

The role of cyclin D1 and vascular endothelial growth factor (VEGF) in radiotherapy response of undifferentiated nasopharyngeal carcinoma

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ABSTRACT

Nasopharyngeal carcinoma (NPC) has a high incidence and mortality rate in Southeast Asia included in Indonesia. Radioresistance is a major obstacle for a successful treatment of NPC. DNA repair in the cell cycle and angiogenesis factors affect the response of tumor cells to radiotherapy. Cyclin D1 that functions in the cell cycle process and vascular endothelial growth factor (VEGF) as an angiogenesis factor are considered to play a role in the occurrence of radioresistance. The objective of this study was to evaluate the association between cyclin D1 and VEGF expressions with radiotherapy response in undifferentiated NPC. This study used a retrospective case control analysis design. Secondary data from medical records of patients diagnosed as undifferentiated NPC who received a complete radiotherapy at the Department of Radiation Oncology, Dr. Hasan Sadikin General Hospital, Bandung. There were 44 samples divided into radiosensitive (22 samples) and radioresistant (22 samples) groups. Immunohistochemical examination of cyclin D1 and VEGF expressions was performed on paraffin blocks of patients' nasopharyngeal biopsy. Data analysis used Chi-Square test with $p \leq 0.05$. Cyclin D1 was expressed strongly in 86.4% of the radioresistant group and 59.1% in the radiosensitive group ($p < 0.05$), VEGF was strongly expressed in 77.3% of the radioresistant group and 54.5% in the radiosensitive group ($p > 0.05$). In conclusion, there is significant association between cyclin D1 expression with radiotherapy response in undifferentiated NPC. However, there is no association between VEGF expression with radiotherapy response.

ABSTRAK

Karsinoma nasofaring merupakan keganasan dengan angka insidensi dan mortalitas tinggi di Asia Tenggara termasuk Indonesia. Radioresistensi menjadi hambatan utama dalam keberhasilan penanganan karsinoma nasofaring. Perbaikan DNA dalam siklus sel dan faktor angiogenesis mempengaruhi respons sel tumor terhadap radioterapi. Siklin D1 yang berperan dalam siklus sel dan *vascular endothelial growth factor* (VEGF) sebagai faktor angiogenesis berperan dalam terjadinya radioresistensi. Penelitian ini bertujuan mengkaji hubungan antara ekspresi siklin D1 dan VEGF dengan respons radioterapi pada karsinoma nasofaring tidak berdiferensiasi. Penelitian ini menggunakan desain analisis *retrospective case control*. Data sekunder yang digunakan berasal dari rekam medis pasien yang didiagnosis sebagai karsinoma nasofaring tidak berdiferensiasi yang mendapat radioterapi lengkap di Departemen Radiasi Onkologi, RSUP Dr Hasan Sadikin Bandung. Terdapat 44 sampel yang dibagi menjadi kelompok radiosensitif (22 sampel) dan radioresisten (22 sampel) berdasarkan respons radioterapi. Deteksi ekspresi siklin D1 dan VEGF dilakukan dengan pemeriksaan imunohistokimia pada blok paraffin sediaan biopsi nasofaring pasien. Analisis data menggunakan uji Chi-Square dengan nilai $p \leq 0.05$. Siklin D1 terekspresi kuat pada 86,4% kelompok radioresisten dan 59,1% pada kelompok radiosensitif ($p < 0.05$), sedangkan VEGF terekspresi kuat pada 77,3% kelompok

radioresisten dan pada 54,5% kelompok radiosensitif ($p > 0.05$). Dapat disimpulkan, terdapat hubungan signifikan antara ekspresi siklin D1 dengan respons radioterapi pada karsinoma nasofaring tidak berdiferensiasi. Akan tetapi tidak terdapat hubungan antara ekspresi VEGF dengan respons radioterapi.

Keywords: cyclin d1- nasopharyngeal carcinoma - radiotherapy – VEGF - response

INTRODUCTION

Nasopharyngeal carcinoma (NPC) is a malignancy originating from the nasopharyngeal epithelium.¹ This malignancy has a high incidence rate in the East and Southeast Asia region. Based on GLOBOCAN 2012, there are 87,000 new cases emerging each year.² In Indonesia, NPC is one of the most common malignancies, ranks the 4th in the most cancer incidence after breast cancer, cervical cancer, and lung cancer, makes the most frequent malignancy in the head and neck region. The incidence of NPC in Indonesia is 6.2 per 100,000 populations per year with 13,000 new cases per year. Undifferentiated nasopharyngeal carcinoma is the most common subtype (90-95%) found in endemic areas such as in Asia including in Indonesia.³

Radiotherapy is the standard choice of undifferentiated nasopharyngeal carcinoma treatment because of its sensitivity to radiotherapy and has difficult anatomical location for surgery.⁴ The radiotherapy has been a chosen treatment since many years and has been performed in various centers in the world. The outcomes of radiotherapy for early stage NPCs are actually quite good with the complete response is about 80-100%. However, the outcomes of the radiotherapy for advanced stage NPCs drops significantly with 5-year survival rate less than 40% due to increased incidence of radioresistance.⁵ Chemoradiation is one of the therapeutic modalities in the advanced stage of NPC, however in some cases chemotherapy becomes contraindication. Therefore, radiotherapy is the only modality in the advance stage of undifferentiated

nasopharyngeal carcinoma.⁶

The occurrence of radioresistance is a multistage process involving many genes. Gene fractions that have functions in cell cycle control, apoptosis/antiapoptosis and DNA repair are presumed to have an important role in radiation-induced DNA damage. However, the mechanism of radioresistance at the molecular level is not fully understood.^{7,8} DNA damage caused by radiation is associated with regulation in the cell cycle.⁹

Cyclin D1 is the most type of cyclin that closely related to the genes involved in the cell cycle. Cyclin D1 is a core protein that bound to CDK4/CDK6 forming the D1-CDK4/6 cyclin complex, which is involved in the phosphorylation of retinoblastoma proteins (pRb) in the G1 and S cell cycle phases and plays an important role in assisting cellular DNA repair.¹⁰

Radioresistance of tumor cells is also associated with angiogenesis process. Rapid tumor growth can lead to hypoxia so that tumor cells will initiate survival mechanism by activating vascular endothelial growth factor (VEGF), which causes increased vascular proliferation and angiogenesis. The newly formed blood vessels facilitate the provision of oxygen and nutrients to tumor cells. This condition can cause radioresistance on tumor cells. The VEGF is an angiogenic protein that plays a role in the growth of endothelial cells and tumor blood vessels.^{10,11} The aim of this study was to evaluate the association between expression of cyclin D1 and VEGF with radiotherapy response in undifferentiated nasopharyngeal carcinoma.

MATERIALS AND METHODS

Sample

The sample of this study was secondary data from medical records of patients diagnosed as undifferentiated nasopharyngeal carcinoma stage IV who only received a complete radiotherapy as they did not meet the requirements for chemotherapy, at the Department of Radiation Oncology, Dr Hasan Sadikin General Hospital, Bandung period 2012-2016. There were 44 samples eligible for inclusion criteria divided into radiosensitive and radioresistant groups based on radiotherapy response according to the agreement of the response evaluation criteria in solid tumors (RECIST). The RECIST criteria divide the radiotherapy response into a complete, partial, stable, and progressive response.¹² Complete responses were incorporated into the radiosensitive group, while the partial, stable and progressive responses were incorporated into the radioresistant group. Both radiosensitive and radioresistant groups consisted of 22 samples for each group. Immunohistochemical examination of cyclin D1 and VEGF was performed on the paraffin block of patient nasopharyngeal taken from biopsy of the radiosensitive and radioresistant groups. Paraffin block preparations were available from medical records Department of Pathology, Dr. Hasan Sadikin General Hospital, Bandung, Indonesia.

Immunohistochemistry (IHC) examination

Cyclin D1 antibody (Clone SP4, Biocare, USA) and VEGF antibody (Clone EP117Y, Biocare, USA) was used as standard procedure of IHC staining. Positively stained tumor cells were counted visually using binocular photon micro-scope

at 400x magnification, and scoring was made according to percentage and intensity of positively stained cells. Cyclin D1 expressions were considered positive if the nuclear was stained and VEGF expressions were considered positive if the cell membrane and cytoplasm was stained. The intensity of cyclin D1 and VEGF immunoexpressions was assessed on a scale of 0-3 (0, colorless; 1+, weak colored; 2+, medium-colored; 3+, strongly colored) and the distribution of cyclin D1 and VEGF immunoexpressions was assessed on a scale of 0-4 (0, negative; +1, <20%; 2+, 20-50%; 3+, 50-80%; 4+, > 80%). The immunoexpression assessment of cyclin D1 and VEGF using Histoscore determined based on the number of multiplication intensities and distributions with a weak expression if total score is 0-4 and strong expressions if total score 6-12.^{10,13} The diagnosis was established by anatomic pathologist at the Department of Anatomic Pathology, Dr. Hasan Sadikin General Hospital, Bandung.

Statistic analysis

The data were presented as frequency or percentage and analyzed using Chi Square test with SPSS 22.0 software for Windows. Significant results were based on statistical calculations when obtained p value ≤ 0.05 .

RESULTS

TABLE 1 shows the characteristics of the study subjects consist of age, sex, stage based on radiotherapy response. Most cases were found in the 40-60 years age range of 24 cases (55%). Males were observed more than females with 32 cases (73%). Most stages were IVB stage with 27 cases (61.4%) and were IV stage with metastasis to lymph nodes with 36 cases (82%).

TABLE 1. Characteristics of subjects based on radiotherapy response

Variable	Radiotherapy response [n (%)]		p
	Sensitive (n=22)	Resistant (n=22)	
Age			
• <40 years	6 (27.3)	7 (31.8)	0.987
• 40-60 years	11 (50.0)	13 (59.1)	
• >60 years	5 (22.7)	2 (9.1)	
Sex			
• Man	14 (63.6)	18 (81.8)	0.176
• Woman	8 (36.4)	4 (18.2)	
Staging			
• IVA			0.987
• T4N0M0	5 (22.7)	3 (13.6)	
• T4N1-2M0	5 (22.7)	4 (18.2)	
• IVB			
• Any T N3 M0	12 (54.5)	15 (68.2)	
• IVC			
• Any T Any N M1	0 (0.0)	0 (0.0)	

TABLE 2 shows the association of cyclin D1 and VEGF expression with radiotherapy response. Cyclin D1 expression statistically showed significant association

($p=0.042$), whereas VEGF expression showed nonsignificant association ($p = 0.112$) with the radiotherapy response.

TABLE 2. Association of immunoeexpression cyclin D1 and VEGF with radiotherapy response

Variable	Radiotherapy response [n(%)]		p
	Sensitive (n=22)	Resistant (n=22)	
Cyclin D1			
• Weak (0-4)	9 (40.9)	3 (13.6)	0.042**
• Strong (6-12)	13 (59.1)	19 (86.4)	
VEGF			
• Weak (0-4)	10 (45.5)	5 (22.7)	0.112
• Strong (6-12)	12 (54.5)	17 (77.3)	

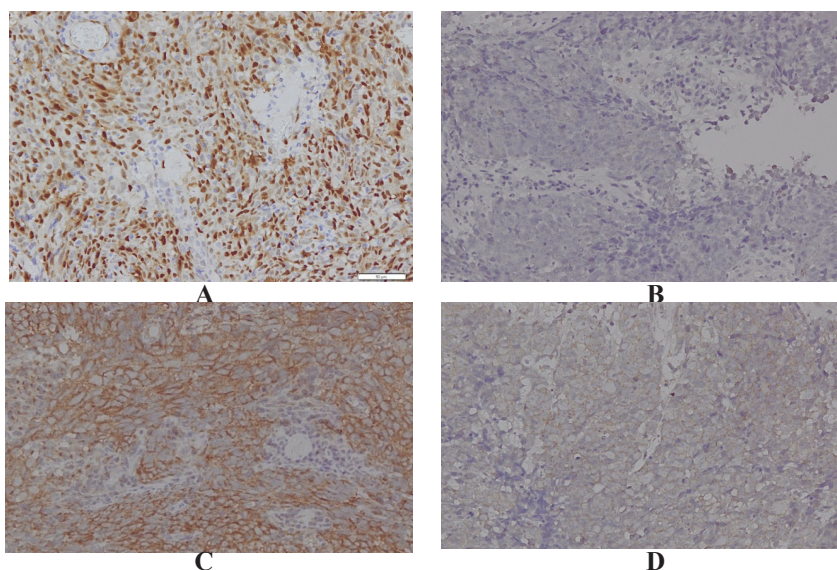


FIGURE 1. Cyclin D1 and VEGF expressions of nasopharyngeal carcinoma (A) Cyclin D1 strong expression, tumor cells were stained in the nuclear (B) Cyclin D1 weak expression (C) VEGF strong expression, tumor cells were stained in the cell membrane and cytoplasm (D) VEGF weak expression. (400X Magnification)

DISCUSSION

In this study, most cases (24 cases) were found in the range 40-60 years old (55%) with males 3 times more than females. This is consistent with the study of Pettersson *et al.*,¹ stated that in high populations of NPC, the incidence increases after the age of 30 years and reaches peak incidence in the range 40-60 years old, with male 2-3 times more than female. In this study, 36 cases (82%) of stage IV NPC have metastasized to lymph nodes. This is consistent with the study of Xuan *et al.*¹⁴ that found 70% of cases of stage IV NPC have metastasized to lymph nodes.

The cyclin D1 expression of all samples in this study showed positive expression, 32 cases (72.7%) showed strong expression and 12 cases (27.3%) showed weak expression. The comparison of cyclin D1 expression in the radiosensitive group and the radioresistant group showed significant result ($p=0.043$). This suggests that there was a significant association between cyclin D1 expression and radiotherapy response. This is likely due to DNA damage by radiation activating the AKT/Cyclin D1/CDK4 pathway. This AKT pathway facilitates the repair of DNA in tumor cells to provide tumor cell survival and subsequently contributes to radioresistance.^{11,15}

The results of this study are consistent with previous studies. The study of Fu *et al.*¹⁰ showed significant differences between strong cyclin D1 expression in the sensitive radiotherapy response group and in the insensitive group ($p<0.05$). Patients with weak cyclin D1 expression are more sensitive to radiation. The strongly expressed cyclin D1 causes G1-S phase shortening in the cell cycle results in uncontrolled cell proliferation and promotes tumor cell growth. Radiation-induced DNA damage can also activate cyclin D1 to assist the process of DNA repair, thus increasing tumor cell survival and results in resistance to radiotherapy.¹⁰ Cyclin D1 depletion in HeLa cervical carcinoma cells and H2009 lung cancer cells significantly increases the sensitivity of cancer cells to radiation. This

suggests cyclin D1 to play a role in repairing damaged DNA.¹⁶ Cyclin D1 is an important key to cell cycle progression through G1 phase associated with poor radiation response. In other study, decreased cyclin D1 in esophageal cancer can slow down tumor cell growth and can reduce the DNA repair capacity of tumor cells. Inhibition of cyclin D1 can prevent DNA repair of tumor cells.¹⁷

In this study, all VEGF immunopositivity showed positive expression, 29 cases (65.9%) showed strong expression and 15 cases (34.1%) showed weak expression. The results of this study show no significant association between VEGF expression with radiotherapy response ($p=0.117$). This may be caused by the hypoxic state that induces HIF α and results in radioresistance occurring through multiple pathways. These pathways are HIF1 α /VEGF pathways, HIF α /CXCL12 pathways, HIF α /Transforming Growth Factor β (TGF β) pathways and HIF α /TGF β /integrin pathways. HIF α /VEGF pathways and HIF α /CXCL12 pathways induce angiogenesis and vasculogenesis. The HIF α /TGF β pathway induces angiogenesis and maintains vascular homeostasis, whereas HIF α /TGF β /integrin pathway may cause endothelial cell survival against radiation effects.¹⁸ In this study, another possible factor that induces angiogenesis, other than VEGF as mentioned above, is more dominant in causing the occurrence of radioresistance. Thus, this study found insignificant association between VEGF with radiotherapy response.

It was explained that in nasopharyngeal carcinoma, the oxygen factor alone is not sufficient to predict radiosensitivity, there are many other biological factors that involve in radiosensitivity.¹⁹ Moreover, the amount of angiogenic factors influence the pathway of radioresistance. Tumor cells with a few angiogenic factors will become radioresistance via hypoxic pathways, whereas tumor cells that have much of angiogenic factors will become radioresistance through intrinsic pathways and ischemic factors. These mechanisms

can cause undetectable VEGF expression in radioresistance.²⁰

The factors mentioned above may induce the occurrence of nasopharyngeal carcinoma metastases into the lymph nodes.²¹ CXCL12 expression is increased in cases of nasopharyngeal carcinoma with lymph node metastasis compared to cases without metastasis to lymph nodes. CXCL12 which forms the bond with CXCR4/CXCR7 may affect migration, invasion and survival of nasopharyngeal carcinoma cells.²² TGF β may serve as a protooncogen at an advanced stage by stimulating angiogenesis and inducing epithelial mesenchymal tumors (EMT) that contribute to tumor cell invasion and metastasis.²³ Expression of integrin α v is significantly associated with lymph node metastasis in nasopharyngeal carcinoma. Integrin is a molecule that involves in the process of invasion, metastasis, angiogenesis and tumor cell survival.¹⁴ In this study, 82% of cases have metastasized to lymph nodes. This suggests that all factors mentioned above may be more dominant than VEGF in causing radioresistance, thus in this study there was no significant association between VEGF and radiotherapy response.

CONCLUSION

There is significant association between cyclin D1 expression with radiotherapy response in undifferentiated nasopharyngeal carcinoma. The stronger cyclin D1 expression, the higher likelihood of radioresistance is observed. However, VEGF expression shows no significant association with radiotherapy response.

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