RESEARCH ARTICLE

Editorial Process: Submission:10/30/2017 Acceptance:01/17/2018

Expression of CDK6 in Oral Squamous Cell Carcinomas

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Abstract

Background: CDK6 is the key factor in regulation of the cell cycle and essential for passage into the G1 phase. It also plays an important role in the development of various tumors. In this cross-sectional study expression of the CDK6 protein in oral squamous cell carcinoma (OSCC) and healthy oral mucosa of controls was assessed to determine relations with malignant transformation and clinicopathologic factors. **Method:** A total of 60 samples, 45 from OSCCs and 15 from healthy tissue, underwent immunohistochemistry for CDK6. Nuclear and cytoplasmic staining of keratinocytes was considered as positive and the percentages of positive cells were calculated. **Results:** Expression of CDK6 was detected in 55.6% of OSCC samples (25 cases) and 13.3% of controls (2 cases), the difference being significant. Mean percentage of CDK6 stained cells was 24.2±29.3 in the OSCC cases and 4.33±2.1 in the control group, again statistically significant. No relationship was detected between CDK6 expression and clinicopathologic factors. **Conclusion:** Overexpression of CDK6 observed in OSCC points to a role for this protein in oral carcinogenesis.

Keywords: CDK6- squamous cell carcinoma- immunohistochemistry

Asian Pac J Cancer Prev, **19 (4)**, 1013-1016

Introduction

Cyclin-dependent kinases (CDKs) are a family of protein kinases that are initially identified based on their regulative role in the cell cycle. They regulate transcription, mRNA processing and differentiation of nerve cells (neurons) (Morgan, 2007). They have a role in cell cycle regulation in eukaryotes.

CDKs are small proteins with a molecular weight of 34 to 40 KD (Morgan, 2007). CDK binds to protein regulators called cyclin. CDKs have a minor kinasic activity, and active kinasic role is only seen in cyclin-CDK complexes (Satyanarayana and Kaldis, 2009).

CDKs phosphorylate their substrates in serine and threonine regions, so they are serine-threonine kinases (Radpour et al., 2009). According to the recent studies on human and mouse genomes, 11 genes encodes CDKs (Elledge and Spottswood, 1991; Engstrom et al., 2006; Forsburg, 2005; Gao et al., 2006; Jaafari Ashkavandi et al., 2010; Malumbres and Barbacid, 2005; Malumbres et al., 2009; Matsushime et al., 1992; Ninomiya-Tsuji et al., 1991; Ogurtsov et al., 2008; Paris et al., 1991; Tsai et al., 1991).

Most of cyclin-CDK complexes control progressing of the cell cycle and membrane transportation. In the cell cycle, after cytoplasmic division of the cell, newly-produced cells can continue proliferation or stop cell division. The undivided cells enter quiescence phase or G0. Those cells which continue dividing enter G1 phase of the next cycle (Malumbres, 2014). Passing from G1 to the S phase is controlled by complex mechanisms in which at least three types of CDKs and their regulators including CdK2, CdK4, and CdK6 play important roles (Besson et al., 2008; Malumbres, 2014).

At the beginning, mitogenic signals lead to cyclin-D synthesis, besides folding and transportation of CdK4 or CdK6 to the nucleus. Two active complexes of CycD-CdK4 or CycD-CdK6 phosphorylate the family members of retinoblastoma proteins (Rb) including PRb, P107, and P130 (Malumbres, 2014). It results in separation of Rb from E2F transcription factors, and subsequently develops the cell cycle from stage G1 into phase S (Serrano et al., 1993).

Any disturbance in regulation of the Rb pathway triggers the carcinogenesis and leads to cancer (Poomsawat et al., 2010). Relevant studies on various tumors such as squamous cell carcinoma (SCC) were more focused on evaluation of the level of these markers and the molecular mechanisms of their activity. Despite the few studies on the relationship between this marker and the clinicopathologic factors, no study was conducted on SCC in this field. Hence, the present study aimed to discuss the relationship between the expression of CDK6 in patients with oral SCC (OSCC) and the clinicopathologic factors.

Materials and Methods

This cross-sectional study was performed on 45 patients with OSCC (grad I, II, and III) including 30 males and 15 females with the mean age of 52.3 years

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who referred to Khalili Hospital, Shiraz, Iran; as well as a control group. A control group was considered as irritation fibrosis consisting of 15 cases including 4 males and 11 females with the mean age of 50.5 years old.

The H and E slides of the available blocks were reviewed, and cases with definite diagnosis and adequate cellular tissue were selected for immunohistochemical staining (IHC). The IHC staining was performed by using Envsion Labled Peroxides System (DAKO; Carpentaria, CA, USA). The samples were fixed in 10% buffered formalin and embedded in paraffin. Then, 4-µm sections were prepared, deparaffinized in xylene, rehydrated in graded alcohol, and washed with distilled water. Antigen retrieval was performed by using DAKO cytomation target retrieval solution (pH=9) for 20 minutes. Endogenous peroxidase activity was inhibited by 3% H_2O_2. The tissue sections were incubated for 30 minutes with anti-CDK6 antibody (Santa Cruz Biotechnology; SD-7961, USA) at a 1/50 dilution.

Normal samples were stained with the same amount of antibody used for staining the tumoral tissues. Omission of primary antibody was employed as negative control, while gastric epithelium was used as positive control for CDK6. Brown nuclear staining and cytoplasmic staining were considered as positive for CDK6.

Two pathologists interpreted the results of immunohistochemistry. Immunoreactivity was expressed by determining the percentage of positive tumor cells. Neoplastic cells were counted in five area at 400x magnification (Aune et al., 2011). The obtained data were analyzed by using SPSS software (version 16; IL, USA). Mann-Whitney test was used to compare the two groups regarding the CDK6 expression. Kruskal-Wallis test was used to compare the CDK6 expression between the case and control group, as well as, among the patients with different grades at different stages.

Table 1. Clinicopathological Profile of 45 Patients with Oral Squamous Cell Carcinoma

Variables		N (%)
Mean Age	52.3	
Sex	Male	30 (66.7%)
	Female	15 (33.3%)
Tumor Size	T1	15 (33.3%)
	T2	20 (44.4%)
	T3	10 (22.2%)
Regional lymph node involvement	N0	28 (62.22%)
	N1	8 (17.78%)
	N2	7 (15.56%)
	N3	2 (4.44%)
TNM Stage	I (well-differentiated)	27 (60%)
	II (moderately-differentiated)	14 (31.11%)
	III (poorly-differentiated)	4 (8.89%)
Histological Grade	I (well-differentiated)	27 (60%)
	II (moderately-differentiated)	14 (31.11%)
	III (poorly-differentiated)	4 (8.89%)

Results

This study investigated the expression of CDK6 in 45 patients with OSCC to find if this marker or other clinicopathologic features are related to this disease. It was done on 45 patients including 30 males and 15 females with the mean age of 52.3 years and 15 controls including 4 males and 11 females with the mean age of 50.5 years. Table 1 shows the clinical data of the studied patients. In the current study, both nuclear and cytoplasmic expressions of CDK6 were observed; however, it was mostly cytoplasmic in normal tissues, and predominantly

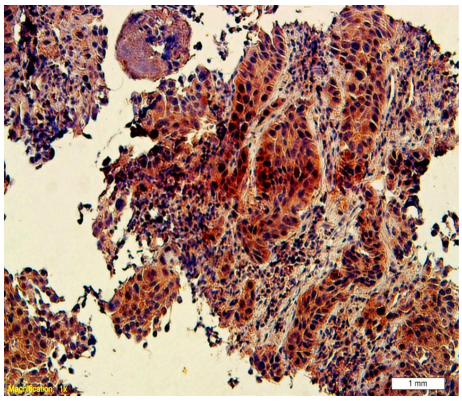

Figure 1. Positive Nuclear and Cytoplasmic Expression of CDK6 in OSCC (200X)

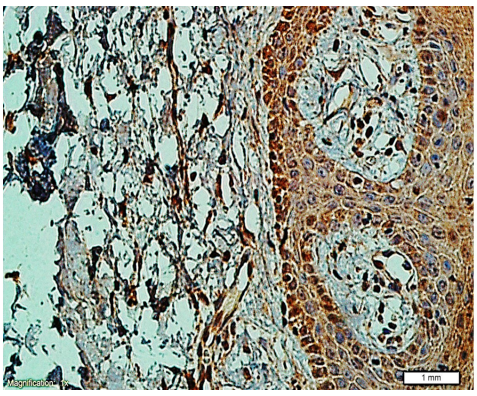

Figure 2. Positive Nuclear and Cytoplasmic Expression of CDK6 in Normal Mucosa (200X)

Table 2. Comparative CDK6- Positivity in Patients with Different Clinicopathologic Characteristics

	Clinicopathologic Factors	CDK6 staining Percentage (Average)	P-value
Size of Lesion	T1	22 ± 25.7	0.23
	T2	30.5 ± 31.9	
	T3	15 ± 29.1	
Lymph Node Involvement	Involved	23.9 ± 29.5	0.96
	Non-involved	24.7 ± 30	
Stage	1	19.3 ± 24.3	0.49
	2	36.7 ± 34.7	
	3	22.7 ± 31.6	
	4	18.9 ± 27.1	
Grade	1	20.4 ± 23.1	0.87
	2	27.1 ± 34.3	
	3	40 ± 48.9	

nuclear in tumoral tissues.

The frequency of CDK6 positivity in the case and control groups was found to be 55.6% (25 cases) and 13.3% (2 cases), respectively, indicating a statistically significant difference between the two (P=0.01). The mean percentage of CDK6 staining in the control group (4.33±2.1) was significantly lower than that in the case groups (24.22±29.3) (P=0.02) (Figure 1 and 2). The results of Mann-Whitney test revealed no relationship between the clinicopathologic characteristics and CDK6 staining (Table 2).

Discussion

Disorientation in cell cycle regulation is a fundamental process in tumor growth. It is a common phenomenon in many types of tumors including OSCC (Dobashi et al., 2004; Mendrzyk et al., 2005).

The present study reported increased incidence of CDK6 (one of the key factors in passing from the G1 to S phase) in OSCC tissue compared with the normal mucosa. Likewise, other studies showed an increased expression of CDK6 in tumors such as medulloblastoma (Mendrzyk et al., 2005) T-Cell Lymphoma and malignant glioma (Tadesse et al., 2015), as well as, OSCC (Chen et al., 1999; Koontongkaew et al., 2000). Increased expression of this marker in OSCC indicates its contribution in the process of carcinogenesis (Poomsawat et al., 2010). In a study, the incidence of CDK6 was observed in 60% of OSCC samples and 8% of normal mucosa slides, which was similar to the results of the present study (Poomsawat et al., 2010).

Many studies reviewed the CDK6 and reported lots of changes including point mutations and genetic rearrangement in the gene of this marker which could be involved in tumorogenesis. In other words, these genetic changes during every stage of DNA transcription can induce tumorogenesis and increase the expression of these markers in tumors.

Another mechanism that increases the CDK6 activity in tumors is the absence of P16 protein expression in these cells (Patel et al., 1997). P16 is a kinase inhibitor that acts as a negative regulator of the cell cycle. Binding of this protein to CDK6 inhibits its binding to D-cyclin, and consequently blocks the G1 phase that inhibits cell proliferation (Patel et al., 1997). These mechanisms (genetic variation in CDK6 and lack of P16 expression) ultimately result in hyper-phosphorylation of Rb protein and passing through G1 phase.

According to our findings, CDK6 expression was observed in both the nucleus and cytoplasm; although, it was chiefly cytoplasmic in normal tissue and mainly nuclear in tumoral tissues. Meanwhile, Poomsawat et al., (2010) observed only nuclear staining of the marker in OSCC samples. They reported the mean staining for these markers to be 0.17% in normal mucosa and 5.04% in OSCC samples, which is lower than our results (Poomsawat et al., 2010). The difference might be due to the differences in the staining patterns, since in our study, the cytoplasmic staining of this marker was significantly higher than the nuclear staining.

Cytoplasmic staining of CDK6 was reported in several studies (Ericson et al., 2003; Poomsawat et al., 2010; Poomsawat et al., 2016); however, the significance and function of the cellular distribution of these markers are still poorly understood. Lower nuclear staining may indicate that a part of the CDK6 activity is regulated by breaking this kinase in the cytoplasmic and nuclear staining; or it can be an indication of a new function of this marker which is still unknown (Kohrt et al., 2009). In line with the first theory asserting the necessity of breaking CDK6 in the cytoplasm to activate it in the nucleus, a study reported the presence of CDK6 in the cytoplasm and nucleus of T-Cell; however, only nuclear CDK6 was active and capable of Rb phosphorylation (Yoon et al., 2010).

Studies proposed some functions for CDK6 in the cytoplasm. For instance, Slomiany et al. claimed that the increased staining of CDK6 in the cytoplasm changed the dynamics of actin filaments and increased the mobility of rat astrocytes (Slomiany et al., 2006). Moreover, Fahraeus and Lane detected CDK6 in folded edges of fibroblast cells and asserted its contribution in the proliferation and movement of these cells (Fahraeus and Lane, 1999).

Recently, the role of CDK6 in cell differentiation has been asserted. According to CDK6 role as a coordinator in cell proliferation and differentiation, this protein is expected to have an active function in both the nucleus and cytoplasm. It means that CDK6 can be involved in regulating transcription in the nucleus and also in remodeling of cytoskeleton in cytoplasm (Kohrt et al., 2009).

Numerous studies showed that CDK6 inhibited cell differentiation (Ericson et al., 2003; Ogasawara et al., 2004). Therefore, higher amount of this marker is expected in higher grades of SCC. However, the present study detected no correlation between the expression of CDK6 and histological grade. Nor was any correlation observed between these markers positivity and clinicopathologic factors. It implies that the expression of these markers : may have no role in prognosis of OSCC patients.

Poomsawat et al., (2020) observed increased staining of this marker at the invasive front of tumor. They suggested this incidence to be probably associated with the motility and invasion of tumor cells. However, the use of excisional and also incisional biopsy samples, in the current study, made it impossible to evaluate the invasive front of tumors in all samples. Hence, further studies are suggested to investigate the relationship between development of this marker at the invasive front of tumors and the clinicopathologic factors. Moreover, examining only the expression of these markers, this study missed evaluation of the mechanisms associated with increased expression. Therefore, assessment of the mechanisms of the increased incidence of these markers is also recommended to be thoroughly studied.

To sum up, the increased expression of CDK6 in OSCC tissue in comparison with normal mucosa, indicated the importance of role of this marker in oncogenesis; which promotes this marker to be used as a treatment target. Furthermore, the significant cytoplasmic incidence of this marker indicated the new function of this marker in the cytoplasm. However, there was no correlation between the

expressions of this marker with clinicopathologic factors, implying that CDK6 would not have a prognostic role.

Acknowledgments

The authors would like to thank the Vice-Chancellery of Research at Shiraz University of Medical Sciences for providing financial support for this study (Grant#90-3648). This manuscript was extracted from the post graduate thesis conducted by Dr. Naghmeh Jeiroodi.

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