Genome-wide analysis of DNA methylation array and microarray in human oral epithelial cells stimulated by arecoline for prolong period - Involvement of aberrant expression of DUSP4 gene in betel quid chewing related oral cancer

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Abstract

1. Introduction

Oral cancer is the eighth most common human cancer. Both environmental and genetic factors induce the development of oral cancer. Although several studies have shown the factors involved in betel quid chewing related oral cancer, the mechanism of causing this type of oral cancer remains elusive. Genetic mutations and aberrant DNA methylations have been suggested to be involved in causing of oral cancer. Unlike genetic mutations, DNA hypermethylations are reversible and can be diagnostic and therapeutic targets. The purpose of this study is detection of novel DNA hypermethylation that can be applied to diagnostic, predictive, interceptive and therapeutic approaches for betel quid chewing related oral cancer.

2. Method and materials

a. Genome-wide analysis

HGEPs were cultured and treated alternating 3 days with 50 μg/ml arecoline and 3 days without arecoline for 1 month. Untreated samples were used as controls. For methylation array, genomic DNA were analyzed using a DNA microarray scanner (Agilent technology). For microarray analysis, a global analysis of mRNA expression was carried out using DNA microarray (human SurePrint G3 Human GE Microarray).

b. Confirmation of the reproducibility in the five candidate genes

In order to confirm the reproducibility of the microarray data, DNA samples were treated with sodium bisulfite and quantitative Methylation Specific PCR (qMSP). Expression of mRNA was analyzed by quantitative reverse transcription polymerase chain reaction (qRT-PCR). Results were compared by Mann-Whitney U test with P-value <0.05 accepted as statistically significant.

c. DUSP4 analysis in Oral cancer cell line

To confirm the DUSP4 expression in oral cancer, four cancer cell lines HSC4, SAS,

BSC-OF and HSY were cultured and treated with 50 μg/ml arecoline alternating 3 days or untreated for 7 days.

d. Pathway analysis

To examine any mechanism in additional to hyper-methylation, phosphorylated JNK (pJNK) in the HGEPs treated with or without arecoline were determined using cell based ELISA.

e. Histological and immunohistochemical and CpG methylation in tissue samples

Twenty tissue samples were obtained from the patients involved in betel quid chewing habit in Sri Lanka. Twenty-two tissue samples (13 OSCC, 9 fibrous polyp) for betel quid non-chewer were retrieved from the archives of HSUH, Japan. Immunohistochemical examination of the tissue samples was performed using anti-DUSP4 antibody. DUSP4 immunoreactivity was assessed in nucleus of epithelial cells. Genomic DNA was extracted from the tissue samples. DNA samples were treated with sodium bisulfite and qMSP was performed.

f. Statistical analysis

The results were compared using the Mann-Whitney U test with p-value <0.05 accepted as statistically significant.

3. Results

CpG island methylation array and global analysis of mRNA reveled 8638 and 455 genes that were more than 2 times hyper-methylated, and their mRNA downregulated respectively in samples stimulated with arecoline for a period of 1 month. Twenty-one of them were tumor suppressor genes, within -1000bp from promoter region. Four candidate genes HNRNPH3, BCL2L11, TFAP2A and DUSP4 with the highest amount of promoter region hypermethylation with downregulated mRNA expression were selected. qMSP showed DUSP4 genes were significantly hypermethylated in HGEPs treated with arecoline for a period of 7 days and 1 month. mRNA expression of DUSP4 was down-regulated in HGEPs

treated with arecoline for 7 days and 1 month. Methylation level of DUSP4 was significantly higher in all cancer cell lines (HSC4, SAS, BSC-OF and HSY) without arecoline treatment as compared to HGEPs. mRNA expression of DUSP4 in untreated BSC-OF cell line was not significantly different from that of HGEPs. BSC-OF showed downregulated DUSP4 mRNA expression after arecoline treatment.

The amount of pJNK was elevated in samples treated with arecoline over a period of 1 month.

IHC staining reveled that DUSP4 expression level was significantly higher in betel quid chewers (66.85 ± 3.0) than in non-chewer OSCC (20.34 ± 4.7). qMSP analysis showed that DUSP4 CpG island was significantly hypermethylated in oral cancer samples obtained from betel quid chewers (89.8 ± 2.6) as compared to those from non-chewer oral cancer cases (44.7 ± 10.6) and the healthy control cases (49.4 ± 13.1).

4. Conclusion

In the present study, genome-wide DNA hypermethylation and global mRNA microarray analysis were carried out using CpG island DNA methylation array and DNA microarray in HGEPs stimulated with arecoline. This is the first report that shows betel quid related hypermethylation of DUSP4 followed by its down regulated expression. DUSP4 hypermethylation can be applied as a target for diagnostic, predictive, interceptive and therapeutic approaches for betel quid chewing related oral cancer.

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1. Introduction

1.1. Oral cancer

Oral cancer is the eighth most common human cancer, with more than 500,000 new cases being diagnosed globally every year. While it represents just over 2% of the global cancer incidence, its 50% fatality rate is a major cause of concern (Siegel et al., 2017). Both environmental and genetic factors induce the development of oral cancer. Association of tobacco smoking, alcohol consumption, diet, living habits, microbial infections and the exposure to a variety of exogenous or endogenous carcinogens are well documented with oral carcinoma (Asthana et al., 2018; Petti, 2009). Not all individuals exposed to these risk factors develop oral carcinoma, additional genetic factors may also contribute to oral carcinoma susceptibility. The environmental factors often induce posterior genetic modifications such as mutations and epigenetics leading to oral cancer. Several kinds of genetic changes are seen in oral cancer such as single nucleotide polymorphism (as in ADH1B, ALDH2, MMP1, FN1, CCNA2, CA9, VEGFC and FAT1) and mutation (as in p53) (Lee et al., 2018; Li et al., 2018; Lin et al., 2018). A number of epigenetic mechanisms including DNA methylation, functional non-coding RNA and histone modifications regulate the expression of different genes. The promoter hypermethylation-mediated silencing of certain tumor suppressor genes occurs without genetic change. Methylation-associated RARB, C3orf14, GPR27, ZNF717 gene silencing and histone H3K9 methylation, are few examples of epigenetic modification (Lai et al., 2014; Lando et al., 2015).

The prevalence of oral cancer is extremely high at approximately 8.7% of cancer incidence in the South and Southeast Asian countries, with 2% worldwide incidence, being the highest globally (Gupta et al., 2016). The habit of betel quid chewing is a major cause of oral cancer in those countries (Lee et al., 2011). Although several studies have shown the factors involved in betel quid chewing related oral cancer, the mechanism of causing this type of oral cancer is still unclear. In a large cohort study in India, all oral cancer developed from potentially malignant oral disorders or precancerous lesions, including oral submucous fibrosis, oral

leukoplakia, erythroplakia, and oral lichenoid lesions; and were seen among users of betel quid, areca nut, or tobacco-based products, or all of these (Gupta et al., 1980).

1.2. Betel quid

Betel quid, a combination of fresh areca nut, slaked lime, fresh betel leaf, and partially dried tobacco, is widely preferred in South and Southeast Asian countries (Fig. 1). The Asian Betel Quid Consortium study found that the prevalence of betel quid and areca nut chewing in the adult population varied from 10·3% in Malaysia to 43·6% in Nepal for men, and from 2·3% in mainland China to 47·8% in Indonesia among women (Lee, et al., 2011). Betel quid have been classified as carcinogenic to human beings (Group 1) by the International Agency for Cancer Research (WHO IARC, 2004). Epidemiological studies have related the habit of betel quid chewing with oral cancer and oral premalignant disorders (Jeng et al., 2001; Lee, et al., 2011). Since arecoline is a major component of betel quid that can be a carcinogen, previous studies have mainly focused on arecoline to elucidate the mechanism of carcinogenesis with betel quid.

1.3. Arecoline

Arecoline (1,2,4,5-tetrahydro-1-methyl-pyridinecarboxylic acid) is a nicotinic acid-based alkaloid, an active component of areca nut, found in betel quid (Fig. 2) (Sharan et al., 2012). Arecoline is genotoxic and might contribute to oral carcinogenesis by facilitating error-prone DNA repair (Ji et al., 2012). DNA repair machinery is an important part of maintaining genomic integrity. Dysregulation of DNA repair resulting in genomic instability is a hallmark of cancer cells that can be associated with arecoline stimulation (Chiba et al., 1998; Kannan et al., 1999). In addition to genetic changes in the tumor-related genes, epigenetic modification plays a vital role in oral carcinogenesis (Takeshima et al., 2008; Tsai et al., 2008). Areca nuts extracts and arecoline induce epigenetic modifications. It has been found that promoter region hypermethylation was followed by loss of mRNA expression of RARB and MGMT in arecoline associated oral cancer (Huang et al., 2010; Lai, et al., 2014). Histone modification is another

epigenetic change by arecoline. Histone protein H3K9 has been shown to be modified by arecoline in human K-562 cell lines and is associated with oral carcinoma (Lin et al., 2011). Collectively, these pieces of evidence suggest the association of betel quid and arecoline to oral carcinogenesis via various epigenetic mechanisms.

1.4. DNA methylation in oral cancer

DNA methylation is one of the several epigenetic mechanisms that cells use to control gene expression. Epigenetic is described as changes in the pattern of gene expression not involving the DNA sequence. Epigenetic events act through chemical modification of DNA and by selectively activating or inactivating genes to determine their expression. DNA methylation and histone modification are two major mechanisms of epigenetic alteration in human cells (Irimie et al., 2018; Shen & Laird, 2013). DNA methylation is an enzymatically catalyzed covalent modification of DNA, occurring typically in the context of cytosine-phosphate-guanine (CpG) dinucleotides. In general, CpG islands, the regions with high CpG content, are demethylated in normal cells. In contrast, regions with an intermediate or low density of CpGs are differentially methylated in some tissues, but not in others (Bird et al., 1985). Changes in methylation of DNA in cancer were first recognized by Feinberg in 1983. In the early days of its recognition, it was thought that epigenetics was linked to a general disruption of the cell cycle, an effect rather than the cause of malignancy (Feinberg & Vogelstein, 1983). The discovery that downregulation of tumor suppressor genes in the absence of a detectable genetic change have led to greater research emphasis on cancer epigenetics (Feinberg, 2001).

Epigenetics refers to the chemical modifications of the DNA leading to selective activation and inactivation of genes thereby influencing their expression. The attachment of the 5-methylcytosine-binding protein to methylated cytosine bases interferes with the binding of transcriptional proteins to gene promoters, halting the expression of that gene. Genes commonly found to be hypermethylated in cancer include tumor suppressors and metastasis-related genes. DNA methylations, unlike genetic mutation, are reversible and can

be a diagnostic and therapeutic target (Ushijima & Asada, 2010). Although several DNA hypermethylation has been detected in oral cancer, little information about it is in the betel quid related oral cancers.

1.5. Purpose of the study

Epigenetic changes are the drivers of oral cancer progression. Earlier studies have analyzed methylation changes in oral precancerous diseases being highly potent for malignant transformation (Cao et al., 2009; Gasco et al., 2002; Kresty et al., 2002; Long et al., 2008). Unlike genetic mutation epigenetic modification is reversible and may be a useful therapeutic target. Hypermethylation on oral epithelium can be a valuable biomarker for prediction of malignant potential of the lesion. Excision of the lesion has been a widely accepted surgical measure to treat these early changes. A key step to improving oral cancer outcomes is identifying the molecular factors driving disease initiation and progression, as these factors represent attractive candidates for targeted therapies (Lubbert et al., 2001; Mack, 2006; Niwa et al., 2013; Schneider & Peek, 2013; Shen et al., 2010; Silverman et al., 2002). A number of approaches exist that enable the large-scale DNA methylation analysis. All of these approaches are based upon any of the three techniques: bisulfite conversion, digestion with methylation sensitive restriction enzymes, and affinity purification of methylated DNA (Zilberman & Henikoff, 2007). DNA microarrays and high-throughput DNA sequencing are commonly used methods of genome-wide study for rapid identification of candidate genes that are associated with carcinogenesis (Kreil et al., 2006). The purpose of this study is the detection of novel DNA hypermethylation that can be applied to diagnostic, predictive, interceptive and therapeutic approaches for betel quid chewing related oral cancer.

2. Materials and Methods

2.1. Genome-wide analysis

Genome-wide analysis in Human Gingival Epithelial Progenitors, pooled (HGEPs) induced by prolonged stimulation with arecoline.

2.1.1. Cell culture (HGEPs)

HGEPs (CELLnTEC advanced cell systems AG, Switzerland) were cultured in CnT-Prime epithelial cell culture medium (CELLnTEC, advanced cell systems AG) containing antibiotics (5% penicillin-streptomycin; Sigma-Aldrich, USA) at 37˚C in an incubator supplied with 5% CO_2. All cells used in this study were at 3 to 5 passages. These samples were treated alternating 3 days with 50 µg/ml arecoline hydrobromide (hereinafter called as arecoline; Sigma-Aldrich, Japan) and 3 days without arecoline for 1 month according to the established method (Fig. 3) (Takai et al., 2016; Uehara et al., 2017). Untreated samples were used as controls.

2.1.2. Methylation array

For methylation array, genomic DNA was extracted from the HGEPs treated with arecoline for 1 month using DNeasy® Blood and Tissue kit (Qiagen, Tokyo, Japan), and sonicated to produce random fragments. One gram of sonicated DNA was incubated with 2 µg of MBD2bt protein. This complex was precipitated using pre blocked nickel magnetic beads. The methylated DNA-enriched DNA fraction was purified using a Qiaquick PCR purification kit (Qiagen) followed by labeling with either cytidine 5-dUTP (Cy 5) or cytidine 3-dUTP (Cy 3). Labeled DNA probes were then mixed and simultaneously hybridized to the human CpG island 224 K array. The DNA samples were analyzed using a DNA microarray scanner (Agilent technology, Santa Clara, USA).

2.1.3. Microarray analysis

For Microarray analysis, total RNA was extracted by the acid guanidine thiocyanate/phenol chloroform method using TRIzol Reagent (Invitrogen Corporation, USA). A global analysis of mRNA expression was carried out using DNA microarray (human SurePrint G3 Human GE Microarray).

2.2. Confirmation of the reproducibility in the candidate genes

From Genome-wide analysis, four candidate genes, named HNRNPH3, BCL2L11, TFAP2A, and DUSP4 were taken. In order to confirm the reproducibility of the methylation array and microarray data, genomic DNA and total RNA were extracted from the cells on 7th and 30th days.

2.2.1. Quantitative methylation-specific polymerase chain reaction

In order to analyze CpG island hypermethylation, the methylation profiles were assessed using a quantitative methylation-specific polymerase chain reaction (qMSP) method. Five hundred ng of genomic DNA was subjected to sodium bisulfite conversion using EpiTect® Fast Bisulfite Conversion kit (Qiagen, USA). For qMSP, two sets of qMSP primers were designed using the MethPrimer (http://urogene.org/methprimer/) (Li & Dahiya, 2002) (Table 1). Bisulfite-converted DNA and a pair of either methylated or unmethylated primers were mixed with KAPA SYBR Fast qPCR Kit (NIPPON Genetics). qMSP was performed on Light Cycler® Nano System (Software version 1.1, Roche Diagnostics, Germany). The qMSP conditions included denaturation at 95ºC for 10 min and 45 cycles of denaturation at 95ºC for 10 sec and annealing at 60ºC for 30 sec. The percentage of DNA methylation in each sample was estimated using the following formula:

$$\text{Methylated DNA (\%)} = \frac{M}{(M+U)} \times 100 = \frac{1}{(1+U/M)} \times 100 = \frac{1}{(1+2^{-\Delta Ct})} \times 100$$

where M and U are the copy number of methylated and unmethylated DNA respectively, and $\Delta Ct = Ct_U - Ct_M$ (Lu et al., 2007; Takai, et al., 2016). Each experiment was conducted in

triplicate on five biologically different culture systems. The result is presented as the percentage of mean ± standard errors of the mean (SE) of DNA methylation.

2.2.2. Quantitative reverse transcription polymerase chain reaction

The expression levels of mRNA in the cultured cells were analyzed by quantitative reverse transcription polymerase chain reaction (qRT-PCR). Two μg of RNA was reverse transcribed (SuperScript Ⅱ Reverse Transcriptase; Invitrogen), according to the manufacturer's instructions using Oligo(dT)$_{12-18}$ primers (Invitrogen). For qRT-PCR, a set of qRT-PCR primer was designed using Primer3 (http://bioinfo.ut.ee/primer3-0.4.0/) (Table 2). cDNA and a pair of primers were mixed with KAPA SYBR Fast qPCR Kit (NIPPON Genetics, Tokyo, Japan). qRT-PCR was performed on Light Cycler® Nano System (Software version 1.1, Roche Diagnostics, Germany). The PCR conditions included denaturation at 95ºC for 10 min and 45 cycles of denaturation at 95ºC for 10 sec and annealing at 60ºC for 30 sec. The expression level of target gene mRNA was standardized against GAPDH mRNA. The relative mRNA expression levels of each sample were calculated as the Ct (the value obtained by subtracting the Ct value of the GAPDH mRNA from the Ct value of the target mRNA) using the ΔΔCt method (Livak & Schmittgen, 2001; Takai, et al., 2016). Each experiment was conducted in three technical and four biological cultures per target gene. Data are expressed as mean ± SE of the ratio of target mRNA to GAPDH mRNA.

2.3. DUSP4 analysis in Oral cancer cell line by qRT-PCR and qMSP

To confirm the DUSP4 expression in oral cancer, cancer cell lines derived from human tongue squamous cell carcinoma (HSC4 and SAS), human basaloid squamous cell carcinoma of the floor of the mouth (BSC-OF) and human papillomavirus-related endocervical adenocarcinoma (HSY) were cultured in Dulbecco's modified eagle's medium (DMEM, Sigma-Aldrich, USA) containing antibiotics (2% penicillin-streptomycin) 10% fetal bovine serum (FBS: Gibco, Invitrogen Corporation, CA) at 37˚C in an incubator supplied with 5% CO_2. Cancer cells were treated with 50 μg/ml arecoline alternating 3 days or untreated for 7

days. Genomic DNA and total RNA were extracted from each sample on the 7th day as described above. qRT-PCR and qMSP were performed in a similar manner to that of HGEPs. To determine the amount of methylation and mRNA expression in cancer cell lines without arecoline treatment, untreated HGEPs were used as controls. In order to determine any further changes in methylation and mRNA expression in cancer cell lines after arecoline treatment, untreated cancer cell lines were used as the control.

2.4. Pathway analysis: phosphorylated c-Jun N-terminal kinase (pJNK)

To examine any mechanism in additional to hypermethylation, cell based Human c-Jun N-terminal kinase (human JNK) phosphorylation ELISA kit (RayBiotech, Inc, USA) was used to detect the phosphorylated JNK (pJNK) in the HGEPs treated with or without arecoline.

2.4.1. JNK inhibitor treatment

HGEPs were cultured in CnT-Prime epithelial cell culture medium for a period of 7 days or 1 month. These cells were divided into four groups based on either treated or untreated with arecoline and/or SP600125 (hereinafter called as JNK inhibitor, Sigma-Aldrich Japan, Tokyo, Japan). JNK inhibitor, at a concentration of 20 μM, was added 1 hour prior to 50 μg/ml arecoline treatment (Lin et al., 2016; Uehara, et al., 2017). These cultures were repeated every three days with or without arecoline and/or JNK inhibitor for the specified time period.

2.4.2. Cell based Enzyme-Linked Immunosorbent Assay (cell ELISA)

Cell based Human JNK phosphorylation ELISA, to detect the pJNK in the cells, was used as per the manufacturer's recommendation. The cells treated and/or untreated with arecoline and/or JNK inhibitor were sub-cultured in 96 well plate at a density of 3×10^4 cells in 100 μl media, incubated with 5% CO_2 at 37˚C. After 24 hrs, these cells were fixed and blocked. Anti-Phospho-JNK (Thr183/Tyr185) or Anti-JNK were pipetted into respective wells and incubated. After the wells were washed, HRP-conjugated anti-mouse IgG was added to the wells. The wells were washed again; a TMB substrate solution was added and incubated in

dark, as a result, blue color developed in proportion to the amount of respective proteins. The stop solution changed the color from blue to yellow and the intensity of the color was determined. Optical absorbance was read at 450nm on Bio Rad 680 microplate reader (Model 680 Microplate reader, RayBiotech, USA).

2.5. Histological and immunohistochemical evaluation of tissue samples obtained from betel quid chewers and non chewers

2.5.1. Clinical characteristic of the patients

Twenty tissue samples were obtained from the patients involved in betel quid chewing habit in Sri Lanka. Twenty-two tissue samples (13 oral squamous cell carcinoma (OSCC), 9 fibrous polyps) were retrieved from the archives of Oral Medicine and Pathology department of Health Sciences University of Hokkaido (HSUH), Japan. Tissue samples from Japanese patients not involved in betel chewing were obtained from those who underwent the oral surgical intervention during the period of 2008 to 2014 in HSUH hospital. This study was approved by the Human genome ethics committee of HSUH (No. 7) and the ethics committee of the Institute of Personalized Medical Science, HSUH (No. 2016-025). Post-surgical tissue sections were already formalin fixed, processed and paraffin embedded following standard protocol.

2.5.2. Immunohistochemical (IHC) analysis

Histopathological examination using routine hematoxylin and eosin staining was performed on the tissue sections at the 5µm thickness to verify the clinical diagnosis. Thereafter, immunohistochemical examination of the tissue samples was performed using anti-DUSP4 antibody. The sections of 5µm thickness were made of the samples and mounted on silane coated slides (New Silane III, Muto pure chemicals co. Ltd, Japan). These slides were deparaffinized in xylene (3 changes every 3 minutes) and rehydrated in a graded alcohol series (100%, 90%, 80% and 70% every 3 minutes). Antigen retrieval was done using 10 mmol

citrate buffer (Citric acid Monohydrate and Tri Sodium Citrate Dihydrate) by heat-induced epitope retrieval method. These slides were maintained at a sub-boiling temperature in a pressure cooker for 3 minutes and bench cooled for further 20 min. Endogenous peroxidase activity was blocked with 3% H_2O_2 in methanol for 10 min. These slides were washed twice for 5 min each with TBS plus 0.025% Triton X-100 with gentle agitation. Specimens were incubated overnight in a humidified chamber at 37ºC with rabbit polyclonal anti-DUSP4 primary antibody (ab72593, diluted to 100× as per manufacturer Abcam, Japan's recommendation). These slides were washed again with TBS plus 0.025% Triton X-100 and treated with rabbit polyclonal secondary antibody for 30 min at room temperature. Reaction products were visualized with diaminobenzidine chromogen concentrate (Dako, USA/Japan) and finally counterstained with hematoxylin.

2.5.3. Scoring the positive cells

DUSP4 immunoreactivity was assessed in the nucleus of epithelial cells; staining was considered evidence of expression. For each sample, at least three fields were equidistantly captured at a magnification of 200×under a light microscope (Olympus BX50, Olympus Corporation). All the cells from the representative fields were counted using the public domain program ImageJ version 1.50b (National Institute of Health, USA). The total number of the positive cells from each examined fields was determined. DUSP4 immunopositivity in each sample was expressed as a composite score by taking the mean of all the cells counted in the selected fields. The composite score was expressed in terms of percentage of the total cells present in those specified fields.

2.6. qMSP in clinical samples

Genomic DNA was extracted from the tissue samples using QIAmp® DNA FFPE tissue kit (Qiagen) following manufacturer's recommendation. Freshly cut 8-15 (depending upon the amount of tissue) tissue sections of 5 μm and not exceeding surface area of 250 mm^2 were subjected to deparaffinization on 1 ml of xylene. These samples were incubated with

proteinase K at 56ºC for 1 hour for lysis and further 1 hour at 90ºC to reverse the formaldehyde modification of nucleic acids. These samples were washed and DNA eluted with recommended buffers. For each sample, 500 ng of purified DNA was subjected to sodium bisulfite conversion and qMSP performed as described earlier. Each experiment was conducted in triplicate and the result is presented as the percentage of mean ± SE of DNA methylation.

2.7. Statistical analysis

All values are expressed as the mean ± SE for the respective groups. Statistical analysis was performed using IBM SPSS Statistical tool for iOS (Version 25; SPSS Inc, USA). The results were compared using the Mann-Whitney U test with a p-value <0.05 accepted as statistically significant.

3. *Results*

3.1. Genome-wide DNA methylation analysis, identification of candidate genes

CpG Island methylation array data suggested 8638 and 7392 genes that were more than 2 times hypermethylated and hypomethylated respectively in samples stimulated with arecoline for a period of 1 month. Global analysis of mRNA revealed 502 upregulated and 455 downregulated genes. Since hypermethylation of tumor suppressor genes followed by down-regulation of their mRNA often induces malignant transformation, these types of genes were selected. Among the genes showing hypermethylation with downregulated expression of their mRNA, 152 genes were within -1000bp from promoter region. Twenty-one of them were tumor suppressor genes. Four candidate genes with the highest amount of promoter region hypermethylation with downregulated mRNA expression were selected. HNRNPH3, BCL2L11, TFAP2A, and DUSP4 were the candidate genes in descending order of amount of promoter region hypermethylation with downregulated mRNA expression (Table 3).

3.2. Reproducibility of microarray data by qMSP and qRT-PCR

In order to check the reproducibility of microarray data and verify the candidate genes in arecoline treated samples, qMSP and qRT-PCR were performed using the primers for the candidate genes. qMSP showed that TFAP2A and DUSP4 genes were significantly hypermethylated in samples treated with arecoline for a period of 7 days (Fig. 4) ($p<0.05$); and DUSP4 in 1 month (Fig. 5) ($p<0.05$). mRNA expression of TFAP2A and DUSP4 were down-regulated whereas that of BCL2L11 was up-regulated in samples treated with arecoline for 7 days (Fig 6) ($p<0.05$). Similarly, HNRNPH3 showed up-regulated whereas DUSP4 showed down-regulated mRNA expression in the period of 1 month (Fig. 7) ($p<0.05$). Overall, the analysis revealed DUSP4 was hypermethylated and downregulated by arecoline treatment within a short interval and remained sustainable over a long period.

3.3. Evaluation of DUSP4 in oral cancer cells

In order to observe whether oral cancer exhibit hypermethylation of DUSP4 with down-regulated expression of its mRNA, the levels of its methylation and mRNA expression were evaluated in oral cancer cell lines. DUSP4 CpG island methylation and mRNA expression of 4 different cancer cell lines were analyzed before and after treatment with arecoline for a period of 7 days. Methylation level of DUSP4 was significantly higher in all cancer cell lines without arecoline treatment as compared to HGEPs (Fig. 8) ($p<0.05$). Upon treatment with arecoline for a period of 7 days, no significant changes were detected in DUSP4 DNA methylation in any of the cancer cell lines (Fig. 9). mRNA expression DUSP4 gene before arecoline treatment on HSC4, SAS and HSY were significantly lower as compared to HGEPs (Fig. 10). mRNA expression of DUSP4 in untreated BSC-OF cell line was not significantly different from that of HGEPs. After treatment with arecoline for 7 days, DUSP4 mRNA showed no further changes in the three cancer cell lines. On the other hand, BSC-OF showed downregulated DUSP4 mRNA expression after arecoline treatment (Fig. 11) ($p<0.05$).

3.4. Cell ELISA to determine the level of pJNK after arecoline treatment

Protein phosphorylation is instrumental in the regulation of protein activity within a cell. It plays important roles in the living cells including proliferation, differentiation, and metabolism. In order to determine the presence of any additional mechanisms of arecoline upon DUSP4, a downstream product of DUSP4, JNK was evaluated. In this study, the amount of phosphorylated JNK (pJNK) was significantly lower ($p<0.05$) in untreated samples as compared to samples treated with arecoline for 1 month (Fig. 12). Furthermore, pJNK was suppressed in samples treated with JNK inhibitor but elevated in samples treated with arecoline over a period of 1 month ($p<0.05$).

3.5. Immunohistochemical staining for DUSP4 in tissue samples obtained from betel quid chewers

In order to determine the expression of DUSP4 in tissue samples, Immunohistochemical (IHC) staining was observed for DUSP4 in oral cancer tissue samples obtained from patients with or without a habit of betel quid chewing habit (Fig. 13). Clinical and histopathological characteristics of the patients are presented in table 4. IHC data was sub-quantitatively analyzed. IHC staining revealed that DUSP4 expression level was significantly higher in betel quid chewers (66.85 ± 3.0) than in non-chewer OSCC (20.34 ± 4.7) (Fig. 14) ($p<0.05$). There was no statistical difference in DUSP4 expression between the non-chewer oral cancer patient and non-chewer healthy controls (17.24 ± 2.74).

3.6. Methylation analysis of DUSP4 CpG island in betel quid chewers

In order to determine the CpG island methylation of DUSP4 in tissue samples, qMSP was performed in DNA samples obtained from oral cancer tissue samples of patients with or without a habit of betel quid chewing habit. qMSP analysis showed that DUSP4 CpG island was significantly hypermethylated in oral cancer samples obtained from betel quid chewers (89.8 ± 2.6) as compared to those from non-chewer oral cancer cases (44.7 ± 10.6) and the healthy control cases (49.4 ± 13.1) (Fig. 15) ($p<0.05$). There was no statistical difference in DUSP4 expression between the non-chewer oral cancer patient and non-chewer healthy controls.

4. *Discussion*

In the present study, global analyses of DNA hypermethylation and mRNA were carried out using CpG island DNA methylation array and DNA microarray in cultured epithelial cells stimulated with arecoline. A candidate gene selected from the microarray data was evaluated for betel quid-related oral cancer using samples obtained from the patients with the habit of betel quid chewing. This is the first report that shows hypermethylation of DUSP4 followed by its down-regulated expression in the betel quid related oral cancer.

4.1. Genome-wide DNA methylation analysis

A genome-wide study is a useful tool for the rapid identification of candidate genes that are associated with carcinogenesis. A number of approaches that enable the large-scale DNA methylation analysis exists, all of which are based upon any of the three techniques: bisulfite conversion, digestion with methylation sensitive restriction enzymes, and affinity purification of methylated DNA (Zilberman & Henikoff, 2007). The Human Epigenome Project used standard sequencing approaches to sequence a massive amount of bisulfite-converted DNA from human tissues and primary cells (Eckhardt et al., 2006). Another study used restriction enzymes and standard cloning and sequencing to analyze almost 14 Mb of unmethylated human DNA and over 8 Mb of methylated DNA (Rollins et al., 2006). These approaches are expensive, labor-intensive and beyond the capabilities of most laboratories despite being highly informative. DNA microarrays and high-throughput DNA sequencing are other methods of genome-wide study mostly used by laboratories and research institutions. High quality commercial oligonucleotide arrays fabricated by commercial industries are available for DNA microarrays in the present day. Bead arrays by Illuminia, short oligonucleotide array by Affimetrix, long oligonucleotide arrays by NimbleGen and Agilent and single nucleotide polymorphism arrays are few among the popular ones. Although each technology has its advantages, the drawbacks should not be overlooked. Illumina bead assay can analyze up to 96 samples at once but only 1536 sites can be assayed simultaneously. Affymetrix arrays are economically favorable but short oligonucleotide probes produce noisier data. To overcome

this limitation longer oligonucleotide arrays are developed by NimbleGen and Agilent which gives cleaner data and dual channel hybridization and makes custom array much inexpensive. Agilent array is a dual channel where two samples are labeled with different fluorescent dyes, such as immunoprecipitated test DNA and control DNA, and are hybridized on a single chip. Hybridizing the test and control samples on the same array overcomes inter-array variation and thus reduces the need for replicates. The major disadvantage of these arrays versus the Affymetrix array is reduced oligonucleotide probe density. However, the longer probes (60-mer) provide a better balance between specificity, sensitivity, and noise than the 25-mers on the Affymetrix array (Kreil, et al., 2006). This translates into array data that require less statistical manipulation. Both NimbleGen and Agilent allow the production of custom arrays. This allows for flexibility in experimental design, as well as in the analysis of DNA methylation in organisms other than mammals and *Arabidopsis* (Zilberman & Henikoff, 2007). In addition to the above-mentioned advantages, there are a few other reasons why this study preferred Agilent technology. The oligonucleotide in this experiment is so designed to avoid the cDNA probe drawbacks (as used in traditional array techniques) and to maximize the specificity for the target gene. In addition, the glass provides an excellent support for attaching the nucleotide sequences, is less sensitive to light, and is non-porous, allowing the use of very small amounts of samples. With these benefits, this study utilized the Agilent technology to analyze the genome-wide methylation of DNA.

4.2. Identification of candidate genes

Genomewide DNA analysis data suggested 8638 genes that were more than 2 times hypermethylated in arecoline treated samples. As the transcription of the gene is closely affected by the promoter regions that are close to the start codon, a search for genes within -1000 bp from promoter region with downregulated mRNA was conducted. Result showed 50 tumor-related genes out of 152 genes. Four candidate genes in descending order of amount of promoter region hypermethylation named HNRNPH3, BCL2L11, TFAP2A and DUSP4 were considered as the candidate genes in this study. DUSP4 gene was significantly

hypermethylated in the HGEPs stimulated with arecoline for both short (7 days) and prolonged periods (1month). DUSP4 mRNA was significantly down-regulated following its hypermethylation. The hypermethylation of CpG island in the gene promoter regions often leads to down-regulated expression of the transcriptional levels (Amormino et al., 2013; Huang, et al., 2010). The down-regulated expression of DUSP4 may be due to their hypermethylation. Therefore, DUSP4 may be a candidate for the crucial gene involved in arecoline related oral cancer.

4.3. DUSP4 gene

4.3.1. Evaluation of DUSP4 methylation in cancer cell lines

The levels of CpG methylation and mRNA expression of DUSP4 was evaluated in oral cancer cell lines. Methylation level of DUSP4 was significantly higher in all cancer cell lines without arecoline treatment as compared to HGEPs. The expression levels of DUSP4 mRNA were significantly lower in the 3 cell lines (HSC4, SAS and HSY) except BSC-OF than the control (HGEPs). The lower level of DUSP4 expression has been confirmed in a number of human cancers including gastric cancer, breast cancer and lymph node cancer implying it to be a tumor suppressor gene (Liu et al., 2013; Schmid et al., 2015; Zhang et al., 2017). Promoter region hypermethylation of DUSP4 followed by reduced expression level of DUSP4 protein and mRNA has been found in astrogliomas and glioma cell lines (Waha et al., 2010). The present data about 3 cell lines were consistent with those previous reports. The hypermethylation of DUSP4 followed by its downregulated mRNA expression may be involved in tumorigenesis of some oral cancers. Interestingly, BSC-OF showed hypermethylation of DUSP4 without down-regulated expression of its mRNA. The arecoline stimulation induced down-regulated expression of DUSP4 in BSC-OF. Previously, tumor suppressor genes such as p21 and p27 were down-regulated via reactive oxygen spices by the stimulation with arecoline (Ji, et al., 2012). Arecoline may stimulate down-regulated expression of DUSP4 via pathways other than its hypermethylation. Further experiments need to clarify this speculation.

4.3.2. CpG island methylation and Immunohistochemical analysis of DUSP4 in tissue samples obtained from betel quid chewers

CpG island methylation level and immunohistochemical expression of DUSP4 gene were examined in the tissues samples obtained from OSCC in betel quid chewers. DUSP4 CpG island was significantly hypermethylated in oral cancer samples obtained from betel quid chewers as compared to those from non-chewer oral cancer cases and the healthy control cases. The results strongly supported an implication from genome-wide analysis data that the hypermethylation of DUSP4 might be a specific phenomenon in the betel quid related oral cancer. Immunohistochemical staining was performed to observe the protein level of DUSP4 in the tissue samples. Unexpectedly, the OSCC samples obtained from betel quid chewer showed that DUSP4 expression level was significantly higher in the OSCC samples obtained from betel quid chewer than those from non-chewers. There are controversial reports about the involvement of DUSP4 in development and progression of cancers. Several reports revealed that DUSP4 may play a role in carcinogenesis and promoting cancer progression (Wang et al., 2003), while others reported that DUSP4 may play a role in cancer suppression (Ichimanda et al., 2018; Kao et al., 2013; Schmid, et al., 2015). A recent paper showed that down-regulated expression of DUSP4 in colorectal cancer cells was related to their progression, although immunohistochemical staining for DUSP4 was stronger and wider in the superficial region of colorectal cancer tissues (Ichimanda, et al., 2018). Immunohistochemical staining for DUSP4 in the tissues may not reflect its in vitro data. p53, a tumor suppressor gene, have shown the same manner as DUSP4. The down-regulated expression of p53 is frequently observed in the cancer cells, while immunohistochemical staining for p53 was more intense in those tissues (Nees et al., 1993). The half-life of the normal p53 protein is too short for their stability to detect in the tissues samples. The mutated p53 can be detected since silencing p53 make their protein stability longer (Nees, et al., 1993). The half-life of the normal DUSP4 protein is not long enough to be stable itself. With a short half-life of ~1/2 hr, there implies a strict protein stability regulation (Hsiao et al., 2016). The down-regulated expression of DUSP4 may induce their protein stability longer.

Further investigations are needed to prove this speculation.

4.3.3. Loss of DUSP4 by arecoline and interaction with JNK pathway

c-Jun N-terminal kinase (JNK), a kinase transcribed by JNK gene is implicated in oncogenic transformation (Kuo et al., 2006). Cell based ELISA in this study showed that the amount of pJNK was significantly higher after arecoline treatment as compared to the controls. JNKs are activated by phosphorylation in the activation loop at residues Thr183/Tyr185. Activated JNK translocates to the nucleus and transactivates c-Jun and other target transcription factors (Cicenas, 2015; Gkouveris & Nikitakis, 2017). They are dephosphorylated and deactivated by MAPK phosphatases like DUSP4 (Cicenas, 2015). Presence of DUSP4 in untreated samples negatively regulates the phosphorylation of JNK in the same manner as the known inhibitors. This result is consistent with a recent study: it has been shown that JNK is a preferred and biologically relevant MAPK target of DUSP4 in diffuse large B cell lymphoma (Schmid, et al., 2015). Loss of DUSP4, after arecoline treatment in this study, might have contributed to uncontrolled phosphorylation thereby activation of JNK.

5. *Clinical implication*

Manipulation of epigenetic changes is a useful mechanism for the prevention of cancer (Feinberg, 2001; Williams et al., 2014). Aberrant DNA methylation due to arecoline is a risk factor for the development of oral cancer. Arecoline related DNA hypermethylation of TSG has been detected as early as in premalignant disorders (Takeshima, et al., 2008). Unlike genetic mutation, epigenetic modification is reversible, it may be useful as a therapeutic target. Application of demethylating agents may be helpful to prevent malignant transformation from precancerous lesions. Clinical trials targeting epigenetic modifications, including DNA methylation and histone deacetylation, have been conducted (Ho et al., 2013). DNA methylation inhibitors, including, 5-aza-2′-deoxycytidine (decitabine), 5-azacytidine (5-aza), 5,6-dihydro-5-azacytidine, zebularine and RNA interference (RNAi) and antisense inhibitors

of DNMT1 have been used as epigenetic targets in cancer therapy (Lubbert, et al., 2001; Mack, 2006; Niwa, et al., 2013; Schneider & Peek, 2013; L. Shen, et al., 2010; Silverman, et al., 2002). Unlike RNA, DNA is a stable molecule and can be easily obtained from tissue samples. This enables the detection of DNA methylation at any time-frame after initial biopsy or after surgery for premalignant or malignant lesions. Detection of aberrant methylation of DUSP4 in an initial lesion may enable to predict future carcinogenic progression. Application of demethylating agent in such pre-malignant conditions can prevent possible malignant transformation.

6. *Conclusion*

In the present study, genome-wide DNA hypermethylation and global mRNA microarray analysis was carried out using CpG island DNA methylation array and DNA microarray in HGEPs stimulated with arecoline. A candidate gene DUSP4, selected from the microarray data was evaluated for betel quid-related oral. This is the first report that shows betel quid related hypermethylation of DUSP4 followed by its down-regulated expression. Methylation-associated DUSP4 silencing and Thr183/Tyr185 associated phosphorylation of JNK are the two mechanisms presented in this study (Fig. 16). This study revealed DUSP4 as a potential candidate target gene affected by arecoline, a betel quid component. DUSP4 hypermethylation can be applied as a target for diagnostic, predictive, interceptive and therapeutic approaches for betel quid chewing related oral cancer.

References

Asthana S, Labani S, Kailash U, Sinha D N, & Mehrotra R. Association of Smokeless Tobacco Use and Oral Cancer: A Systematic Global Review and Meta-Analysis. Nicotine Tob Res 2018.

Bird A, Taggart M, Frommer M, Miller O J, & Macleod D. A fraction of the mouse genome that is derived from islands of nonmethylated, CpG-rich DNA. Cell 40:91-99, 1985.

Cao J, Zhou J, Gao Y, Gu L, Meng H, Liu H, & Deng D. Methylation of p16 CpG Island Associated with Malignant Progression of Oral Epithelial Dysplasia: A Prospective Cohort Study. Clinical Cancer Research 15:5178-5183, 2009.

Chiba I, Muthumala M, Yamazaki Y, Uz Zaman A, Iizuka T, Amemiya A, Shibata T, Kashiwazaki H, Sugiura C, & Fukuda H. Characteristics of mutations in the p53 gene of oral squamous-cell carcinomas associated with betel-quid chewing in Sri Lanka. Int J Cancer 77:839-842, 1998.

Cicenas J. JNK inhibitors: is there a future? MAP Kinase 4:2015.

de Faria Amormino S A, Arao T C, Saraiva A M, Gomez R S, Dutra W O, da Costa J E, de Fatima Correia Silva J, & Moreira P R. Hypermethylation and low transcription of TLR2 gene in chronic periodontitis. Hum Immunol 74:1231-1236, 2013.

Eckhardt F, Lewin J, Cortese R, Rakyan V K, Attwood J, Burger M, Burton J, Cox T V, Davies R, Down T A, Haefliger C, Horton R, Howe K, Jackson D K, Kunde J, Koenig C, Liddle J, Niblett D, Otto T, Pettett R, Seemann S, Thompson C, West T, Rogers J, Olek A, Berlin K, & Beck S. DNA methylation profiling of human chromosomes 6, 20 and 22. Nature Genetics 38:1378, 2006.

Feinberg A P. Cancer epigenetics takes center stage. Proceedings of the National Academy of Sciences 98:392-394, 2001.

Feinberg A P, & Vogelstein B. Hypomethylation of ras oncogenes in primary human cancers. Biochemical and Biophysical Research Communications 111:47-54, 1983.

Gasco M, Bell A K, Heath V, Sullivan A, Smith P, Hiller L, Yulug I, Numico G, Merlano M, Farrell P J, Tavassoli M, Gusterson B, & Crook T. Epigenetic inactivation of 14-3-3 σ in oral carcinoma: Association with p16INK4asilencing and human papillomavirus negativity. Cancer Res 62:2072-2076, 2002.

Gkouveris I, & Nikitakis N G. Role of JNK signaling in oral cancer: A mini review. Tumour Biol 39:1010428317711659, 2017.

Gupta P C, Mehta F S, Daftary D K, Pindborg J J, Bhonsle R B, Jalnawalla P N, Sinor P N, Pitkar V K, Murti P R, Irani R R, Shah H T, Kadam P M, Iyer K S, Iyer H M, Hegde A K, Chandrashekar G K, Shiroff B C, Sahiar B E, & Mehta M N. Incidence rates of oral cancer and natural history of oral precancerous lesions in a 10-year follow-up study of Indian villagers. Community Dent Oral Epidemiol 8:283-333, 1980.

Ho A S, Turcan S, & Chan T A. Epigenetic therapy: use of agents targeting deacetylation and methylation in cancer management. OncoTargets and therapy 6:223-232, 2013.

Hsiao W-Y, Lin Y-C, Liao F-H, Chan Y-C, & Huang C-Y. Dual-Specificity Phosphatase 4 Regulates STAT5 Protein Stability and Helper T Cell Polarization*. PLoS One 10:e0145880, 2016.

Huang S H, Lee H S, Mar K, Ji D D, Huang M S, & Hsia K T. Loss expression of O6-methylguanine DNA methyltransferase by promoter hypermethylation and its relationship to betel quid chewing in oral squamous cell carcinoma. Oral Surg Oral Med Oral Pathol Oral Radiol Endod 109:883-889, 2010.

Ichimanda M, Hijiya N, Tsukamoto Y, Uchida T, Nakada C, Akagi T, Etoh T, Iha H, Inomata M, Takekawa M, & Moriyama M. Downregulation of dual-specificity phosphatase 4 enhances cell proliferation and invasiveness in colorectal carcinomas. Cancer Science 109:250-258,

2018.

Irimie I A, Ciocan C, Gulei D, Mehterov N, Atanasov G A, Dudea D, & Berindan-Neagoe I. Current Insights into Oral Cancer Epigenetics. International Journal of Molecular Sciences 19:2018.

Jeng JH, Chang MC, & LJ H. Role of areca nut in betel quid-associated chemical carcinogenesis: current awareness and future perspectives. Oral Oncology 37:477-492, 2001.

Ji W T, Yang S R, Chen J Y, Cheng Y P, Lee Y R, Chiang M K, & Chen H R. Arecoline downregulates levels of p21 and p27 through the reactive oxygen species/mTOR complex 1 pathway and may contribute to oral squamous cell carcinoma. Cancer Sci 103:1221-1229, 2012.

Kannan K, Munirajan A K, Krishnamurthy J, Bhuvarahamurthy V, Mohanprasad B K, Panishankar K H, Tsuchida N, & Shanmugam G. Low incidence of p53 mutations in betel quid and tobacco chewing-associated oral squamous carcinoma from India. Int J Oncol 15:1133-1136, 1999.

Kao D D, Oldebeken S R, Rai A, Lubos E, Leopold J A, Loscalzo J, & Handy D E. Tumor necrosis factor-alpha-mediated suppression of dual-specificity phosphatase 4: crosstalk between NFkappaB and MAPK regulates endothelial cell survival. Mol Cell Biochem 382:153-162, 2013.

Kreil D P, Russell R R, & Russell S. Microarray Oligonucleotide Probesl In*Methods in Enzymology* (Vol. 410, pp. 73-98). Academic Press. 2006, 73-98.

Kresty L A, Mallery S R, Knobloch T J, Song H, Lloyd M, Casto B C, & Weghorst C M. Alterations of p16INK4a and p14ARF in patients with severe oral epithelial dysplasia. Cancer Res 62:5295-5300, 2002.

Kuo R C, Lin C Y, & Kuo M Y. Prognostic role of c-Jun activation in patients with areca quid chewing-related oral squamous cell carcinomas in Taiwan. J Formos Med Assoc 105:229-234,

2006.

Lai Z L, Tsou Y A, Fan S R, Tsai M H, Chen H L, Chang N W, Cheng J C, & Chen C M. Methylation-associated gene silencing of RARB in areca carcinogens induced mouse oral squamous cell carcinoma. Biomed Res Int 2014:378358, 2014.

Lando M, Fjeldbo C S, Wilting S M, B C S, Aarnes E K, Forsberg M F, Kristensen G B, Steenbergen R D, & Lyng H. Interplay between promoter methylation and chromosomal loss in gene silencing at 3p11-p14 in cervical cancer. Epigenetics 10:970-980, 2015.

Lee C H, Ko A M, Warnakulasuriya S, Yin B L, Sunarjo, Zain R B, Ibrahim S O, Liu Z W, Li W H, Zhang S S, Kuntoro, Utomo B, Rajapakse P S, Warusavithana S A, Razak I A, Abdullah N, Shrestha P, Kwan A L, Shieh T Y, Chen M K, & Ko Y C. Intercountry prevalences and practices of betel-quid use in south, southeast and eastern Asia regions and associated oral preneoplastic disorders: an international collaborative study by Asian betel-quid consortium of south and east Asia. Int J Cancer 129:1741-1751, 2011.

Lee W, Hsiao J, Ou C, Huang C, Chang C, Tsai S, Chen K, Huang J, Wong T, Lai Y, Wu Y, Hsueh W, Wu S, Yen C, Chang J, Lin C, Weng Y, Yang H, Chen Y, & Chang J. The influence of pre-diagnosis alcohol consumption and the polymorphisms of ethanol metabolizing genes on the survival of head and neck cancer patients. Cancer Epidemiology Biomarkers & Prevention 2018.

Li L C, & Dahiya R. MethPrimer: designing primers for methylation PCRs. Bioinformatics 18:1427-1431, 2002.

Li Y, Wang Y, Sun H, Zhang Y, Li H, Cong X, Yin W, & Song W. Association Between Matrix Metalloproteinase-1, 2, 3 Polymorphisms and Oral Cancer Risk: A Meta-Analysis. Genet Test Mol Biomarkers 22:456-464, 2018.

Lin P C, Chang W H, Chen Y H, Lee C C, Lin Y H, & Chang J G. Cytotoxic effects produced by arecoline correlated to epigenetic regulation in human K-562 cells. J Toxicol Environ

Health A 74:737-745, 2011.

Lin S H, Chiou S J, Ho W T, Chuang C T, Chuang L Y, & Guh J Y. Arecoline-induced pro-fibrotic proteins in LLC-PK1 cells are dependent on c-Jun N-terminal kinase. Toxicology 344-346:53-60, 2016.

Lin Y M, Shao J, Yin X H, Huang C, Jia X W, Yuan Y D, Wu C J, Zhen E M, Yao Z X, Zeng X T, & Liu R H. Meta-Analysis Results on the Association Between TP53 Codon 72 Polymorphism With the Susceptibility to Oral Cancer. Front Physiol 9:1014, 2018.

Liu Y, Du F, Chen W, Yao M, Lv K, & Fu P. Knockdown of dual specificity phosphatase 4 enhances the chemosensitivity of MCF-7 and MCF-7/ADR breast cancer cells to doxorubicin. Exp Cell Res 319:3140-3149, 2013.

Livak K J, & Schmittgen T D. Analysis of relative gene expression data using real-time quantitative PCR and the 2(-Delta Delta C(T)) Method. Methods 25:402-408, 2001.

Long N K, Kato K, Yamashita T, Makita H, Toida M, Hatakeyama D, Hara A, Mori H, & Shibata T. Hypermethylation of the RECK gene predicts poor prognosis in oral squamous cell carcinomas. Oral Oncology 44:1052-1058, 2008.

Lu L, Katsaros D, Rigault de la Longrais I A, Sochirca O, & Yu H. Hypermethylation of let-7a-3 in Epithelial Ovarian Cancer Is Associated with Low Insulin-like Growth Factor-II Expression and Favorable Prognosis. Cancer Res 67:10117-10122, 2007.

Lubbert M, Wijermans P, Kunzmann R, Verhoef G, Bosly A, Ravoet C, Andre M, & Ferrant A. Cytogenetic responses in high-risk myelodysplastic syndrome following low-dose treatment with the DNA methylation inhibitor 5-aza-2'-deoxycytidine. Br J Haematol 114:349-357, 2001.

Mack G S. Epigenetic cancer therapy makes headway. J Natl Cancer Inst 98:1443-1444, 2006.

Nees M, Homann N, Discher H, Andl T, Enders C, Herold-Mende C, Schuhmann A, & Bosch F X. Expression of Mutated p53 Occurs in Tumor-distant Epithelia of Head and Neck Cancer

Patients: A Possible Molecular Basis for the Development of Multiple Tumors. Cancer Res 53:4189-4196, 1993.

Neha Gupta R G, Arun Kumar Acharya, Basavaraj Patthi, Venkatesh Goud, Somanath, & Reddy A G, Ashish Singla. Changing Trends in oral cancer – a global scenario. Nepal J Epidemiol 6:613-619, 2016.

Niwa T, Toyoda T, Tsukamoto T, Mori A, Tatematsu M, & Ushijima T. Prevention of *Helicobacter pylori*-Induced Gastric Cancers in Gerbils by a DNA Demethylating Agent. Cancer Prevention Research 6:263-270, 2013.

Petti S. Lifestyle risk factors for oral cancer. Oral Oncology 45:340-350, 2009.

Rollins R A, Haghighi F, Edwards J R, Das R, Zhang M Q, Ju J, & Bestor T H. Large-scale structure of genomic methylation patterns. Genome Res 16:157-163, 2006.

Schmid C A, Robinson M D, Scheifinger N A, Muller S, Cogliatti S, Tzankov A, & Muller A. DUSP4 deficiency caused by promoter hypermethylation drives JNK signaling and tumor cell survival in diffuse large B cell lymphoma. J Exp Med 212:775-792, 2015.

Schneider B G, & Peek R M. Gastric Cancer Prevention by Demethylation. Cancer Prevention Research 6:253-256, 2013.

Sharan R N, Mehrotra R, Choudhury Y, & Asotra K. Association of betel nut with carcinogenesis: revisit with a clinical perspective. PLoS One 7:e42759, 2012.

Shen H, & Laird P W. Interplay between the cancer genome and epigenome. Cell 153:38-55, 2013.

Shen L, Kantarjian H, Guo Y, Lin E, Shan J, Huang X, Berry D, Ahmed S, Zhu W, Pierce S, Kondo Y, Oki Y, Jelinek J, Saba H, Estey E, & Issa J-P J. DNA Methylation Predicts Survival and Response to Therapy in Patients With Myelodysplastic Syndromes. Journal of Clinical Oncology 28:605-613, 2010.

Siegel R L, Miller K D, & Jemal A. Cancer Statistics, 2017. CA Cancer J Clin 67:7-30, 2017.

Silverman L R, Demakos E P, Peterson B L, Kornblith A B, Holland J C, Odchimar-Reissig R, Stone R M, Nelson D, Powell B L, DeCastro C M, Ellerton J, Larson R A, Schiffer C A, & Holland J F. Randomized controlled trial of azacitidine in patients with the myelodysplastic syndrome: a study of the cancer and leukemia group B. J Clin Oncol 20:2429-2440, 2002.

Takai R, Uehara O, Harada F, Utsunomiya M, Chujo T, Yoshida K, Sato J, Nishimura M, Chiba I, & Abiko Y. DNA hypermethylation of extracellular matrix-related genes in human periodontal fibroblasts induced by stimulation for a prolonged period with lipopolysaccharide derived from Porphyromonas gingivalis. J Periodontal Res 51:508-517, 2016.

Takeshima M, Saitoh M, Kusano K, Nagayasu H, Kurashige Y, Malsantha M, Arakawa T, Takuma T, Chiba I, Kaku T, Shibata T, & Abiko Y. High frequency of hypermethylation of p14, p15 and p16 in oral pre-cancerous lesions associated with betel-quid chewing in Sri Lanka. J Oral Pathol Med 37:475-479, 2008.

Tsai Y S, Lee K W, Huang J L, Liu Y S, Juo S H, Kuo W R, Chang J G, Lin C S, & Jong Y J. Arecoline, a major alkaloid of areca nut, inhibits p53, represses DNA repair, and triggers DNA damage response in human epithelial cells. Toxicology 249:230-237, 2008.

Uehara O, Takimoto K, Morikawa T, Harada F, Takai R, Adhikari B R, Itatsu R, Nakamura T, Yoshida K, Matsuoka H, Nagayasu H, Saito I, Muthumala M, Chiba I, & Abiko Y. Upregulated expression of MMP-9 in gingival epithelial cells induced by prolonged stimulation with arecoline. Oncol Lett 14:1186-1192, 2017.

Ushijima T, & Asada K. Aberrant DNA methylation in contrast with mutations. Cancer Sci 101:300-305, 2010.

Waha A, Felsberg J, Hartmann W, von dem Knesebeck A, Mikeska T, Joos S, Wolter M, Koch A, Yan P S, Endl E, Wiestler O D, Reifenberger G, Pietsch T, & Waha A. Epigenetic downregulation of mitogen-activated protein kinase phosphatase MKP-2 relieves its growth suppressive activity in glioma cells. Cancer Res 70:1689-1699, 2010.

Wang H-y, Cheng Z, & Malbon C C. Overexpression of mitogen-activated protein kinase phosphatases MKP1, MKP2 in human breast cancer. Cancer Lett 191:229-237, 2003.

WHO International Agency for Research on Cancer. IARC monographs on the evaluation of carcinogenic risks to humans, vol 85. Betel-quid and areca-nut chewing and some areca-nut-derived nitrosamines. Lyon, France: International Agency for Cancer Research, June, 2004. http://monographs.iarc.fr/ENG/ Monographs/vol85/mono85.pdf

Williams S D, Hughes T E, Adler C J, Brook A H, & Townsend G C. Epigenetics: a new frontier in dentistry. Aust Dent J 59 Suppl 1:23-33, 2014.

Zhang R, Wang G, Zhang P F, Zhang J, Huang Y X, Lu Y M, Da W, Sun Q, & Zhu J S. Sanguinarine inhibits growth and invasion of gastric cancer cells via regulation of the DUSP4/ERK pathway. J Cell Mol Med 21:1117-1127, 2017.

Zilberman D, & Henikoff S. Genome-wide analysis of DNA methylation patterns. Development 134:3959-3965, 2007.

Table1. Primer sequence used for DNA CpG island methylation analysis

Genes	Description	Gene Bank No.	Primers (5' - 3')	Product size (bp)
HNRNPH3	Heterogeneous nuclear ribonucleoprotein H3	NM_012207	MF: TTTCGAGGTATTGACGGC	122
			MR: GCGAAATCCGAATTTCTTAA	
			UF: AGTTTTTGAGGTATTGATGGT	122
			UR: CACACAAAATCCAAATTTCTTAA	
BCL2L11	BCL2 like 11	NM_207002	MF: GTTATAGTTAGGGCGGTGACG	166
			MR: AACGATTATTAACAAACTCGCC	
			UF: TTGTTATAGTTAGGGTGGTGATGG	166
			UR: AAACAATTATTAACAAACTCACC	
TFAP2A	Transcription factor AP-2 alpha (Activating enhancer binding Protein 2 alpha)	NM_0010322	MF: TTAGGAGAGTTTTTAGGAGATAGCG	141
			MR: CCAAACCACCTATAAAACGTACG	
			UF: TAGGAGAGTTTTTAGGAGATAGTGA	141
			UR: ACCAAACCACCTATAAAACATACAC	

DUSP4	Dual specificity phosphatase 4	NM_001394	MF: TTATAGTTTATTCGAGTAGGGTTCG MR: CTTTATTCCGAACGAAACGAC	175
			UF: TATAGTTTATTTGAGTAGGGTTTGG UR: TACCTTTATTCCAAACAAAACAAC	176

Table 2. Primer sequence used for mRNA expression analysis

Genes	Primers (5' - 3')	Product size (bp)
HNRNPH3	F: TGACGCTAGTGATGGGACAG	198
	R: TGTTTCCCCAGAGCATTTTC	
BCL2L11	F: GCCCCTACCTCCCTACAGAC	202
	R: TATGGTGGTGGCCATACAAA	
TFAP2A	F: GAGGTCCCGCATGTAGAAGA	162
	R: GAAGACTTCGTTGGGGTTCA	
DUSP4	F: AAAGGCGGCTATGAGAGGTT	193
	R: CACTGCCGAGGTAGAGGAAG	

Table 3. Genes in descending order of level of methylation within -1000 bp from promoter region, with downregulated mRNA level

Gene name	Highest level of methylation	mRNA expression level
HNRNPH3	13.309802	-2.139767506
BCL2L11	13.02451	-2.068169089
TFAP2A	8.274107	-2.143928268
DUSP4	6.6753793	-2.885458237
ASRGL1	6.208294	-2.0324136
IREB2	5.5387263	-2.401547
SPON2	5.516695	-2.0112731
IKBKB	5.2696276	-2.4076849
NOL7	4.192739	-2.1528997
GULP1	4.0700736	-2.0129784
TPD52L2	4.0485516	-3.3593135
EZH2	2.9464529	-2.2147154
HNRNPUL1	2.8281174	-2.0143383
THOC1	2.8184586	-2.0148385
SASH1	2.8090777	-2.5348494
PTPRK	2.7381675	-2.3931631
CIRBP	2.6457407	-2.53629
CDH1	2.3483205	-2.0095231
KIF1B	2.1369853	-2.0469993
PRPF4B	2.092156	-2.197274

Table 4. Characteristics of patients and tissue samples

	Betel Quid Non-Chewer (Healthy control)	Betel Quid Non-Chewer (Oral Cancer)	Betel Quid Chewer (Oral Cancer)
Number of samples (n)	9	13	20
Gender (M: F)	5:4	7:6	15:5
Age in years (Range; Mean+/-SE)	21-82 ; 54.22 +/- 6.18	29-82 ; 54.71 +/- 7.25	35-81; 55.4 +/- 3.13
Histopathological Diagnosis (n)	Fibrous polyp of oral tissue (9)	Well Diff OSCC (9) Mod Diff OSCC (4)	Well Diff OSCC (11) Mod Diff OSCC (9)
DUSP4 positive score (Mean+/-SE)	17.23 +/- 2.74	20.33 +/- 4.75	66.84 +/- 3
DUSP4 CpG methylation (Mean+/-SE)	49.41 +/- 13.11	44.68 +/- 10.64	89.83 +/- 2.67

SE: Standard Error; M: Male; F: Female; Well Diff OSCC: Well Differentiated Squamous Cell Carcinoma; Mod Diff OSCC: Moderately Differentiated Squamous Cell Carcinoma;

Figure Legend

Fig. 1. Betel quid components. a) *Areca catechu* plant produces areca nut fruit, a component of betel quid preparation, b) edible component of areca nut fruit, c) dried and cut pieces of areca nut, d) betel quid preparation composed of areca nut, slaked lime, fresh betel leaf and partially dried tobacco.

Fig. 2. Chemical structure of arecoline. Arecoline (1,2,4,5- tetrahydro-1- methyl-pyridinecarboxylic acid), a nicotinic acid-based alkaloid is the active component of areca nut, found in betel quid.

Fig. 3. Cell culture condition. HGEPs were cultured in CnT-Prime epithelial cell culture medium containing antibiotics (5% penicillin-streptomycin) at 37˚C in an incubator supplied with 5% CO_2. These samples were treated alternating 3 days with (+) 50 µg/ml arecoline and 3 days without (-) arecoline for 1 month. Untreated samples were used as controls.

Fig. 4. CpG island methylation of candidate genes in HGEPs stimulated with arecoline for a period of 7 days. Value represents Mean±SE (n=5). The results show promoter region of CpG island of TFAP2A and DUSP4 gene were significantly hypermethylated in arecoline treated samples than in control. $p<0.05$.

Fig. 5. CpG island methylation of candidate genes in HGEPs stimulated with arecoline for a period of 1 month. Value represents Mean±SE (n=5). The results show promoter region of CpG island of DUSP4 gene was significantly hypermethylated in arecoline treated samples than in control. $p<0.05$.

Fig. 6. mRNA expression of candidate genes in HGEPs stimulated with arecoline for a period of 7 days. Data is presented as a ratio of expression of target gene to GAPDH. Value

represents Mean±SE (n=4). The results show TFAP2A and DUSP4 and were significantly downregulated; BCL2L11 was significantly upregulated in arecoline treated samples than in control. $p<0.05$.

Fig. 7. mRNA expression of candidate genes in HGEPs stimulated with arecoline for a period of 1 month. Data is presented as a ratio of expression of target gene to GAPDH. Value represents Mean±SE (n=4). The results show HNRNPH3 was significantly upregulated and DUSP4 was significantly downregulated in arecoline treated samples than in control. $p<0.05$.

Fig. 8. CpG island methylation of DUSP4 gene in cancer cell lines without arecoline treatment. HGEPs were taken as controls. Value represents Mean±SE (n=5). The results show promoter region of CpG island of DUSP4 gene was significantly hypermethylated in cancer cell lines, $p<0.05$.

Fig. 9. CpG island methylation of DUSP4 gene in cancer cell lines treated with arecoline for a period of 7 days. Untreated samples were taken as control. Value represents Mean±SE (n=5). The results show no changes in the promoter region of CpG island of DUSP4 gene in arecoline treated cancer cell lines as compared to respective controls. $p<0.05$.

Fig. 10. mRNA expression of DUSP4 gene in cancer cell lines without arecoline treatment. HGEPs were taken as control. Value represents Mean±SE (n=4). DUSP4 mRNA expression was significantly lower in HSC4, SAS and HSY cancer cell lines without arecoline treatment as compared to HGEP, $p<0.05$.

Fig. 11. mRNA expression of DUSP4 gene in cancer cell lines after 7 days arecoline treatment. Untreated samples were taken as controls. Value represents Mean±SE (n=4). DUSP4 mRNA expression was significantly lower in BSC-OF cancer cell line with arecoline treatment as compared to control, $p<0.05$.

Fig 12. Amount of pJNK in cells stimulated with arecoline and JNK inhibitor. The amount of phosphorylated JNK in samples stimulated with arecoline for 1 month was significantly higher than the control. JNK inhibitor can significantly inhibit the amount of pJNK formed after arecoline stimulation. Value represents Mean±SE (n=8). $p<0.05$.

Fig. 13. Histopathological and DUSP4 Immunohistochemical study of the samples obtained from betel quid chewer and non chewer patients. a) Low magnification HE staining of fibrous polyp (betel quid non chewer healthy control), b) Low magnification HE staining of oral squamous cell carcinoma (betel quid non chewer oral cancer), c) Low magnification HE staining of oral squamous cell carcinoma (betel quid chewer oral cancer), d) High magnification HE staining of (a), e) High magnification HE staining of (b), f) High magnification HE staining of (c), g) High magnification IHC staining of (a) nucleus of a very less number of cells are stained with anti-DUSP4 antibody in healthy controls, e) High magnification IHC staining of (b) increasing number of nucleus of cells are stained with anti-DUSP4 antibody in oral cancer cells of betel quid non chewers, f) High magnification IHC staining of (c), most of the cells are stained with anti-DUSP4 antibody in samples obtained from betel quid chewers. Scale bar 50μm.

Fig. 14. DUSP4 positive cells in samples obtained from betel quid chewers and non-chewers. IHC analysis showed that number of DUSP4 positive cells was significantly higher in samples obtained from oral cancer who have a habit of chewing betel quid as compared to oral cancer and normal tissue who do not have a habit of betel quid chewing. Value represents Mean±SE. $p<0.05$.

Fig. 15. DUSP4 CpG island methylation in samples obtained from betel quid chewers and non-chewers. DUSP4 was significantly methylated in samples obtained from oral cancer who have a habit of chewing betel quid as compared to oral cancer and normal tissue who do not have a habit of betel quid chewing. Value represents Mean±SE. $p<0.05$.

Fig. 16. Pathways of arecoline associated oral carcinogenesis. Arecoline acts as an agonist at muscarinic acetylcholine receptors (M1-M4). Activation of these receptors initiates a cascade of signaling molecules via various pathways. Methylation associated DUSP4 silencing and pJNK associated transactivation of transcription factors are the two mechanisms presented in this study. Both mechanisms promote malignant transformation of the cell.