D1 expression.
(B) Immunohistochemical features of subjects with negative cyclin D1 expression.

Table 1. Clinical characteristics of the subjects.

Subjects Characteristics	n (%)
Age, mean (SD)	44.87 (13.964)
Gender, Male	27 (87.1)
Performance status (ECOG)	
- 0	25 (80.6)
- 1	6 (19.4)
Domicile	
- Jakarta	11 (35.5)
- Outside Jakarta	20 (64.5)
Smoking habit	11 (35.5)
Duration of chief complaint	
- >6 months	20 (64.5)
- ≤6 months	11 (35.5)
Signs and symptoms	
- Mass in the neck	29 (93.5)
- Olfactory disturbance	21 (67.7)
- Visual disturbance	13 (41.9)
- Ear disturbance	17 (54.8)
- Neurology symptoms	3 (9.7)
- Pain	20 (64.5)
Histopathology	
- WHO-I	1 (3.2)
- WHO-II	1 (3.2)
- WHO-III	29 (93.6)
Stage T	
- T2	7 (22.6)
- T3	4 (12.9)
- T4	20 (64.5)
Stage N	
- N0	1 (3.2)
- N1	1 (3.2)
- N2	12 (38.7)
- N3	17 (54.8)
Metastasis location	
- Liver	3 (9.7)
- Lung	7 (22.6)
- Bone	28 (90.3)
Amount of metastasis	
- 1 location	25 (80.7)
- >1 location	6 (19.3)
Comorbidity	
- Hypertension	1 (3.2)
- Diabetes mellitus	1 (3.2)
- Heart disease	0 (0.0)
- Renal disease	0 (0.0)
Number of cycle cisplatin used	
- Cisplatin <4x	3 (9.6)
- Cisplatin ≥4x	27 (90.4)

decreased kidney function (eGFR <60 mL/min/1.73 m^2) during treatment were given carboplatin in place of cisplatin.

Distribution of Subjects based on Treatment Response

Based on the treatment response using the RECIST 1.1 criteria, 1 subject with had complete response, 13 subjects partial response, 9 subjects had a stable disease, and 8 subjects had a progressive disease. Based on the treatment response, the subjects were divided into two groups: respond (partial and complete response) and not respond (stable disease or progressive disease). Fourteen subjects (45.2%) were classified as respond and 16 subjects (54.8%) did not respond.

Cyclin D1 Expression and Treatment Response

The assessment was carried out by assessing the percentage of cells that had the cyclin D1 expression and the degree of intensity of the cyclin D1 expression compared to positive controls (**Figure 1**). An assessment of the cyclin D1 expression used the h-score method. After obtaining the h-score for all subjects, the cut-off value was determined using the (Receiver Operating Characteristic) ROC Curve of the entire study sample. From 31 subjects, we reported 15 subjects with positive cyclin D1 expression. This study reported that higher proportion of cyclin D1 positive was noted in respond group compared with not respond (66.7% vs. 33.3%, $p = 0.032$). The mean h-score also significantly different between respond and non-respond (116.24 (SD 57.80) vs. 77.97 (SD 45.27), $p<0.05$).

DISCUSSION

This study included homogeneous subjects of stage IV B NPC patients. However, patients with the same stage had different responses to the treatment and recurrence rate.[3,12] Based on the clinical conditions, most also had the same characteristics; for example, all subjects had a good performance status or either 0 (80.6%) or 1 (19.4%), because the requirement to get a combination chemotherapy is a good performance status (0 or 1). Recondo et al.[13]

reported that a performance status of 0 or 1 is related to a good response to treatment. The presence of other comorbidities that could affect the patient's condition, such as diabetes mellitus and hypertension, were assessed. The subjects of this study mostly (93.6%) did not have comorbidities.

Subjects in this study mostly lived outside Jakarta (64.5%), which reflected that this study not only described patients living in Jakarta, but it also described patients from various regions. This was possible because Cipto Mangunkusumo Hospital is a national referral center with adequate facilities for the management of NPC, especially diagnostic facilities (MRI) and treatment (radiotherapy).

The majority of subjects were men (87.1%) and the average age of the subjects at the time of diagnosis was 44.87 years. This was in accordance with the literature and previous research in Indonesia, that cases of NPC were dominated by men.[14] Adham et al.[15] reported that men were 2.4 times more likely to have NPC than women. Hayati et al.[1] also reported in 2017 that men (73.8%) were more likely than women to be diagnosed with NPC, with a median age at diagnosis of 46 years.

Most of the subjects came after the chief complaint lasted more than six months. Jayalie et al.[16] conducted a study of NPC patients in Cipto Mangunkusumo General Hospital and reported that more than half of the subjects had a complaint duration of six months or less before a diagnosis was made. It differs from this study because in this study, the subjects included were patients with the stage of metastasis. In this stage, the duration of complaints was longer than in the lower stage.[16] The chief complaint that brought them to a health facility was a mass in the neck, and other complaints included a nasal disorder (nasal congestion, nosebleeds) and impaired vision (double vision, decreased vision), similar to the results of Jayalie et al.[16]

In this study, the location of metastasis in most subjects (90.3%) was the bone, followed by the lungs and liver. This is in accordance with the literature, which states that bone is the most common metastatic location in NPC.[17,18]

In this study, some subjects experienced a decline in kidney function (eGFR <60 ml/min/1.73 m^2), so cisplatin was replaced with carboplatin. Cisplatin is the main choice in patients with head and neck cancers, but its toxicity to the kidney limits its use. Several studies have shown that carboplatin in head and neck cancers, including NPC, exhibits the same efficacy. A non-inferiority study in Thailand involving 206 locally advanced stage NPC patients reported the same efficacy between cisplatin and carboplatin in concurrent or adjuvant administration.[19] Kua et al.[20] reported that the response rate of carboplatin did not differ from that of cisplatin. Based on these data, the use of carboplatin is not inferior to the use of cisplatin as an alternative for individuals with renal impairment.

Proportion of the Cyclin D1 Expression

In this study, most subjects (48.4%) had a positive cyclin D1 expression. Studies that assessed the cyclin D1 expression in NPC are limited. Various studies in the west included the categorization of NPC into squamous neck and head cell carcinoma. The results of this study show a higher expression than previous publications abroad, which showed that 30–50% of cases had a cyclin D1 expression.[4] This study showed similar result. Hwang et al.[21] also reported that the expression of cyclin D1 in NPC patients who experienced recurrence was 66%.

The increase in the cyclin expression in cancer can be caused by the amplification of the CCND1 gene, the translocation of the CCND1 gene (t11.14) (q13; q32), or a cyclin D1 protein degradation defect.[22] Most of the increase in the cyclin D1 expression is caused by CCND1 gene amplification. Increasing the CCND1 gene promoter plays a role in amplification, such as of NF-κB, PI3K-akt, EGFR, and the β-catenin-LEF1 pathway.[23]

An increased expression of cyclin D1 in cancer can be stimulated by various growth factors. A study on prostate cancer showed that an excessive expression of EGFR would cause an increase in the number of mRNA and cyclin D1 protein. Breast cancer that has the Her2 expression will also be associated with an increase in the cyclin D1 expression.[23] The

expression of LMP1 due to an EBV infection in nasopharyngeal squamous cells will be followed by the expression and phosphorylation of EGFR. In NPC, 60–90% of cases have the EGFR expression. This was followed by the upregulation of the cyclin D1 gene promoter through an increase in heterodimer c-Jun/Jun B.[24]

The high proportion of metastatic NPC subjects who have the cyclin D1 expression provides an opportunity for drugs to work on the cyclin D-CDK4/6 pathway to inhibit tumor proliferation. Until now, the study of CDK 4/6 inhibitors in NPC has been limited. Hsu et al.[25] conducted a palbociclib administration test in animals with amplified CCND1 gene NPC, showing the effect of inhibiting tumor growth and reducing EBV titers. Jiao et al.[26] provided a case report on NPC patients with pulmonary metastasis and an excess amplification of CDK 4/6 who were given palbociclib, and they showed a partial response after 6 months of treatment. They triggered further research regarding the use of palbociclib in advanced NPC patients.

Cyclin D1 Expression and Treatment Response

The group of subjects who responded to treatment had a higher proportion of the cyclin D1 expression than the group of subjects who did not respond. This is contrary to the previous studies of Noel et al[10] and Biliran et al[11], which states that the cyclin D1 expression is higher in NPC patients who do not respond compared to those who do respond to treatment.

Nonetheless, these results are supported by several other studies. In vitro study of Akervall et al.[27] showed that the mean concentration inhibiting growth of 50% of the cells (ID50) was lower in cell lines with higher cyclin D1 expression. Seiler et al.[8] conducted a study on bladder cancer and reported that the cyclin D1 expression in the lymph nodes demonstrated a good response to platin-based chemotherapy compared to a group of subjects who had a negative/weak cyclin D1 expression.

The Paradoxical Effect of Cyclin D1 on DNA Repair

Several studies explain the ambiguous/ paradoxical effect of cyclin D1. Different types of cancer cells can influence the different roles of cyclin D1 in response to the administration of cytostatic drugs, although the mechanism underlying these responses is still unknown.[28]

A highly excessive expression of cyclin D1 is reported to cause chromosomal instability in some cancers (breast, neck, head cancer) because they are forced to enter the S phase immediately, obstructing the DNA repair process.[22]

An excessive expression of cyclin D1 also can trigger an excessive expression of protein in the DNA repair process. The excessive stimulation of RAD51 will cause toxicity to DNA, causing a disruption to the DNA repair mechanism and DNA instability.[22] Richardson et al.[29] reported that an excessive RAD51 expression would lead to genome instability due to aneuploid chromosome formation and changes to the chromosome arrangement.

Some reports identify differences in the cyclin D1 response to the degree of DNA damage. Severe DNA damage, such as due to chemotherapy or high-dose radiation, will be followed by a sharp decrease in cyclin D1 levels, which will prevent cells from entering the S phase. This will cause cells to stop proliferation and die. Meanwhile, the low degree of DNA damage is not enough to decrease cyclin D1 levels sharply, so the cell continues to divide. With low-grade DNA damage, cyclin D1 will be found in the cell nucleus, and it has a role in DNA repair activity. In addition, cyclin D1 will activate the expression of the DNA repair protein, RAD51.[22]

Myklebust et al.[30] also reported that an increase in the cyclin D1a and D1b isoforms had a different response to 5-fluorouracil. In the study, it was reported that the expression of the cyclin D1a isoform in colorectal cancer subjects receiving 5-fluorouracil responded better to treatment than subjects with the cyclin D1b expression. This can occur because of an imbalance between the ability of DNA repair and synthesis.[23]

Strengths and Limitations of Research

This study is the first to assess the cyclin D1 expression, specifically in cases of metastatic NPC in Indonesia. Informations obtained from this study can be a further basis for our understanding of the cyclin D1 pathway in the biology process of NPC. This study has a limitation in that until

now, the cut-off value to determine the cyclin D1 expression as positive has not been agreed upon in the international community.

CONCLUSION

Cyclin D1 positive were expressed in half of advanced NPC subjects (48.4%). There are significant mean differences in the cyclin D1 expression between the group of subjects who responded compared with who did not respond to platinum 5FU ($p = 0.048$). Further studies are needed to clarify the paradoxical effects of cyclin D1 in NPC focusing the mechanism that explaining this finding. Studies still needed before cyclin D1 could be widely used as predictor for chemotherapy response in advanced NPC.

CONFLICT OF INTEREST

The authors declare that there is no conflict of interest concerning the publication of this paper.

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