RESEARCH COMMUNICATION

Cyclin D1 Amplification in Tongue and Cheek Squamous Cell Carcinomas

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Abstract

Introduction: Several molecular markers have been studied for their usefulness as prognostic markers in oral squamous cell carcinoma (OSCC). One such molecular marker is cyclin D1 which is a proto-oncogene located on 11q13 in humans. Objective: To explore the feasibility of using cyclin D1 as a prognostic marker in tongue and cheek SCC by the fluorescent-in-situ hybridization (FISH) method. Methods: Fifty paraffin-embedded samples (25 each of cheek and tongue SCCs) were obtained from the archives of the Oral Pathology Diagnostic Laboratory. Sociodemographic data, histopathologic diagnoses, lymph node status and survival data were obtained from the Malaysian Oral Cancer Database and Tissue Bank System (MOCDTBS)coordinated by the Oral Cancer Research and Coordinating Centre (OCRCC), University of Malaya. The FISH technique was used to detect the amplification of cyclin D1 using the Vysis protocol. Statistical correlations of cyclin D1 with site and lymph node status were analyzed using the Fisher exact test. Kaplan-Meier and Log Rank (Mantel-Cox) test were used to analyze cyclin D1 amplification and median survival time. Results: Positive amplification of cyclin D1 was detected in 72% (36) of OSCCs. Detection of positive amplification for cyclin D1 was observed in 88% (22) and 56% (14) of the tongue and cheek tumors, respectively, where the difference was statistically significant (P=0.012). Lymph node metastasis of cheek SCCs showed a trend towards a significant association (P= 0.098) with cyclin D1 amplification whereas the lymph node metastasis of tongue SCC was clearly not significant (P= 0.593).There was a statistically significant correlation between cyclin D1 positivity and survival rate (P=0.009) for overall SCC cases and (P<0.001) for cheek SCC cases. Conclusion: The present study found that cyclin D1 amplification may differ in different subsites of OSCC (tongue vs cheek) and its positive amplification implies an overall poor survival in OSCCs, particularly those arising in cheeks.

Key words: Oral SCC - tongue - cheek - fluorescent-in-situ hybridization

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Introduction

Oral squamous cell carcinoma (OSCC) together with Squamous cell carcinoma (SCC) of the head and neck is ranked as the sixth most common malignancy worldwide (Parkin et al., 1999). It is predominantly a disease of the old age and occurs generally in the fifth and sixth decades (Siar et al., 1990). Nonetheless, younger patients (less than 45 years) (Llewellyn et al., 2003), contracting the disease with or without major risk factors have been reported (Chen et al., 1999; Chitapanarux et al., 2006). Well recognized risk factors include tobacco (Warnakulasuriya et al., 2005) and alcohol (Petti and Scully, 2005) usage. However, in South-East Asia, betel quid chewing has contributed to the prevalence of OSCC in certain ethnic groups (Scully and Bedi, 2000; Zain and Ghazali, 2001).

The treatment for OSCC includes surgery (Petruzzelli et al., 2003), radiation (Yao et al., 2005) or combination of both (Kasperts et al., 2005). However, prognosis of OSCC has not improved much over the past decades (Woolgar et al., 1999). It is generally believed that prognosis is better if the disease is detected, diagnosed and treated early. Besides possible diagnostic delay (Allison et al., 1998), poor prognosis could be due to inherent weakness in current prognostic benchmarks such as the TNM system and histopathological grading. Thus several molecular markers have been studied for

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their usefulness as prognostic markers in OSCC.

One such molecular marker is cyclin D1 which is a proto-oncogene. Cyclin D1 is located on 11q13 in humans. This region is commonly amplified in several types of cancer including SCC (Sauter et al., 2000). Amplification by cytogenetic methods and overexpression by immunohistochemistry (IHC) of cyclin D1 is found in 20 to 40% and 40 to 80% of HNSCCs respectively. These genetic abnormalities are significantly correlated to the clinical behavior of the tumor such as aggressive tumor growth, recurrence, poor prognosis and lymph node metastasis (Shinozaki et al., 1996; Koyomoto et al., 1997; Nagasawa et al., 2001; Miyamoto et al., 2002; Namazie et al., 2002; Miyamoto et al., 2003; Myo et al., 2005). Therefore the amplification of cyclin D1 may be a valuable biologic marker of poor prognosis, tumor aggressiveness and recurrence (Shinozaki et al., 1996; Koyomoto et al., 1997; Nagasawa et al., 2001; Namazie et al., 2002).

Southern blot hybridization, polymerase chain reaction (PCR) and IHC methods have been used to evaluate cyclin D1 deregulation (Miyamoto et al., 2002). Inherent problems are present when these methods are used in evaluating molecular genetic markers in OSCCs. OSCC specimens are too small to yield adequate purified DNA for analysis by Southern blot technique. Although, PCR is suitable for use with small amounts of DNA but contamination with normal cells may cause misinterpretation of data. The disadvantage of IHC is that it cannot identify genetic abnormalities such as chromosome rearrangements, amplifications and deletions. Moreover, the presence of antigen specific antibodies is critical in the analysis with IHC. Conversely, fluorescence in situ hybridization (FISH) can be used to overcome these disadvantages, as this technique requires very little tumor tissue, rapid, does not use purified DNA and the results are not affected by the DNA degradation (Myo et al., 2005). FISH technique uses metaphase or interphase chromosomes to detect gene loci and to obtain numerical information such as translocation, rearrangement, deletion and amplification involving specific chromosomal regions as loss or gain of fluorescent signals in a variety of solid tumors including OSCCs having small specimens (Miyamoto et al., 2002; 2003). Miyamoto et al., (2003) states that cyclin D1 amplification to be a more reliable prognostic indicator than cyclin D1 overexpression in OSCCs.

OSCC occurs in different sub-sites in the oral cavity with differences in their clinical behavior and prognosis. Many studies have reported these differences in clinical behavior and prognosis in SCC of tongue and cheek (Sathyan et al., 2006; Liao et al., 2010). Therefore in the present study we explored the feasibility of using cyclin D1 as a prognostic marker in tongue and buccal mucosa SCC using the FISH method, looking at the association between cyclin D1 amplification with sites, lymph node metastasis based on tumor sites and also survival time.

Materials and Methods

Samples

This was a cross-sectional study utilizing 50 formalin fixed, paraffin embedded tissue (FFPE) samples of OSCC which were retrieved from the archives of the Oral Pathology Diagnostic Laboratory, Faculty of Dentistry, University of Malaya after obtaining approval from the Faculty of Dentistry, Medical Ethics Committee, University of Malaya (MEC ethics approval number is DF OS0905/0017)

OSSC patients from the year 2004 to 2010 who have not received any previous treatment such as radiotherapy or chemotherapy were included in this study. There were 25 samples of cheek SCC and 25 samples of tongue SCC.

Socio-demographic, Histopathology and Survival Period Data

Socio-demographic data such as age, gender, ethnic group and habits was obtained from the Malaysian Oral Cancer Database and Tissue Bank System(MOCDTBS) (Zain et al., 2005) coordinated by the Oral Cancer Research and Coordinating Centre (OCRCC). The histopathological diagnoses and lymph node status were also obtained from the MOCDTBS which have been re-evaluated by AR and an oral pathologist (RBZ). The survival period of all cases was also obtained from MOCDTBS.

Preparation of Samples

The FFPE tumor tissues were identified for each case using the archived diagnostic slide as reference and the areas marked on the slides to ensure the presence of tumor areas in the respective blocks. One new section was further stained with Haematoxylin and Eosin as reference slide and the other following sections of 4 μm thickness were cut and placed on slides. These sections were incubated at 37°C overnight and deparaffinized by washing in xylene, rehydrated in graded ethanol and distilled water.

FISH Analysis of cyclin D1 amplification

After incubation in 0.2 M HCl at room temperature for 20 minutes, the sections were heat-pretreated in citrate buffer (2 × SSC, pH 7.0) at 80°C for 30 minutes. They were then digested with protease buffer at 37°C for 80 minutes, rinsed in 2 × SSC at room temperature for 3 minutes and dehydrated in graded ethanol (70, 85, and 100%) for 2 minutes each.

The SO LSI cyclin D1 DNA probe (Vysis, Inc. Downers Grove, IL, USA) which hybridizes to band 11q13 of human chromosome was used. The centromeric probe for chromosome 11 (alpha satellite) was used for dual color FISH. For each slide, 1-μl of probe was mixed with 2-μl purified H2O and 7μl LSI hybridization buffer and applied to the dry slide. The tissue area was coverslipped and sealed with rubber cement. The slides

were then incubated in a moist chamber (Hybridizer Instrument for in situ hybridization, DAKO, S2450, Denmark) for denaturation at 82°C for 5 minutes and hybridization at 37°C for about 16 hours.

Post hybridization washes were performed the following day using 0.4 × SSC/0.3% NP -40 at 73°C for 2 minutes to remove non-specifically bound probe and in 2 × SSC/0.1% NP-40 at room temperature for 2 minutes and after application of 5 μL of mounting medium containing 4',6'-diamidino-2-phenylindole (DAPI), the tissue area was coverslipped. These slides were viewed under a fluorescence microscope (BX 16, OLYMPUS, Tokyo, Japan).

Evaluation of FISH technique and image analysis

Evaluation of the preparation was performed by counting at least 200 nuclei per slide, according to the criteria described by Hopman et al. (1988). Enumeration of the florescent signals was performed in at least 200 nuclei per slide under objective power of 100X, using an Olympus florescent microscope BX61 equipped with single band sets for DAPI, Fluorescein Isothiocyanate (FITC) and spectrum Orange to discriminate the color signals of green for chromosome 11 centromeric DNA and orange for cyclin D1 during scoring. Images for documentation were then captured by using a spectral imaging camera and processed by case data manager expo 5.0. If the signals ratio of the orange signals to the green signals is 2 or more than 2 then it was considered positive amplification.

Statistical analysis

The data were entered and analyzed using SPSS 18 software (SPSS, Chicago, IL). Chi square or Fisher exact tests were performed to investigate the association between cyclin D1 amplification with tumor sites and lymph node metastasis based on tumor sites whereas Kaplan Meier and Log rank test were employed to compare the median survival time between positive and negative cyclin D1 amplification. Median survival time indicates the time at which the survival is 50% or half of the patients die. Level of significance was set at P less than 0.05.

Results

The socio-demographic and clinico-pathological characteristics of the samples based on tumor site are given in Table 1. Twenty-three cases (46%) in this study showed no regional lymph node metastasis (N0), whereas 27 (54%) had positive lymph node metastasis (N+ve).

Overall, the cyclin D1 amplification was positive in 72% (36 cases) and negative in 28% (14) of cases. For tongue SCC, 88% (22) was positive for cyclin D1 amplification compared to only 56% (14) of cheek SCC. This finding is statistically significant (P = 0.012) (Table 2).

The cyclin D1 amplification was positive in 10 cases (45.5%) and 12 cases (54.5%) of tongue SCC with and without lymph node metastasis respectively whereas, only 1 case (33.3%) and 2 cases (66.7%) of tongue SCC with and without lymph node metastasis were negative for cyclin D1 amplification respectively. These findings are statistically not significant (P = 1.000) (Table 2).

In cheek SCC, positive cyclin D1 amplification was found in 11 cases (78.6%) and 3 (21.4%) with and without lymph node metastasis respectively whereas, 5 cases (45.5%) and 6 cases (54.5%) with and without lymph node metastasis were negative for cyclin D1 amplification respectively. These findings are also not statistically significant (P=0.115) (Table 2).

In patients with positive cyclin D1 amplification, the survival rate was 47.2%, while for those patients with negative amplification the survival rate was higher at 57.1%. Kaplan- Meier survival analysis shows the

Table 1. Socio-demographic and Clinico-pathologic Characteristics of Samples based on Tumor Site (Tongue vs Cheek SCC)

Characteristics	Tumor site		
	Tongue SCC[a]	Cheek SCC[a]	Total[b]
Mean Age[d]	59.24 (14.45)[c]	59.96 (12.38)[c]	60.34 (13.36)[c]
Range[d]	26 - 81	39 - 94	26 - 94
Ethnicity			
Indian	12 (48)	22 (88)	34 (68)
Malay	7 (28)	3 (12)	10 (20)
Chinese	6 (24)	-	6 (12)
Gender			
Male	12 (48)	7 (28)	19 (38)
Female	13 (52)	18 (72)	31 (62)
Risk Habits			
Present	23 (92)	24 (96)	47 (94)
Absent	2 (8)	1 (4)	3 (6)
Lymph node metastasis (pN)			
N0	14 (56)	9 (36)	23 (46)
N+ve	11 (44)	16(64)	27 (54)

[a](n = 25) Frequency (%); [b](n = 50) Frequency (%); [c](SD); [d]years

Table 2. Association Between the Amplification of Cyclin D1 and Tumor Sites, Lymph Node Metastasis and Cyclin D1 Amplification based on Tumor Site (Tongue and Cheek SCC)

Tumor site	Cyclin D1 amplification			
	Positive	Negative	Total	p-value
Tongue	22 (88%)	3 (12%)	25 (100%)	0.012[a]
Cheek	14 (56%)	11 (44%)	25 (100%)	
Total	36 (72%)	14 (28%)	50 (100%)	
Tongue				
N0	12 (54.5%)	2 (66.7%)		1.000[b]
N+ve	10 (45.5%)	1 (33.3%)		
Total	22 (88%)	3 (12%)	25 (100%)	
Cheek				
N0	3 (21.4%)	6 (54.5%)		0.115[b]
N+ve	11 (78.6%)	5 (45.5%)		
Total	14 (56%)	11 (44%)	25 (100%)	

[a]Chi-square test was used; [b]Fisher exact test was used

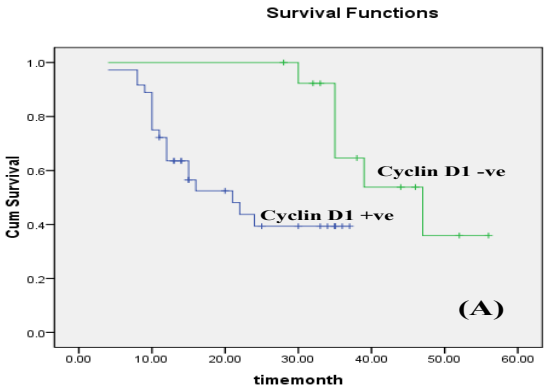

Figure 1. Median Survival Time for the sample (n=50)

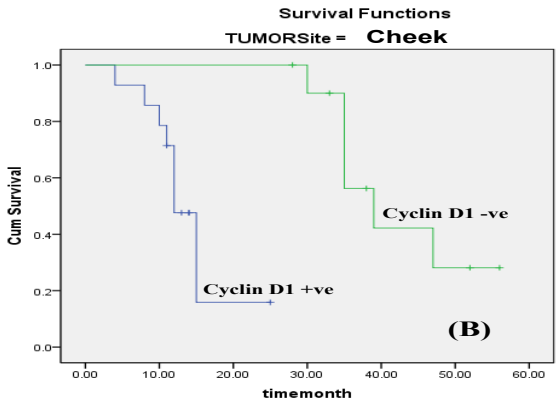

Figure 2. Median Survival Time of Patients with Cheek Cancer (n=25)

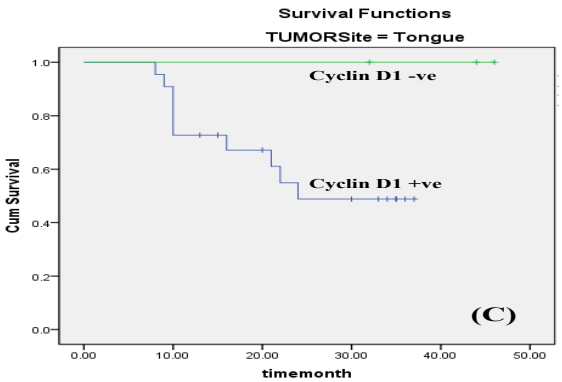

Figure 3. Median Survival Time of Patients with Tongue Cancer (n=25)

overall median survival time was 35 months. For overall SCC, log rank test shows the median survival times were 47 months and 21 months for patients with negative and positive amplifications respectively and the difference was statistically significant (P =0.009) (Figure 1). For cheek SCC, the median survival times was also significantly longer in patients with cyclin D1 negative amplifications (39 months) than in positive (12 months) (P <0.001)(Figure 2). However, for tongue SCC, the median survival time could not be estimated for patients with cyclin D1 negative amplifications as all cases were censored, thus statistical comparison using log rank test was not possible (Figure 3).

Discussion

In the present study, we explored the feasibility of using cyclin D1 as a prognostic marker in tongue and cheek SCC by the FISH method. Patients with HNSCCs, including oral cavity, esophagus, lung and adenocarcinoma of the breast show frequent genetic alteration in chromosome 11q13 loci (Berenson et al., 1989; Kitagawa et al., 1991; Mori et al., 1992; Roelofs et al., 1993; Williams et al., 1993; Merdith et al., 1995; Bekri et al., 1997; Muller et al., 1997). In HNSCC, the amplification of 11q13 may be an important biologic marker for poor prognosis (Merdith et al., 1995). Various oncogenes present in 11q13 amplified region are SEA, EMS1, FGF4, FGF3, protein phosphatase 1 α and cyclin D1 (Schuuring 1995). Several studies have established that the size of 11q13 amplicon varies in different cases but the amplicon always include cyclin D1. Therefore among the putative oncogenes present in the 11q13 region, cyclin D1 is most likely target for 11q13 amplification and is important for the development and progression of HNSCC as it is consistently amplified and overexpressed in tumor cells with 11q13 amplification (Bartkova et al., 1995; Izzo et al., 1998; Jin et al., 1998; Akervall et al., 2002; Hvang et al., 2002).

Cyclin D1 is an important regulator of G1 to S-phase transition in numerous cell types from diverse tissues. Binding of cyclin D1 to its kinase partners, the cyclin dependent kinases 4 and 6 (CDK4/6), results in the formation of active complexes that phosphorylates the Retinoblastoma tumor suppressor protein (Rb). Hyperphosphorylation of Rb results in the release of Rb-sequestered E2F transcription factors and the subsequent expression of genes required for entry into S-phase. More recently, cyclin D1 has also been shown to act as a cofactor for several transcription factors in a CDK independent manner (Coqueret, 2002).

The overexpression of cyclin D1 protein may lead to the unrepaired DNA damage, accumulation of genetic errors and a selective growth advantage for altered cells (Myo et al., 2005). In fibroblasts, the overexpression of cyclin D1 protein induces cellular transformation and genetic instability (Lovec et al., 1994; Asano et al., 1995).

In the present study, cyclin D1 amplification was positive in 72% OSCC cases. Other studies have reported positive cyclin D1 amplification as low as 16% in SCC of oral cavity (Nimeus et al., 2004) to 56.5% in SCC of tongue (Fuji et al., 2001). The variation in positive cyclin D1 amplification in these studies may be due to the reason that some studies include all subsites of SCC of oral cavity (Miyamoto et al., 2003; Myo et al., 2005) whereas others have studied only a particular subsite (Fuji et al., 2001).

The clinical behaviour of tongue and cheek SCC has been shown to be different in different studies (Bell et al., 2007; Shaw et al., 2009). Patients with cheek

SCC have higher propensity for skin and bone invasion and distant metastasis than those of tongue cancer. Conversely, patients with tongue SCC showed a higher rate of lymph node metastasis to the neck than those with cheek cancer (Liao et al., 2010). These differences in the clinical behaviour of SCC of different subsites may be due to different molecular genetic pathways. In the present study, cyclin D1 amplification occurred mostly in tongue SCC which is 88% compared to only 56% in cheek SCC.

The lymph node metastasis of tongue SCC was not significantly associated with the amplification of cyclin D1 (P=1.000). However, Miyamoto et al., (2002, 2003) states that the cyclin D1 numerical aberrations are significantly associated with an invasive tumor phenotype and pathologic lymph node status. Moreover, Myo et al., (2005) has concluded that the aberration in cyclin D1 numbers to be valuable in identification of patients at high risk of late lymph node metastasis in stage I and II OSCCs. Since the sample size of the present study was small (25 cases of cheek SCC), further studies with larger sample size are recommended to evaluate and confirm the association between cyclin D1 amplification and lymph node status.

Log-Rank (Mantel-Cox) test showed that the median survival time for all patients with OSCCs is significantly different between cyclin D1 positive and negative patients (P = 0.009) (Figure 1A). Moreover, the Log-Rank (Mantel-Cox) test of median survival time for patients with cheek cancer was significantly different between cyclin D1 positive and negative patients (P<0.001) (Figure 1B). Lui et al., (2004) and Sathyan et al., (2006) reported a significant association between the expression of cyclin D1 and survival. Eventhough, their studies evaluated the overexpression of the cyclin D1 protein whereas the present study evaluated the amplification of cyclin D1 gene; we further support Lui et al., (2004)'s and Sathyan et al., (2006)'s proposal that cyclin D1 is an important prognostic marker of survival in cheek cancers.

Fuji et al., (2001) showed a significant 5-year disease-free survival rate between cyclin D1 amplification positive and negative patients with tongue SCC whereas, in the present study, the median survival time could not be estimated for patients with cyclin D1 negative amplifications in tongue SCC as all cases were censored (Figure 1C).

In conclusion the present study found that cyclin D1 amplification is higher in tongue cancer as compared to cheek cancer. The shorter median survival time in patients with positive amplification in cheek cancer implies an over all poor survival in cheek SCC with amplification of cyclin D1. Therefore, cyclin D1 may be considered as a prognostic marker for cheek cancer. However, further studies with larger sample size and evaluation of cyclin D1 in different subsites of OSCC are required.

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