研 究 者：Shajedul Islam（所属：Health Sciences University of Hokkaido）

研究題目：DNA hypermethylation of *sirtuin 1*（*SIRT1*）caused by betel quid chewing—a possible predictive biomarker for malignant transformation

目　的：

Arecoline, the major alkaloid of areca nut, is known to induce oral carcinogenesis, however, its mechanism is still needed to elucidate. *SIRT1* has been identified as playing a role in the maintenance of epithelial integrity, and its alteration is often related to carcinogenesis. In the present study, in order to characterize the association between chronic arecoline stimulation and carcinogenesis the mRNA expression, DNA methylation, and protein levels of *SIRT1* in human gingival epithelial progenitor cells（HGEPs）, stimulated with arecoline was assessed. In addition, DNA methylation levels of *SIRT1* were assessed in paraffin-embedded tissue samples of oral squamous cell carcinoma（OSCC）obtained from BQ chewing and non-chewing patients and in tissue samples from healthy control subjects.

対象および方法：

HGEPs cells were treated with arecoline at a concentration of 50μg/ml. The culture was replaced every 3 days, alternating with and without arecoline for 30days. Untreated samples were used as controls. Total RNA was extracted from the HGEPs and cDNA was prepared from the RNA. mRNA expression levels of *SIRT1* were analyzed by quantitative real-time PCR. DNA was extracted from the HGEPs and treated with sodium bisulfite. DNA methylation analysis was performed by quantitative methylation-specific PCR（q-MSP）. Proteins were extracted and analyzed by Western blotting. DNA was also extracted from the buccal swabs of BQ chewers and treated with sodium bisulfite. DNA methylation analysis was assessed by using q-MSP. DNA methylation levels of *SIRT1* were assessed by using qMSP in paraffin-embedded tissue samples of OSCC obtained from BQ chewing and non-chewing patients and in tissue samples from healthy control subjects. Statistical analysis was performed on a database using IBM SPSS Statistics 23（IBM, Armonk, NY）. Mann-Whitney U test was performed between groups of arecoline-treated and untreated samples. Pearson's chi-squared test was used to analyze gender differences in groups of tissue samples（BQ chewers OSCC, BQ non-chewers OSCC, and healthy controls）and in buccal smear samples（control and BQ chewer）. One-way ANOVA test was performed to analyze the age and DNA methylation level differences in tissue samples and buccal smear sample subjects. Multivariable regression analysis was performed with Bonferroni adjusted p values for multiple comparisons in tissue samples of OSCC and healthy control subjects, and buccal smear samples of BQ chewers and

non-chewers subjects. Results with p values of <0.05 were considered to be statistically significant.

結果および考察：

Results of the present study demonstrated that *SIRT1* in OSCC is hypermethylated and that the methylation levels were significantly higher in the OSCC of BQ chewers than in that of non-chewers. Our *in vitro* model showed that 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. These results suggest that *SIRT1* is involved in oral cancer caused by BQ chewing and that hypermethylation of *SIRT1* in the oral mucosa of BQ chewers may be a predictive marker for detecting early events in multistage carcinogenesis.

Although hypermethylation of *SIRT1* has been reported in several cancer tissues, this is the first demonstration of hypermethylation of *SIRT1* in OSCC. 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. Therefore, we employed an in vitro model of a daily BQ chewing habit that we showed previously to contain hypermethylated genes. The cells were stimulated with arecoline, a major component of BQ, for a prolonged period according to our previous protocol. 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. *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. 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). MeCP2 has been shown to interact with DNA methyltransferase 1 (DNMT1) and recruits the latter to induce *SIRT1* promoter methylation. 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. TGF-β is likely to silence *SIRT1* epigenetically by inducing the MeCP2 expression, although other possible mechanisms cannot be ruled out. These findings may provide an underlying molecular mechanism of the effect of arecoline on DNA hypermethylation. 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. The duration of chewing habits was correlated positively to the frequency of *SIRT1* hypermethylation in our data. This observation may support the previous paper that showed increasing the years of quid chewing habits was positively associated with oral cancer, wherein *SIRT1* hypermethylation may play an important role in the process of their development. Together, these findings indicate that DNA hypermethylation of *SIRT1* in epithelium of BQ chewers may be an early event involved in oral carcinogenesis. Previous reports confirmed DNA hypermethylation in precancerous lesions and oral cancer with the habits of BQ chewing. 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 clinically 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 tumor suppressor genes（TSGs）in smears of buccal mucosa, could be useful for the detection of early changes caused by BQ chewing habits. Cigarette smoking and alcohol consumptions are other risk factors for oral cancer. Those habits also cause alteration of DNA methylation. In fact, previous studies demonstrate clear evidence that development of oral cancer follows the same biological pathways irrespective of the source of carcinogenic exposure. Therefore, 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.

In conclusion, our data demonstrate 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 are necessary to confirm our findings, which might lead to a better understanding of the molecular basis of oral carcinogenesis induced by various environmental exposures.

成果発表：（予定を含めて口頭発表、学術雑誌など）

Publications

1. Islam S, et al. DNA hypermethylation of sirtuin1（SIRT1）caused by betel quid chewing-

a possible predictive biomarker for malignant transformation. *Clinical Epigenetics*. [**IF : 5.69**].

2. Islam S, et al. How Each Component of Betel Quid Is Involved in Oral Carcinogenesis : Mutual Interactions and Synergistic Effects with Other Carcinogens- a Review Article. *Current Oncology Reports*. [**IF : 3.94**].
3. Islam S, et al. Sirtuin-1 and oral cancer (Review). *Oncology Letters*. [**IF : 1.87**].

Book Chapter

4. Islam S, et al. Impacts of Sirtuin1 and Sirtuin3 on Oral Carcinogenesis. *In : Sirtuin Biology in Medicine : Targeting New Avenue of Care in development, Aging, and Disease*. Elsevier Science Publishing Co Inc, ISBN 978-0-12-814118-2 (in press).

Conference Presentations

1. Japanese Association for Dental Research- (66th Annual Meeting ; November 17-18, 2018 ; Hokkaido, Japan).
2. International Association for Dental Research- (97th General Session ; June 19-22, 2019 ; Vancouver, Canada).
3. Japanese Cancer Association- (78th Annual Metting ; September 26-28, 2019 ; Kyoto, Japan).
4. Japan Society of Clinical Oncology- (57th Annual Meeting ; October 24-26, 2019 ; Fukuoka, Japan).