Involvement of sirtuin 1 (SIRT1) in betel quid chewing related oral cancer

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SUMMARY

The habit of betel quid chewing is considered to be one of predominant risk factors for the induction of oral cancer, one of the leading malignancies in South Asian countries. Betel quid often consists of a mixture of betel leaf, areca nut, and slaked lime, with or without tobacco. Each component in betel quid may individually, synergistically, and coordinately participate in carcinogenesis. However, the underlying molecular mechanisms for the development of oral cancer remain unclear. Other environmental risk factors, such as tobacco smoking and alcohol consumption, often leading to oral cancer by inducing genetic and epigenetic modification. In addition to genetic modifications, DNA hypermethylation is one of most common epigenetic events observed in oral cancer. In fact, DNA hypermethylation of several tumor suppressor genes has been detected in precancerous lesions and oral cancers. Precancerous lesions with DNA hypermethylation followed by transcriptional downregulation of the tumor suppressor genes have a high risk of malignant transformation. However, few publications have identified DNA hypermethylation in betel quid-related oral cancer and precancerous lesions. Areca nut is one of the basic components in betel quid and contains several active chemical ingredients. One of major chemicals is arecoline and is considered to be the most significant procarcinogen present in betel quid. Previous studies have demonstrated that arecoline had a comprehensive effect on cellular gene expression and promote oral cancer. Arecoline may promote oral cancer by inducing transcriptional downregulation of the tumor suppressor genes and, that this downregulation is possibly induced by DNA hypermethylation. It is, however, the role of arecoline in DNA hypermethylation followed by downregulated transcriptional levels has not been clarified.

Sirtuins (SIRTs) are class III histone deacetylase family protein, comprising seven members, SIRT1-7. SIRT1 was the first family member to be discovered and is the most studied molecule. *SIRT1* is predominantly located in the nucleus, also in the cytosol and, targets both histone and non-histone cellular substrates. Dysregulation of *SIRT1* expression has previously described in many human malignancies including oral cancer. However, the physiological relevance of *SIRT1* in betel quid-related oral cancer remains unexplored. Betel quid-related oral cancer is often preceded by the development of precancerous lesions, characterized by the disruption of epithelial integrity and, consequently, the transformation to

invasive cancer. *SIRT1* has been identified as playing a role in the maintenance of epithelial integrity and contributing to the prevention of both the invasion and metastasis potential of the oral epithelium. From these observations, we hypothesize that decreased *SIRT1* expression may occur in oral cancer induced by betel quid chewing habit. Since the downregulated expression of *SIRT1* has been attributed to DNA hypermethylation, we hypothesize that DNA hypermethylation of *SIRT1* may be observed followed by its transcriptional downregulated expression in betel quid chewing oral cancer patients.

In the present study, we analyzed whether the hypermethylation of *SIRT1* followed by its transcriptional downregulation in the human gingival epithelial cells could be caused by arecoline, a major component of betel quid. In addition, we investigated the methylation status of *SIRT1* in smear samples of macroscopically healthy buccal mucosa from subjects with a habit of betel quid chewing. Furthermore, we examined the methylation status of *SIRT1* in paraffin-embedded tissue samples of oral squamous cell carcinoma (OSCC) obtained from betel quid chewing and non-chewing patients and in tissues samples from healthy control subjects. Our *in vitro* model showed that, DNA hypermethylation is involved in *SIRT1* transcriptional downregulation following chronic stimulation with arecoline. The habits of betel quid chewing and the duration of chewing years are positively correlated with DNA methylation frequency of the *SIRT1* gene of oral epithelia. We also revealed that *SIRT1* was significantly hypermethylated in tissue samples of OSCC from betel quid chewers and non-chewers than in oral mucosa from healthy control subjects. Results also showed that hypermethylation of *SIRT1* was significantly higher in OSCC from betel quid chewing patients than in that from non-chewing patients. Collectively, these results suggest that *SIRT1* is involved in the oral cancer caused by betel quid chewing, and that hypermethylation of *SIRT1* in the oral mucosa of betel quid chewers could be a predictive marker for detecting early events of multistage carcinogenesis.

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1. INTRODUCTION

Oral cancer is the fifteenth most frequent and major cause of death from cancer around the world (Ferlay *et al.*, 2015). The highest prevalence rate of oral cancer has been reported in South Asian countries, including India, Pakistan, Sri Lanka, and Bangladesh, where it is the third most common and fifth leading cause of cancer death, followed by breast, lung, stomach, and cervical cancer (Ferlay *et al*., 2015). The mean age of occurrence of this disease in different parts of the oral cavity ranges between 51 and 55 years or above (Lee *et al*., 2011). Globally, this condition is more predominant in males than in females; in South Asian countries, it is the most common malignancy and third major cause of deaths in males, followed by lung and stomach cancer (Ferlay *et al*., 2015; Lee *et al*., 2011). The etiologies of oral cancer include betel quid chewing, smoking, alcohol consumption, genetic predisposition, and viruses, including human papillomavirus (HPV) (IARC, 2004; Chiba, 2001). The site of occurrence of this disease depends on region-specific epidemiological risk factors. In South Asian countries, the cheek (buccal mucosa) and gingiva are the leading sites of involvement, whereas, in western societies, the tongue is most commonly affected (Chiba, 2001). The high prevalence of oral cancer at characteristic sites in South Asian population may be attributed to the habit of BQ chewing, which is one of the main etiological factors for the development of this cancer and has been a significant threat to public health in these areas (IARC, 2004; Chiba *et al.*, 1998; Chiba, 2001). The term 'quid' is defined as "a substance, or mixture of substances, placed in the mouth or chewed and remaining in contact with the mucosa, usually containing one or both of the two basic ingredients, tobacco and/or areca nut, in a raw or any manufactured or processed form" (Zain *et al*., 1999). Thus, BQ may be considered as any quid comprising of betel leaf and a combination of areca nut and slaked lime, with or without tobacco. The principal components of BQ and the prevalence of BQ chewing habits are shown in Figure 1 (Islam *et al*., 2019a). It has been described that different components of BQ act on the oral epithelium and induces generic and epigenetic alterations. Subsequently, these alterations lead to the initiation and promotion of oral carcinogenesis in the oral mucosa (Chiba, 2001; Islam *et al*., 2019a). However, the roles of the components of BQ in oral carcinogenesis remain unclear.

Areca nut is one of the basic ingredients wrapped in a betel leaf along with the other components of the BQ. The chemical composition of areca nut varies, and may include carbohydrates, fats, proteins, fiber, polyphenols, tannins, alkaloids, and various trace elements as the major constituents (Cox *et al*., 2004; Warnakulasuriya, 2002). Alkaloids, polyphenols, and tannins may be responsible for the areca nut-associated effects on oral mucosa. The major areca nut alkaloids are arecoline, arecaidine, guvacaine, and guvacoline, wherein, arecoline is the most abundant alkaloid and considered to be the most important procarcinogen present in areca nut (Cox *et al*., 2004; Warnakulasuriya, 2002). Arecoline undergoes a nitrosation reaction and gives rise to a variety of BQ-specific nitrosamines (BSNAs) within the acidic environment of the oral cavity and stomach. The formation of these nitrosamines induces oxidative stress by interacting with DNA, proteins or other macromolecules, and contributes to carcinogenesis in the oral mucosa, including epithelium and submucosal connective tissues (Islam *et al*., 2019a). However, the mechanisms by which nitrosamines interact with DNA or other macromolecules and exert their carcinogenic activities remain unclear. Arecoline, the main alkaloid present in the areca nut, induces chromatin relaxation by inhibiting poly (ADP-ribose) polymerase (PARP), a family of proteins involved in DNA repair and maintenance of genomic stability (Saikia *et al*., 1999). The relaxation of the chromatin structure by arecoline allows for the interaction of nitrosamines with DNA resulting in the formation of adducts. The formation of these DNA adducts may induce genomic alterations and the epigenetic silencing of tumor suppressor genes (TSGs) (Saikia *et al*., 1999; Islam *et al*., 2019a). It is, however, the role of arecoline in DNA hypermethylation followed by downregulated transcriptional levels of TSGs has not been clarified.

DNA methylation is one of the several epigenetic mechanisms that cells use to control gene expression (Abiko *et al*., 2014). DNA methylation is an enzymatically catalyzed covalent modification of DNA, occurring typically in the context of Cytosine-Guanine (CpG) islands. The epigenetic alteration most studied in the human cancer cell is DNA methylation. The number of genes with aberrant methylation in the cancer cell is still unknown, but it is estimated that approximately 5% (approximately 1,500–2,000) of the human genome can be aberrantly methylated in a cancer cell (Bird, 1986; Esteller, 2007, 2008). Typically, there is hypermethylation of TSGs and hypomethylation of oncogenes. The silencing of TSGs

through hypermethylation appears to be especially important in progression to cancer, whereas hypomethylation is linked to chromosomal instability and loss of imprinting (Baylin *et al*., 2016). Therefore, aberrant DNA methylation without the presence of genetic alterations is now widely recognized as either a causative or correlative event in carcinogenesis (Esteller, 2007, 2008). Environmental risk factors, for instance, tobacco smoking, alcohol drinking, and BQ chewing often promote oral cancer by inducing genetic and epigenetic alterations (IARC, 2004; Petti *et al.*, 2008). Both genetic and epigenetic factors often work together in affecting multiple cellular pathways in cell-cycle regulation, DNA repair, apoptosis, cell-to-cell adhesion and carcinogen metabolism which leads to tumor progression (Bailoor *et al*., 2015). In fact, it is well-documented that precancerous oral lesions with DNA hypermethylation followed by the transcriptional downregulation of TSGs have a high risk of malignant transformation (Shridhar *et al*., 2016; Takuma *et al*., 2010). Hence, hypermethylation on oral epithelium can be one of the valuable markers for prediction of malignant potential of the lesions.

The sirtuins (SIRTs) family protein are class III histone deacetylases (HDACs), comprised of seven members, SIRT1-7. SIRTs are widely expressed in normal tissues and involved in several biological processes (Carafa *et al*., 2016; Sebastian *et al*., 2012; Islam *et al*., 2019b). *SIRT1* was the first family member to be discovered and is the most studied molecule. The functional activity of *SIRT1* is dependent on availability of nicotinamide adenine dinucleotide (NAD^+). High levels of NAD+ induce *SIRT1* activity, whereas high NADH levels inhibit its function (Wang *et al.*, 2008). *SIRT1* is predominantly located in the nucleus and, also in the cytosol (Carafa *et al*., 2016). Due to *SIRT1* localization, it is capable of deacetylating lysine residues on both histone and non-histone proteins, which is thought to affect their stability, transcriptional activity, and translocation (Wang *et al*., 2008). Deacetylation of histones by *SIRT1* has been shown to induce chromatin condensation, whereas acetylation by histone acetyltransferases (HATs) causes chromatin decondensation (Wang *et al*., 2008). This balance is crucial for normal cellular functions, and any disturbance of it will be related to cancer (Gray *et al*., 2001). *SIRT1*-mediated deacetylation of non-histone proteins has been suggested to be more important in cancer than histones (Carafa *et al*., 2016; Sebastian *et al*., 2012). However, in tumor biology, *SIRT1* seems to play contradictory roles and dysregulation of *SIRT1* expression has frequently been reported in many

human malignant diseases including oral cancer (Islam *et al*., 2019b). Although, it remains controversial whether *SIRT1* acts as a tumor suppressor or promoter in oral cancer and, preclinical data are inconclusive to address such debate.

BQ-related oral cancer is often preceded by the development of precancerous lesions, characterized by disruption of epithelial integrity and consequently, transforming to invasive cancer. Interestingly, it was reported that stable expression of *SIRT1* aids in maintaining epithelial integrity, and this contributes to the prevention of both invasion and metastasis. However, the involvement of *SIRT1* in BQ-related oral cancer has not been clarified. These observations suggest that decreased *SIRT1* expression may occur in oral cancer induced by BQ chewing habit. Since the downregulated expression of *SIRT1* has been attributed to DNA hypermethylation, we hypothesize that DNA hypermethylation of *SIRT1* may be observed followed by its transcriptional downregulated expression in BQ chewing oral cancer patients.

In the present study, in order to characterize the association between chronic arecoline stimulation and carcinogenesis, we investigated whether the hypermethylation of *SIRT1* followed by its transcriptional downregulation in the human gingival epithelial cells could be caused by arecoline. In addition, we examined the methylation status of *SIRT1* in smear samples of macroscopically healthy buccal mucosa from subjects with a habit of BQ chewing. Furthermore, we analyzed the methylation status of *SIRT1* in paraffin-embedded tissue samples of oral squamous cell carcinoma (OSCC) obtained from BQ chewing and non-chewing patients and in tissues samples from healthy control subjects for clarifying the role of *SIRT1* in carcinogenesis.

2. MATERIALS AND METHODS

1. Ethics statement

All participants in the study provided written informed consent, and the study was approved by the Institutional Review Boards of the Ethics Committee on Human Genetic Research at the Health Sciences University of Hokkaido, Japan (Number# 2016-025) and the Ethical Committee at the University of Peradeniya, Sri Lanka (Number # 7/2004).

2. Cell culture and arecoline exposure

Human gingival epithelial progenitors (HGEPs) cells, primary keratinocytes derived from healthy gingival epithelium, were purchased from CELLnTEC Advanced Cell Systems (Basel, Switzerland) and cultured in CnT-Prime epithelial culture medium (CELLnTEC Advanced Cell Systems) at 37 °C in a humidified atmosphere of 95% air and 5% CO_2. HGEPs cells were spread onto 100 mm tissue culture plates at a density of 4.0×10^4 cells/mL. Arecoline (arecoline hydrobromide) was purchased from Sigma-Aldrich (St. Louis, MO). Following overnight incubation, the HGEPs cells were treated with arecoline at a concentration of 50 μg/mL. The concentration of arecoline used in this study was as described in previous experiments (Uehara *et al*., 2017). Briefly, arecoline at the concentration of 50 µg/mL had no cytotoxic effect on the cells stimulated, even for a prolonged period, the method of alternating between 3 days with 50 µg/mL of arecoline and 3 days without arecoline for 1 month was selected. The untreated samples were used as controls. The flow chart of cell-cultured is briefly shown in Figure 2.

1): Quantitative methylation-specific polymerase chain reaction

Genomic DNA was extracted from the HGEPs cells using DNeasy® Blood & Tissue Kit (Qiagen), following the manufacturer's instructions. The extracted DNA samples were treated with sodium bisulfite using the EpiTect® Plus Bisulfite Kits (Qiagen). DNA methylation of the *SIRT1* gene was analyzed using SYBR green-based quantitative methylation-specific polymerase chain reaction (qMS-PCR) after checking the existence of CpG islands around the promoter region by UCSC Genome Browser (http://genome.ucsc.edu/index.html) (Fig. 3). Two sets of primers were used; one for methylated and one

for unmethylated DNA sequences (Oliveira *et al*., 2014). The primers used for *SIRT1* gene were shown in Table 1. For PCR, the bisulfite-treated DNA template was mixed with KAPA SYBR FAST qPCR Kit and a pair of primers. The PCR conditions included initial incubation at 50 °C for 2 min, denaturing at 95 °C for 10 min, and 50 cycles of denaturing at 95 °C for 15 s and annealing at 58 °C for 1 min. After PCR amplification, a dissociation curve was generated to confirm the size of the PCR product. The percentage of DNA methylation in a sample was estimated using the following formula:

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

where M is the copy number of methylated DNA, U is the copy number of unmethylated DNA, and $\Delta Ct = Ct_U - Ct_M$ (Katsaros *et al*., 2007). Each experiment was performed in triplicate. Data are expressed as mean ± standard deviation (SD).

2): Real-time quantitative reverse-transcription polymerase chain reaction

Total RNA was extracted from the HGEPs cells using the RNeasy® Mini Kit (Qiagen) following the manufacturer's instructions. Total RNA was reverse transcribed into cDNA using a ReverTra Ace® qPCR RT Master Mix (Toyobo, Osaka, Japan). The cDNA levels were measured using the LightCycler® Nano System (Roche Diagnostics, Basel, Switzerland). The primers used for *SIRT1* gene expression analysis were shown in Table 2 (Li *et al*., 2016; Murray *et al*., 2013). For real-time quantitative reverse transcription-polymerase chain reaction (qRT-PCR), cDNA was mixed with KAPA SYBR FAST qPCR Kit (Nippon Genetics, Tokyo, Japan) and a pair of primers. The PCR conditions included initial incubation at 50 °C for 2 min, denaturing at 95 °C for 10 min, 40 cycles of denaturing at 95 °C for 15 s and annealing at 60 °C for 1 min. The relative expression of each mRNA was 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 ΔΔCq method (Livak *et al*., 2001). Specifically, the amount of target mRNA relative to GAPDH mRNA is expressed as $2^{-(\Delta Ct)}$. Each experiment was performed in triplicate. Data are expressed as mean ± standard error (SE) of the ratio of the target mRNA to GAPDH mRNA.

3): Western blotting analysis

Proteins were extracted from the HGEPs cells using lysis buffer [50 mM Tris HCL, pH 7.5; 10 mM EDTA, pH 7.5; 165 mM NaCl; 10 mM NaF; 1 % Nonidet P-40; 1 mM PMSF; 1 mM $NaVO_3$; 10 μg/mL leupeptin; and 10 μg/mL aprotinin]. The lysis reaction was carried out for 1 h at 4°C. The samples were centrifuged at 15,000 rpm for 30 minutes at 4°C, and the supernatant was used as sample. Protein concentration quantified by Lowry's protein assay. Fifteen micrograms of protein samples were used for western blotting analysis. Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) was carried out in pre-cast gels (4-20% gradient of polyacrylamide; Mini-PROTEAN TGX Gels; Bio-Rad, Hercules, CA, USA). After electrophoresis, gels were transferred electrophoretically onto polyvinylidene difluoride membranes (Immobilon-P; Millipore, Bedford, MA, USA) and blocked for 1 h with Tris-buffered saline (TBS) containing 5% skimmed milk. Blocked membranes were washed twice with TBS containing 0.05% Tween-20 solution.

The primary antibodies used were: mouse monoclonal antibody against SIRT1 (dilution 1:1000, #ab110304; Abcam, Cambridge, MA, USA) and rabbit monoclonal antibody against GAPDH (dilution 1:5000, #CST5174; Cell Signaling Technology, Danvers, Massachusetts, USA). Membranes were incubated with the primary antibody overnight at 4°C, washed three times with TBS containing 0.05% Tween-20 solution, and incubated with a horseradish peroxidase-conjugated secondary antibody (dilution 1:10,000; Jackson Immuno-Research Laboratories Inc, West Grove, PA, USA) for 1 h at room temperature. Bands of SIRT1 and GAPDH were visualized by the enhanced chemiluminescence system (Clarity Max™ Western ECL Substrate; Bio-Rad) and LuminoGraph I (ATTO Corporation, Tokyo, Japan), and recorded using ImageSaver6 software (ATTO Corporation). Expression levels of SIRT1 and GAPDH in cells treated with or without arecoline were quantified by analyzing the intensity of each band using CS Analyzer4 software (ATTO Corporation). Each experiment was performed in triplicate. Data are expressed as mean ± SE of the ratio of the target protein to GAPDH protein.

3. Buccal smear samples and clinicopathological data collection

Prior to sample collection, the nature of the study was fully explained to all participants. Information obtained from the interview included socioeconomic and demographic characteristics, personal and family

histories, risk factors for oral cancer, such as lifestyle, alcohol drinking, tobacco smoking, and BQ chewing including its frequency, and the added use of betel leaf, areca nut, slaked lime, and tobacco. Clinic attendees of at least 20 years of age and with the ability to complete the questionnaires by an interview and clinical oral examinations were eligible to participate. The presence of oral mucosal lesions was evaluated and documented by a registered dentist, based on the recommendations of the World Health Organization (Kramer *et al.*, 1978). Participants having oral mucosal lesions or any systemic disorders (such as diabetes, immune-compromised, or genetic diseases) were excluded from this study. A convenience sample of 70 study subjects was recruited and classified into two groups: controls (45 healthy, non-chewers) and BQ chewers (25 healthy, chewers). Both the controls and BQ chewer groups were non-smokers and non-drinkers. Subjects who had chewed one BQ per day for at least 6 months were considered as chewers. The brief description of the study subject, study design, and sample collection were shown in Figure 4.

For oral cancer screenings, samples for biomarker testing should be easily available. Thus, the present study used samples obtained by buccal smear, which is noninvasive and easy to perform and which may assist in screening for oral cancers, particularly in areas with limited resources. Buccal smear samples were collected by research staff accordingly manufacturer instructions (Qiagen). Briefly, the participants were restricted in eating and drinking for 30 minutes prior to collection, and it was verified that the participant's mouth was empty. The swab sticks were removed from the package carefully to avoid contaminating the tip of the swab with gloves or against any surface. The swab was firmly rubbed and rotated the swab along the inside of the cheek for 5-10 times and, to ensure that the entire tip was in contact with the cheek, this step was repeated on the other cheek. The swab stick was removed from the mouth, being careful not to touch swab tips with any other surface such as teeth, lips, or other surfaces. The swab was placed directly into the tube containing the DNA stabilizing reagent Gentra® Puregene® buccal cell kits (Qiagen), and the tube was labeled with identifying information. Samples were stored at minus 20 degrees Celsius until shipment on ice to HSUH for testing.

1): Quantitative methylation-specific polymerase chain reaction

Genomic DNA was extracted from the buccal smear samples using Gentra® Puregene® buccal cell kits (Qiagen), following the manufacturer's instructions. The extracted DNA samples were treated with sodium bisulfite using the EpiTect® Plus Bisulfite Kits (Qiagen) and DNA methylation of the *SIRT1* gene was performed using the methods described previously (Katsaros *et al*., 2007). Each experiment was performed in triplicate. Data are expressed as mean ± SD of the DNA methylation.

4. Tumor specimen and tissue collection

Oral squamous cell carcinoma (OSCC) and normal oral mucosa tissue samples were obtained from patients treated surgically. Twelve OSCC tissue samples were obtained from patients with BQ chewing habit in Sri Lanka. Twenty-two OSCC tissue samples were obtained from Japanese patients without BQ chewing habits, and 13 normal oral mucosae were obtained from individuals who underwent oral surgical intervention from 2008 to 2014 at the Health Sciences University of Hokkaido (HSUH) Hospital. The postsurgical tissue sections were formalin-fixed, processed, and paraffin-embedded following standard protocols. None of these patients received chemotherapy or radiotherapy prior to tumor resection. Data on patient demographics were retrieved from the archives of the Oral Medicine and Pathology Department at HSUH, Japan. The study design and sample collection are briefly shown in Figure 5.

1): Quantitative methylation-specific polymerase chain reaction

Genomic DNA was extracted from the tissue samples using DNeasy® Blood & Tissue Kit (Qiagen, Venlo, Netherlands), following the manufacturer's instructions. The extracted DNA samples were treated with sodium bisulfite using the EpiTect® Plus Bisulfite Kits (Qiagen) and DNA methylation of the *SIRT1* gene was performed using the method described previously (Katsaros *et al*., 2007). Each experiment was performed in triplicate. Data are expressed as mean ± SE of the DNA methylation.

5. Statistical analysis

Statistical analysis was performed on a database using IBM SPSS Statistics 23 (IBM, Armonk, NY). Comparisons between two groups were performed using the Mann-Whitney U test. Multiple variable

comparisons were performed by using multivariable regression analysis. Results with *p*-values of *<0.05* were considered to be statistically significant.

3. RESULTS

1: The effects of arecoline on *SIRT1* DNA methylation, mRNA expression, and protein production

In order to confirm whether the hypermethylation of *SIRT1* caused by BQ chewing downregulates the transcriptional level of *SIRT1* in HGEPs cells, HGEPs cells were stimulated with arecoline, a major component of BQ. The methylation level of *SIRT1* in cells treated with arecoline at a concentration of 50 μg/mL was significantly increased compared to that of control cells (Fig. 6a). The expression levels of *SIRT1* mRNA in the cells treated with arecoline were significantly decreased compared to the control group (Fig. 6b). To determine whether the mRNA expression levels of *SIRT1* relates to its protein production, further analysis was performed using western blotting to investigate the effects of arecoline on SIRT1 protein production. The protein levels of SIRT1 (bands of 120 kDa) was reduced by arecoline treatment in HGEPs cells compared to that in controls. On the other hand, the protein levels of GAPDH (bands of 37 kDa) was the same in all cells (Fig. 7a). The ratio of intensities of SIRT1 to GAPDH (SIRT1/GAPDH) in controls was considered to be 100%. The ratio of intensities of SIRT1/GAPDH in control and arecoline HGEPs cells were 100±16.2% and 40.1±3.3%, respectively. SIRT1 protein levels were reduced by arecoline in HGEPs cells (Fig. 7b). These results indicate that *SIRT1* mRNA transcription is suppressed by arecoline, resulting in decreased protein production in HGEPs. Together, these results demonstrate that DNA hypermethylation is involved in *SIRT1* transcriptional downregulation in HGEPs cells following chronic stimulation with arecoline.

2: Subject characteristics and profiling of *SIRT1* DNA methylation

A total of 70 adult participants were enrolled in the current study, including 14 males and 56 females. Based on the history of oral habits, the participants were classified into two groups: controls (45; healthy, BQ non-chewers) and BQ chewers (25; healthy, chewers). The mean age of controls and BQ chewers were 35.5±13.6 years and 39.0±11.3 years, respectively. The mean chewing years in the BQ chewers group was 7.3±10.3 years. Of the 25 BQ chewers, 5 (24%) has used BQ for 6 months or more, 14 (56%) chewed for 1-10 years, and 5 (20%) has used BQ for more than 10 years. The mean DNA methylation

level of *SIRT1* in controls and BQ chewers were 4.0 ± 4.6 and 16.5 ± 23.7, respectively. Detailed demographics of controls and BQ chewer groups are summarized in Table 3.

3: The effects of betel quid chewing on *SIRT1* DNA methylation status

In this study, we investigated possible correlations between BQ chewing habits and the DNA methylation status of the *SIRT1* promoter region in oral mucosal epithelium. Multivariable regression analysis was conducted using DNA methylation as a dependent variable, and age, sex, and BQ chewing habit as independent variables. The results showed that BQ chewing habit was the only significant predictor of *SIRT1* DNA methylation level (Table 4; Fig. 8 A). This result suggests that DNA hypermethylation of *SIRT1* is observed in macroscopically healthy BQ chewers epithelium before their clinical changes.

4: The effects of betel quid components on *SIRT1* DNA methylation status

A total of 25 participants were enrolled in the BQ chewers group, including 5 (20%) males and 20 (80%) females. Of the 25 BQ chewers, 7 (18%) have used BQ in the combination of betel leaf and areca nut, and 18 (72%) chewed BQ containing betel leaf, areca nut, and slaked lime. Based on the history of BQ components, the participants were classified into two groups: group 1 (7; betel leaf and areca nut) and group 2 (18; betel leaf, areca nut, and slaked lime). The detail demographics description was shown in Table 5.

We investigated the possible correlation of BQ components and the DNA methylation level of *SIRT1* in smear samples of BQ chewers. Multivariable regression analysis was conducted using DNA methylation as a dependent variable, and groups (group 1 and group 2) as independent variables. The results showed that no significant differences in *SIRT1* DNA methylation levels were observed among groups and the frequency of quid chewing habits (Table 6). Together, these data suggest that DNA hypermethylation of *SIRT1* could be caused by synergistic effects of BQ components onto the oral epithelium.

5: The effects of chewing years on *SIRT1* DNA methylation status

Further, among the BQ chewers, we investigated the possible correlation of *SIRT1* DNA methylation level and chewing years. The mean chewing years were 7.3±10.3 years. Multivariable regression analysis was

conducted using DNA methylation as a dependent variable, and age, sex, and chewing years as independent variables. It was revealed that the duration of chewing habit was only significantly correlated to the levels of *SIRT1* DNA methylation (Table 7; Fig. 8 B). These results demonstrate that BQ chewing and the duration of BQ chewing habits are positively correlated with DNA methylation frequency of the *SIRT1* gene of oral epithelia (Fig. 8 A-B).

6: DNA methylation status of *SIRT1* in oral squamous cell carcinoma (OSCC) obtained from BQ chewing and non-chewing patients

We examined the promoter methylation status of *SIRT1* in OSCC tissue samples obtained from BQ chewing and non-chewing patients, and oral mucosa samples from healthy control subjects. The demographic data of the participants are listed in Table 8. Multivariable regression analysis was conducted using DNA methylation as a dependent variable, and age, sex, groups (analysis I, BQ chewers *vs* healthy controls; analysis II, BQ non-chewers *vs* healthy controls; analysis III, BQ chewers *vs* BQ non-chewers) as independent variables. Analysis I investigates BQ chewing associated with OSCC, while analysis II investigates BQ non-chewing with OSCC. Analysis III investigates BQ chewing and non-chewing associated with OSCC. Analysis I revealed that *SIRT1* was significantly hypermethylated in tissue samples of OSCC from BQ chewers than in oral mucosa from healthy control subjects (Table 9; Fig. 9 A). Analysis II showed that *SIRT1* was also significantly hypermethylated in tissue samples of OSCC from BQ non-chewers than in oral mucosa from healthy control subjects (Table 10; Fig. 9 B). Analysis III explored that *SIRT1* was significantly hypermethylated in tissue samples of OSCC from BQ chewers than in that of OSCC of BQ non-chewers (Table 11; Fig. 9 C). Collectively, these results showed that *SIRT1* was significantly hypermethylated in tissue samples of OSCC from BQ chewers and BQ non-chewers than in oral mucosa from healthy control subjects. Results also showed that hypermethylation of *SIRT1* was significantly higher in OSCC from BQ chewing patients than in that from BQ non-chewing patients (Fig. 9 A-C).

4. DISCUSSION

In the present study, our *in vitro* model showed that the hypermethylation is followed by downregulation of the transcriptional level of *SIRT1*. A higher level of methylation of *SIRT1* was observed in smear samples obtained from macroscopically healthy buccal mucosa in BQ chewers than in non-chewers. Results also demonstrated DNA hypermethylation of *SIRT1* in OSCC, and the methylation levels were significantly higher in the OSCC of BQ chewers than in that of non-chewers. These results suggest that *SIRT1* is involved in the oral cancer caused by BQ chewing, and that hypermethylation of *SIRT1* in the oral mucosa of BQ chewers could be a predictive marker for detecting early events of multistage carcinogenesis.

Although hypermethylation of *SIRT1* has been reported in several cancer tissues (Frazzi *et al*., 2016, 2017; Lisbosa *et al*., 2011), the hypermethylation of *SIRT1* in OSCC has not been demonstrated. We confirmed the occurrence of *SIRT1* hypermethylation in OSCC of BQ chewers and non-chewers. We also found that the hypermethylation level of *SIRT1* was significantly higher in OSCC of patients with BQ chewing habits than in those of non-chewing habits. These results indicate that the DNA hypermethylation of *SIRT1* caused by BQ chewing is involved in BQ-related OSCC. It was not known whether the hypermethylation of *SIRT1* caused by BQ chewing is linked to *SIRT1* transcription. The extraction of RNA from paraffin-embedded tissue samples remains extremely challenging, and no consensus or standardized isolation method has been described (Bohmann *et al*., 2009). Therefore, we employed an *in vitro* model of a daily BQ chewing habit that we showed previously to contain hypermethylated genes (Takai *et al*., 2016). The cells were stimulated with arecoline, a major component of BQ, for a prolonged period according to our previous protocol (Uehara *et al*., 2017). We confirmed that significantly high level of methylation of *SIRT1* was observed followed by downregulated expression of *SIRT1* transcription and protein expression. This hypermethylation of *SIRT1* may cause the downregulated expression of *SIRT1* observed in OSCC. These results support previous findings suggesting *SIRT1* as a tumor suppressor (Chen *et al*., 2014; Kang *et al*., 2018). *SIRT1* has been reported to play a role in maintaining epithelial integrity by inducing the expression of epithelial-cadherin. Downregulation of *SIRT1* expression may weaken epithelial-epithelial interaction leading to malignant transformation of the epithelia (Chen *et al*., 2014;

Kang *et al*., 2018). The hypermethylation of *SIRT1* caused by arecoline in BQ chewers epithelium may be related to the instability of epithelial-epithelial interactions causing malignant transformation. It is still unknown how arecoline causes the hypermethylation of *SIRT1*. The promoter region of *SIRT1* possesses a potential regulator of epigenetic factors, methyl-CpG-binding protein 2 (MeCP2) (Volkmann *et al*., 2013). MeCP2 has been shown to interact with DNA methyltransferase 1 (DNMT1) and recruits the latter to induces *SIRT1* promoter methylation (Volkmann *et al*., 2013). Arecoline was previously documented to promote oral submucosal fibrosis and the progression to oral cancer through pathways involved in transforming growth factor-beta (TGF-β) production (Khan *et al*., 2012; Volkmann *et al*., 2013). TGF-β is likely to silence *SIRT1* epigenetically by inducing the MeCP2 expression, although other possible mechanisms cannot be ruled out (Volkmann *et al*., 2013). TGF-β has shown to decrease miR-30a-3p expression, a negative regulator for MeCP2. Where overexpression of MeCP2 affects *SIRT1* promoter methylation and subsequent epigenetic silencing (Volkmann *et al*., 2013). The formation of nitrosamines in BQ chewing epithelium supposed to be one of causative factor for DNA hypermethylation followed by epigenetic silencing (Saikia *et al*., 1999). Arecoline induces the formation of nitrosamines inside the oral mucosa of BQ chewers (Sundqvist *et al*., 1991). In fact, it is well-documented that the nitrosation of arecoline at neutral pH yielded approximately four times more areca nut specific nitrosamines than at acidic or alkaline pH (Nair *et al*., 2004). These nitrosamines could be related to downregulated transcriptional levels of *SIRT1*, as well as, other TSGs that were often observed in oral cancer of BQ chewing. Further studies are warranted to clarify this hypothesis.

From these data, we hypothesized that the methylation level of *SIRT1* in healthy oral epithelium of BQ chewing subjects is higher than that of non-chewing subjects. We showed that the methylation level of *SIRT1* in smear samples obtained from macroscopically healthy buccal mucosa of BQ chewers is significantly higher than that in the samples of BQ non-chewers. In addition, the duration of chewing habits was correlated positively to the frequency of *SIRT1* hypermethylation. This observation may support the previous paper that showed increasing the years of quid chewing habits were positively associated with oral cancer (Chen *et al*., 2017; Guha *et al*., 2014; Wang *et al*., 2017), wherein *SIRT1* hypermethylation may play an important role in the process of their development. The possible

involvement of *SIRT1* in BQ-related oral cancer may explain by its inhibitory effects on TGF-β pathway (Chen *et al*., 2014). *SIRT1* shows negatively regulate TGF-β pathway and its associated downstream molecules (Chen *et al*., 2014). These findings may explain the reasons why TGF-β and its associated downstream molecules are overexpressed in BQ-related oral cancer compared to BQ non-chewing oral cancers (Khan *et al*., 2012; Meng *et al*., 2011; Pant *et al*., 2016; Xiao *et al*., 2008). Wherein arecoline caused *SIRT1* downregulation, and that this downregulated expression failed to prevent TGF-β-induced malignant transformation in oral epithelium of BQ chewers. Collectively, these findings indicate that DNA hypermethylation of *SIRT1* caused by the habits BQ chewing is involved in malignant transformation potential of oral epithelium.

Although, previously published reports confirmed DNA hypermethylation in precancerous lesions and oral cancer with the habits of BQ chewing (Chakraborty *et al*., 2017; González-Ramírez *et al*., 2011; Huang *et al*., 2013; Kaur *et al*., 2010; Shridhar *et al*., 2016; Takuma *et al*., 2010). It is, however, no studies have shown alteration of DNA methylation in the macroscopically healthy epithelium of BQ chewers. To be the best of our knowledge, this is the first report that showed DNA hypermethylation in clinical healthy oral epithelium of BQ chewers. This result indicates that DNA hypermethylation may be caused by some carcinogens as an early event of carcinogenesis before their clinical changes. Therefore, examination of *SIRT1* hypermethylation, as well as, other TSGs in smears of buccal mucosa could be useful for the detection of early changes caused by BQ chewing habits.

Samplings from buccal mucosa and saliva are the two most common non-invasive methods for genetic, epigenetic, and proteomic studies (Theda *et al*., 2018). However, salivary ribonucleases rapidly degrade epithelial cell RNA during collection, and usable RNA has not been extracted from scrapings of buccal mucosa (Ceder *et al*., 1985). Therefore, DNA methylation analysis using buccal smear samples may be used as a molecular screen for oral cancer, particularly in areas with limited resources. A few previous studies investigated interactions between BQ chewing habits and DNA methylation using smear samples from buccal OSCC (Huang *et al*., 2013; Yang *et al*., 2018). Those studies analyzed DNA methylation levels with participants having combined habits of BQ chewing, tobacco smoking, and alcohol drinking (Huang *et al*., 2013; Yang *et al*., 2018). Therefore, those studies might not reflect on the process of

carcinogenesis induced by habit of BQ chewing. Our study is the first report that shows increased levels of DNA methylation in healthy buccal mucosa samples obtained from BQ chewers. Cigarette smoking and alcohol consumption are other risk factors for oral cancer (Lee *et al*., 2013). Those habits also cause alteration of DNA methylation (Lee *et al*., 2013; Wang *et al*., 2017). In fact, previous studies demonstrate clear evidence that development of oral cancer follows the same biological pathways irrespective of the source of carcinogenic exposure (Dysvik *et al*., 2006; Lunde *et al*., 2010). The hypermethylation of *SIRT1* may be a target for the prediction of oral carcinogenesis caused by those habits, as well as BQ chewing. Further investigations are needed to examine this hypothesis.

5. CONCLUSIONS

In conclusion, our data demonstrated for the first time that DNA hypermethylation of *SIRT1* occurs in OSCC and normal oral mucosa obtained from BQ chewers and that the methylation status in buccal smear samples might be considered as an applicable routine oral screening procedure in high-risk populations, particularly in relation to BQ induced oral cancers. Further studies will, therefore, be necessary to confirm our findings, which might lead to a better understanding of the molecular basis of oral carcinogenesis induced by various environmental exposures.

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TABLES

Table 1. Primer sequences used in *sirtuin 1* (*SIRT1*) methylation levels analysis

Gene	Accession ID	Primers (5' - 3')	Product size (bp)	Tm (°C)	Reference
SIRT1	NM_012238 (transcript variant 1)	MF: GGCGAATTTGGTTGTATTATACG	110 bp	62.2	Oliveria *et al*., 2014
		MR: GAACGAAAACTATTACGTCTACCG			
		UF: GGGGTGAATTTGGTTGTATTATATG	112 bp		
		UR: AAACAAAAACTATTACATCTACCACT			

MF: methylated forward; MR: methylated reverse; UF: unmethylated forward; UR: unmethylated reverse; bp: base pair

Table 2. Primer sequences used in *sirtuin 1* (*SIRT1*) gene expression levels analysis

Gene	Forward primer	Reverse primer	Reference
SIRT1	GCGATTGGGTACCGAGATAA	TTGCATGTGAGGCTCTATCC	Murray *et al*., 2013
GAPDH	GTGAAGGTCGGAGTCAAC	GTTGAGGTCAATGAAGGG	Li *et al*., 2016

Table 3: Characteristics of human participants and buccal smear samples

	Control (non-chewer)	**Betel quid chewer**	**Total**
Samples, N (%)	45 (64.2)	25 (35.7)	70 (100)
Male, N (%)	9 (20)	5 (20)	14 (20)
Female, N (%)	36 (80)	20 (80)	56 (80)
Age (mean ± SD)	35.5 ± 13.6	39.5 ± 12.0	
Duration of betel quid chewing habits			
≥ 6-months, N (%)	-	6 (24)	
1-10 years, N (%)	-	14 (56)	25 (35.7)
> 10-years, N (%)	-	5 (20)	
Chewing years (mean ± SD)		7.3 ± 10.3	
SIRT1 DNA methylation level (mean ± SD)	4.0 ± 4.6	16.5 ± 23.7	

NOTE: Data are expressed as mean ± standard deviation (SD)

Table 4: Multivariable regression analysis of *sirtuin 1* (*SIRT1*) DNA methylation level (total buccal smear samples)

Variables	**B**	**Standard error**	**Beta**	**t**	***p*-value**
Age	.219	.134	.183	1.630	.108
Sex (Male: 1, Female: 2)	3.107	4.314	.080	.720	.474
Betel quid chewers *vs* non-chewers	11.633	3.645	.358	3.192	**.002**

NOTE: Values in bold represent statistical significance ($p<0.05$).

B: unstandardized coefficients; Beta: standardized coefficients.

Table 5: Characteristics of betel quid components in smear samples of betel quid chewers

	Group 1	**Group 2**	**Total**
Samples, N (%)	7 (28)	18 (72)	25 (100)
Male, N (%)	1 (4)	4 (16)	5 (20)
Female, N (%)	6 (24)	14 (56)	20 (80)
Total betel quid chewing (mean ± SD)	2673 ± 3414.8	5581.6 ± 7846.2	
SIRT1 DNA methylation level (mean ± SD)	7.0 ± 4.9	20.3 ± 27.0	

NOTE: Data are expressed as mean ± standard deviation (SD)

NOTE: Group 1, betel leaf and areca nut; Group 2; betel leaf, areca nut, and slaked lime

Table 6: Multivariable regression analysis of *sirtuin 1* (*SIRT1*) DNA methylation level on betel quid components

Variables	**B**	**Standard error**	**Beta**	**t**	***p*-value**
Group 1 *vs* Group 2	10.618	10.342	.205	1.027	.316
Quid number	.001	.001	.354	1.771	.091

Group 1, betel leaf and areca nut; Group 2, betel leaf, areca nut, and slaked lime

B: unstandardized coefficients; Beta: standardized coefficients.

Table 7: Multivariable regression analysis of *sirtuin 1* (*SIRT1*) DNA methylation level (betel quid chewer samples)

Variables	**B**	**Standard error**	**Beta**	**t**	***p*-value**
Age	.189	.396	.095	.478	.638
Sex (Male: 1, Female: 2)	9.529	9.782	.164	.974	.341
Chewing years	1.280	.455	.558	2.814	**.010**

NOTE: Values in bold represent statistical significance ($p<0.05$).

B: unstandardized coefficients; Beta: standardized coefficients.

Table 8: Characteristics of patients and tissue samples

	BQ chewers OSCC	**BQ non-chewers OSCC**	**Healthy controls**	**Total**
	Sri Lankan patients	**Japanese patients**		
Samples, N (%)	12 (25.5)	22 (46.8)	13 (27.7)	47 (100)
Male, N (%)	9 (75)	9 (40.9)	6 (46.2)	24 (51.1)
Female, N (%)	3 (25)	13 (59.1)	7 (53.8)	23 (48.9)
Age	56.3 ± 15.9	61.2 ± 15.2	58.5 ± 17.8	
SIRT1 DNA methylation level	41.4 ± 13.4	22.2 ± 14.3	11.2 ± 6.6	

NOTE: data are expressed as mean ± standard deviation (SD).

BQ: betel quid; OSCC: oral squamous cell carcinoma.

Table 9: Multivariable regression analysis of *sirtuin 1* (*SIRT1*) DNA methylation level (BQ chewers OSCC *vs* healthy controls)

Variables	B	Standard error	Beta	t	*p*-value
Analysis I					
Age	.167	.138	.150	1.212	.239
Sex (Male: 1, Female: 2)	6.228	4.783	.169	1.302	.207
BQ chewers OSCC *vs* healthy controls	28.721	4.349	.793	6.604	.000

NOTE: Values in bold represent statistical significance ($p<0.05$).

BQ: betel quid; OSCC: oral squamous cell carcinoma

B: unstandardized coefficients; Beta: standardized coefficients.

Table 10: Multivariable regression analysis of *sirtuin 1* (*SIRT1*) DNA methylation level (BQ non-chewers OSCC *vs* healthy controls)

Variables	**B**	**Standard error**	**Beta**	**t**	***p*-value**
Analysis II					
Age	.169	.124	.208	1.362	.183
Sex	6.643	3.967	.255	1.675	.104
BQ non-chewers OSCC *vs* healthy controls	11.837	4.076	.444	2.904	**.007**

NOTE: Values in bold represent statistical significance ($p<0.05$).

BQ: betel quid; OSCC: oral squamous cell carcinoma

B: unstandardized coefficients; Beta: standardized coefficients.

Table 11: Multivariable regression analysis of *sirtuin 1* (*SIRT1*) DNA methylation level (BQ chewers OSCC *vs* BQ non-chewers OSCC)

Variables	B	Standard error	Beta	t	*p*-value
Analysis III					
Age	.055	.161	.051	.340	.736
Sex	6.577	5.101	.201	1.289	.207
BQ chewers OSCC *vs* BQ non-chewers OSCC	17.132	5.392	.501	3.177	**.003**

NOTE: Values in bold represent statistical significance ($p<0.05$).

BQ: betel quid; OSCC: oral squamous cell carcinoma

B: unstandardized coefficients; Beta: standardized coefficients.

FIGURES

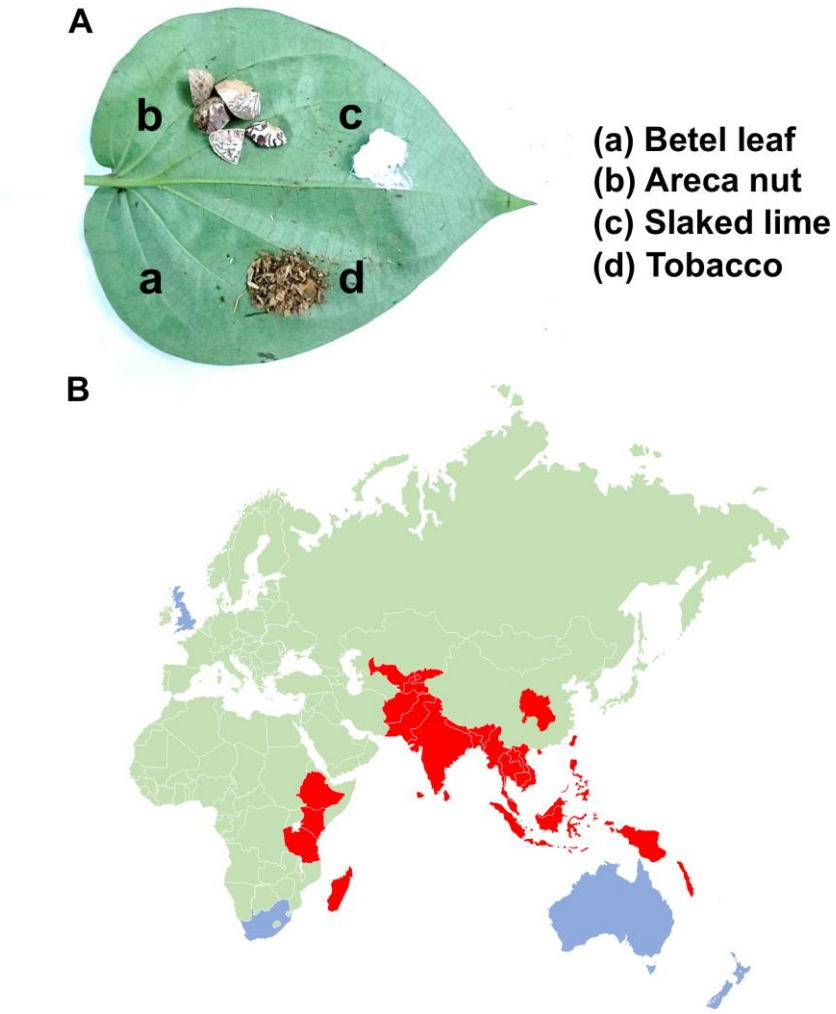

Figure 1. The major ingredients of betel quid and regions where betel quid chewing is prevalent (modification from Islam *et al.,* 2019b). (A) the major ingredients of betel quid include the following: (a) betel leaf; (b) areca nut; (c) slaked lime, most commonly prepared in paste form; and (d) tobacco components (sun-dried or fermented). (b) The red areas on the map depict regions where betel quid chewing is prevalent in South Asia and the South Pacific Islands. The blue colored-areas represent the countries comprising habitual betel quid chewers originating from the Indian subcontinent; the countries include United Kingdom (mainly at Yorkshire, Birmingham, Leicester, East, and West London), South Africa (Durban and Johannesburg), Australia and New Zealand.

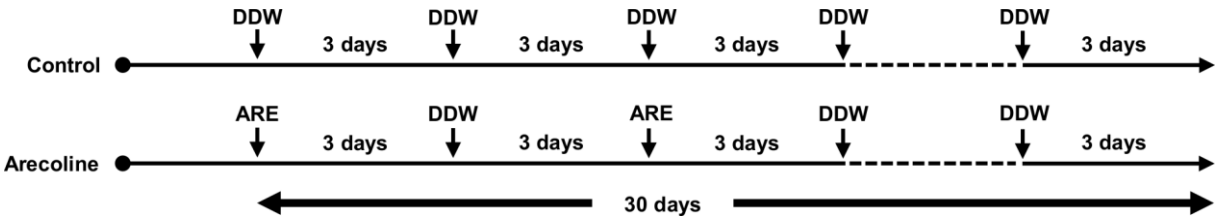

Figure 2. Flow chart of cell culture. Human gingival epithelium progenitors (HGEPs), cells were treated with arecoline at a concentration of 50 μg/mL. The culture media was replaced every 3 days, alternating media with and without arecoline for 30 days. Untreated samples were used as controls. DDW, double-distilled water; ARE, arecoline

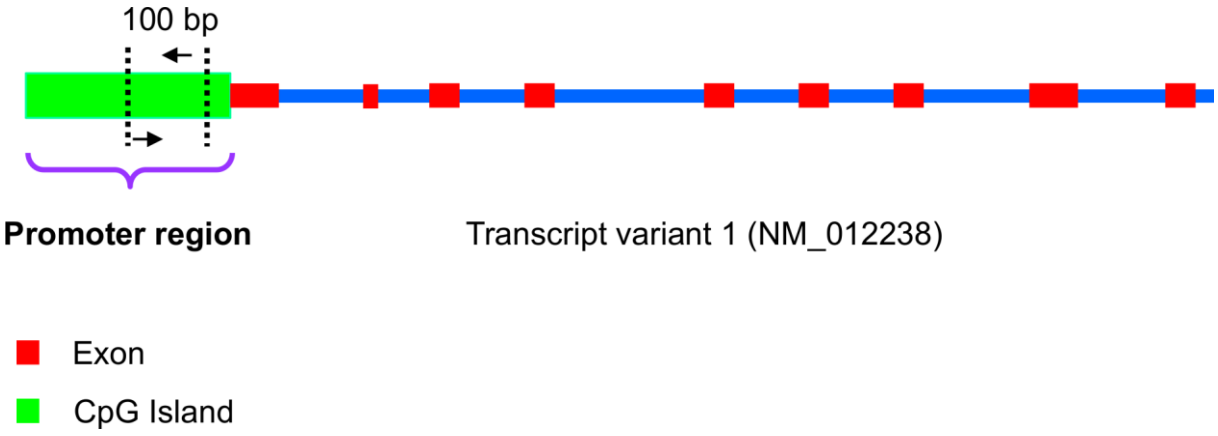

Figure 3. Designing and identification of CpG island around the promoter region of the *sirtuin 1* (*SIRT1)* gene. *SIRT1* has three transcription variants. Transcription variants 1 (NM_O12238) has 9 exons. The CpG islands were located close to promoter areas at the transcription start sites of *SIRT1*. The product is approximately 100 base pairs in length.

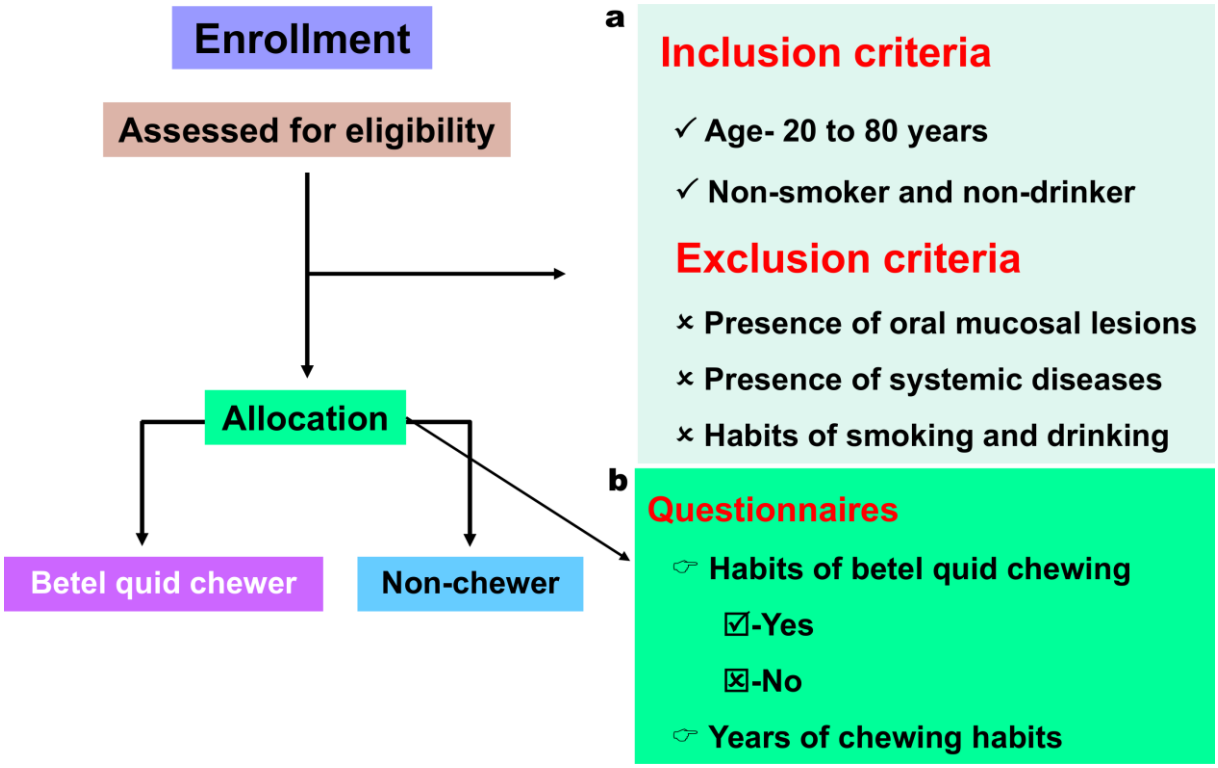

Figure 4. Buccal smear samples collection and distribution. (a) presence of oral mucosal lesions, systemic diseases, and habits of smoking and drinking subjects were eliminated followed by inclusion and exclusion criterions. (b) buccal smear samples of betel quid chewers and non-chewers were obtained from Sri Lankan participants by asking questionnaires.

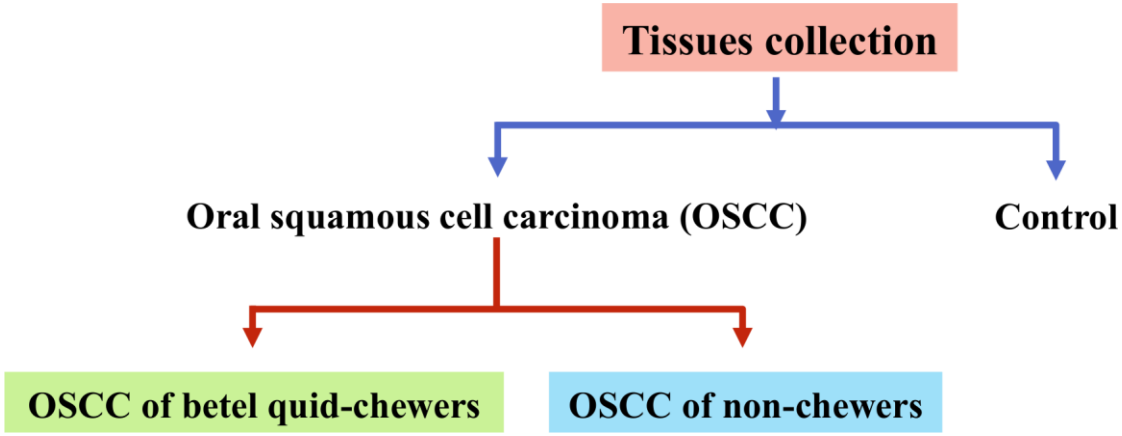

Figure 5. Tissue samples collection and distribution. Paraffin-embedded tissues samples of oral squamous cell carcinoma (OSCC) grouped according to the habits of patients. The OSCC of BQ chewers tissue samples obtained from Sri Lankan patients and OSCC of non-chewers were from Japanese patients. The controls are defined as tissues from healthy patients without the presence of oral lesions.

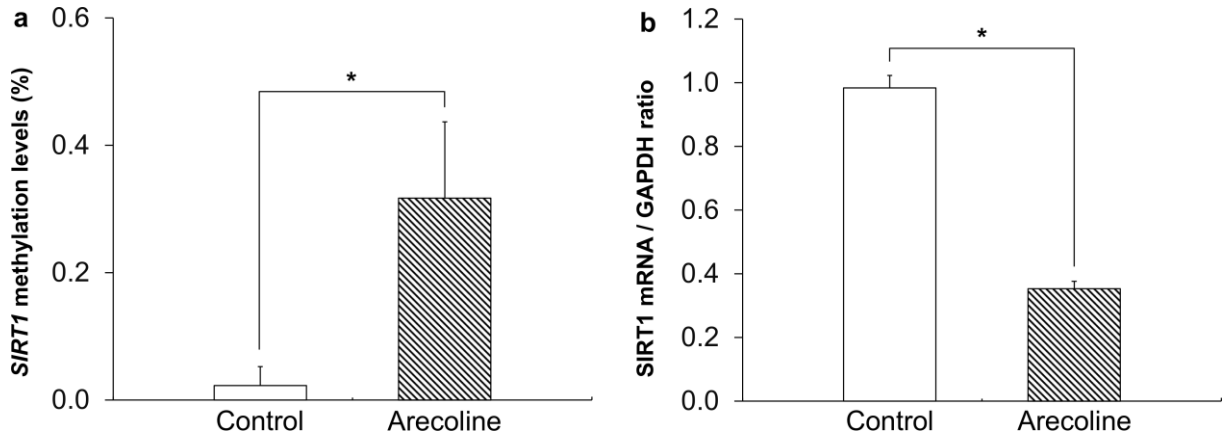

Figure 6. (a) DNA methylation levels of *sirtuin 1* (*SIRT1*) in human gingival epithelial progenitors (HGEPs) cells. The levels of *SIRT1* DNA methylation in cells treated with 50 μg/mL arecoline were significantly increased compared to that in control cells. (b) *SIRT1* mRNA expression levels in HGEPs cells. The expression levels of *SIRT1* mRNA in cells treated with arecoline at 50 μg/mL was significantly decreased compared to that in control cells. A value of $p<0.05$ was considered to be statistically significant (n=4).

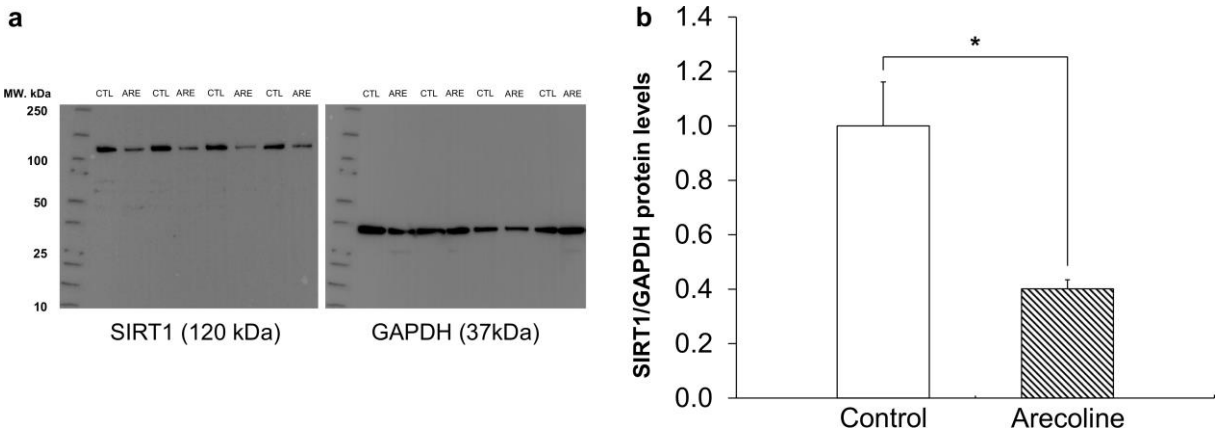

Figure 7. (a) sirtuin 1 (SIRT1) protein production in human gingival epithelial progenitors (HGEPs) cells. The production of SIRT1 protein (bands of 120 kDa) was reduced by arecoline treatment compared to controls. As a control, the levels of GAPDH (bands of 37 kDa) were similar in all cells. (b) The SIRT1/GAPDH protein expression ratio in HGEPs cells. The ratio of intensities of SIRT1 to GAPDH in control cells was considered to be 100%. The ratio of intensities of SIRT1/GAPDH in control cells and arecoline-treated cells was 100±16.2% and 40.1±3.3%, respectively. The SIRT1 protein levels were reduced by arecoline treatment. A value of $p<0.05$ was considered to be statistically significant (n=4). CTL, control; ARE, arecoline.

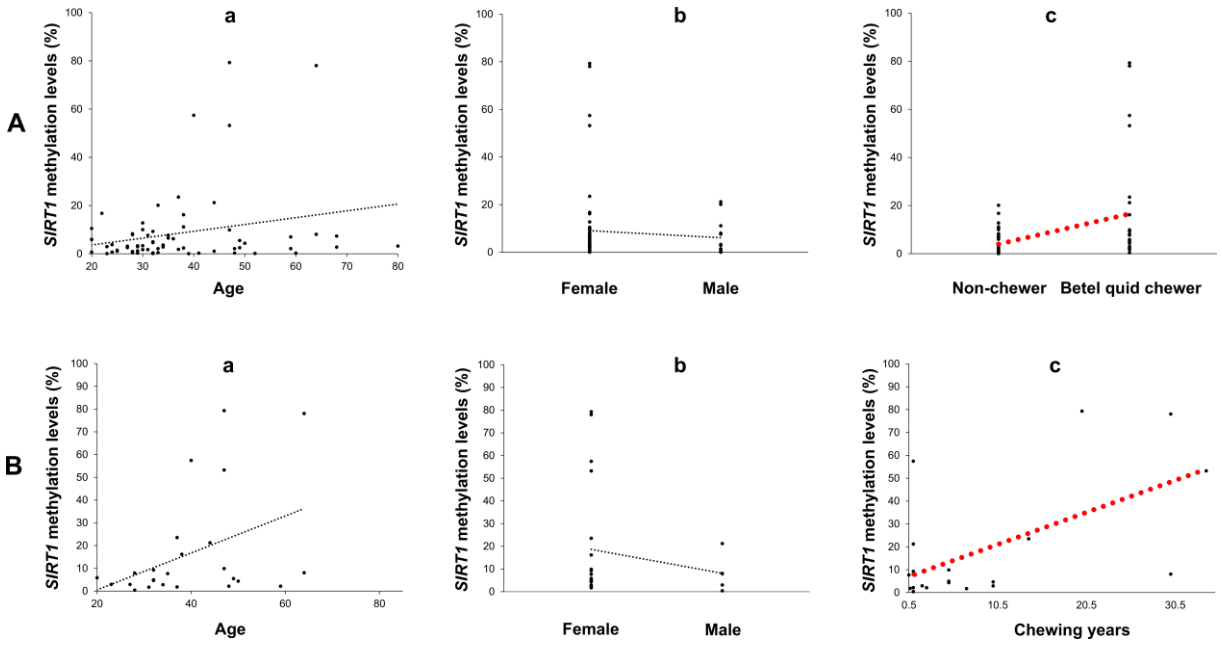

Figure 8. (A) *sirtuin 1* (*SIRT1*) DNA methylation levels in buccal smear samples of betel quid chewers and non-chewers. No significant correlation was observed in terms of age and gender to groups of betel quid chewers and non-chewers (a,b). Betel quid chewing habit was the only significant predictor of *SIRT1* DNA methylation level (c). (B) *SIRT1* DNA methylation levels in buccal smear samples of betel quid chewers. The chewing years of betel quid were significantly correlated to the levels of *SIRT1* DNA methylation level (c). A value of $p<0.05$ was considered to be statistically significant.

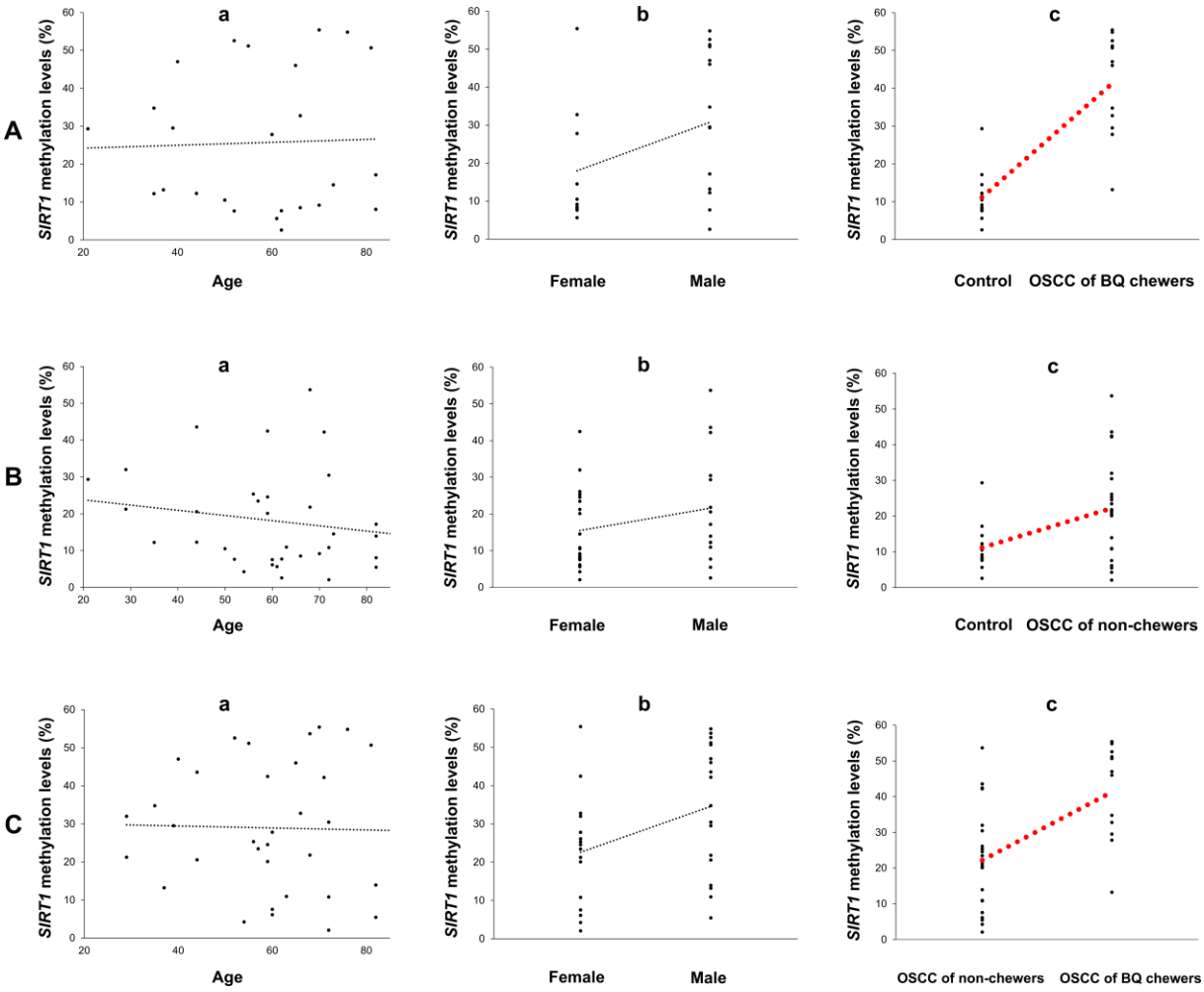

Figure 9. (A) *sirtuin 1* (*SIRT1*) DNA methylation levels in controls and oral squamous cell carcinoma (OSCC) of betel quid (BQ) chewers. *SIRT1* was significantly hypermethylated in tissue samples of OSCC from BQ chewers than in oral mucosa from healthy control subjects. (B) *SIRT1* DNA methylation levels in controls and OSCC of non-chewers. *SIRT1* was significantly hypermethylated in tissue samples of OSCC from non-chewers than in oral mucosa from healthy control subjects. (C) *SIRT1* DNA methylation levels in oral OSCC of non-chewers and OSCC of BQ chewers. *SIRT1* was significantly hypermethylated in tissue samples of OSCC from BQ chewers than in that of OSCC of non-chewers. A value of $p<0.05$ was considered to be statistically significant.