RESEARCH COMMUNICATION

Genetic Alteration in Oral Squamous Cell Carcinoma Detected by Arbitrarily Primed Polymerase Chain Reaction

Pitsapaporn Wangpermtam[1], Sirima Sanguansin[1*], Songsak Petmitr[2], Phaibul Punyarit[3], Woranut Weerapradist[4]

Abstract

Oral cancer ranks as one of the top ten cancers in Thailand. Molecular carcinogenesis of this disease remains unknown. The purpose of this report was to identify the genetic alteration profile in Thai oral squamous cell carcinoma (OSCC) patients using arbitrarily primed PCR and to determine the association between genetic alterations and clinico-pathological characteristics. Band alteration profiles in the 33 OSCC tissues were compared with corresponding normal tissues amplified from 60 arbitrary primers using arbitrarily primed polymerase chain reaction (AP-PCR) were identified with 12 primers. Among these, 45 band patterns presented the alteration ranged from 36% to 88%. Primer AD15 at 750 base pairs (AD15-750bp) was found to have both the highest band alteration (88%) and the highest band loss (37%). The highest DNA band amplification was found in primer AX11-1300bp (52%). Primer AX-11 at 1300 base pairs at the altered frequency of 52% was significantly associated with smoking (p=0.007), and primer N20 at 800 base pairs showed association with low grade tumors (p=0.030). Our results indicate that AP-PCR is a useful technique for detecting genetic alteration in oral squamous cell carcinomas and to provide various genetic alternative data.

Keywords: genetic alteration - oral SCC - arbitrarily primed PCR

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Introduction

The oral cavity is one of the human body parts that has many important functions involving breathing, talking, chewing, and swallowing. Several diseases and injuries of the oral cavity can cause the impairment of the structure and result in low life quality. Now, oral cancer is still a frightening disease and is ranked as one of the top ten cancers worldwide as well as in Thailand (Attasara and Buasom, 2010; Duvvuri and Myers, 2009; Parkin et al., 2005). It ranks the fourth most common cancer in Thai males and the eighth in females (Attasara and Buasom, 2010).

Oral squamous cell carcinoma (OSCC) represents more than 90% of all oral cancers. This cancer type derives from the flat cells (squamous cells) that cover the surfaces of the mouth such as the buccal mucosa, tongue, and lips (Brinkman and Wong, 2006). However, the exact cause of oral cancer is unknown, but it is believed that predisposing factors such as smoking, alcohol consumption, poor general and oral health, socio-economic factors as low income level, genetic and aging play important roles in the etiopathogenesis of the disease (Scully, 2011). Although many studies identified various genetic aberrations in OSCC, appropriate genetic markers to manage individual oral cancer patients are needed to be elucidated.

Arbitrarily primed polymerase chain reaction (AP-PCR), a DNA fingerprinting method, provides highly reproducible patterns of amplified DNA fragments, which faithfully reflect differences in the relative abundance or sequence of the templates. The basis of AP-PCR is the amplification of genomic DNA with arbitrary primers under conditions in which low specific priming is encouraged. This technique allows the detection of genetic alteration in cancer by comparison in band intensity between tumor DNA and paired normal DNA which reflects genetic alterations. Several cancer studies report genetic alteration by this technique. The alterations in band intensity by AP-PCR among colorectal cancer were used as data to detect the allelic loss and gain in the colorectal tumor genome (Peinado et al., 1992). The amplification in DNA mapped to chromosome 6p12 adjacent to the pim-1 oncogene was associated with shorter disease-free survival among non small cell lung cancer patients (De Juan et al., 1998). In addition, genetic aberration in breast cancer detected by AP-PCR has been reported (Pakeetoot et al., 2007). Moreover, increasing in band intensity detected by arbitrary primers in cervical cancer was associated with clinico-pathological feature (Paditaporn et al., 2007). Nowadays, no study has reported the genetic alteration

*[1]Department of Oral Biology, [4]Department of Oral and Maxillofacial Pathology, Faculty of Dentistry, [2]Department of Tropical Nutrition and Food Science, Faculty of Tropical Medicine, Mahidol University, [3]Department of Clinical Pathology, Army Institute of Pathology, Bangkok, Thailand *For correspondence: dtssu@mahidol.ac.th*

in oral squamous cell carcinoma by AP-PCR method. Our study aims to identify genetic alterations in Thai oral squamous cell carcinoma patients by using arbitrarily primed PCR and to determine the association between genetic alterations and clinico-pathological characteristics of these patients.

Materials and Methods

Specimens

Fresh oral squamous cell carcinoma tissues and corresponding normal tissues were obtained from 33 patients who underwent surgery at the Ear, Nose and Throat (E.N.T) unit at Pramongkutklao Hospital, Bangkok, Thailand. None of the patients received any therapeutic treatment, either chemotherapy or radiation before operation. Sample collection was performed according to protocols approved by the Pramongkutklao Hospital Research Committee and Mahidol University Institutional Review Board (MU-IRB2010/170.0806). Immediately after surgery, the tissues were soaked with normal saline and brought to a pathologist at the Department of Clinical Pathology to separate tumor tissue and normal tissue with the size of 0.5 × 0.5 × 0.5 cm. each and kept separately in microtubes before frozen snapping in liquid nitrogen and stored at -80°C. The tissues were embedded in Optimum Cutting Temperature medium (O.C.T), cut under cryostat tissue sections and continuously stained with hematoxylin-eosin for microscopic examination by a pathologist. The histopathology of tumor was classified according to the TNM system of the American Joint Committee on Cancer (AJCC). Tumor tissues presenting more than 90% tumor cells and corresponding normal tissues were used in this study.

DNA extraction

Genomic DNA was prepared by proteinase K and salting out method as described previously (Miller et al., 1988, Blin and Stafford, 1976). DNA quantification was then measured by a spectrophotometer, NanoDrop ND-1000. The ratio of absorbance at the wavelength of 260 nm and 280 nm being more than 1.5, as a high DNA purity, was accepted. The working concentration of DNA (20 ng/μl) was adjusted with deionized water before continuous use in this study.

AP-PCR analysis

A set of 1,200 arbitrary primers (Operon, USA) was screened using the DNAsis program. Primers with nucleotide sequences having 80% homology to sense strands of human 18S and 28S rDNAs were excluded. After screening, 60 arbitrary primers were selected for this study (Table 1). The detection of genetic alterative pattern was carried out by AP-PCR based on protocols of William et al. (1990) performed under stringency conditions. Each 25 μl of AP-PCR mixture containing 1X PCR reaction buffer (10 mM Tris-HCl pH 9.0, 50 mM KCl, 1.5 mM $MgCl_2$), 1.0 mM $MgCl_2$, 1 unit of recombination Taq DNA Polymerase (illustra^{tm}, GE Healthcare, UK), 0.2 mM of each dNTP, 20 µM arbitrary primer and 100 ng DNA was amplified by a programmed Gene Amp PCR System 9700. Forty-five cycles (denatured at 94°C for 1 min, annealed at 36°C for 1 min, and extended at 72°C for 2 min) were performed for the hybridization between the arbitrary primers to sequences dispersed throughout genomic DNA continuing to amplification. Total AP-PCR products were then electrophoresed in 1.4% agarose gel at 90 volts for 1 h and stained with ethidium bromide. Gel imaging was visualized and recorded under UV light by G: BOX HR system (SYNGENE, USA) with 3 seconds and 100 mini-seconds exposure time. DNA band patterns and the increase or decrease in band intensity, referred to as band loss and band amplification, were compared between tumor DNA and paired normal DNA.

Statistical analysis

The frequency of change in band intensity referred to as genetic alteration in amplified DNA with each primer was collected and analyzed. The association between band alterations and clinico-pathological characteristic including age, sex, smoking, alcohol consumption, clinical stage of the disease, histopathological features, status of cervical lymph node, and disease-free survival was analyzed by SPSS program (version 17.0). Chi-square test was used to determine the association among these parameters and p value <0.05 was considered as a statistically significant difference. Disease-free survival was carried out with patients whose clinical evolution was followed up within two years.

Results

The band patterns of 33 samples from OSCC DNA and corresponding normal DNA detected by AP-PCR method with 60 primers were analyzed and represented as DNA band amplification and DNA band loss. The results showed that 12 of 60 arbitrary primers (AB19, AD15, AG11, AO5, AU1, AX11, BB3, D15, N20, S3, S10, S13) provided genetic alteration ranging between 36% to 88% with 45 different band sites. DNA band amplification ranged between 18% to 52%, while DNA band loss ranged between 6% to 37%. Among these data, primer AD15 at 750 base pairs resulted in both the highest band alteration (88%) (Figure 1) and the highest band loss (37%) (Figure

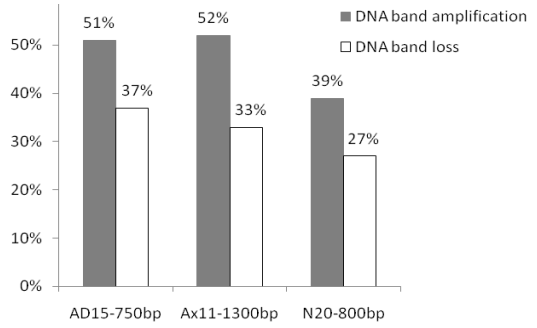

Figure 1. Genetic Alteration Frequency. Primer AD15-750bp showed both the highest DNA band alteration (88%) and the highest DNA band loss (37%). Primer AX11-1300bp revealed the highest DNA band amplification (52%). DNA band alteration (66%) detected by primer N20-800bp showed 39% in DNA band amplification that associated with histopathological feature

Table 1. The Association Between Clinico-Pathological Data and DNA Band Alteration Detected by Primer N20-800bp in 33 Samples

Parameters	Classification of DNA band alteration							
	Band amplification		p-value	OR (95% C.I.)	Band loss		p-value	OR (95% C.I.)
	+	-			+	-		
Age								
≤50	3	6	1.00	1.4(0.28-7.11)	3	6	0.67	0.6(0.12-3.52)
>50	10	14			6	18		
Sex								
Male	9	16	0.68	1.7(0.35-8.88)	8	17	0.39	0.3(0.03-2.90)
Female	4	4			1	7		
Alcohol consumption								
Yes	9	15	1.00	0.7(0.15-3.54)	8	16	0.38	4.0(0.42-37.8)
No	4	5			1	8		
Smoking								
Yes	8	16	0.42	0.4(0.08-1.91)	7	17	1.00	1.4(0.23-8.72)
No	5	4			2	7		
Clinical stage								
I-II	7	14	0.46	2.0(0.46-8.53)	6	15	1.00	0.8(0.16-4.18)
III-IV	6	6			3	9		
Histopathology								
PD + MD	5	15	0.03†	4.8(1.06-21.7)	5	15	1.00	1.3(0.28-6.30)
WD	8	5			4	9		
Cervical lymph nodes								
+	9	6	1.00	1.0(0.22-4.72)	2	8	0.68	0.5(0.09-3.40)
-	4	14			7	16		
Disease free survival								
<1 year	5	12	0.22	2.4(0.57-10.0)	4	13	0.70	1.4(0.31-6.89)
1-2 years	8	8			5	11		

OR, Odds ratio; C.I., confidence interval; PD, poor differentiation; MD, moderate differentiation; WD, well differentiation; † $p < 0.05$

Table 2. The Association Between Clinico-Pathological Data and DNA Band Alteration Detected by Primer AX11-1300bp in 33 Samples

Parameters	Classification of DNA band alteration							
	Band amplification		p-value	OR (95% C.I.)	Band loss		p-value	OR (95% C.I.)
	+	-			+	-		
Age								
≤50	5	4	1.00	0.8(0.17-3.72)	1	8	0.21	5.7(0.61-53.2)
>50	12	12			10	14		
Sex								
Male	15	10	0.11	0.2(0.03-1.33)	10	15	0.21	0.2(0.02-2.01)
Female	2	6			1	7		
Alcohol consumption								
Yes	14	10	0.25	2.8(0.56-14.0)	9	15	0.68	2.1(0.35-12.4)
No	3	6			2	7		
Smoking								
Yes	16	8	0.00†	16(1.69-151.1)	10	14	0.21	5.7(0.60-53.2)
No	1	8			1	8		
Clinical stage								
I-II	10	11	0.72	1.5(0.36-6.44)	7	14	1.00	1.0(0.22-4.50)
III-IV	7	5			4	8		
Histopathology								
PD + MD	8	12	0.15	3.3(0.76-14.8)	6	14	0.71	1.4(0.33-6.34)
WD	9	4			5	8		
Cervical lymph nodes								
+	6	4	0.70	1.6(0.36-7.38)	5	5	0.24	2.8(0.60-13.4)
-	11	12			6	17		
Disease free survival								
<1 year	7	10	0.30	2.3(0.58-9.64)	3	14	0.07	4.6(0.95-22.8)
1-2 years	10	6			8	8		

OR, Odds ratio; C.I., confidence interval; PD, poor differentiation; MD, moderate differentiation; WD, well differentiation; † $p < 0.05$

2), whereas primer AX11 at 1300 base pairs provide the highest percentage in DNA band amplification (52%) (Figure 1).

The association between genetic alteration identified with 12 arbitrary primers and clinico-pathological data in OSCC was analyzed statistically. The results showed

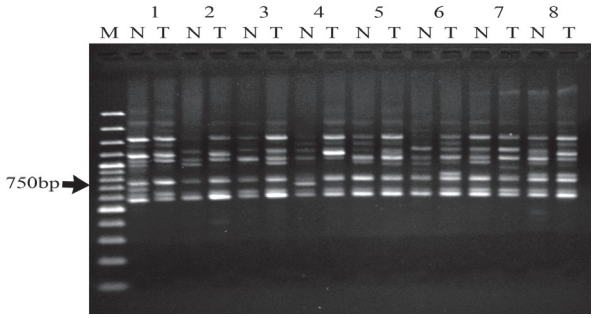

Figure 2. DNA Band Pattern Amplified by AD 15 Primer on 1.4% Agarose Gel after Stained with Ethidium Bromide. The highest band alterations (88%) and band loss (37%) was detected at 750 base pairs band size (arrow). Band amplification was shown in sample number 2, 3 and 4.Band loss was shown in sample number 5 and 7.(M;DNA ladder marker, N; normal DNA, T; tumor DNA)

that only two primers, N20 and AX11, showed statistical significance amongst those analyzed. DNA band amplification detected by N20 at 800 base pairs (N20-800) was significantly associated with histopathological features (p=0.030) and was found to be more associated with low grade (well differentiation) than high grade (moderate and poor differentiation) (Table 1). The incidence of the detectable band amplification at this site was 39%. Another DNA band amplification in AX11 at 1300 base pairs (AX11-1300) with the incidence of 52% showed significant association with smoking (p=0.007) and the amplified band intensity showed a higher relation to smoking than nonsmoking (Table 2).

Discussion

It is accepted worldwide that cancer is still one of the leading diseases. The cause of the disease development is unknown but it is believed that genetic instability is involved in this event (Kupferman et al., 2006). Even though numerous research studies were conducted to identify alterative genes, the genetic alterative data seems to be inadequate to keep our life far from this disease. Thus, AP-PCR was proposed as a genetic data finding method. This method, explained by Welsh and McClelland (1990) and McClelland and Welsh (1994) as a simple and fast technique without prior specific sequence information required, was widely used in many types of cancers. The present study demonstrated the use of this technique in oral squamous cell carcinoma. We found genetic alteration with either an increase or decrease in DNA band intensity in OSCC samples compared with the paired normal ones when detected by 12 of 60 arbitrary primers. Similarly, Pakeetoot et al. (2007) reported the detected DNA band alteration in 13 of 60 random primers in breast cancer, while Paditaporn et al. (2007) screened cervical cancer with 54 arbitrary primers. Our study demonstrated that the incidence in the variation of genetic changes ranged from 36% to 88% with the incidence in band amplification ranging between 18% to 52%, and band loss ranging between 6% to 37%. Although the arbitrary primers in detecting the genetic alteration among OSCC, breast cancer and cervical cancer were different, the incidence in these alterations were similar. In breast cancer, the incidence in band loss intensity detected by six primers ranged from 23% to 40% with the highest band loss detected by B12 primer, while that in band amplification intensity detected by eight primers ranged from 27% to 80% showed the highest incidence in D15 primer (Pakeetoot et al., 2007). In addition, Paditaporn et al. (2007) screened cervical cancer with 54 arbitrary primers. The highest in band alteration was detected in seven primers. Among the detected primers, the incidence in band loss intensity ranged from 51.9% to 75.9% and that in band amplification intensity ranged from 24.1% to 70.4%. The association study between fragment alterations in genomic DNA amplified by this technique and clinical data showed that U8 primer amplified fragment was significantly associated with cervical cancer stage II and AE3 primer loss fragment showed significant association with lower age of cervical cancer patient (Paditaporn et al., 2007). In this study, we analyzed the association between 45 alterative band sites produced by 12 random primers with provided clinico-pathological data. Only two sites produced by two different random primers, N20 and AX11, showed significant association with low grade OSCC cancer and OSCC patients who smoked, respectively.

The useful genetic alterative data from AP-PCR technique was further analyzed in several cancers. Chariyalertsak et al. (2005) discovered high frequencies in alterative DNA fragments amplified from AX11 primer and used this information to identify and localize the altered genomic fragments. They could localize DNA amplification fragment on chromosome 2p25.3 and chromosome 7q11.23. Furthermore, significant association between DNA amplification on chromosome 2p25.3 and poorly differentiated cholangiocarcinoma tumor was reported. Pongstaporn et al. (2006) demonstrated that DNA amplified fragment from AE11 primer was mapped on chromosome 10q24.3 and further identified as IVS-4 of the glutathione-S-transferase Omega2 gene and polymorphism in the GSTO2 gene was associated with ovarian cancer risk. Sareeboot et al. (2010) could map DNA amplified fragment form AB19 primer on chromosome 13q31.1 associated with poor prognosis in colorectal cancer. Bankovic et al. (2010) studied non-small cell lung carcinoma and reported that DNA fragments amplified with five random primers by AP-PCR demonstrated altered mobility in polyacrylamide gel electrophoresis. The informative sequences provided several identified genes relevant to histological grade and tumor stage. Although AP-PCR is a useful technique for screening the frequency in DNA band patterns referred to as genetic alteration, these DNA fragments should be identified for finding the specific genes associated with cancer. However, it should be noted that AP-PCR is a sensitive technique, and inadequately prepared DNA resulting in DNA impurity, uncontrolled reagents, e.g., buffer conditions, enzyme quality, primer preparation, and PCR conditions, can produce unreliable results (Navaro et al., 1999).

In conclusion, AP-PCR is a useful technique for

detecting genetic alterations in Thai oral squamous cell carcinoma and can provide various genetic alternative data.

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