ISSN: 3032-3134 (ONLINE)
InaJBCS | Indonesian Journal of Biomedicine and Clinical Sciences

Association between CDK4 expression and overall survival of osteosarcoma patients

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https://doi.org/10.22146/inajbcs.v56i01.11896

Submitted: 2023-10-30
Accepted : 2024-01-05

ABSTRACT

Osteosarcoma is the most common primary bone tumor malignancy, accounting for 30 - 80% of all primary bone tumors. It is presented in a bimodal distribution manner with the age of onset divided into two groups, 10-20 and >60 y.o. Various factors have significance in the patient's prognosis, including the expression of cyclin-dependent kinase 4 (CDK4). This CDK4 has an essential role in the pathogenesis of osteosarcoma through inactivation of the Rb gene, which is associated with the patient's survival. This study was conducted to evaluate the correlation between CDK4 expression and the survival of osteosarcoma patients. It was a cross-sectional study involving 50 patients diagnosed with osteosarcoma based on clinical, radiological, and histopathological examination. Available formalin-fixed paraffin-embedded (FFPE) samples were retrieved for immunohistochemical (IHC) staining of CDK4. The survival data was collected from medical records. CDK4 expression and survival data were analyzed statistically using the Kaplan-Meier curve. Out of 50 subjects, CDK4 was found to be expressed in 38 samples (76%). The group with negative CDK4 showed a slightly longer overall survival (by 0.2 mo) than the positive CDK4 group. However, these results were not statistically significant (p = 0.821). In conclusion, the overexpression of CDK4 may not directly affect the survival rate in osteosarcoma. Other factors need to be considered to understand the complexity of the disease.

ABSTRAK

Osteosarkoma merupakan keganasan tumor tulang primer yang paling umum terjadi, mencakup 30 - 80% dari seluruh tumor tulang primer. Pola distribusi osteosarcoma berbentuk bimodal dengan onset umur dibagi menjadi dua kelompok, yaitu 10-20 dan >60 tahun. Berbagai faktor berperan penting dalam prognosis pasien, termasuk ekspresi dari *cyclin-dependent kinase* 4 (CDK4). CDK4 memiliki peran penting dalam patogenesis osteosarkoma melalui inaktivasi gen Rb, yang berhubungan dengan survival pasien. Penelitian ini dilakukan untuk mengkaji hubungan antara ekspresi CDK4 dan survival pada pasien osteosarkoma. Penelitian ini merupakan penelitian potong lintang yang melibatkan 50 pasien yang didiagnosis osteosarkoma berdasarkan pemeriksaan klinis, radiologi, dan histopatologi. Sampel *formalin-fixed paraffin-embedded* (FFPE) yang tersedia diambil untuk pewarnaan imunohistokimia (IHC) CDK4. Data survival dikumpulkan dari rekam medis. Ekspresi CDK4 dan data survival dianalisis secara statistik menggunakan kurva Kaplan-Meier. Dari 50 subjek penelitian, ekspresi CDK4 didapatkan pada 38 (76%) sampel. Keseluruhan survival pada kelompok CDK4 negatif adalah 0,2 bulan lebih lama dibandingkan kelompok CDK4 positif; namun tidak menunjukkan perbedaan yang bermakna secara statistik (p = 0,821). Simpulan, ekspresi CDK4 yang berlebihan tidak secara langsung mempengaruhi tingkat kelangsungan hidup pada osteosarkoma. Faktor-faktor lain perlu dipertimbangkan untuk memahami kompleksitas penyakit ini.

Keywords:
CDK4 expression;
osteosarcoma;
survival;
bone cancer;
prognosis

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INTRODUCTION

Osteosarcoma is the most common type of primary bone cancer. It accounts for 30 - 80% of all primary bone tumors.[1] The incidence rate is 5.6 cases per 1,000,000 people annually.[2] Between the ages of 15 and 19, the number of cases of osteosarcoma has increased to an estimated 8 - 11 per 1,000,000 people annually. Data from Dr. Cipto Mangunkusumo Hospital, Jakarta demonstrated that a total of 219 cases of osteosarcoma were recorded between 1995 and 2007, averaging 16.8 cases annually. Osteosarcoma was responsible for 70.59% of all bone malignancies, making it the most common type. The highest number of cases occurred during the second decade of life.[3] Osteosarcoma is a type of cancer that mostly affects the bones of the body. It is more common in men than women, with a ratio of 3:2. This is because men have a longer period of bone growth than women. The most common age range for osteosarcoma is between 10 and 25 yr. However, it can also occur in people who are 60 y.o. or older, which is why it is known as a cancer with bimodal distribution.[4] Before the advent of effective chemotherapy, the overall 2-yr survival rate for patients with osteosarcoma was between 15% and 20% after undergoing surgical resection and radiotherapy.[5]

Uncontrolled cell proliferation and growth are key characteristics of cancer. Cyclin-dependent kinases (CDKs) play a vital role in regulating the cell cycle. However, due to genetic and epigenetic changes that affect their regulatory pathways, CDKs are often overexpressed or overactive in tumors. This leads to a loss of checkpoint integrity, resulting in uncontrolled cell growth and malignant transformation.[6,7] Among CDKs, cyclin-dependent kinase-4 (CDK4) is a type of kinase that plays a crucial role in regulating the G1-S phase of the cell cycle. It does this by deactivating a tumor suppressor protein called retinoblastoma (Rb) in both cancer cells and during cell division. When stimulated to proliferate, CDK4 combines with cyclin D1 to induce the phosphorylation of Rb (pRb) and weaken its gene suppressor function. This leads to pRb no longer being able to bind to the transcription factor E2F, which in turn promotes the transcription of multiple cell cycle and anti-apoptotic genes, ultimately causing cancer cells to grow.[8]

In osteosarcoma, Rb and p53 gene abnormalities were commonly observed. The Rb protein family, has three distict binding domains and interacts with the E2F transcription factor family, a regulatory protein. The E2F transcription family consists of six members, with each E2F member having a DNA binding and dimerization domain. E2F-1 to E2F-5, which stimulate transcription, binds to various Rb protein families, including pRb, pRb2/p130, and p107, and is regulated by the cell cycle. E2F-1, E2F-2, and E2F-3 bind to pRb, while E2F-4 and E2F-5 bind to p107 or p130. By adolescence, 70% of all osteosarcomas contain Rb mutations. Alteration in mesenchymal progenitor cells and initiation of osteosarcoma due to loss of Rb and p53 have been demonstrated *in vivo*.[8]

Cyclin-dependent kinase-4 has recently been identified as a potential therapeutic target in several types of cancer, including human breast cancer, liposarcoma, glioblastoma, and melanoma. Due to its significance in cancer cells, CDK4 inhibitors become one of the promising candidates for the treatment of various cancers.[9] Several investigators conducted immunohistochemical studies using a human osteosarcoma tissue microarray to evaluate CDK4 expression and their correlation with pathological characteristics and clinical significance in patients with osteosarcoma. Immunohistochemistry staining revealed that the nucleus of

osteosarcoma cells was immunoreactive with CDK4, consistent with in vitro study where CDK4 expressed predominantly in the nucleus of osteosarcoma cells using immunofluorescence tests.[7]

This study aimed to investigate an association between the expression of CDK4 and the survival of patients with osteosarcoma. Several findings suggest that CDK4 plays a crucial role in suppressing tumor suppressor gene function, and it may determine the survival of patients with malignancies.

MATERIAL AND METHODS

Samples and data collection

This cross-sectional study was conducted at the Department of Anatomical Pathology of Dr. Sardjito General Hospital in Yogyakarta, from January 1, 2012, to June 30, 2020. The study involved patients who were clinically, radiologically, and pathologically diagnosed with osteosarcoma. Fifty eligible patients' formalin-fixed paraffin-embedded (FFPE) samples were collected for the study. Two pathologists reviewed and reclassified all pathological specimens according to the WHO Classification of Tumors of Soft Tissue and Bone. The research protocol has been reviewed and approved by the Medical and Health Research Ethics Committee, Faculty of Medicine, Public Health and Nursing, Universitas Gadjah Mada/Dr. Sardjito General Hospital (KE/FK/1393/EC/2019).

Immunochemistry staining

Samples of FFPE were tested for osteocalcin (R&D; Biocare Medical, USA) to confirm the diagnosis of osteosarcoma. Subsequently, the samples were sliced into 3µm thickness for CDK4 immunostaining examination. Sections of FFPE were incubated, deparaffinized, and rehydrated. Antigen retrieval was conducted with the use of a decloaking chamber (Biocare Medical, USA). The mouse monoclonal antibody against CDK4 (Biocare Medical, USA) was used by diluting in phosphate buffer saline by a 1:100. Diamino-benzidine was applied to visualize the positive cells.

Interpretation of CDK4 immunostaining results

An independent pathologist evaluated CDK4-positive cells in a high-power field under a light microscope to assess CDK4 expression using the average method. The assessment was performed by two independent pathologists. The evaluation of CDK4 expression was adapted from Zhou *et al.*[7] by dividing the result into six groups: 0, no stained found in the tumor cell nucleus; 1+: positively stained in <10% of tumor cell nucleus; 2+: positively stained in 10% -25% of the tumor cell nucleus; 3+: positively stained in 26% -50% of the tumor cell nucleus; 4+: positively stained in 51% -75% of the tumor cell nucleus; 5+: positively stained in > 75% of the tumor cell nucleus. Tumors with a staining score of ≥3 were categorized into groups with overexpressed CDK4, and ≤2 were classified with underexpressed CDK4.

Statistical analysis

The association of CDK4 with overall survival was analyzed using the Kaplan-Meier curve. A p-value < 0.05 was considered statistically significant.

RESULTS

The demographic and clinicopathological features of the research subjects are summarized in TABLE 1. Two observers' data from the measurement of CDK4 were tested for Cohen's Kappa reliability to determine the consistency of measurements. The results showed good consistency with

a value of 0.673, which falls within the range of 0.61-0.80. The study consisted of 50 patients whose average age was 25.44 y.o. Out of these patients, 31 (62%) were younger than 25 y.o., 16 (32%) were aged between 25-59 y.o., and three patients (6%) were over 59 y.o. The study also included 32 male patients (64%) and 18 female patients (36%). Furthermore, 39 patients (78%) had primary tumors in their extremities, while 11 patients (22%) had primary tumors not located in the extremities area (TABLE 1). Out of the 50 samples assessed for histological grading, 46 of them (92%) were classified as high-grade osteosarcomas, while only 4 samples (8%) were classified as low-grade osteosarcomas. The majority of the osteosarcoma types were conventional osteosarcomas, which included osteoblastic and chondroblastic subtypes (FIGURE 1). Each subtype accounted for 13 samples, which is 26% of the total samples assessed. Based on the radiological examination, 23 out of 50 samples (46%) showed metastases, while the remaining 27 samples (54%) showed no evidence of metastasis. At the time of sample collection, 32 patients (64%) had already passed away, while the remaining 18 patients (36%) were still alive as of June 2020 (TABLE 1).

TABLE 1. Demographic and clinicopathological features of the subjects

Characteristics	n (%)
Sex	
• Male	32 (64.0)
• Female	18 (36.0)
Age group (yr)	
• < 25	31 (62.0)
• 25-59	16 (32.0)
• ≥ 60	3 (6.0)
Subtype	
• Osteoblastic	13 (26.0)
• Fibroblastic	4 (8.0)
• Chondrobalstic	13 (26.0)
• Giant cell rich	5 (10.0)
• Epithelioid	2 (4.0)
• Teleangiectatic	3 (6.0)
• Fibroblastic with chondroblastic parts	1 (2.0)
• Giant cell rich with fibroblastic parts	1 (2.0)
• Giant cell rich with chondroblastic parts	1 (2.0)
• Extraskeletal	3 (6.0)
• Parosteal	2 (4.0)
• Periosteal	1 (2.0)
• Low-grade intraosseous (central)	1 (2.0)

TABLE 1. Demographic and clinicopathological features of the subjects (cont.)

Characteristics	n (%)
Metastasis	
• Without metastasis	27 (54.0)
• Metastasis	23 (46.0)
Location	
• Extremity	39 (78.0)
• Non-extremity	11 (22.0)
Histologic grading	
• Low grade	4 (8.0)
• High grade	46 (92.0)
Pathologic staging	
• Unstaging	20 (40.0)
• Staging	30 (60.0)
T (Size)	
• Tx	0 (0.0)
• T0	0 (0.0)
• T1	5 (10.0)
• T2	25 (50.0)
• T3	0 (0.0)
N (Lymph nodes)	
• Nx	23 (46.0)
• N0	6 (12.0)
• N1	1 (2.0)
M (Metastasis)	
• Mx	29 (58.0)
• M0	0 (0.0)
• M1a	0 (0.0)
• M1b	1 (2.0)
Status	
• Alive	32 (64.0)
• Died	18 (36.0)

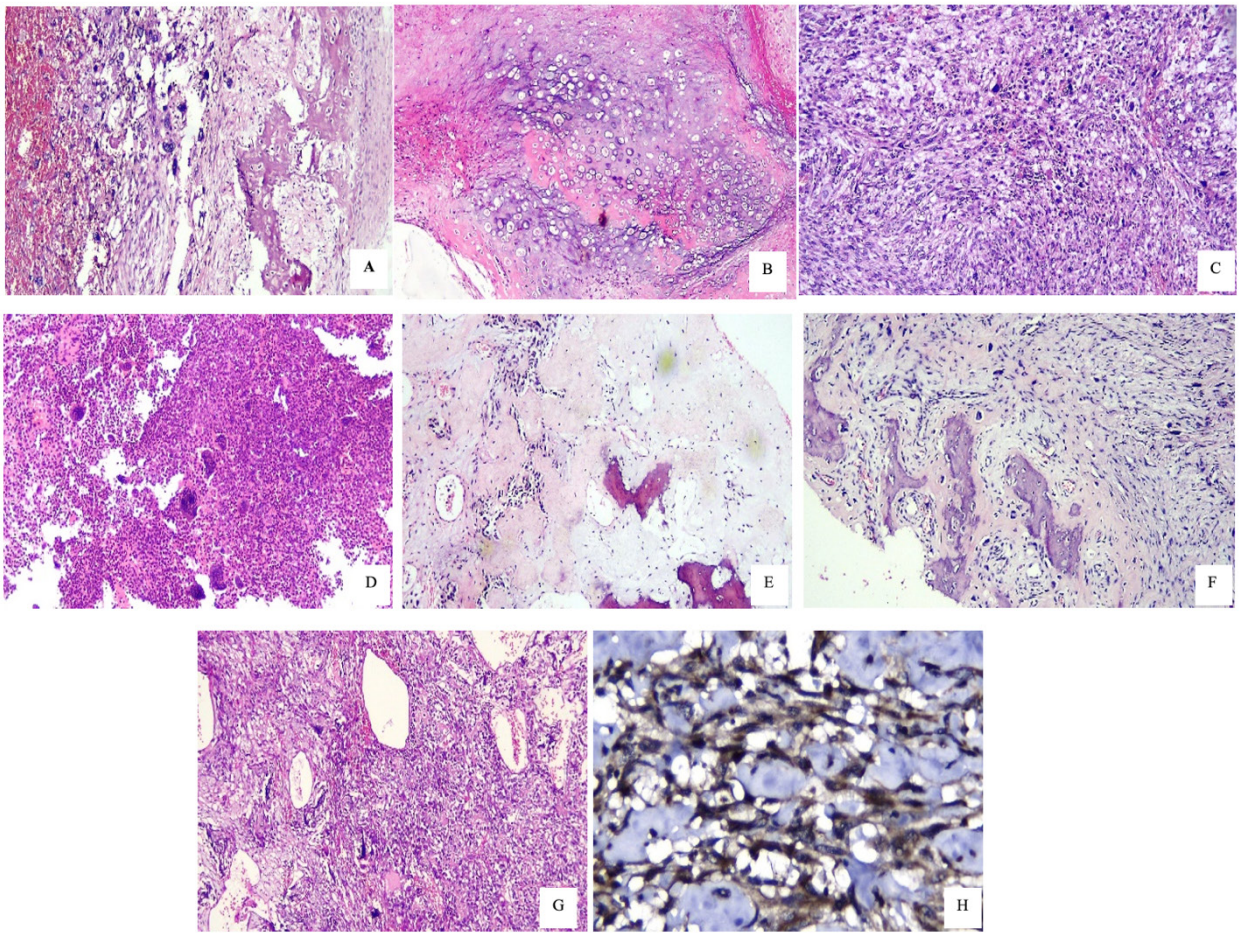

FIGURE 1. Representative images of osteosarcoma subtype. Osteoblastic osteosarcoma (HE 100x); B. Chondroblastic osteosarcoma (HE 100x); C. Fibroblastic osteosarcoma (HE 100x); D. Giant cell-rich osteosarcoma (HE 100x); E. Periosteal osteosarcoma (HE 100x); F. Parosteal osteosarcoma (HE 100x); G. Telangiectatic osteosarcoma (HE 100x); H. Immunohistochemical staining of osteocalcin expressed in the cytoplasm of tumor cells (original magnification 400×).

No significant relationship in CDK4 expression with all external variable was observed in this study (TABLE 2) including sex (p = 1.000), histological grading (p = 1.000), metastasis (p = 0.750), age (p = 0.729), pathological staging (p = 0.427), tumor location (p = 0.424), and mortality status (p = 0.309).

A study for up to 90 mo from 50 osteosarcoma patients with complete clinical information to investigate the association between CDK4 expression and overall survival in osteosarcoma patients was conducted. Of 50 samples, 38 (76%) expressed CDK4, while 12 (24%) did not. Among those expressing CDK4, low expression was found in 20 samples (52.6%), and high expression of CDK4 was found in 18 samples (47.4%) (FIGURE 2).

The survival of from 6 mo to 3 yr were presented in FIGURE 3. Overall survival in the patients with negative CDK4 stain was 23.6 mo, while the patients with positive CDK4 stain was 23.4 mon (TABLE 3). Overall survival in the negative CDK4 group was 0.2 mo longer than in the positive CDK4 group (FIGURE 4). However, it did not show a significant difference (p = 0.821) (TABLE 3). This study also found that CDK4 was not a significant predictor of mortality (p = 0.823) (TABLE 4).

TABLE 2. Characteristics of study subjects with CDK4 expression

Characteristics	CKD4 expression		p
	Negative	Positive	
Total subjects	12 (24.0)	38 (76.0)	
Sex			
• Male	8 (66.7)	24 (63.2)	1.000**
• Female	4 (33.3)	14 (36.8)	
Age group			
• < 25	7 (58.3)	24 (63.2)	
• 25-59	4 (33.3)	12 (31.6)	0.729***
• ≥ 60	1 (8.3)	2 (5.3)	
Metastasis			
• Without metastasis	6 (50.0)	21 (55.3)	0.750*
• Metastasis	6 (50.0)	17 (44.7)	
Location			
• Extremity	8 (66.7)	31 (81.6)	0.424**
• Non-extremity	4 (33.30	7 (18.4)	
Histologic Grading			
• Low grade	1 (8.3)	3 (7.9)	1.000**
• High grade	11 (91.7)	35 (92.1)	
Pathologic staging			
• Localized	7 (100)	21 (91.3)	
• Regional	0 (0.0)	1 (4.3)	0.427***
• Distant	0 (0.0)	1 (4.3)	
Status			
• Alive	6 (50.0)	26 (68.4)	0.309**
• Died	6 (50.0)	12 (31.6)	

*:Chi-square; **:Fisher exact test; ***:Mann Whitney

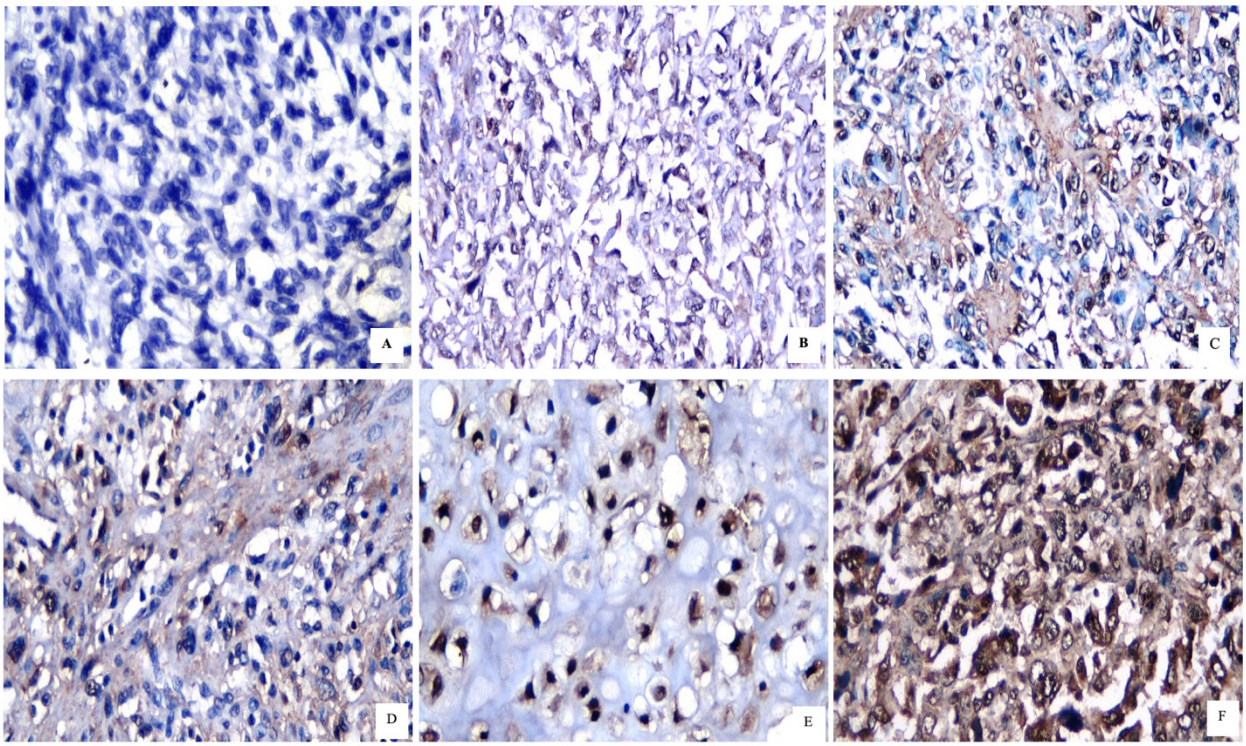

FIGURE 2. Representative images of different immunohistochemical staining intensities of CDK4. Based on the percentage of cells with positive nuclear staining, CDK4 staining patterns were categorized into six groups: 0, no nuclear staining (A); 1+: <10% of positive cells (B); 2+, 10%-25% of positive cells (C); 3+, 26%-50% of positive cells (D); 4+, 51%-75% of positive cells (E); and 5+, >75% of positive cells (F). Original magnification 400×.

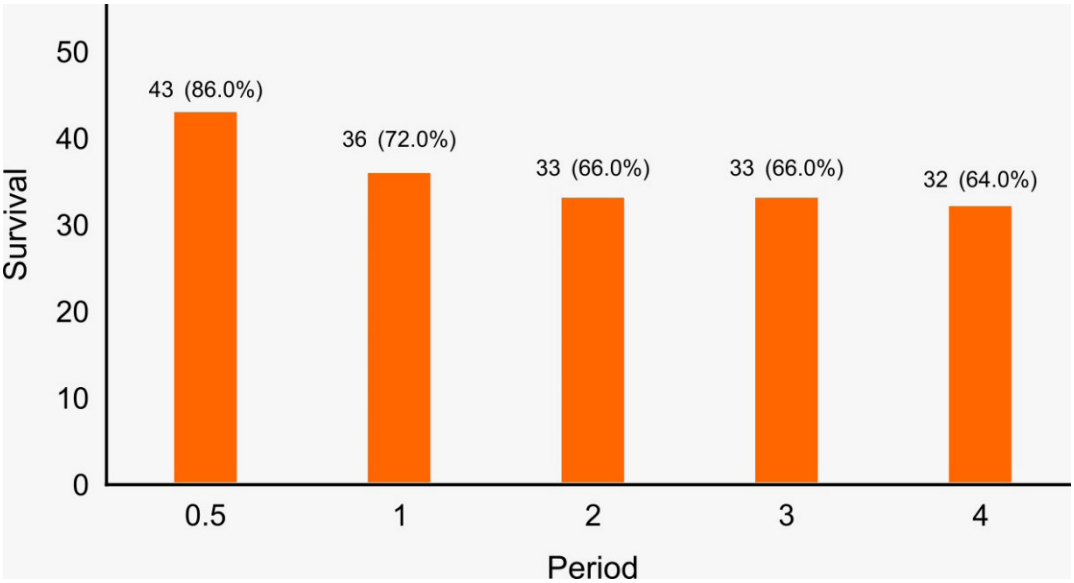

FIGURE 3. Overall survival of patients with osteosarcoma at the Departement of Anatomical Pathology, Sardjito General Hospital, Yogyakarta 2012-2020

TABLE 3. Kaplan Meier test

Variable	Expression	Event [n (%)]	Mean survival	p
CDK4	Negative	6 (50.0)	23.6	0.821
	Positive	12 (31.6)	23.4	

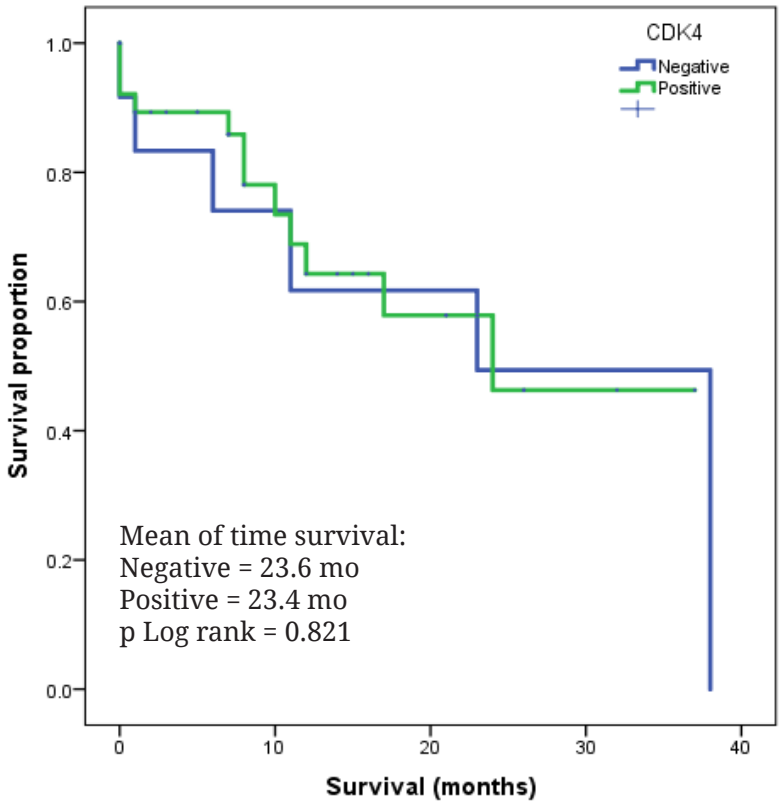

FIGURE 4. Kaplan-Meier curve of osteosarcoma patient survival based on CDK4 expression (positive and negative)

TABLE 4. Cox regression test

Variable	Expression	Univariate Cox regression		
		p	HR	95% CI
CDK4	Negative Positive	0.823	0.89	0.31-2.54

DISCUSSION

In recent years, CDK4 has been found to be a potential therapeutic target in several types of cancer, including human breast cancer, liposarcoma, glioblastoma, and melanoma. This finding has prompted the invention of a CDK4 inhibitor, considered a promising treatment for many types of cancer.[10] Jiang *et al.*[11] reported that CDK4 expression in tumor cell nuclei could be a potential marker for developing nasopharyngeal cancer and its poor prognosis. Zhou *et al.*[7] who were the first to determine the expression of CDK4 in osteosarcoma also reported that most of the osteosarcoma tissue is highly expressed CDK4, in which overexpression of CDK4 is associated with decreased survival rates. The CDK4 expression of the non-survivor samples was significantly higher than the CDK4 expression in the survivor samples. CDK4 can mediate cell proliferation and cell migration, affecting the progression of cancer, increasing the potential for metastasis, worsening prognosis, and affecting the response to chemotherapy; thus, it will be very promising if CDK4 inhibitor was developed as a treatment for osteosarcoma. It is hoped that using CDK4 inhibitors will improve the prognosis and survival of osteosarcoma patients.[7]

In this study, no significant relationship between CDK4 expression and all external variables, including sex, age, tumor location, histological grading, pathological staging, metastasis, and mortality was observed ($p > 0.05$). The survival of osteosarcoma patients for 6 mo; 1; 2; and 3 yr was 86.0; 72.0; 66.0; and 66.0%, respectively. This result was lower at 6 mo and 1 yr but higher at 2 yr and 3 yr in comparison to the results of a study at Shenzhen Second People's Hospital, which had an overall survival of 6 mo; 1 yr, 2 yr, and 3 yr of 96.87; 92.96; 61.71; and 42.18%, respectively.[12] The overall survival of this study is also lower than the results from other studies in other countries, with a relatively high 5-yr overall survival. EURAMOS (European and American Osteosarcoma Study) showed an overall survival of 79 and 71% for 3 and 5 yr, respectively.[13] Other than that, this study also has lower overall survival than a study in Taiwan, which showed a 5-yr survival rate of 67.7%.[14] This result is most likely due to therapy as Mirabello *et al.*[15] performed a clinical trial in the early 1980s, where chemotherapy administration before and after definitive surgical resection resulted in a significant increase to around 70% in the 5-yr survival rate.

Overall survival in the negative CDK4 group was 0.2 mo longer than the positive CDK4 group. However, it was not statistically significant ($p= 0.821$). Furthermore, CDK4 was also not significant predictor of mortality ($p= 0.823$). Overexpression of CDK4 does not significantly indicate a lower survival rate, and underexpression of CDK4 does not significantly indicate a higher survival rate. Some authors reported that complete resection of pulmonary metastatic lesions in patients with osteosarcoma and pulmonary metastases can help predict their survival. The survival rate is influenced not only by a single factor but also by a combination of other factors, including metastasis, tumor location, grading and staging, complications, and environments.[16]

CONCLUSION

In conclusion, CDK4 is highly expressed in osteosarcoma. However, the overexpression of CDK4 may not directly affect the survival rate in osteosarcoma. Other factors need to be considered to understand the complexity of the disease.

ACKNOWLEDGEMENT

The researcher states that it has no competing interests.

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