



# Relation between Different Stages of Oral Cancer and Aberrant Methylation in Taiwan: Preliminary Report

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## ABSTRACT

During the past decade oral cancer has become the fourth cause of death in Taiwan. Screening and early diagnosis may increase the survival rate. Thus we aim to improve the techniques of early detection of oral cancer.

Aberrant DNA methylation patterns are common in oral cancer and others. In this study, we focus on BRAF and CALCA gene which play an important role of cell growth and have never been used as a specific biomarker for oral cancer survey before. The purpose of this study is to confirm the correlation between the sample types and the trends of methylation level of BRAF gene and CALCA gene, making early detection more accurate by using specific biomarker in the future.

## INTRODUCTION

In the report of the Taiwan Executive Yuan, Department of Health, oral cancer have become the fourth common cause of cancer death in Taiwan. Previous studies have found that after surgical treatments, the patients who had early stages (stage I and stage II) of oral cancer has a higher 5-year survival rate (81.9%) than the later ones (stage III and stage IV, 23-25%)<sup>1,2</sup>. This indicated the importance of early detection of carcinogenesis for high survival rate.

Aberrant DNA methylation patterns are common in oral cancer and others<sup>3,4</sup>. When the DNA have been methylated, especially in promotor region, the transcription may be stably repressed<sup>5</sup>. Methylation of tumor suppressor genes has been frequently reported to be involved in carcinogenesis<sup>6,7</sup>. There are about 18 genes that have already been reported with aberrant methylation in oral cancer tissue<sup>8</sup>. Being more stable and easily amplifiable than other protein biomolecules<sup>9</sup>, the status of DNA methylation may become an ideal biomarker for early cancer detection.

BRAF plays a key role as an intermediary in the RAS-RAF signaling cascade, a pathway responsible for normal cell growth, differentiation, and survival. BRAF is the main activator of MEK<sup>10</sup>. Recently, the researcher has found out that mutated BRAF causes overactive downstream signaling via MEK and ERK<sup>11</sup>. This leads to excessive cell proliferation and survival which are independent of growth factors. Other study showed examples of mutated BRAF in other cancer type<sup>12</sup>. In view of the close correlation between this gene and oncogenesis, analysis of methylation status on BRAF gene may provide more information of other epigenetic ways other than mutation.

CALCA is a gene that codes for three related, but different, polypeptides, calcitonin, katecalcitonin and  $\alpha$ -calcitonin gene-related peptide ( $\alpha$ -CGRP). Previous studies showed that CALCA has a correlation with ovarian cancer, cervical cancer and prostate cancer<sup>13,14,15</sup>. Furthermore,  $\alpha$ -CGRP has been shown to enhance angiogenesis through its interactions with CALCA receptor components on vascular endothelial cells that stimulate endothelial cell growth and migration as well as promote capillary-like tube formation in vitro. These data showed potential correlations between CALCA and the other cancer type. In this study, we investigated the methylation patterns in the different tissue from the same patient.

## RECENT RESULTS

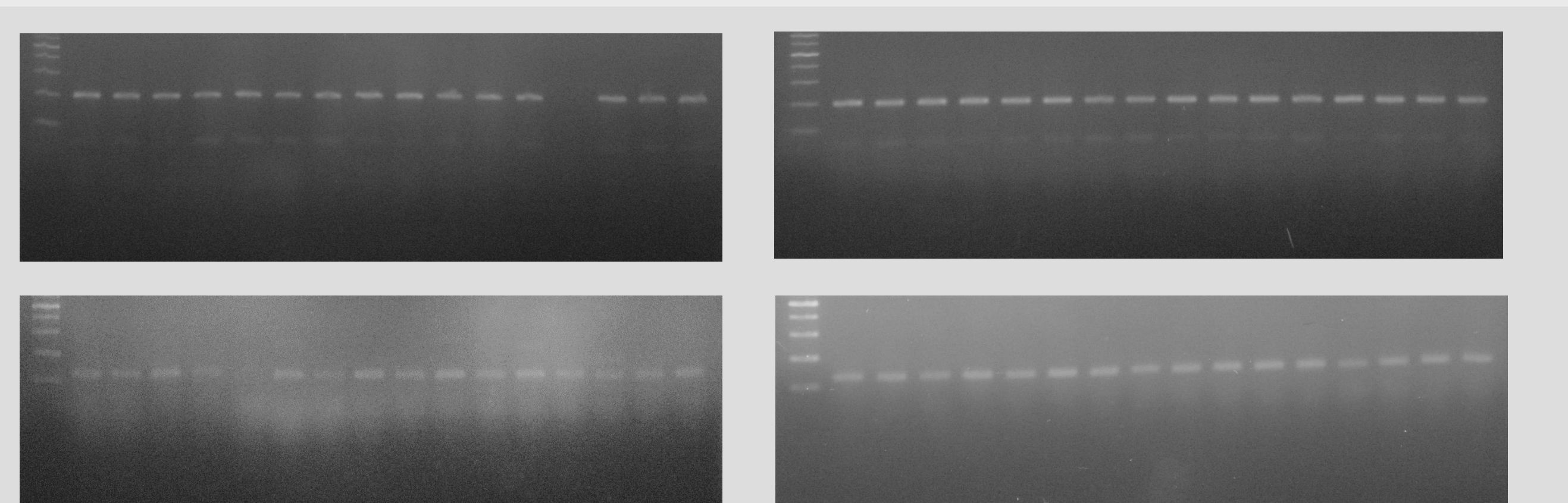


Figure 1. These are the PCR product of 8 patient (each patient has 4 kinds of tissue sample) using biotin primer for confirmation before pyrosequencing. (the upper two panels are BRAF gene, the lower two for CALCA)

Both of these data indicated that the primer design is ready for use in pyrosequencing.

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## METHODS AND MATERIALS

### Patients and Tissue samples

The tissue samples were obtained from patients of single medical center and were divided into three parts: tumor, nearby and surrounding (normal) tissue. Then, the tissue was deposited in liquid nitrogen for DNA and RNA extractions. Each one about 300 mg. The data of patient collected include: age, gender, risk factors (alcohol/ betel nut/ cigarette), clinical and pathological stage, and operation.

### DNA Isolation and Methylation Analysis

All sample tissues DNA are extracted with Genra® Puregene® Tissue Kit. Then we used the Pyrosequencing™ Assay Design Software to design the primer. The specific of these primer is that specific bases would change in later steps (Bisulfite treatment and PCR). And the methylated cytosine will remain unchanged, but the normal cytosine will convert to uracil, make it easier for us to analyze the methylation status of the sequence. To prevent the conversion in the primer sets region, forward primer should avoid G, reverse primer should avoid C, and the Inner (Sequencing) primer design should depend on the direction. The genomic DNA obtained from oral epithelial cells or tissue samples was subjected to bisulfite treatment using EZ DNA Methylation-Gold™ Kit (ZYMO RESEARCH CORP.) according to the manufacturer's instruction. After thermal denaturation and bisulfite treatment, the DNA was applied to PCR using primer sets designed to amplify regions of interest with GoTaq® Green Master Mix M7122 (Promega): BRAF forward primer, 5'-TTCCTTTACTTACTACACCTCAGATATATTTCTTC-3'; reverse primer, 5'-GCATCTCAGGGCCAAAAATTTAAT-3'. PCR amplifications were performed as follows: 95 °C for 5 min; followed by 45 cycles of 95 °C for 1 min, 55.5 °C for 30 sec, and 72 °C for 45 sec, and ended with an extension of 72 °C for 5 min and quick chill to 8 °C on a Program Temp Control System PC-320 (ASTEC). CALCA forward primer, 5'-GGCGAGGGTTCAAACCA-3'; reverse primer, 5'-ATGGAAATGGTGGGGGATG-3'. PCR amplifications were performed as follows: 95 °C for 5 min; followed by 45 cycles of 95 °C for 1 min, 50.7 °C for 30 sec, and 72 °C for 45 sec, and ended with an extension of 72 °C for 5 min and quick chill to 8 °C on a Program Temp Control System PC-818A/S (ASTEC). Products amplified with both types of primers were examined on 2% agarose gel.

### Pyrosequencing

Pyrosequencing is sequencing by synthesis and is a technique for accurate and consistent analysis of DNA sequences. BRAF biotin-labeled reverse primer, 5'-GCATCTCAGGGCCAAAAATTTAAT-3'; inner (sequencing) primer, 5'-GTCTAGCTACAGTGAAATCT-3'; CALCA biotin-labeled forward primer, 5'-GGCGAGGGTTCAAACCA-3'; sequencing primer, inner (sequencing) primer 5'-GGGAATTTGAGGGCAGTTT-3'. BRAF Biotin-PCR amplifications were performed as follows: 95 °C for 5 min; followed by 45 cycles of 95 °C for 1 min, 56.5 °C for 30 sec, and 72 °C for 45 sec, and ended with an extension of 72 °C for 5 min and quick chill to 8 °C. CALCA Biotin-PCR amplifications were performed as follows: 95 °C for 5 min; followed by 45 cycles of 95 °C for 1 min, 53.5 °C for 30 sec, and 72 °C for 45 sec, and ended with an extension of 72 °C for 5 min and quick chill to 8 °C. After confirmation, all PCR products will be sent to Sung, Huang-Mo, an Assistant Prof. of the College of Bioscience and Biotechnology, NCKU, to perform pyrosequencing.

## DISCUSSION

We conducted a series of experiments to examine the possible sets of the primers which can be used in regular PCR and the Methylation Specific PCR at the same time.

For future work, we are going to do the pyrosequencing to determine the methylation level between the sample types.

Finally, we perform a statistical analysis of the C/T ratio of the sample, to investigate the methylation status between different samples from same patient and also the correlation between the sample types and the trends of the methylation level of BRAF gene and CALCA gene.

## CONCLUSIONS

Aberrant DNA methylation patterns are common in oral cancer and others. In this study, we focus on BRAF and CALCA gene, both of them play an important role in cell growth and have never been used as a specific biomarker of the oral cancer before.

The correlation between the sample types and the trends of the methylation level of BRAF gene and CALCA gene, making early detection of cancer more accurate by using specific biomarker in the future.

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