RESEARCH ARTICLE

Alu Hypomethylation in Smoke-Exposed Epithelia and Oral Squamous Carcinoma

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Abstract

    <u>Background</u>: Alu elements are one of the most common repetitive sequences that now constitute more than 10% of the human genome and potential targets for epigenetic alterations. Correspondingly, methylation of these elements can result in a genome-wide event that may have an impact in cancer. However, studies investigating the genome-wide status of Alu methylation in cancer remain limited. <u>Objectives</u>: Oral squamous cell carcinoma (OSCC) presents with high incidence in South-East Asia and thus the aim of this study was to evaluate the Alu methylation status in OSCCs and explore with the possibility of using this information for diagnostic screening. We evaluated Alu methylation status in a) normal oral mucosa compared to OSCC; b) peripheral blood mononuclear cells (PBMCs) of normal controls comparing to oral cancer patients; c) among oral epithelium of normal controls, smokers and oral cancer patients. <u>Materials and Methods</u>: Alu methylation was detected by combined bisulfite restriction analysis (COBRA) at 2 CpG sites. The amplified products were classified into three patterns; hypermethylation (^{m}C^{m}C), partial methylation (^{u}C^{m}C+^{m}C^{u}C), and hypomethylation (^{u}C^{u}C). <u>Results</u>: The results demonstrate that the %^{m}C^{m}C value is suitable for differentiating normal and cancer in oral tissues (p=0.0002), but is not significantly observe in PBMCs. In addition, a stepwise decrease in this value was observed in the oral epithelium from normal, light smoker, heavy smoker, low stage and high stage OSCC (p=0.0003). Furthermore, receiver operating characteristic (ROC) curve analyses demonstrated the potential of combined %^{m}C or %^{m}C^{m}C values as markers for oral cancer detection with sensitivity and specificity of 86.7% and 56.7%, respectively. <u>Conclusions</u>: Alu hypomethylation is likely to be associated with multistep oral carcinogenesis, and might be developed as a screening tool for oral cancer detection.

Keywords: Alu element - hypomethylation - oral cancer - smoke-exposed epithelia

Introduction

    Oral squamous cell carcinoma (OSCC) is the most frequent malignant neoplasm of the oral cavity which represents approximately 3% of all malignancies affecting humans (Yasusei et al., 2004; Song et al., 2011). OSCC accounts for more than five hundred thousand newly diagnosed cases every year worldwide (Massimo et al., 1995; Massimo et al., 2012). Generally, the highest incidence rates of oral cancer are found in South-East Asia, and Central and Eastern Europe for both males and females (Ahmedin et al., 2011). Because of its high mortality and low cure rate, OSCC represents a major global public health and socioeconomic problem (Massimo et al., 2012). At present, OSCC still lacks reliable diagnostic and prognostic molecular markers.

    Cancers including OSCC are now known to develop and progress through a series of genetic and epigenetic alterations (Lingen et al., 2011; Saintingny et al., 2011). While on one hand genetic aberrations constitute irreversible changes (increased copy number) or mutations in the DNA coding sequences resulting in overexpression/ increased activity or inactivation, of key oncogenes and tumor suppressor genes, respectively (Lingen et al., 2011; Saintigny et al., 2011). On the other hand, promoter hypermethylation of tumor suppressor gene and genome-wide hypomethylation are the main features commonly associated epigenetics events (Chalitchagorn et al., 2004; Kitkumthorn and Mutirangura, 2011; Song et al., 2011). Of interest though, both types of alterations are now thought to occur in the transition of normal oral epithelium to premalignant lesion and to overt carcinomas (Diez-Perez et al., 2011; Lingen et al., 2011). Furthermore, with recent data suggesting that smoking related oral premalignant conditions might be associated with genome-wide hypomethylation (Demarini, 2004; Ian et al., 2007; Subbalekha et al., 2009) further investigation can likely afford the possibility of identifying novel molecular markers of OSCC.

    Genome-wide hypomethylation can occur on

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interspersed repetitive sequences (IRS) and are dispersed throughout the genome. Long Interspersed Element 1 (LINE-1) and Alu accounting for the majority of IRS can likely represent key targets for genome-wide methylation that can lead to abnormal epigenetic events and consequently cancer. However, while the methylation status of LINE-1 is now reported to be widespread in many cancers, corresponding analysis of Alu methylation remains sparse and unclear (Debra et al., 2007; Moore et al., 2008; Hou et al., 2010; Wilhelm et al., 2010; Pobsook et al., 2011).

Fundamentally, Alu elements are Short Interspersed Elements (SINEs), widely dispersed with a notably high copy number (~500,000 copies) and accounting for ~10% of the human genome (Rubin et al., 1980). Thus, Alu elements can represent likely targets for genome-wide methylation (Xiang et al., 2010; Nakkuntod et al., 2011). In general though, Alu elements are normally methylated and transcriptionally inactive, but in certain stress-induced conditions, for example cellular heat shock, can lead to demethylation (hypomethylation) of CpG islands and activate Alu transcription (Peter et al., 2008). Although Alu transcripts are not protein encoding, nonetheless they can regulate associated gene expression, affecting processes such gene recombination, chromosome translocation, nucleosome formation and genome evolution that impacts genomic instability (Alexandros et al., 2008; Ana et al., 2009; Kristy et al., 2009).

While reduction in Alu methylation levels have been observed in several cancers (breast, colon, stomach, liver, lung, ovarian, urinary bladder, prostate gland) (Choi et al., 2007; 2009; Rodriguez et al., 2008; Watts et al., 2008; Yoo et al., 2008; Bollati et al., 2009; Daskalos et al., 2009; Lee et al., 2009; Park et al., 2009; Cho et al., 2010; Hehuang et al., 2010; Hou et al., 2010; Kwon et al., 2010; Xiang et al., 2010; Yoshida et al., 2011), those occurring in OSCC have not been reported. Here, we evaluated and compared levels and pattern of Alu methylation levels in formalin-fixed and paraffin-embedded (FFPE) specimens of normal and OSCC,and in peripheral blood mononuclear cells (PBMCs). Furthermore, we compared this emerging data with that of oral rinse samples from control patients and those with OSCC with known smoking habit that may provide new knowledge of Alu methylation in the pathogenesis of oral cancer.

Materials and Methods

Samples

In this study, samples were retrieved from 3 patient cohorts collected during January-December 2011. The demographics of these patient samples were collected from the available answer from questionnaires and records (Table 1). The patient cohorts that were used in this study include:

Cohort 1

FFPE archived cases (9 OSCC and 22 normal oral mucosa) were derived from the Faculty of Dentistry, Chulalongkorn University. From each retrieved case, 3-5 sections of approximately 5 μm-thickness, were

Table 1. Demographic Data of All Sample Groups

Sample groups	N	Male:Female	Age (Average±SD)
Paraffin-embedded tissue			
Normal	22	6:16	47.59±13.87
Oral cancer	9	5:4	64.33±14.76
PBMCs			
Normal	31	14:17	48.28±11.78
Oral cancer	36	16:20	63.03±11.58
Oral rinse			
Normal (Non-smoker)	42	12:31	48.37±11.65
Light smoker	42	36:6	41.09±8.06
Heavy smoker	24	19:5	55.21±9.66
Oral cancer	43	21:22	60.40±12.95
Low stage (I+II)	14	5:9	63.79±11.68
High stage (III+IV)	29	16:13	58.76±13.41

prepared onto clean microscopic glass slides. One section underwent haematoxylin and eosin (H&E) staining, which than used for confirmatory histopathological evaluation by a pathologist (NK). All oral cancer samples consisting of at least 80% tumor cells were included for analysis.

Cohort 2

The PBMCs were derived from 36 patients with OSCC (36) and normal controls (31) and patients. The collection was carried out at three centers (Rajavithi Hospital, Bangkok; Buddhachinaraj Hospital, Bangkok; Faculty of Dentistry, Chulalongkorn University, Bangkok). Patients who had prior chemotherapy or radiotherapy were excluded. From each patient, six mL of blood was collected in heparinized tube, which than after underwent Ficoll-Hypaque centrifugation to separate the PBMCs which were used as source for DNA extraction.

Cohort 3

A total of 153 oral rinse samples were collected. Sample groups included normal, which was essentially sub-divided into non-smoker (42) and light to heavy smokers (66). The remaining group constituted samples from patients with histopathologically confirmed OSCC (43). Oral rinse from OSCC patients was collected prior to any treatment. All oral rises was done with 10 mL of sterile 0.9% normal saline solution and after gargling for 15 sec, solutions underwent centrifugation, and the cell pellet underwent DNA extraction within 24 hours of collection (see below). Total oral cancers were classified into 2 groups depended on patient pathological status including low and high stage oral cancer.

All participating subjects in cohorts 2 and 3 were given a self-administered questionnaire to collect medical history and information on smoking, prior to sample collection. Smoking consumption as number of years smoked, number of cigarettes smoked daily, age at which patient started smoking and the numbers of years since quitting, were carefully recorded. However, total smokers were divided into light and heavy smoker groups base on the average mean of pack/year value as previously described (Godtfredsen et al., 2004). After completing the questionnaire, patients underwent clinical examination by an oral surgeon (KS) and confirmation of patient histopathology by a pathologist (NK), prior to oral
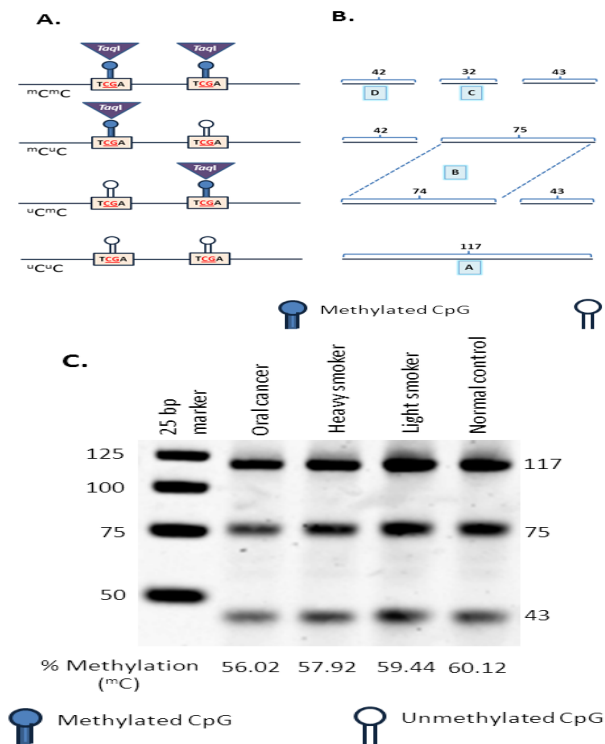
Figure 1. Alu Methylation Pattern in COBRA Alu Method. Amplified products digested with *TaqI* are represented in 3 patterns; hypermethylation ($^mC^mC$) 42/43 base pair (bp), partial methylation ($^mC^uC+^uC^mC$) 74/75 bp and hypomethylation ($^uC^uC$) 117 bp. **A)** *TaqI* can digest only methylated CpG; **B)** different pattern of amplified product after *TaqI* digestion including A, B, C, and D which used calculate % methylation; and **C)** Gel electrophoresis showing 3 size of *TaqI* digested amplified product (117bp, 75 bp and 43 bp) in the indicated groups of oral rinse samples

rinse was collection. All samples were obtained under a protocol approved by the Ethics Committee, Faculty of Dentistry, Chulalongkorn University, Thailand (approval number: 7/2010) and signed informed consent.

DNA extraction

Cells from oral rinses and PBMCs were centrifuged at 4°C at 2500 rpm for 15 min. Next, the supernatant from oral rinse samples was discarded and the resulting cell pellets resuspended in sterile PBS. For PBMCs, after standard Ficoll-Hypaque centrifugation, the layer corresponding to PBMCs was removed and placed in PBS for washing. Washed cells were centrifuged at 4°C at 2,500 rpm for 15 min and the resulting pellet underwent DNA extraction with 1 mL of extraction buffer supplemented with 10% SDS and proteinase K 0.5 mg/mL. For FFPE tissue sections, these first underwent de-paraffination in xylene prior to lysing the tissue off the slide with 1 mL of extraction buffer and than transferring the extracts to a clean eppendorf tube. All the lysed extracts were first incubated at 50°C for 72h, and then 0.5 mL of phenol-chloroform solution was added to each before mixing thoroughly. After, the mixtures were centrifuged at 4°C at 14000g for 15 min and for each sample, the resulting clear upper phase was carefully removed and transferred to a clean eppendorff tube and the DNA precipitated by adding 10M ammonium acetate and absolute ethanol.

The precipitated DNA was than centrifuged at 14000g, washed with 70% ethanol and after air drying, the pellet was re-suspended in distill water and used for COBRA Alu anaysis.

Combined bisulfite restriction analysis of Alu (COBRA Alu)

All DNA samples were converted to bisulfite DNA by using sodium bisulfate as previously described (Chalitchagorn et al., 2004). Briefly, a total of 1 µg of DNA of each sample first underwent denaturation in 0.22 M NaOH at 37°C for 10 min and after the addition of 10 mM hydroquinone (Sigma-Aldrich, Singapore) and 3M sodium bisulfite (pH 5.0) samples underwent an additional incubation at 50°C for 16-20h. After, DNA was recovered using the Wizard DNA Clean-Up Kit (Promega, Madison, WI) following the manufacturer's protocol. DNA samples were eluted from the columns by distilled water and precipitated with sodium acetate and 100% ethanol as indicated previously. Then, COBRA Alu was performed as previously described (Kitkumthorn et al., 2012; Sirivanichsunthorn et al., 2013). Briefly, the modified DNA pellets were resuspended in distilled water 1 µL of this was subject to 45 cycles of PCR using forward (GGCGCGGTGGTTTACGTTTGTAA) and reverse (TTA ATAAAAACGAAATTTCACCATATTAACCAAAC) primers with an annealing temperature of 53°C. After, all amplified products were than digested with 2U of *TaqI* in *TaqI* buffer (MBI Fermentas, Glen Burnie, MD) overnight at 65°C. The digested products were identified by 8% non-denaturing polyacrylamide gel electrophoresis and visualized with SYBR green.

Alu methylation analysis and calculation

The amplified products of DNA samples from the 3 patient cohorts were classified into 3 types depended on the methylation pattern of the 2 CpG dinucleotides. These are the hypermethylated ($^mC^mC$), partial methylated ($^uC^mC$ and $^mC^uC$) and hypomethylated loci ($^uC^uC$). After enzyme digestion, three product size (117bp, 75bp, 43bp) depending on the methylation status of the loci are generally detected as shown in Figure 1. Then, band intensities can be measured and quantitated by a phosphoimager using ImageQuant Software (Molecular Dynamics, GE Healthcare, Slough, UK). Next, the percentage of each methylation pattern can be calculated using the following; First, the intensity of each band is divided by bp of DNA length; %117/117=A, %75(74)/74.5=B, %42(43)/42.5=D and C (represent of $^mC^mC$)=D-B. After that, the percentage of methylation was calculated as following formula; $^mC=100\times(2D)/(2A+2D)$, $^mC^mC=100\times C/(A+B+C)$, $^uC^mC+^mC^uC=100\times B/(A+B+C)$ and $^uC^uC=100\times A/(A+B+C)$.

DNA extracted from HeLa, DauDi and JurKat cell lines were used as positive controls in the experiments and for inter-assay adjustments.

Statistical analysis

Statistical analysis was performed using SPSS software for Windows version 17.0 (SPSS Inc., Chicago, IL). Analysis of variance (ANOVA) and independent
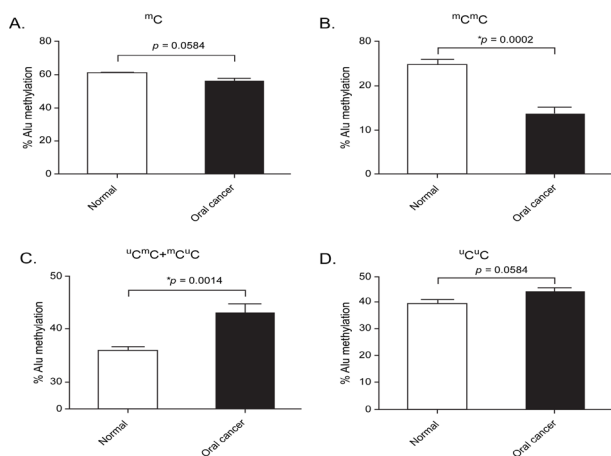
Figure 2. Comparisons of COBRA Alu Methylation Levels in FFPE Derived Normal Oral Mucosa and Oral Cancer Tissue
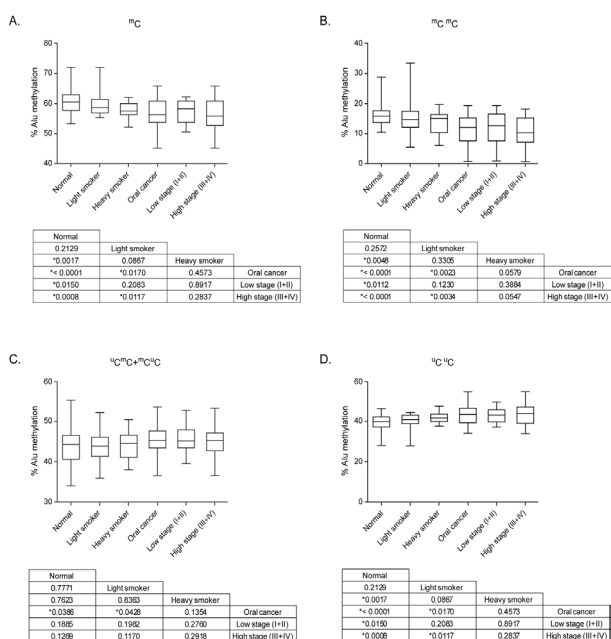

Figure 4. Comparisons of Alu Methylation Levels in Oral Rinses from Normal Controls, Light Smokers, Heavy Smokers, Total Oral Cancer, Low Stage Oral Cancer and High Stage Oral Cancer

sample t-test was performed to calculate significant differences in normal oral epithelium and oral cancer epithelium. All p values were obtained by two sided and values <0.05 were considered to be statistically significant. A receiver-operating characteristic (ROC) curve was used to test the feasibility of the COBRA Alu method of analysis of methylation status could distinguish between normal oral mucosa and oral cancer.

Results

Alu methylation status comparing normal oral mucosa and oral cancer tissues

In this analysis, we observed the frequency of each Alu methylation pattern compare between normal oral mucosa and oral cancer FPPE tissue samples. The results as shown in Table 1 and Figure 2 (A-D), indicate that overall methylation levels (^mC) in oral cancer, was lower
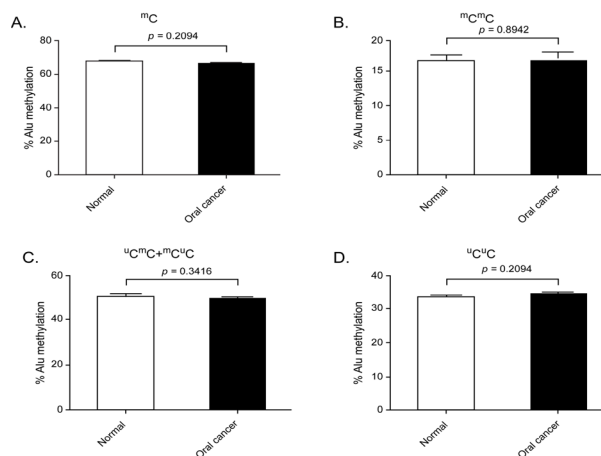

Figure 3. Comparison of Alu Methylation Levels in PBMCs from Normal and Oral Cancer Patients

than normal oral mucosa (p=0.0584). Moreover, when same comparison was done using %$^mC^mC$ values, this difference was significantly lower in oral cancer tissue (p<0.0002). In contrast, the partial methylation levels and the %$^uC^uC$ values in oral cancer tissues was higher than normal oral mucosa with p value=0.0014 and 0.0584. Overall, the data suggest that Alu hypomethylation were found in oral cancer tissue especially when observed in %$^mC^mC$ value.

Comparisons of Alu methylation status between PBMCs from normal and oral cancer patients

As our previous analysis indicated that there was a clear difference in the methylation levels between normal oral mucosa and oral cancer tissue, we questioned if we would find a similar trend in PBMCs isolated from independent groups of normal and oral cancer patients. As shown when performing this analysis, Alu methylation levels and pattern were found to be only different between the two groups of PBMCs (p=0.2094; Figure 3). However, the decrease of methylation level was not found in the comparison of %$^mC^mC$ and %$^uC^mC+^mC^uC$.

Comparison of Alu methylation status in oral rinse samples from normal, light smoker, heavy smoker and oral cancer patients

Since our analysis using PBMCs showed only a marginal difference between normal and oral cancer patients, we sought to address if DNA from oral rinse may hold value. Cellular material from oral rinse from normal, smokers (light and heavy) and oral cancer patients was used to extract DNA and perform methylation analysis. The overall methylation level and the p value decreased respectively, from normal oral epithelium, light smoker (p=0.2129), heavy smoker (p=0.0017) and oral cancer (p<0.0001). Moreover, the ^mC level decreased from low stage (stage I and II) to high stage (stage III and IV) oral cancer (p=0.0150 and p=0.0008), respectively (Table 2). Conversely, hypomethylation pattern is observed to be highly elevated in patients with oral cancer and those who exposed smoking related carcinogens than in normal oral epithelium. However, no significant difference in the analysis of partial methylation pattern was observed. (Figure 4).

Table 2. Percentage of Alu Methylation Levels in All Sample Groups

| Sample groups		Level (Average±SD)			
		^{m}C	$^{m}C^{m}C$	$^{u}C^{m}C+^{m}C^{u}C$	$^{u}C^{u}C$
Paraffin-embedded tissue	Normal	60.62±5.65	24.63±6.79	35.99±3.04	39.38±5.65
	Oral cancer	56.46±4.77	13.65±4.57	42.81±5.16	43.54±4.77
PBMCs	Normal	66.68±3.52	16.25±6.11	50.43±5.26	33.32±3.52
	Oral cancer	65.56±3.67	16.48±7.89	49.08±6.16	34.44±3.67
Oral rinse	Normal (Non-smoker)	60.41±3.37	16.44±3.93	43.97±4.30	39.59±3.37
	Light smoker	59.48±3.39	15.26±5.40	44.22±3.73	40.52±3.39
	Heavy smoker	57.81±2.52	13.53±3.80	44.28±3.13	42.19±2.52
	Oral cancer	56.50±4.84	11.08±5.27	45.50±3.43	43.42±4.84
	Low stage (I+II)	57.42±3.69	11.56±5.63	45.86±3.59	42.58±3.69
	High stage (III+IV)	56.17±5.31	10.84±5.18	45.33±3.40	43.83±5.31
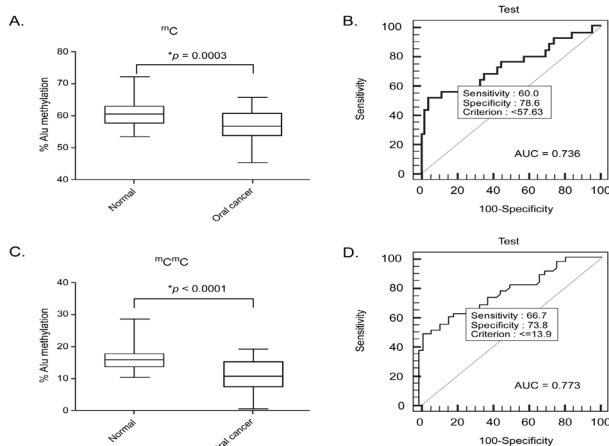
Figure 5. ROC Curve Analysis of Pair Data between Normal and Oral Cancer. Combined sensitivity (^{m}C or $^{m}C^{m}C$) calculated by 100%-[false negative ^{m}C×false negative $^{m}C^{m}C$] and combined specificity (^{m}C or $^{m}C^{m}C$) calculated by Specificity ^{m}C×Specificity $^{m}C^{m}C$. (AUC represent for Area under curve)

Receiver Operating Characteristic (ROC) curve analysis of ^{m}C and $^{m}C^{m}C$ methylation pattern

Since our data suggested that ^{m}C and $^{m}C^{m}C$ show the highest significant value, we chose to further analysis this sub-set. We selected methylation and hypermethylation results to perform ROC curve to assess if this was able to discriminate normal and oral cancer tissues. As shown in Figure 5, ^{m}C pattern demonstrated a sensitivity and specificity of 60.0% and 78.6%, respectively. In the same way, 66.7% sensitivity and 73.8% specificity was observed for the $^{m}C^{m}C$ pattern. However, sensitivity and specificity determined individually for ^{m}C and $^{m}C^{m}C$ are not suitable for use as tool for oral cancer detection. Fortunately, the combination of these two markers did improve the diagnostic power of the oral cancer detection (86.68% sensitivity and 56.68% specificity). With a high percentage of sensitivity, the methylation level of ^{m}C or $^{m}C^{m}C$ in oral rinse sample has high potential for use as a screening tool for oral cancer detection from the oral rinse specimen.

Discussion

In this study, we have used COBRA Alu analysis, which is highly capable of detecting methylation levels of Alu elements at 2 CpG loci. This alone is the key advantage of this technique, providing information on multiple CpG loci rather than 1. For example, in the FFPE derived tissue, we not only found hypomethylation in both of the CpG loci ($^{m}C^{m}C$), we were also able to examine the increase in $^{u}C^{u}C$ pattern which we determined to hold grater significance.

Since a previous study reported that Alu hypomethylation was correlated with aging when assessed in PBMCs (Jintaridth and Mutirangura, 2010), we chose to follow this approach for oral cancer, since it is not as invasive as tissue biopsy. Although our data did show that methylation was datable, the result of methylation patterns showed no significant changes. Nonetheless, decreasing trend in methylation level and increasing hypomethylation loci same as paraffined-embeded tissue were noted. The results are far from conclusive and this might have occurred by the possibility that the PBMCs could be with a high proportion of normal PBMCs and with very few circulating cancer cell DNA in the patient sample.

Finally, we observed notable differences in oral rinse samples which its self represents an excellent cost-effective and non-invasive technique for sample collection. We also investigated the component of potentially malignant condition arising from a smoking habit. Our results demonstrate a significant reduction in Alu methylation level in oral rinse samples from non-smoker, light smoker, heavy smoker and cancer patients (Figure 4). The level of Alu methylation is noted to be stepwise decrease concordant with the potentially malignant changes of the oral epithelium. Although the majority of the oral rinse contains cells from the normal oral epithelium, in smokers and cancer patients the oral rinse can likely include dysplastic/cancer squamous cells (Subbalekha et al., 2009; Wangsri et al., 2012). The DNA from dysplastic/cancer cells is capable to show reduced the Alu methylation level. This observation is confirmed by the Alu methylation level in high stage cancer is lower than low stage cancer, and collectively demonstrating the sensitivity of the COBRA Alu method of analysis.

The association between smoking status and Alu hypomethylation in tumors suggest that tobacco exposure may be causing genome-wide damage and contributing in epigenetic events including Alu methylation status. Smoking has been associated to promote methylation of several genes in different cancer, for example, SFRP in head and neck squamous cell carcinoma (HNSCC) (Marsit et al., 2006) and TSLC1/IGSF4 in non-small cell lung cancer (Kikuchi et al., 2006). Although smoking has not been previously shown directly to cause genome-

wide hypomethylation, there are reports suggesting that smoking can be associated with vitamin B12 reduction, which is required for the normal synthesis of S-adenyl-methionine (Gabriel et al., 2006), an important protein involved in methyl-transferase pathway. This may provide a clue in better understanding the association between Alu hypomethylation and smoking.

   The result of this study clearly demonstrates that Alu methylation level and pattern in oral cancer was readily datable in oral rinse sample than in tissues or PBMCs. Here, we proposed to use the sample from oral rinse technique for developing a test for oral cancer detection. Supporting this is that when we performed ROC curve to evaluate the sensitivity and specificity for this test, we observe high sensitivity in combined ^mC or $^mC^mC$ methylation pattern, implying that, this technique may be suitable for oral cancer screening. However, some limitations of this study could be concerned. Firstly, in case of OSCC with ulcer, the results may disturbed by some blood cells contamination. Secondly, our experiment had limited sample size and unmatched age of the participants among normal, smoker and oral cancer patients. Therefore, further investigation should be age consideration and larger sample size evaluation. In conclusion, Alu methylation might be beneficial method for screening oral cancer in oral rinse sample.

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