ตำแหน่งของไลน์-1 ที่มีระดับเมทิลต่ำกว่าปกติ สำหรับการตรวจหามะเร็งในช่องปากชนิดสแควมัสเซลล์คาร์ซิโนมา

นางเกศกัญญา สัพพะเลข

วิทยานิพนธ์นี้เป็นส่วนหนึ่งของการศึกษาตามหลักสูตรปริญญาวิทยาศาสตรดุษฎีบัณฑิต
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LINE-1 HYPOMETHYLATION LOCI
FOR DETECTION ORAL SQUAMOUS CELL CARCINOMA

Mrs. Keskanya Subbalekha

A Thesis Submitted in Partial Fulfillment of the Requirements

for the Degree of Doctor of Philosophy Program in Oral Biology

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Thesis Title	LINE-1 HYPOMETHYLATION LOCI FOR DETECTION ORAL SQUAMOUS CELL CARCINOMA
By	Mrs. Keskanya Subbalekha
Field of Study	Oral Biology
Thesis Advisor	Assistant Professor Atiphan Pimkhaokham, D.D.S., Ph.D.
Thesis Co-advisor	Professor Apiwat Mutirangura, M.D., Ph.D.
Thesis Co-advisor	Associate Professor Prasit Pavasant, D.D.S.,Ph.D.

Accepted by the Faculty of Dentistry, Chulalongkorn University in Partial Fulfillment of the Requirements for the Doctoral Degree

..... Kanok Sorat .....

Associate Dean for Communication and Technology Affairs
Acting on behalf of Dean of the Faculty of Dentistry
(Assistant Professor Kanok Sorathesn, D.D.S., M.S.)

THESIS COMMITTEE

..... Tussanee Yongchaitrakul ..... Chairman
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..... P. Atiph ..... Thesis Advisor
(Assistant Professor Atiphan Pimkhaokham, D.D.S., Ph.D.)

..... Apiwat Mutirangura ..... Thesis Co-advisor
(Professor Apiwat Mutirangura, M.D., Ph.D.)

..... Prasit Pava ..... Thesis Co-advisor
(Associate Professor Prasit Pavasant, D.D.S., Ph.D.)

..... Budsaba R ..... External Member
(Associate Professor Budsaba Rerkamnuaychoke, D.M.Sc.)

..... Shanop Shuangshoti ..... Member
(Associate Professor Shanop Shuangshoti, M.D.)

..... Oranart ..... Member
(Assistant Professor Oranart Matangkasombut, D.D.S., Ph.D.)

เกศกัญญา สัพพะเลข : ตำแหน่งของไลน์-1 ที่มีระดับเมทิลต่ำกว่าปกติ สำหรับการตรวจหามะเร็งในช่องปากชนิดสแควมัสเซลล์คาร์ซิโนมา (LINE-1 HYPOMETHYLATION LOCI FOR DETECTION ORAL SQUAMOUS CELL CARCINOMA) อ.ที่ปรึกษา: ผศ.ทพ.ดร. อาทิพันธุ์ พิมพ์ขาวขำ, อ.ที่ปรึกษาร่วม: ศ.นพ.ดร. อภิวัฒน์ มุทิรางกูร, รศ.ทพ.ดร. ประสิทธิ์ ภวสันต์, 72 หน้า.

การศึกษานี้มีวัตถุประสงค์เพื่อ (1) ศึกษาระดับเมทิลของไลน์-1ทั้งจีโนมในมะเร็งช่องปากชนิดสแควมัสเซลล์คาร์ซิโนมา (2) ศึกษาว่าสามารถตรวจพบระดับเมทิลที่ต่ำกว่าปกติของไลน์-1ในน้ำบ้วนปากของผู้เป็นมะเร็งช่องปากชนิดสแควมัสเซลล์คาร์ซิโนมาได้หรือไม่ (3) ศึกษาระดับเมทิลของไลน์-1ในแต่ละตำแหน่งของมะเร็งช่องปากชนิดสแควมัสเซลล์คาร์ซิโนมา โดยในการศึกษาระดับเมทิลของไลน์-1ทั้งจีโนมใช้วิธีcombined bisulfite restriction analysis of LINE-1s (COBRALINE-1) และศึกษาระดับเมทิลของไลน์-1แต่ละตำแหน่งด้วยCOBRA unique to LINE-1 (CU-L1) ผลการศึกษาพบว่ามะเร็งช่องปากชนิดสแควมัสเซลล์คาร์ซิโนมามีระดับเมทิลของไลน์-1ทั้งจีโนมต่ำกว่าเยื่อบุผิวปกติของช่องปาก และสามารถตรวจพบระดับเมทิลที่ต่ำกว่าปกติของไลน์-1 ในน้ำบ้วนปากของผู้เป็นมะเร็งช่องปากชนิดสแควมัสเซลล์คาร์ซิโนมาได้ ระดับเมทิลต่ำกว่าปกติของไลน์-1ไม่สัมพันธ์กับระดับขั้น(stage) ลักษณะทางจุลพยาธิ (histological grade) ตำแหน่งของมะเร็ง และประวัติการเคี้ยวหมาก สูบบุหรี่ และการดื่มอัลกอฮอล์ ส่วนระดับเมทิลของไลน์-1แต่ละตำแหน่งมีความแตกต่างกันและยังมีความแตกต่างกันในผู้ป่วยแต่ละรายด้วย แต่ไม่สัมพันธ์กับลักษณะทางคลินิกและจุลพยาธิ อย่างไรก็ตามผู้ป่วยแต่ละรายมีความผิดปกติของระดับเมทิลอย่างน้อย 1 ตำแหน่งจากจำนวนที่ศึกษาทั้งหมด 14 ตำแหน่ง โดยสรุป มะเร็งช่องปากชนิดสแควมัสเซลล์คาร์ซิโนมามีระดับเมทิลของไลน์-1ทั้งจีโนมต่ำกว่าปกติ และสามารถตรวจพบความผิดปกตินี้ในน้ำบ้วนปากของผู้ป่วยได้ด้วยวิธี COBRALINE-1 การเปลี่ยนแปลงระดับเมทิลของไลน์-1แต่ละตำแหน่งไม่เท่ากันและแตกต่างกันในผู้ป่วยแต่ละรายโดยไม่มีลักษณะจำเพาะ ความผิดปกตินี้ไม่ขึ้นกับลักษณะทางคลินิกและจุลพยาธิของมะเร็งช่องปากชนิดสแควมัสเซลล์คาร์ซิโนมา

สาขาวิชา ชีววิทยาช่องปาก
ปีการศึกษา 2551

ลายมือชื่อนิสิต เกศกัญญา สัพพะเลข
ลายมือชื่ออาจารย์ที่ปรึกษา
ลายมือชื่ออาจารย์ที่ปรึกษาร่วม
ลายมือชื่ออาจารย์ที่ปรึกษาร่วม

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KEY WORDS: LINE-1/ HYPOMETHYLATION/ ORAL SQUAMOUS CELL CARCINOMA/ ORAL RINSE/ COBRA

KESKANYA SUBBALEKHA: LINE-1 HYPOMETHYLATION LOCI FOR DETECTION ORAL SQUAMOUS CELL CARCINOMA. THESIS ADVISOR: ASST. PROF. ATIPHAN PIMKHAOKHAM, D.D.S., Ph.D., THESIS CO-ADVISORS: PROF. APIWAT MUTIRANGURA, M.D., Ph.D., ASSOC. PROF. PRASIT PAVASANT, D.D.S., Ph.D., 72 pp.

This study aimed to (i) investigate genome-wide LINE-1 methylation level of oral squamous cell carcinomas (OSCCs); (ii) clarify whether LINE-1 hypomethylation can be detected in oral rinses of OSCC patients (iii) study the pattern of methylation loss in specific LINE-1s. The combined bisulfite restriction analysis of LINE-1s (COBRALINE-1) and the modified COBRALINE-1 called COBRA unique to LINE-1 (CU-L1) were used to investigate genome-wide LINE-1s and specific LINE-1s, respectively. OSCCs revealed lower methylation levels of LINE-1s than normal oral epithelium and this hypomethylation could be detected in oral rinses of OSCC patients. There was no significant difference in the levels of genome-wide LINE-1 hypomethylation among tumors with different stages, histological grades, locations, history of betel chewing, smoking and/or alcohol consumption. Interestingly, methylation levels of each specific LINE-1 varied among loci. Each individual OSCC had different aberrant methylation levels in each specific LINE-1s. However, each individual OSCC had this aberration at least one of the 14 studied intronic LINE-1s. Moreover, there was still no significant difference in the specific LINE-1 methylation levels among clinico-pathological features of OSCCs. In conclusion, OSCCs possessed genome-wide LINE-1 hypomethylation and this alteration could be detected from oral rinses of OSCC patients by a simple technique, COBRALINE-1. The alteration of methylation levels in each specific LINE-1 was not equally distributed and had no specific character. The aberrant LINE-1 methylation was independent to clinico-pathological features of OSCCs.

Field of study: Oral Biology — Student's signature Keskanya Subbalekha

Academic year: 2008 — Advisor's signature P. Atiphan

Co-advisor's signature Apiwat Mutirangura

Co-advisor's signature Prasit Pavasant

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TABLE OF CONTENTS

TABLE OF CONTENTS (continued)

LIST OF FIGURES

LIST OF TABLES

LIST OF ABBREVIATIONS

COBRA	combined bisulfite restriction analysis
LINE-1	long intersperse nuclear element-1
COBRALINE-1	COBRA of genome-wide LINE-1
CU-L1	COBRA of unique LINE-1
OSCC	oral squamous cell carcinoma
HNSCC	head and neck squamous cell carcinoma
WBC	white blood cell
ANOVA	analysis of variance
DNA	deoxyribonucleic acid
CpG	dinucleotide containing cytosine and guanine, respectively

CHAPTER I
INTRODUCTION

Background and rationale

Oral cancer is an aggressive disease occurs in oral cavity and causes morbidity and mortality. The malignant cells may originate from any normal cells in the mouth, including epithelium, connective tissue, cartilage, bone, muscle, salivary gland, nerve, and vascular system. They can invade nearby structures and metastasis to distant organs. The treatments of the progressive stage diseases are still complicated and leave miserable disabilities. Many patients have poor quality of life due to difficulties in eating, chewing, swallowing, speaking and unaesthetic looking. Moreover, second primary tumors or recurrence were reported frequently. The 5-year survival rate is still low, approximately 50% in early stage and less than 20% in late stage. Early detection and diagnosis are very important ways for better results of the treatment and lesser the physical and mental disabilities. The etiology and pathological mechanisms of this disease are still unclear; however many evidences support the roles of environment and genes. Smoking, alcohol drinking and betel chewing are well-documented environmental factors causing higher risk to develop oral cancers. The genetic aberrations in oral cancers include the changes in genetic and epigenetic mechanisms controlling gene expression. Genetic alterations refer to the changes in nucleotide sequences, for example point and gross mutations, can cause malignant transformation. Epigenetic modification is a mechanism controlling gene expression without affecting the DNA sequences. In recent years, DNA methylation has become intensive investigation of epigenetics in cancers. Paradoxical alterations of DNA methylation, local hypermethylation of certain genes as well as global (genome-wide) hypomethylation, are reported in some cancers such as hepatocellular carcinoma and urothelial carcinoma. Increased methylation in promoter can inactivate tumor suppressor genes. However, the role of global hypomethylation, the decreased methylation levels of the entire genome, is less documented. The DNA hypomethylation may induce genomic instability and lead to malignant transformation.

Generally, methylation in genome locates in CpG dinucleotides, which are dispersed throughout the whole genome, in both noncoding repetitive sequences and genes (2-5). However, it seems that methylation in repetitive elements constitute the major part for the reason that repetitive elements comprise about 45% of the human genome (6, 7). LINE-1s (long intersperse nuclear element-1s) are the most abundant retrotransposons in the human genome (8) which are highly repetitive mobile DNA sequences distributed across the entire genome. Recent study demonstrated that LINE-1 hypomethylation related to genome hypomethylation (9-11). Hypomethylation of LINE-1s has been reported in many types of cancers including neuroendocrine tumors (12), carcinoma of the breast, lung, liver, esophagus, stomach, colon, urinary bladder prostate, and head and neck (8, 10, 13-19). Moreover, hypomethylation levels of LINE-1s can be used as a prognostic marker for epithelial ovarian cancers (20) cervical cancers (21) and hepatocellular carcinoma (22). Normal tissues from different organs possess different LINE-1 methylation levels and ranges (10). In addition, carcinogenic tissues have significantly lower levels of LINE-1 methylation than their normal tissue counterparts except in cancer of kidney, thyroid and lymph node (10). These data implied that methylation levels may be important for cellular function. Surprisingly, methylation levels of each LINE-1 in specific location varied in the same tissue type (23). Nevertheless, head and neck cancer cell lines revealed positive correlations of specific LINE-1s methylation levels with each other and with the genome-wide levels but differed from normal oral epithelium (23). Therefore, although global hypomethylation can generally deplete LINE-1 methylation levels, LINE-1 methylation in each location can be influenced differently. However, global methylation in normal oral tissues and malignancies is still to be clarified.

This study aims to investigate global methylation level in oral epithelium and evaluate global hypomethylation in oral squamous cell carcinomas (OSCCs). The combined bisulphite restriction analysis (COBRA) of genome-wide LINE-1s (COBRALINE-1) was used. Moreover, methylation levels of LINE-1s in some specific loci were also studied, using COBRA unique to LINE-1 (CU-L1) technique which the primers were designed for amplifying specific LINE-1s.

Research questions

1. Whether LINE-1 methylation levels in normal oral epithelium differ from those of normal blood leukocytes.

2. Do OSCCs possess genome-wide LINE-1 hypomethylation?

3. Whether the genome-wide LINE-1 hypomethylation correlates with clinico-pathological features of OSCCs.

4. Whether the aberrant methylation levels of LINE-1s can be detected in oral rinses of OSCC patients.

5. Whether the loss of methylation of LINE-1s in OSCCs is locus specific.

Objectives

1. To investigate LINE-1 methylation levels, both genome-wide and specific loci, in normal oral epithelium and OSCCs.

2. To clarify the relationship of global hypomethylation levels in various clinicopathological features of OSCCs.

3. To detect LINE-1 hypomethylation in oral rinses of OSCC patients.

Hypothesis

1. Normal oral epithelium possesses different levels of LINE-1 methylation from normal white blood cells.

2. OSCCs acquire genome-wide LINE-1 hypomethylation.

3. Genome-wide LINE-1 hypomethylation in OSCCs correlates with the clinico-pathological features.

4. Genome-wide LINE-1 hypomethylation can be detected in oral rinses of OSCC patients.

5. Loss of methylation of LINE-1s in OSCCs is locus specific.

Key words

oral squamous cell carcinomas (OSCCs), oral rinses, global hypomethylation, methylation, long intersperse nuclear element-1s (LINE-1s), combined bisulphite restriction analysis (COBRA)

Definition

Hypomethylation is methylation levels that lower than methylation levels found in specimens collected from normal individuals.

Hypermethylation is methylation levels that higher than methyaltion levels found in specimens collected from normal individuals.

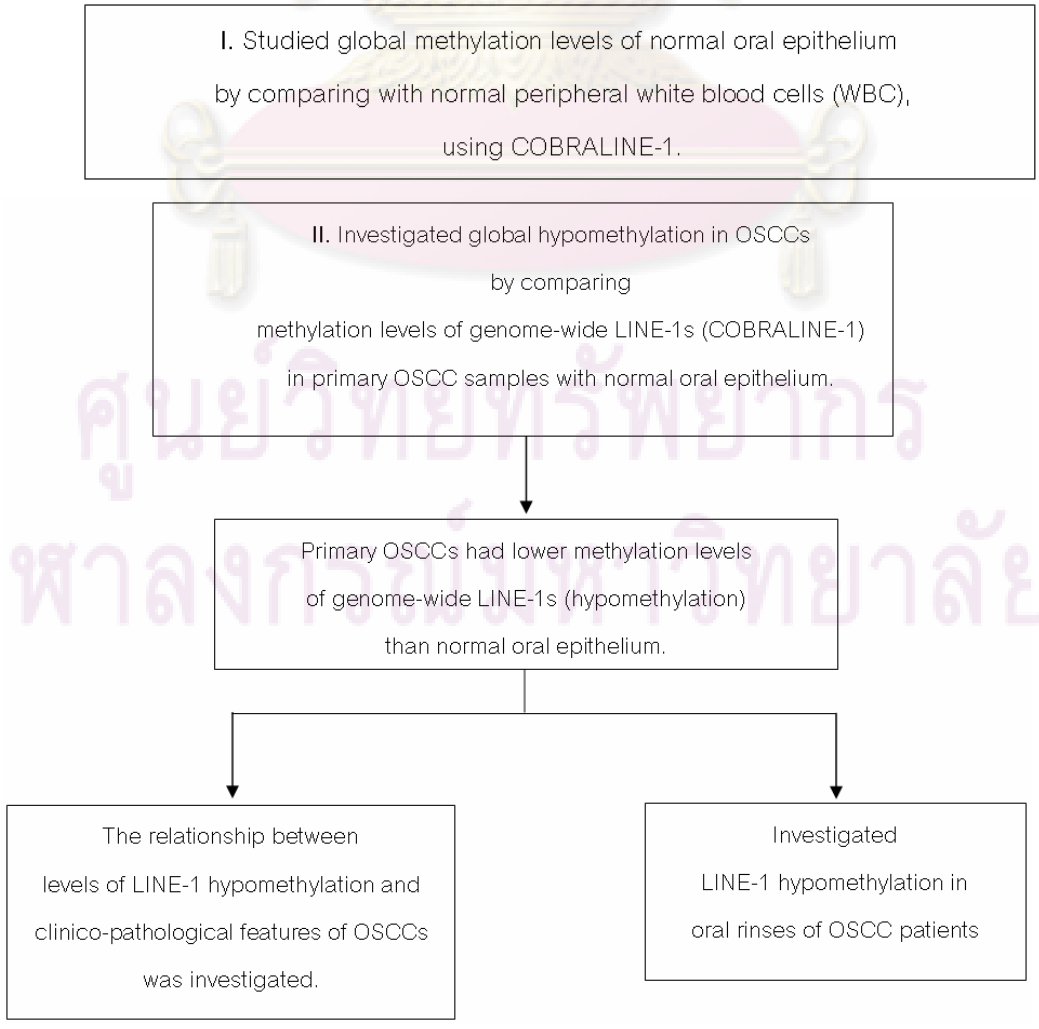
Expected benefit

1. If LINE-1 hypomethylation presents in OSCCs and/or relates to clinicopathological characters of OSCCs, it may be used as a biomarker for OSCCs.

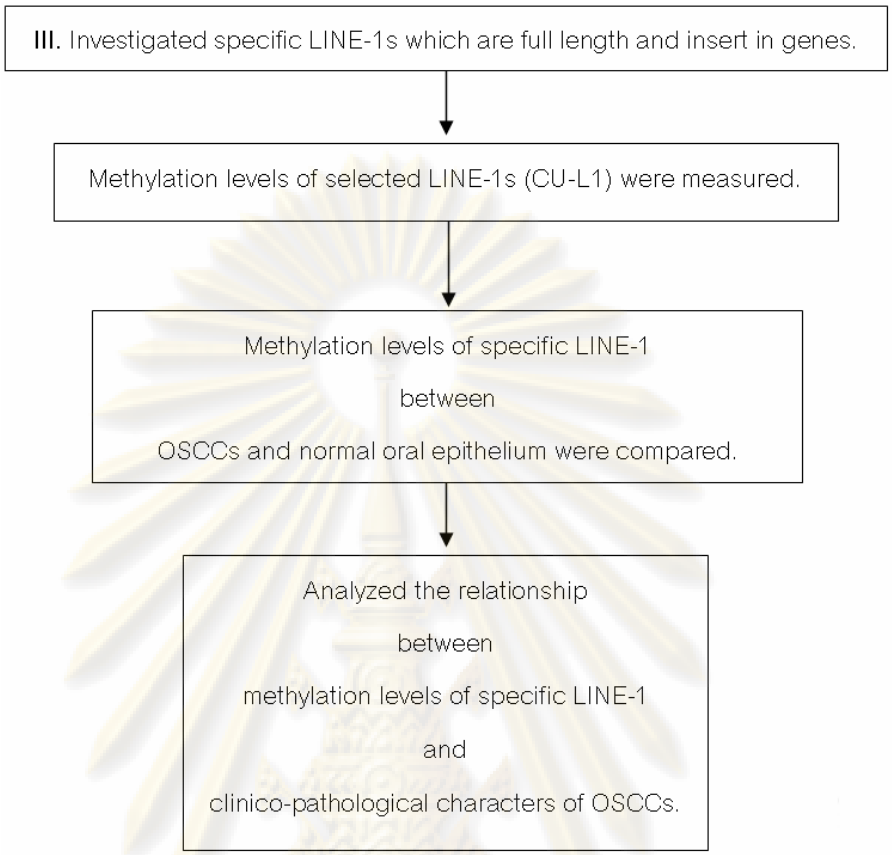
2. If the aberrant methylation levels of LINE-1s can be detected in oral rinses of OSCC patients, a sensitive, non invasive technique for detection of OSCCs can be developed.

3. The better understanding of the molecular pothogenesis of OSCCs may leads to earlier and more accurate diagnosis, effective treatment and prevention of OSCCs.

Research methodology framework



Research methodology framework (continued)



CHAPTER II
REVIEWS AND RELATED LITERATURES

Oral cancers

Introduction

Oral cancers, according to the International Classification of Diseases, ninth revision (ICD9), refer to malignant neoplasm that develop at the lip (ICD9 140), tongue (ICD9 141), gum (ICD9 143), floor of mouth (ICD9 144), cheek mucosa, vestibule of mouth, palate, uvula, retromolar area (ICD9 145) and oropharynx (ICD9 146). Oral cancers can be classified by cellular origins. Carcinoma is a cancer originating from epithelium, while sarcoma has mesenchymal origin, including muscle, connective tissue, neurovascular system, cartilage or bone. The most common cancer in the oral cavity is squamous cell carcinoma (24). Oral squamous cell carcinoma (OSCC) is an aggressive disease; it can invade nearby structures, metastases to distant organs and cause lethality. It also has highly recurrent rate and second primary lesion can be frequently detected.

Epidemiology

Commonly, OSCCs occur in individuals older than 40 years of age, but currently, the incidence in people younger than 40 years of age is increasing (25). In 2002, cancers of the oral cavity were found to be the 9th ranking among global cancers by site. They occurred in about 274 000 patients, not only in developing countries but also in the developed ones (26). The incidence of oral cancers has demographic variation. While 40,000 new cases are record in the European Union (27) it accounts 2-4% of all cancers diagnosed and approximately 30,000 cases occur annually in the United States (28). In Thailand, it ranges from 3.1 to 8.4 per 100,000. Generally, males had higher incidence than females, except in Khon Kaen Registry (Table 2.1) (29). Despite arrival of molecular biology leading to effective treatment in many types of cancers, the treatment results of oral cancers are still not satisfactory. The standard treatment, surgery, not only causes facial disfiguration and difficulty in eating and speaking, but also achieve a low five-year survival rate, which improved by only 5%

(from 54% in 1974-1976 to 59% in 1995-2000) (28). Although OSCCs are easily seen, most patients were detected in advanced stages which results in poor survival rates (30). Early detection and diagnosis is important and results in better outcome of treatment (31).

Table 2.1 Incidence of oral cancers in Thailand (29).

Registry	Incidence (per 100,000)	
	Female	Male
Bangkok	3.1	4.4
Chiang Mai	5.3	7.3
Khon Kaen	7.3	3.6
Songkhla	4.5	8.4

Etiology

The development of OSCCs is still unclear; however it is a multifactorial process influenced by environmental effects as well as patient's genetic predisposition (32). Carcinogenesis environments, including viral infection such as Epstein-Barr virus (EBV), human papillomavirus (HPV) (33, 34); chemical agents such as paint fumes, plastic by products, wood dust, asbestos, gasoline fumes, methyl isocyanate (35) and formaldehyde (36) have been considered as possible risk factors. Chronic inflammations such as irritation from poorly fitting dentures and poor oral hygiene also have been implicated (37). Smoking, the use of tobacco products, excessive alcohol consumption and betel chewing have been well-documented as major risk factors in OSCCs (29, 34, 37, 38). Numerous genetic events that alter normal function of genes are also discovered in OSCCs. These genetic modifications include both genetic and epigenetic mechanisms. Genetic mechanism influences gene expression by sequence of the nucleotides. The alteration of DNA sequence; known as mutation in proto-oncogenes or tumor suppressor genes results in malignant transformation, uncontrollable proliferation and invasion of tumor cells (39-41). Epigenetic mechanism controls gene activity in the absence of DNA sequence change. Over the recent years, DNA methylation is one of the extensive studied epigenetic alterations in cancers.

Paradoxically aberrant methylation in cancers, promoter hypermethylation and global hypomethylation occurred in the same cancer. In OSCCs, hypermethylation of tumor suppressor genes such as *p16*, O6-methylguanine-DNA methyltransferase (*MGMT*) (42) (43), E-cadherin (44), *p14* (45), adenomatous polyposis coli (*APC*) (46) has been reported. However, status of global hypomethylation is still to be clarified.

Clinicopathological features

The prognosis for patients with OSCC depends on both histological subtype (grade) and clinical extent (stage) of tumor. The grading of a tumor is the microscopic determination of the differentiation of the tumor cells. Three histological features of OSCC cells are well-, moderately-, and poorly-differentiation. Well-differentiated lesions generally have a less aggressive biologic course and better prognosis than poorly differentiated lesions. The clinical staging of OSCCs is known as the TNM system (table 2.2). T is a measure of the primary tumor size, N is an estimation of the regional lymph node metastasis, and M is a determination of distant metastases. As the clinical stage advances from I to IV, prognosis worsens (47).

Table 2.2 TNM staging system for OSCC (47)

Stage I	Stage II	Stage III	Stage IV
T1N0M0	T2N0M0	T3N0M0	All N2
		T1N1M0	All N3
		T2N1M0	All T4
		T3N1M0	All M1

Description and abbreviations

T-Tumor T1-tumor less than 2 cm in diameter

T2-tumor 2-4 cm in diameter

T3-tumor greater than 4 cm in diameter

T4-tumor invades adjacent structures

N-Node N0-no palpable nodes

N1-ipsilateral palpable nodes

N2-contralateral or bilateral nodes

N3-fixed palpable nodes

M-Metastasis M0-no distant metastasis

M1-clinical or radiographic evidence of metastasis

Detection and diagnosis

Unfortunately, most of OSCCs were found in advanced stages even if they located in easily seen area, which result in high morbidity and mortality rate. The 5-year survival rate is still low, but it may be improved if the patient was early detected and underwent treatment before the spreading of cancer to the lymph node. The gold standard of diagnosis is histological study of scalpel-biopsied tissue which requires expertise. Nevertheless, oral examination by well-trained staff can help screening intra-oral pathology. In recent years, many technique were developed, such as methylene blue (48), tolonium chloride (49) or toluidine blue staining (50-52); chemiluminescence (53); exfoliative cytology using brush biopsy (54-56) or oral scraping (57); studies of salivary biochemistry (58, 59); and also molecular biology including tumor markers (60-

63). But none can replace surgical biopsy, only adjunctive screening tools. Therefore, it is important to further explore and to improve non-invasive methods for reliable early detection oral malignancies.

Treatment

Goals of treatment consist of removal of cancer load, maintenance of quality of life, and prevention of secondary cancer. Classically, treatment involves surgical removal the primary tumor and the metastasized lymph nodes, while non-metastasized lymph nodes are frequently removed to prevent tumor spreading (64, 65). Pre-operative and/or post-operative radiotherapy combine with surgery can improve success rate of treatment (66). Chemotherapy may be used as adjuvant therapy in advanced cases. However, the outcomes of treatment are still unsatisfied due to the high morbidity and mortality rates. The patients always have poor quality of life after tumor ablation and suffer from difficulties in chewing, swallowing, speaking and facial disfigurement (67). The radiotherapy results in rampant dental caries, jaw stiffness, xerostomia, poor intra-oral wound healing and osteoradionecrosis of the jaw bones (68-70). Though the innovations in cancer treatment modalities including immunotherapy and gene therapy have progressed and been able to improve treatment outcomes in many kinds of cancers, but in oral cancers, this modality is still a trial. Nowadays, researchers are trying hard to study molecular biology of cancers in order to better understand the pathological mechanisms which may lead to treatment success. Unfortunately, the molecular biology of oral cancers is still a mystery. However, the global alteration may give more information for oral cancer pathological processes.

Epigenetics in cancers

Generally, expression of genes is controlled by genetic and/or epigenetic mechanisms. Genetic mechanism refers to sequence of the nucleotides, including adenine (A), thymine (T), cytosine (C), and guanine (G). Epigenetics refers to heritable phenotypic alterations in the absence of DNA sequence changes. DNA methylation is one of the most commonly occurring epigenetic events taking place in the mammalian genome (71). Aberrant DNA methylation, including promoter hypermethylation of tumor

suppressor genes and paradoxically, genome-wide (global) hypomethylation has been reported in many kinds of cancers including head and neck cancers (17).

DNA methylation in cancers

DNA methylation is an evolutionally conserved mechanism to regulate gene expression in mammals. In vertebrates, addition of methyl group at 5-carbon position of cytosine (72) usually occurs at the 5'cytosine in CpG dinucleotides (73, 74) (Figure 2.1). It has shown to be associated with transcriptional silencing of the genes in normal development (75). The DNA methylation is maintained by heritability after DNA replication (76, 77). Distinct DNA methylation patterns are developmentally and tissue specific, both in overall 5-methylcytosine content and in the sites at specific genes (78-81).

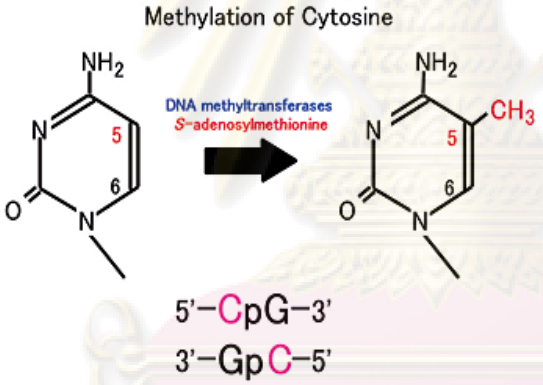


Figure 2.1 Methyl cytosine. Addition of methyl group at 5-carbon position of cytosine usually occurs at the 5'cytosine in CpG dinucleotides (82).

Cytosine methylation has a number of functions, including X chromosome inactivation, genomic imprinting, immobilization of mammalian transposons, suppression of transcriptional noise and maintaining genomic stability (83-87). Methylation may inactivate one or both alleles of the tumor suppressor genes in sporadic cancers and can potentially act as a second hit during the development of hereditary cancers (Figure 2.2) (83, 88). Methylation that occurs within gene deficient regions, such as in pericentromeric heterochromatin, appears crucial for maintaining the conformation and integrity of the chromosome (4, 5). Methylation has also been proposed as a genome defence against surreptitious mobile genetic elements (89).

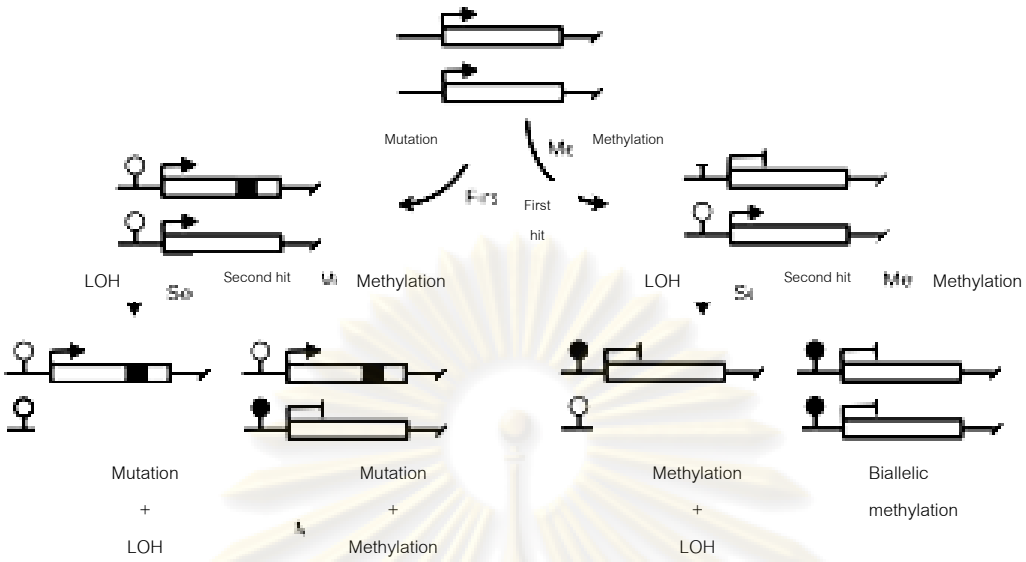


Figure 2.2 Diagram demonstrated methylation as a second hit. Epigenetic and genetic mechanisms may act in combination to cause inactivation of gene during the development of hereditary cancers (83).

Many studies demonstrated that epigenetic mutations involving an imbalance in cytosine methylation are detected in cancers. It is well established that two kinds of changes in the DNA methylation pattern occur in many cancers, regional hypermethylation of specific genes and global hypomethylation. These imbalances can present together in a single tumor, though the net effect is usually a decrease in total methylation levels (89-91). This paradoxical coexistence of a global decrease in methylation with regional hypermethylation implies that independent and different processes are responsible for hypomethylation and hypermethylation. If these defects precede malignancy, indicating that they are not simply a consequence of the malignant state. In case of methylation imbalance contributes directly to tumor initiation, the alteration should occur in early stages of cancer or in premalignant cells. If it contributes directly to tumor progression, methylation defects should increase in frequency and/or severity coordinately with increasing malignancy grades.

While hypermethylation inactivates tumor suppressor genes, global losses of methylation in cancer may lead to the alterations in the expression of proto-oncogenes critical to carcinogenesis (75, 92). It may also facilitate chromosomal instability (4, 5, 93-

97) and may activate the latent retrotransposons (16, 98-101). The extent of genome wide hypomethylation in tumors parallels closely to the degree of malignancy, though it is tumor type dependent. In breast, ovarian, cervical, brain and prostate tumors, for example, hypomethylation increases progressively with increasing malignancy grade (90, 102-105). Thus hypomethylation may serve as a biological marker with prognostic value. The human genome is not methylated uniformly and contains regions of unmethylated segments interspersed by methylated regions (71). Genome-wide hypomethylation has been demonstrated by downregulation of methylated CpG dinucleotides, which disperse throughout the whole genomes both in noncoding repetitive sequences and genes. However, hypomethylation of the repetitive sequences, such as LINE seems to constitute the major part of the global hypomethylation of the cancer genome (11, 106).

LINE-1 retrotransposons

Mammalian transposable elements compose of DNA transposons and retrotransposons (Figure 2.3). DNA transposons encode a transposase activity and generally move through DNA intermediate by a cut and paste mechanism utilizing the transposase. Although roughly 3% of the human genome is composed of DNA transposons, they are remnants or fossils of ancient elements and it is unlikely that any remain transpositionally active. Retrotransposons encode a reverse transcriptase activity and move by a copy and paste process involving RNA intermediate thus the original retrotransposon is maintained *in situ* where it is transcribed. The transcript is then reverse transcribed and integrated into a new genomic location. Approximately 42% of the human genome composes of retrotransposons and although most of these elements are inactive, some retain the ability of retrotransposition. Retrotransposable elements can be classified as autonomous retrotransposons when they encode certain proteins necessary for their mobility and nonautonomous retrotransposons such as Alu, processed pseudogenes and SVA elements which do not encode any protein. There are two classes of autonomous retrotransposons, LTR (long terminal repeat) and non-LTR retrotransposons. LINEs (long interspersed nucleotide elements) are non-LTR

retrotransposons and comprise 21% of the human genome. There are inactive LINE elements such as LINE-2 and active LINE elements, such as LINE-1 (107).

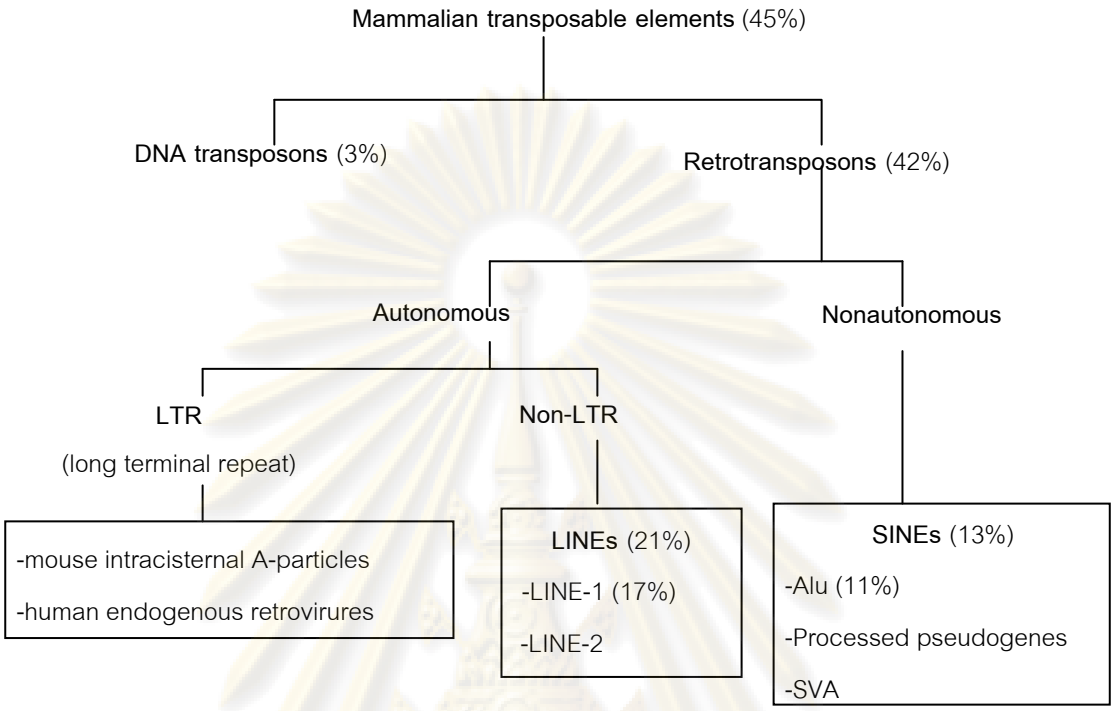


Figure 2.3 Mammalian transposable elements. Transposable elements comprise about 45% of human genome and compose of transposons and retrotransposons. LINE-1 is a kind of autonomus, non-LTR retrotransposons and comprise about 17% of human genome (107).

LINE-1 retrotransposons, the most abundant sequences in human genomes are self-replicating human transposable elements. Over evolutionary time, they have not only expanded greatly in number but also have other roles. Some of which are quite useful to the organisms whereas others are detrimental to individual members of the species. They are estimated 600,000 copies and comprise of at least 17% of the human genomes. Some of these elements are within genes (107). Over 75% of human genes contain at least one LINE-1 insertion, usually as part of introns, 5′UTR sequences or 3′UTR sequences (108). Most LINE-1 elements are retrotransposition defective because they are 5′ truncated; contain internal rearrangement and harbor mutations within their open reading frames (6). Full-length LINE-1s are about 2,000 copies, but only 30-60

copies may be competent for transposition (89, 109). When the full length, non-mutated LINE-1 is transcribed and then reverse transcribed, it might integrate in and disrupt important gene functions (8, 108). Germline mutations where LINE-1 retrotranspositions impair the functional gene are known in several hereditary disorders, including the factor VIII in hemophilia A, the dystrophin gene in Duchenne muscular dystrophy, the fukutin gene in Fukuyama-type congenital muscular dystrophy, the cytochorme b_{558} heavy chain gene in X-linked chronic granulomatous disease, and the type IV collagen genes in Alport syndrome (110). DNA methylation at the CpG site in LINE-1 promoter is the normal mechanism for silencing of its potentially harmful retrotransposing activity in the mammalian genome (111, 112). In addition, hypomethylation of LINE-1 promoter can cause genome instability by inactivating the tumor suppressor genes such as *APC* in colon cancer or by activating the oncogenes such as *c-MYC* in breast cancer (113, 114) And there is also evidenced that hypomethylation of LINE-1 can cause chromosome instability (115, 116). Hypomethylation of LINE-1s has been reported in several malignancies, including neuroendocrine tumors (12), carcinoma of the breast, lung, liver, esophagus, stomach, colon, urinary bladder prostate, and head and neck (8, 10, 13-19). Moreover, hypomethylation levels of LINE-1s can be used as a prognostic marker for epithelial ovarian cancers (20), cervical cancers (21) and hepatocellular carcinoma (22). Full length LINE-1 is 6 kb and contains a 5′ untranslated region (5′UTR), a 1 kb ORF1 that encodes a nucleic acid binding protein, a 4 kb ORF2 which encodes a protein with endonuclease and reverse transcriptase activities, allowing their mobilization in genomes through an RNA intermediate, a 3′ untranslated region (3′UTR), a poly(A) tail (Figure 2.4). Within LINE-1 5′UTR, they contain not only a sense strand promoter for their own transcription, but also an antisense promoter (ASP) (117). This ASP has been shown to provide an alternative transcription start site for a number of human genes including *c-MET*, a receptor tyrosine kinase whose activation can lead to transformation and tumorigenicity in a variety of tumors (118-120). Since LINE-1 elements are constituted most of the human genome and distributed across the entire genome, LINE-1 sequences are well suited to study changes in genome methylation.

Figure 2.4 Diagram illustrated full-length LINE-1 element. Full length LINE-1 is 6 kb and contains a 5′ untranslated region (5′UTR), a 1 kb ORF1 that encodes a nucleic acid binding protein, a 4 kb ORF2 which encodes a protein with endonuclease (EN) and reverse transcriptase activities (RT), C represents a conserved cytosine-rich motif, a 3′ untranslated region (3′UTR), and a poly(A) tail. LINE-1 elements are often flanked by 7-20 bp target site duplications (TSD) (107)

In previous study demonstrated that COBRALINE-1 could efficiently evaluate the genome-wide methylation status of LINE-1s in genomic DNA and it represents the whole genome methylation status (10, 121). In addition, the methylation levels of genome-wide LINE-1s varied among types of normal tissues and also had variation in ranges but did not depend on age and gender (10). Some tissues such as thyroid and esophagus demonstrated wider ranges of the methylation levels than others. Moreover, most of cancer tissues including head and neck cancers have hypomethylation of LINE-1s, comparing with their normal tissue counterparts except cancers of kidney, thyroid and lymph mode (Figure 2.5). This evidence supports that LINE-1 methylation level is specific to tissue types and the hypomethylation of LINE-1s is also specific to types of cancers. However, the information of LINE-1 methylation in oral cancers; mostly are OSCCs, the malignant tumor of oral epithelial origin, has still not well established. In order to study methylation levels of LINE-1s in OSCCs, the same type of tissues should be compared. Therefore, normal oral epithelium should be used.

Interestingly, although sequences of every LINE-1s are homologous, methylation levels of individual LINE-1at each locus are different. The study of 17 selected full-length intronic LINE-1s (using CU-L1 technique) revealed different methylation levels among specific LINE-1s in each normal individual and also different between normal oral epithelium and normal WBC. However, HNSCCs occupied lower methylation levels than normal oral epithelium almost all studied loci, except LINE-1 at SPOCK3 locus (Figure 2.6) (23). Therefore it was interesting to clarify the methylation character of specific LINE-1s in OSCCs in an attempt to seek for molecular markers.

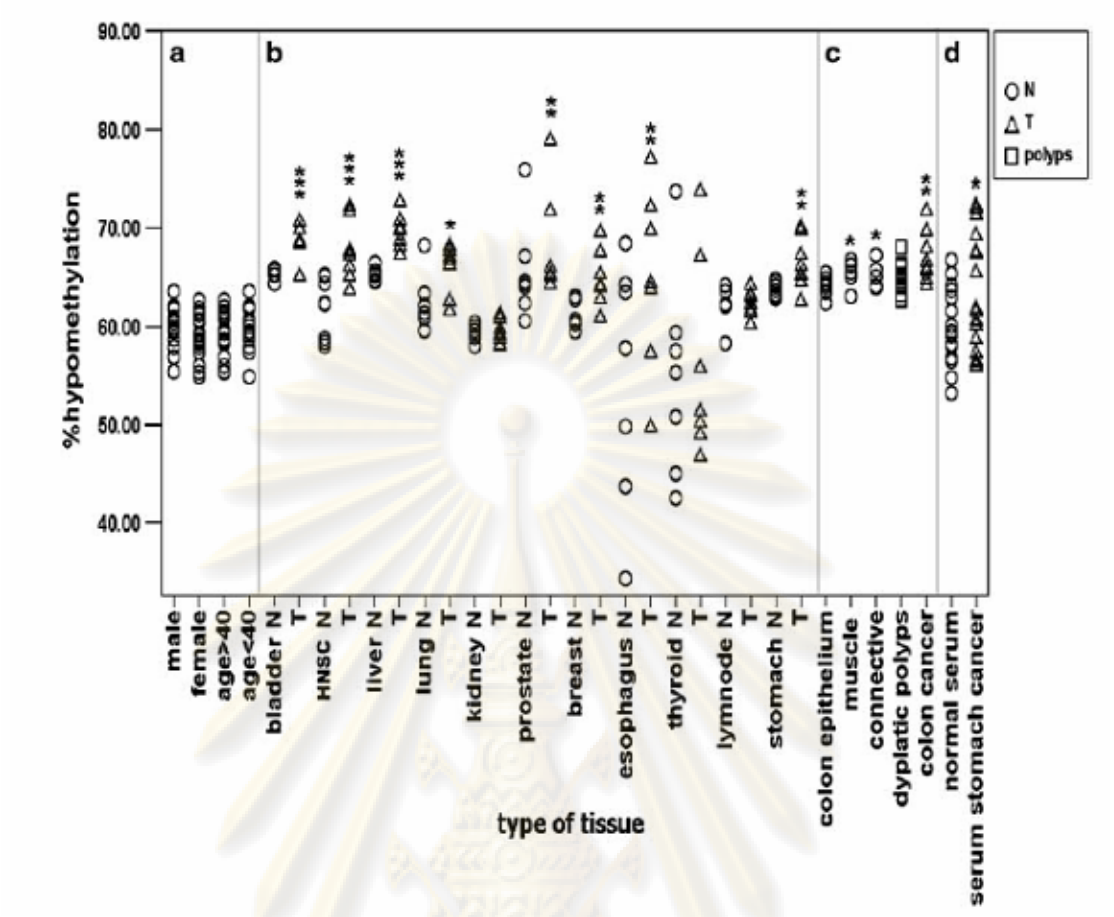


Figure 2.5 LINE-1 hypomethylation levels in several tissue types (10). Circles, triangles, and squares are levels of COBRALINE-1 from normal, malignant, and premalignant tissues, respectively. The vertical axis displays percentage levels of LINE-1 hypomethylation. Sample types are labeled. (a–d) are the hypomethylation levels of leukocytes, cancers, microdissected colonic tissues, and sera, respectively. Single, double, and triple asterisks indicate significant differences in hypomethylation levels between normal tissues and the tested samples at $P<0.05$, <0.01, and <0.001, respectively. HNSC stands for head and neck squamous cell. N and T are normal and malignant tissues, respectively.

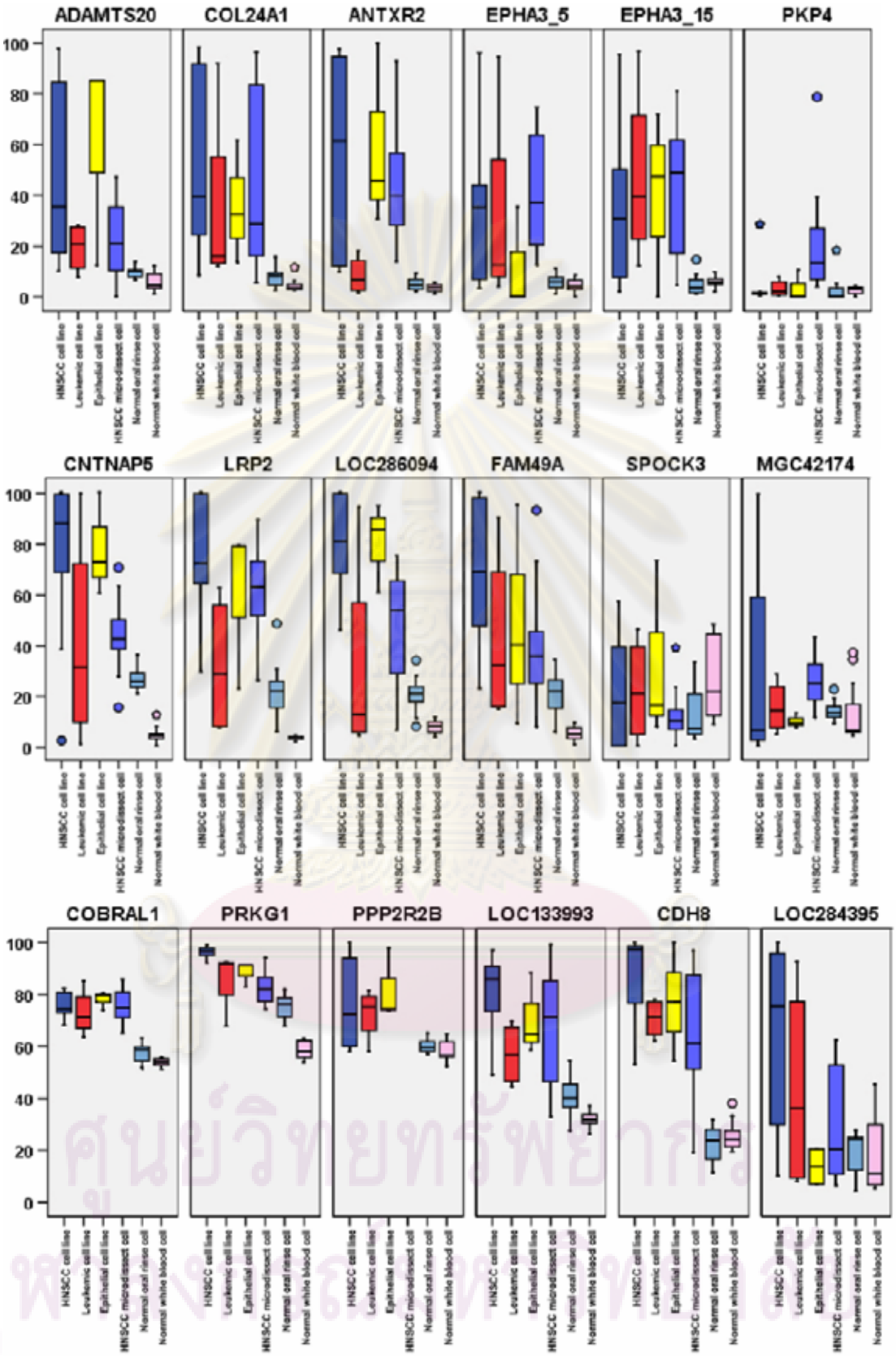


Figure 2.6 (continue to next page for Figure legend)

Figure 2.6 Mean of hypomethylation compared among each cell type within all 17 specific LINE-1s (CU-L1) and COBRALINE-1 (COBRAL1). Vertical axis represents levels of hypomethylation. Each bar represents hypomethylation levels of each cell type, including HNSCC cell lines, leukemic cell lines, epithelial cell lines, HNSCC microdissected cells, normal oral rinse cells and normal white blood cells. CU-L1 methylation varied in levels and ranges among loci and among tissue types. COBRALINE-1 had narrower range of methylation levels than CU-L1. Interestingly all studied malignancies have genome-wide hypomethylation than both kinds of normal cells (23).

CHAPTER III
MATERIALS AND METHODS

Samples

Normal oral epithelium

Because of the availability of shed oral epithelium, the normal oral epithelial cells can be collected from oral rinses of healthy volunteers who have no intra-oral lesions (55, 122, 123). Twenty milliliters of sterile 0.9% sodium chloride solution were rinsed and gargled for 15 seconds then spitted into a sterile 50-ml sterile closed container and kept at 4°C until processed to collect DNA, within 1 hour.

Primary OSCC tissues

Specimens from patients diagnosed and histological confirmed to be
OSCC were collected at the time patients receiving surgical excision. The specimens were kept in sterile phosphate buffered saline (PBS) at -30°C until processed to collect DNA.

OSCC oral rinses

Twenty milliliters of sterile 0.9% sodium chloride solution were rinsed and gargled for 15 seconds by patients presented OSCC lesions. Then the rinsed solution was spitted into a sterile 50-ml sterile closed container and kept at 4°C until processed to collect DNA, within 1 hour.

Genomic DNA extraction

Cells in oral rinses (from normal individuals or OSCC patients) were pelleted by centrifuging at 2500g for 15 minutes at 4°C. The supernatant was discarded and cell pellets were washed twice in sterile PBS. Then the cell pallets were placed in 1% SDS/proteinase K 0.5 mg/ml DNA extraction buffer and incubated at 50°C overnight. OSCC tissues were thawed on ice. After the thawed tissues were washed twice in sterile PBS, they were chopped into small pieces and placed in 1% SDS/proteinase K 0.5 mg/ml DNA extraction buffer, incubated at 50°C overnight. The digested cell pellets or

tissues and fluids were then subjected to phenol-chloroform extraction and ethanol precipitation. The precipitated DNA was resuspended in Tris-EDTA treated water.

COBRA

This quantitative technique is used to determine methylation level in small amounts of DNA. COBRA consists of a standard sodium bisulphite treatment followed by polymerase chain reaction (PCR), then restriction digestion and quantitation.

Sodium bisulphite treatment

Principle

Bisulphite deaminates unmethylated cytosines and converts them to uracils, but leaving methylated cytosines unchanged (Figure 3.1, 3.2). After bisulphite treatment, the methylated sequence can be differentiated from unmethylated sequence by further analysis, such as sequencing, methylation specific PCR, restriction enzyme analysis.

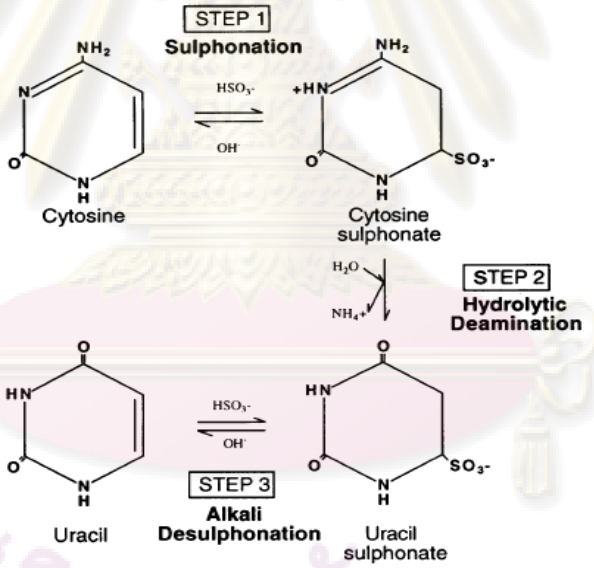


Figure 3.1 Schematic diagram of the bisulphite conversion reaction.

The deamination of cytosine by sodium bisulphite involves the following steps: (step 1) addition of bisulphite to the 5-6 double bond of cytosine, (step 2) hydrolytic deamination of the resulting cytosine-bisulphite derivative to give a uracil-bisulphite derivative, and (step 3) removal of the sulphonate group by a subsequent alkaline treatment, to give uracil (124).

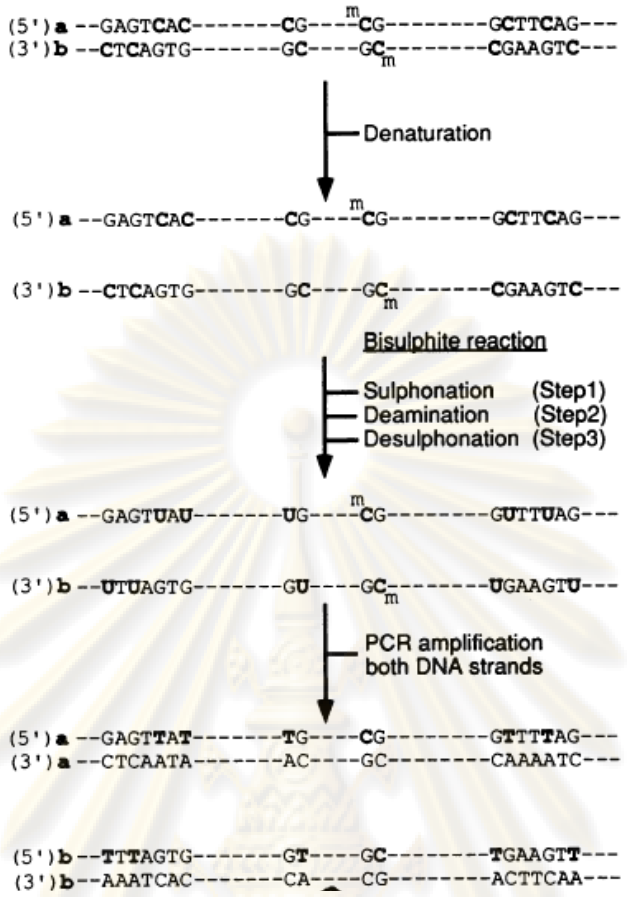


Figure 3.2 Complementary DNA strand after bisulfite reaction. After the bisulphite reaction, the two DNA strands are no longer complementary and therefore can be amplified independently. The two complementary strands in the original DNA are labeled as (a) and (b). Cytosine residues and their corresponding uracil and thymine conversion products are shown in bold type (124).

Technique

Genomic DNA 2 µg in 50 µl water was denatured in 0.2 M NaOH at 37°C for 10 minutes, and then incubated with 30 µl of 10 mM hydroquinone and 520 µl of 3 M sodium bisulphite at 50°C, 16-20 hours. After that, bisulphite-treated DNA was desalted with DNA Clean-Up system (Promega, Madison, WI). Subsequently, it was desulfonated by 0.3 M NaOH and precipitated with ethanol. Finally the DNA was resuspended in 20 µl of water (125).

PCR and specific restriction enzyme digestion

Principle

After DNA is treated with sodium bisulphite, PCR is performed to amplify LINE-1 sequences. In this step the bisulphite converted uracils in DNA sequence will be amplified as thymines, whereas unconverted cytosines will be amplify as cytosines. Thus after PCR, the DNA sequences which contain unmethylated cytosines will be changed from their original sequences; while the ones that contain methylated cytosines will retain their original sequences. Then the PCR products will be digested with restriction enzyme that is specific to methylated or unmethylated sequence (Figure 3.3).

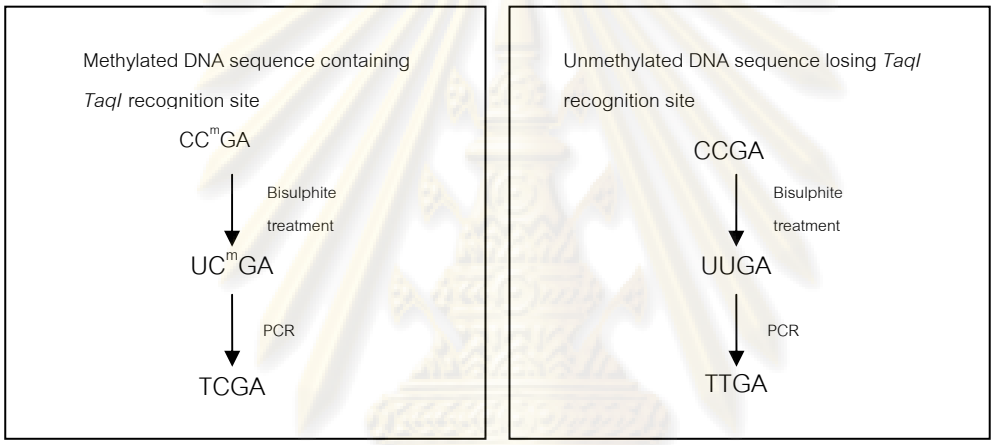


Figure 3.3 Example of restriction site of *Taq*I enzyme. This enzyme recognizes TCGA sequence. After PCR amplifying sodium bisulphite treated DNA; the methylated DNA retains the cutting site whereas unmethylated DNA loses the cutting site.

Technique

The 5′UTR of LINE-1.2 from NCBI Accession number M80343 was used for COBRALINE-1 (10) and specific LINE-1s were selected by blat using L1.2 sequence to http://genome.ucsc.edu. Full length intronic LINE-1s with the representative COBRALINE-1 CpG dinucleotides were selected; from these criteria, 17 specific LINE-1s were selected (22). All selected LINE-1 were listed in table 3.1. Bisulphite-treated DNA 0.2 µg were subjected to 35 cycles of PCR with a couple of primers, as listed in table 3.2 (23). These DNA were denatured at 95 °C, 1 minute, annealed at of 53 °C, 1

minute and extended at 72 °C, 1 minute. The PCR amplicon sizes were 160 bp and approximately 300-500 bp for COBRALINE-1 and CU-L1, respectively. Then the PCR amplicons were digested in 10 µl reaction volume with 2 U of *Taq*I and 8 U of *Tas*I in 1X *Taq*I buffer (MBI fermentas, Flamborough, Ontario, Canada) at 65°C overnight, then electrophoresed in 8% nondenaturing polyacrylamide gel and stained with SYBR$^{\bar{I}}$ green I nucleic acid gel stain (Sigma-Aldrich, St. Louis, Missouri).

*Taq*I restriction enzyme recognize TCGA sequence which C was protected from bisulphite conversion by methylation, while *Tas*I restriction enzyme recognize AATT sequence which the last T was unmethylated CpG before treated with bisulphite. For COBRALINE-1, the methylated amplicons can be digested by *Taq*I and yielded two 80 bp fragments, whereas unmethylated amplicons can be digested by *Tas*I and yielded 62 bp and 98 bp fragments (Figure 3.4, 3.5). CU-L1 amplicons contain both representative CpG dinucleotides and usually have additional *Taq*I site(s) and *Tas*I site(s). Therefore, there are more methylated, unmethylated and also control bands which has no candidate CpG restriction sequence (Figure 3.6 and Table 3.2). However, methylation levels of each methylated or unmethylated bands have linear correlations in the same sample (23).

Table 3.1 General characteristics of the selected specific LINE-1s (23).

Gene	Gene location	LINE-1 location	LINE-1 orientation
COL24A1	1p22.3	intron 24	Antisense
FAM49A	2p24.3-2p24.2	intron 2	Sense
CNTNAP5	2q14.3	intron 11	Antisense
PKP4	2q24.1	intron 1	Sense
LRP2	2q31.1	intron 19	Antisense
MGC42174	2q37.1	intron 8	Antisense
EPHA3	3p11.1	intron 5	Antisense
EPHA3	3p11.1	intron 15	Antisense
ANTXR2	4q21.21	intron 16	Antisense
SPOCK3	4q32.3	intron 7	Antisense
LOC133993	5q12.3	intron 3	Antisense
PPP2R2B	5q32	intron 8	Antisense
LOC286094	8q24.22	intron 1	Sense
PRKG1	10q21.1	intron 9	Sense
ADAMTS20	12q12	intron 7	Antisense
CDH8	16q21	intron 7	Antisense
LOC284395	19q12	intron 1	Antisense

Table 3.2 Oligonucleotide sequences and amplicon sizes for CU-L1 and COBRALINE1 (23)

Gene	COBRA unique and LINE-1 sequence oligoes	Size(bp)	Methylated bands (bp)	Unmethylated bands (bp)
COL24A1	GTTAAAGGGTTAAGAATGTGTGTAG GTAAAACCCTCCGAACCAAATATAAA	336	47,151,60,54,80	294,98
FAM49A	GTTTTAAAAAAAAATAAAGTTGG GTAAAACCCTCCGAACCAAATATAAA	385	41,151,113,80	287,98
CNTNAP5	GATTAAATTTTAATTGAATTAGAG GTAAAACCCTCCGAACCAAATATAAA	403	43,151,60,53,80	289,98
PKP4	GGTATGATTTTAAAAAAAGAGAT GTAAAACCCTCCGAACCAAATATAAA	392	48,211,53,80	294,98
LRP2	GGTATATAATTTTTATGGTGTTG GTAAAACCCTCCGAACCAAATATAAA	435	44,150,60,53,80	289,98
MGC42174	ATTGAGGTGTATTAAGAGATGGA GTAAAACCCTCCGAACCAAATATAAA	553	181,60,53,80	276, 98
EPHA3-IVS5	TGTTATTGGAATATATGGAGATT GTAAAACCCTCCGAACCAAATATAAA	386	42,151,60,53,80	288,98
EPHA3-IVS15	TAAGGATAAAAATTTTTGAAGTT GTAAAACCCTCCGAACCAAATATAAA	464	60,150,60,53,80	305,98
ANTXR2	TATTGAGTATTAATTATGTATTTAGTAT GTAAAACCCTCCGAACCAAATATAAA	416	28,150,60,53,80	273,98
SPOCK3	GTGTAATTTTTTTAGATTTTGTAG GTAAAACCCTCCGAACCAAATATAAA	492	300,60,36,17,80	262,98
LOC133993	TTAGGATATTTTTTATTTTGGGA GTAAAACCCTCCGAACCAAATATAAA	446	101,264,80	347,98

Table 3.2 Oligonucleotide sequences and amplicon sizes for CU-L1 and COBRALINE1 (continued) (23).

Gene	COBRA unique and LINE-1 sequence oligoes	Size(bp)	Methylated bands (bp)	Unmethylated bands (bp)
PPP2R2B	GGGGAAAAAATTGAAAGTT	590	8,24,151,60,53,80	270,98
	GTAAAACCCTCCGAACCAAATATAAA			
LOC286094	TATGTAAGTATGGAAATTTGAGG	429	43,151,60,53,80	290,98
	GTAAAACCCTCCGAACCAAATATAAA			
PRKG1	AAAATTTTTAGTTGTTAAATGG	374	152,60,53,80	247,98
	GTAAAACCCTCCGAACCAAATATAAA			
ADAMTS20	AAGTTGTGTGGTTTTTTGTAAAT	468	81,151,60,36,17,80	328,98
	GTAAAACCCTCCGAACCAAATATAAA			
CDH8	GGATTTGGGAGTTGGATAGTTAG	405	21,211,53,38	276,56
	GTAAAACCCTCCGAACCAAATATAAA			
LOC284395	GAGAAATAGAATAGGTATGATTGATAA	473	23,151,60,53,80	270,98
	GTAAAACCCTCCGAACCAAATATAAA			
Genomewide	CCGTAAGGGGTTAGGGAGTTTTT	160	80	62, 98
(COBRA LINE-1)	RTAAAACCCTCCRAACCAAATATAAA			

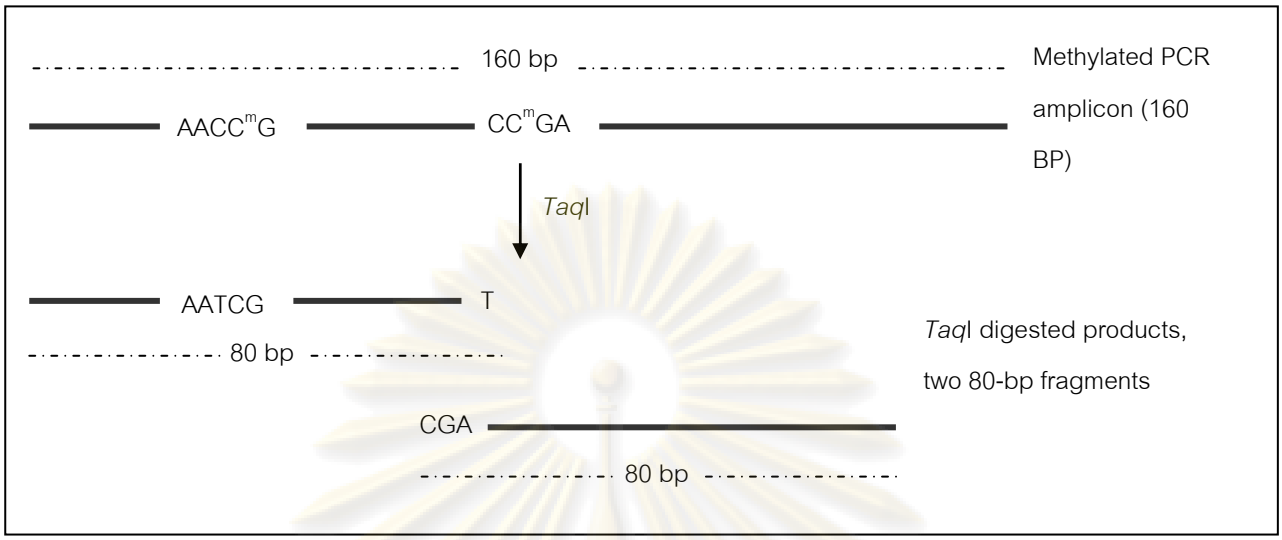


Figure 3.4 COBRALINE-1 PCR amplicon with *Taq*I recognition site (TCGA nucleotide sequences). After bisulfite treatment and PCR, methylated CCGA will be converted to TCGA (*Taq*I site). A methylated 160-bp amplicon of COBRALINE-1 yields two 80-bp *Taq*I-digested fragments.

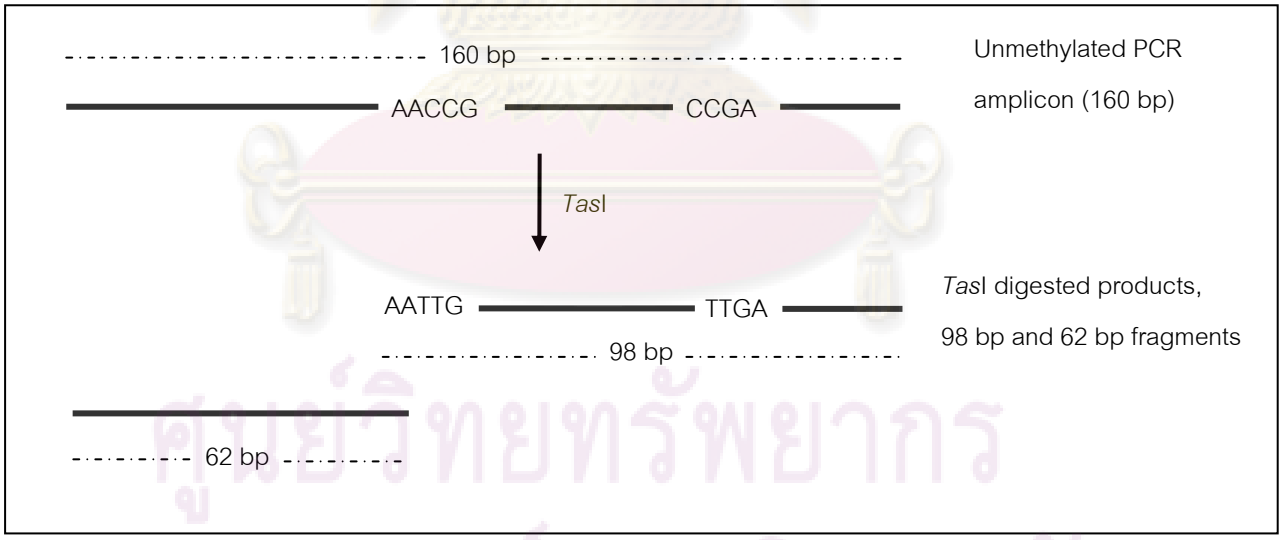


Figure 3.5 COBRALINE-1 PCR amplicon with *Tas*I recognition site (AATT nucleotide sequences). After bisulfite treatment and PCR, unmethylated AACCG will be converted to AATTG (*Tas*I site). An unmethylated 160-bp amplicon of COBRALINE-1 yields a 98-bp and a 62-bp *Tas*I-digested fragment.

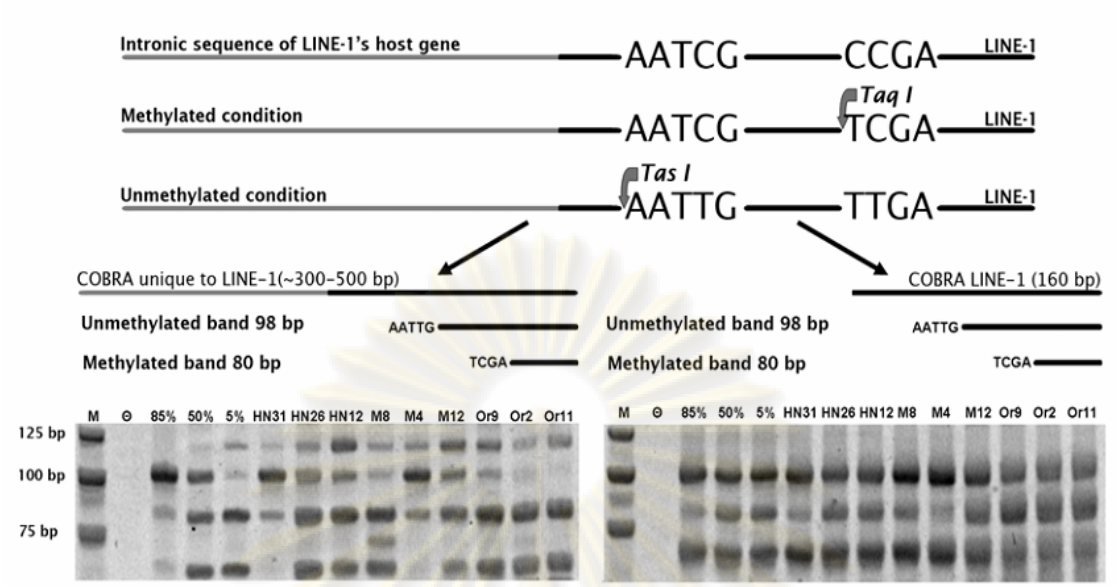


Figure 3.6 The schematic representation and examples of CU-L1 (left) and COBRALINE-1 (right). LINE-1 sequence in relation with 5' unique sequence is shown. AACCG and CCGA are LINE-1 sequences; when treated with bisulfite and PCR, unmethylated AACCG will be converted to AATTG (*Tas*I site) and methylated CCGA to TCGA (*Taq*I site). The amplicon sizes of CU-L1 are approximately 300 to 500 bp while of COBRALINE-1 are 160 bp. After digestion, COBRALINE-1 yielded 62- and 98-bp *Tas*I-digested unmethylated LINE-1 sequences and 80-bp *Taq*I-digested methylated LINE-1 sequences. CU-L1 usually has additional *Taq*I site(s) and AATT sequences. Therefore, there are more methylated and unmethylated bands. A typical example of results from COBRALINE-1 and CU-L1 are also shown. The ranges of intensity between methylated and unmethylated bands of CU-L1 were wider than COBRALINE-1. M is standard size marker, O is negative control. Several samples of HN (head and neck squamous cell carcinoma cell lines) are demonstrated (23).

Methylation levels of LINE-1

Intensities of DNA fragments in the electrophoresed gel were measured by PhosphorImager, using Image Quant Software (Molecular Dynamics, Pharmacia Amersham). LINE-1 methylation level was calculated as a percentage of the intensity of the methylated LINE-1 digested by *Taq*I, devided by the sum of unmethylated LINE-1

digested by *Tas*I and the *Taq*I- positive amplicons (Figure 3.7) (10). The same preparation of genomic DNA from HeLa, Daudi, and K562 cell lines was used as positive controls in all the experiments and to adjust for interassay variations.

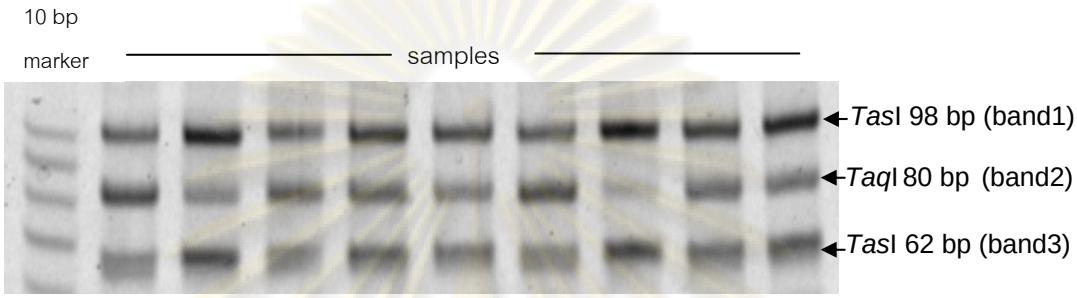


Figure 3.7 Schematic illustrations of digested PCR products and methylation level quantitation. Digested LINE-1 PCR products on the electrophoresed nondenaturing polyacrylamide gel were quantitated for methylation levels. The LINE-1 methylation level was calculated as a percentage (the intensity of methylated (*TaqI*-positive, 80 bp) amplicon, divided by the sum of the unmethylated (*Tas*I-positive, 98 bp and 62 bp) amplicons and the methylated amplicons) (10).

$$\% \text{ methylation} = \frac{\text{Band 2}}{\text{Band 1+2+3}} \times 100$$

Statistical analysis

Statistical analyses were performed using SPSS software for Windows 11.5 (SPSS Inc., Chicago, IL). Values were calculated using Student's *t*-test, analysis of variance (ANOVA), Brown-Forsythe test or Kruskal-Wallis test as indicated. A *p*-value of <0.05 was considered significant.

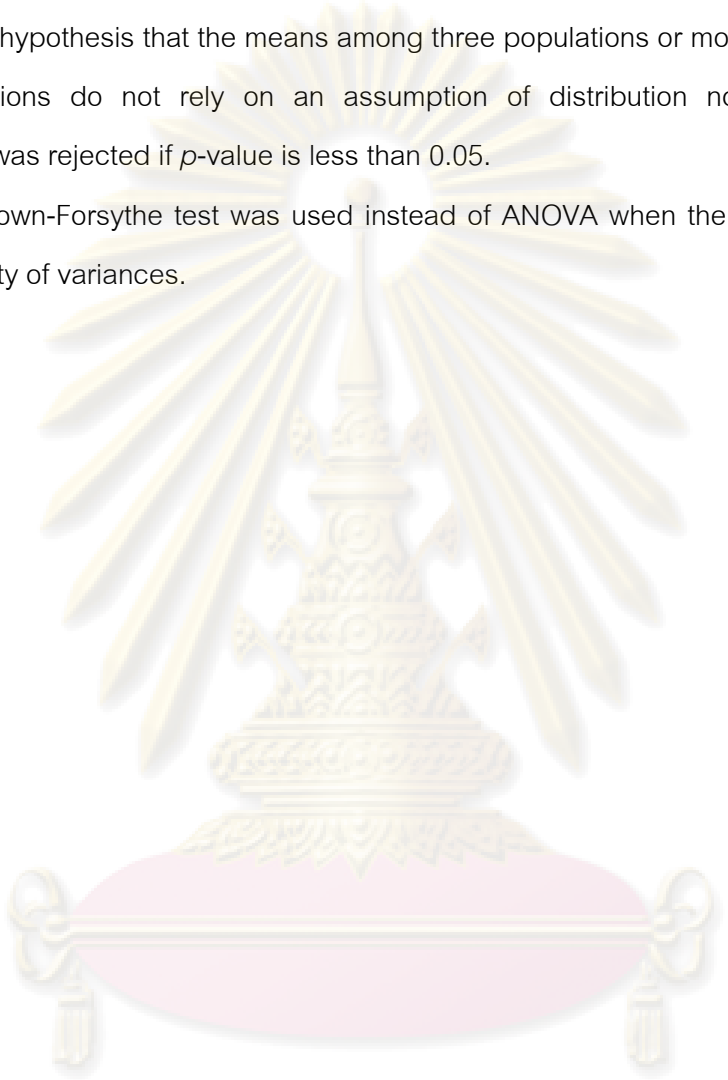
Student's *t*-test was used to test the null hypothesis that the means of two normally distributed populations are equal. And also both populations must be assumed to have equal variances. The null hypothesis was rejected if *p*-value is less than 0.05.

One-way ANOVA was used to test the null hypothesis that the means among three populations or more are equal. This analysis was used under the assumption of (1)

population is independent, (2) the distributions in each population are normal and (3) the variance should be the same (equal variances). The null hypothesis was rejected if *p*-value is less than 0.05.

The Kruskal-Wallis test is a nonparametric analysis. This method was used to test the null hypothesis that the means among three populations or more are equal when the populations do not rely on an assumption of distribution normality. The null hypothesis was rejected if *p*-value is less than 0.05.

Brown-Forsythe test was used instead of ANOVA when the populations have heterogeneity of variances.



CHAPTER IV
RESULTS

Methylation status of genome-wide LINE-1s in normal oral epithelium

Even though males and females possess different sets of sex chromosome and aging may influence the methylation levels (126); previous study exhibited that levels of LINE-1 methylations in WBC did not depend on gender or age (10). In order to clarify this phenomenon in oral tissues, methylation levels between genders and between the young (not older than 40 years of age) and the elderly (older than 40 years of age) were studied, using COBRALINE-1. Normal oral rinses were collected from 37 volunteers (13 males and 24 females); mean methylation levels ± SD were 42.61% ± 3.50 and 41.33% ± 2.36 in males and females, respectively. Of these 37 volunteers, 22 were classified as the young group (mean age was 30.45 years, range 20-40 years) and 15 as the elderly group (mean age was 61.73 years, range 42-75 years). Mean methylation levels ± SD were 42.13% ± 3.12 and 41.26% ± 2.37 in the young and the elderly, respectively. There was no significant difference of mean methylation levels between males and females (*p*-value = 0.191) and between the young and the elderly (*p*-value = 0.37) using Student's *t*-test (table 4.1). However, when compared with normal WBCs (mean methylation level ± SD = 46.15% ± 1.48, N = 12, (23)); methylation levels of normal oral epithelium (mean methylation level ± SD = 41.77% ± 2.83, N = 37) was lower than of normal WBCs significantly (*p*-value < 0.001, figure 4.1).

Table 4.1 Methylation status of genome-wide LINE-1s in normal oral epithelium

	N	% methylation of LINE-1s				*p*-value
		Mean	S.D.	Minimum	Maximum	
Total	37	41.77	2.83	36.83	49.46	
Between genders						0.191
Males	13	42.61	3.50	37.69	49.46	
Females	24	41.33	2.36	36.83	46.97	
Between age groups						0.37
40 years old or younger	22	42.13	3.12	36.83	49.46	
Older than 40 years old	15	41.26	2.37	37.69	48.07	

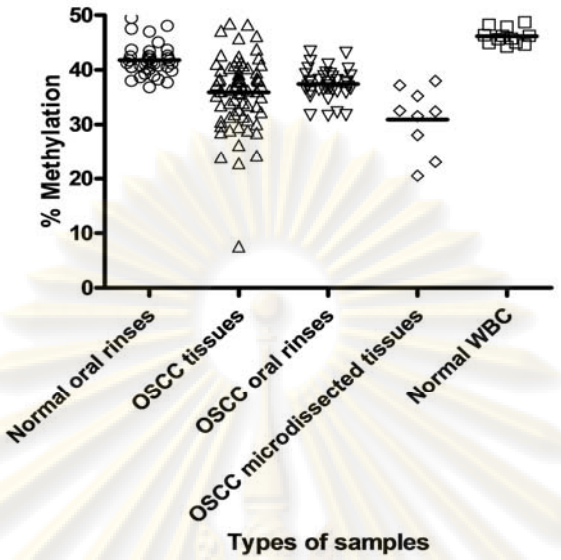


Figure 4.1 Methylation levels of genome-wide LINE-1s in each group of samples. The horizontal line indicates the mean of methylation levels. Normal oral rinses (n=37), OSCC tissues (n=69), OSCC oral rinses (n=38), OSCC microdissected tissues (n=9) and normal WBCs (n=12) had mean methylation levels ± SD of 41.78% ± 2.84, 35.88% ± 6.60, 37.53% ± 2.61, 30.95% ± 6.03, and 46.15% ± 1.48, respectively. Normal oral rinses had lower methylation level than normal WBCs significantly (p-value < 0.01). All OSCC samples had lower methylation levels than normal oral rinses, significantly (p-value < 0.01) but no difference from each others (p-value > 0.05).

Genome-wide LINE-1 hypomethylation in primary OSCC tissues and oral rinses of OSCCs patients

Primary OSCC tissues were obtained from 69 OSCC patients (32 males and 37 females); mean methylation levels ± SD were 35.73% ± 6.36 and 36.01% ± 6.88 in males and females, respectively. OSCC oral rinses were from 38 OSCC patients (24 males and 14 females); mean methylation levels ± SD were 37.87% ± 2.98 and 39.95% ± 1.75 in males and females, respectively. There was also no difference of LINE-1s methylation levels between genders in OSCC oral rinses and OSCC tissues (p-value = 0.297 and

0.862, respectively using Student's *t*-test). Therefore, LINE-1 methylation levels in normal and malignant oral tissues have no difference between genders. However, methylation levels of LINE-1s in primary OSCC tissues and in oral rinses of OSCC patients (mean methylation levels ± SD were 35.88% ± 6.6 and 37.53% ± 2.61, respectively) were lower than those of normal oral rinses, significantly (*p*-value < 0.001, Figure 4.1, Table 4.2, 4.3). Surprisingly, methylation levels of OSCCs from primary lesions and from oral rinses were not different (*p*-value = 0.518). Consequently, OSCCs possessed genome-wide hypomethylation of LINE-1s and this alteration could be detected in oral rinses of OSCC patients.

In order to clarify whether the contamination of normal cells in OSCC tissues and OSCC oral rinses may affect levels of methylation, the genomic DNA from 9 OSCC microdissected samples which had more homogeneity of cancerous cells were also included in this analysis. Although methylation levels of OSCC microdissected samples (mean methylation level ± SD was 30.95% ± 6.03) were lower than others, but it was not statistically different from primary OSCC tissues and OSCC oral rinses (*p*-value > 0.05, Figure 4.1, Table 4.2, 4.3). This finding suggested that normal cells had little or no effect on hypomethylation levels of cancerous cells.

Table 4.2 Methylation levels of genome-wide LINE-1s in each sample group

Types of samples	N	Methylation level (%)			
		Mean	S.D.	Minimum	Maximum
normal oral rinses	37	41.78	2.84	36.83	49.46
OSCC primary tissues	69	35.88	6.60	7.62	47.13
OSCC oral rinses	38	37.53	2.61	31.60	43.36
OSCC microdissected	9	30.95	6.03	20.51	37.96
normal WBCs	12	46.15	1.48	44.21	48.74

Table 4.3 Comparisons of methylation levels among groups

p-value	normal oral rinses	OSCC tissues	OSCC oral rinses	OSCC microdissected	normal WBCs
normal oral rinses		0.00000005*	0.000000032*	0.00547*	0.00000037*
OSCC tissues	0.00000005*		0.518	0.361	0.000000000*
OSCC oral rinses	0.000000032*	0.518		0.106	0.000000000*
OSCC microdissected	0.00547*	0.361	0.106		0.00048206*
normal WBCs	0.00000037*	0.000000000*	0.000000000*	0.00048206*	

* The mean difference is significant at < .05 level.

Genome-wide LINE-1 hypomethylation is independent from clinico-pathological features of OSCCs

Mean methylation levels in tumor stage I (36.62% ± 6.81), II (37.47% ± 3.84), III (35.40% ± 7.78) and IV (36.43% ± 4.96) did not differ from each others, *p*-value = 0.681 (one-way ANOVA, Figure 4.2). Tumors which had histological features of well-differentiated, moderately-differentiated, and poorly-differentiated cells had no different levels of methylation, *p*-value = 0.924 (Kruskal-Wallis test), mean methylation levels were 36.19% ± 6.06, 37.05% ± 4.28, and 36.50% ± 3.86, respectively (Figure 4.3). OSCCs occurred at tongue, gum, buccal mucosa, floor of the mouth, palate, lip, or oropharynx had no difference of mean methylation levels, *p*-value = 0.464 (Kruskal-Wallis test), mean methylation levels were 36.33% ± 6.57, 35.88% ± 5.88, 36.37% ± 4.42, 35.67% ± 5.74, 39.07% ± 2.00, 37.30% ± 5.74, and 43.11, respectively. Patients, who did not smoke, drink alcohol or chew betel quid had no significant difference of methylation levels with patients who exposed to any of these risk factors, *p*-value = 0.427 (Brown-Forsythe test) (Table 4.4).

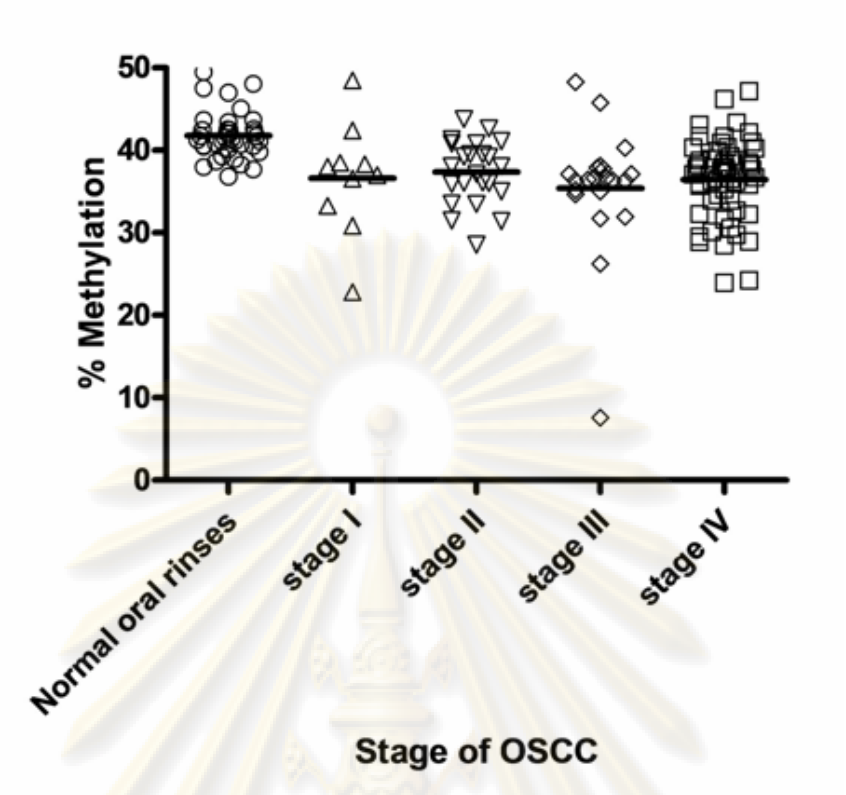


Figure 4.2 Methylation levels of genome-wide LINE-1s in each cancer stage. All stages showed hypomethylation levels. The mean methylation levels did not statistically differ among stages (ANOVA *p*-value = 0.681). Normal oral rinses (n=37), OSCC stage I (n=10), II (n=23), III (n=21) and IV (n=53) had mean methylation levels ± SD of 41.78% ± 2.84, 36.62% ± 6.81, 37.47% ± 3.84, 35.40% ± 7.78, and 36.43% ± 4.96, respectively.

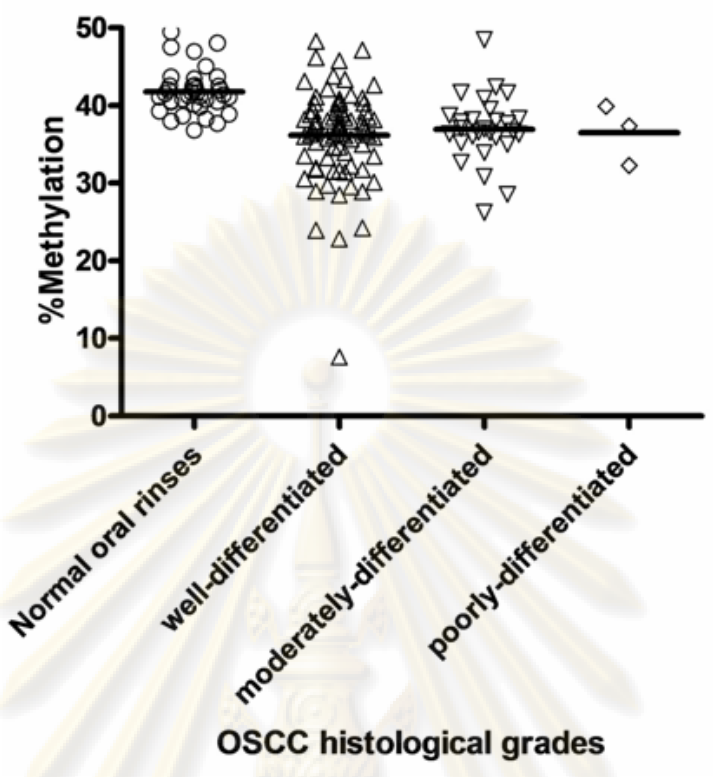


Figure 4.3 Methylation levels of genome-wide LINE-1s in each histological grade. All three grades of malignant cells had hypomethylation. There was no statistical difference in methylation levels among grades (Kruskal-Wallis test *p*-value = 0.924). Means methylation levels ± SD of normal oral rinses (n=37), well-differentiated (n=75), moderately-differentiated (n=28), and poorly-differentiated (n=3) were 41.78% ± 2.84, 36.19% ± 6.06, 37.05% ± 4.28, and 36.50% ± 3.86, respectively.

Table 4.4 Genome-wide LINE-1 methylation levels in different clinical stages, histological grades, tumor locations and risk factors

	N (%)	% Methylation			
		Mean	SD	95% CI	*p*-value
OSCC samples*	107 (100)				
Stage					0.681
I	10 (9.35)	36.62	6.81	(31.75 - 41.49)	
II	23 (21.50)	37.47	3.84	(35.81 - 39.13)	
III	21 (19.63)	35.40	7.78	(31.85 - 38.94)	
IV	53 (49.53)	36.43	4.96	(35.07 - 37.80)	
Histological grading**					0.924
Well-differentiated	75 (70.09)	36.19	6.06	(34.87 - 37.63)	
Moderately-differentiated	28 (26.17)	37.05	4.28	(35.39 - 38.71)	
Poorly-differentiated	3 (2.80)	36.50	3.86	(26.91 - 46.10)	
Location					0.464
Tongue	30 (28.04)	36.33	6.57	(33.88 - 38.78)	
Gum	24 (22.43)	35.88	5.88	(33.40 - 38.37)	
Buccal mucosa	21 (19.63)	36.37	4.42	(34.35 - 38.38)	
Floor of mouth	16 (14.95)	35.67	5.74	(32.61 - 38.73)	
Palate	8 (7.48)	39.07	2.00	(37.40 - 40.73)	
Lip	7 (6.54)	37.30	5.74	(31.99 - 42.61)	
Oropharynx	1 (0.93)	43.11	†	†	
Risk factors					0.427
No	18 (16.82)	36.84	6.63	(33.54 - 40.13)	
Betel	30 (28.04)	35.54	4.60	(33.82 - 37.25)	
Smoking	13 (12.15)	38.77	4.62	(35.98 - 41.57)	
Alcohol	6 (5.61)	32.21	12.77	(18.81 - 45.61)	
Smoking+alcohol	34 (31.78)	36.55	4.08	(35.12 - 37.97)	
Smoking+betel	5 (4.67)	39.70	2.95	(36.03 - 43.36)	
Smoking+alcohol+betel	1 (0.93)	34.47	†	†	

*OSCC samples include OSCC tissues (n=69) and OSCC oral rinses (n=38)

**Data was not available in one case

† Data could not obtained due to n< 2 and were excluded from the statistical analysis

Methylation levels of specific LINE-1s vary among location and individuals

Methylation levels of the same normal oral epithelium sample differed among 17 studied loci. There were methylation level variations, for example LINE-1s at PKP4, EPHA3, COL24A1 introns were nearly completely methylated, whereas LINE-1s at PPP2R2B and PKG1 owned about 30-50% methyation. In addition, the ranges of methylation levels were also different among loci. While most of specific LINE-1 had about 5% range, LINE-1 at SPOCK3, LRP2, FAM49, Loci284395 and Loci286094 had wider range, about 20-30% (Figure 4.4).

Characteristics of LINE-1 hypomethylation in OSCCs

Specific LINE-1s were studied in OSCC tissues; the informative 14 loci were selected. We found that methylation levels of OSCC tissues had wider range than normal oral epithelium. However, most of OSCC tissues demonstrated lower methylation levels from normal oral epithelium, except at CNTNAP5 locus which OSCCs were hypermethylated. Moreover, some OSCC samples owned hypomethylation and some owned hypermethylation of LINE-1s at MGC42174 locus (Figure 4.4).

When means of methylation levels of each locus were compared between normal oral epithelium and OSCC tissues, 12 of 14 loci had significant differences (Student t-test, *p*-value < 0.05) (Table 4.5). Interestingly, 2 loci which were hypermethylated and hyper/hypomethylated; CNTNAP5 and MGC42174, respectively had no statistical difference of the means.

Although each OSCC did not show alteration of LINE-1 methylation levels in all loci, surprisingly; each individual possessed at least 1 aberrant methylation-level locus (data not shown). The aberrant methylation in all 14 loci revealed no correlation with clinico-pathological features of OSCCs (ANOVA, *p*-value >0.05).

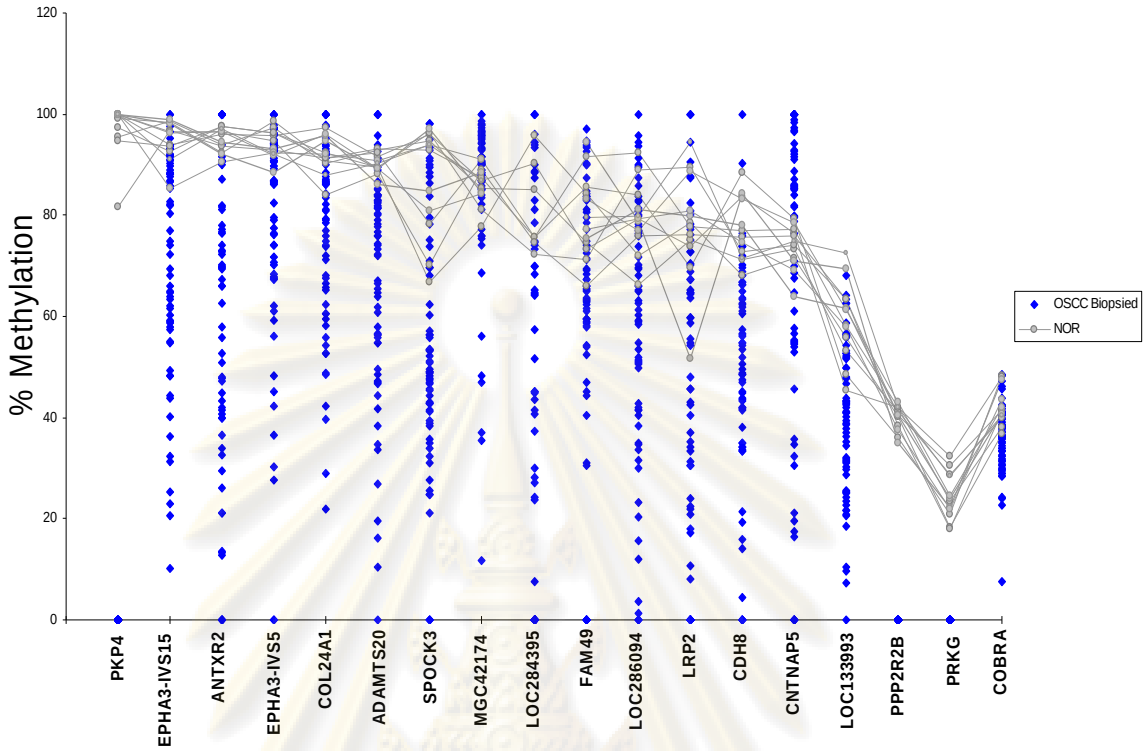


Figure 4.4 Methylation levels of specific LINE-1s. NOR is normal oral epithelium. OSCC tissues are primary OSCC tissues. The value at 0 means that the DNA could not successful amplified, not the 0% methylation.

Table 4.5 Compare means of methylation levels of specific LINE-1 in each locus

Loci	Type	N	Mean of methylation levels (%)	S.D.	*p*-value
CNTNAP5	normal	12	73.79	4.33	0.223
	OSCC	65	68.94	30.13	
ANTXR2	normal	12	94.86	2.38	0.000
	OSCC	64	64.90	27.79	
FAM49	normal	12	79.72	8.41	0.003
	OSCC	62	68.93	18.14	
COL24A1	normal	12	92.00	3.72	0.000
	OSCC	68	77.24	18.57	
ADAMTS20	normal	12	90.19	2.07	0.000
	OSCC	67	67.88	21.92	
LOC	normal	7	81.36	9.07	0.002
284395	OSCC	42	62.74	26.74	
LOC	normal	12	79.54	6.92	0.000
286094	OSCC	65	60.32	23.93	
LRP2	normal	12	78.02	10.90	0.000
	OSCC	53	56.30	24.32	
CDH8	normal	12	77.38	6.30	0.000
	OSCC	68	48.51	22.03	
LOC	normal	12	59.25	7.83	0.000
133993	OSCC	67	40.14	14.50	
MGC42174	normal	12	86.14	3.90	0.694
	OSCC	67	85.23	16.48	
SPOCK3	normal	12	87.09	10.73	0.000
	OSCC	64	57.43	21.45	
EPHA3-	normal	12	95.02	4.01	0.000
IVS15	OSCC	68	71.16	22.46	
EPHA3-	normal	12	94.15	2.84	0.000
IVS5	OSCC	65	81.54	18.03	

CHAPTER V
CONCLUSION AND DISCUSSION

Conclusion

Methylation levels of genome-wide LINE-1s in normal oral epithelium differed from those of normal blood leukocytes but did not depend on age and genders. The results from this study not only confirmed that age and genders had no influence to methylation levels of genome-wide LINE-1, but also this kind of epigenetic mechanisms was specific to tissue types, as the methylation levels in 2 types of normal tissues; normal oral epithelium and normal WBCs were significantly different. OSCCs, like most of other malignancies, also possess genome-wide LINE-1 hypomethylation. Interestingly, the hypomethylation of genome-wide LINE-1s can be detected in oral rinses of OSCCs patients, at the same level of those detected in OSCC primary tissues. However, this epigenetic aberration (both found in OSCC tissues and OSCC oral rinses) does not depend on tumor stages, histological grades, sites of tumor or the well-documented risk factors, including smoking, alcohol abused and betel chewing.

When specific LINE-1s were studied, normal oral epithelium showed different methylation levels of specific LINE-1 among individuals and loci. Some loci owned high methylation levels, while others acquired lower levels. This finding revealed that not all LINE-1s were completely methylation. Each individual of normal oral epithelium occupied different methylation levels in the same locus, and it also differed from other loci. Although the entire genome methylation levels were decreased in OSCCs but this alteration did not distribute equally in every LINE-1s. In OSCCs, alteration of methylation levels of specific LINE-1s studied here had no specific pattern. Each individual OSCC had aberrant methylation levels in different loci and different magnitudes. Although most of OSCCs lost methylated CpG, some gained methylated CpG. Thus the alterations of methylation of LINE-1s in cancerous tissues were not specific to locus. Moreover, the aberrant LINE-1 methylations were not influenced by clinical stages, histological grades, tumor sites and risk factors. However, methylation levels of specific LINE-1s were significantly different between normal oral epithelium and OSCCs tissues.

Discussion

The efficiency of current treatment modalities for OSCCs depends strongly on the time of diagnosis, with better chance of survival and less morbidity if a tumor has been detected at an early stage. Thus, there is an urgent need for rapid and efficient early detection methods. Detection of cancers in the oral cavity still requires expertise. Up till now, the accurate diagnosis of oral cancers depends on surgical biopsy and histological studies which are difficult to apply in large populations. However, there are attempts to develop simple and reliable tools for early discovery of oral cancers. During this decade, the use of saliva or mouthwashes/oral rinses for malignancy detection has been a focus of interest. Evidence suggested that epithelial cells in saliva provide suitable materials for head and neck squamous cell carcinomas (HNSCCs) genetic analysis (123). Exfoliated oral mucosal cells and also malignant cells can be easily collected via saliva or oral rinses. This procedure is not invasive, not expensive, and does not require expertise. Moreover, the shed cancer cells in saliva and primary cancerous tissues had the same results of microsatellite alterations (55), and aberrant promoter methylation (127). Saliva or oral rinses of HNSCC patients exhibited telomerase activity (122), increased mitochondrial DNA content (128), and promoter hypermethylation (129). Loss of heterozygosity (LOH) was found in mouthwashes of OSCC patients but not found in those of healthy individuals (130). Comprehensive salivary analysis revealed an overall altered salivary composition in OSCCs (58, 59) and also an increase in tumor markers including Cyfra 21-1, tissue polypeptide antigen, CA125, and IL-8 (60, 61). Three species of bacteria in saliva were found to be increased in OSCC patients (131). Salivary transcriptome study revealed elevation of 7 transcripts including DUSP1, H3F3A, IL1B, IL8, OAZ1, SAT, and S199P in OSCCs (132, 133). Accordingly, biomarkers in oral rinse represent a possible screening tool capable to apply in massive population to identify high risk individuals instead of current screening methods which require specialties. However, the above biomarkers are still not efficiently applied in clinical screening. This may be due to the nature of cancer development is a multistep process; the use of specific markers may be insufficient for detection. Thus, this study was performed in order to seek for a biomarker which can

detect malignant change at any steps, whether early or late stage; and the global hypomethylation might be a good candidate.

Epigenetic alterations including global (genome-wide) hypomethylation were reported in many types of cancers (134-137). Although there were evidences that supported the epigenetic involvement in oral malignancies, global hypomethylation has not been reported yet (138-141). Some studies revealed global hypomethylation in HNSCCs which included carcinomas in oral cavity, nose, sinuses, pharynx, and larynx (18, 19). But previous study pointed out that methylation level of LINE-1s, which reflect global methylation levels, had tissue specification. Different kinds of tissue from the same organ system possess different methylation levels, for instance esophagus and stomach, or bladder and kidney (10). Furthermore, tumors of larynx/hypopharynx and oral cavity had different levels of soluble CD44 (62), as well as the incidence of LOH (142). Accordingly, we proposed that OSCCs which originate from mucosa in oral cavity may have different methylation levels from carcinomas originate from other locations in head and neck regions. For that reason, we studied LINE-1 hypomethylation in OSCC patients by using oral epithelium of normal individuals as controls. The easiest and noninvasive way to collect oral epithelium is from oral rinses. From this study, LINE-1 hypomethylations could detect OSCCs not only at early occurrence but also at any hidden site of the oral cavity and in any histological type. It was independent of smoking, alcohol consumption and betel chewing. This finding differed from the study of Smith *et* al., which reported that global hypomethylation in HNSCC associated with smoking, alcohol consumption and stage (17). Therefore, our LINE-1 methylation study suggested that OSCCs may have different natures from HNSCCs and the methylation levels of LINE-1s are specific to types of tissues. Perhaps the global loss of methylation in OSCC occurs since early onset of carcinogenesis and does not continue with the tumor progression is another possibility contributes to the reason that LINE-1 hypomethylation did not relate to clinico-pathological status. In spite of the fact that oral rinses of OSCC patients consist of few shed cancerous cells and contaminated with normal epithelium and some immune cells; surprisingly, the hypomethylation could be detected in oral rinses of OSCC patients and did not statistically differ from those found in primary OSCC tissues and OSCC microdissected samples. Thus, COBRALINE-1 of

OSCC oral rinses could reflect the majority of OSCC methylation levels. Consequently, COBRALINE-1 of oral rinse appears to have a role in oral cancers screening. However, the sensitivity and specificity of this technique in identification of OSCCs are to be proved.

In contrast to general believe that LINE-1 should have been completely methylated, the results from this study in normal oral epithelium revealed partial methylation levels of genome wide LINE-1s. In addition, the lower methylation levels of some CU-L1s in normal oral epithelium proved that not all LINE-1s are completely methylated. Another interesting finding was that although the methylation levels of genome-wide LINE-1s had significantly decreased in OSCCs but, the methylation did not loss evenly in all loci. From the study of 14 selected specific LINE-1s in OSCCs, some loci were hypermethylated and some were hypomethylated and some were within normal range. The alterations of methylation level were not the same in every OSCCs. Each individual OSCC had aberration in different loci and in different degrees. These findings suggested that methylated CpG dinucleotides of 5' LINE-1 had randomly changed in OSCCs. Even though a little proportion of specific LINE-1s was studied, in OSCCs we found that at least one locus showed aberrant methylation. These aberrations also had no correlation with clinico-pathological features. Then CU-L1 may be useful in detection OSCCs and may increase sensitivity power of the test from COBRALINE-1.

In conclusion, this study revealed that OSCCs had hypomethylation of LINE-1s and this aberration could be found in oral rinses of the patients. Our findings suggested the potential use of COBRALINE-1 of oral rinses as a non invasive tool for OSCCs detection. However, CU-L1 may provide more accuracy information of OSCCs. These simple PCR techniques still need further study in an attempt to improve sensitivity and specificity, eventually becomes a reliable investigation technique.

Future study

Since CU-L1 is a very interesting technique for detection aberrant methylation of specific LINE-1s and oral rinse can be used as a source of oral cancer cells collection. Further study of CU-L1 in oral rinses of OSCC patients may give some more accuracy and reliability for developing a simple and non-invasive screening or diagnostic tool. However, CU-L1 amplifies specific LINE-1 which has less copy than genome-wide LINE-1s; more amount of cancerous DNA may be needed. The amount of cancerous DNA collected from patient's oral rinses depend on shed cancerous cells and aberrant methylation levels may be masked by those of normal cells, therefore CU-L1 in oral rinses may give a less sensitive result. The large numbers of OSCC oral rinse samples may be required to warrant the sensitivity and specificity of this test before apply as a screening modality.

Another interesting aspect may be the methylation levels of leukocytes of cancerous patients, since leukocytes contribute to host defense mechanisms against malignant cells. Also the methylation status of LINE-1s in precancerous lesions is not reported. Moreover, the mechanisms and roles of loss or gain methylated CpG dinucleotides of specific LINE-1s in malignancies are still needed elucidation.

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VITA

I, Mrs. Keskanya Subbalekha was born on 7th May 1970 in Chonburi Province, Thailand. I got married to Dr. Sissanu Subbalekha and have 2 sons, Surawish and Panuwach. I received the degree of Doctor of Dental Surgery from Chulalongkorn University, Thailand in 1994. I finished the Oral and Maxillofacial Surgery Residency Training program from Faculty of Dentistry, Chulalongkorn University in 1997 and was a diplomate in Thai Board of Oral and Maxillofacial Surgery in 1998. After working as a teacher and an oral and maxillofacial surgeon in Department of Oral and Maxillofacial Surgery, Faculty of Dentistry, Chulalongkorn University since 1996, then I started studying for the degree of Doctor of Philosophy in Oral Biology at Faculty of Dentistry, Chulalongkorn University in 2003. The research components of this degree were performed at the Research Unit of Mineralized Tissue, Faculty of Dentistry and at the Center of Excellence in Molecular Genetics of Cancer and Human Diseases, Department of Anatomy, Faculty of Medicine, Chulalongkorn University. At present, I continue my work as a teacher and an oral and maxillofacial surgeon in Department of Oral and Maxillofacial Surgery, Faculty of Dentistry, Chulalongkorn University.