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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ADHIKARI Bhoj Raj

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Graduate School of Dentistry,
Health Sciences University of Hokkaido

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Abstract

1. Introduction

Oral cancer is the eighth most common cancer in humans. Both environmental and genetic factors induce the development of oral cancer. Although several studies have demonstrated the factors involved in betel quid chewing-related oral cancer, the mechanism by which this cancer is caused remains elusive. Genetic mutations and aberrant DNA methylations have been suggested to be involved in the development of oral cancer. Unlike genetic mutations, DNA hypermethylations are reversible and can be used as diagnostic and therapeutic targets. The objective of this study was to detect novel DNA hypermethylations that can be applied to diagnostic, predictive, interceptive, and therapeutic approaches for betel quid chewing-related oral cancer.

2. Materials and methods

a. Genome-wide analysis

Human gingival epithelial progenitors (HGEPs) were cultured and treated with a 3-day alternating regimen with/without arecoline (50 μg/ml) for 1 month. Untreated samples were used as controls. DNA microarrays were used for genomic DNA analysis (methylation array) and global analysis of mRNA expression levels (microarray) in the samples.

b. Reproducibility of the methylation array and microarray data

For the reproducibility of the methylation array and microarray data, the DNA samples were treated with sodium bisulfite and quantitative methylation-specific polymerase chain reaction (qMSP) was performed; mRNA expression was analyzed by quantitative reverse transcription polymerase chain reaction (qRT-PCR).

c. Analysis of dual-specificity phosphatase 4 (DUSP4) gene in oral cancer cell line

To examine the expression of DUSP4 gene in oral cancer, four cancer cell lines HSC4, SAS, BSC-OF, and HSY were cultured and treated with an alternating regimen of arecoline (50 μg/ml) for 3 days each for a total of 7 days.

d. Signaling pathway analysis

To examine the presence of any other mechanism, in addition to hypermethylation, the levels of phosphorylated c-Jun N-terminal kinase (pJNK) in the HGEPs treated with or without arecoline were determined using cell based enzyme-linked immunosorbent assay (ELISA).

e. Histological and immunohistochemical evaluations, and CpG methylation analysis

Twenty oral tissue samples from cancer patients with betel quid chewing habit were obtained from Sri Lanka, and 22 samples (13, oral cancer; 9, fibrous polyp) from betel quid non-chewers were retrieved from the archives of Health Sciences University of Hokkaido (HSUH), Japan. Immunohistochemical examinations of the tissue samples were performed using anti-DUSP4 antibody. DUSP4 immunoreactivity was assessed in the nuclei of the epithelial cells. Genomic DNA was extracted from the tissue samples, which were then treated with sodium bisulfite and subjected to qMSP.

f. Statistical analysis

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

3. Results

CpG island methylation array and global analysis of mRNA revealed 8638 and 455 genes with more than 2-fold increase in methylation and downregulation of mRNA expression respectively, in HGEP samples stimulated with arecoline for a period of 1 month. Among them, 21 were tumor suppressor genes within -1000bp in the promoter region. Four candidate genes (HNRNPH3, BCL2L11, TFAP2A, and DUSP4) with the highest amounts of hypermethylation in their promoter regions and downregulated levels of mRNA expression were selected. Significant hypermethylation and downregulation in the expression levels of the *DUSP4* gene were noted in the HGEPs treated with arecoline for a period of 7 days and 1 month. Methylation levels of *DUSP4* were significantly higher in all four cancer cell lines without arecoline treatment when compared to the HGEPs. The mRNA expression level of *DUSP4* in the untreated BSC-OF cell line was not significantly different from that in the HGEPs; however, the levels were downregulated after arecoline treatment. pJNK levels were

also elevated in samples treated with arecoline for a period of 1 month.

Immunohistochemical (IHC) staining revealed significantly higher expression levels of DUSP4 in betel quid chewers (66.85 ± 3.0) when compared to the non-chewing oral cancer patients (20.34 ± 4.7). CpG islands in DUSP4 were significantly hypermethylated in samples obtained from betel quid chewers (89.8 ± 2.6) when compared to those from the non-chewing oral cancer patients (44.7 ± 10.6) and healthy controls (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. To the best of our knowledge, this is the first report to demonstrate hypermethylation of DUSP4 followed by the downregulation of its mRNA expression levels in betel quid-related oral cancer. Our findings indicate that DUSP4 hypermethylation can be applied as a target for diagnostic, predictive, interceptive, and therapeutic approaches in these cancers.