การเปรียบเทียบระดับเมทิเลชันของไลน์-1 ในส่วนประกอบของเลือดกับน้ำบ้วนปากของผู้ป่วยมะเร็งเซลล์สความัสในช่องปากแต่ละราย

นางสาว ตวงทอง พบสุข

วิทยานิพนธ์นี้เป็นส่วนหนึ่งของการศึกษาตามหลักสูตรปริญญาวิทยาศาสตรมหาบัณฑิต
สาขาวิชาศัลยศาสตร์ช่องปากและแม็กซิลโลเฟเชียล ภาควิชาศัลยศาสตร์
คณะทันตแพทยศาสตร์ จุฬาลงกรณ์มหาวิทยาลัย
ปีการศึกษา 2553

INTRA-INDIVIDUAL COMPARISON OF LINE-1 METHYLATION LEVELS IN PERIPHERAL BLOOD COMPONENTS WITH ORAL RINSE OF ORAL SQUAMOUS CELL CARCINOMA PATIENTS

Miss Tuangtong Pobsook

A Thesis Submitted in Partial Fulfillment of the Requirements
for the Degree of Master of Science Program in Oral and Maxillofacial Surgery
Department of Oral and Maxillofacial Surgery
Faculty of Dentistry
Chulalongkorn University
Academic Year 2010

Thesis Title	INTRA-INDIVIDUAL COMPARISON OF LINE-1 METHYLATION LEVELS IN PEIPHERAL BLOOD COMPONENTS WITH ORAL RINSE OF ORAL SQUAMOUS CELL CARCINOMA PATIENTS
By	Miss Tuangtong Pobsook
Field of Study	Oral and Maxillofacial Surgery
Thesis Advisor	Keskanya Subbalekha, D.D.S., Ph.D.
Thesis Co-Advisor	Professor Apiwat Mutirangura, M.D., Ph.D.

Accepted by the Faculty of Dentistry, Chulalongkorn University in Partial Fulfillment of the Requirements for the Master's Degree

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ตวงทอง พบสุข : การเปรียบเทียบระดับเมทิเลชันของไลน์-1 ในส่วนประกอบของเลือดกับน้ำบ้วนปากของผู้ป่วยมะเร็งเซลล์สความัสในช่องปากแต่ละราย.

(INTRA-INDIVIDUAL COMPARISON OF LINE-1 METHYLATION LEVLES IN PERIPHERAL BLOOD COMPONETS WITH ORAL RINSE OF ORAL SQUAMOUS CELL CARCINOMA PATIENTS) อ. ที่ปรึกษาวิทยานิพนธ์หลัก : อ.ทพญ.ดร. เกศกัญญา สัพพะเลข, อ. ที่ปรึกษาวิทยานิพนธ์ร่วม : ศ.นพ.ดร. อภิวัฒน์ มุทิรางกูร, 152 หน้า.

มะเร็งเซลล์สความัสในช่องปาก (Oral squamous cell carcinoma) เป็นมะเร็งที่พบได้ร้อยละ 90 ของผู้ป่วยมะเร็งช่องปากทั้งหมด ปัจจุบันอัตราการรอดชีวิตของผู้ป่วยยังน้อยกว่าร้อยละ 50 สาเหตุหนึ่งเนื่องจากรอยโรคมักได้รับการวินิจฉัยล่าช้า เมื่อลุกลามไปมากแล้ว การตรวจพบรอยโรคได้ตั้งแต่ในระยะเริ่มแรก ด้วยวิธีที่ไม่ยุ่งยากอาจเพิ่มอัตราการรอดชีวิตของผู้ป่วยได้ ปัจจุบันพบว่าเมทิเลชัน (Methylation) ของไลน์-1 (LINE-1s) ซึ่งเป็นกรดนิวคลีอิกที่เกิดซ้ำ (Repetitive sequences) ในจีโนม (Genome) มีระดับต่ำลงในเนื้อเยื่อที่เป็นมะเร็งหลายชนิด และสามารถตรวจได้จากน้ำบ้วนปากของผู้ป่วยมะเร็งในช่องปากอีกด้วย การศึกษานี้มีวัตถุประสงค์เพื่อเปรียบเทียบระดับเมทิเลชันของไลน์-1 ในเลือดของผู้ป่วยมะเร็งเซลล์สความัสในช่องปากกับคนปกติ และศึกษาความแตกต่างระหว่างระดับเมทิเลชันของไลน์-1ในเลือด และน้ำบ้วนปากของผู้ป่วยแต่ละคน

จากการศึกษาพบว่าเมทิเลชันของไลน์-1ในบัฟฟีโคท (Buffy Coat) และพลาสมา (Plasma) ของผู้ป่วยมะเร็ง (37 คน) มีระดับสูงกว่ากลุ่มควบคุม (45 คน) อย่างไม่มีนัยสำคัญ ซึ่งตรงข้ามกับในน้ำบ้วนปากที่ระดับของเมทิเลชันจะต่ำกว่าคนปกติอย่างมีนัยสำคัญ และพบว่าระดับเมทิเลชันของไลน์-1 ในน้ำบ้วนปากของคนปกติจะสูงกว่าระดับในบัฟฟีโคท และพลาสมาของคนเดียวกัน ส่วนในผู้ป่วยมะเร็ง ดีเอ็นเอ (DNA) จากทั้งสามแหล่งมีระดับเมทิเลชันไม่ต่างกัน นอกจากนี้ยังพบว่าการใช้เทคนิคคอบบราไลน์-1 (COBRA LINE-1) สามารถให้ข้อมูลเกี่ยวกับรูปแบบ (Pattern) ของเมทิเลชันได้อีกด้วย จากข้อมูลที่เพิ่มเติมขึ้นมาดังกล่าวพบว่าในผู้ป่วยมะเร็ง ระดับของผลผลิตดีเอ็นเอ (DNA product) ขนาด 98 คู่เบส (base pair, bp) ซึ่งเป็นผลผลิตจากรูปแบบของไลน์-1ที่สูญเสียเมทิเลชันทั้งหมด (Complete unmethylated LINE-1 sequence) จะสูงกว่าในคนปกติอย่างมีนัยสำคัญเมื่อตรวจจากดีเอ็นเอทั้งสามแหล่ง และพบว่าระดับของผลผลิตดังกล่าวในคนเดียวกันยังไม่แตกต่างกันอีกด้วย ส่งผลให้การใช้น้ำบ้วนปากที่มีดีเอ็นเอจากเซลล์ชนิดอื่นปะปน มีความไว (Sensitivity) และความจำเพาะ (Specificity) สูงขึ้นถึงประมาณร้อยละ 80-90 ซึ่งสูงกว่าการใช้ระดับเมทิเลชันเพียงอย่างเดียวโดยไม่คำนึงถึงรูปแบบเมทิเลชัน จึงมีแนวโน้มว่าระดับของผลผลิตขนาด 98 คู่เบสในน้ำบ้วนปากซึ่งสามารถเก็บจากผู้ป่วยได้โดยง่าย อาจช่วยให้ตรวจพบมะเร็งช่องปากได้ตั้งแต่ระยะแรก นำไปสู่การเฝ้าระวังโรค การพยากรณ์โรคที่ถูกต้อง ซึ่งจะช่วยเพิ่มอัตราการรอดชีวิต และคุณภาพชีวิตของผู้ป่วยเหล่านี้ได้

ภาควิชา ศัลยศาสตร์ ลายมือชื่อนิสิต ตวงทอง พบสุข

สาขาวิชา ศัลยศาสตร์ช่องปากและแม็กซิลโลเฟเชียล ลายมือชื่อ อ.ที่ปรึกษาวิทยานิพนธ์หลัก เกศกัญญา สัพพะเลข

ปีการศึกษา 2553 ลายมือชื่อ อ.ที่ปรึกษาวิทยานิพนธ์ร่วม

5176111032 : MAJOR ORAL AND MAXILLOFACIAL SURGERY
KEYWORDS : ORAL SQUAMOUS CELL CARCINOMA (OSCC) / LONG INTERSPERSED NUCLEAR ELEMENT-1 (LINE-1) / METHYLATION / COMPLETE UNMETHYLATION / PERIPHERAL BLOOD COMPONENTS / ORAL RINSE

TUANGTONG POBSOOK : INTRA-INDIVIDUAL COMPARISON OF LINE-1 METHYLATION LEVELS IN PERIPHERAL BLOOD COMPONETS WITH ORAL RINSE OF ORAL SQUAMOUS CELL CARCINOMA PATIENTS. THESIS ADVISOR : KESKANYA SUBBALEKHA, D.D.S., Ph.D., THESIS CO-ADVISOR : PROF APIWAT MUTIRANGURA, M.D., Ph.D., 152 pp.

Oral squamous cell carcinoma (OSCC), an aggressive epithelial malignancy, is the most common oral cancer, accounting for at least 90% of total cases. Survival has remained at less than 50% and quality of life has been poor. This outlook is due to delayed diagnosis when the disease has already reached an advance stage. Thus, early detection and non-invasive screening tool can be crucial for each patient. Many studies have shown significant decreased methylation levels of long interspersed nuclear element-1s (LINE-1s) in many types of cancerous tissue, including oral rinse of oral squamous cell carcinoma.

This study aimed to explore the methylation levels of LINE-1s in the buffy coat and plasma of OSCC patients. Moreover, the level of buffy coat, plasma, and oral rinse within an individual were also investigated.

Here, LINE-1 hypomethylation level in oral rinse of OSCC patients (N= 37) was confirmed. In buffy coat and plasma of the cancer patients, the levels were insignificantly increased when they were compared to those of controls (N=45). Moreover, new information of partial methylation pattern of LINE-1s was also reported, suggesting the different gaining and losing methylation processes between normal and cancer. By including this important knowledge with overall methylation level, it was found that the levels of complete unmethylated (98 bp product) LINE-1s were significantly higher in all three DNA sources of OSCC patients. Not only that, complete unmethylated LINE-1s was an improved biomarker for distinguishing cancer from normal DNA, in the presence of other contaminated DNA types. With simply and non-invasively obtained oral rinse sample, the level of LINE-1 complete unmethylated sequence was a distinctive candidate for detecting early stage OSCC and provided sensitivity as well as specificity reaching 80-90%. Therefore, a better survival and quality of life of OSCC patients might be achieved.

Department : ORAL AND MAXILLOFACIAL SURGERY Student's Signature .....
Field of Study : ORAL AND MAXILLOFACIAL SURGERY Advisor's Signature .....
Academic Year : 2010 Co-Advisor's Signature .....

ACKNOWLEDGEMENTS

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Last but not least, I thank my family for every virtue in me and for giving me strength and reassurance during these years of hard work.

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LIST OF ABBREVIATIONS

LINE-1s	Long interspersed nuclear element-1s
COBRA	Combined bisulfite restriction analysis
COBRA LINE-1	Combined bisulfite restriction analysis of LINE-1
OSCC	Oral squamous cell carcinoma
HNSCC	Head and neck squamous much cell carcinoma
CTC	Circulating tumor cells
CNAPS	Circulating nucleic acid in plasma and serum
NOR	Normal oral rinse, oral rinse of normal subjects
OSCCR	Oral squamous cell carcinoma rinse, oral rinse of oral squamous cell carcinoma patients
NBC	Normal buffy coat, buffy coat of normal subjects
OSCC BC	Oral squamous cell carcinoma buffy coat, buffy coat of oral squamous cell carcinoma patients
NPM	Normal plasma, plasma of normal subjects
OSCC PM	Oral squamous cell carcinoma plasma, plasma of oral squamous cell carcinoma patients
NWBC	Normal white blood cells
OSCC WBC	Oral squamous cell carcinoma white blood cells, white blood cells of oral squamous cell carcinoma patients
WSU-HN	Head and neck squamous cell carcinoma cell lines
ROC	Receiver-operating characteristic
AUC	Area under the curve

CHAPTER I

INTRODUCTION

Background and Rationale

Oral squamous cell carcinoma (OSCC), an aggressive epithelial malignancy, is the most common oral cancer.[1-4] Each year, more than 500,000 people worldwide are diagnosed with the squamous cell carcinoma of head and neck, the sixth most common neoplasm in the world, in which over 90% arising in the oral cavity.[1, 5] Despite numerous advances in treatment utilizing recent protocols for surgery, radiation, and chemotherapy, the long-term survival has remained at less than 50% for the past 50 years.[6, 7] This outlook is due to the fact that oral cancer is often diagnosed when the disease has already reached an advance stage; 56.2% reached stage IV disease when they were first diagnosed.[8] The 5-year survival of early-stage oral cancer is approximately 80%, while it drops to 19% for late-stage disease.[5] In Thailand, this rate estimated from available data is about 20-40% mainly due to the lack of knowledge about malignancy, cultural and socioeconomic background, patient referral, and the fear of surgery including scalpel biopsy to obtain diagnosis. In addition, a number of patients chose to have traditional herbal treatment rather than surgical procedure. These may be the reasons for delayed diagnosis or treatment, resulting in poor survival.[9] Moreover, OSCC is particularly dangerous because its association to second primary tumor is higher than other malignancies; 35% chances of developing new tumor after the first one is treated.[10] Until now, to obtain diagnosis, the gold standard still histopathological examination of suspicious tissue from surgical biopsy.[11] However, surgical manipulation including incisional biopsy increases risk of cancer cell dissemination into blood circulation.[12, 13] Thus, early detection by non-invasive screening tool can be crucial and persuasive for each patient. Though many studies tried to develop simple techniques for early detection of precancerous and cancerous lesions, including clinical examination with toluidine blue or methylene blue staining[14], chemiluminescence[15], tissue fluorescence[14], exfoliative cyotology by brush biopsy[16], and studies of saliva biochemistry[17, 18], none of these techniques provides consistently high accuracy or significantly impact routine protocol.[11] Additionally, the standard treatment of oral malignancy is surgical ablation with or without adjunctive radiation and chemotherapy. This also results in

miserable quality of life from facial disfiguration, compromised mastication efficiency, impotent pronunciation, swallowing difficulty, and also emotional instability.

Epigenetics, which was first introduced in 1942, came to our interest increasingly in the topic of pathological development. This term, epigenetics, contemporarily refers to the modification of the genome that are heritable during cell division but do not involve a change in the DNA sequence. In other words, it describes heritable changes in gene expression that are not simply attributable to nucleotide sequence variation.[19-21] These days, it is accepted that epigenetics is one of the important features of development and, possibly, progression of several pathological conditions and diseases, such as atherosclerosis, autoimmune diseases, hereditary disorders, and especially cancer. Currently, roles of epigenetics in carcinogenesis are concerned as etiological and predictive factors, which lead us to the new page of molecular studies in determining diagnosis, pathogenesis, and prognosis of many types of cancer[22], including cancer of colon, breast, lung, esophagus, stomach, liver, kidney, prostate, ovarian, and head and neck.[23] By using molecular makers which contain either genetic or epigenetic information concerning the malignancies, many benefits can be achieved. DNA methylation, a modification of DNA in which the methyl group was transferred to DNA sequence, has critical roles in controlling gene activities and architecture of the nucleus of the cell. In normal condition, it is important for maintaining health and preventing diseases. Today, DNA methylation is the best-known epigenetic marker in cancer which both increase and decrease of its level played an important part in initiation and progression of benign cell transforming to an invasive cancer.[23] The cancer genome is frequently characterized by hypermethylation of specific gene promotors concurrently with an overall decrease in the level of 5-methyl cytosine. This hypomethylation of the genome largely affects the intergenic (non-gene) regions of the DNA, particularly repetitive sequences, and transposable elements.[23-28]

LINE-1s, long interspersed nuclear element-1s, methylation was investigated and concluded to have a representative value of genome-wide methylation.[11, 28, 29] Normal tissues from different organs showed a variety of DNA methylation levels[30, 31] and the difference in LINE-1 methylation from several tissue origins was

accounted for this phenomenon.[28] Therefore, it can be inferred that methylation levels of LINE-1s are tissue specific. Many studies have shown significant difference between LINE-1 methylation levels from many types of cancerous tissue and their normal tissue counterparts.[11, 28, 32-34] Interestingly, the hypomethylation of LINE-1s was found not only in the representative cancerous tissue but also the bodily fluid including whole blood,[35] serum,[28, 34] and plasma.[36] Moreover, this phenomenon was also found in oral rinses of oral squamous cell carcinoma patients.[11] Although previous data revealed the tissue-specific character of LINE-1 methylation levels, those methodologies used a pool of samples to represent the specific group. There is no data comparing methylation level in oral rinse with those in two components of peripheral blood- the buffy coat and plasma, within an individual. Furthermore, the methylation levels in buffy coat and plasma of oral squamous cell carcinoma patients have not been reported.

This study aimed to explore the methylation levels of LINE-1s in the two components of peripheral blood which were buffy coat and plasma of oral squamous cell carcinoma patients. Moreover, LINE-1 methylation levels in oral rinse, buffy coat and plasma, of the same individual in both non-cancer subjects and oral squamous cell carcinoma patients were also investigated.

Research Questions

1. Whether LINE-1 methylation in the two components of peripheral blood, buffy coat and plasma, of OSCC patients differ from those of control subjects.

2. Whether LINE-1 methylation within each control subject differs among different DNA samples, oral rinse, buffy coat and plasma.

3. Whether LINE-1 methylation within each OSCC patient differs among different DNA samples, oral rinse, buffy coat and plasma.

Objectives

1. To investigate methylation levels of LINE-1s in buffy coat and plasma, of OSCC patients.

2. To study the differences between LINE-1 methylation levels in oral rinse, buffy coat, and plasma intra-individually.

Hypothesis

Hypothesis 1

Ho : Methylation levels of LINE-1s in peripheral blood components, which were buffy coat and plasma, of OSCC patients were not significantly different from those of control subjects.

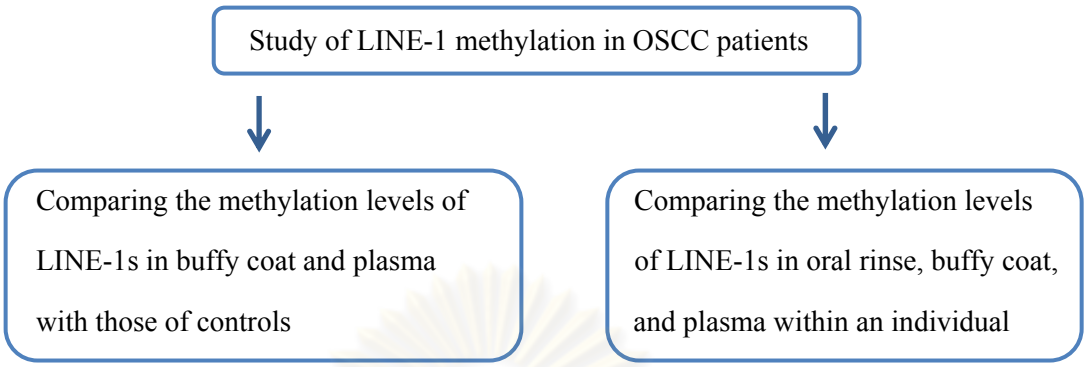
Ha : Methylation levels of LINE-1s in peripheral blood components, which were buffy coat and plasma, of OSCC patients were significantly different from those of control subjects.

Hypothesis 2

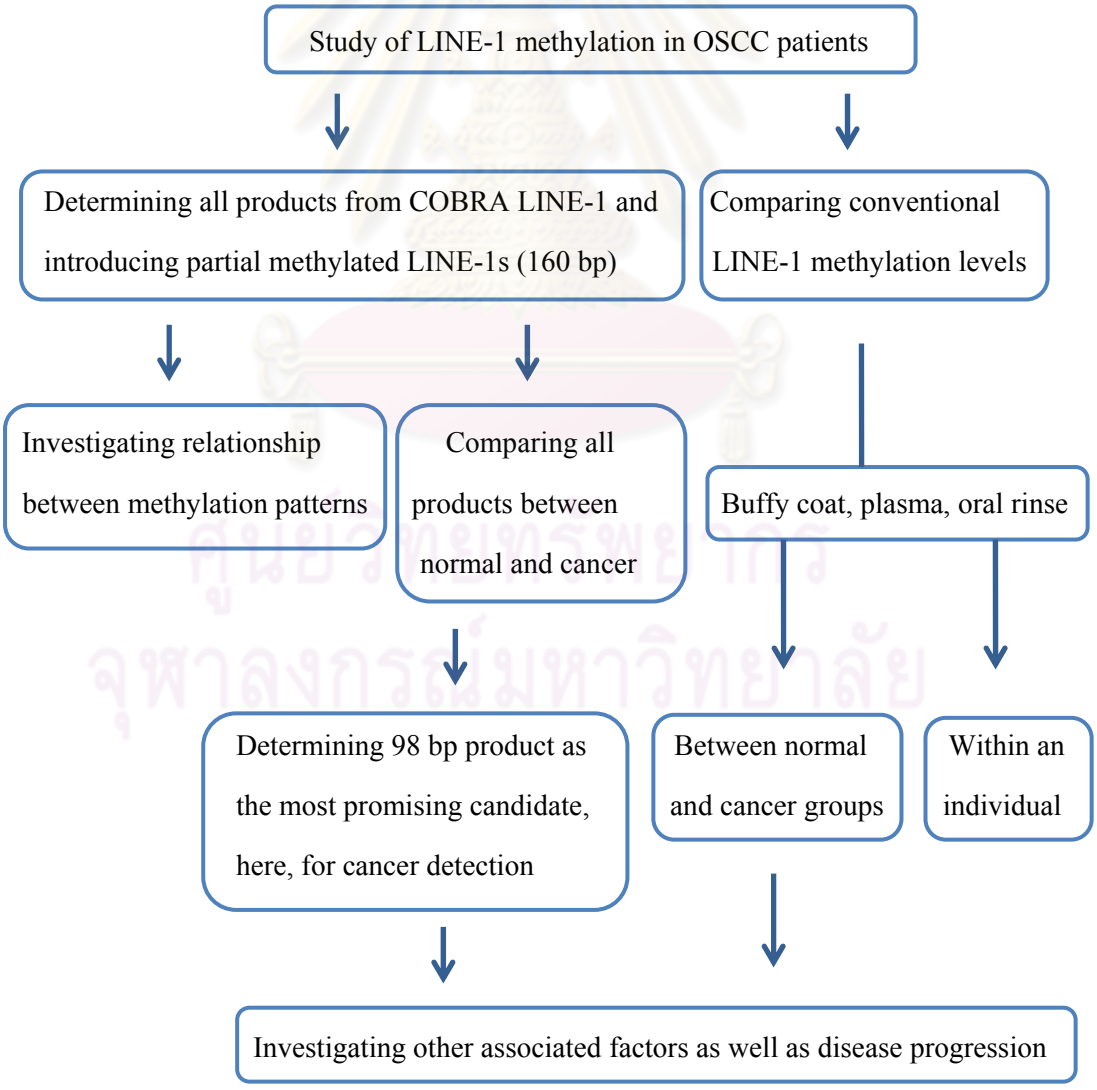
Ho : Methylation levels of LINE-1s in oral rinse, buffy coat, and plasma were relatively the same when compared intra-individually.

Ha : Methylation levels of LINE-1s in oral rinse, buffy coat, and plasma were significantly different when compared intra-individually.

Methodology Framework Proposed



Methodology Framework Accomplished



Keywords

Oral squamous cell carcinoma (OSCC), Long interspersed nuclear element-1s (LINE-1s), Hypomethylation, Complete unmethylation (98 bp), COBRA LINE-1, Buffy coat, Plasma, Oral rinse

Type of Research

Analytical cross-sectional research, Translational research

Expected Benefits

1. Changes of LINE-1 methylation and complete unmethylation (98 bp) in OSCC are clarified and molecular pathogenesis by losing and gaining methylation of OSCC is better understood.
2. Complete unmethylation of LINE-1s in oral rinse, which demonstrated the best performance as biomarker, can eventually be used for early detection of OSCC and result in an improved survival as well as quality of life of OSCC patients.
3. With the new knowledge of non-cell type-specific in complete unmethylation (98 bp), more tests using pool of mixed DNA samples with higher sensitivity and specificity may further be developed.
4. By COBRA LINE-1, new calculation method with partial methylation pattern included can be adopted for investigation of other types of cancer.

Time Schedule and Administration

Table 1 Schedule and administration of study year 2008

Activities	Study year 2008											
	May	Jun	Jul	Aug	Sep	Oct	Nov	Dec	Jan	Feb	Mar	Apr
1. Preliminary												
1.1 Review literatures	←	—	—	—	—	—	→					
1.2 Research planning			←	—	—	→						
1.3 Proposal development					←	—	—	—	—	—	—	→
1.4 Proposal Presentation												
1.5 Ethics committee												
2. Data collection and processing												
3. Data analysis												
4. Data report												
4.1 Publication development												
4.2 Thesis development and defense												

Table 2 Schedule and administration of study year 2009

Activities	Study year 2009											
	May	Jun	Jul	Aug	Sep	Oct	Nov	Dec	Jan	Feb	Mar	Apr
1. Preliminary												
1.1 Review literatures												
1.2 Research planning												
1.3 Proposal development												
1.4 Proposal Presentation	↔											
1.5 Ethics committee		↔										
2. Data collection and processing		←	—	—	—	—	—	—	—	→		
3. Data analysis								←	—	—	—	—
4. Data report												
4.1 Publication development												
4.2 Thesis development and defense												

Table 3 Schedule and administration of study year 2010

Activities	Study year 2010											
	May	Jun	Jul	Aug	Sep	Oct	Nov	Dec	Jan	Feb	Mar	Apr
1. Preliminary												
1.1 Review literatures												
1.2 Research planning												
1.3 Proposal development												
1.4 Proposal Presentation												
1.5 Ethics committee												
2. Data collection and processing												
3. Data analysis	→	→	→	→	→	→	→	→	→	→	→	→
4. Data report												
4.1 Publication Development	←	—	—	—	—	→					↔	→
4.2 Thesis Development and defense							←	—	—	—	→	

Budget

DNA extraction reagents	Baht 8,000
Plasma extraction kit	Baht 42,000
Bisulfite treatment kit	Baht 46,500
PCR reagents	Baht 13,000
Restriction enzyme kit (*Taq*I, *Tas*I)	Baht 12,000
Electrophoresis reagents	Baht 7,000
Blood collection devices	Baht 1,000
Appendorfs, tubes, pipette and tips	Baht 23,000
Total	Baht 152,500

CHAPTER II

LITERATURE REVIEW

Oral Squamous Cell Carcinoma (OSCC)

Oral squamous cell carcinoma (OSCC) is defined as an invasive epithelial neoplasm with varying degrees of squamous differentiation and a propensity to early and extensive lymph node metastasis. Although cancer of the oral cavity includes one that extends from the lips to the palatoglossal folds and may be either epithelial, mesenchymal, or haematolymphoid, more than 90% of malignant neoplasms of the oral cavity is squamous cell carcinoma of the lining mucosa.

Incidence and Epidemiology

OSCC occurs predominantly in the fifth and sixth decades of life. Mostly, males are affected more often than females because of their heavier habits of both tobacco and alcohol. However, this ratio is shifting to the opposite direction due to increasing number of female smokers. There is a significant increase in incidence in younger population, particularly males in many developed and developing countries especially those in the west.[37] In Thailand, the estimated incidence rate of oral cancer is 5.2 and 4.6 per 100,000 population in males and females respectively. Such high incidence rate is observed in Songkhla, Khon Kaen, and increasingly in Rayong where industrial operation has been started later than others. In northern and north-eastern part of Thailand, such as Chiang Mai and Khon Kaen, betel quid chewing which is a known contributing factor is common in female villagers. In the south, such as Songkhla, a relationship of radium contaminated well water consumption was found associated with upper digestive tract including oral cavity neoplasm.[38] At the global level, the estimates of worldwide incidence, new cases and mortality are varied among different global regions as well as the international organizations collecting cumulative data. Higher incidence of oral and pharyngeal cancer, compared to other types of cancer, is in Indian and African countries, especially the male population.[39] According to World Health Organization (WHO) report in 2005, oral and oropharyngeal cancer is the eleventh most common cancer worldwide. Several studies

reported head and neck cancer as the sixth most common cancer, in which more than 90% is squamous cell carcinoma of the oral cavity.[2, 40]

Etiology

The cause of OSCC is not exactly known, however, it is proposed to be multifactorial. It is likely that more than a single contributing factor is needed to produce such a malignancy. Both extrinsic factors, including as tobacco smoking, alcohol consumption, and betel quid chewing, as well as intrinsic factors, such as systemic conditions and genetic disorders, may play different roles in pathogenesis.[1, 41]

Tobacco smoking and smokeless tobacco

Of all factors believed to contribute to oral cancer, tobacco is regarded as the most important. About 80% of OSCC patients are smokers.[42] Furthermore, it is found that smokers who continue smoking after the diagnosis of this disease have two to six times greater risk in developing second primary carcinoma of aerodigestive tract than smokers who quit smoking.[41] Not only that it is dose dependent, OSCC development also depends on types of smoking. Pipe, cigar smoking, and smokeless tobacco carry a greater risk than does cigarette smoking. The habit of reverse smoking which the burning end is held inside the mouth considerably elevates one's risk for oral cancer.[42]

Alcohol consumption

Alcohol, although not believed to be a carcinogen itself, appears to add to the risk of oral cancer development when consumed excessively.[41, 42] It is well-established that the combination of alcohol and tobacco abuse over long periods may increase risk by a factor of 15 or more.[41] It is found that smoking together with alcohol consumption were responsible for rising incidence of oral cancer in young people in northern Thailand.[8] In addition, nutritional deficiencies, associated with heavy alcohol consumption, contribute to an increased risk of cancer development.[41]

Betel quid/ betel nut chewing

Betel quid is a compound of natural substances, areca palm nuts, betel leaf, staked lime, and tobacco leaf, which is chewed for psychostimulating effect. Betel

quid or betel nut chewing is most common in Asia and found associated with significant development of precancerous lesions. Beyond our awareness, more than 600 million persons worldwide chew these quids on a regular basis.[41] Reactive oxygen species in the betel nut can be involved in tumor initiation process by compromising oral mucosa and salivary fluid. Thus other toxic chemical can penetrate and damage normal biological process.[3] Fifty percent of oral cancers are attributable to betel chewing, in high chewing-populated areas.[43] The use of betel quid together with smoking increase risk of oral cancer by 8-15 times, when betel quid habit without smoking reach about 1-4 times.[44]

Iron deficiency

Iron deficiency, especially the severe and chronic form is associated with elevated risk of OSCC of the posterior mouth and oropharynx. People who are deficient in iron tend to have impaired cell mediated immunity. Further, iron is essential to the normal functioning of epithelial cells of the upper digestive tract which includes oral cavity. In deficiency states, these epithelial cells turn over more rapidly and produce an atrophic or immature oral mucosa.[41]

Vitamin-A deficiency

Excessive keratinization of the mucous membrane is one of the results of vitamin-A deficiency. Many researchers have suggested a productive and preventive role in oral precancer and cancer. Long-term therapy with retinoic acid and betacarotene is found associated with regression in the severity of dysplasia within such lesions.[41]

Candidal infection

Oral candidiasis is frequently cited as an oral precancerous lesion,[45] especially hyperplastic type. Certain strains have been shown to produce nitrosamines, chemicals that implicate in carcinogenesis, however, to this date, the evidence to suggest this role is largely circumstantial.

Oncogenic viruses: Human papillomaviruses (HPVs)

Viral agents capable of integration into the host's genome may be particularly dangerous and potentially could commandeer the host's ability to regulate normal growth and proliferation of infected cell. The oncogenic viruses may immortalize the host cell or disrupt genetic material thereby facilitating malignant transformation.[41] In recent meta-analysis, HPV genomic DNA was detected in approximately 26% of all head and neck squamous cell carcinoma (HNSCC) by sensitive polymerase chain reaction (PCR)-base method.[46] HPVs not only contribute to oral cancer development, but also to carcinoma of the pharyngeal tonsil, larynx, esophagus, and genital organs as well. HPV subtypes 16, 18, 31, and 33 are the strains most closely associated with dysplasia and OSCC.[41]

Oncogenes and tumor suppressor genes

Oncogenes and tumor suppressor genes can be acted on by a variety of causative agents. Normal genes or proto-oncogenes are transformed into activated oncogenes through the actions of viruses, irradiation, or chemical carcinogens. Once activated, they may stimulate production of genetic material through amplification and overexpression of the involved genes. As a result, a wide range of neoplasms, including OSCC are initiated. Tumor suppressor genes, on the other hand, allow tumor production indirectly when they become inactivated or mutated. Genetic aberrations commonly identified in OSCC include abnormalities of the ras, myc, and epidermal growth factor (EGFR) oncogenes, and the p53, pRb, p16 and E-cadherin tumor suppressor genes.[1, 3, 41]

Pathogenesis

Oral cancer, like most of other malignancies, arises from the change of normal cell cycle control affected mainly from genetic events. Conceptually, oral cancer progress through two biologic stages. The first stage is loss of cell cycle control through increased proliferation and decreased apoptosis. This appears in form of carcinoma in situ where an increased number of dividing cells can be seen in all levels of the epithelium. The second stage is increased tumor cell motility, leading to invasion and metastasis. In this process, neoplastic epithelial cells penetrate the

basement membrane and invade underlying tissues and further reach regional lymph nodes.[42] Each etiological factor including genetic, epigenetic, and environmental factors play parts in different roles from normal transformation to precancerous lesion and eventually cancer development.[47] In 1953, Slaughter et al proposed the changed epithelial cells of upper aerodigestive tract, including oral mucosa, as a model of "field cancerization". In this term, oral mucosa can sustain initial injury from repeated exposure to carcinogen but it contains accumulation the affected genetic alteration. When large area is exposed, the risk of developing multiple tumors from separate clones is high.[48] Braakhuis et al proposed progression model in 2004 to further explain cellular processes, also relating with the clinical appearances. The basal cells with genetic defects divide, accumulate more changes from carcinogenic insult, and push normal cells aside.[49] Unfortunately, existing abnormalities are macroscopic invisible and probably microscopic undetectable. These facts result in incomplete tumor resection as well as occurrence of cancer at another site.[47]

Clinical Features

The most common site for intraoral carcinoma is the tongue, usually the posterior lateral and ventral surfaces. Floor of the mouth is affected almost as frequently in men but is involved much less commonly in women. Other sites of frequent involvement include soft palate, gingival, buccal mucosa, labial mucosa, and hard palate respectively. OSCC can present in a various clinical presentation, including exophytic, endophytic, indurated, ulcerative, leukoplakic (white lesion), erythroplakic (red lesion), and erythroleukoplakic (red-and-white lesion) mass.[41] Symptoms associated with the lesions include pain, referred pain to the ear, malodor from the mouth, bleeding, neck swelling, difficulty in speaking, opening of the mouth, chewing, swallowing, and weight loss. Small OSCC usually presents with asymptomatic minimal findings, therefore in suspicious lesions, high attention is needed especially if the patients have tobacco and alcohol habits. Extremely advanced cancers present as ulceroproliferative growth with necrosis area and extension to surrounding structures, such as bone, muscle, and skin.[37, 50]

OSCC of the tongue is more common in younger patients. It is typically asymptomatic but when deep invasion occurs, pain and dysphagia may be the chief

complaints of the patients. Most erythroplakic patches that appear on the tongue are either in situ or invasive OSCC. The most common location of cancer of the tongue is posterior-lateral border, accounting for as many as 45% tongue lesions. In contrast, it is hardly occurs on dorsum or tip of the tongue. The lesions of the tongue are more troublesome than the others because of their silent progression in the area that is difficult to visualize. As a result, the lesions are more often advanced or have metastasized regionally by the time of discovery. OSCC of the floor of mouth usually presents as a painless, non-healing, and indurated ulcer. The lesion occasionally infiltrates surrounding soft tissue and limits tongue mobility. In the palate, it commonly occurs on the soft palate whereas hard palate carcinoma is relatively rare. Lesion on this area generally presents as asymptomatic red and white plaque or ulcerated and keratotic mass. Lesions of the buccal mucosa and lesions on the gingival each account for approximately 10%.[42] In betel quid chewers, the buccal indurated margin or verrucous growth will be seen upon the preferred side of chewing.

Radiographic Features

Abnormal radiographic findings are evident when neoplastic cells invade and destroy underlying bone. However, the lesion may be either severely painful or completely painless. Bone involvement is characterized by an irregular, “moth-eaten” radiolucency with ill-defined or ragged margins, which must be differential diagnosed from osteomyelitis of the jaw bone.[41] The invasion of OSCC to alveolar ridge may cause destruction of the teeth-supporting bone producing “teeth floating in space” appearance.

Histopathologic Features

OSCC arises from dysplastic epithelium and is characterized histopathologically by invasive islands and cords of malignant squamous epithelial cells. Invasion is represented by irregular projection of pathologic epithelium through the basement membrane and into subepithelial connective tissue. Individual squamous cells and sheets or islands of cells are seen to be thriving as independent entities within the connective tissues, without attachment to the surface epithelium. Characteristics of the cancerous cells are shown as hyperchromatic nuclei, increased

nuclear-cytoplasmic ratio, cellular and nuclear polymorphism as well as mitotic figures. The product of epithelial cells, keratin, is found as keratin pearls within the epithelium.[41] The neoplastic cells may surround and destroy blood vessels and invade into the lumina of veins or lymphatics. A significant inflammatory host response is usually found surrounding the nests of invading tumor cells. Lymphocytes, plasma cells, and macrophages may be seen in large numbers.[42]

Histopathological degree is ranged in grading from low grade (well differentiated OSCC or grade I) to high grade (poorly differentiated OSCC or grade III or IV). In low-grade OSCC, tumor cells are mature enough to closely resemble its tissue of origin while in high-grade OSCC, the cells present with more pleomorphism but less or no keratin production. Because of most OSCC are moderately or well-differentiated lesions therefore keratin pearls and individual cell keratinization are usually evident.

Metastasis

Normally, OSCC will metastasize to regional lymph nodes, however it sometimes spread through bloodborne routes to lungs, liver, or bone. Like other malignancies, metastasis of OSCC requires basic steps of biologic events which are invasion through the basement membrane, entrance into lymphatics or blood vessels, survival of cells in the vessels against numerous immune cells, escape from the circulation to new tissue, as well as implantation and establishment of self-staining colony. OSCC in the anterior floor of the mouth, anterior alveolar ridge, anterior buccal mucosa, and lower lip will metastasize firstly to submental triangle lymph node of the same side (level I). OSCC located more posterior in the floor of mouth, in the tongue, posterior buccal mucosa, and posterior alveolar ridge will spread to the submandibular triangle (level II). OSCC that appears in the retromolar trigone, tonsillar fossa, and pharyngeal tongue will often first affect the jugulodigastric lymph node (level III).[51] Maxillary and oropharyngeal OSCC tend to metastasize through jugulodigastric or retropharyngeal node group.

In South Asia, more than two-thirds of the patients with buccal mucosa or gingival cancers present with submandibular lymph node enlargement and more than three-fourths of the patients with tongue, floor of mouth cancers present with neck

swelling, implying clinically obvious lymph node metastasis.[37] It is unfortunately that such distant metastasis is often occult at the time of discovery of the primary lesion.[5] In stage I, II, and III, incidence of distant metastasis of oral cancer is approximately 3% whereas it reaches 10% in stage IV patients. Further examinations consist of an x-ray of lungs and liver tests.[52]

Staging

According to the sixth edition (2002) of American Joint Committee on Cancer (AJCC), oral squamous cell carcinoma is classified into four stages according to clinical findings of tumor size (T), lymph node involvement (N), and distant metastasis (M).[53]

- *Primary Tumor Size (T)*

Tx: No available information on primary tumor

T0: No evidence of primary tumor

Tis: Only carcinoma in situ at primary site

T1: Tumor 2 cm or less in greatest diameter

T2: Tumor more than 2 cm but not more than 4 cm in greatest diameter

T3: Tumor more than 4 cm in greatest diameter

T4: (Lip-vermilion border) Tumor invades through cortical bone, inferior alveolar nerve, floor of the mouth or skin of face i.e. chin or nose

T4a: (Oral cavity) Tumor invades adjacent structures e.g. through cortical bone, into deep (extrinsic) muscle of tongue (genioglossus, hyoglossus, patatoglossus, and styloglossus), maxillary sinus, skin of face

T4b: Tumor invades masticatory space, pterygoid plates, or skull base and/or encases internal carotid artery

Superficial erosion alone of bone or tooth socket by gingival primary is not sufficient to classify a tumor as T4.

- *Regional Lymph Node Involvement (N Stage)*

Nx: Regional lymph nodes cannot be assessed

N0: No regional lymph node metastasis

N1: Metastasis in a single ipsilateral node, 3 cm or less in greatest dimension

N2: metastasis in -

N2a: Single ipsilateral lymph node more than 3 cm but not more than 6 cm in greatest dimension

N2b: Multiple ipsilateral lymph nodes, none more than 6 cm in greatest dimension

N2c: Bilateral or contralateral lymph nodes, none more than 6 cm in greatest dimension

N3: Metastasis in a lymph node more than 6 cm in greatest dimension

- *Distant Metastasis (M Stage)*

Mx: Presence of distant metastasis cannot be assessed

M0: No distant metastasis

M1: Distant metastasis is present

- *TNM Clinical Staging*

Table 4 TNM clinical staging categories

Stage	**T**	**N**	**M**
Stage 0	Tis	N0	M0
Stage I	T1	N0	M0
Stage II	T2	N0	M0
Stage III	T3	N0	M0
	T1	N1	M0
	T2	N1	M0

Stage	T	N	M
Stage IVA	T3	N1	M0
	T4a	N0	M0
	T4a	N1	M0
	T1-T4a	N2	M0
Stage IVB	Any T	N3	M0
	T4b	Any N	M0
Stage IVC	Any T	Any N	M1

Differential Diagnosis

Oral potentially malignant (previously termed as premalignant) lesion can resemble the malignant status. Among all, erythroplakia, leukoplakia, and lichenoid lesions are the most important.[1] Ulcerative lesion, especially, on top of erythroplakia or aggressive candidiasis may also look alike. The depressed, irregularly shaped, ulcerated central area with a surrounding "rolled" border of normal, red or white mucosa happens, the lesion may be similar to granulomatous lesions, such as deep fungal infection, tuberculosis, tertiary syphilis, Crohn's disease, and chronic traumatic ulcers. In the palate and contiguous tissues, midline granuloma and necrotizing sialometaplasia would be serious diagnostic considerations. Careful history taking is especially important, and biopsy findings are crucial to confirm the diagnosis.[41]

Diagnostic Procedures

The gold standard in obtaining the definite diagnosis is still lesion biopsy. Incisional biopsy without breaking marginal biological border of tumor spread is highly recommended. In patients with both obvious primary lesion in the oral cavity and enlarged cervical lymph node, the biopsy is always taken from the primary site. Special studies that use monoclonal antibodies directed against cytokeratins may be needed to distinguish high-grade OSCC from other malignancies.

Additionally, chemiluminescence and tissue fluorescence are reported as potential diagnostic tool in detection of oral cancer.[14, 15] Tissue staining and

exfoliative cytology from brush biopsy are also used as diagnostic aids. The molecular changes which are observable in circulating plasma or serum, and body fluids[11], can be representatives or markers for oral cancer. These are simple and non-invasive or minimally invasive procedures for early detection and tumor surveillance.[28, 34, 35] More information regarding tumor marker development as well as potential markers in clinical practice is further reviewed in the next section. Although various biological markers have been proposed, their accuracies are still doubted. So far, none has high impact on routine clinical care. Comprehensive histopathological staging of pathological specimens is still an important determinant of postoperative management and prognosis prediction.[5] TNM staging, grade, and depth of tumor invasion remain important factors in predicting the course of disease.[54]

Treatment and Prognosis

Today, surgery, radiotherapy, and chemotherapy are still the mainstays of cancer treatment. Not only that, immunotherapy, gene therapy, and antiangiogenic therapy with high hope are becoming more and more popular these days. However, they have not been routinely used.[51] Surgery remains the most successful therapeutic modality for OSCC. Extension of excision depends on invasion pattern of the tumor to surrounding structure. Normally wide excision is recommended. As a result, facial disfiguration and loss of important structures in of the masticatory system are the primary post-operative complications. In case of lymph node involvement, several types of neck dissection should be concerned. Nevertheless, indication for neck dissection and the type of neck dissection, especially in the N0 neck is still a controversy. In general, OSCC that locates anterior to circumvallate papilla, a supraomohyoid neck dissection should be considered. For oropharyngeal lesions, either level I to IV or level II to IV lymph node should be dissected in case of involvement. There was a report that occult node metastasis is about 20-30% though it is found negative clinically and radiographically. However, some investigation yielded lower percent and therefore, they suggested that only primary tumor with high chance of metastasis should undergo an elective neck dissection in clinically N0 cases.

The clinical stage of the disease guides the treatment of OSCC. Post-surgical radiotherapy is indicated when tumor is too large to be included in the best surgical margin, or there is evidence of palpable lymph nodes.[41] Chemotherapy is used in combined drug protocol to help kill tumor cell in a greater proliferative rate than tumor cell population. These adjuctive therapies produce significant side effect, such as mucositis, xerostomia, and trismus, leading to pain, difficulty in speaking, dysphagia, dental caries, and osteoradionecrosis. In most of the cases, the patients' quality of life is certainly compromised.

A statistically significant relationship among histologic grading, tumor size, and locoregional involvement and survival rates were reported whereas gender and risk factors were found unrelated.[2] Nonetheless, it was reported that the most important factor in the prognosis of OSCC is the status of the cervical lymph nodes. The cure rates drop to nearly half with regional lymph node involvement. Clinical staging seems to correlate much better with prognosis than microscopic grading, thus early detection of the pathology gives a better stage and improve overall prognosis.

Cancer Biomarkers

Definition and Classification of Cancer Biomarkers

A biomarker is defined as "A characteristic that is objectively measured as an indicator of normal biological processes, pathogenic processes, or pharmacological response to a therapeutic intervention", according to the US National Institute of Health's (NIH) Working Group and the Biomarkers Consortium (http://www.biomarkersconsortium.org). In terms of cancer, NIH's National Cancer Institute (NCI) describes it as "A biological molecule found in blood, other body fluids, or tissue that is a sign of a normal or abnormal process, or of a condition or disease". Generally speaking, cancer biomarker is any substance that can be measured in the body or body's product and can identify the presence, process or status of cancer. It can be released from the cancer itself or it can be a response of the body to the cancer, such as antibody.

One of the goals in cancer research nowadays includes finding markers that can be used to detect, predict, monitor the disease and eventually improve overall

quality of life. Not only that, cancer biomarker study is an approach to understand carcinogenesis[55] and probably lead to prevention and personalized treatment for each cancer patient.

Table 5 Classification of cancer biomarker

Based on Objective and Disease Status	Based on Nature of Biomolecules	Based on Other Criteria
•Screening Biomarkers •Diagnostic Biomarkers •Prognostic Biomarkers •Predictive Biomarkers •Monitoring or Surveillance Biomarkers	•Genetic Biomarkers - DNA - RNA •Epigenetic Biomarkers - Methylation •Proteomic Biomarkers •Glyco Biomarkers	•Pathogenic Biomarkers - Viral - Bacterial •Imaging Biomarkers •Biochemical Markers - Oncofetal antigens - Cancer antigens - Hormones - Tissue receptors - Enzymes - Serum/tissue protein

Modified from Mishra,[56] Malati,[57] and American Cancer Society (http://www.cancer.org)

Until now, no uniform classification has been established. However, many consensuses, institutes, and reviews have classified cancer markers by several grounds. For instance, based on purpose, screening biomarker is used for identifying cancer in people with no symptoms of disease or finding cancer in its earliest stage. However, predictive biomarker is the determinant of cancer treatment effectiveness. Moreover, these signature molecules can be classified based on their nature, for example, DNA, RNA, protein, and carbohydrate. Viral agents associated with the lesion are also counted, such as Epstein-Barr Virus (EBV) as a marker of nasopharyngeal carcinoma and Human Papillomavirus (HPV) as a marker of cervical as well as oral cancers. Some authors have suggested imaging, sometimes conjunctively to other information, as a marker of cancer. For example, prostate specific antigen (PSA) in correlation with bio-imaging data might be used to detect prostate cancer. Furthermore, a well-known mammogram is extensively used for routine breast cancer screening. It must be remembered that carcinogenesis is multi-step process with many factors involved therefore some markers in these categories are overlapping (Table 5).

Characteristics of Cancer Biomarkers

An ideal cancer marker should be, first, highly sensitive and specific for particular type of cancer. If not, it should be universal for all type of malignancies. Second, it should serve its primary purpose. In other words, it should be able to detect cancer at early stage as well as transforming premalignant lesion (screening marker) or accurately correlate with advancement of clinical tumor status (prognostic marker). Third, it is best to obtain from simple and non-invasive intervention. Moreover, its benefits should be applicable to both men and women of all ages and races. Further, marker test should be easily reproducible and standardize among laboratories. Additionally, small measurement error or biological variation should not affect capability of the markers. Last, examination method ought to be cost-effective.[55-57] Although no such marker has achieved all prerequisites, several markers met certain qualifications.

Examples of Cancer Markers in Clinical Practice

From the earliest days of human chorionic gonadotropin (HCG) identifying pregnancy as well as gestational trophoblastic disease (cancer of the placenta), or the discovery of Bence Jones protein and tumor specific antigen carcinoembryonic antigen (CEA) relating to colon carcinoma in 1965, to this present day, substantial number of studies were dedicated to bringing the cancer markers to clinical practice. Although there was no marker definitively for routine usage in the global picture, many of them are reliable and widely used for screening, detecting, predicting, and monitoring the malignancy.

A few examples of regularly mentioned and commonly used cancer markers are raised below.[56, 58, 59]

- Prostate specific antigen (PSA)

PSA is small glycoprotein with protease activity which is specific for prostate tissue. In 60-70% of males with prostate cancer, PSA highly increased compared to the minimal level in normal adults. However, recent report showed that PSA was not found in every patient with prostate cancer. More molecular markers regarding this cancer are currently investigated and validated.

- Alpha-fetoprotein (AFP)

In healthy adults, who are not pregnant, rarely have detectable AFP in blood. Increased AFP level is often associated with liver cancer and its roles of early detection, diagnosis, and monitoring were demonstrated. Additionally, AFP has been approved by US Food and Drugs Administration (FDA) for diagnosis and monitoring of testicular cancer.

- Carcinoembryonic antigen (CEA)

CEA is a part of cell membrane. It is shed into blood of over 50% of patients with breast, colon, lung, gastric, ovarian, pancreatic, and uterine cancer. The increased level of CEA is tended to be used for prognostic prediction of colorectal cancer.

- Cancer antigen (CA)-125

This FDA-approved is used for diagnosis and monitoring of women with ovarian cancer. The elevated serum is detected in over 75% of the patients. Therefore, CA-125 was proposed to be useful for primary and recurrence tumor detection and therapy. However, CA-125 test is affected by pregnancy and menstruation cycle.

- Estrogen receptor (ER)

Normally, ER is found in the nucleus of breast and ovary tissues. It was reported with uses of identifying the patients who responded to the treatment of breast cancer.

These mentioned markers, as well as the following examples: CA 19-9, B-type natriuretic peptide (BNP), transferrin receptor, insulin-like growth factor (IGF-II), troponin T, interleukin-2 receptor, and insulin-like growth factor-binding protein (IGFBP), have been approved the US food and drug administration (FDA).[55, 58]

Oral Squamous Cell Carcinoma Markers

Significance of oral squamous cell carcinoma markers

Although definitive diagnosis of cancers is determined by histopathology of the obtained OSCC-suspected tissue sample, surgical intervention in biopsy procedure may promote dissemination of cancer cells into circulation, increasing chances of metastasis. Incisional biopsy, though believed to be a proper manipulation for cancer tissue sampling, Kusukawa et al demonstrated that 20% of OSCC patients, who went through incisional biopsy procedure, had transient detectable cytokeratin 19 (CK19),

which is important filament making up cytoskeleton of normal and malignant cells of epithelial origin, in their blood stream. On the contrary, CK19 was not detected in any patient undergone excisional biopsy.[12] However, in clinical practice, choosing incisional or excisional does not rely on only this fact. Tumor size, location as well as further treatment are the main considerations. Dyavanagoudar et al also reported RT-PCR detecting CK19 in blood of 16 % of OSCC patients (4 in 25 patients), 15 minutes after incisional biopsy was done. CK19 was not found in controls, submucosal fibrosis and leukoplakia. Despite CK19 was detected after incisional biopsy in only stage III and IV OSCC, this does not change the fact that surgical intervention disrupts connective tissue and basement membrane, major physical barriers to migration of tumor cells,[13] and possibly increases risk of cancer spread at any stage. Moreover, as minimally invasive as it was performed without anesthesia, fine needle aspiration for cytology test may not prevent risk of tumor cell dissemination and second tumor development.[60] Additionally, a study in animal model (29 rats) showed that although complete tumor resection was done beyond clinical margin of the lesion, it might not be able to prevent spread of cancer, determined by detection of cancer DNA in blood during the operation.[61] Simple and least invasive procedures, such as saliva or oral rinse collection, mucosal swab, as well as blood drawn, to obtain OSCC-specific information are more attractive for OSCC patients.

Moreover, with biopsy process, choosing and manipulating tissue site from part of the lesion are subjective to operator and assessing the specimen is subjective to pathologist. It is definitive if biopsy result is positive. However, missed invading cancer cells left in the intraoral remaining lesion results in misinterpretation as dysplasia or other potentially malignant lesion.[1] Therefore, treatment as planned may not be sufficient. Slaughter et al also reported an incidence of OSCC together with cancer elsewhere (11% of total OSCC cases).[48] In addition, oral cancer is particularly dangerous because of its high risk of developing the second primary tumor. According to The Oral Cancer Foundation, patients who survive the first encounter have up to a twenty-fold increased risk of second cancer development (www.oralcancerfoundation.org). Close investigation as well as monitoring are very important.

Further, although oral cavity is simply accessible and oral lesion is easily noticed. Most of the OSCC cases have reached stage IV at the time of diagnosis. One of the reasons is patient ignorance or effortless to seek proper treatment. Also, in small health care services where facilities and resources are not readily equipped, surgical biopsy may not be performed in the first detection visit. Patient referral as well as impression of surgery obtaining tissue sample can discourage the patients and even delay diagnosis.

Survival of the patients depends on locoregional and distant metastasis.[1, 4] However, occult node investigation is difficult. Traditionally, staging of the neck is performed by physical examination and imaging. Sentinel node biopsy (lymphoscintigraphy), though coming of interests, had no evidence of superiorly to conventional imaging in occult node identification.[4] Correct evaluation of lymph node status and treatment plan, especially in T1 and T2, may help eliminate cancer spread or spare the patients from unnecessary neck dissection procedure which certainly improve quality of life. Moreover, since TNM staging is not individualized and its improvement is needed, relying on alone does not provide proper treatment for every patient. The seventh edition of new TNM staging by American Joint Committee on Cancer (AJCC), launched in 2010, has mentioned a misunderstanding in M category of the old version (2002) and proposed new modifications for several cancers. This edition also emphasized on the new direction of personalized medicine.[62] Details in genetic or epigenetic level may be useful for a tailor-made treatment of each patient. In these mentioned circumstances, routine blood drawn and saliva-based sample collection method to achieve supported information would become more persuasive for both the patients and health care providers.

Sources of DNA for Oral Squamous Cell Carcinoma Markers

To determine the presence of OSCC, source of DNA representing genetic or epigenetic alterations of the malignancy is bodily fluid in direct contact, draining or bathing the tumor lesion.

- Oral rinse and saliva

Exfoliated cancerous oral epithelia especially from ulcerative surface of the lesion or naked DNA can be obtained in saliva or by rinsing oral cavity with normal

isotonic saline solution. Directly onto the lesion, mucosal swab can pick up cells representing cancer. Apart from the cells themselves, changes resulting from cell transformation can also be found in saliva as well as salivary rinse. These saliva-based fluids contain not only DNA but also RNA, and many macromolecules, especially proteins, such as enzymes, cytokines, and growth factors, resulting from carcinogenesis. Some examples of reviewed proteomic marker candidates in saliva included cancer antigen 125 (CA 125), carcinogenic embryonic antigen (CEA), squamous cell carcinoma associated antigen (SCC), matrix metalloproteinase-2 (MMP-2), MMP-11, interleukin 6 (IL-6), interleukin 8 (IL-8), Cyfra 21-1, p53 antibodies, telomerase, tumor necrosis factor-alpha (TNF-α), transferrin, α-amylase.[63-65]

Saliva and saliva rinse have particular benefits for OSCC marker over other types of sample. They can be repeatedly collected, stored and transported easier than other sample types. With the least invasive collection protocol and only a few simple equipments required, the patient compliance is high and the cost is low. Importantly, as it has direct contact to the lesion, it tends to be the great representative of OSCC. In addition, saliva-based fluids were reported in consideration with not only oral cancer, but also breast[66] and ovarian cancer[67] as well.

- Circulatory blood

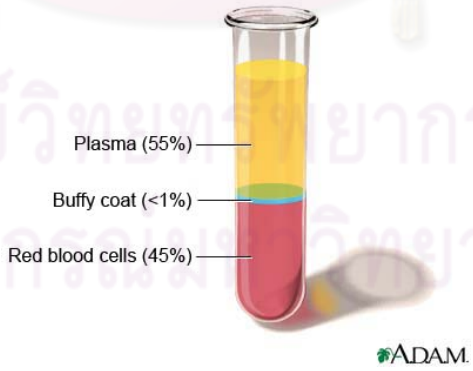


Figure 1 **Blood components.** Blood samples collected with anticoagulant will be seen in 3 separate layers, plasma, buffy coat containing white cells and platelets, and packed red blood cells. *(picture from http://www.pennmedicine.org)*

Blood circulating throughout the body contains plasma as liquid component and formed elements consisting of red blood cells, white blood cells, and platelets. After blood collection with anticoagulant and centrifugation, blood components are separated into 3 layers; plasma (top layer), buffy coat (middle layer), and red blood cells (bottom layer).

The buffy coat layer is composed with platelet and white blood cells. Mononuclear leukocytes such as lymphocytes can be mixed with the platelet. Since granulocytes have a little higher density, they reside in the bottom of the buffy layer, next to the below red cells. As the density of carcinoma cells were lighter than red cells and most of them were lighter than polymorphonuclear cells, the majority of cancer cells spreading into the blood lay above or in lymphocyte/platelet layer of the buffy coat.[68]

Circulating tumor cells (CTC or CTCs), dislodging from the lesion, could be detected in several types of solid malignancy. Despite a few different names were used to describe metastatic cells in peripheral blood, the terminology “CTC” was mostly used in general. Additionally, tumor cells in bone marrow, reservoir of cells from which they might re-circulate into circulation, were specifically named as disseminated tumor cells (DTC or DTCs).[69] In head and neck cancer, detection of preoperative CTC as well as DTC and intra-operative CTC, by immunohistochemistry and RT-PCT, were significantly related to duration of disease-free period (disease-free survival) and metastasis-free period (metastasis-free survival). These findings suggested that, before surgical treatment, the presence of CTC and DTC could predict development of locoregional recurrence and distant metastasis.[70] An important leap of CTC knowledge was the relationship of CTC and breast cancer metastasis, which benefited in real-life application. In 68 patients receiving chemotherapy and endocrine therapy, Liu et al reported a strong correlation between CTC number and progression of metastatic breast cancer, which was routinely monitored by radiographic imaging. More importantly, CTC results were obtained many weeks before disease was radiographically detectable and the number of CTC more than or equal to five cells could predict shorter survival.[71]

Not only CTC, but the white blood cells, residing in buffy coat component, also carry cancer DNA in blood. Generally, “vertical transfer” (from parental to daughter cells) of oncogenic information, such as oncogene transmission, is well-known. Additionally, there is “horizontal transfer” which carcinogenic information is transmitted from eukaryotic cell fragments to other eukaryotic cells. This mechanism can be involved with virus because of its integration ability. More importantly, without viral agent playing parts, this horizontal transfer can occur by direct uptake of cancer DNA in apoptotic bodies particularly by phagocytic cells such as white blood cells, fibroblasts, and endothelial cells. Normally, as an immune defense of the body, abnormal DNA would be destroyed. However, in malfunction of body check point, the cancer DNA can survive and results in higher rate of mutation and metastasis. This oncogenic transfection from dissemination in the blood is known as “genometastasis” hypothesis.[72-75]

Several reviews explained circulating nucleic acids in plasma and serum (CNAPS) and the release mechanism into circulation, which may responsible for the horizontal transfer and metastasis. Not only cancer cell apoptosis or necrosis that plays some parts,[72, 73] but active viable tumor cell shedding also can produce detectable amount of cancer in plasma and serum.[75] In OSCC study, Hamana et al demonstrated the possible role of alleic imbalances in serum DNA as a predictive tool for OSCC prognosis. From 64 OSCC patients, alleic imbalances were found in 59% of tissue samples and 52% of serum samples, preoperatively. Four-week postoperatively, the patients who were negative for alleic imbalances had no recurrence. However, six patients who were still positive died from distant metastasis within less than a year follow-up.[76] Detection of human papillomavirus (HPV) in serum was shown with role in monitoring metastasis of head and neck squamous cell carcinoma patients.[77]

Garcia-Olmo et al was the first to study CTC at the same time with cell-free tumor DNA in plasma. In this animal model, tumor progression was associated with the increased free DNA in plasma as well as CTC. However, after cancer cells had been inoculated into the rats, DNA in plasma could be detected earlier than CTC. Though cancer DNA in blood indicates risk of metastasis, in this study, there was no significant relationship between detected DNA in blood and metastasis.[78] Apart from

its benefits in cancers, CNAPS is also useful for prenatal diagnosis, monitoring organ transplant and in acute medicine practice.[74]

Only few comparative studies between plasma and serum, in terms of DNA sources, have been done.[79] Nonetheless, the results usually suggested that serum was more labile than plasma,[80] most likely due to the release of cellular constituents upon inclusion of blood cells into the clot. For quantitative estimation of DNA, the use of plasma, obtained by centrifugation of whole blood with EDTA, was recommended.[81]

Development and Validation of Efficient Markers

Obtaining efficient markers requires many well-strategized studies. For early detection, Pepe et al has proposed five-phase guideline as systematic approach to develop, evaluate, and validate the screening markers.[82]

Table 6 Phases of screening marker development

Phase	Objectives
Phase I Preclinical exploratory phase	Promising directions identified
Phase II Clinical assay and validation phase	Ability of assay established
Phase III Retrospective/longitudinal phase	- Ability to detect preclinical disease determined - "Screen positive rule" identified
Phase IV Prospective screening phase	- Extent and characteristics of disease detected by the test identified - False positive rate identified
Phase V Prospective randomized trial phase	- Impact of screening on reducing burden of disease determined

Adapted from Pepe, 2001[82]

Briefly, in phase I, suspected markers are compared between normal and cancer tissues. In phase II, a clinical protocol, such as non-invasive clinical sample collection, is developed and tested. In phase III of longitudinal study, suspected markers are constantly evaluated in healthy people monitoring for occurrence of

cancer. In phase IV, asymptomatic and high risk individuals are screened and those who have positive test are followed if cancer really develops. Finally, in phase V, the test is applied to large population to see whether the markers have an impact on disease-related morbidity and mortality (Table 6).[82]

Biomarker assays for cancer detection should have certain characteristics. Sensitivity and specificity, usually referred as accuracy in clinical term, are the most important performance. "Sensitivity" or "true positive rate" refers to the proportion of cases with confirmed diseased (by gold standard) who test positive for biomarker among total confirmed cases. It tells us how good a marker test is at correctly identifying people who have disease. "Specificity" or "true negative rate" refers to the proportion of controls subjects (confirmed without disease) who test negative for biomarker among total confirmed normal controls. It tells us how good a marker test is at correctly identifying people who are well. Additionally, positive and negative predictive values refer to the chance that positive and negative test result, respectively, will be correct (Table 7).[55, 83, 84]

Table 7 Contingency table of accuracy determination

		Condition *(as determined by "Gold Standard")*		
		Positive	Negative	
Test Outcome	Positive	True Positive	False Positive	⇨ Positive predictive value
	Negative	False Negative	True Negative	⇨ Negative predictive value
		⇩ Sensitivity	⇩ Specificity	

To determine sensitivity and specificity from the continuous raw data, receiver-operating characteristic (ROC) curve is widely and reliably used.[84, 85] It provides a statistical method to assess diagnostic accuracy and gives not only these two characteristics, but also area under the curve (AUC) to be easily compared with

other marker tests. The closer AUC is approaching 1, the higher accuracy the test provides. In addition, cut-off values are also generated from ranges of data. The optimal one is conjugated with the optimal sensitivity and specificity as well as maximum AUC (Fig. 2).[84]

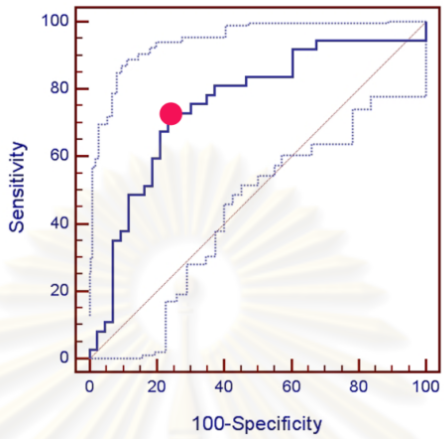


Figure 2 **Receiver-operating characteristic (ROC) curve.** Red dot indicates the optimal sensitivity and specificity as well as cut-off value of the test.

In addition, the requirement for performance of the test varies with the intended uses. High sensitivity is crucial for diagnosis and monitoring diseases. For screening, specificity is extremely required. Further, since carcinogenesis is multistep process in which many factors and mechanisms involved, single marker usually cannot provide enough accuracy or information. A panel or set of multiple markers, used together, may be developed and proved. An improvement of sensitivity and specificity was reported for head and neck squamous cell carcinoma.[86, 87] A group of markers complementing the accuracy of each other may be used in two ways. In a series testing, one marker is tested after the previous marker gives positive or negative result. In a parallel testing, all markers are tested at the same time and the result is interpreted according to criteria. For instance, the cancer patient must have positive result from all tests or two out of three.[55]

Despite cancer biomarkers are gaining enormous interest and becoming more popular now, it is not the purpose of marker to replace standard biopsy procedure. Biomarkers should be selected carefully and used wisely to help with screening, diagnosis, and monitor recurrence of the disease. For example, at the first detection of abnormal or premalignant lesion, biomarker test may be performed. The patients with

positive result should further have biopsy. Moreover, biomarker tests are suitable for routine screening in people without symptoms or monitoring recurrence. There are risks of incorrect result from marker test. In case of false-positive result, a normal individual whose test confirms as cancer, the person would encounter great anxiety and unnecessary invasive procedures. In case of false-negative result, a cancer patient whose test confirms as normal, the proper treatment will be delayed and probably results in cancer metastasis.

Genetics and Epigenetics of Cancer

The term "genetics" generally refers to status in which DNA base-pair sequence is concerned. Today, it is well-known that the underlying basis of cancer is a cumulative series of genetic alterations leading to deregulated cell growth, particularly alterations that provide a selective growth advantage to tumor cells. Genetic alterations, the changes of DNA sequence, happen in many ways including deletion, insertion, recombination, and amplification of some parts of certain genes. For example, proto-oncogenes, genes involved in regulations of normal cell proliferation, become oncogenes and lead to abnormal cell growth and differentiation in carcinogenesis. In addition, alterations of tumor suppressor genes, genes that block abnormal cell proliferation, were related to cancer development.

However, natural events of embryogenesis and differentiation, which are controlled by specific patterns of gene expression in specific tissues and organs, proceed without any changes in DNA sequences. Also, the transformation of normal cells to precancerous or cancerous cells was thought to act the similar way. These ideas gave rise to the modern epigenetic concept. The term "epigenetics", contrarily to genetics, describes modifications of DNA that are not involved in DNA sequence changes. The forms of epigenetic modification, occurring in every normal human cell and influencing on gene expression, are known as DNA methylation, histone deacetylation and RNA interference. In the complex molecular basis, chromatin, a bead-on-string of double stranded DNA and histone core (Fig. 3), may be activated or silenced in each part of it. In other words, every cell in human organism has the same instruction manual, but different cell types are using different chapters. Any error of epigenetic modifications, which makes cells read the wrong chapters, may affect

normal gene expression and cause pathological conditions. Recently, it is accepted that not only genetic, but epigenetic changes also play an important role in development of several diseases especially cancer.[88, 89]

Furthermore, epigenetic epidemiology and markers may have some benefits over genetic markers. Since genetics is sequence-concerned, finding the specific sequences involving in carcinogenesis is more like "finding needle in a haystack". Flanagan also pointed out that epigenetic characters had greater variability across the genome, cell types and individuals. Also with continuous variable, such as DNA methylation range from 0-100%, a better statistic analysis as well as disease risk assessment are allowed. Additionally, the association between epigenetics and level of gene expression provides more understanding in biological mechanism, especially carcinogenesis.[90] As opposed to irreversible state of genetics, epigenetic alteration in normal cell process of homeostasis and development is reversible. This finding benefits in pharmacological study for treatment of pathological conditions.[91]

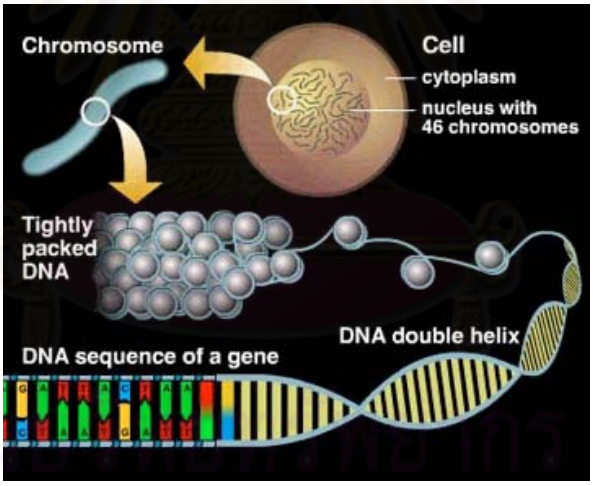


Figure 3 **From DNA to cell.** DNA, a unit of pentose sugar, phosphate group, and nitrogenous base, is arranged in double stranded helix, wound around histones to form nucleosomes. Nucleosomes are organized into solenoids, which in turn make up the long chromatin loops. These loops are tightly packed and become chromosome. Genes, the basic unit of inheritance, are contained in chromosomes and consist of DNA. *(picture from http://www.sciencemuseum.org.uk)*

DNA Methylation

DNA methylation is a modification of DNA molecule itself in which methyl groups are transferred from the methyl donor, S-adenosylmethionine, to the 5' carbon of cytosine ring in cytosine nucleotides by enzyme DNA methyltransferase (DNMT) (Fig. 4A and 4B).

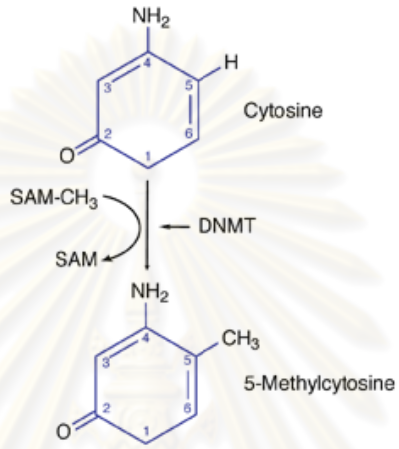


Figure4A **Enzymatic reaction of DNA methylation.** Methyl group (CH_3) is transferred from the methyl donor, S-adenosylmethionine, to the 5' carbon of cytosine ring of cytosine nucleotides by DNA methyltransferase (DNMT).

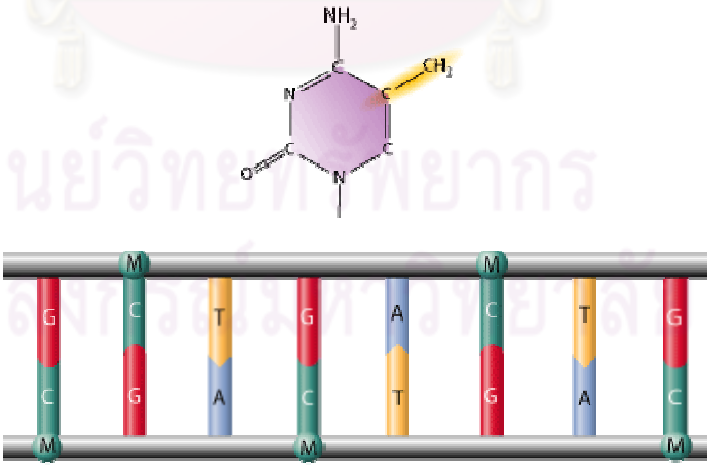


Figure 4B **DNA methylation of CpG dinucleotides.**[88] DNA methylation mostly occurs in CpG dinucleotides where cytosine base precedes guanine.

Mainly, the methylated cytosines are in “CpG or CG dinucleotides” where cytosine (C) precedes guanine (G). In normal cells, about 70% of the CpG dinucleotides in the mammalian genome are methylated and most of them are found primarily in repetitive sequences, the sequences that occur repeatedly and generally in non-gene regions of the genome. In fact, 45% of all CpG dinucleotides in the whole genome are in repetitive elements. Just 7% are within CpG islands, which are clusters of CpG dinucleotides found in specific gene areas, and these CpGs are usually unmethylated.[85, 92-95]

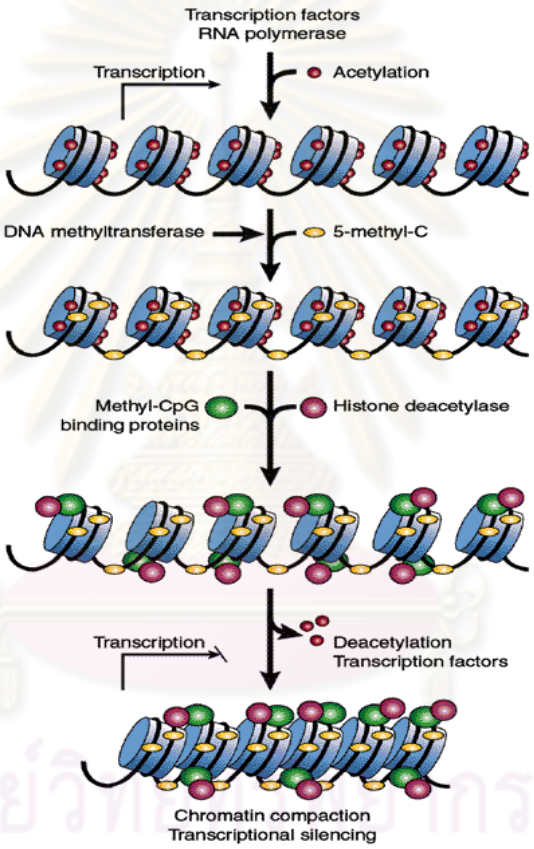


Figure 5 **Transcriptional gene silencing by DNA methylation.** DNA methylation condenses chromatin structure, inhibiting histone acetylation and binding of transcriptional complexes. These mechanisms contribute to gene silencing or switching off. *(picture from http://www.med.ufl.edu/biochem/keithr/research.html)*

Function of DNA methylation

The change from cytosine to 5-methylcytosine by DNA methylation is associated with transcriptional silencing (switching off) of some genes, in concert with alteration

in chromatin structure. This process is important in development,[96] protection against intragenomic parasites,[97] X-inactivation,[98] mental health,[99] and cancer. One important example of regulatory role of DNA methylation is genomic imprinting which control the expression of some genes during development. For instance, most of the cases, both the paternal and maternal alleles of a gene are expressed however, some gene expressions depend on only one allele. This is where methylation plays a part; inactive another allele that it is not needed.[100] Since methylation directly switches off gene expression by deacetylating histones or preventing the binding of transcription factors, deacetylated histones bind DNA very tightly resulting in loss of transcription capacity or gene expression control (Fig. 5).

De Novo methylation and inheritance of DNA methylation pattern

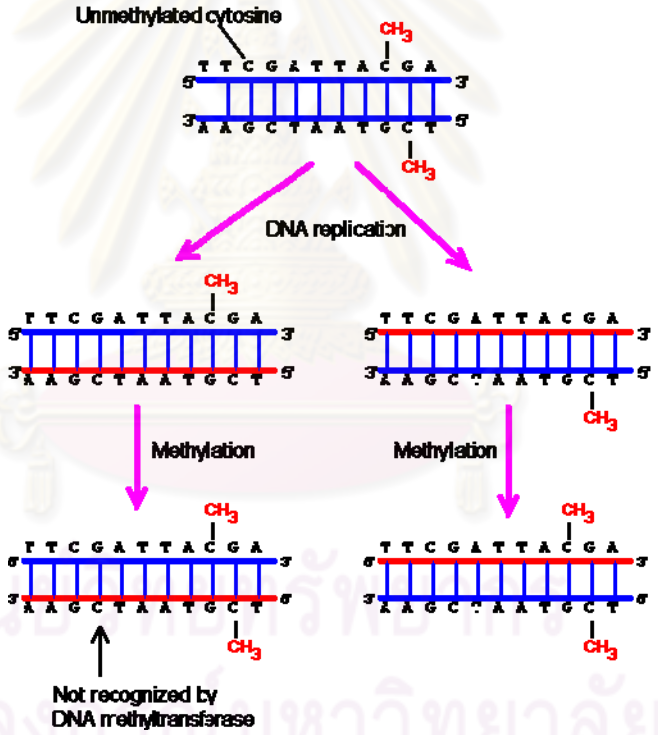


Figure 6 **Inheritance of the DNA methylation pattern.** The DNA methylatransferase1 (DNMT1) can methylate only the CpG sequence paired with the methylated CpG. It takes action in daughter cells. Hence, the original pattern can be maintained after DNA replication. *(picture from http://www.web-books.com)*

During early development, germ cell methylation patterns are erased by an initial wave of global demethylation near the eight-cell stage of blastocyst formation.

During the implantation stage, methylation patterns are rapidly re-established following a wave of de novo methylation, a new methylation at the beginning, by enzyme DNMT3a and DNMT3b. The amount and pattern of methylation are tissue and cell specific which are relatively stable afterwards.[101-103] In addition, any type of cells has its own methylation pattern so that a unique set of proteins may be expressed to perform functions specific for this cell type. Thus, during cell division, the methylation pattern should also pass over to daughter cells. This process is achieved by DNMT1, which can methylate only the CpG sequence paired with methylated CpG (Fig.6).

DNA methylation and cancer

The methylation pathway is directly related to major chronic conditions including heart disease, diabetes mellitus, Alzheimer's disease, Parkinson's disease, Down's syndrome, autism, and certainly cancer. Also, it was recognized for more than twenty years that DNA methylation in tumor cells are altered from that of normal cells.[104, 105] In this case, methylation changes can exhibit in two ways, hypermethylation of specific regions and global (genome-wide) hypomethylation.[19, 24, 87, 95, 99, 104, 106-108] These imbalances can be presented together in a single tumor, though the net effect is usually a decrease in total methylation level.[92] Upon our basic knowledge, if methylation imbalances contribute directly to tumor initiation, the alterations should occur in early stages of cancer or in premalignant cells. Also, if the imbalances contribute to tumor progression, methylation defects should increase in frequency and/or severity coordinately with increasing malignancy grades. It is found that methylation defects are present in cells before the onset of obvious malignancy, sometimes months or years, is clinically detectable, therefore it cannot be explained as only consequence of a deregulated cancer cells.[26, 35, 92] As a result, usage of methylation as biomarkers will be useful for detecting cancer earlier and less invasively than currently available methods, and thereby enhancing the success of treatment. Further, in breast, ovarian, cervical, and brain tumor, hypomethylation increases progressively with increasing malignancy grade.[109-112] Thus, hypomethylation may serve as a prognostic indicator as well.

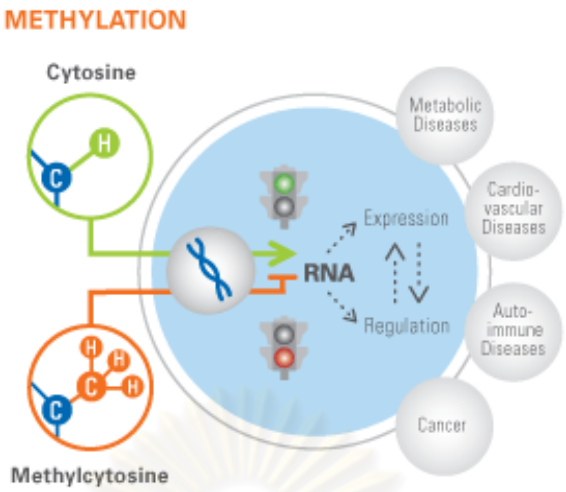


Figure 7 **Schematic diagram of DNA methylation and diseases.** Different cytosine methylation states act as a switch-on and switch-off of genes and transposable elements which result in numerous biological processes and diseases. *(picture from http://marketing.appliedbiosystems.com)*

Promoter hypermethylation in tumor tissues is a common event in the development of many types of cancer, including head and neck squamous cell carcinoma (HNSCC).[108, 113] Not only that, global genomic hypomethylation in tumor tissue is also a signature in a wide variety of malignancies, ranging from solid tumors, such as breast, colon, oral, and lung cancer, to cancer of the blood.[24, 114, 115] Moreover, it may evolve progressively in multistage carcinogenesis.[116] Normally, hypermethylation occurs chiefly in specific CpG island and gene promoter region of tumor suppressor genes, metastasis-related genes, and DNA-repair genes.[19, 117] In the past, Knudson's two-hit hypothesis of tumor suppressor gene inactivation by methylation was proposed. Methylation may inactivate one or both alleles of the tumor suppressor genes in "sporadic" (acquired) cancer and can potentially act as a second hit during the development of "hereditary" cancer.[118, 119] Global hypomethylation which is the deplete of methylation levels of the whole genome, on the contrary to hypermethylation, occurs not only in transcription control regions of the genes, such as promoter, but importantly also in non-coding repetitive sequences such as retrotransposons.[24, 34, 35] One of the causal roles of this hypomethylated event is believed to be the decrease in methyltransferase activity. The consequence of genome-wide hypomethylation of chromosome, which is mostly occurs in repetitive

DNA that normally heavily methylated,[120] is an induction of chromosmal instabilbity in carcinogenesis.[92, 121] As a result, the formation of abnormal chromosomal structures is likely to happen.[24, 122] A link between hypomethylation and the stability of whole chromosome arms was reported in several conditions including the human Immunodeficiency-Centromeric Instability-Facial Anomalies (ICF) syndrome[123] and cancers such as hepatocellular as well as prostate carcinoma.[124, 125]

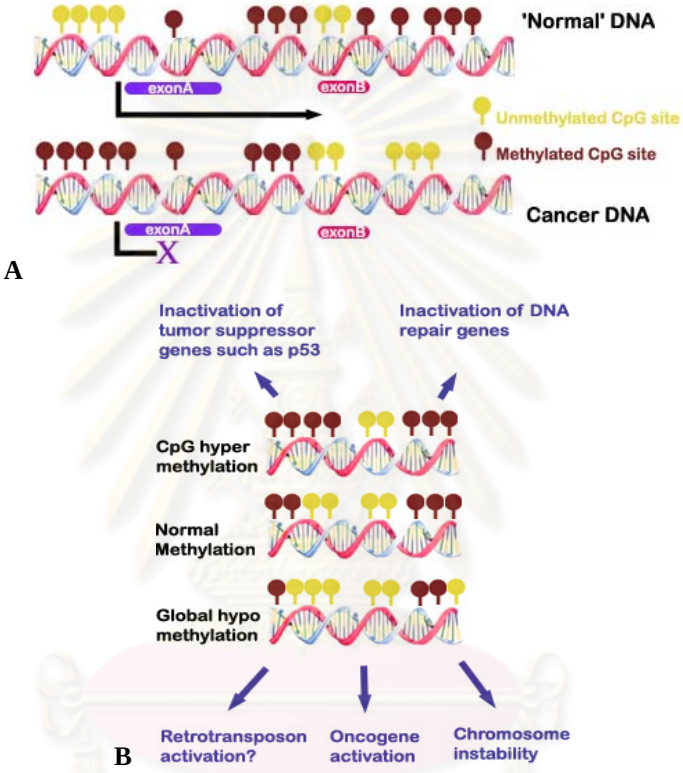


Figure 8 **DNA hyper- and hypomethylation. A** Cancer cells are characterized by hypermethylation of CpG islands and general DNA hypomethylation. **B** Hypermethylation of specific region, such as p53 tumor suppressor gene or DNA repair gene, together with global hypomethylation due to epigenetic modification of genome have dramatic effect on the stability of DNA which leading to neoplastic transformation. *(picture from http://www.cellscience.com)*

In addition, not only does DNA in nucleus carry the valuable information for functioning of the human body, mitochondrial DNA, RNA, and proteins are gaining interests. However, despite the similar biological information provided by RNA and

proteins, more chemical and biological stability as a source for molecular diagnosis is obtained via nuclear methylation profiles.[87]

Apart from epigenetic, specifically DNA methylation, genetic abnormalities, certainly play some parts. As mentioned earlier, these genetic alterations include several types of mutations. In head and neck cancer, frequently investigated genetic characters are point mutation, loss of heterozygosity (LOH) or allelic loss on chromosome arms, and microsatellite instability (MSI) which is an increased frequency of deletion of microsatellite repeats.[126, 127] Genetic and epigenetic mechanisms can cooperate directly and indirectly in cancer. For example, direct cooperation includes complete inactivation of tumor suppressor genes by methylation of one allele and either deletion or mutation of the other. Indirectly, aberrant loss of methylation in the pericentromeric regions of chromosomes 1 and 16 is associated with abnormalities of these chromosomes, including loss and gain of the whole chromosome arm (Fig. 8).[128] It is realized that this cooperation affects multiple cellular pathways, such as cell cycle regulation, DNA repair, apoptosis, angiogenesis, and cell-to-cell adhesion, during the process of tumor growth and progression.[126, 127]

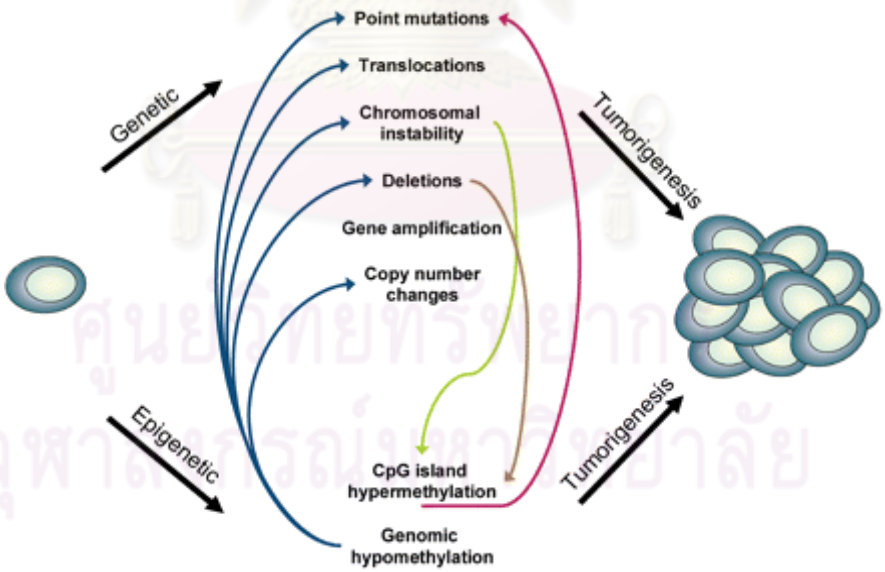


Figure 9 **A genetic-epigenetic mechanism model of human cancer pathogenesis.** 90-95% of all cancers, uniformly exhibit both genetic and epigenetic defects genome-wide, and these mechanism show substantial interaction.[128]

Long interspersed nuclear element-1s (LINE-1s) and genome-wide hypomethylation

A human genome contains the repeats of nucleotides and the unique sequences or single-copy DNA which primarily are genes, specific regions of nucleotides that are expressed to yield functional products. Apart from the genes, in the genome, there are many regions believed to play some parts in maintaining human body in normal functions as well, including repetitive sequences (Fig. 10).

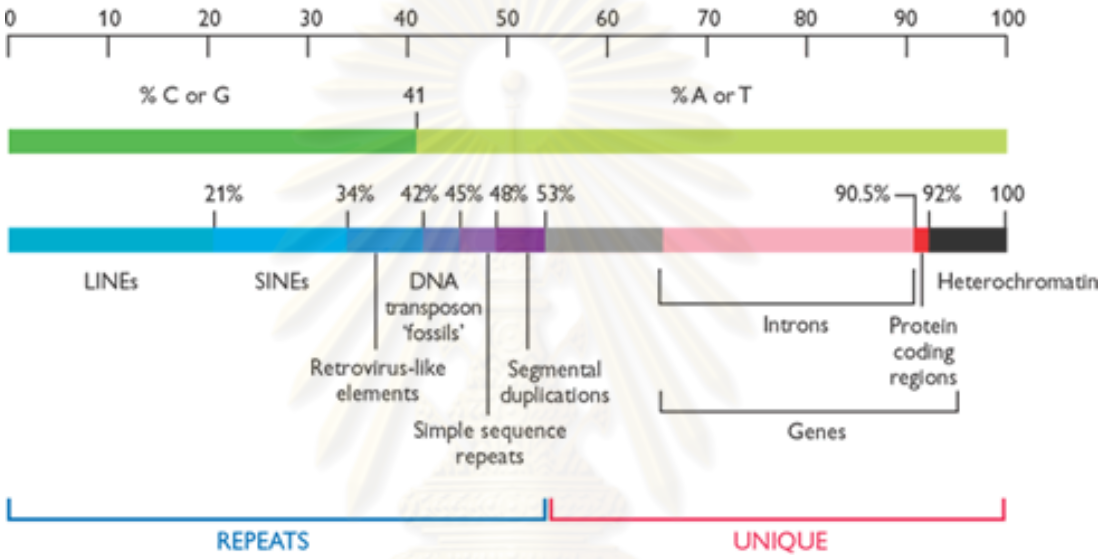


Figure 10 **A perspective of genomic composition.** Human genome has 53% of repetitive sequences. Almost half of the repeats are the LINEs family which accounts for approximately 21% of whole genome.[100]

Repetitive sequences, the same sequences that present in many copies, often thousands of times, are subclassified into two groups, satellites and interspersed repeats. Satellite repeats are cluster together in certain chromosome location, where they occur in tandem; the repeat units are placed immediately next to each other. Interspersed or dispersed repeats tend to be scattered throughout the genome in an apparently random fashion.[89, 117] The two types of interspersed repeats include retrotransposons, sequences of DNA that can move in the genome by being transcribed to RNA and then back to DNA by reverse transcriptase, and DNA transposons which do not necessarily require RNA intermediate for transposition. When a DNA copy of retroviral RNA is synthesized by viral reverse transcription,

there will be repetitive sequences of hundreds nucleotides at both ends of the proviral DNA. They are called long terminal repeats, LTR, and involved in integration and subsequent transcription of proviral DNA (Fig. 11).[100]

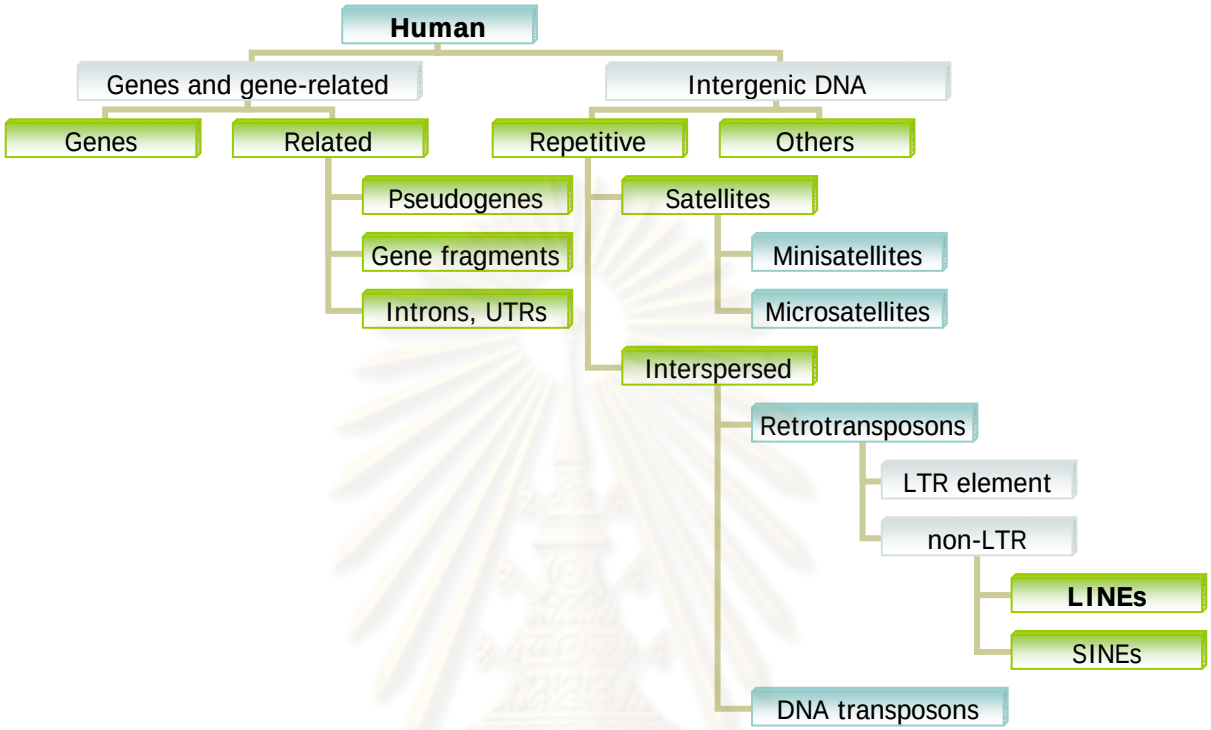


Figure 11 The organization of human genome.[100, 117]

Long interspersed nuclear elements, (LINEs), are the most common subfamily of retrotransposon, composing about 21% of human genome in approximately 850,000 copies (Fig. 10).[100] Among all of the LINEs discovered, long interspersed nuclear element-1s, LINE-1s, takes the majority of more than 516,000 copies or approximately two third of the LINEs.[117]

Full length of LINE-1 retrotransposon have two open reading frames, one which encodes a nucleic acid binding protein and a second which encodes a protein with endonuclease and reverse transcriptase activities, allowing their mobilization in genomes through an RNA intermediate. LINE-1 retrotransposon present at target site of chromosomal DNA is transcribed into mRNA by RNA polymerase and maturation process. The opening frame of LINE-1, ORF2 protein, cleaves the one DNA strand at the target site which is AT-rich and usually similar to consensus TTAAAA (Fig. 12).

The cleaved strand dissociates and binds to polyA tail of LINE-1 mRNA. With DNA primer, LINE-1 is then converted back into DNA by reverse transcription. Cleavage of another DNA strand occurs then the whole process of new LINE-1 copy formation and integration into target site are completed (Fig. 13). Though it is not completely understood, it becomes more and more accepted that the evolution of transposable elements interact in a complex way with other aspect of the whole genome dynamics. LINE-1 and other transposable element families probably provide a variety of advantages to the host genome.[129] However, if transposable elements integrate themselves in growth-regulating genes, they might disrupt important functions of those genes. LINE-1 mutational insertions in sporadic cancers have been found in colon tumor which *APC* gene is disrupted and breast tumor which *CMYC* gene is disrupted.[130, 131] However, the deleterious effect of LINE-1s in cancer may not require transposition. It has been suggested that because of the typically strong activity of 5'LTRs or promoters of LINE-1s, hypomethylation mediated transcriptional activation of LINE-1s could also disrupt the nearby genes. Luckily, the promoters of most LINE-1s have been deleted.[92] In cancer cells, LINE-1 expression

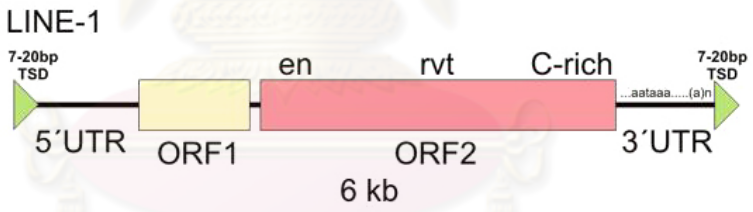


Figure 12 **Functional components of LINE-1 (6 kb).** The ORF2 contains endonuclease (en), reverse transcriptase (rvt) domain as well as a cysteine-rich domain (C-rich). 5' untranslated region (5'UTR) contains also internal promoter for RNA polymerase II (in a usual gene, promoter is upstream 5'UTR). 3' untranslated region (3'UTR) contains canonical polyadenylation signal (AATAAA) and a polyA tail (that is also normally absent from the ordinary genes, and is only added to mRNA by action of polyA polymerase). LINE-1 is flanked by target site duplication that arises during the target primed reverse transcription.

and retrotransposition cause transcriptional deregulation, insertional mutation, DNAbreaks, and an increased frequency of recombinations, contributing to genomic instability.[132]

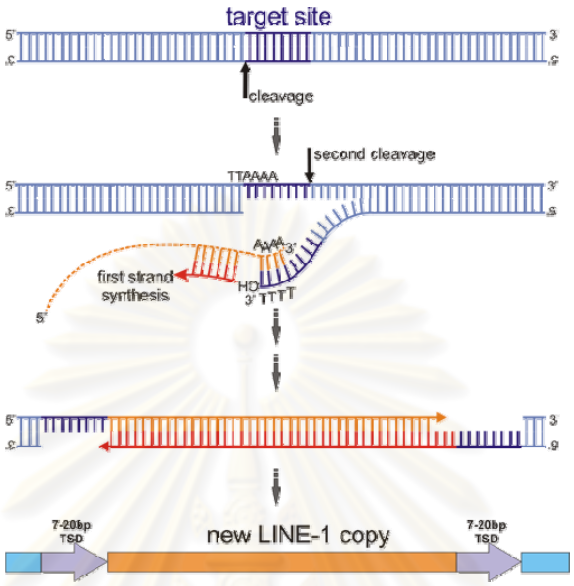


Figure 13 **The movement of LINE-1 retrotransposon.** A new LINE-1 copy is formed and integrated into target site.

In the transposable elements, including LINE-1s, DNA methylation commonly occurs and plays a key role in suppressing the movement of the elements. Since such LINE-1 transposition mentioned above can have negative impact on genome, it might therefore be understood that the genome have evolved mechanisms for limiting the movement of their transposable elements.[117] From tissue, sera, and plasma studies, hypomethylaiton of LINE-1s was reported to be associated with incidence of many types of cancers, such as carcinomas of urinary bladder, liver, lung, prostate gland, stomach, colon, breast, and head and neck including OSCC.[11, 28, 36] Moreover, progression of epithelial ovarian cancer[33] and prostate adenocarcinoma[106] were found correlating with hypomethylation of LINE-1s as well. Furthermore, previous studies have described the hypomethylation of genomic repetitive sequences, particularly LINE-1s, as a marker of the global genomic hypomethylation, in these malignancies.[25, 29, 81, 133-135] Thus examination of methylation at LINE-1 regions has served as a proxy for measuring global methylation levels.[11, 28, 114]

In the past few decades, many studies have been conducted to investigate the association between methylation level with various factors, for instance, age, sex, smoking habit, alcohol consumption, folate, and HPV infection. The results were still controversial and inconclusive due to a variety of methodology, sample size, laboratory technique, and the fact that it was almost impossible to evaluate such factor independently from others. Since aging is the most common risk factor for the development of most adult malignancies, it has been assumed that an age- and mutagen-related mutations account for increased cancer incidence in the older populations.[136] Evidences suggest that aging is accompanied by the accumulation of cells with aberrant CpG island methylation in normal-appearing tissues.[136, 137] In colorectal cancer, this age-dependent methylation accounts for the majority of aberrantly methylated genes, and this process is active in multiple other tissues as well.[136] A longitudinal study comparing intra-individually with 12 and 16-year follow up indicated that methylation changed over time.[137] However, some reports had contradictory findings.[11, 28, 138-142] Additionally, the cross sectional and longitudinal studies investigating normal DNA extracted from peripheral leukocytes revealed no difference in the level of LINE-1 methylation between the elderly and the young.[11, 138] An interesting report of repetitive sequences has shown that, though insignificantly, LINE-1 methylation in peripheral blood mononuclear cells (PBMC) of normal people was gaining accordingly to the increased age. More importantly, at the specific age of 40 to 59, LINE-1s significantly increased, suggesting different mechanisms of losing or gaining methylation at different age ranges.[143]

Further, several studied have shown that LINE-1 methylation was independent on gender.[28, 143] It was also reported that by using the same protocol, LINE-1 methylation levels in OSCC tissue and oral rinse from both normal subjects and OSCC patients were not different between male and female.[11] Folate is known to play an essential role in one-carbon transfer involving remethylation of homocysteine to methionine, which is a precursor of S-adenosylmethionine, the primary methyl group donor for most biological methylations.[144] A diet deficient in B12 and folate can results in hypomethylation[136] which links to carcinogenesis.[35] However, the studies in colorectal cancer and hepatocellular carcinoma suggested otherwise.[142, 144, 145] LINE hypomethylation in head and neck cancer was reported with more

pronounced in HPV-negative than HPV-positive tumors.[146] Nevertheless, this finding needed to be repeatedly validated.

Cell type specific of LINE-1 methylation

Not only there are differences in methylation level among individuals, but analysis of the total base composition of DNA from various types of tissue also revealed considerable tissue specific and cell specific in the extend of methylated cytosine residues within the same normal individual.[30, 92] The data also showed a greater relationship between tissues and cell types that had the same development pathways or similar phenotype. For instance, two skeletal tissues sampled from different part of the body showed similarity to each other while their methylation levels differ from other tissue types.[116] This might be due to the fact that though methylation of CpG dinucleotides is heritable from parent to daughter cells, it alters with mammalian development and differentiation.[96]

Resembling methylation from whole genome analysis, the levels of LINE-1 methylation were significantly different among various tissue types, both in normal and malignant groups. However, the distribution of LINE-1 hypomethylation within a normal tissue type between individuals was consistently clustered within 5% range.[28] Considering the distribution of LINE-1 hypomethylation level, each malignancy displays a certain degree of correlation with the methylation status of its normal tissue. Similar findings were also found in highly repeated sequences including EcoRI and Alu families.[111] However, the levels of methylation among tissue types in the same individual are still needed to be clarified.

Quantitative analysis of DNA and LINE-1 methylation

Quantitative methylation profiling is gaining momentum in various disease states such as inflammation, cardiovascular disease and diabetes, and, importantly, cancer.[81] Among techniques studying methylation, the sodium bisulfite method was introduced in 1992 and proven to be beneficial in analyzing 5-methyl-cytosine content in clinical DNA samples. With reliability, it can be used as a part of diagnostic technology.[103] To productively determine methylation level of DNA, these days, many protocols are based on the same key procedure of modifying DNA with sodium

bisulfite as the first step and subsequently amplifying DNA by polymerase chain reaction (PCR) with specific primers. Examples of these techniques include combined bisulfite restriction analysis (COBRA), methylation-specific PCR (MSP), bisulfite genomic sequencing PCR (BSP), and methylation-sensitive single nucleotide primer extension (MS-SNuPE).[91, 147, 148]

COBRA is widely used for obtaining methylation content in DNA with high accuracy and reliability.[149-151] Not only that, it is easy to be performed, simply reproducible, applicable to small amount of DNA, compatible with paraffin-embedded section as well as cost-effective.[150] The modified protocol of PCR-based COBRA for quantitatively and effectively evaluating LINE-1, called COBRA LINE-1, was reported.[28] There are three main processes of COBRA LINE-1; determination of methylated loci by bisulfite treatment, product amplification by PCR and followed by restriction enzyme digestion, specific to different methylation statuses of the two cytosines. Although mutation at those cytosines would cause miscalculation of methylation, this kind of error does not affect the investigated level due to the large number of LINE-1s in the genome. Therefore, despite relying on only two loci in each LINE-1 sequence, COBRA LINE-1 can present methylation level of thousands LINE-1s as well as represent genome-wide methylation. Incomplete conversion and minor DNA degradation in bisulfite treatment may impair the measured methylation.[91, 148] Still, an application of LINE-1 methylation level from COBRA LINE-1 as a surrogate for global methylation level was also proved.[149, 151] In addition, many studies have confirmed the use and efficiency of COBRA LINE-1 in determining methylation level of LINE-1s in tissues as well as body fluids of normal and cancer patients.[11, 28, 32-35, 106, 143, 147, 151, 152] Principles and details of each step of COBRA LINE-1 are further elaborated in the next chapter.

CHAPTER III

RESEARCH METHODOLOGY

Cases and Controls

Patients who were histopathological-confirmed with oral squamous cell carcinoma (OSCC) and had not undergone any treatment were included in "OSCC group" of this study. Patients with prior chemotherapy or radiation were excluded. In this group, cases were obtained from three centers; Otolaryngology Institute of Thailand, Rajavithi Hospital, Department of Surgery, Buddhachinaraj Hospital and Department of Oral and Maxillofacial Surgery, Faculty of Dentistry, Chulalongkorn University, during June to December, 2009.

At Rajavithi Hospital, where most of OSCC cases in this study were obtained, approximately 40 cases of head and neck squamous cell carcinoma were examined on the collecting dates (Monday, Tuesday, and Thursday morning clinic hour). However, lesions in some of the cases originated in nasopharynx, oropharynx, or outer lower lip, not oral cavity. Thus, those patients were not included. Moreover, some of the patients already had blood investigation from the referring centers therefore those cases were discarded due to ethical concern. As a result, there were 22 OSCC cases from this collaboration. Nine more cases came from Buddhachinaraj Hospital and six more cases were from Faculty of Dentistry, Chulalongkorn University (Table 8).

Healthy volunteers and patients, who had no pathology concerning cell proliferation and cell changes, with similar general backgrounds to OSCC patients, were included as "control group" (non-cancer or normal group). All of 45 non-cancer people in this study were from Faculty of Dentistry, Chulalongkorn University (Table 8).

For all participants, demographic data, history of smoking, alcohol consumption, betel nut chewing, medical condition, familial history of cancer and oral examination were recorded. Additionally, for OSCC group, neck examination, histopathological study and stage of disease were documented. Every subject was provided with research information, risks, benefits, and protocols approved by the Ethics Committee, Faculty of Dentistry, Chulalongkorn University, Bangkok. After

receiving consent forms, which had been completed by each participant, sample collection process was begun.

Table 8 Centers for sample collection

Department	**Control (N)**	**OSCC (N)**
Department of oral surgery, Faculty of dentistry, Chulalongkorn University	45	6
Otolaryngology Institute of Thailand, Rajavithi Hospital	-	22
Department of surgery, Buddhachinaraj Hospital	-	9
OSCC = Oral squamous cell carcinoma		

The data collection process including history review, extraoral and intraoral examination, data record, sample collection as well as information and consent form presentation were performed by the author of this study, for most participants. However, two of twenty two OSCC patients from Rajavithi Hospital were processed by one resident colleague at the Department of Otolaryngology. For all nine patients from Buddhachinaraj Hospital, this process as well as sample transportation were performed by one staff of the Department of Surgery. For all samples collected, every step of laboratory procedures and data analysis was completed only by the author.

Sample Collection

Two types of clinical sample, oral rinse and peripheral blood, were collected from both OSCC and control groups. From each peripheral blood sample, buffy coat and plasma components were separated and studied as two different DNA sources.

Blood samples of OSCC patients were drawn during hematologic laboratory investigation approximately 2 weeks prior to surgical treatment. Those of other subjects were collected according to his or her consent. Each of 9-ml CBC tube with EDTA as an anticoagulative agent was filled with 6 milliliters of whole blood sample drawn from median cubital vein. It was stored at 4˚C until processed within 24 hours.

On the same day as the blood collection, oral rinse samples were also obtained. In each participant, 10 milliliters of sterile 0.9% normal saline solution was gargled for 20 seconds then transferred into a sterile 15-ml closed container. It was also kept at 4˚C until processed within 24 hours.[11]

Genomic DNA Extraction

Eleven head and neck squamous cell carcinoma (HNSCC) cell lines (WSU-HN), including WSU-HN 4, 6, 8, 12, 13, 17, 19, 22, 26, 30 and 31, were also included in this study. Before DNA extraction, each blood samples were centrifuged at 1600 g for 10 minutes at 4˚C and the plasma portion was separated from the whole blood. Then, genomic DNA was extracted from three clinical sources; oral rinse, buffy coat and plasma, as well as WSU-HNs. Next, all extracted DNA samples were subjected to polymerase chain reaction (PCR) based technique, quantitatively investigating LINE-1 methylation, called combined bisulfite restriction analysis of LINE-1 (COBRA LINE-1).

- **Oral Rinse**

Cells in the oral rinses from non-cancer and OSCC patients were pelleted by centrifuging at 2500 rounds per minute (rpm) for 10 minutes at 4˚C. The supernatant was discarded and cell pellets were washed twice in sterile PBS. The cell pellets were placed in a mixture of extraction buffer (Lysis II) and 10% SDS. Proteinase K was added and the mixture was incubated at 50˚C for two nights. The digested cell pellets were then subjected to phenol-chloroform-isoamyl alcohol and centrifuged at 14000 rpm for 15 minutes. Only the upper phase was added with ammonium acetate with cold absolute ethanol and centrifuged at 14000 rpm for 15 minutes. After that, the DNA precipitate was washed with 70% ethanol, dried, suspended in Tris-EDTA treated water and stored at -20˚C.

- **Buffy Coat**

After plasma was separated, extraction buffer (Lysis I) was added to the remaining portion of buffy coat and red blood cells. The mixture was centrifuged at 1000 g for 8 minutes at 4˚C and the supernatant was discarded. These processes were repeated. The cell pellets were placed in a mixture of extraction buffer (Lysis II) and

10% SDS. Proteinase K was added and the mixture was incubated at 50˚C for two nights. The digested cell pellets were then subjected to phenol-chloroform-isoamyl alcohol and centrifuged at 4000 rpm for 5 minutes. Further processes were done in the same protocol as oral rinse.

- **Plasma**

The transferred plasma was processed with QIAamp DNA blood mini kit (Valencia, CA) following manufacturer's protocol. The plasma was added with protease and AL buffer. After incubation at 56˚C for 10 minutes, cold absolute ethanol was added. A part of mixture was transferred to spin column inside the collection tube and centrifuged at 15700 g at 16˚C for 1 minute. This step was repeated until total original mixture was centrifuged and the solution in collection tube was discarded after each centrifugation. AW1 buffer was added and the same spinning was repeated again. After AW2 was added, the mixture was centrifuged at 13000 rpm for 3 minutes and Tris-EDTA treated water was finally used to dilute the plasma DNA left in spin column. If DNA extraction of plasma was not applicable after plasma separation from the blood, the separated fresh plasma samples were stored immediately at -20˚C.[153]

- **WSU-HN Cell Lines**

Suspension of cell pellets in PBS was centrifuged at 3000 rpm for 10 minutes. The supernatant was discarded and the pellets were placed in a mixture of extraction buffer (Lysis II) and 10% SDS. RNase was added and the mixture was incubated at 37˚C for 1 hour. Proteinase K was then added and 3 hours of incubation at 50˚C was begun. Precipitation by phenol-chloroform-isoamyl alcohol was performed as described for oral rinse until DNA was suspended in Tris-EDTA treated water.

COBRA LINE-1

PCR-based combined bisulfite restriction analysis (COBRA) of LINE-1, COBRA LINE-1, consists of three main steps; sodium bisulfite treatment, LINE-1 amplification by PCR and specific restriction enzyme digestion by *Taq*I and *Tas*I enzymes. The overall process is described in Figure 14.

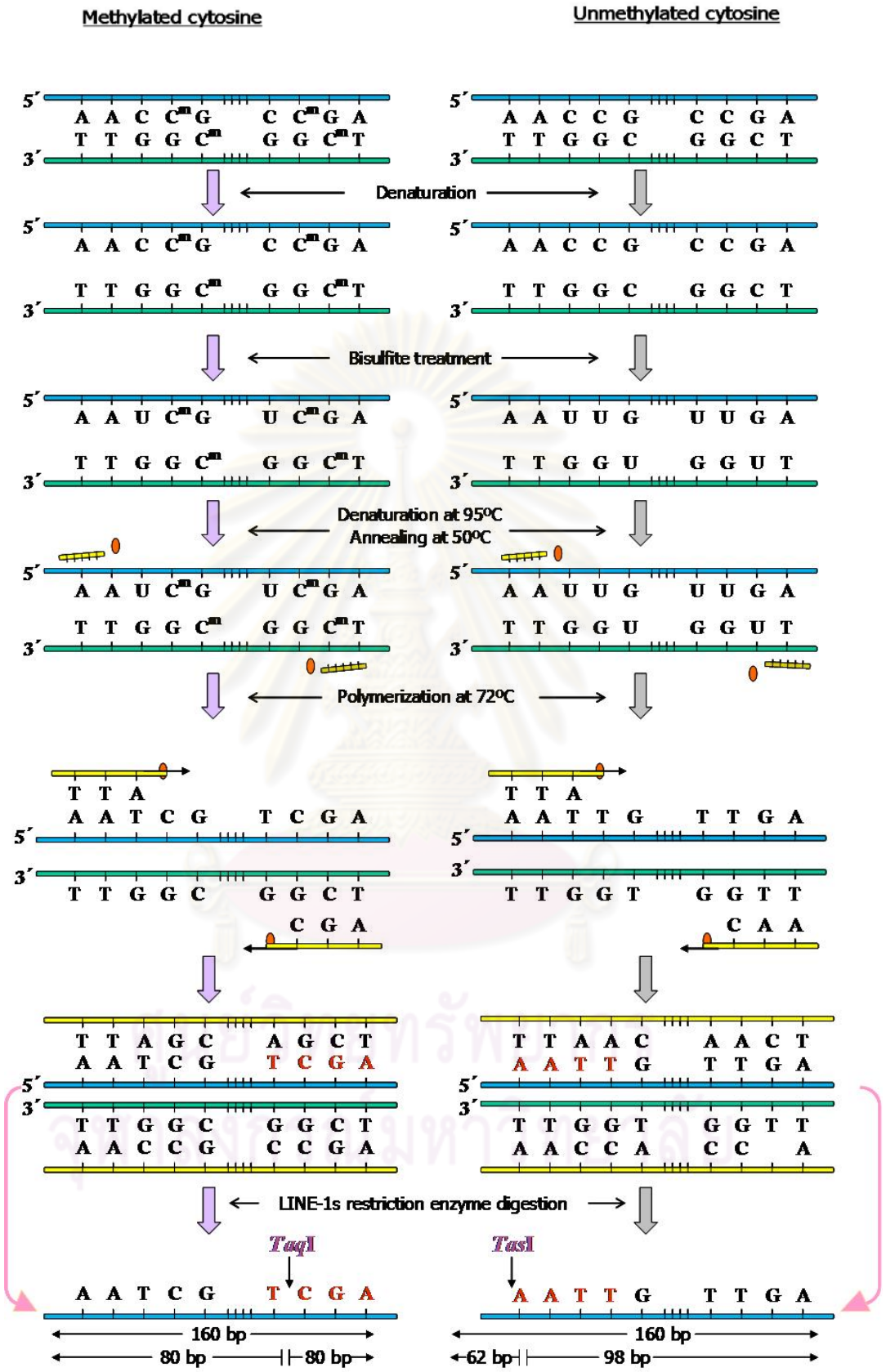


Figure 14 **COBRA LINE-1.** The main three steps are bisulfite treatment, PCR amplification, and specific enzyme digestion.

- **Bisulfite Treatment**

Principle of bisulfite treatment

Sodium bisulfite method is ideal for mapping the normal and aberrant pattern of methylation thus it will be used here to evaluate the methylation status of LINE-1s in the whole genome.[11, 28] In this process, bisulfite deaminates unmethylated cytosines and converts them to uracils (Fig. 15) and leaves methylated cytosines unchanged.[20, 91, 149, 150, 154] In other words, this step turns epigenetic event into detectable genetic character. Therefore, after bisulfite treatment, the methylated sequence can be differentiated from unmethylated sequence by further analysis, such as sequencing, methylation specific PCR, or restriction enzyme analysis.

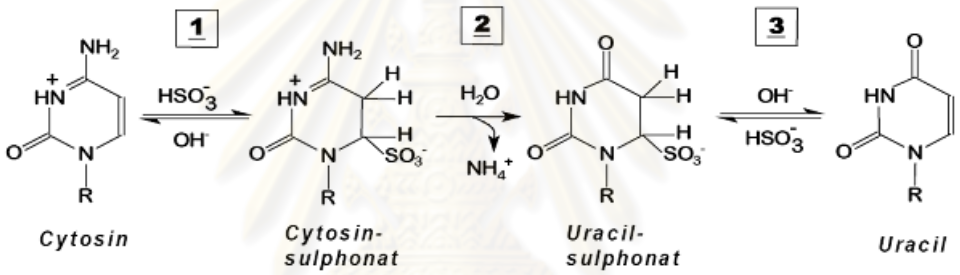


Figure 15 **Schematic diagram of the bisulfite conversion reaction.** The deamination of cytosine by sodium bisulfite involves the following 3 steps: addition of bisulfite to the 5-6 double bond of cytosine, hydrolytic deamination of the resulting cytosine-bisulfite derivative to give a uracil-bisulfite derivative and removal of the sulphonate group by a subsequent alkaline treatment to give uracil.

Bisulfite treatment technique

Bisulfite conversion was performed by EZ DNA Methylation-Gold™ Kit (Zymo Research, www.zympresearch.com) following manufacturer instruction. CT conversion reagent (130 µl) was added to 20 µg of DNA. The DNA mixtures were incubated at 98˚C for 10 minutes, and 64˚C for 2.5 hours, then held at 4˚C. M-Binding buffer (600 µl) was added to each Zymo-Spin™ Column and then DNA mixture was added. After inverting several times, the columns were centrifuged at full

speed for 30 seconds then the flow-through was discarded. M-Wash buffer (100 µl) was added to each column then spinning process began again. M-Desulphonation buffer (200 µl) was added and the mixture was left at room temperature for 15-20 minutes. All mixtures were spun for 30 seconds then flow-through was discarded. M-Wash buffer (200 µl) was added in the column then spinning process was repeated. The column matrix was placed in appendorf then 10 µl of M-Elution buffer was added directly into the column matrix. After a brief spinning, eluted DNA was ready for amplification.

- **Polymerase Chain Reaction (PCR)**

Principle of PCR

Polymerase chain reaction (PCR) is a mean of replicating a short, specific DNA sequence so that millions of copies of the sequence are made within a short period of time. The primary advantage of PCR is that it can be used with extremely small quantities of DNA. This process contains three basic steps: DNA denaturing at high temperature, primer hybridization at a low temperature, and primer extension (DNA replication) at an intermediate temperature and requires four components: two primers, DNA polymerase, a large number of free DNA nucleotides, and genomic DNA sample. Genomic DNA is first heated to a relatively high temperature so that it denatures and becomes single-stranded. It is then exposed to a large quantity of primers, which anneal to the complementary bases as it is cooled down. The DNA is then heated to an intermediate temperature and with the presence of large number of free DNA bases, a new DNA strand is synthesized by DNA polymerase. When the heating-cooling-heating cycle is repeated, the newly synthesized DNA serves as a template for further synthesis and the primer-bounded DNA products are amplified geometrically (Fig. 16).[89] In PCR, after bisulfite treatment, the bisulfite converted uracils will be amplified as thymines, whereas unconverted cytosines will be amplified as cytosines.

PCR technique

After bisulfite treatment, 1 µl DNA was subjected to 35-cycle PCR with two LINE-1 primers, LINE-1-F (5'-CCGTAAGGGGTTAGGGAGTTTTT-3') and LINE-

1-R (5'-RTAAAACCCTCCRAACCA AATATAAA-3'). In 24 µl of master mixture with the primers, there were also magnesium chloride, dNTP and Hotstart Taq. PCR cycling condition was 1 minute of 95˚C denaturation, 1 minute of 50˚C annealing of primers, and 1 minute of 72˚C extension.[11, 28] Then the PCR product amplicons, 160 bp of full length LINE-1, were digested in restriction enzyme digestion.

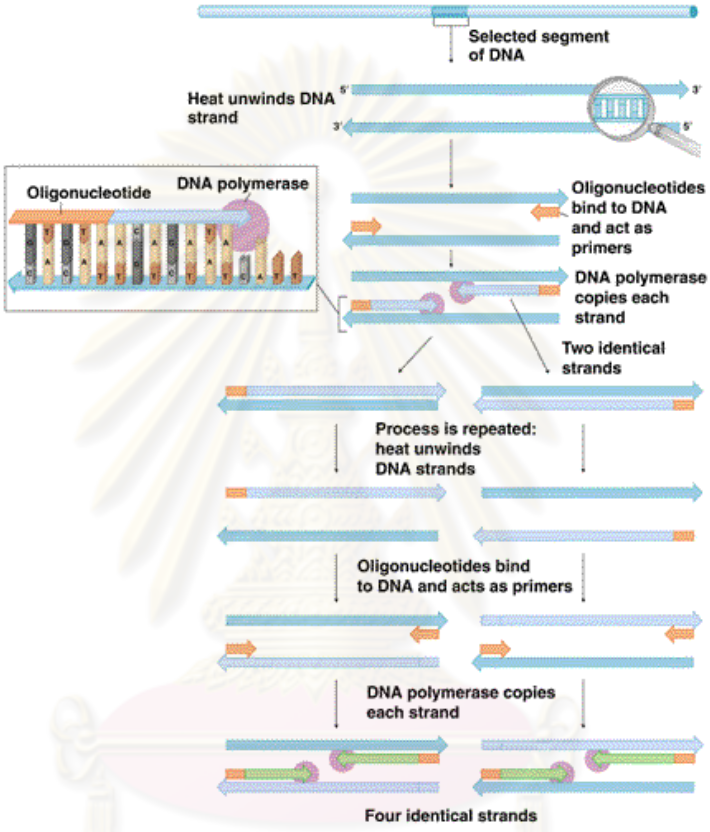


Figure 16 **Amplification of DNA by PCR.** The starting double-stranded DNA is heated to separate the strands (denaturation) and then cooled to allow primers, usually oligonucleotides of 15 to 20 bases, to bind to each strand of DNA (annealing). DNA polymerase from *Thermus aquaticus* (*Taq* polymerase) is used to synthesize new DNA strands starting from the primers, resulting in the formation of two new DNA molecules. The process then repeated for multiple cycles, resulting in the exponentially amplified of target DNA.

- **Specific Restriction Enzyme Digestion**

Principle of specific restriction enzyme digestion

Specific restriction enzyme digestion allows fragmentation of DNA by using restriction enzyme that recognizes specific sequence DNA. *Taq* I restriction enzyme recognizes TCGA sequence which C was protected from bisulfite conversion by methylation, while *Tas* I restriction enzyme recognizes AATT sequence which the last T was unmethylated CpG before treated with bisulfite. When digesting the LINE-1 amplicons (160 bp) with *Taq* I and *Tas* I enzymes, the methylated amplicons, *Taq* I positive, yield two 80 bp DNA fragments (Fig. 17), whereas the unmethylated amplicons, *Tas* I positive yield 62 and 98 bp fragments (Fig. 18). The intensities of these amplicons will be calculated for the methylation level of LINE-1s.

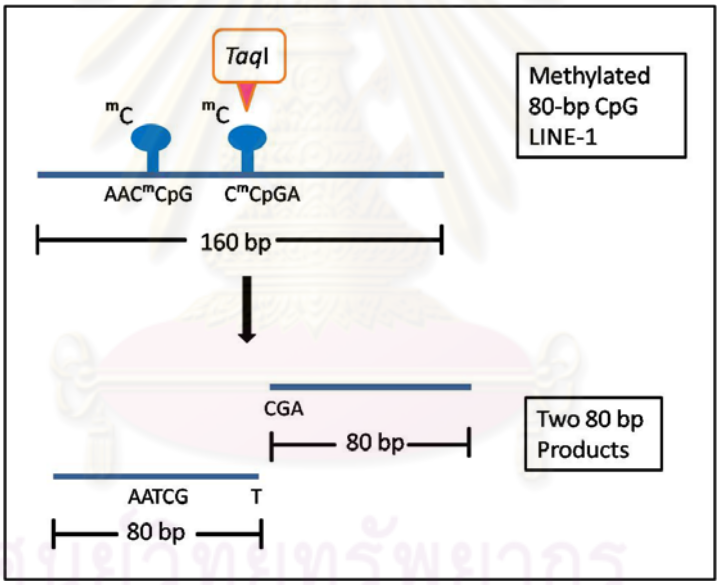


Figure 17 **COBRA LINE-1 PCR amplicon with *Taq*I recognition site (TCGA nucleotide sequences).** After bisulfite treatment and PCR, methylated CCGA will be converted to TCGAA methylated 160-bp amplicon of COBRA LINE-1 yields two 80-bp *Taq*I-digested fragments.[11, 28] Methylated cytosines are demonstrated by blue oval marks.

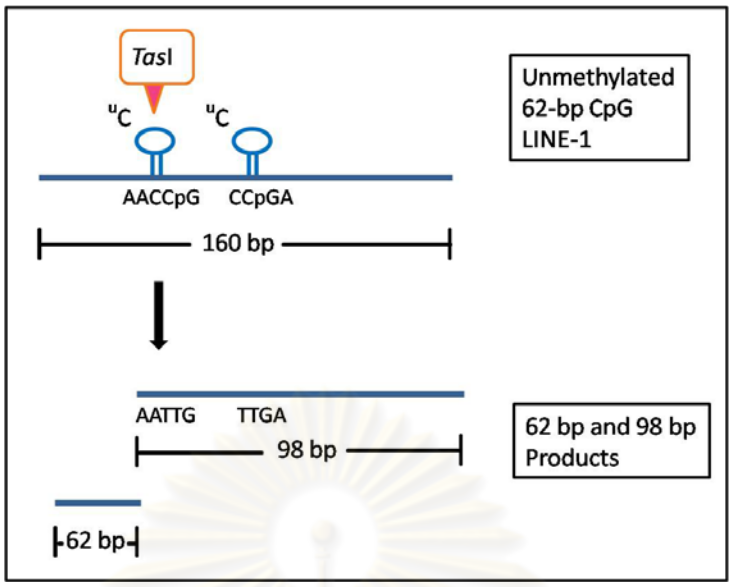


Figure 18 **COBRA LINE-1 PCR amplicons with TasI recognition site (AATT nucleotide sequences).** After bisulfite treatment and PCR, unmethylated AACCG will be converted to AATTG (TasI site). An unmethylated 160-bp amplicon of COBRA LINE-1 yields a 98-bp and a 62-bp TasI-digested fragment.[11, 28] Unmethylated cytosines are shown by hollow oval marks.

Specific restriction enzyme restriction technique

The 2 µl of PCR amplicons were digested in 8 µl of a mixture of *Taq* I, *Tas* I, NE buffer 3 and BSA under overnight incubation at 65˚C.

Methylation Analysis

- **Electrophoreses**

After digesting the LINE-1 amplicons with *Taq*I and *Tas*I enzymes, LINE-1 products were then electrophoresed in 8% nondenaturing polyacrylamide gel, at 130 voltage for approximately 40 minutes in order to separate different sizes of cut products.

- **LINE-1 Methylation Calculation**

Intensities of DNA fragments in the gel which had been stained with SYBR green nucleic acid gel stain were measured by PhosphoImager using Image Quant

Software. LINE-1 methylation was calculated as a percentage of the intensity of methylated LINE-1s digested by *Taq*I (80 bp) divided by the sum of the unmethylated LINE-1s digested by *Tas*I (98 and 62 bp) and *Taq*I positive amplicons. Then, statistical analysis was performed as indicated.

Statistical Analysis

Statistical analyses for inter-group comparisons were performed by SPSS software for Windows version 17.0. The different between LINE-1 methylation levels in each DNA source of OSCC patients were compared with those of controls by independent *t*-test. Studies of contributing factors and progression of the cancer were completed by *t*-test or one-way ANOVA as indicated. Repeated measurement in SigmaStat® for Windows version 2.03 (SPSS Inc., Chicago, IL) was used for intra-individual comparison. Significance was determined when *p*-value was lower than 0.05.

CHAPTER IV

RESULTS

Part I: Demographic Information, History and Examination

Table 9 Group frequencies

		Control (N)	**OSCC (N)**
Total		45	37
Oral rinse		45	37
Buffy coat		44	35
Plasma		45	35
Gender	Male	12	17
	Female	33	20
Age	Range	27-82	39-82
	Mean	49	62.5
History of smoking	Currently or previously smoke	7	14
	Never smoke	36	23
	Missing from total	2	-
History of drinking	Currently or previously drink	10	18
	Never drink	33	19
	Missing from total	2	-
History of betel chewing	Currently or previously chew	0	19
	Never chew	42	18
	Missing from total	3	-

Table 10 OSCC sub-group frequencies

		OSCC (N)
Location of lesion	Tongue, floor of mouth	18
	Gingiva	8
	Buccal mucosa	5
	Lip	1
	Multiple-area coverage	5
Tumor size	T1	7
	T2	11
	T3	5
	T4	13
	Missing from total	1
Lymph node involvement	N0	23
	N1-3	13
	Missing from total	1
Metastasis	Mx	14
	M0	22
	M1	0
	Missing from total	1
Stage	Stage I	7
	Stage II	7
	Stage III	6
	Stage IV	16
	Missing from total	1
Grade	Well-differentiated	11
	Moderately-differentiated	11
	Poorly-differentiated	5
	Missing from total	10

Part II: New Knowledge of Methylation Pattern to Improve OSCC Detection

1. Discovering Partial Methylation by COBRA LINE-1

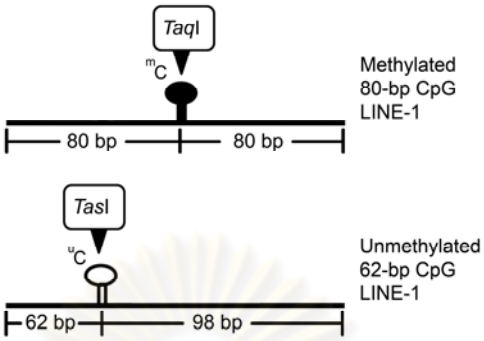


Figure 19 **Specific enzymatic restrictions by *Taq*I and *Tas*I enzymes.** *Taq*I is specific to methylated cytosine (black oval mark) at 80 bp location of 160 bp full length LINE-1s. *Tas*I is specific to unmethylated cytosine (hollow oval mark) at 62 bp location.

COBRA LINE-1 technique, used in this study, was benefited from specific enzyme digestion by *Taq*I and *Tas*I restriction enzymes. As mentioned earlier, LINE-1 amplicon for COBRA was 160 bp with 2 specific sites for *Taq*I and *Tas*I. *Taq*I is specific for methylated CpG at 80 bp and *Tas*I is specific for unmethylated CpG at 62 bp in full length LINE-1s (Fig. 19). Originally, 2 patterns of LINE-1 methylation were considered in COBRA LINE-1 concept, complete methylation which was the methylation at both 62-bp and 80-bp CpG, and complete unmethylation which was the loss of methylation at both 62-bp and 80-bp CpG. Therefore, it was understood that the product from *Taq*I cutting LINE-1s were two 80 bp amplicons (Fig. 20A) and from *Tas*I cutting LINE-1s were 98 and b2 bp amplicons (Fig. 20B).

In 2008, Phokaew reported that methylation of LINE-1s at different loci were distinctive. Complete unmethylation pattern was more common in cancer. In normal genome, complete and partially methylated sequences were more frequent.[32] Moreover, in this study, it was also found that there was the product size of 160 bp from COBRA LINE-1 as well. Although this product size might be noticed before, it has not been taken into consideration. As a result of 160 bp, it was likely that there were 4 possible patterns including partial methylation of each LINE-1 sequence to be cut. Partial methylated LINE-1 sequence could present in 2 forms, resulting in

different product amplicons. In case of unmethylated 62-bp CpG with methylated 80-bp CpG sequences, the cut products were 62, 18, and 80 bp (Fig. 20C). Sequence with methylated 62-bp CpG and unmethylated 80-bp CpG sequences yielded 160 bp uncut amplicon (Fig. 20D). All products from COBRA LINE-1, in this proposed concept, were 160, 98, 80, 62 and 18 bp. However, in reality, the size of 18 bp product was very small and contributing only insignificant percentage to the total intensity. The short sequence of 18 bp also made it difficult to be accurately identified from electrophoresed.

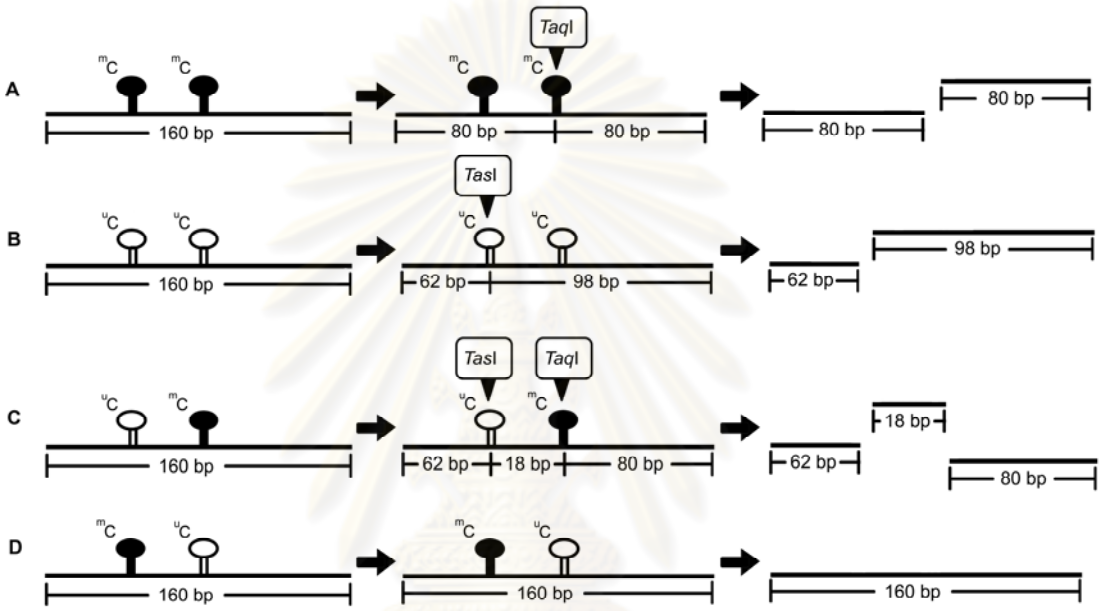


Figure 20 **Four different methylation patterns yielding different cut products from specific cuts by *Taq*I and *Tas*I.** The pattern of LINE-1 methylation is determined by methylation status of 2 CpG locations (at 62 and 80 bp). Methylated cytosines are shown by black oval marks and unmethylated cytosine by the hollow ones. Originally, only pattern A and B were considered on the basis of COBRA LINE-1. Complete methylation pattern (A) yielded 80 bp product. Complete unmethylation (B) yielded 62 and 98 bp. However, in fact, there were 2 forms of partial methylation pattern (C and D) as well. Pattern C could be cut by both enzymes, giving 62, 18 and 80 bp. Pattern D could not be split by any enzymes, providing 160 bp uncut product.

As a result, 18 bp was discarded, leaving 4 sizes of amplicon, 160, 98, 80 and 62 bp, as the counted COBRA LINE-1 products. The level of each fragment was calculated by the intensity of that product divided by sum of all product intensities (Fig. 21). COBRA LINE-1 technique could provide not only methylation level, but also additional information regarding methylation pattern.

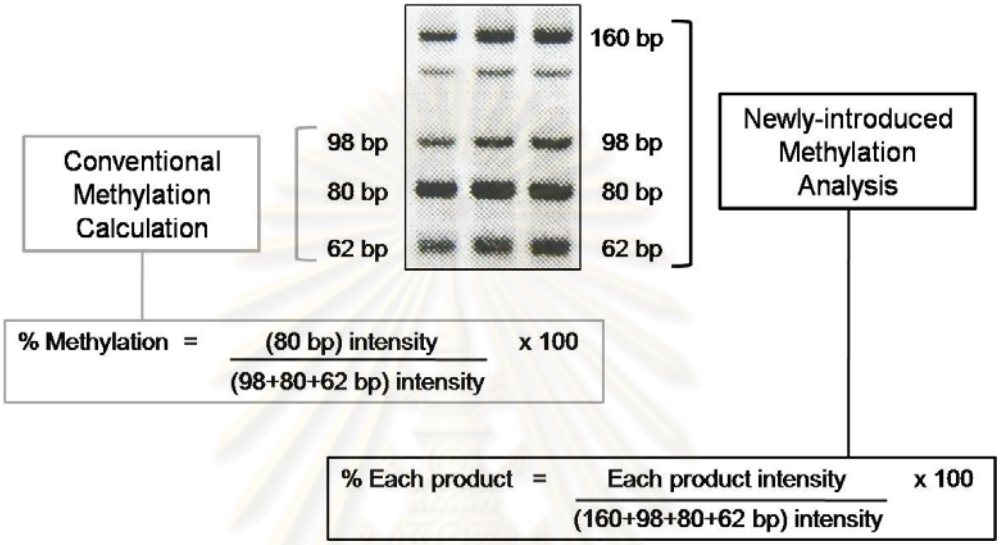


Figure 21 **Newly-introduced methylation calculation which included 160 bp from partial methylation pattern.** Percentage of product is equal to that product intensity divided by sum of all product intensities. Conventional methylation calculation method is also shown on the left.

2. Correlation of Methylation, Unmethylation, and Partial Methylation

To further understand LINE-1 partial methylation in normal and cancer, oral rinses from normal controls (normal oral rinse, NOR) and a panel of head and neck squamous cell carcinoma cell lines (WSU-HN) were compared in terms of correlation between COBRA LINE-1 products, 80 bp from methylation in LINE-1, 98 bp from complete unmethylation of LINE-1, and 160 bp from partial methylation of LINE-1 sequence. Interestingly, not only correlations between normal and cancer were different, but each of them was also statistically significant.

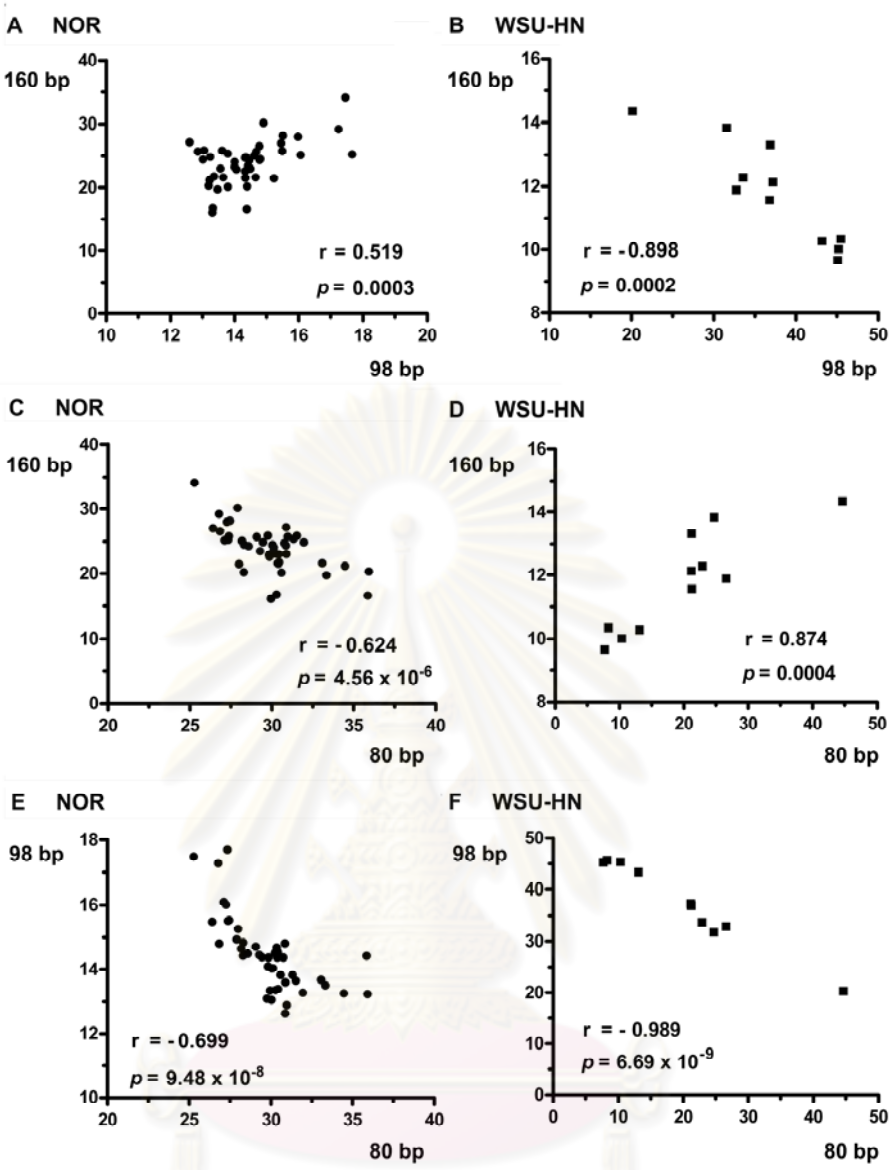


Figure 22 **Correlations between 160 bp and 98 bp (A and B), 160 bp and 80 bp (C and D), as well as 98 bp and 80 bp product (E and F) in normal oral rinse (NOR) and head and neck squamous cell carcinoma cell lines (WSU-HN).** Correlations among the three products; 160 bp, 98 bp and 80 bp, representing partial, complete unmethylation and methylation pattern, respectively, in normal and cancer cells were different.

In NOR, the level of 160 bp was directly correlated with the level of 98 bp (p = 0.0003, r = 0.519) (Fig. 22A). In contrast, these two fragments were inversely correlated in WSU-HN (p = 0.0002, r = -0.898) (Fig. 22B). Moreover, the levels of

160 bp and 80 bp of NOR were inversely correlated (p = 4.56 x 10^{-6}, r = -0.624) (Fig. 22C), whereas, in WSU-HN, these two fragments were directly correlated (p = 0.0004, r = 0.874) (Fig. 22D). In addition, the correlation of 98 bp and 80 bp was inverse in both NOR and WSU-HN (p = 9.48 x 10^{-8}, r = -0.699 and p = 6.69 x 10^{-9}, r = -0.989, respectively) (Fig. 22E and 22F).

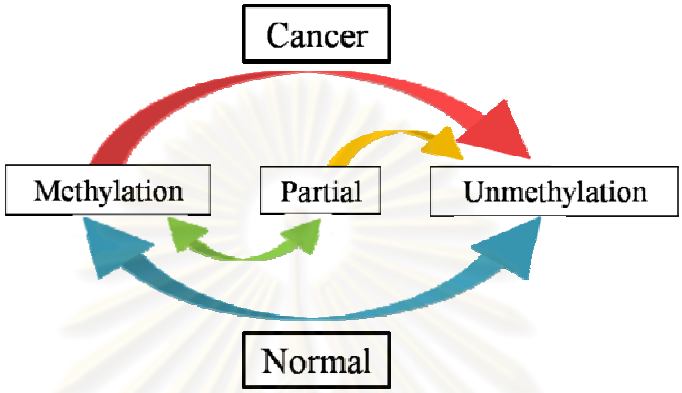


Figure 23 **A diagram representing hypothesized LINE-1 methylation dynamics comparing normal and cancer genomes.** The inverse correlation between 2 patterns suggested the changeable status between those patterns (demonstrated by arrows). Since correlations in normal and cancer were not the same, loosing and gaining methylation in normal cells and in carcinogenesis were different. There was no reported regarding methylation change in carcinogenesis as a reversible process. Thus, one-way arrow is shown in cancer.

The direct correlations suggested that partial methylation (160 bp) represented loss of methylation (98 bp) in normal DNA (Fig. 22A) but reflected methylation (80 bp) in cancer (Fig. 22D). The inverse correlations between two patterns might suggest that one pattern, with decreasing methylation, lost methylation to another one, with increasing methylation, and vice versa. In normal DNA, interchangeable methylation status was found between partial loss of methylation and methylation (Fig. 22C and Fig. 23, green arrow) and between methylation and unmethylation (Fig. 22E and Fig. 23, blue arrow). In cancer, processes of losing or gaining methylation were different from normal cells. Additionally, since the reversible process in carcinogenesis was hugely doubted and never been supported, here, methylation loss was shown as one-

way flow in cancer. In other words, from inverse correlations in cancer, unmethylated sequences were derived from partially methylated (Fig. 22B and Fig. 23, orange arrow) and methylated LINE-1 sequences (Fig. 22F and Fig. 23, red arrow).

3. Differences of COBRA LINE-1 Products between Normal and Cancer

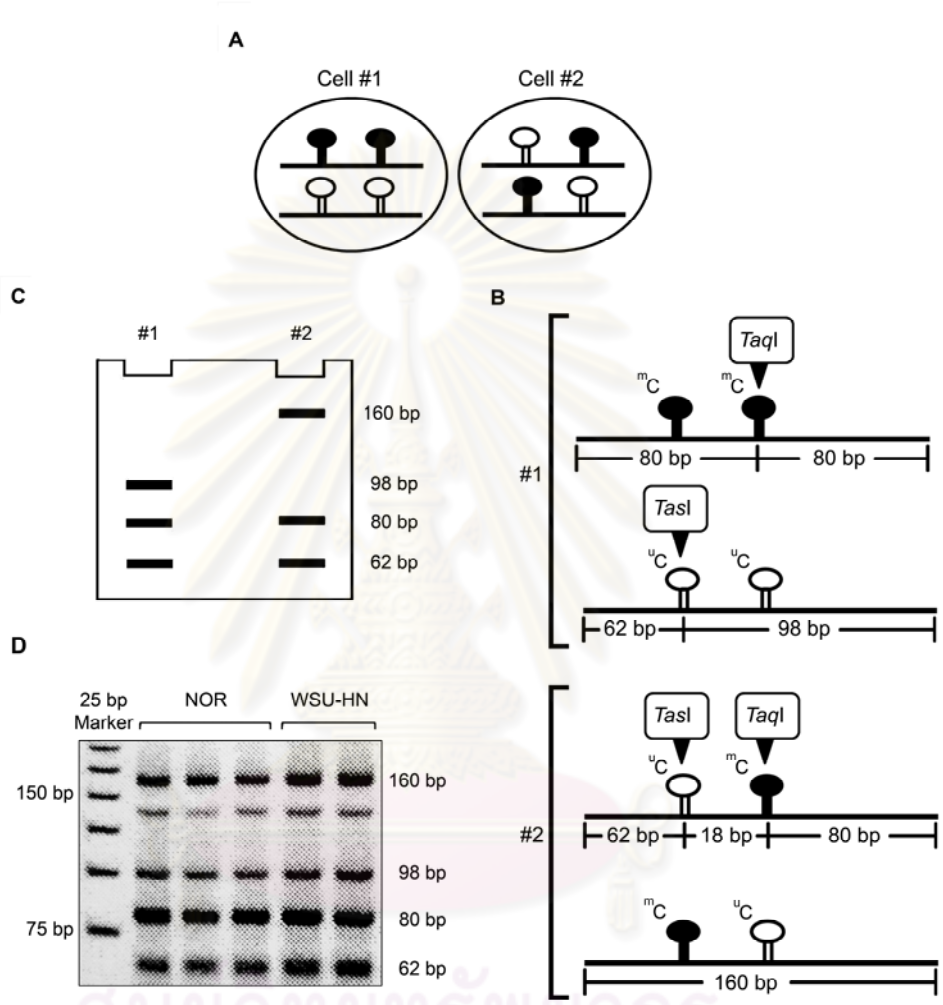


Figure 24 **Different cells having the same methylation level but different cut products.** Cell #1 and #2 in A have the same methylation level of 50%. However, patterns in those cells are different, resulting in different enzyme cut sites (B) and different products (C and D). Additional to methylation level, methylation pattern is also important.

Moreover, LINE-1 methylation level is average value of LINE-1s across whole genome. As a result, different cells having different methylation patterns may result in the same methylation level. In Figure 24A, regardless of methylation pattern, methylation

level, conventionally calculated by percentage of 80 bp/(98 bp+80 bp+62 bp), in cell #1 and #2 were approximately 50%. However, the cut products from both cells were different (Fig. 24B and 24C). Thus LINE-1 methylation alone might be not highly sufficient for cancer representation. Using oral rinse of OSCC patients as the model, percentage of 160, 98, 80, and 62 bp product amplicons, as well as overall methylation level were further compared between normal and cancer.

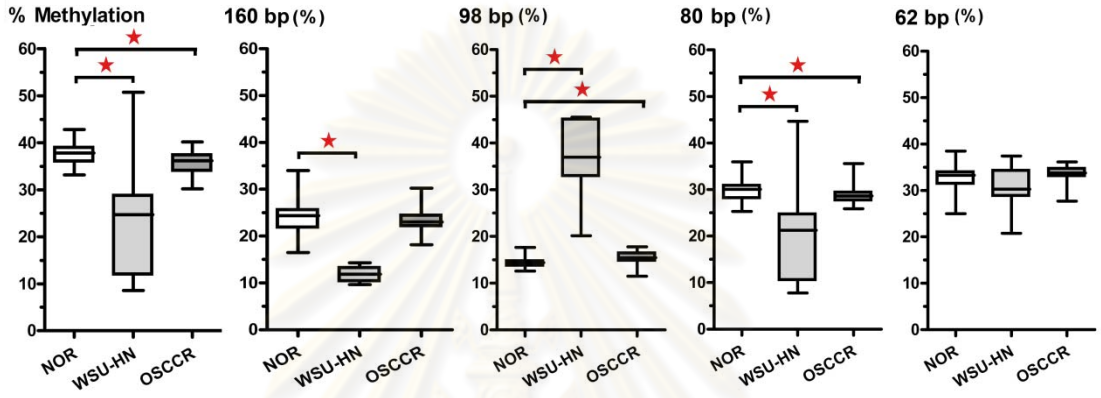


Figure 25 **Methylation level and all COBRA LINE-1 products in normal oral rinse (NOR), head and neck squamous cell carcinoma cell lines (WSU-HN) and OSCCR (OSCC oral rinse).** Regarding clinical samples, NOR was significantly different from both WSU-HN and OSCCR in conventional methylation, 98 and 80 bp levels. Stars indicate statistical significance.

Similar to previous report, LINE-1 methylation level in normal oral rinse, NOR, (mean ± SD = 37.65% ± 2.50) was significantly higher than in head and neck squamous cell carcinoma cell lines, WSU-HN, (23.18% ± 12.04) (p = 0.0026). Interestingly, the percentage of 160 bp from partial methylation in NOR (23.83% ± 3.46) was also significantly higher than in WSU-HN (11.77% ± 1.60) ($p = 1.07 \times 10^{-15}$). Therefore, normal DNA had more partially methylated LINE-1 sequences than squamous cell carcinoma DNA. Moreover, the levels of 80 bp in NOR (29.88% ± 2.31) was higher than in WSU-HN (20.23% ± 10.55) as well (p = 0.013). On the contrary, the level of 98 bp from complete unmethylation of WSU-HN (37.13% ± 7.66) increased significantly when compared with NOR (14.42 ± 1.16%), $p = 1.75 \times 10^{-6}$ (Fig. 25).

Comparing oral rinse of OSCC patients (OSCC rinse, OSCCR) with NOR, the results were similar to those in WSU-HN. Methylation level of LINE-1s in OSCCR (35.69% ± 2.36) was significantly lower than that of NOR (37.65% ± 2.50), $p = 0.001$. Level of 80 bp in OSCCR (28.71% ± 1.77) were also significantly lower than that in NOR (29.88% ± 2.31), $p = 0.014$. On the other hand, 98 bp in OSCCR (15.50% ± 1.37) was significantly higher than that in NOR (14.42% ± 1.16), $p = 0.0002$. However, 160 bp level, from partial methylation, did not provide significant difference between NOR and OSCCR (23.83% ± 3.46 and 23.30% ± 2.17, respectively), $p = 0.404$. Additionally, percentage of 62 bp in NOR (33.27% ± 2.97) was not different from those in both WSU-HN (30.87% ± 4.48) and OSCCR (33.49% ± 1.68), $p = 0.116$ and 0.689 respectively. Conclusively, only LINE-1 methylation, 98 and 80 bp levels gave significance between NOR and both types of cancer DNA (Fig. 25).

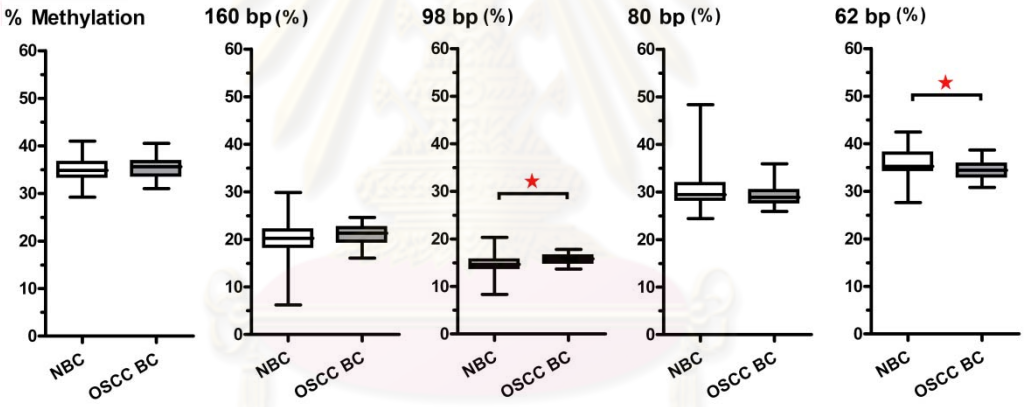


Figure 26 **Methylation level and all COBRA LINE-1 products in normal buffy coat (NBC) and OSCC buffy coat (OSCC BC).** Differences between NBC and OSCC BC were found in 98 and 62 bp.

Information in normal buffy coat (NBC) and buffy coat of OSCC patients (OSCC BC) were further studied. It was found that the level of 98 and 62 bp were able to distinguish OSCC BC (15.65% ± 1.08 and 34.55% ± 2.15, respectively) from NBC (14.64% ± 2.11 and 35.70% ± 2.92, respectively), $p = 0.0073$ and 0.047, respectively. In NBC, LINE-1 methylation (34.97% ± 2.45), 160 bp (19.85% ± 3.80), and 80 bp (30.50% ± 4.19) were not different from those of OSCC BC (35.37% ± 2.23,

20.93% ± 2.25, and 29.14% ± 2.00, respectively), p = 0.458, 0.139, and 0.06, respectively (Fig. 26).

All COBRA LINE-1 products were then analyzed in the plasma samples as well. In normal plasma (NPM), the level of 98 bp (14.83% ± 1.93), 80 bp (29.83% ± 2.25), and 62 bp (35.11% ± 3.38) differed significantly from those of OSCC (16.07% ± 1.39, 28.49% ± 1.44, and 33.39% ± 1.89, respectively), p = 0.0018, 0.0018, and 0.0051, respectively. On the other hand, methylation level (35.39% ± 2.73) and 160 bp (21.46% ± 3.50) in NPM were not statistically different from those in OSCC PM (35.56% ± 1.91 and 22.48% ± 2.52, respectively), p = 0.761 and 0.130, respectively (Fig. 27).

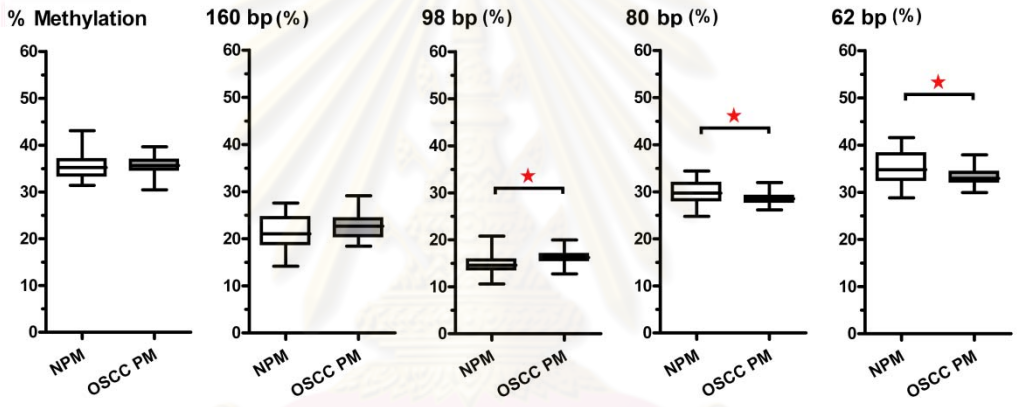


Figure 27 **Methylation level and all COBRA LINE-1 products in normal plasma (NPM) and OSCC plasma (OSCC PM).** Differences between NPM and OSCC PM were found in 98, 80 and 62 bp.

4. Difference of COBRA LINE-1 Products between Matched Pairs

Previous studies reported no association between methylation level and age or gender.[11] However, to eliminate methylation information possibly influenced from these 2 factors, normal subjects and OSCC patients were matched by identical age and gender. Accordingly, 16 pairs were matched.

Analyzed by t-test, in NOR, methylation (38.46% ± 2.82), 98 bp (14.30% ± 1.26) and 80 bp (30.15% ± 2.75) were significantly different from those of matched OSCCR (35.14% ± 2.51, 15.82% ± 1.01 and 28.15% ± 1.31, respectively), p = 0.0013,

0.0002, and 0.0085, respectively (Fig. 28A, 28C, and 28D). Level of 160 bp and 62 bp provided insignificance between normal (24.19% ± 2.61 and 32.56% ± 1.60, respectively) and OSCCR (23.43% ± 1.51 and 33.60% ± 1.31, respectively), p = 0.2621 and 0.0510, respectively (Fig. 28B and 28E). Significances found when cases were matched were also found in methylation level, 98 bp, and 80 bp in total population analysis (cases unmatched) (Fig. 25).

In NBC, only 98 bp (14.87% ± 1.25), and 80 bp (29.94% ± 1.79) differed significantly from those of matched OSCC BC (16.06% ± 0.78, and 28.24% ± 1.19), p = 0.0026, and 0.0012, respectively (Fig. 28C and 28D). On the contrary, methylation (36.04% ± 2.30), 160 bp (20.55% ± 2.68) and 62 bp (35.17% ± 1.69) in NBC were not different from those of OSCC BC (34.42% ± 2.02, 22.01% ± 1.68 and 34.13% ± 1.51, respectively), p = 0.0541, 0.1253, and 0.1135, respectively (Fig. 28A, 28B and 28E). When cases were unmatched, 98 bp level also showed significance (Fig. 26). However, 80 bp which provided insignificant difference before (Fig. 26) could differentiate NBC from OSCC BC when age and gender were matched (Fig. 28D). On the contrary, 62 bp in buffy coat which provided significance in total-case comparison (Fig. 26) was unable to separate normal and cancer upon matched cases (Fig. 28E).

In NPM, only level of 160 bp (20.64% ± 2.69), 98 bp (15.09% ± 2.22), and 80 bp (30.65% ± 1.81) were significantly different from those of matched OSCC PM (23.11% ± 2.06, 16.35% ± 0.73, and 28.29% ± 1.34, respectively), p = 0.0224, 0.0417, and 0.001, respectively (Fig. 28B, 28C, and 28D). Insignificantly, in NPM, methylation level (36.81% ± 3.29) and 62 bp (34.19% ± 3.59) were different from those of OSCC PM (35.63% ± 1.42 and 32.68% ± 1.15, respectively), p = 0.2496 and 0.1694, respectively (Fig. 28A and 28E). When cases were unmatched, 98 and 80 bp level also showed significance (Fig. 27). However, 160 bp which gave insignificant difference in total-case analysis (Fig. 27) provided significance when age and gender were matched (Fig. 28). On the other hand, 62 bp in plasma could not differentiate normal from OSCC when cases were matched (Fig. 28E), unlike when age and gender were discarded (Fig.27).

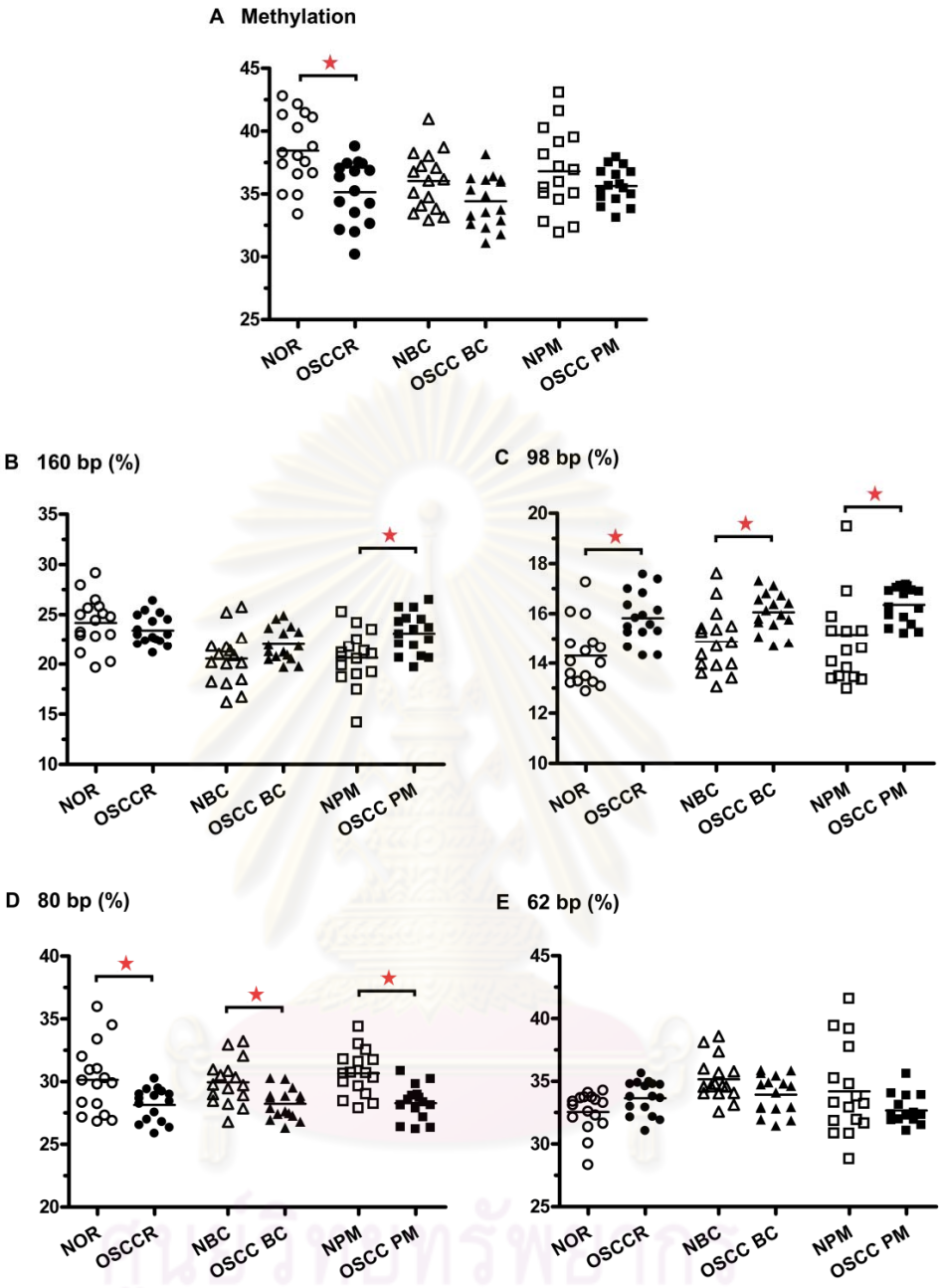


Figure 28 **Methylation level and all COBRA LINE-1 products in normal oral rinse (NOR) compared with OSCC oral rinse (OSCCR) and normal buffy coat (NBC) compared with OSCC buffy coat (OSCC BC) of 16 matched pairs.** Only 98 and 80 bp provided significant differences between all sample types of normal and OSCC.

Table 11 Levels and changed levels of COBRA LINE-1 products in normal and cancer DNA

Types of Sample	Levels	All Obtained Cases				16 Matched Cases			
		Normal (mean, %)	**OSCC** (mean, %)	**Change**	***t*-test**	**Normal** (mean, %)	**OSCC** (mean, %)	**Change**	**Paired *t*-test**
OR	**Meth**	37.65	35.69	↓	0.0005	38.46	35.14	↓	0.0013
	160 bp	23.83	23.31	↓	0.4042	24.19	23.43	↓	0.2621
	98 bp	14.42	15.50	↑	0.0002	14.30	15.82	↑	0.0002
	80 bp	29.88	28.71	↓	0.0140	30.15	28.15	↓	0.0085
	62 bp	33.27	33.49	↑	0.6894	32.56	33.66	↑	0.0510
BC	**Meth**	34.97	35.37	↑	0.4576	36.04	34.42	↓	0.0541
	160 bp	19.85	20.93	↑	0.1395	20.55	22.01	↑	0.1253
	98 bp	14.64	15.65	↑	0.0073	14.87	16.06	↑	0.0026
	80 bp	30.51	29.14	↓	0.0603	29.95	28.24	↓	0.0012
	62 bp	35.70	34.55	↓	0.0470	35.17	34.13	↓	0.1135
PM	**Meth**	35.39	35.56	↑	0.7612	36.81	35.63	↓	0.2496
	160 bp	21.46	22.48	↑	0.1303	20.64	23.11	↑	0.0224
	98 bp	14.83	16.07	↑	0.0018	15.09	16.35	↑	0.0417
	80 bp	29.83	28.49	↓	0.0018	30.65	28.29	↓	0.0010
	62 bp	35.11	33.39	↓	0.0051	34.19	32.68	↓	0.1694

Arrows determined changes from normal percentage

Red indicated statistical significance

Meth = Methylation level

OR = Oral rinse

BC = Buffy coat

PM = Plasma

At this point, the level of 98 bp, from complete unmethylation pattern, demonstrated the most reliable result, capability of distinguishing between normal and OSCC by all three types of DNA and by matched-cases analysis as well as when age

and gender were not considered. The level of 80 bp also provided good potential in depicting OSCC from normal by all three DNA types in 16 matched cases. Methylation level, originally calculated, showed detecting ability only by oral rinse sample.

5. Improving Accuracy in DNA-contaminated Oral Rinse Samples

Though previous study, investigating LINE-1 methylation level in oral rinse, suggested that methylation level in OSCCR was significantly lower than that in NOR, about 64% of OSCCR samples had LINE-1 methylation level overlapping with the range of NOR values.[11] The same situation was also found in this study. LINE-1 methylation, in more than three quarter of OSCCR, was within NOR range. Therefore, sensitivity of methylation level from COBRA LINE-1, conventionally analyzing only 2 patterns of total methylation and unmethylation, in oral rinse for detecting OSCC was still limited. This incidence might be due to the contamination of other DNA types, particularly from normal white blood cells (NWBC) and possibly OSCC white blood cells (OSCC WBC), in oral rinses of OSCC patients. Also, NWBC could be found in normal saliva. Unfortunately, since LINE-1 methylation level in each tissue varies,[28] the levels in contaminated cancer samples were deviated and unable to represent OSCC accurately.

Using buffy coat as a model of white blood cells, intra-individual comparisons by paired *t*-test between buffy coat and oral rinse DNA were performed in each person in normal and OSCC population. Interestingly, 98 bp in NBC (14.64% ± 2.11) was not different from NOR (14.41% ± 1.17) within a normal individual ($p = 0.454$) and 98 bp in OSCC BC (15.72% ± 1.05) was almost the same as OSCCR (15.50% ± 1.40), $p = 0.452$ (Fig. 29C). The same phenomenon was found in 80 bp as well (Fig. 29D). Individually, 80 bp level in NBC (30.51% ± 4.19) and NOR (29.92% ± 2.32) were not different ($p = 0.444$) and neither as OSCC BC (29.14% ± 2.00) and OSCCR (28.68% ± 1.82), $p = 0.332$ (Fig. 29D). These suggested that, with 98 and 80 bp, there would be no discrepancy when using oral rinse sample contaminated with different DNA types. Despite LINE-1 methylation in OSCC BC (35.37% ± 2.23) was relatively the same as in OSCCR (35.71% ± 2.39), $p = 0.565$, methylation in NBC (34.97% ± 2.45) was significantly different from NOR (37.63% ± 2.52), $p = 1.24 \times 10^{-5}$ (Fig. 29A).

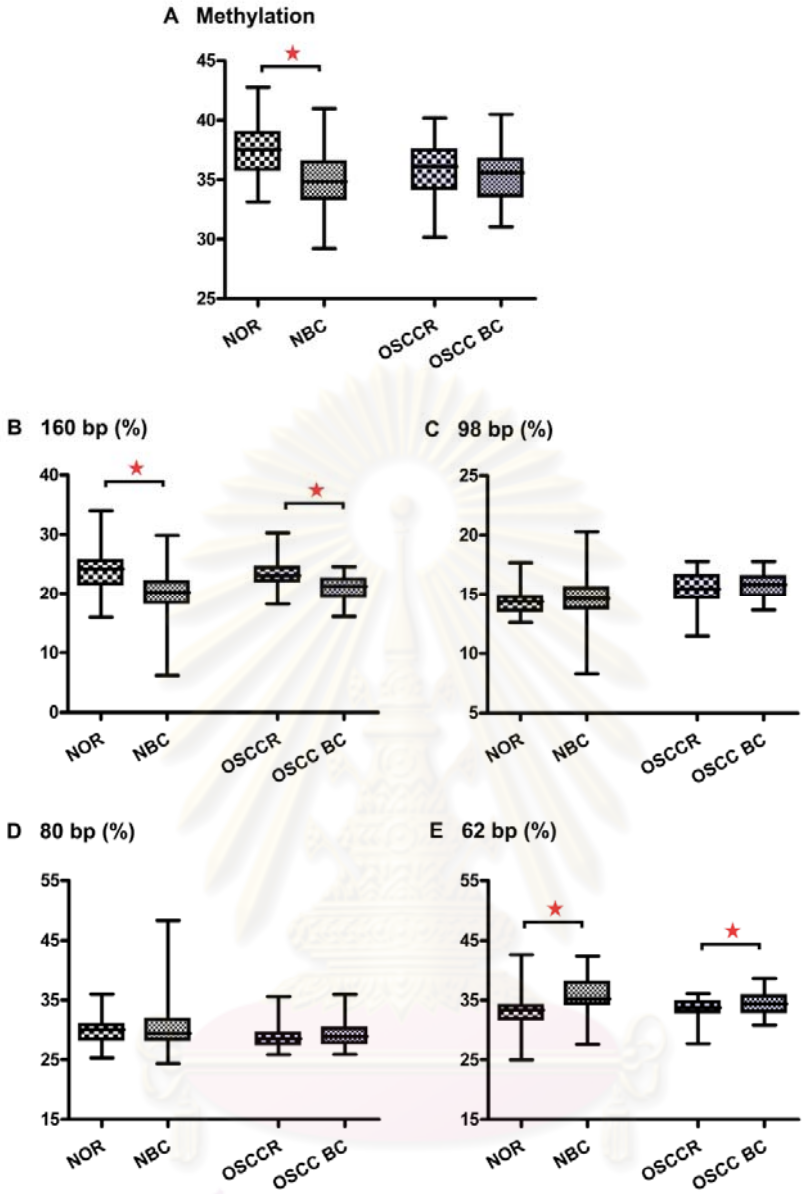


Figure 29 **Intra-individual comparison of oral rinse sample with the buffy coat in both normal and OSCC.** Considering 98 and 80 bp, there was no differences between oral rinse and buffy coat in both normal and OSCC groups (C and D). On the contrary, cell-type specific event was generally observed in 160 (B) and 62 bp (E) as well as conventional methylation level in only normal group (A).

On the other hand, 160 bp and 62 bp had intra-individual discrepancy among different DNA types in oral rinse. The level of 160 bp in NBC (19.85% ± 3.80) and NOR (23.69% ± 3.37) as well as OSCC BC (20.93% ± 2.45) and OSCCR (23.37% ±

2.20), were different within the same individual, p = 1.16 x 10^{-6} and 0.0001, respectively (Fig. 29B). Additionally, the level of 62 bp in NBC (35.70% ± 2.92) and NOR (33.35% ± 2.96) as well as OSCC BC (34.55% ± 2.15) and OSCCR (33.45% ± 1.72), were also different in the same person, p = 1.86 x 10^{-5} and 0.0281, respectively (Fig. 29E), resulting in decreased sensitivity of using oral rinse samples.

It was striking that the level of 98 bp and 80 bp provided significant differences between normal and cancer DNA; NOR and OSCCR, NOR and WSU-HN, NBC and OSCC BC as well as NPM and OSCC PM, but presented the same level among DNA from different sources in both normal and OSCC individual. Thus, 98 and 80 bp amplicons from COBRA LINE-1 had the highest potential detecting OSCC especially by oral rinse contaminated by several other DNA types.

6. Sensitivity and Specificity of OSCC marker candidates

Receiver-operating characteristic (ROC) curve is favorably used to determined important information regarding accuracy of the tests; area under the curve (AUC), sensitivity, and specificity. The more AUC is approaching 1, the higher sensitivity and specificity the test provides. Here, since the products that provided significant differences between normal and OSCC by clinically-obtained samples; oral rinse, buffy coat, and plasma, they were further analyzed with the ROC. Therefore, conventional LINE-1 methylation level, 98 bp, 80 bp, and 62 bp product levels were examined. To determine sensitivity and specificity of the tests, OSCCR was evaluated against NOR. Additionally, OSCC BC and OSCC PM were also evaluated against NBC and NPM, respectively. Analysis of WSU-HN countering NOR were shown as well (Fig. 30).

Among these analyses, 98 bp level revealed the most promising result. It provided AUC of 1 with remarkable sensitivity and specificity of 100% (p = 0) in distinguishing WSU-HN from NOR. Further, when comparing normal and cancer DNA by clinically-obtained samples; oral rinse, buffy coat, and plasma, 98 bp showed the highest AUC (0.763, 0.713, and 0.744 respectively). Sensitivity and specificity provided by oral rinse sample were 72.97% and 75.56% respectively, by buffy coat sample were 67.65% and 74.42% respectively, and by plasma sample were 75.00% and 75.56%, respectively (Table 12).

Table 12 Receiver-operating characteristic (ROC) curve of measurements among various types of sample

Samples	Levels	ROC				
		AUC	*p*-value	Sensitivity (%)	Specificity (%)	Cut-off Value[a]
WSU-HN[b]	**Methylation**	0.909	0.0001	90.91	100	≤ 30.09
N = 11	**98 bp**	1	0	100	100	> 17.6768
	80 bp	0.905	0.0001	90.91	95.56	≤ 26.63
	62 bp	0.678	0.1276	63.64	82.22	≤ 31.15
OSCCR[b]	**Methylation**	0.707	0.0003	78.38	53.33	≤ 37.4979
N = 37	**98 bp**	0.763	0.0001	72.97	75.56	> 14.7963
	80 bp	0.676	0.0038	83.78	60.00	≤ 29.7017
	62 bp	0.589	0.1640	67.57	55.56	> 33.38
OSCC BC[c]	**Methylation**	0.551	0.4459	58.82	60.47	> 35.11
N = 35	**98 bp**	0.713	0.0004	67.65	74.42	> 15.50
	80 bp	0.601	0.1221	82.35	46.51	≤ 30.30
	62 bp	0.632	0.0364	42.86	84.09	≤ 33.94
OSCC PM[d]	**Methylation**	0.557	0.3851	80.00	40.00	> 34.19
N = 35	**98 bp**	0.744	0.0001	75.00	75.56	> 15.55
	80 bp	0.673	0.0041	77.78	57.78	≤ 29.12
	62 bp	0.640	0.0249	91.67	40.00	≤ 36.36

Gray band indicated statistical significance from independent *t*-test and ROC

[a] Cut-off value indicated cancer

[b] ROC data was obtained from analyses with oral rinses of control group (N = 45)

[c] ROC data was obtained from analyses with buffy coat of control group (N = 44)

[d] ROC data was obtained from analyses with plasma of control group (N = 45)

WSU-HN = Head and neck squamous cell carcinoma cell lines

OSCCR = Oral rinses of oral squamous cell carcinoma patients

OSCC BC = Buffy coat of oral squamous cell carcinoma patients

Table 13 Receiver-operating characteristic (ROC) curve of measurements among oral rinse samples and biopsied tissues from the previous cohort

Samples	Level (%)	ROC				
		AUC	*p*-value	Sensitivity (%)	Specificity (%)	Cut-off Value[a]
OSCCR[b]	**Methylation**	0.621	0.1558	48.15	100	< 42.7815
N = 27	**98 bp**	0.926	0.0001	92.59	88.73	> 15.5175
	80 bp	0.683	0.0191	59.26	86.05	> 31.561
	62 bp	0.624	0.0833	62.96	64.44	≤ 32.52
Biopsied	**Methylation**	0.661	0.0919	52.38	100	≤ 31.982
Tissue[b]	**98 bp**	0.967	0.0001	95.24	88.37	> 15.5175
N = 21	**80 bp**	0.605	0.2595	42.86	90.70	≤ 26.9659
	62 bp	0.585	0.2897	38.10	82.22	> 34.73

Gray band indicated statistical significance from independent *t*-test and ROC

[a] Cut-off value indicated cancer

[b] ROC data was obtained from analyses with oral rinses of control group (N = 45)

OSCCR (N = 27) and OSCC biopsied tissues (N = 21) from the previous study were re-analyzed by ROC curve. Among values investigated, 98 bp level also gave the highest AUC in both OSCCR (0.926) and OSCC biopsied tissues (0.967) providing sensitivity of 92.59% and 95.24% respectively and specificity of 88.73% and 88.37% respectively (Table 13).

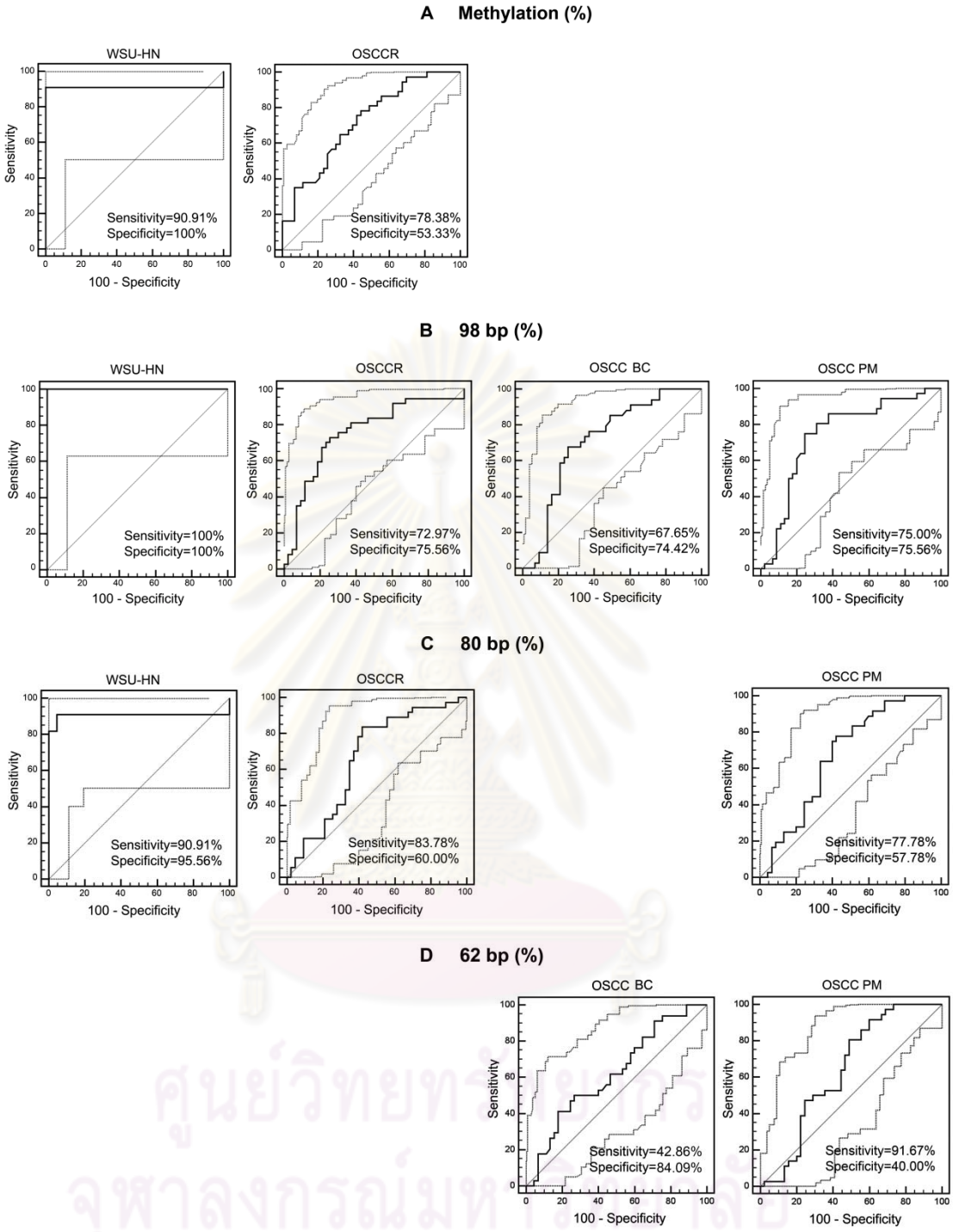


Figure 30 **ROC curves presenting sensitivity and specificity of the test measuring conventional LINE-1 methylation level (A), 98 bp level (B), 80 bp level (C) and 62 level (D).** Only curves of levels providing significances by *t*-test and ROC, which were depicted in Table 12, were shown here.

Part III: Comparison between Original LINE-1 Methylation Level and Newly-introduced LINE-1 Complete Unmethylation (98 bp)

1. Methylation Levels and 98 bp Levels in Oral Rinse, Buffy Coat and Plasma, of OSCC Patients

1.1 Normal Controls VS OSCCs

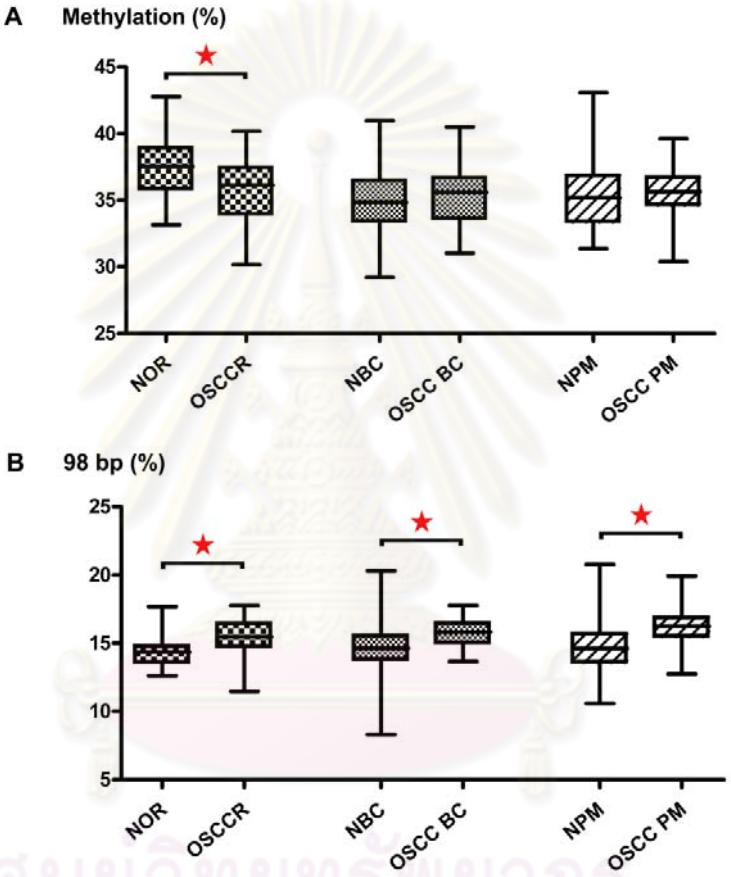


Figure 31 **Methylation levels (A) and complete unmethylation levels (B) between normal and OSCC in oral rinse, buffy coat and plasma.** Significant higher level of 98 bp in OSCC was detected by all DNA sources (B). Conventional hypomethylation (A) in oral rinse was confirmed.

As reported in Part II and Table 11, the level of 98 bp product amplicon in OSCCR was significantly higher than that in NOR, on the contrary to conventional methylation level which was significantly lower. However, while methylation level

did not provide significance in buffy coat and plasma, 98 bp in OSCC BC as well as OSCC PM were significantly increased, compared to NBC and NPM, respectively (Fig. 31).

1.2 Normal Controls VS Stage I OSCCs

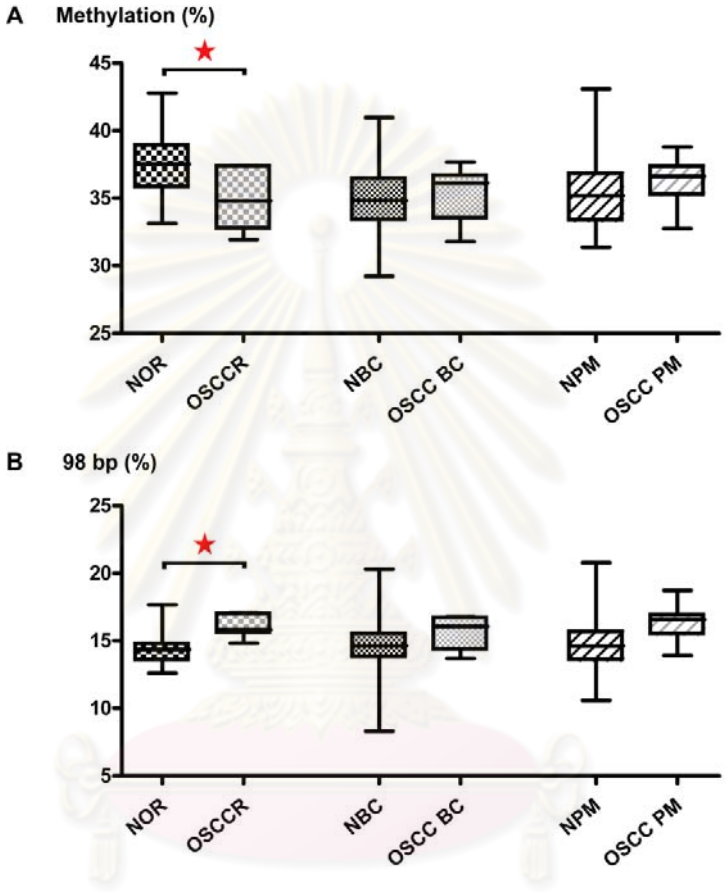


Figure 32 **Methylation levels (A) and complete unmethylation levels (B) between normal and only stage I OSCC in oral rinse, buffy coat and plasma.** Conventional methylation and 98 bp in only oral rinse could significantly differentiate OSCC from normal.

Additionally, to determine whether LINE-1 methylation and 98 bp could detect OSCC in its early stage, only stage I OSCC was compared with controls. Methylation level of stage I OSCC decreased significantly only in oral rinse sample (NOR = 37.65% ± 2.50, OSCCR = 34.81% ± 2.20, p = 0.0066). Nonetheless, methylation level stage I OSCC BC (35.18% ± 2.17) and stage I OSCC PM (36.25% ± 1.89) were insignificantly higher than their normal controls (34.97% ± 2.45 and

35.39% ± 2.73, respectively), p = 0.8382 and 0.4282, respectively (Fig. 32A). These findings were the same as comparison between normal and all-stage OSCC (Fig. 31A).

In oral rinse, 98 bp of stage I patient group (16.01% ± 0.81) was significantly higher than that of NOR (14.42% ± 1.16), p = 0.0011. However, despite giving significant result regardless of stage, 98 bp in stage I OSCC BC (15.60% ± 1.25) and stage I OSCC PM (16.36% ± 1.48) did not differ significantly from those of NBC (14.64% ± 2.11) and NPM (14.83% ± 1.93), p = 0.2459 and 0.0502, respectively (Fig. 32B).

2. Intra-Individual Comparison of LINE-1 Methylation and 98 bp Levels in Oral Rinse, Buffy Coat, and Plasma

Only subjects whose all three types of samples could be obtained were analyzed for intra-individual difference of LINE-1 methylation and 98 bp level. Unlike one-way ANOVA used to compare between pooled samples, repeated measurement was performed to determined difference in each subject.

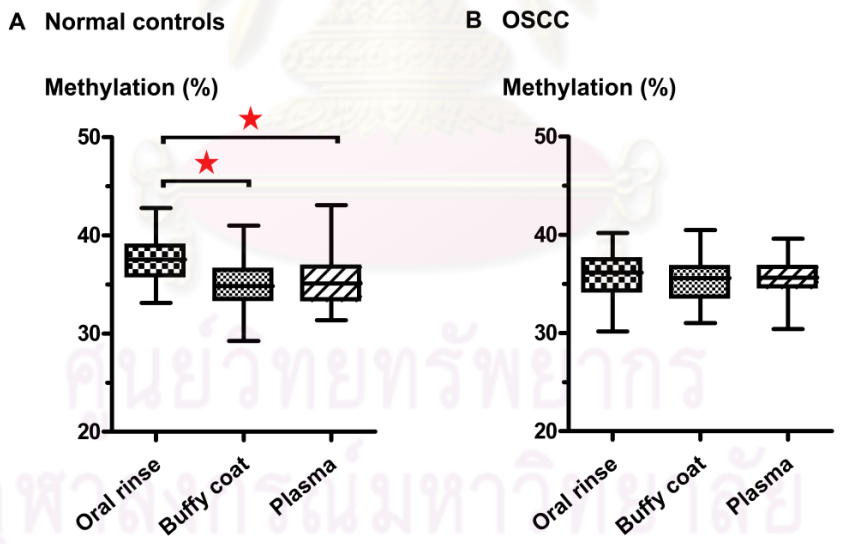


Figure 33 **Intra-individual comparison between methylation levels in oral rinse, buffy coat and plasma in normal controls (A) and OSCC (B).** In normal subjects, methylation level in oral rinse was significantly higher than those of buffy coat and plasma. There was no difference between methylation in the 3 OSCC samples.

Within 44 normal individuals, the level in oral rinse (37.64% ± 2.52) was significantly higher than that in both buffy coat (34.97% ± 2.45), p = 0.001, and plasma (35.35% ± 2.74), p = 0.001. The level in buffy coat was not significantly lower than that in plasma (p = 0.754) (Fig. 33A). Within 35 OSCC individuals, LINE-1 methylation level in oral rinse (35.71% ± 2.39) was the highest and gradually decreased in plasma (35.56% ± 1.91) and buffy coat (35.37% ± 2.23), respectively. There was no statistical significance among three types of sample (Fig. 33B).

In NOR, 98 bp level (14.41% ± 1.17) was slightly lower than that in NBC (14.64% ± 2.11), and NPM (14.84% ± 1.95), respectively. The differences among all were not significant, p = 0.448 (Fig. 34A). In OSCC individuals, 98 bp level in OSCCR (15.50% ± 1.40) was lower than that in OSCC BC (15.72% ± 1.05), and OSCC PM (16.07% ± 1.39), respectively. There was no significance among three types of sample, p = 0.240 (Fig. 34B), similarly to methylation level in OSCC.

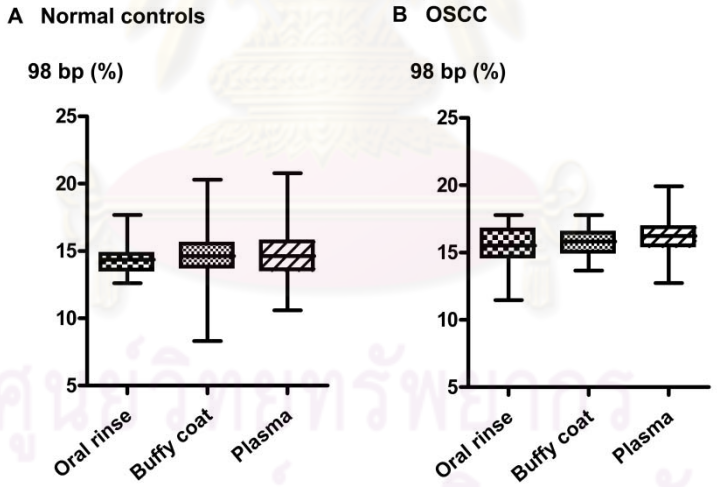


Figure 34 **Intra-individual comparison between 98 bp in oral rinse, buffy coat and plasma, in normal controls (A) and OSCC (B).** In both normal and OSCC groups, different DNA sources had similar levels of 98 bp.

3. Analyses of Possible Relating Factors

3.1 Gender

Considering gender, *t*-test was performed to determine if LINE-1 methylation and complete unmethylation levels were different among the group of normal males and normal females. Male OSCC patients (OSCC male) were compared with female OSCC patients (OSCC female).

3.1.1 Oral Rinse

Between normal groups, there was no significant difference between LINE-1 methylation level in oral rinse of male (38.26 ± 2.61%) and female (37.42% ± 2.46), $p = 0.324$. Among OSCCs, methylation of OSCC male (34.75% ±1.72) was significantly lower than that of female (36.49% ± 2.57), $p = 0.0233$ (Fig. 35A).

The level of 98 bp in oral rinse of normal male (14.68% ± 1.48) was slightly higher than that of female (14.33% ± 1.03), $p = 0.378$. Also in OSCC group, no significant difference was found between 98 bp level in male (15.60% ±1.11) and female (15.42% ± 1.58), $p = 0.690$ (Fig. 35B).

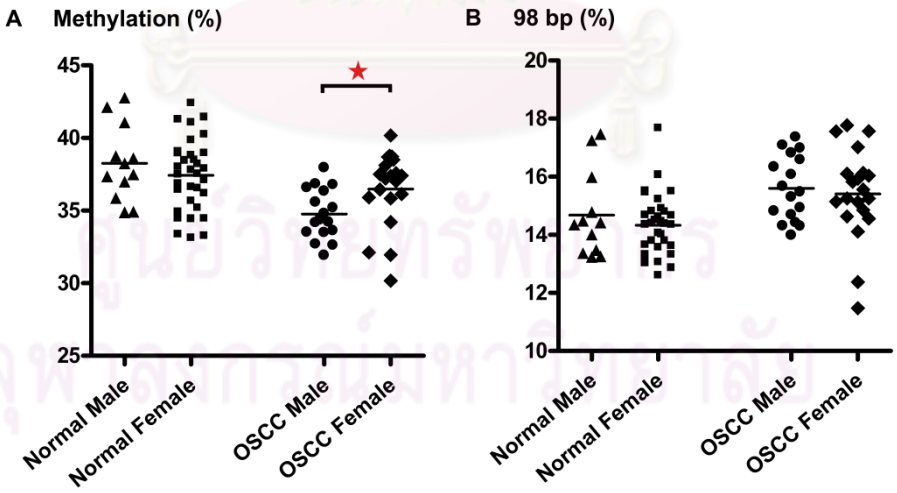


Figure 35 **Methylation (A) and 98 bp levels (B) in oral rinse of normal and OSCC male and female.** Conventional methylation level in oral rinse of OSCC male was significantly lower than that of OSCC female.

3.1.2 Buffy Coat

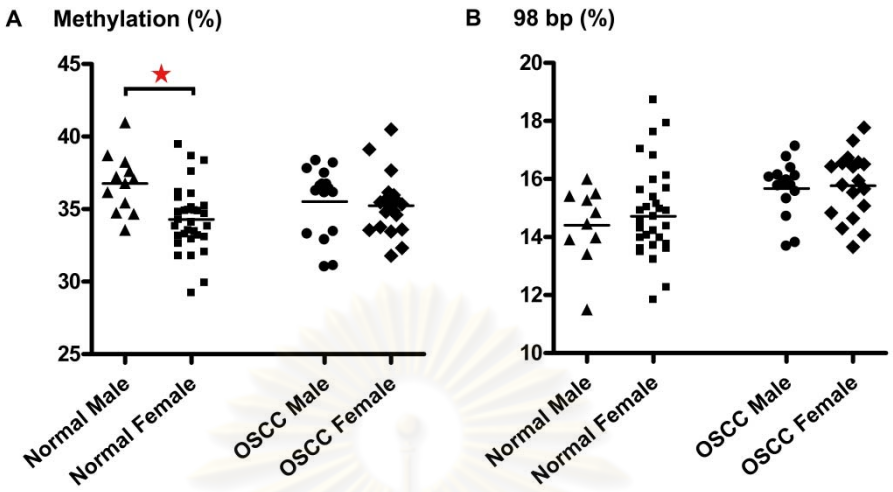


Figure 36 **Methylation (A) and 98 bp levels (B) in buffy coat of normal and OSCC male and female.** Conventional methylation level in buffy coat of normal female was significantly lower than that of normal male.

In normal group, methylation level of LINE-1s in buffy coat of male (36.78% ± 2.03) was significantly higher than that of female (34.30% ± 2.27), p = 0.0019. On the contrary, in OSCC group, the level in buffy coat of male was (35.53% ± 2.39) and female (35.24% ± 2.14) were relatively the same, p = 0.701 (Fig. 36A).

The level of 98 bp in buffy coat of normal male (14.41% ± 2.82) was not significantly different from that of normal female (14.72% ± 1.82), p = 0.378. Also in OSCC group, the level in buffy coat of male (15.67% ± 0.96) was almost the same as that of female (15.77% ± 1.14), p = 0.969 (Fig. 36B).

3.1.3 Plasma

LINE-1 methylation in plasma of normal male (36.83% ± 2.97) was significantly higher than that of normal female (34.87% ± 2.48). p = 0.0314. In OSCC group, the level in plasma of male (34.91% ± 2.07) was lower than that of female (36.10% ± 1.63). Nevertheless, the difference between OSCC subgroups did not reach significance, p = 0.066 (Fig. 37A).

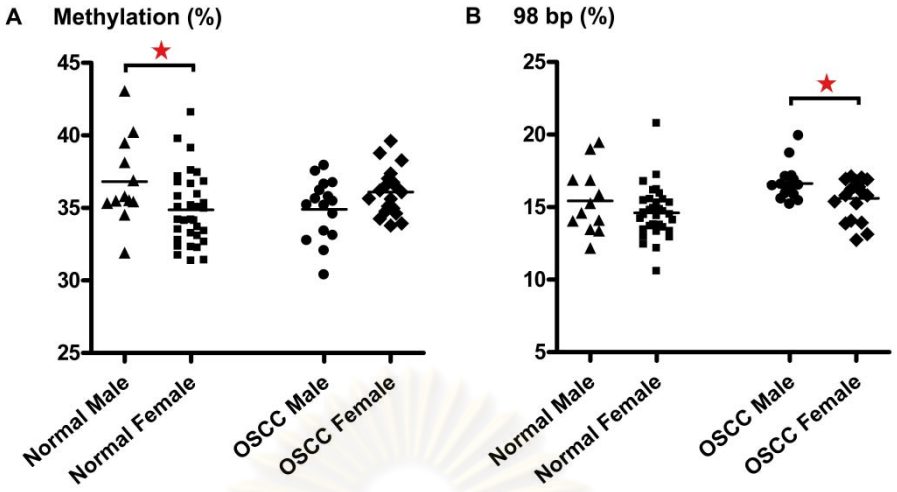


Figure 37 **Methylation (A) and 98 bp levels (B) in plasma of normal and OSCC male and female.** Normal female had significantly lower methylation in plasma than normal male.

Levels of 98 bp in plasma of normal male (15.44% ± 2.27) was slightly higher than that of normal female (14.61% ± 1.77), p = 0.205. There was significant difference between OSCC male (16.63% ± 1.21) and OSCC female (15.61% ± 1.39), p = 0.0286 (Fig. 37B).

3.2 Age

Pearson's correlation was performed to determine correlation of LINE-1 methylation and complete unmethylation (98 bp) with age.

3.2.1 Oral Rinse

LINE-1 methylation was not significantly correlated with age in both normal, p = 0.3 (Fig. 38A) and OSCC group, p = 0.987 (Fig. 38B). The level of 98 bp in oral rinse was correlated with age (p = 0.017, r = 0.357) (Fig. 39A). However, no correlation was found in oral rinse of OSCC (p = 0.066) (Fig. 39B).

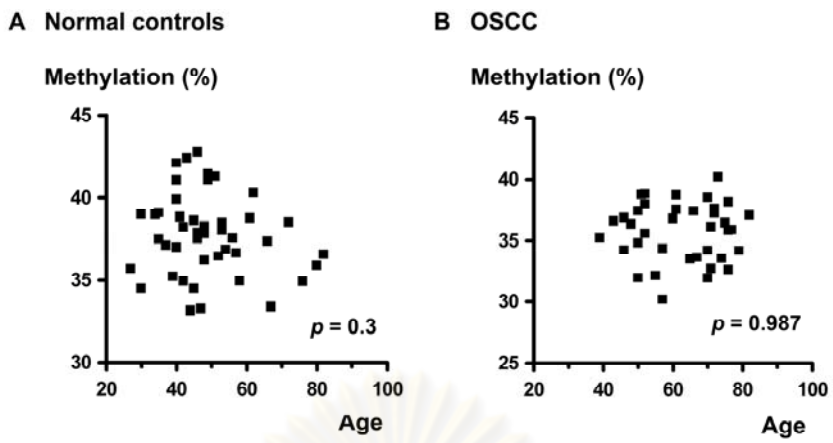


Figure 38 **Correlations between methylation level in oral rinse and age in normal controls (A) and OSCC (B).** There was no correlation between methylation in oral rinse and age in both normal and OSCC groups.

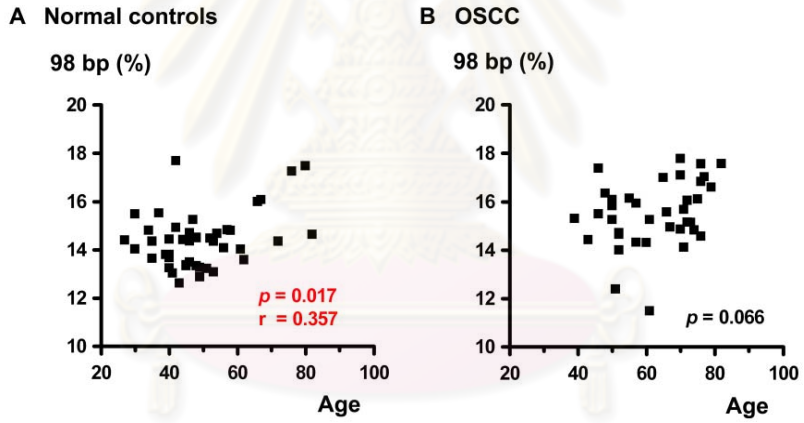


Figure 39 **Correlations between 98 bp level in oral rinse and age in normal controls (A) and OSCC (B).** The level of 98 bp product in oral rinse was positively correlated with age in normal group.

3.2.2 Buffy Coat

There was no correlation between LINE-1 methylation level and age in normal (p = 0.78) and OSCC group (p = 0.295) (Fig. 40). Similar to oral rinse, in normal controls, 98 bp in buffy coat was correlated with age (p = 0.005, r = 0.418, respectively). However, no correlation was found in buffy coat of OSCC (p = 0.625) (Fig. 41).

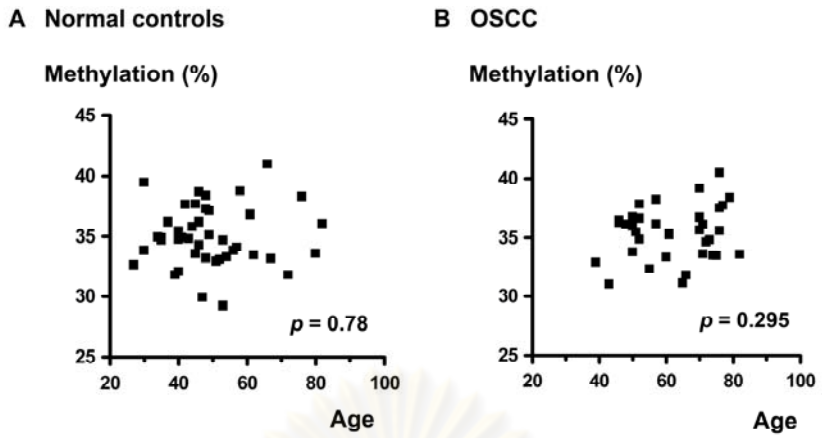


Figure 40 **Correlations between methylation level in buffy coat and age in normal controls (A) and OSCC (B).** Neither methylation in buffy coat of controls nor of OSCC was correlated with age.

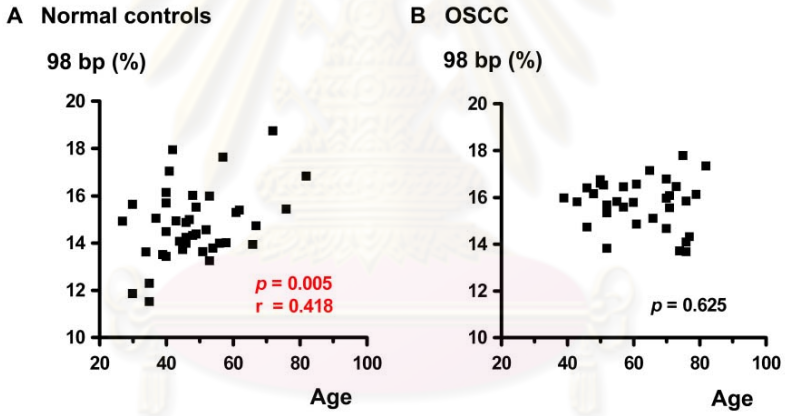


Figure 41 **Correlations between 98 bp level in buffy coat and age in normal controls (A) and OSCC (B).** The level of 98 bp in buffy coat of controls was correlated with age.

3.2.3 Plasma

In plasma of normal controls, LINE-1 methylation was significantly correlated with age (p = 0.01, r = 0.391). However, in OSCC patients, there was no such correlation, p = 0.872 (Fig. 42). There was no correlation between 98 bp in plasma and age in both normal and OSCC samples, p = 0.114 and 0.113, respectively (Fig. 43).

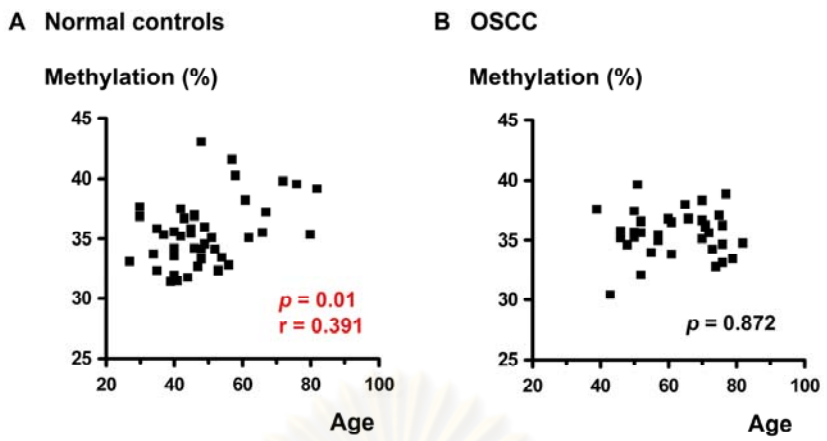


Figure 42 **Correlations between methylation level in plasma and age in normal controls (A) and OSCC (B).** In normal controls, conventional LINE-1 methylation in plasma was correlated with age.

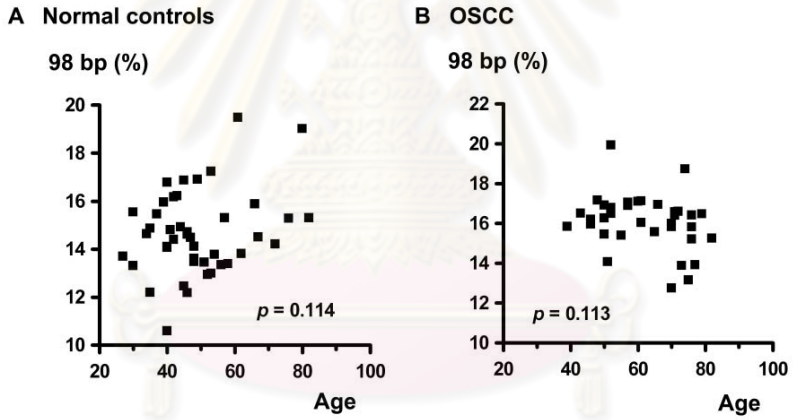


Figure 43 **Correlations between 98 bp level in plasma and age in normal controls (A) and OSCC (B).** There was no relationship between 98 bp in plasma and age in both normal and OSCC groups.

3.3 History of Smoking

As smoking is a well-known contributing factor, *t*-test was used to determine significant differences between subjects with history of smoking (smoker group) and without history of smoking (non-smoker group) in both normal and OSCC groups separately. Subjects who previously smoked but currently abstain from the habit were categorized in smoker group.

3.3.1 Oral Rinse

In oral rinse, LINE-1 methylation level of normal non-smoker (37.82% ± 2.40) was higher than normal smoker (37.60% ± 2.99). Among OSCC patients, non-smoker group (36.17% ± 2.40) had higher methylation than smoker group (34.90% ± 2.17). There was no significance between subgroups, p = 0.826 and 0.116, respectively (Fig. 44A).

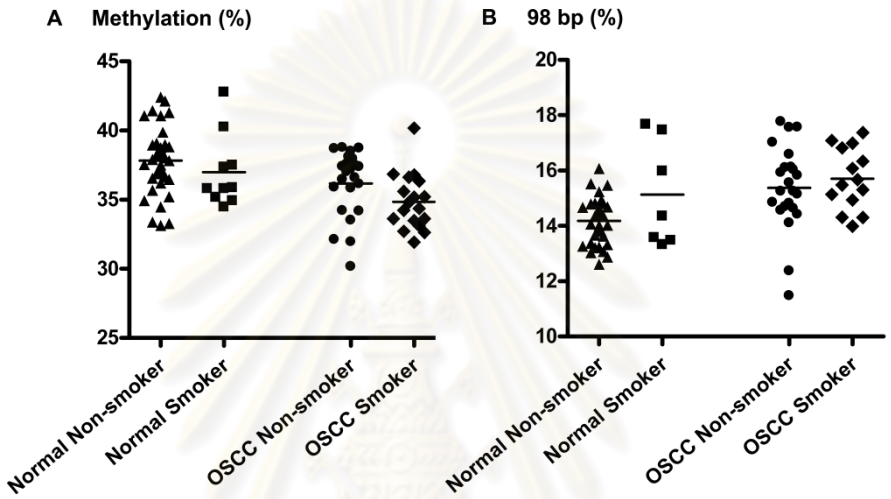


Figure 44 **Methylation (A) and 98 bp levels (B) in oral rinse of normal and OSCC smoker and non-smoker.** There was no difference between smoker and non-smoker in both normal and OSCC groups, regarding methylation as well as 98 bp levels in oral rinse.

The levels of 98 bp in smoker subgroups of both normal (15.13% ± 1.90) and OSCC (15.70% ± 1.11) were higher than the non-smoker subgroups (14.18% ± 0.81 and 15.38% ± 1.52, respectively). Also, the complete unmethylation levels in both groups provided insignificant difference, p = 0.239 and 0.495, respectively (Fig. 44B).

3.3.2 Buffy Coat

In buffy coat, unlike in oral rinse, LINE-1 methylation level increased in normal smoker (35.71% ± 2.80), compared to normal non-smoker (34.72% ± 2.39). In OSCC, the patients who smoked had (35.68% ± 2.09) higher

methylation level than patients who did not (35.19% ± 2.34). There was no significance between subgroups (p = 0.336 and 0.531) (Fig. 45A).

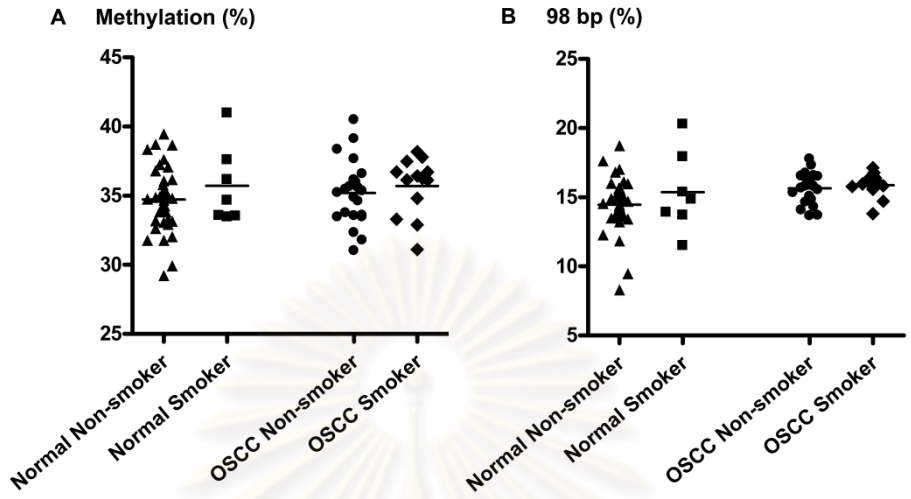


Figure 45 **Methylation (A) and 98 bp levels (B) in buffy coat of normal and OSCC smoker and non-smoker.** There was no difference between smoker and non-smoker in both normal and OSCC groups, regarding methylation as well as 98 bp levels in buffy coat.

Similar to in oral rinse, the level of 98 bp in buffy coat increased in smokers (Normal = 15.37% ± 2.91, OSCC = 15.88% ± 0.89), compared to non-smokers (Normal = 14.45% ± 1.99, OSCC = 15.63% ± 1.14). The differences were not significant (p = 0.309 and 0.355, respectively) (Fig. 45B).

3.3.3 Plasma

LINE-1 methylation level in plasma of normal smoker (35.66% ± 0.62) was relatively the same as non-smoker group (35.24% ± 2.96). Among OSCC, methylation of smokers (35.71% ± 1.33) was also close to the level of non-smoker (35.47% ± 2.21). There was no significance between smoking and non-smoking experience (p = 0.71 and 0.723, respectively) (Fig. 46A).

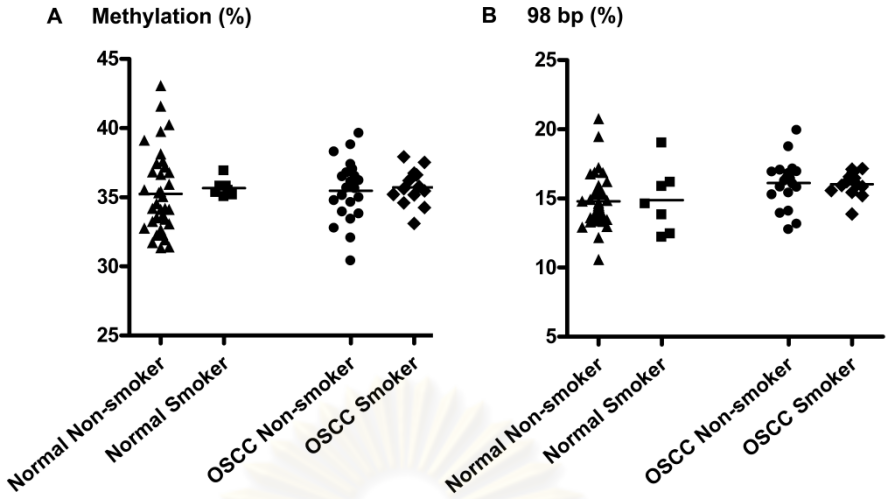


Figure 46 **Methylation (A) and 98 bp levels (B) in plasma of normal and OSCC smoker and non-smoker.** There was no difference between smoker and non-smoker in both normal and OSCC groups, regarding methylation as well as 98 bp levels in plasma.

In plasma, 98 bp level in normal non-smoker (14.78% ± 1.92) and OSCC non-smoker (16.10% ± 1.64) were almost the same as in normal smoker (14.87% ± 2.38) and OSCC smoker (16.02% ± 0.89), respectively (p = 0.915 and 0.870) (Fig. 46B).

3.4 History of alcohol consumption

Independent sample t-test was used to determine significance between subjects with and without history of alcohol consumption. In control group, subjects with previous and current history of alcohol consumption were classified in “normal drinker” group. Subjects without history of alcohol consumption were classified in “normal non-drinker”. OSCC patients who previously and currently drank alcohol were classified in “OSCC drinker” group and the patients who never drank were classified in “OSCC non-drinker”.

3.4.1 Oral Rinse

Methylation level of LINE-1s in oral rinse of normal non-drinker (37.79% ± 2.21) and normal drinker (37.77% ± 3.33) were the same, p = 0.982. The decreased methylation level was found in OSCC drinker (35.05% ± 2.37), compared

with OSCC non-drinker (36.31% ± 2.25). However, there was no significance in OSCC group, $p = 0.107$ (Fig. 47A).

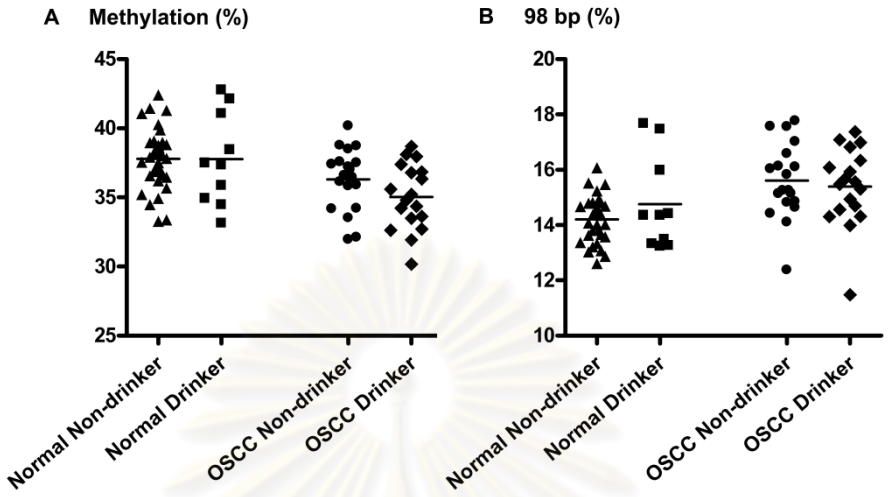


Figure 47 **Methylation (A) and 98 bp levels (B) in oral rinse of normal and OSCC drinker and non-drinker.** There was no difference between drinker and non-drinker in both normal and OSCC groups, regarding methylation as well as 98 bp levels in oral rinse.

The level of 98 bp in oral rinse of normal non-drinker (14.20% ± 0.82) was lower than the level of normal drinker (14.75% ± 1.70). In cancer, the level of 98 bp of OSCC non-drinker (15.61% ± 1.35) was slightly more than that in OSCC drinker (15.38% ± 1.42). Statistical significance was not found in normal group ($p = 0.163$) or OSCC group ($p = 0.620$) (Fig. 47B).

3.4.2 Buffy Coat

LINE-1 methylation levels in buffy coat of normal drinker (35.24% ± 3.08) and OSCC drinker (35.85% ± 2.38) were insignificantly higher than those of normal non-drinker (34.75% ± 2.27) and OSCC non-drinker (34.93% ± 2.04), respectively, $p = 0.510$ and 0.227 (Fig. 48A).

In buffy coat, 98 bp of normal non-drinker was averagely 14.45% ± 2.05. In normal drinker, the level increased to 15.12% ± 2.50. OSCC non-drinker (15.73% ± 1.18) also had lower level of 98 bp, compared to OSCC drinker (15.72% ±

0.93). The differences did not reach significance, p = 0.396 and 0.766, respectively (Fig. 48B).

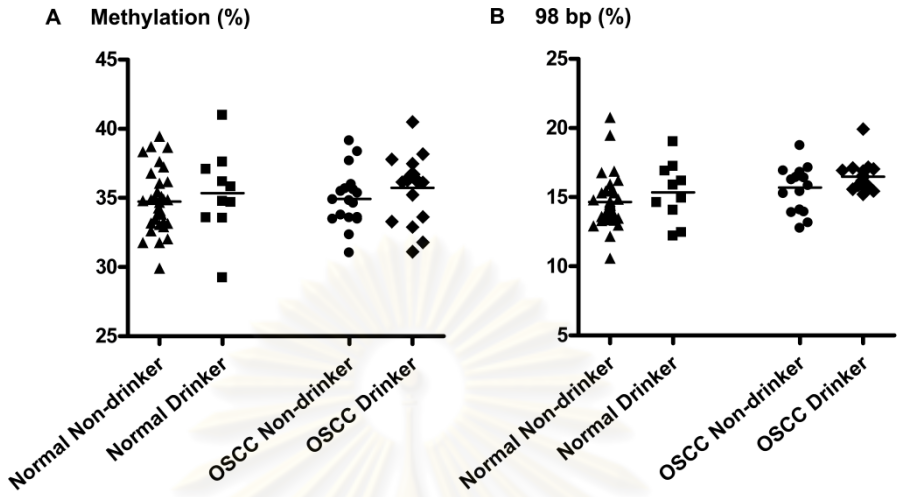


Figure 48 **Methylation (A) and 98 bp levels (B) in buffy coat of normal and OSCC drinker and non-drinker.** There was no difference between drinker and non-drinker in both normal and OSCC groups, regarding methylation as well as 98 bp levels in buffy coat.

3.4.3 Plasma

Methylation level of LINE-1s in plasma of normal non-drinker was 35.55% ± 2.90. The level decreased in normal drinker (34.50% ± 1.85) without significance, p = 0.289. In OSCC group, methylation in OSCC drinker (35.67% ± 1.47) and OSCC non-drinker (35.45% ± 2.29) were almost the same, p = 0.732 (Fig. 49A).

Like it was found in buffy coat, the level of 98 bp in plasma of normal drinker (15.34% ± 2.14) was higher than normal non-drinker (14.63% ± 1.92). Unmethylation of LINE-1s in OSCC drinker (16.48% ± 1.08) was also higher than in OSCC non-drinker (15.69% ± 1.57). Nonetheless, there was no statistical significance in both normal and OSCC groups, p =0.326 and 0.093 (Fig. 49B).

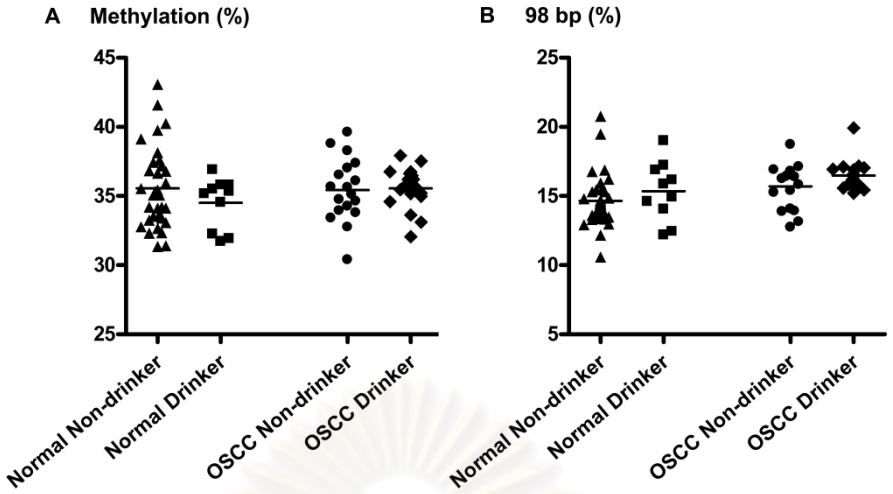


Figure 49 **Methylation (A) and 98 bp levels (B) in plasma of normal and OSCC drinker and non-drinker.** There was no difference between drinker and non-drinker in both normal and OSCC groups, regarding methylation as well as 98 bp levels in plasma.

3.5 History of Betel Nut Chewing

History of betel nut chewing was analyzed by *t*-test. In control group, no one had previous or current history of betel chewing. Therefore, influence of betel chewing on methylation or unmethylation levels could not be generated. OSCC patients who had previous and current history of betel chewing were classified in "OSCC chewer" and the patients with no history were classified in "OSCC non-chewer".

3.5.1 Oral Rinse

LINE-1 methylation level in oral rinse in OSCC chewer (36.49% ± 2.50) was significantly higher than in OSCC non-chewer (34.86% ± 1.94), $p = 0.0341$ (Fig. 50A). Also, 98 bp in oral rinse of OSCC chewer (15.65% ± 1.53) was higher than OSCC non-chewer (15.35% ± 1.20). However, the difference was not significant, $p = 0.510$ (Fig. 50B).

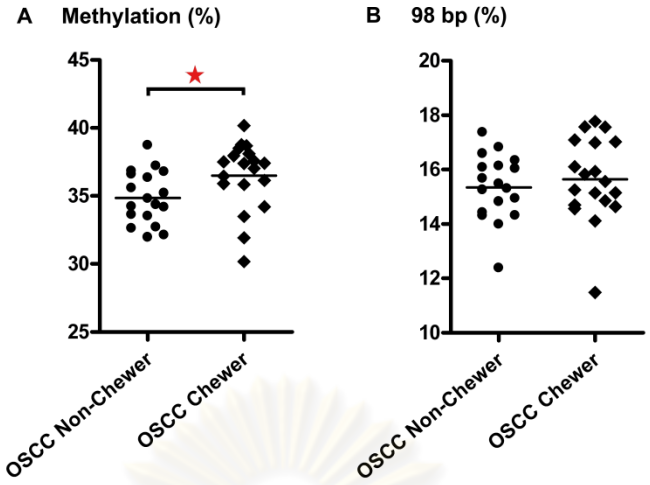


Figure 50 **Methylation (A) and 98 bp levels (B) in oral rinse of OSCC chewer and non-chewer.** Methylation level in oral rinse of OSCC chewer was significantly higher than that of OSCC non-chewer.

3.5.2 Buffy Coat

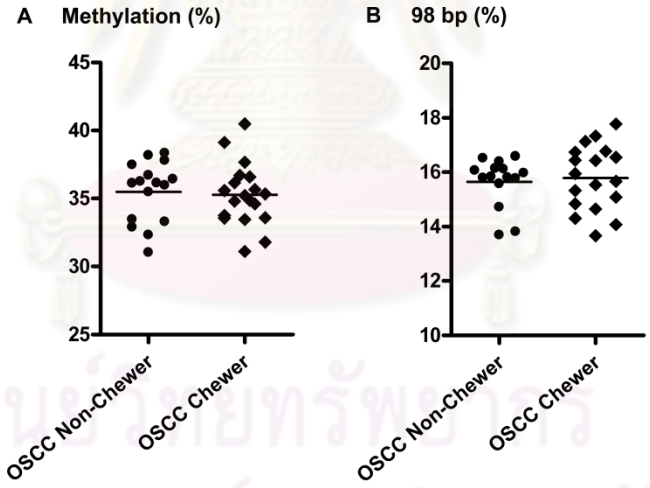


Figure 51 **Methylation (A) and 98 bp levels (B) in buffy coat of OSCC chewer and non-chewer.** Regarding methylation as well as 98 bp levels in buffy coat, there was no difference between chewer and non-chewer.

Methylation level of LNE-1 in buffy coat of OSCC chewer (35.27% ± 2.29) was almost the same as the level of OSCC non-chewer (35.49% ± 2.23), p = 0.773 (Fig. 51). Similarly, the level of 98 bp in OSCC chewer (15.79% ± 1.19) was not different from that of OSCC non-chewer (15.65% ± 0.89), p = 0.947 (Fig. 51B).

3.5.3 Plasma

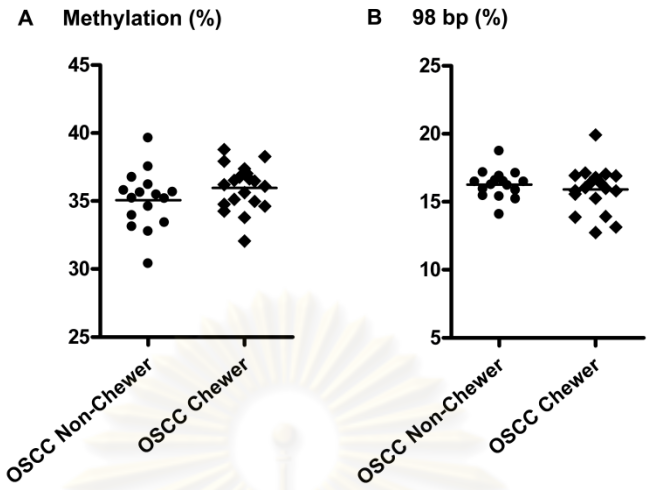


Figure 52 Methylation (A) and 98 bp levels (B) in plasma of OSCC chewer and non-chewer. Regarding methylation as well as 98 bp levels in plasma, there was no difference between chewer and non-chewer.

In plasma, between OSCC chewer and non-chewer, neither LINE-1 methylation level nor 98 bp provided significant difference, p = 0.175 and 0.458, respectively. LINE-1 methylation in non-chewer was 35.07% ± 2.12 and slightly increased in the patients with history of betel chewing (35.96% ± 1.67) (Fig. 52A). The level of 98 bp in OSCC non-chewer was 16.27% ± 1.03 and it reduced in OSCC chewer to 15.91% ± 1.65 (Fig. 52B).

3.6 Tumor Size (T)

One-way ANOVA was performed to determine difference of LINE-1 methylation and LINE-1 complete unmethylation among different tumor sizes in OSCC patients.

3.6.1 Oral Rinse

Methylation level of LINE-1s in oral rinse of OSCC patients declined respectively from T3 (36.02% ± 2.47), T4 (35.93% ±2.49), T2 (35.72% ± 2.55) to T1 (34.81% ± 2.20). There was no significance among different sizes of the lesion, p = 0.775 (Fig. 53A). The level of 98 bp in oral rinse increased respectively

from T4 (15.17% ± 1.66), T2 (15.39% ± 1.42), T3 (15.90% ± 1.25) to T1 (16.01% ± 0.81). Like found in methylation level, 98 bp among different tumor sizes did not provide significance, $p = 0.562$ (Fig. 53B).

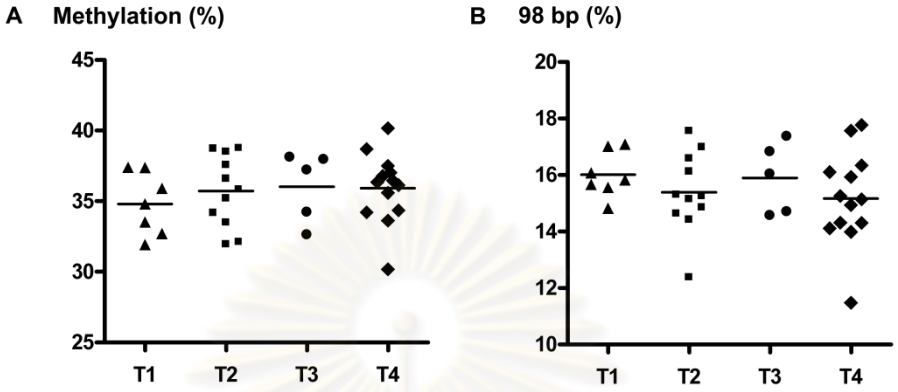


Figure 53 **Methylation (A) and 98 bp levels (B) in oral rinse of OSCC patients with different classified tumor sizes (T1-T4).** Methylation and 98 bp levels in oral rinse were not different among different tumor sizes.

3.6.2 Buffy Coat

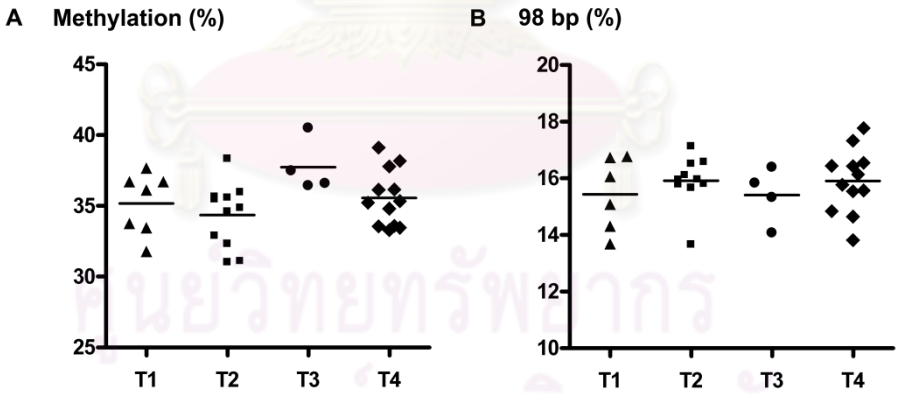


Figure 54 **Methylation (A) and 98 bp levels (B) in buffy coat of OSCC patients with different classified tumor sizes (T1-T4).** Methylation and 98 bp levels in buffy coat were not different among different tumor sizes.

In buffy coat, methylation level of LINE-1s in T1 (35.18% ± 2.17), T2 (34.35% ± 2.26), T3 (37.75% ± 1.88), and T4 (35.56% ± 1.99) were not significantly different, $p = 0.071$ (Fig. 54A). Similarly, 98 bp levels in T1 (15.44% ±

1.29), T2 (15.91% ± 0.91), T3 (15.40% ± 0.99), and T4 (15.90% ± 1.12) were not statistical significant, $p = 0.711$ (Fig. 54B).

3.6.3 Plasma

LINE-1 methylation level in plasma of T1 was 36.25% ± 1.89 and declined in T2 (35.78% ± 2.61), T4 (35.41% ± 1.01), and T3 (34.14% ± 1.89), respectively. The differences were not significant, $p = 0.368$ (Fig. 55A). LINE-1 complete unmethylation, 98 bp, in plasma of T3 was 16.79 ± 2.13% and gradually reduced in T1 (16.36% ± 1.48), T4 (16.03% ± 1.32), and T2 (15.70% ± 1.25), respectively. Again, the differences were not significant, $p = 0.570$ (Fig. 55B).

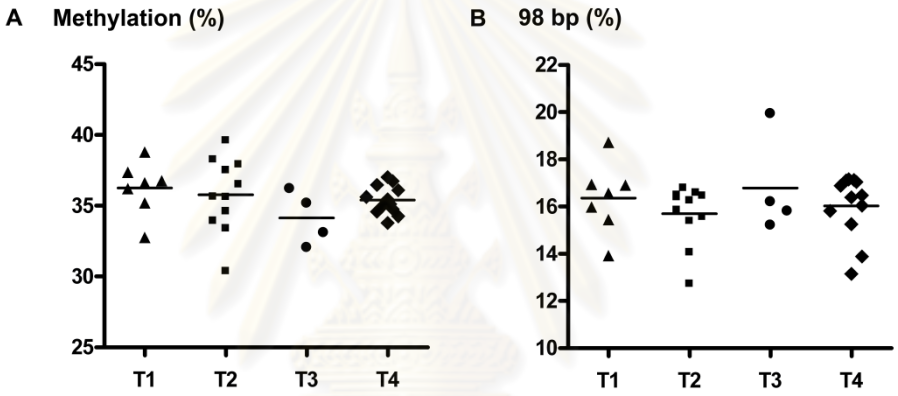


Figure 55 **Methylation (A) and 98 bp levels (B) in plasma of OSCC patients with different classified tumor sizes (T1-T4).** Methylation and 98 bp levels in plasma were not different among different tumor sizes.

3.7 Lymph Node Involvement (N)

Lymph node involvement was classified into two subgroups according to the absence (N0) or presence (N1-3) of involvement.

3.7.1 Oral Rinse

In oral rinse, LINE-1 methylation levels in N0 and N1-3 group were 35.42% ± 2.48 and 36.09% ±2.24, respectively (Fig. 56A). The level of 98 bp in N0 and N1-3 group were 15.56% ± 1.51 and 15.40% ± 1.14, respectively (Fig. 56B).

There was no statistical significance in methylation (p = 0.423) and 98 bp level (p = 0.735) between patients with and without lymph node involvement (Fig. 56).

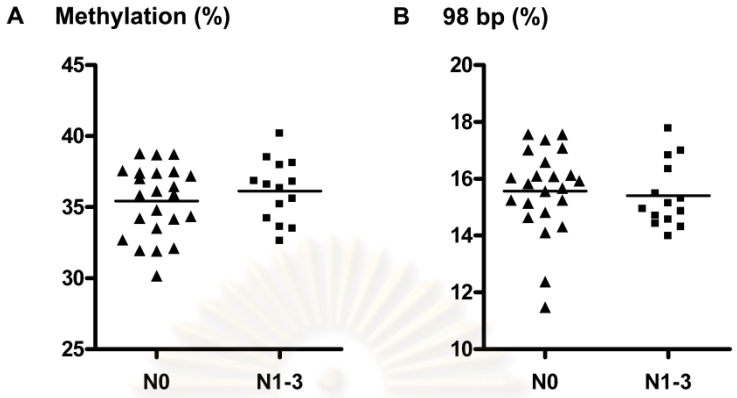


Figure 56 **Methylation (A) and 98 bp levels (B) in oral rinse of OSCC patients with (N1-3) and without (N0) lymph node involvement.** Methylation and 98 bp levels in oral rinse did not differ between presence and absence of lymph node enlargement.

3.7.2 Buffy Coat

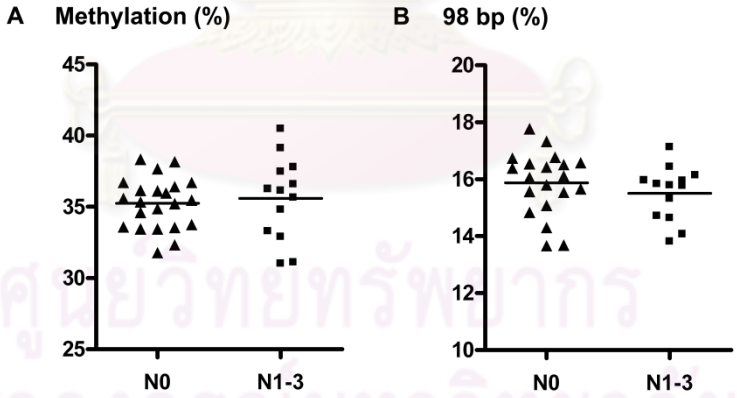


Figure 57 **Methylation (A) and 98 bp levels (B) buffy coat of OSCC patients with (N1-3) and without (N0) lymph node involvement.** Methylation and 98 bp levels in buffy coat were not different between presence and absence of lymph node enlargement.

In buffy coat, LINE-1 methylation in N0 and N1-3 were 35.24% ± 1.79 and 35.53% ± 3.02, respectively (Fig. 57A). The levels of 98 bp in both

subgroups were 15.87% ± 1.11 and 15.50% ± 0.95, respectively (Fig. 57B). No significance was found in either methylation ($p = 0.765$) or complete unmethylation ($p = 0.341$).

3.7.3 Plasma

In plasma, LINE-1 methylation in N0 and N1-3 were 35.77% ± 1.65 and 35.15% ± 2.42, respectively (Fig. 58A). In these groups, 98 bp were 16.12% ± 1.22 and 16.00% ± 1.70, respectively (Fig. 58B). The differences were insignificant ($p = 0.386$ and 0.812, respectively)

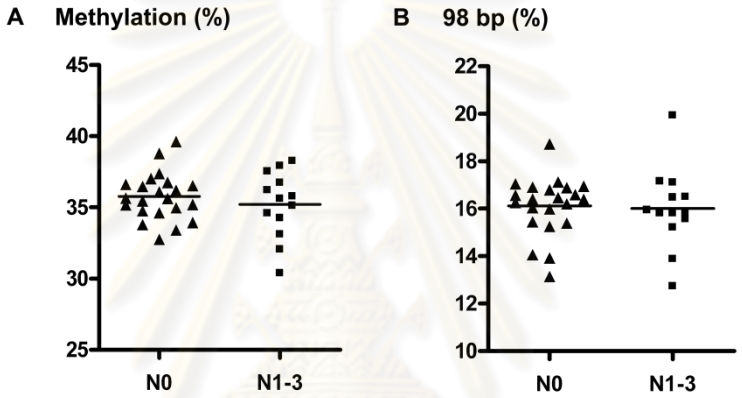


Figure 58 **Methylation (A) and 98 bp levels (B) plasma of OSCC patients with (N1-3) and without (N0) lymph node involvement.** Methylation and 98 bp levels in plasma of the patients with and without lymph node condition were not different.

3.8 Metastasis

Metastatic status was obtained from 36 patients. Twenty two of them had no metastasis (M0). In M0, LINE-1 methylation levels in oral rinse, buffy coat, and plasma were 36.33% ± 1.72, 35.64% ± 2.21, 35.72% ± 1.65, respectively. The levels of 98 bp in oral rinse, buffy coat, and plasma were 15.62% ± 1.20, 15.40% ± 1.21, 16.27% ± 1.63, respectively. The status could not be assessed in 14 patients (Mx). In Mx, methylation of LINE-1s in oral rinse, buffy coat, and plasma were 34.60% ± 2.94, 34.87% ± 2.34, and 35.28% ± 2.38, respectively. The levels of 98 bp in these sample sources of Mx were 15.32% ± 1.67, 16.28% ± 0.39, and 15.77% ± 0.95,

respectively. Secondary tumor was not found in any of the patient in this study therefore methylation level of LINE-1s was not analyzed according to the presence or absence of metastasis.

3.9 Stage

3.9.1 Oral Rinse

LINE-1 methylation level in oral rinse of OSCC patients was increasing respectively according to the progressed stage of disease. In stage I, stage II, stage III, and stage IV, the levels were 34.81% ± 2.20, 35.59% ± 2.92, 35.88% ± 1.61, 35.98% ± 2.58, respectively. However, the differences were not statistically significant, $p = 0.762$ (Fig. 59A).

Unlike LINE-1 methylation level which was increasing respectively according to the progressed stage of disease, 98 bp level was lowest stage IV (15.06% ± 1.45) and increased respectively in stage II (15.39% ± 1.65), stage I (16.01% ± 0.81) and stage III (16.23% ± 1.21). The differences were not statistically significant, $p = 0.238$ (Fig. 59B).

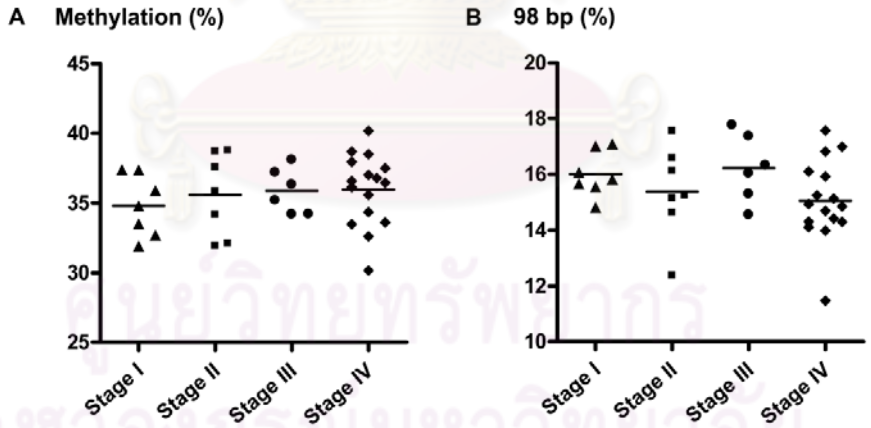


Figure 59 Methylation (A) and 98 bp levels (B) in oral rinse of OSCC patients with different classified stages. Conventional LINE-1 methylation and 98 bp levels in oral rinse were not different among different stages.

3.9.2 Buffy Coat

In stage III, methylation level of LINE-1s was 37.01% ± 2.94. It reduced respectively in stage II (35.31% ± 1.80), stage I (35.18% ± 2.17), and stage IV (34.88% ± 2.21). The differences did not reach significance, $p = 0.346$ (Fig. 60A).

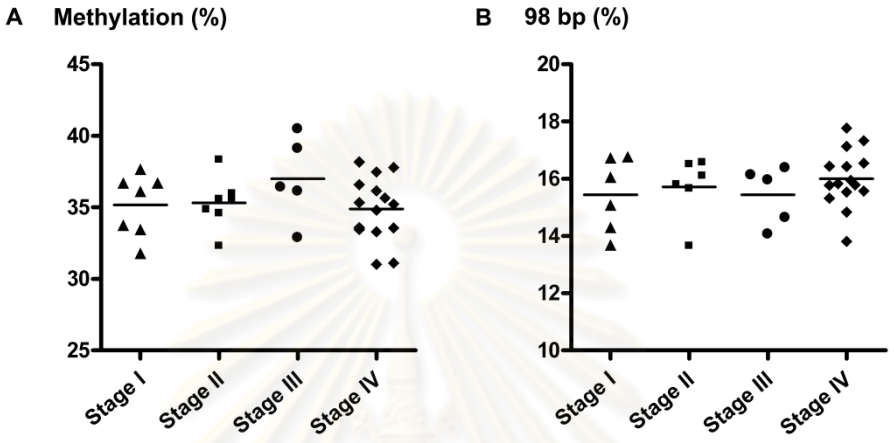


Figure 60 Methylation (A) and 98 bp levels (B) in buffy coat of OSCC patients with different classified stages. Conventional LINE-1 methylation and 98 bp levels in buffy coat were not different among different stages.

In the most progressed disease, stage IV, 98 bp in buffy coat was 16.00% ± 1.00. The level gradually declined in stage II (15.72% ±1.07), stage III (15.439% ± 1.02) and stage I (15.442% ± 1.29), respectively. There was no significance among different stages, $p = 0.628$ (Fig. 60B).

3.9.3 Plasma

Ranking from the highest to lowest in plasma methylation level, there were stage I (36.25% ± 1.89), stage III (35.72% ± 1.16), stage II (35.63% ± 2.06), and stage IV (35.13% ± 2.16), respectively. No significance was found among groups, $p = 0.665$ (Fig. 61A).

The level of 98 bp in plasma of stage I (16.36% ± 1.48), stage III (16.17% ± 0.58), stage II (16.00% ± 0.96), and stage IV (15.95% ± 1.79) were relatively the same, $p = 0.940$ (Fig. 61B).

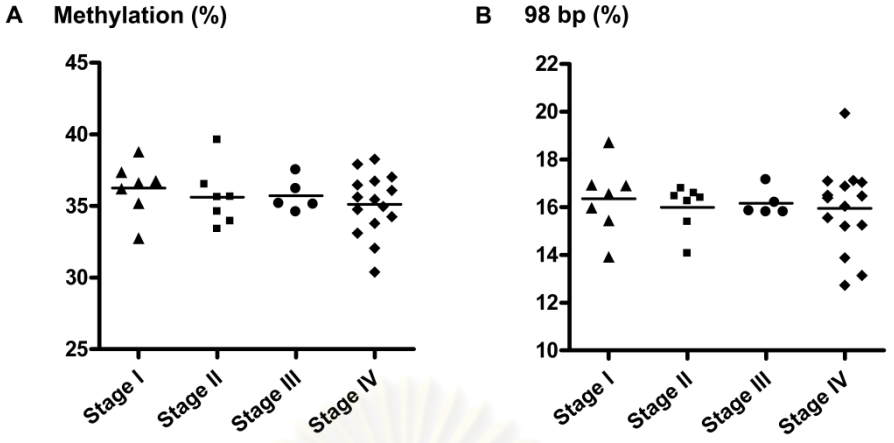


Figure 61 **Methylation (A) and 98 bp levels (B) in plasma of OSCC patients with different classified stages.** In plasma, LINE-1 methylation and 98 bp levels did not differ among different stages.

3.10 Histopathological grading

3.10.1 Oral Rinse

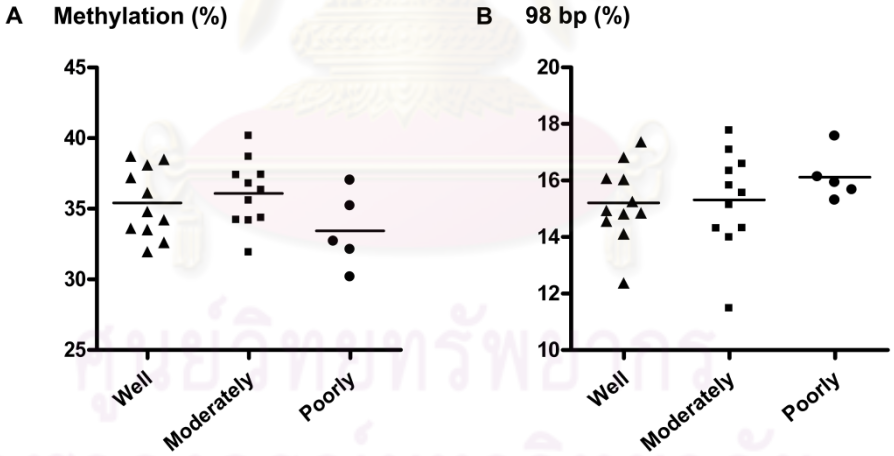


Figure 62 **Methylation (A) and 98 bp levels (B) in oral rinse of OSCC patients with different histopathological grading.** Conventional methylation and 98 bp levels in oral rinse of patients with different grading were not statistically different.

LINE-1 methylation level in oral rinse of moderately differentiated group was highest (36.10% ± 2.35). In well differentiated and poorly differentiated,

the levels were lower (35.40% ± 2.45 and 33.45% ± 2.69, respectively). The differences were not significant, $p = 0.155$ (Fig. 62A).

In oral rinse, the level of 98 bp of the most advanced histological change, poorly differentiated, was the highest (16.12% ± 0.87). It reduced accordingly in moderately differentiated (15.31% ± 1.75) and well differentiated cases (15.20% ± 1.37). Like methylation level in oral rinse, these differences were not significant, $p = 0.499$ (Fig. 62B).

3.10.2 Buffy Coat

Methylation level of LINE-1s was declining according to the advanced histological change. In well differentiated, moderately differentiated, and poorly differentiated group, methylation levels were 36.14% ± 2.11, 35.92% ± 2.36, and 34.21% ± 1.81, respectively. Significance was not found among groups, $p = 0.267$ (Fig. 63A).

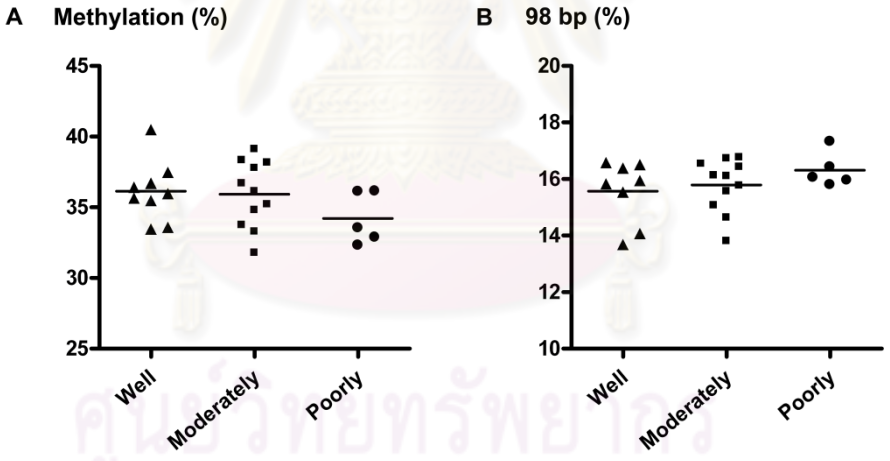


Figure 63 Methylation (A) and 98 bp levels (B) in buffy coat of OSCC patients with different histopathological grading. Conventional methylation and 98 bp levels in buffy coat of patients with different grading were not statistically different.

The level of 98 bp in buffy coat of OSCC patients was increasing accordingly to the advancement of disease. In well differentiated, moderately differentiated, and poorly differentiated group, these levels were 15.57% ± 1.11,

15.78% ± 0.94, and 16.41% ± 0.61, respectively. Significance was also not found, p = 0.398 (Fig. 63B).

3.10.3 Plasma

In plasma, LINE-1 methylation levels were relatively the same among well, moderately, and poorly differentiated OSCC (p = 0.947). The levels in these 3 subgroups were 35.79% ± 2.19, 35.67% ± 1.24, and 35.48% ± 1.40, respectively (Fig. 64A).

The levels of 98 bp in well differentiated cases (15.65% ± 1.66), moderately differentiated (16.33% ± 0.94), and poorly differentiated (16.02% ± 0.77) were not significantly different, p = 0.477 (Fig. 64B).

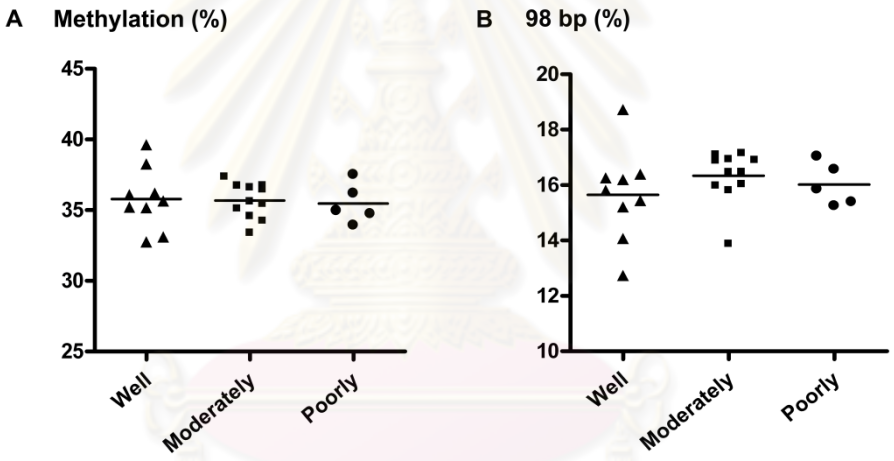


Figure 64 **Methylation (A) and 98 bp levels (B) in plasma of OSCC patients with different histopathological grading.** Conventional methylation and 98 bp levels in plasma of patients with different grading were not statistically different.

CHAPTER V

DISCUSSION

A certain part of cancer research was dedicated to biomarker development for screening and early detection. Though cancer, OSCC especially, status relies solidly on histopathological examination, minor surgical manipulation by incisional biopsy may not prevent dissemination of cancer cells into the circulation, resulting in micrometastasis.[12] Moreover, especially in the areas where medical and dental facilities are not well equipped, surgical biopsy as well as cytology investigation, which need special skills, may not be performed at once. Patient referral and impression of up-coming surgery may discourage the patients and delay proper treatments. Occult node and second primary tumor evaluation are also difficult.[1, 4] Missing information of lymph node and cancer cells unintentionally left in other parts of biopsied specimen can result in misdiagnosis as well as inadequate treatment modalities. To alleviate this issue, markers from circulatory blood, plasma, and serum became more attractive because disease information, from circulating tumor cells escaping from primary site as well as cell-free DNA or RNA, could be obtained.[22] In oral cavity, saliva and salivary rinse have gained enormous attention due to its least invasiveness, direct contact to oral cancer, entire-lesion coverage, high patient compliance, simple collection and storage, reproducibility by everyone, and cost-effectiveness.[155] Surprisingly, saliva-based markers were associated with not only cancer of oral cavity, but also breast[66] as well as ovarian cancer.[67] Additionally, self-collected oral rinses from 3,377 women could serve as a source of DNA representing breast cancer.[66] Moreover, biomarkers may provide more information regarding suitable treatment options for each patient. TNM staging is not the best treatment indicator for all. New improvement of it by American Joint Committee on Cancer (AJCC), launched in 2010, has discussed flaws of the old version, proposed solutions and new ideas for several cancer types, and supported the trend of personalized medicine.[62]

Genome-wide or global hypomethylation in many types of cancer cells was confirmed.[21, 28, 33, 35, 106, 151] This alteration was detected in DNA derived from peripheral blood components of cancer patients as well.[35] Since LINE-1s is the

majority of repetitive sequence, methylation level of LINE-1s can be used as surrogate for the global level.[11, 28, 29] Also, the decrease of LINE-1 methylation was detected in many types of malignancies[11, 25, 28, 32-34, 81, 152] and correlated with progressiveness of some cancers,[28, 34, 152] suggesting the potential role of LINE-1 sequence for cancer detection. Though this phenomenon was additionally reported in oral rinse of OSCC patients,[11] LINE-1 methylation in circulatory blood of OSCC has not been demonstrated. Normally, buffy coat contains platelet and leukocytes. In cancer, since tumor cells have almost the same density as white blood cells, small amount of them also resided in the buffy layer of blood. Several studies have demonstrated detection of circulating tumor cells (CTC) in buffy coat layer and implication of CTC for predicting prognosis as well as adjusting treatment modalities. Moreover, some of the white blood cells of the patients may carry cancerous DNA from horizontal transfection as well.[73, 75] The plasma portion of blood has circulating free cancer DNA (known as circulating nucleic acid in plasma and serum, CNAPS) from cancer cell apoptosis or active process of the cancer[75] which might also be targeted for OSCC.

The primary purpose of this study was to elucidate LINE-1 methylation in peripheral blood components, buffy coat and plasma, of OSCC patients and further investigate the difference of LINE-1 methylation in three types of sample intra-individually. Moreover, this study also discovered that there was another product, uncut 160 bp amplicon, from COBRA LINE-1. This size of product could result from partial methylation which was never previously included in methylation calculation. Therefore COBRA LINE-1 products and analysis of methylation patterns were further studied and clarified.

Whereas most quantitative methylation techniques cannot distinguish specific LINE-1 methylation pattern, here, COBRA LINE-1 could classify LINE-1 sequences, depending on methylation status of 62- and 80-CpG, into the following four classes; methylation at both locations (complete methylation) (Fig. 65A), unmethylation at both locations (complete unmethylation) represented by “98 bp” (Fig. 65B), unmethylation at 62-CpG and methylation at 80-CpG (partial methylation) (Fig. 65C), and methylation at 62-CpG and unmethylation at 80-CpG (uncut partial methylation) represented by “160 bp” (Fig. 65D).

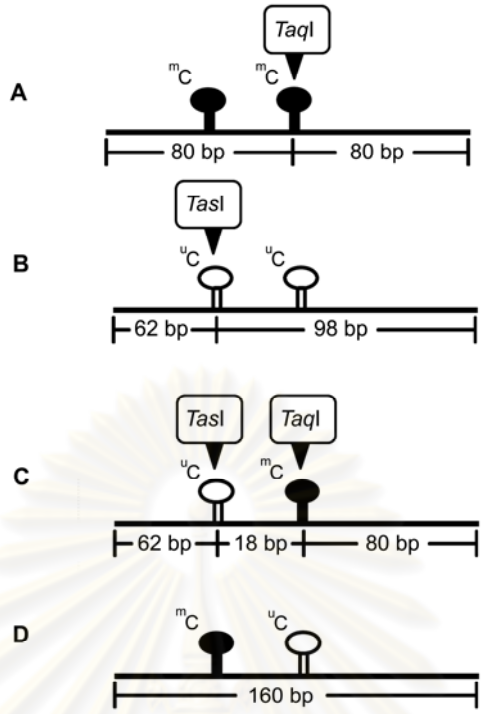


Figure 65 **Four methylation patterns in LINE-1 sequence yielding different products from COBRA LINE-1.** Complete methylated LINE-1 pattern is demonstrated in A. Complete unmethylated pattern is shown in B. Partial methylated sequence can be found in two switching forms in C and D.

Partially methylated LINE-1 sequences were previously noticed in both cancer and normal genomes.[32] Here, these sequences were taken into consideration and calculation formula. Interestingly, when the levels of uncut partial methylation (160 bp) were investigated with methylated (80 bp) or unmethylated sequences (98 bp), their correlations in normal genomes were the opposite of those in cancer. In cancer, partially methylated sequences were inversely correlated with unmethylated sequences but directly correlated with methylated sequences. Simply explained, in cancer, decreasing level of partial methylated sequences due to methylation loss resulted in gaining level of unmethylated sequences (Fig. 22-23, p. 64-65). In addition, cancer cells had less partially methylated sequences than normal cells. Therefore, in cancer, hypomethylated LINE-1 sequences were possibly derived by methylation loss from both partially methylated and methylated LINE-1s. In normal genomes, both partially methylated and unmethylated LINE-1 sequences were

inversely correlated with methylated sequences. The inversed correlation might be due to the interchanging of methylated forms of LINE-1s with partially methylated LINE-1s in normal genomes (Fig. 22-23, p. 64-65). These data suggested that mechanism of losing or gaining methylation in normal cells was likely to be different from carcinogenesis.

Moreover, along with conventional LINE-1 methylation, in this study, all four sizes of COBRA LINE-1 product were analyzed between normal and OSCC samples. While LINE-1 methylation in oral rinse of OSCC patients was significantly decreased, LINE-1 methylation levels in buffy coat and plasma were gained, however, insignificantly. Oral rinses containing exfoliated epithelial cells directly from the lesion could be a better presenter than DNA in buffy coat and plasma. Interestingly, LINE-1 complete unmethylation, 98 bp, in all sample types were increased significantly in OSCC, compared to their normal counterparts. The same escalation happened in all types of sample indicated that the level of 98 bp corresponded greatly with the presence of cancer, even in circulatory blood drawn from remote area.

When gender was considered, there was no difference between LINE-1 methylation in oral rinse of male and female in control group. This result supported the previous study.[11] However, unlike it was found before,[11] methylation in OSCC males differed from OSCC females. This finding might result from different history of smoking and alcohol consumption between male and female patients. Among 20 OSCC females, 19 of them had no history of smoking and 16 of them had no history of alcohol consumption, while the majority of OSCC male smoked and drank. Though it was shown here that there was no significance between methylation in oral rinse of the patients with and without these contributing factors, apparently, the patients who smoked or drank had lower methylation level in oral rinse.

In oral rinse of normal controls, the levels of 98 bp were correlated with age. In spite of this incidence, the degree of correlation was low. To reassure that the ability of 98 bp in differentiating OSCC from normal group was not influenced from age, 16 pairs of normal and OSCC were matched by identical age and gender. Among all values investigated, 98 and 80 bp levels were capable of significantly differentiating normal and cancer by all types of DNA sources.

The recent study revealed that, in normal subjects, LINE-1 methylation in peripheral blood mononuclear cells (PBMC) was not associated with age.[143] Here, similar finding was also found in the buffy coat of controls. Interestingly, it was shown that, in OSCC group, both methylation and 98 bp in all types of sample were not correlated with age.

Varying degrees of sensitivity and specificity in oral cancer tests especially by saliva-based and oral rinse sample were reported. In a proteomic study, mammary serine protease inhibitor (Maspin) and Cyclin D1 (CycD1) in saliva were reported with sensitivity and specificity reaching 100% in detecting OSCC. However, only 19 tongue cancer was included. In the study, cut-off value was calculated by mean plus/minus SD of healthy controls. Sensitivity and specificity of markers were then calculated by fraction of positive or negative test that correctly classified each patient.[156] When sensitivity and specificity are involved, receiver-operating characteristic (ROC) curve is a reliable tool[84, 157] and must be generated.[55]

A prediction model constructed by salivary mRNA showed sensitivity and specificity of 91% for oral cancer detection.[158] Though probably protected by association with macromolecules in saliva,[159] RNA is susceptible to rapid degradation outside the body if not immediately stabilized after sampling. DNA is more stable and easily extracted from biological fluids as well as tissues.[160] Moreover, difficulty for frequent usage of saliva is the collection step, especially for proteomics. It was suggested that saliva collection should be strictly followed standard protocols; in terms of food or drink refraining, current medicines, hormonal status, timing, repeat sampling, duration or even seating position.[161, 162] Salivary rinse with sterile normal saline or distill water, targeted for molecular study, can be performed more conveniently and spontaneously.

Genetic and epigenetic alterations including DNA methylation available from exfoliated cell in oral rinse of head and neck squamous cell carcinoma (HNSCC) were also reported. However, only few have discussed sensitivity and specificity for clinical setting. In which demonstrated, mostly were in moderate levels. For instance, in the study of nine genes, while *MMPI* from tissue sample could identify HNSCC with sensitivity and specificity slightly above 90%, in salivary rinse, sensitivity of this gene

decreased to 20%.[158] Hypomethylation of LINE-1s in oral rinse of OSCC patients was demonstrated.[11] However, sensitivity and specificity for using it as cancer detection tool were still not high.

One major obstacle when using oral rinse of OSCC patients as a source for biomarker was the fact that oral rinse harbored not only cancerous cells but also white blood cells as well as normal epithelial cell from other parts of oral cavity. Moreover, oral rinse of the controls also contained small amount of normal white blood cells as cellular component of saliva. The previous study has shown wide-range and varied LINE-1 methylation in different cell types, possessing cell-type specific character.[28] Unfortunately, such contamination in oral rinse samples could distract the represented methylation level and decrease accuracy of the test. Collection method such as mucosal swab directly onto the lesion may increase cancer-normal ratio in OSCC samples. However, from study of microsatellite alterations in HNSCC, tumor DNA was detected in swab samples slightly more than in the rinses, without statistical significance.[16] Additionally, mucosal swab is also subjective to operator.

Strikingly, confirmed by intra-individual comparisons, 98 bp levels in different DNA types within each individual were relatively the same. This phenomenon highly suggested that 98 bp was not only specific to cancer, it also represented each individual as a whole, not cell-type specific. Therefore, although the sample contained various types of DNA, 98 bp level could still provide high accuracy of the test (Fig. 34, p. 82). Within normal individuals, LINE-1 methylation in oral rinse was significantly higher than those in blood components. While methylation in blood components barely changed between normal and cancer, the level in oral rinse of OSCC was apparently decreased, compared to its level in normal controls. This might result in relatively the same methylation levels between three sources in OSCC (Fig. 33, p. 81).

The level of 98 bp showed more potential than methylation level in differentiating cancer from normal controls even though these samples were contaminated with other DNA types. Exceptionally, the level of 98 bp was the only value independent from various age and sex and provided significance between 16 matched cases in all three sources of DNA. Not only that, 98 bp only in oral rinse was

also capable of detecting OSCC in early stage (Fig. 32, p. 80). When sensitivity and specificity was measured, as expected, the oral rinse sample provided higher percentage than buffy coat and plasma. Compared with methylation level, the test using 98 bp for cancer detection gave higher optimal sensitivity and specificity up to 70-80% by oral rinse of this sample set and reaching 90% by oral rinse of the last cohort. Indisputably, the level of 98 bp demonstrated outstanding performance as marker for early detection of OSCC by easily-collected oral rinse samples.

Tangkijvanich reported that serum LINE-1 hypomethylation was positively correlated with not only tissue LINE-1 hypomethylation but also with the increased tumor size, resulting in poorer survival of hepatocellular carcinoma.[34] In this study of OSCC, neither LINE-1 methylation nor level of 98 bp in any type of sample related to progression or advancement of the lesion, determined by tumor size (T), lymph node involvement (N), stage, and histopathological grading. They might not predict OSCC aggressiveness or serve as prognostic markers. These findings supported the study by Subbalekha et al, in 2009.[11]

This study showed no significant difference between subjects with and without smoking or drinking. However, in oral rinse, methylation level in smokers was reduced and 98 bp was gained, suggesting the possible role of smoking in carcinogenesis by methylation alteration. In buffy coat, both methylation and 98 bp were increased in smokers as well as drinkers, corresponding with the increased levels in buffy coat of OSCC patients, compared to controls. Thus, changes in methylation and complete unmethylation level from these contributing factors might be found in buffy coat as well. Unexpectedly, the patients with history of betel nut chewing, which was one of the risks factor for oral cancer especially in Asian countries, had significantly higher oral rinse methylation than the patients without chewing experience. Previous studies regarding methylation of LINE-1s and chewing habit are still limited. Larger sample size in well-designed studies may help clarify the effect of these etiological factors. From this study, the roles of smoking, alcohol, and betel were still undetermined.

In biomarker research, determining controls and cases is crucial. Malignancy change in high risk individual may be missed and results in false positive result. In

addition, contributing factors which have influence on the marker can deflect result of non-cancer population. In this study, none of the controls developed premalignant lesion or OSCC during 1-year follow-up. Also, influence of risk factor such as smoking on 98 bp did not diminish its ability for detection. Nevertheless, more study should be performed on 98 bp, especially in oral rinse, for example, matched pair test with large sample size and double blinded experiment categorizing unknown cases and controls. Previous studies reported different levels of conventional LINE-1 methylation between premalignant lesions or carcinoma in situ and cancer of colon,[28] ovary,[33] and uterine cervix.[152] The level of 98 bp in potentially malignant lesions of the oral cavity and in other types of cancer should also be investigated. Evaluation of 98 bp complete unmethylation before, after tumor resection, and during followed-up period is also challenging. Additionally, if only 98 bp as a marker may not serve highest accuracy, panel of markers together with 98 bp should also be considered and developed. Lastly, step-by-step methylation analysis of COBRA LINE-1 protocol also needs to be standardized and generalized to be simply and correctly managed every laboratory facility.

CHAPTER VI

SUMMARY

In conclusion, this study demonstrated LINE-1 hypermethylation in buffy coat and plasma of OSCC patients, contrarily to LINE-1s in oral rinse which was hypomethylated. Intra-individually, in normal population, LINE-1 methylation in oral rinse was higher than in blood components. In OSCC, LINE-1 methylation level was relatively the same, regardless of DNA sources. Moreover, in this study, partial methylation patterns were first taken into consideration and calculation. Correlation of partial methylation of LINE-1 sequences with methylated and unmethylated sequences in cancer and normal genomes were inversed. Thus, methylation loss of LINE-1 sequences in cancer and normal cells may result from different processes. In addition to overall level, the pattern of methylation was crucial for better understanding mechanisms of global hypomethylation in cancer. With this information, the percentage of complete unmethylated LINE-1, represented by 98 bp, was a highly improved biomarker for distinguishing OSCC from normal DNA, both from cancerous oral epithelial cells as well as blood components drawn from remote area. Strikingly, levels of 98 bp from different DNA sources in one individual were not different, no cell-specific character. Therefore, here, LINE-1 complete unmethylation was the best candidate for detecting the presence OSCC in its early stage by easily-collected oral rinse sample contaminated with a pool of mixed-DNA, with sensitivity and specificity from ROC curve reaching 80-90%.

These findings are important for OSCC marker discovery. Nonetheless, further effort should be made to verify ability of 98 bp. Double blinded experiment and prospective study on bigger sample size of healthy individuals monitoring for cancer development should be performed. LINE-1 complete unmethylation levels between potentially malignant lesion and OSCC also needed to be investigated. Additionally, it is recommended that the levels and ranges in other types of cancer be clarified.

REFERENCES

[1] Scully, C. and Bagan, J. Oral squamous cell carcinoma overview. Oral Oncol 45 (April-May 2009):301-308.

[2] Arduino, P. G., et al. Clinical and histopathologic independent prognostic factors in oral squamous cell carcinoma: A retrospective study of 334 cases. J Oral Maxillofac Surg 66 (August 2008):1570-1579.

[3] Mehrotra, R. and Yadav, S. Oral squamous cell carcinoma: Etiology, pathogenesis and prognostic value of genomic alterations. Indian J Cancer 43 (April-June 2006):60-66.

[4] Munsell, D. S. Is sentinel node biopsy a better predictor of survival than conventional imaging for patients with T1/T2 squamous cell cancer of the oral cavity and N0 neck disease? The International Journal of Head and Neck Surgery (2007).

[5] Kumar, V., Abbas, A. K. and Fausto, N. Robbins and cotran pathologic basis of disease. 7th. Philadelphia: Elsevier Saunders, 2005.

[6] Vokes, E. E., Weichselbaum, R. R., Lippman, S. M. and Hong, W. K. Head and neck cancer. N Engl J Med 328 (January 1993):184-194.

[7] Forastiere, A., Koch, W., Trotti, A. and Sidransky, D. Head and neck cancer. N Engl J Med 345 (December 2001):1890-1900.

[8] Iamaroon, A., et al. Analysis of 587 cases of oral squamous cell carcinoma in northern thailand with a focus on young people. Int J Oral Maxillofac Surg 33 (January 2004):84-88.

[9] Kerdpon, D. and Sriplung, H. Factors related to delay in diagnosis of oral squamous cell carcinoma in southern thailand. Oral Oncol 37 (February 2001):127-131.

[10] Lingen, M. W., Kalmar, J. R., Karrison, T., Speight, P. M. Critical evaluation of diagnostic aids for the detection of oral cancer. Oral Oncol 44 (January 2008):10-22.

[11] Subbalekha, K., et al. Detection of line-1s hypomethylation in oral rinses of oral squamous cell carcinoma patients. Oral Oncol 45 (Feb 2009):184-191

[12] Kusukawa, J., Suefuji, Y., Ryu, F., Noguchi, R., Iwamoto, O. and Kameyama, T. Dissemination of cancer cells into circulation occurs by incisional

biopsy of oral squamous cell carcinoma. Journal of Oral Pathology & Medicine 29 (2000):303-307.

[13] Dyavanagoudar, S., Kale, A., Bhat, K. and Hallikerimath, S. Reverse transcriptase polymerase chain reaction study to evaluate dissemination of cancer cells into circulation after incision biopsy in oral squamous cell carcinoma. Indian J Dent Res 19 (October-December 2008):315-319.

[14] Kalmar, J. R. Advances in the detection and diagnosis of oral precancerous and cancerous lesions. Oral Maxillofac Surg Clin North Am 18 (November 2006):465-482.

[15] Ram, S. and Siar, C. H. Chemiluminescence as a diagnostic aid in the detection of oral cancer and potentially malignant epithelial lesions. Int J Oral Maxillofac Surg 34 (2005):521-527.

[16] Spafford, M. F., et al. Detection of head and neck squamous cell carcinoma among exfoliated oral mucosal cells by microsatellite analysis. Clin Cancer Res 7 (March 2001):607-612.

[17] Westra, W. H. and Califano, J. Toward early oral cancer detection using gene expression profiling of saliva: A thoroughfare or dead end? Clin Cancer Res 10 (December 2004):8130-8131.

[18] Shpitzer, T., Bahar, G., Feinmesser, R. and Nagler, R. M. A comprehensive salivary analysis for oral cancer diagnosis. J Cancer Res Clin Oncol 133 (September 2007):613-617.

[19] Shaw, R. The epigenetics of oral cancer. Int J Oral Maxillofac Surg 35 (February 2006):101-108.

[20] Coleman, W. B. and Rivenbark, A. G. Quantitative DNA methylation analysis: The promise of high-throughput epigenomic diagnostic testing in human neoplastic disease. J Mol Diagn 8 (May 2006):152-156.

[21] Feinberg, A. P. The epigenetics of cancer etiology. Semin Cancer Biol 14 (December 2004):427-432.

[22] Glazer, C. A., Chang, S. S., Ha, P. K. and Califano, J. A. Applying the molecular biology and epigenetics of head and neck cancer in everyday clinical practice. Oral Oncol 45 (April-May 2009):440-446.

[23] Esteller, M. Epigenetics in cancer. N Engl J Med 358 (March 2008):1148-1159.

[24] Ehrlich, M. DNA methylation in cancer: Too much, but also too little. Oncogene 21 (August 2002):5400-5413.

[25] Ehrlich, M. DNA methylation and cancer-associated genetic instability. Adv Exp Med Biol 570 (2005):363-392.

[26] Das, P. M. and Singal, R. DNA methylation and cancer. J Clin Oncol 22 (November 2004):4632-4642.

[27] Jones, P. A. DNA methylation and cancer. Cancer Res 46 (February 1986):461-466.

[28] Chalitchagorn, K., et al. Distinctive pattern of line-1 methylation level in normal tissues and the association with carcinogenesis. Oncogene 23 (November 2004):8841-8846.

[29] Suter, C. M., Martin, D. I. and Ward, R. L. Hypomethylation of l1 retrotransposons in colorectal cancer and adjacent normal tissue. Int J Colorectal Dis 19 (March 2004):95-101.

[30] Ehrlich, M., et al. Amount and distribution of 5-methylcytosine in human DNA from different types of tissues of cells. Nucleic Acids Res 10 (April 1982):2709-2721.

[31] Gama-Sosa, M. A., Wang, R. Y., Kuo, K. C., Gehrke, C. W. and Ehrlich, M. The 5-methylcytosine content of highly repeated sequences in human DNA. Nucleic Acids Res 11 (May 1983):3087-3095.

[32] Phokaew, C., Kowudtitham, S., Subbalekha, K., Shuangshoti, S. and Mutirangura, A. Line-1 methylation patterns of different loci in normal and cancerous cells. Nucleic Acids Res 36 (October 2008):5704-5712.

[33] Pattamadilok, J., et al. Line-1 hypomethylation level as a potential prognostic factor for epithelial ovarian cancer. Int J Gynecol Cancer 18 (July-August 2008):711-717.

[34] Tangkijvanich, P., Hourpai, N., Rattanatanyong, P., Wisedopas, N., Mahachai, V. and Mutirangura, A. Serum line-1 hypomethylation as a potential prognostic marker for hepatocellular carcinoma. Clin Chim Acta 379 (April 2007):127-133.

[35] Hsiung, D. T., et al. Global DNA methylation level in whole blood as a biomarker in head and neck squamous cell carcinoma. Cancer Epidemiol Biomarkers Prev 16 (January 2007):108-114.

[36] Aparicio, A., et al. Line-1 methylation in plasma DNA as a biomarker of activity of DNA methylation inhibitors in patients with solid tumors. Epigenetics 4 (April 2009).

[37] Barnes, L., Eveson, J. W., Reichard, P. and Sidransky, D. Pathology and genetics of head and neck tumours. World health organization classification of tumours. Lyon: IARC Press, 2005.

[38] Sriplung, H., Wiangnon, S., Sontipong, S., Sumitsawan, Y. and Martin, N. Cancer incidence trends in thailand, 1989-2000. Asian Pac J Cancer Prev 7 (April-June 2006):239-244.

[39] Ferlay, J., Shin, H., Bray, F., Forman, D., Mathers, C., Parkin, D. M. Estimates of worldwide burden of cancer in 2008: Globocan 2008. Int J Cancer 127 (2010):2893-2917.

[40] Epstein, J. B., Zhang, L., Rosin, M. Advances in the diagnosis of oral premalignant and malignant lesions. J Can Dent Assoc 68 (2002):617-621.

[41] Neville, B. W., Damm, D. D., Allen, C. M. and Bouquot, J. E. Oral and maxillofacial pathology. 3rd. St. Louis: Saunders Elsevir, 2009.

[42] Regezi, J. A., Sciubba, J. J. and Jordan, R. C. K. Oral pathology clinical pathological correlation. 5th. St. Louis: Saunders Elsevier, 2008.

[43] Petti, S. Lifestyle risk factors for oral cancer. Oral Oncol 45 (April-May 2009):340-350.

[44] Katzenellenbogen, R. A., Baylin, S. B. and Herman, J. G. Hypermethylation of the dap-kinase cpg island is a common alteration in b-cell malignancies. Blood 93 (June 1999):4347-4353.

[45] Pontes, H. A. R., et al. Oral candidiasis mimicking an oral squamous cell carcinoma: Report of a case. Gerodontology

[46] Kreimer, A. R., Clifford, G. M., Boyle, P. and Franceschi, S. Human papillomavirus types in head and neck squamous cell carcinomas worldwide: A systematic review. Cancer Epidemiol Biomarkers Prev 14 (Feb 2005):467-475.

[47] Choi, S. and Myers, J. N. Molecular pathogenesis of oral squamous cell carcinoma: Implications for therapy. J Dent Res 87 (January 2008):14-32.

[48] Slaughter, D. P., Southwick, H.W., Smejkal, W. Field cancerization in oral stratified squamous epithelium; clinical implications of multicentric origin. Cancer 6 (September 1953):963-968.

[49] Braakhuis, B. J., Brakenhoff, R. H., Leemans, C. R. A genetic progression model of oral cancer: Current evidence and clinical implications. J Oral Pathol Med 33 (2004):317-322.

[50] Abrahm, J. L., et al. Cancer principles & practice of oncology. 7th. Philadelphia: Lippincott Williams & Wilkins, 2005.

[51] Marx, R. E. and Stern, D. Oral and maxillofacial pathology a rationale for diagnosis and treatment. Carol Stream: Quintessence Publishing Co, Inc, 2003.

[52] Betka, J. Distant metastases from lip and oral cavity cancer. ORL J Otorhinolaryngol Relat Spec 63 (July-August 2001):217-221.

[53] Patel, S. G. and Shah, J. P. Tnm staging of cancers of the head and neck: Striving for uniformity among diversity. CA: A Cancer Journal for Clinicians 55 (2005):242-258.

[54] Woolgar, J. A. Histopathological prognosticators in oral and oropharyngeal squamous cell carcinoma. Oral Oncol 42 (March 2006):229-239.

[55] Kumar, S., Mohan, A. and Guleria, R. Biomarkers in cancer screening, research and detection: Present and future: A review. Biomarkers 11 (2006):385-405.

[56] Mishra, A. and Verma, M. Cancer biomarkers: Are we ready for the prime time? Cancers 2 (2010):190-208.

[57] Malati, T. Tumour makers : An overview. Indian Journal of Clinical Biochemistry 22 (2007):17-31.

[58] Ludwig, J. A. and Weinstein, J. N. Biomarkers in cancer staging, prognosis and treatment selection. Nat Rev Cancer 5 (November 2005):845-856.

[59] Sturgeon, C. M. Practice guidelines for tumour marker use in the clinic. Ned Tijdschr Klin Chem Labgeneesk 32 (2007):76-78.

[60] Uchida, N., Suda, T., Inoue, T., Fujiwara, Y. and Ishiguro, K. Needle track dissemination of follicular thyroid carcinoma following fine-needle aspiration biopsy: Report of a case. Surg Today 37 (2007):34-37.

[61] Garcia-Olmo, D. C., Gutierrez-Gonzalez, L., Samos, J., Picazo, M. G., Atienzar, M. and Garcia-Olmo, D. Surgery and hematogenous dissemination: Comparison between the detection of circulating tumor cells and of tumor DNA in plasma before and after tumor resection in rats. Ann Surg Oncol 13 (August 2006):1136-1144.

[62] Sobin, L. H. and Compton, C. C. Tnm seventh edition: What's new, what's changed. Cancer 116 (2010):5336-5339.

[63] Anastasios K. Markopoulos, E. Z. M., Georgios Tzimagiorgis. Salivary markers for oral cancer detection. The Open Dentistry Journal 4 (2010):172-178.

[64] Nagler, R. M. Saliva as a tool for oral cancer diagnosis and prognosis. Oral oncology 45 (2009):1006-1010.

[65] Wu, J.-Y., et al. Potential biomarkers in saliva for oral squamous cell carcinoma. Oral oncology 46 (2010):226-231.

[66] Liang, X., et al. Whole-genome amplification of oral rinse self-collected DNA in a population-based case-control study of breast cancer. Cancer Epidemiol Biomarkers Prev 16 (August 2007):1610-1614.

[67] Di-Xia, C., Schwartz, P. E. and Fan-Qin, L. I. Saliva and serum ca 125 assays for detecting malignant ovarian tumors. Obstetrics & Gynecology 75 (1990).

[68] Powell, M. E. A. Formation and morphology of the 'buffy coat' and measurement of the density of blood and tumour cells. Nature 193 (March 1962):1047-1050.

[69] Pantel, K., Brakenhoff, R. H., Brandt, B. Detection, clinical relevance and specific biological properties of disseminating tumour cells. Nat Rev Cancer 8 (May 2008):329-340.

[70] Partridge, M., Brakenhoff, R., Phillips, E., Ali, K., Francis, R., Hooper, R., Lavery, K., Brown, A., Langdon, J. Detection of rare disseminated tumor cells identifies head and neck cancer patients at risk of treatment failure. Clin Cancer Res 9 (November 2003):5287-5294.

[71] Liu, M. C., et al. Circulating tumor cells: A useful predictor of treatment efficacy in metastatic breast cancer. J Clin Oncol 27 (November 2009):5153-5159.

[72] Conti, M. Cancer determining information transmission and circulation. Cancer Metastasis Rev 26 (March 2007):215-220.

[73] Bergsmedh, A., et al. Horizontal transfer of oncogenes by uptake of apoptotic bodies. Proc Natl Acad Sci U S A 98 (May 22 2001):6407-6411.

[74] Tsang, J. C. and Lo, Y. M. Circulating nucleic acids in plasma/serum. Pathology 39 (April 2007):197-207.

[75] Garcia-Olmo, D., Garcia-Olmo, D. C., Ontanon, J. and Martinez, E. Horizontal transfer of DNA and the "Genometastasis hypothesis". Blood 95 (January 2000):724-725.

[76] Thomas, E., Shaw, R. J. and Risk, J. M. Monitoring of circulating tumour-associated DNA as a prognostic tool for oral squamous cell carcinoma. Br J Cancer 93 (2005):960-960.

[77] Capone, R. B., et al. Detection and quantitation of human papillomavirus (hpv) DNA in the sera of patients with hpv-associated head and neck squamous cell carcinoma. Clin Cancer Res 6 (November 2000):4171-4175.

[78] Garcia-Olmo, D. C., Gutierrez-Gonzalez, L., Ruiz-Piqueras, R., Picazo, M. G. and Garcia-Olmo, D. Detection of circulating tumor cells and of tumor DNA in plasma during tumor progression in rats. Cancer Lett 217 (January 2005):115-123.

[79] Ogawa, M. Differentiation and proliferation of hematopoietic stem cells. Blood 81 (June 1993):2844-2853.

[80] Ng, E. K., et al. Mrna of placental origin is readily detectable in maternal plasma. Proc Natl Acad Sci U S A 100 (April 2003):4748-4753.

[81] Jurgens, B., Schmitz-Drager, B. J. and Schulz, W. A. Hypomethylation of l1 line sequences prevailing in human urothelial carcinoma. Cancer Res 56 (December 1996):5698-5703.

[82] Pepe, M. S., et al. Phases of biomarker development for early detection of cancer. J Natl Cancer Inst 93 (July 2001):1054-1061.

[83] Loong, T. W. Understanding sensitivity and specificity with the right side of the brain. BMJ 327 (September 2003):716-719.

[84] Soreide, K. Receiver-operating characteristic (roc) curve analysis in diagnostic, prognostic and predictive biomarker research. Journal of Clinical Pathology (2008).

[85] Laird, P. W. The power and the promise of DNA methylation markers. Nat Rev Cancer 3 (April 2003):253-266.

[86] Spafford, M. F., et al. Detection of head and neck squamous cell carcinoma among exfoliated oral mucosal cells by microsatellite analysis. Clinical Cancer Research 7 (March 2001):607-612.

[87] Carvalho, A. L., et al. Evaluation of promoter hypermethylation detection in body fluids as a screening/diagnosis tool for head and neck squamous cell carcinoma. Clinical Cancer Research 14 (January 2008):97-107.

[88] Sugimura, T. and Ushijima, T. Genetic and epigenetic alterations in carcinogenesis. Mutat Res 462 (April 2000):235-246.

[89] Jorde, L. B., Carey, J. C., Bamshad, M. J. and White, R. L. Medical genetics. 3rd. St. Louis: Mosby, 2003.

[90] Flanagan, J. M. Human methylome variation and the rise of epigenetic epidemiology. Current Pharmacogenomics and Personalized Medicine 8 (2010):89-91.

[91] Brena, R. M. and Huang, T. H. M. Quantitative assessment of DNA methylation: Potential applications for disease diagnosis, classification, and prognosis in clinical settings. J Mol Med 84 (2006):365-337.

[92] Costello, J. F. and Plass, C. Methylation matters. J Med Genet 38 (May 2001):285-303.

[93] Rollins, R. A., et al. Large-scale structure of genomic methylation patterns. Genome Res 16 (Febuary 2006):157-163.

[94] Das, R., et al. Computational prediction of methylation status in human genomic sequences. Proc Natl Acad Sci U S A 103 (July 2006):10713-10716.

[95] Ehrlich, M. DNA methylation: Normal development, inherited diseases, and cancer. J Clin Ligand Assay 23 (2000):144-146.

[96] Shiota, K. DNA methylation profiles of cpg islands for cellular differentiation and development in mammals. Cytogenet Genome Res 105 (2004):325-334.

[97] Walsh, C., Chaillet, J. and Bestor, T. Transcription of iap endogenous retroviruses is constrained by cytosine methylation. Nat Genet 20 (1998):116 – 117.

[98] Li, E. Chromatin modification and epigenetic reprogramming in mammalian development. Nat Rev Genet 3 (September 2002):662-673.

[99] Robertson, K. D. and Wolffe, A. P. DNA methylation in health and disease. Nat Rev Genet 1 (October 2000):11-19.

[100] Cooper, G. M. and Hausman, R. E. The cell. 4th. A molecular approach. Washington D.C.: ASM Press, 2007.

[101] Razin, A. and Shemer, R. DNA methylation in early development. Hum Mol Genet 4 (1995):1751-1755.

[102] Stoeber, K., et al. Immunoassay for urothelial cancers that detects DNA replication protein mcm5 in urine. Lancet 354 (October 1999):1524-1525.

[103] Calladine, C. R., Drew, H. R., Luisi, B. F. and Travers, A. A. Understanding DNA the molecule & how it works. 3rd. San Diego: Elsevier Academic Press, 2004.

[104] Feinberg, A. P. and Tycko, B. The history of cancer epigenetics. Nat Rev Cancer 4 (February 2004):143-153.

[105] Goelz, S. E., Vogelstein, B., Hamilton, S. R. and Feinberg, A. P. Hypomethylation of DNA from benign and malignant human colon neoplasms. Science 228 (April 1985):187-190.

[106] Cho, N. Y., et al. Hypermethylation of cpg island loci and hypomethylation of line-1 and alu repeats in prostate adenocarcinoma and their relationship to clinicopathological features. J Pathol 211 (February 2007):269-277.

[107] Baylin, S. B., Esteller, M., Rountree, M. R., Bachman, K. E., Schuebel, K. and Herman, J. G. Aberrant patterns of DNA methylation, chromatin formation and gene expression in cancer. Hum Mol Genet 10 (April 2001):687-692.

[108] Jones, P. A. and Baylin, S. B. The fundamental role of epigenetic events in cancer. Nat Rev Genet 3 (June 2002):415-428.

[109] Kim, Y. I., et al. Global DNA hypomethylation increases progressively in cervical dysplasia and carcinoma. Cancer 74 (August 1994):893-899.

[110] Qu, G., Dubeau, L., Narayan, A., Yu, M. C. and Ehrlich, M. Satellite DNA hypomethylation vs. Overall genomic hypomethylation in ovarian epithelial tumors of different malignant potential. Mutat Res 423 (January 1999):91-101.

[111] Gama-Sosa, M. A., et al. The 5-methylcytosine content of DNA from human tumors. Nucleic Acids Res 11 (October 1983):6883-6894.

[112] Narayan, A., et al. Hypomethylation of pericentromeric DNA in breast adenocarcinomas. Int J Cancer 77 (September 1998):833-838.

[113] Eng, C., Herman, J. G. and Baylin, S. B. A bird's eye view of global methylation. Nat Genet 24 (February 2000):101-102.

[114] Hoffmann, M. J. and Schulz, W. A. Causes and consequences of DNA hypomethylation in human cancer. Biochem Cell Biol 83 (June 2005):296-321.

[115] Roman-Gomez, J., et al. Promoter hypomethylation of the line-1 retrotransposable elements activates sense/antisense transcription and marks the progression of chronic myeloid leukemia. Oncogene 24 (November 2005):7213-7223.

[116] Sakamoto, H., et al. Cell type-specific methylation profiles occurring disproportionately in cpg-less regions that delineate developmental similarity. Genes Cells 12 (October 2007):1123-1132.

[117] Brown, T. A. Genome. 2nd. New York: John Wiley & Sons Inc., 2002.

[118] Grady, W. M., et al. Methylation of the cdh1 promoter as the second genetic hit in hereditary diffuse gastric cancer. Nat Genet 26 (September 2000):16-17.

[119] Jones, P. A. and Laird, P. W. Cancer epigenetics comes of age. Nat Genet 21 (February 1999):163-167.

[120] Yoder, J. A., Walsh, C. P. and Bester, T. H. Cytosine methylation and the ecology of intragenomic parasites. Trends Genet 13 (1997):335-340.

[121] Eden, A., Gaudet, F., Waghmare, A. and Jaenisch, R. Chromosomal instability and tumors promoted by DNA hypomethylation. Science 300 (April 2003):455.

[122] Dunn, B. K. Hypomethylation: One side of a larger picture. Ann N Y Acad Sci 983 (March 2003):28-42.

[123] Gaudet, F., et al. Induction of tumors in mice by genomic hypomethylation. Science 300 (April 2003):489-492.

[124] Wong, N., Lam, W. C., Lai, P. B., Pang, E., Lau, W. Y. and Johnson, P. J. Hypomethylation of chromosome 1 heterochromatin DNA correlates with

q-arm copy gain in human hepatocellular carcinoma. Am J Pathol 159 (August 2001):465-471.

[125] Schulz, W. A., et al. Genomewide DNA hypomethylation is associated with alterations on chromosome 8 in prostate carcinoma. Genes Chromosomes Cancer 35 (September 2002):58-65.

[126] Fan, C. Y. Epigenetic alterations in head and neck cancer: Prevalence, clinical significance, and implications. Curr Oncol Rep 6 (March 2004):152-161.

[127] Bjornsson, H. T., Fallin, M. D. and Feinberg, A. P. An integrated epigenetic and genetic approach to common human disease. Trends Genet 20 (August 2004):350-358.

[128] Brena, R. M. and Costello, J. F. Genome-epigenome interactions in cancer. Hum Mol Genet 16 Spec No 1 (April 2007):R96-105.

[129] Sellis, D., Provata, A. and Almirantis, Y. Alu and line1 distributions in the human chromosomes: Evidence of global genomic organization expressed in the form of power laws. Mol Biol Evol 24 (November 2007):2385-2399.

[130] Maraschio, P., Zuffardi, O., Dalla Fior, T. and Tiepolo, L. Immunodeficiency, centromeric heterochromatin instability of chromosomes 1, 9, and 16, and facial anomalies: The icf syndrome. J Med Genet 25 (March 1988):173-180.

[131] Chen, R. Z., Pettersson, U., Beard, C., Jackson-Grusby, L. and Jaenisch, R. DNA hypomethylation leads to elevated mutation rates. Nature 395 (September 1998):89-93.

[132] Belgnaoui, S. M., Gosden, R. G., Semmes, O. J. and Haoudi, A. Human line-1 retrotransposon induces DNA damage and apoptosis in cancer cells. Cancer Cell Int 6 (2006):13.

[133] Kazazian, H. H., Jr. and Moran, J. V. The impact of l1 retrotransposons on the human genome. Nat Genet 19 (May 1998):19-24.

[134] Santourlidis, S., Florl, A., Ackermann, R., Wirtz, H. C. and Schulz, W. A. High frequency of alterations in DNA methylation in adenocarcinoma of the prostate. Prostate 39 (May 1999):166-174.

[135] Takai, D., Yagi, Y., Habib, N., Sugimura, T. and Ushijima, T. Hypomethylation of line1 retrotransposon in human hepatocellular

carcinomas, but not in surrounding liver cirrhosis. Jpn J Clin Oncol 30 (July 2000):306-309.

[136] Issa, J. P. Aging, DNA methylation and cancer. Crit Rev Oncol Hematol 32 (September 1999):31-43.

[137] Bjornsson, H. T., et al. Intra-individual change over time in DNA methylation with familial clustering. JAMA 299 (June 2008):2877-2883.

[138] Bollati, V., et al. Decline in genomic DNA methylation through aging in a cohort of elderly subjects. Mech Ageing Dev 130 (April 2009):234-239.

[139] Romanov, G. A. and Vanyushin, B. F. Methylation of reiterated sequences in mammalian dnas. Effects of the tissue type, age, malignancy and hormonal induction. Biochim Biophys Acta 653 (April 1981):204-218.

[140] Drinkwater, R. D., Blake, T. J., Morley, A. A. and Turner, D. R. Human lymphocytes aged in vivo have reduced levels of methylation in transcriptionally active and inactive DNA. Mutat Res 219 (January 1989):29-37.

[141] Rath, P. C. and Kanungo, M. S. Methylation of repetitive DNA sequences in the brain during aging of the rat. FEBS Lett 244 (February 1989):193-198.

[142] Figueiredo, J. C., et al. Global DNA hypomethylation (line-1) in the normal colon and lifestyle characteristics and dietary and genetic factors. Cancer Epidemiol Biomarkers Prev 18 (April 2009):1041-1049.

[143] Jintaridth, P. and Mutirangura, A. Distinctive patterns of age-dependent hypomethylation in interspersed repetitive sequences. Physiol Genomics (February 2010):194-200.

[144] Brunaud, L., et al. Effects of vitamin b12 and folate deficiencies on DNA methylation and carcinogenesis in rat liver. Clin Chem Lab Med 41 (August 2003):1012-1019.

[145] Kim, Y. I. Folate and DNA methylation: A mechanistic link between folate deficiency and colorectal cancer? Cancer Epidemiol Biomarkers Prev 13 (April 2004):511-519.

[146] Richards, K. L., et al. Genome-wide hypomethylation in head and neck cancer is more pronounced in hpv-negative tumors and is associated with genomic instability. PLoS One 4 (2009):e4941.

[147] Yang, A. S., Estecio, M. R., Doshi, K., Kondo, Y., Tajara, E. H. and Issa, J. P. A simple method for estimating global DNA methylation using bisulfite pcr of repetitive DNA elements. Nucleic Acids Res 32 (2004):e38.

[148] Laird, P. W. Principles and challenges of genome-wide DNA methylation analysis. Nat Rev Genet 11 (2010):191-203.

[149] Zhenggang Xiong, P. W. L. Cobra: A sensitive and quantitative DNA methylation assay. Nucleic Acids Res 25 (1997):2532-2534.

[150] Liu, Z.-J. and Maekawa, M. Polymerase chain reaction-based methods of DNA methylation analysis. Analytical Biochemistry 317 (2003):259-265.

[151] Mutirangura, A. Quantitative pcr analysis for methylation level of genome: Clinical implications in cancer. Asian Biomedicine 1 (2007):121-128.

[152] Shuangshoti, S., Hourpai, N., Pumsuk, U. and Mutirangura, A. Line-1 hypomethylation in multistage carcinogenesis of the uterine cervix. Asian Pac J Cancer Prev 8 (2007):307-309.

[153] Bruns, D. E., Ashwood, E. R., Burtis, C. A. Fundamentals of molecular diagnostics. Missouri: Saunders Elsevier, 2007.

[154] Oakes, C. C., La Salle, S., Robaire, B. and Trasler, J. M. Evaluation of a quantitative DNA methylation analysis technique using methylation-sensitive/dependent restriction enzymes and real-time pcr. Epigenetics 1 (July-September 2006):146-152.

[155] Pink, R., et al. Saliva as a diagnostic medium. Biomed Pap Med Fac Univ Palacky Olomouc Czech Repub 153 (2009):103-110.

[156] Shpitzer, T., et al. Salivary analysis of oral cancer biomarkers. Br J Cancer 101 (October 2009):1194-1198.

[157] Wong, D. T. Towards a simple, saliva-based test for the detection of oral cancer. Expert Review of Molecular Diagnostics 6 (2006):267-272.

[158] Lallemant, B., et al. Clinical relevance of nine transcriptional molecular markers for the diagnosis of head and neck squamous cell carcinoma in tissue and saliva rinse. BMC Cancer 9 (2009):370.

[159] Park, N. J., Li, Y., Yu, T., Brinkman, B. M. N. and Wong, D. T. Characterization of RNA in saliva. Clin Chem 52 (June 2006):988-994.

[160] Ovstebo, R., Kierulf, P. and Huag, K. B. F. Circulating mucleic acids in blood as biomarkers. Norsk Epidemiology 16 (2006):41-49.

[161] Nagler, R., Bahar, G., Shpitzer, T. and Feinmesser, R. Concomitant analysis of salivary tumor markersโ€”a new diagnostic tool for oral cancer. Clinical Cancer Research 12 (2006):3979-3984.

[162] Chiappin, S., Antonelli, G., Gatti, R. and De Palo, E. F. Saliva specimen: A new laboratory tool for diagnostic and basic investigation. Clinica Chimica Acta 383 (2007):30-40.

Appendices

Appendix A

- Data collection sheet for OSCC patients

ตารางบันทึกข้อมูลผู้ป่วย Oral Cancer

...../...../.....

ชื่อ	HN	เพศ	อายุ	CC (symptom+duration)	ปัจจัยเสี่ยง: บุหรี่	ปัจจัยเสี่ยง: สุรา	ปัจจัยเสี่ยง: หมาก	Family Hx of cancer	Hx of Herbal Tx	PMH	การตรวจภายในช่องปาก: appearance	การตรวจภายในช่องปาก: location	การตรวจภายในช่องปาก: size (mm.)	TNM Stage	Histo grading (differentiation): well	Histo grading (differentiation): moderately	Histo grading (differentiation): poorly	Dx	Tx
ตัวอย่าง	51-111111	ญ	50	ปวด บวมใต้คาง มา 2 เดือนแล้ว	x	/	/	x	/	DM	swelling with deep ulceration	floor of mouth (Lt)	10 x 8 x 5	T1N1M0	/	x	x	squamous cell carcinoma	hemiglossect, SOHND

Biopsy experience : เคย biopsy ไปหนึ่งครั้ง เจ็บมากโดยเฉพาะตอนกลืนอาหาร แผลหายช้า

Etc : ตั้งแต่อายุ 20 ปี, เคี้ยวหมากมา 20 ปี เลิกไปนาน 2 ปีแล้ว
controlled DM มานาน 5 ปี ใช้ยา chlorpropamide 250 mg 1x1
neck node -ve
moderate plaque and calculus, mild gingival inflammation

หมายเหตุ : บันทึก / แทน ใช่ x แทน ไม่ใช่ - แทน ไม่มีข้อมูล หรือ ประเมินไม่ได้
การวัดขนาดให้ใช้ขนาดเท่าที่ประเมินได้จากการตรวจช่องปากทางคลินิกเท่านั้น

- Data collection sheet for normal controls

ตารางบันทึกข้อมูล Control Subjects

...../...../.....

ชื่อ	HN	เพศ	อายุ	CC & PI	PMH	ปัจจัยเสี่ยง			Family Hx of Cancer	Extraoral Examination	Intraoral examination		X-Ray	Dx / Imp	Tx
						ดื่มสุรา	สูบบุหรี่	หมากพลู			Appearance & Location	size (mm.)			
ตัวอย่าง	52-111111	ช	35	มีปุ่มกระดูกโหนกลางเพดานปาก ขัดขวางการใส่ฟันปลอม	none	x	/	x	/	WNL	large and lobulated bony exostosis at the middle of palate	30x30x15	-	torus palatinus	torectomy under GA

Biopsy experience : ไม่เคยได้รับการ biopsy ในร่างกายมาก่อน

Etc : ตื่นเต้นบ้างเป็นครั้งคราว
มีคางปืนมะเร็งปอด เสียชีวิต 3 ปีแล้ว
mild plaque and calculus, mild gingival inflammation

หมายเหตุ : บันทึก / แทน ใช่ x แทน ไม่ใช่ - แทน ไม่มีข้อมูล หรือ ประเมินไม่ได้
การวัดขนาดให้ใช้ขนาดเท่าที่ประเมินได้จากการตรวจช่องปากทางคลินิกเท่านั้น

- Informed consent sheet for all participants (in Thai)

ข้อมูลและรายละเอียดเกี่ยวกับการทำวิจัยที่ใช้ประกอบการพิจารณาเข้าร่วมโครงการ

(Informed Consent)

มะเร็งเซลล์สความัส หรือมะเร็งเยื่อบุผิวในช่องปากเป็นเนื้องอกชนิดร้ายแรงในช่องปากที่พบได้มากที่สุดในปัจจุบัน ผู้ป่วยมักมาด้วยอาการบวม ปวด มีแผลเรื้อรัง ลุกลามไปอย่างรวดเร็ว ในการรักษานั้น ต้องผ่าตัดภายใต้การดมยาสลบ ตัดเนื้อเยื่อ และกระดูกที่เป็นมะเร็งรวมถึงเนื้อเยื่อปกติรอบๆออกด้วย ผู้ป่วยมักมาพบทันตแพทย์เมื่อรอยโรคมีขนาดใหญ่ ลุกลามมากแล้ว ทำให้ในการรักษาดังกล่าวต้องตัดเนื้อเยื่อ และอวัยวะในรวมถึงนอกช่องปากเป็นบริเวณกว้าง อาจส่งผลให้ใบหน้าผิดรูปร่าง เคี้ยว และกลืนลำบาก หรือพูดไม่ชัด ปัจจุบันจะใช้การตัดชิ้นเนื้อบางส่วนไปตรวจเพื่อช่วยวินิจฉัยโรคแต่ ผู้ป่วยส่วนใหญ่กลัว จึงหลีกเลี่ยงการมาพบทันตแพทย์ ทำให้ตรวจพบเมื่อรอยโรคลุกลามไปมากแล้ว ดังนั้นหากตรวจพบผู้ป่วยได้ในระยะแรก จึงน่าจะทำให้การรักษามี ประสิทธิภาพ ลดโอกาสสูญเสียอวัยวะ และผู้ป่วยมีคุณภาพชีวิตที่ดีขึ้น

การวิจัยครั้งนี้จึงมีวัตถุประสงค์เพื่อพัฒนาวิธีตรวจคัดกรองมะเร็งที่ไม่ยุ่งยาก และเจ็บปวดน้อยที่สุด สำหรับผู้ป่วย โดยตรวจในระดับดีเอ็นเอจากน้ำบ้วนปาก และเลือดของผู้ป่วยที่เป็นมะเร็งเยื่อบุผิวในช่องปากเปรียบเทียบกับในคนปกติ

กลุ่มผู้เข้าร่วมการวิจัยจะแบ่งออกเป็น 2 กลุ่มได้แก่ กลุ่มผู้ป่วยที่เป็นมะเร็งเยื่อบุผิวในช่องปาก จำนวน 30 คน และกลุ่มคนปกติ โดยในที่นี้หมายถึงผู้ป่วยอื่นๆที่ต้องรับการรักษาด้วยการผ่าตัด แต่ไม่เป็นมะเร็งจำนวน 30 คน เมื่อผู้ป่วยตัดสินใจเข้าร่วมการวิจัยจะทำการซักประวัติ ตรวจบริเวณศีรษะ ลำคอ และภายในช่องปาก หลังจากนั้นจะทำการเก็บเลือด และน้ำบ้วนปากของทั้งผู้ป่วยมะเร็ง และผู้ป่วยปกติ ในวันที่ผู้ป่วยมารับการเจาะเลือดเพื่อตรวจร่างกายก่อนเข้ารับการผ่าตัด โดย

- ในการเจาะเลือด 1 ครั้งนั้นจะเก็บเลือดเพิ่มจากปกติอีก 6 มิลลิลิตร ซึ่งจะไม่ก่อให้เกิดอันตราย ต่อสุขภาพของผู้ป่วย
- ให้ผู้ป่วยอมกลั้วน้ำเกลือ 10 มิลลิลิตร ในช่องปาก 30 วินาที แล้วบ้วนกลับใส่หลอดเก็บ น้ำบ้วนปาก

หลังจากนั้นผู้วิจัยจะนำเลือด และน้ำบ้วนปากไปศึกษาต่อในห้องปฏิบัติการ

การเข้าร่วมการวิจัยครั้งนี้ไม่ได้เสี่ยงต่อการเกิดโรค ไม่ได้ทำให้โรคที่เป็นอยู่ลุกลามมากขึ้น ไม่เกิด อันตรายต่อสุขภาพใดๆ ไม่ทำให้เกิดความเจ็บปวดเพิ่มขึ้น ไม่ต้องได้รับยาเพิ่มเติม ผู้ป่วยสามารถกลับบ้าน ได้เลย และไม่ต้องกลับมาทำซ้ำในส่วนนี้อีก การวิจัยนี้ไม่มีผลต่อแผนการรักษาเดิมของผู้ป่วย ผู้ป่วยที่ เข้าร่วมโครงการวิจัยจะได้รับการรักษา และติดตามผลตามปกติ ไม่เสียค่าใช้จ่าย และไม่มีค่าตอบแทน

ประโยชน์ที่ได้จากการวิจัยครั้งนี้คือมีส่วนร่วมในการพัฒนาค้นคว้าเกี่ยวกับวิธีการตรวจวินิจฉัยโรคมะเร็งเยื่อบุผิวในช่องปาก ทำให้สามารถตรวจพบผู้ป่วยได้ในระยะแรก การรักษาง่ายขึ้น มีประสิทธิภาพ เพิ่มขึ้น ลดความพิการ และอาการแทรกซ้อนต่างๆ ผู้ป่วยจะมีคุณภาพชีวิตที่ดีขึ้น

การเข้าร่วมในการวิจัยครั้งนี้เป็นโดยสมัครใจ ผู้ป่วยสามารถที่จะปฏิเสธการเข้าร่วม หรือถอนตัวออกจากการวิจัยได้ทุกขณะโดยไม่ต้องได้รับโทษ หรือสูญเสียประโยชน์ซึ่งพึงได้รับ ข้อมูลต่างๆที่อาจนำไปสู่การเปิดเผยตัวของผู้เข้าร่วมการวิจัยจะได้รับการปกปิด ยกเว้นว่าจะได้รับคำยินยอมไว้โดยกฎระเบียบ และกฎหมายที่เกี่ยวข้องเท่านั้น จึงจะเปิดเผยสู่สาธารณชนได้ ในกรณีที่ผลการวิจัยได้รับ การตีพิมพ์ ชื่อ และที่อยู่ของผู้เข้าร่วมการวิจัยจะได้รับการปกปิดอยู่เสมอ ผู้เข้าร่วมการวิจัย หรือผู้แทนตามกฎหมายจะได้รับแจ้งในทันทีหากมีข้อมูลใหม่ซึ่งอาจใช้ประกอบการตัดสินใจของผู้ร่วมการวิจัยว่าจะยังเข้าร่วมโครงการวิจัยต่อไปหรือไม่ ทั้งนี้ผู้กำกับดูแลการวิจัย ผู้ตรวจสอบ คณะกรรมการพิจารณาจริยธรรม และคณะกรรมการที่เกี่ยวข้องกับการควบคุมยา สามารถเข้าไปตรวจสอบบันทึกข้อมูลทางการแพทย์ของ ผู้เข้าร่วมการวิจัยเพื่อเป็นการยืนยันถึงขั้นตอนในการวิจัยทางคลินิก และข้อมูลอื่นๆโดยไม่ละเมิดเอกสิทธิ์ ในการปิดบังข้อมูลของผู้เข้าร่วมการวิจัยตามกรอบที่กฎหมาย และกฎระเบียบได้อนุญาตไว้ นอกจากนี้ โดยการเซ็นให้ความยินยอม ผู้เข้าร่วมการวิจัย หรือผู้แทนตามกฎหมายมีสิทธิตรวจสอบและมีสิทธิที่จะ ได้รับข้อมูลเช่นกัน

หากตรวจสอบพบว่าข้อมูลที่ได้รับไม่เป็นความจริงผู้วิจัยอาจต้องยกเลิกการเข้าร่วมในโครงการวิจัย ของผู้เข้าร่วมการวิจัยนี้

ขอขอบคุณในความร่วมมือ หากมีข้อสงสัย ปัญหา หรือข้อมูลเพิ่มเติมสามารถติดต่อได้ที่ ทพญ. ตวงทอง พบสุข ภาควิชาศัลยศาสตร์ คณะทันตแพทยศาสตร์ จุฬาลงกรณ์มหาวิทยาลัย

- Consent Form form for all participants (in Thai)

เอกสารยินยอมเข้าร่วมการวิจัย (Consent Form)

การวิจัยเรื่อง การเปรียบเทียบระดับเมทิเลชันของไลน์วันในส่วนประกอบของเลือดกับน้ำบ้วนปากของผู้ป่วยมะเร็งเซลล์สความัสในช่องปากแต่ละราย

ก่อนที่จะลงนามในใบยินยอมให้ทำการวิจัยนี้ ข้าพเจ้าได้รับการอธิบายจากผู้วิจัยถึงวัตถุประสงค์ของการวิจัย วิธีการวิจัย อันตราย หรืออาการที่อาจเกิดขึ้นจากการวิจัย หรือจากยาที่ใช้ รวมทั้งประโยชน์ที่จะเกิดขึ้นจากการวิจัยอย่างละเอียด และมีความเข้าใจดีแล้ว

ผู้วิจัยรับรองว่าจะตอบคำถามต่างๆ ที่ข้าพเจ้าสงสัยด้วยความเต็มใจไม่ปิดบังซ่อนเร้นจนข้าพเจ้าพอใจ

ข้าพเจ้าเข้าร่วมโครงการวิจัยนี้โดยสมัครใจ ข้าพเจ้ามีสิทธิที่จะบอกเลิกการเข้าร่วมในโครงการวิจัยนี้เมื่อใดก็ได้และการบอกเลิกการเข้าร่วมการวิจัยนี้ จะไม่มีผลต่อการรักษาโรคที่ข้าพเจ้าจะพึงได้รับต่อไป

ผู้วิจัยรับรองว่าจะเก็บข้อมูลเฉพาะเกี่ยวกับตัวข้าพเจ้าเป็นความลับ และจะเปิดเผยได้เฉพาะในรูปที่เป็นสรุปผลการวิจัย การเปิดเผยข้อมูลเกี่ยวกับตัวข้าพเจ้าต่อหน่วยงานต่างๆ ที่เกี่ยวข้องกระทำได้เฉพาะกรณีจำเป็น ด้วยเหตุผลทางวิชาการเท่านั้น

ผู้วิจัยรับรองว่าหากเกิดอันตรายใดๆ จากการวิจัยดังกล่าว ข้าพเจ้าจะได้รับการรักษาพยาบาลโดยไม่คิดมูลค่า

ข้าพเจ้าได้อ่านข้อความข้างต้นแล้ว และมีความเข้าใจดีทุกประการ และได้ลงนามในใบยินยอมนี้ด้วยความเต็มใจ

ลงนาม.....ผู้ยินยอม

(.....)

ลงนาม.....พยาน

(.....)

ลงนาม.....พยาน

(.....)

ลงนาม.....หัวหน้าโครงการวิจัย

(..... ตวงทอง พบสุข .....)

วันให้คำยินยอมเข้าร่วมวิจัย วันที่.....เดือน.....พ.ศ.....

ข้าพเจ้าไม่สามารถอ่านหนังสือได้ แต่ผู้วิจัยได้อ่านข้อความในใบยินยอมนี้ให้แก่ข้าพเจ้าฟังจนเข้าใจดีแล้ว ข้าพเจ้าจึงลงนาม หรือประทับลายนิ้วหัวแม่มือขวาของข้าพเจ้าในใบยินยอมนี้ด้วยความเต็มใจ

ลงนาม.....ผู้ยินยอม

(.....)

ลงนาม.....พยาน

(.....)

ลงนาม.....พยาน

(.....)

ลงนาม.....หัวหน้าโครงการวิจัย

(..... ตวงทอง พบสุข .....)

วันให้คำยินยอมเข้าร่วมวิจัย วันที่.....เดือน.....พ.ศ.....

ในกรณีที่ผู้ถูกทดลองยังไม่บรรลุนิติภาวะ จะต้องได้รับการยินยอมจากผู้ปกครองหรือผู้อุปการะโดยชอบด้วยกฎหมาย

ลงนาม.....ผู้ยินยอม

(.....)

ลงนาม.....พยาน

(.....)

ลงนาม.....พยาน

(.....)

ลงนาม.....หัวหน้าโครงการวิจัย

(..... ตวงทอง พบสุข .....)

วันให้คำยินยอมเข้าร่วมวิจัย วันที่.....เดือน.....พ.ศ.....

- Withdrawal form in case drop-out is demanded (in Thai)

<u>เอกสารยกเลิกการยินยอมเข้าร่วมวิจัย (Withdrawal Form)</u>

การวิจัยเรื่อง การเปรียบเทียบระดับเมทิเลชันของไลน์วันในส่วนประกอบของเลือดกับน้ำบ้วนปากของผู้ป่วยมะเร็งเซลล์สความัสในช่องปากแต่ละราย

เหตุผลในการยกเลิกการยินยอมเข้าร่วมวิจัย

❑ ย้ายภูมิลำเนา

❑ ไม่สะดวกในการเดินทาง

❑ เหตุผลอื่น

ลงนาม.....ผู้ยกเลิกการยินยอม

(.....)

ลงนาม.....พยาน

(.....)

ลงนาม.....พยาน

(.....)

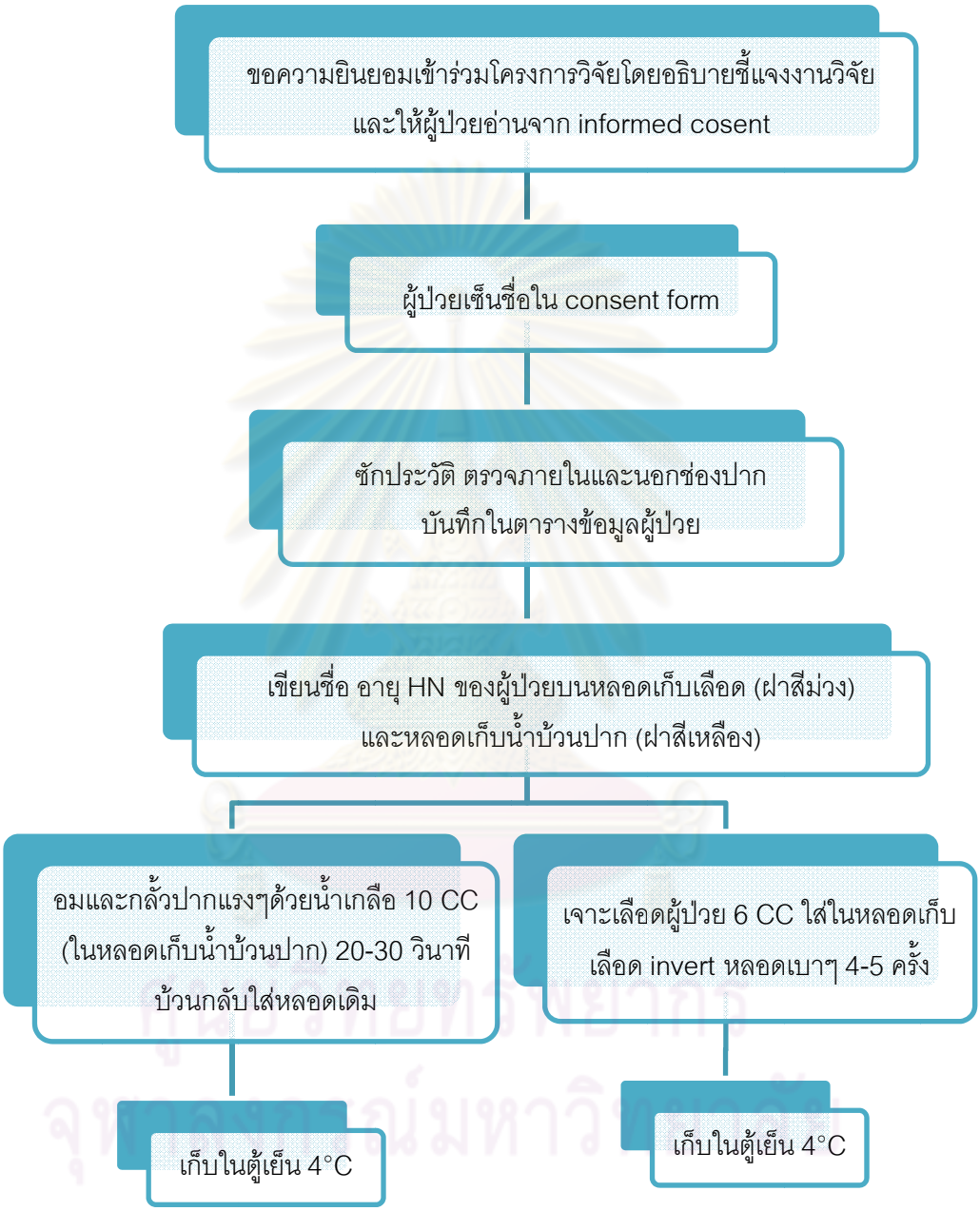
ลงนาม.....หัวหน้าโครงการวิจัย

(..... ตวงทอง พบสุข .....)

วันยกเลิกการยินยอมเข้าร่วมวิจัย วันที่.....เดือน.....พ.ศ. .....

- Sample collection protocol for Rajavithi and Buddhachinaraj hospital

ขั้นตอนการเก็บตัวอย่างผู้ป่วย Oral Cancer และ Non-Cancer (Control)



ขอบพระคุณในความร่วมมือ

ทญ. ตวงทอง พบสุข โทร 081-751-4517

Oral and Maxillofacial Surgery, Chulalongkorn University

Appendix B

Statistics Output

Table 1 Descriptive analysis of all COBRA LINE-1 products

Descriptive Statistics

Type		N	Minimum	Maximum	Mean	Std. Deviation
NOR	Meth	45	33.14	42.78	37.6487	2.49534
	bp160	45	15.99	34.03	23.8317	3.46387
	bp98	45	12.61	17.68	14.4218	1.16128
	bp80	45	25.31	35.95	29.8770	2.31006
	bp62	45	24.99	42.63	33.2739	2.97358
	Valid N	45				
OSCC OR	Meth	37	30.17	40.18	35.6934	2.36542
	bp160	37	18.18	30.27	23.3055	2.16603
	bp98	37	11.48	17.77	15.5011	1.36971
	bp80	37	25.86	35.60	28.7132	1.77458
	bp62	37	27.70	36.13	33.4944	1.68025
	Valid N	37				
WSU-HN	Meth	11	8.57	50.73	23.1773	12.04337
	bp160	11	9.65	14.34	11.7718	1.59870
	bp98	11	20.17	45.54	37.1282	7.66013
	bp80	11	7.77	44.69	20.2291	10.55012
	bp62	11	20.80	37.43	30.8673	4.48093
	Valid N	11				
NWBC	Meth	44	29.23	40.98	34.9736	2.45156
	bp160	44	6.22	29.85	19.8457	3.79518
	bp98	44	8.31	20.30	14.6357	2.10709
	bp80	44	24.40	48.31	30.5061	4.18834
	bp62	44	27.60	42.42	35.7011	2.92439
	Valid N	44				
OSCC WBC	Meth	35	31.02	40.49	35.3720	2.22915
	bp160	35	16.08	24.59	20.9298	2.24804
	bp98	35	13.66	17.77	15.6492	1.07746
	bp80	35	25.91	35.90	29.1358	2.00356

	bp62	35	30.82	38.69	34.5462	2.15104
	Valid N	35				
N PM	Meth	45	31.36	43.07	35.3929	2.72740
	bp160	45	14.17	27.56	21.4558	3.49517
	bp98	45	10.59	20.78	14.8264	1.92642
	bp80	45	24.78	34.38	29.8333	2.24963
	bp62	45	28.81	41.58	35.1142	3.37761
	Valid N	45				
OSCC PM	Meth	35	30.40	39.62	35.5583	1.91158
	bp160	35	18.39	29.11	22.4849	2.51987
	bp98	35	12.74	19.93	16.0743	1.39305
	bp80	35	26.14	31.96	28.4909	1.44005
	bp62	35	29.91	37.91	33.3917	1.89378
	Valid N	35				

Table 2 Correlations between 160 bp and 98 bp in normal and cancer

Correlations

Type			bp160	bp98
NOR	bp160	Pearson Correlation	1	.519**
		Sig. (2-tailed)		.00025909
		N	45	45
	bp 98	Pearson Correlation	.519**	1
		Sig. (2-tailed)	.000	
		N	45	45
OSCC OR	bp160	Pearson Correlation	1	.240
		Sig. (2-tailed)		.152
		N	37	37
	bp98	Pearson Correlation	.240	1
		Sig. (2-tailed)	.152	
		N	37	37

WSU-HN	bp160	Pearson Correlation	1	-.898**
		Sig. (2-tailed)		.00017546
		N	11	11
	bp98	Pearson Correlation	-.898**	1
		Sig. (2-tailed)	.000	
		N	11	11
Bx Tissue	bp160	Pearson Correlation	1	-.629**
		Sig. (2-tailed)		.002
		N	21	21
	bp98	Pearson Correlation	-.629**	1
		Sig. (2-tailed)	.002	
		N	21	21
OR (last cohort)	bp160	Pearson Correlation	1	-.403*
		Sig. (2-tailed)		.037
		N	27	27
	bp98	Pearson Correlation	-.403*	1
		Sig. (2-tailed)	.037	
		N	27	27
NWBC	bp160	Pearson Correlation	1	.590**
		Sig. (2-tailed)		.000
		N	44	44
	bp98	Pearson Correlation	.590**	1
		Sig. (2-tailed)	.000	
		N	44	44
OSCC WBC	bp160	Pearson Correlation	1	.637**
		Sig. (2-tailed)		.000
		N	35	35
	bp98	Pearson Correlation	.637**	1
		Sig. (2-tailed)	.000	
		N	35	35

**. Correlation is significant at the 0.01 level (2-tailed).

*. Correlation is significant at the 0.05 level (2-tailed).

Table 3 Correlations between 160 bp and 80 bp in normal and cancer

Correlations

Type			bp160	bp80
NOR	bp160	Pearson Correlation	1	-.624**
		Sig. (2-tailed)		.0000045602
		N	45	45
	bp80	Pearson Correlation	-.624**	1
		Sig. (2-tailed)	.000	
		N	45	45
OSCC OR	bp160	Pearson Correlation	1	-.528**
		Sig. (2-tailed)		.001
		N	37	37
	bp80	Pearson Correlation	-.528**	1
		Sig. (2-tailed)	.001	
		N	37	37
WSU-HN	bp160	Pearson Correlation	1	.875**
		Sig. (2-tailed)		.00042526
		N	11	11
	bp80	Pearson Correlation	.875**	1
		Sig. (2-tailed)	.000	
		N	11	11
Bx Tissue	bp160	Pearson Correlation	1	-.291
		Sig. (2-tailed)		.201
		N	21	21
	bp80	Pearson Correlation	-.291	1
		Sig. (2-tailed)	.201	
		N	21	21
OR (last cohort)	bp160	Pearson Correlation	1	-.504**
		Sig. (2-tailed)		.007
		N	27	27
	bp80	Pearson Correlation	-.504**	1
		Sig. (2-tailed)	.007	
		N	27	27

NWBC	bp160	Pearson Correlation	1	-.819**
		Sig. (2-tailed)		.000
		N	44	44
	bp80	Pearson Correlation	-.819**	1
		Sig. (2-tailed)	.000	
		N	44	44
OSCC WBC	bp160	Pearson Correlation	1	-.667**
		Sig. (2-tailed)		.000
		N	35	35
	bp80	Pearson Correlation	-.667**	1
		Sig. (2-tailed)	.000	
		N	35	35

**. Correlation is significant at the 0.01 level (2-tailed).

Table 4 Correlations between 98 bp and 80 bp in normal and cancer

Type			bp98	bp80
NOR	bp98	Pearson Correlation	1	-.699**
		Sig. (2-tailed)		.000000094819
		N	45	45
	bp80	Pearson Correlation	-.699**	1
		Sig. (2-tailed)	.000	
		N	45	45
OSCC OR	bp98	Pearson Correlation	1	-.718**
		Sig. (2-tailed)		.000
		N	37	37
	bp80	Pearson Correlation	-.718**	1
		Sig. (2-tailed)	.000	
		N	37	37
WSU-HN	bp98	Pearson Correlation	1	-.990**
		Sig. (2-tailed)		.0000000066887
		N	11	11
	bp80	Pearson Correlation	-.990**	1
		Sig. (2-tailed)	.000	
		N	11	11

Bx Tissue	bp98	Pearson Correlation	1	-.397
		Sig. (2-tailed)		.075
		N	21	21
	bp80	Pearson Correlation	-.397	1
		Sig. (2-tailed)	.075	
		N	21	21
OR (last cohort)	bp98	Pearson Correlation	1	.404*
		Sig. (2-tailed)		.037
		N	27	27
	bp80	Pearson Correlation	.404*	1
		Sig. (2-tailed)	.037	
		N	27	27
NWBC	bp98	Pearson Correlation	1	-.743**
		Sig. (2-tailed)		.000
		N	44	44
	bp80	Pearson Correlation	-.743**	1
		Sig. (2-tailed)	.000	
		N	44	44
OSCC WBC	bp98	Pearson Correlation	1	-.687**
		Sig. (2-tailed)		.000
		N	35	35
	bp80	Pearson Correlation	-.687**	1
		Sig. (2-tailed)	.000	
		N	35	35

**. Correlation is significant at the 0.01 level (2-tailed).

*. Correlation is significant at the 0.05 level (2-tailed).

Table 5 Independent sample test of NOR and WSU-HN

		Levene's Test for Equality of Variances		t-test for Equality of Means			
						95% Confidence Interval	
		F	Sig.	Sig. (2-tailed)	Mean Difference	Lower	Upper
Meth	Equal variances assumed	29.734	.000	.000	14.47141	10.66073	18.28209
	Equal variances not assumed			.002560	14.47141	6.36091	22.58190
160 bp	Equal variances assumed	3.465	.068	1.066389×10^{-15}	12.05986	9.90094	14.21878
	Equal variances not assumed			.000	12.05986	10.62653	13.49318
98 bp	Equal variances assumed	39.866	.000	.000	-22.70636	-25.03894	-20.37378
	Equal variances not assumed			.0000017477	-22.70636	-27.85916	-17.55356
80 bp	Equal variances assumed	26.654	.000	.000	9.64788	6.27886	13.01691
	Equal variances not assumed			.013	9.64788	2.54097	16.75480
62 bp	Equal variances assumed	2.463	.122	.116	2.40667	.17797	4.63536
	Equal variances not assumed			.116	2.40667	-.68478	5.49811

Table 6 Independent sample test of NOR and OSCCR

		Levene's Test for Equality of Variances		t-test for Equality of Means			
						95% Confidence Interval	
		F	Sig.	Sig. (2-tailed)	Mean Difference	Lower	Upper
Meth	Equal variances assumed	.001	.981	.001	1.95526	.87867	3.03186
	Equal variances not assumed			.000	1.95526	.88399	3.02654
160 bp	Equal variances assumed	5.608	.020	.424	.52621	-.77721	1.82963
	Equal variances not assumed			.404	.52621	-.72330	1.77573
98 bp	Equal variances assumed	1.289	.260	.00022731	-1.07927	-1.63544	-.52309
	Equal variances not assumed			.000	-1.07927	-1.64563	-.51291
80 bp	Equal variances assumed	2.290	.134	.014	1.16376	.24243	2.08510
	Equal variances not assumed			.012	1.16376	.26553	2.06199
62 bp	Equal variances assumed	3.685	.058	.689	-.22044	-1.31421	.87333
	Equal variances not assumed			.674	-.22044	-1.26172	.82084

Table 7 Independent sample test of NBC and OSCC BC

		Levene's Test for Equality of Variances		t-test for Equality of Means			
						95% Confidence Interval	
		F	Sig.	Sig. (2-tailed)	Mean Difference	Lower	Upper
Meth	Equal variances assumed	.115	.736	.458	-.39836	-1.46090	.66417
	Equal variances not assumed			.453	-.39836	-1.44965	.65293
160 bp	Equal variances assumed	2.363	.128	.139	-1.08414	-2.52981	.36154
	Equal variances not assumed			.119	-1.08414	-2.45342	.28515
98 bp	Equal variances assumed	4.816	.031	.012	-1.01353	-1.79365	-.23341
	Equal variances not assumed			.00729176	-1.01353	-1.74444	-.28263
80 bp	Equal variances assumed	5.237	.025	.079	1.37031	-.16369	2.90430
	Equal variances not assumed			.06025	1.37031	-.06084	2.80146
62 bp	Equal variances assumed	1.591	.211	.047	1.15498	-.02273	2.33269
	Equal variances not assumed			.047	1.15498	.01696	2.29300

Table 8 Independent sample test of NPM and OSCC PM

		Levene's Test for Equality of Variances		t-test for Equality of Means			
						95% Confidence Interval	
		F	Sig.	Sig. (2-tailed)	Mean Difference	Lower	Upper
Meth	Equal variances assumed	3.518	.064	.7612	-.16540	-1.24495	.91416
	Equal variances not assumed			.751	-.16540	-1.19947	.86868
160 bp	Equal variances assumed	5.710	.019	.146	-1.02908	-2.42355	.36539
	Equal variances not assumed			.13029	-1.02908	-2.36897	.31081
98 bp	Equal variances assumed	2.741	.102	.00182	-1.24784	-2.01709	-.47859
	Equal variances not assumed			.001	-1.24784	-1.98723	-.50845
80 bp	Equal variances assumed	9.758	.003	.003	1.34248	.47258	2.21237
	Equal variances not assumed			.00178	1.34248	.51707	2.16788
62 bp	Equal variances assumed	20.036	.000	.008	1.72251	.45353	2.99148
	Equal variances not assumed			.00514	1.72251	.53301	2.91201

Table 9 Descriptive analysis of 16 matched pairs by identical age and gender

Descriptive Statistics

	N	Minimum	Maximum	Mean	Std. Deviation
ORmeth	16	33.37	42.78	38.4575	2.82183
ORmethOSCC	16	30.17	38.77	35.1387	2.50534
BCmeth	16	32.92	40.98	36.0394	2.30295
BCmethOSCC	16	31.11	38.17	34.4200	2.01896
PMmeth	16	31.92	43.07	36.8056	3.28809
PMmethOSCC	16	33.11	37.93	35.6325	1.42134
OR160	16	19.64	29.13	24.1856	2.60911
OR160 OSCC	16	21.14	26.38	23.4300	1.50590
BC160	16	16.22	25.79	20.5500	2.67731
BC160 OSCC	16	19.72	24.93	22.0106	1.68139
PM160	16	14.17	25.27	20.6388	2.69248
PM160 OSCC	16	19.68	26.49	23.1088	2.06267
OR98	16	12.87	17.25	14.3019	1.25501
OR98 OSCC	16	14.30	17.57	15.8169	1.00991
BC98	16	13.07	17.62	14.8706	1.24915
BC98 OSCC	16	14.71	17.33	16.0594	.78117
PM98	16	12.98	20.78	15.1563	2.23085
PM98 OSCC	16	15.21	17.16	16.3531	.72615
OR80	16	26.81	35.95	30.1475	2.75487
OR80 OSCC	16	25.86	30.22	28.1494	1.31086
BC80	16	26.80	33.22	29.9475	1.79292
BC80 OSCC	16	26.32	30.30	28.2419	1.19434
PM80	16	27.88	34.38	30.6513	1.80640
PM80 OSCC	16	26.21	30.85	28.2925	1.34306
OR62	16	28.32	34.25	32.5613	1.59851
OR62 OSCC	16	31.05	35.62	33.6000	1.30588
BC62	16	32.57	38.59	35.1656	1.68894
BC62 OSCC	16	31.46	35.98	34.1263	1.56914
PM62	16	28.81	41.58	34.1856	3.58586
PM62 OSCC	16	31.06	35.59	32.6825	1.15065
Valid N (listwise)	16				

Table 10 Independent sample test of 16 matched pairs by identical age and gender

				95% Confidence Interval of the Difference		Sig. (2-tailed)
		Mean	Std. Deviation	Lower	Upper	
Pair 1	ORmeth	3.31875	3.35838	1.52919	5.10831	.0013
Pair 2	BCmeth	1.61938	3.09926	-.03210	3.27085	.0541
Pair 3	PMmeth	1.17312	3.91803	-.91465	3.26090	.2496
Pair 4	OR160	.75563	2.59403	-.62664	2.13789	.2621
Pair 5	BC160	-1.46062	3.59890	-3.37834	.45709	.1253
Pair 6	PM160	-2.47000	3.88270	-4.53895	-.40105	.0224
Pair 7	OR98	-1.51500	1.24784	-2.17993	-.85007	.0002
Pair 8	BC98	-1.18875	1.31648	-1.89025	-.48725	.0026
Pair 9	PM98	-1.19688	2.15009	-2.34258	-.05117	.0417
Pair 10	OR80	1.99813	2.64258	.58999	3.40626	.0085
Pair 11	BC80	1.70563	1.70606	.79653	2.61472	.0012
Pair 12	PM80	2.35875	2.30654	1.12968	3.58782	.0010
Pair 13	OR62	-1.03875	1.95881	-2.08253	.00503	.0510
Pair 14	BC62	1.03938	2.47358	-.27870	2.35745	.1135
Pair 15	PM62	1.50313	4.16506	-.71628	3.72253	.1694

Table 11 LINE-1 methylation of 44 controls and 35 OSCC patients for intra-individual comparison

	type	N	Mean	Std. Deviation	Minimum	Maximum
ORmeth	normal	44	37.6345	2.52291	33.14	42.78
	OSCC	35	35.7097	2.39268	30.17	40.18
BCmeth	normal	44	34.9736	2.45156	29.23	40.98
	OSCC	35	35.3720	2.22915	31.02	40.49
PMmeth	normal	44	35.3464	2.74081	31.36	43.07
	OSCC	35	35.5583	1.91158	30.40	39.62

Table 12 LINE-1 complete unmethylation (98 bp) level of 44 controls and 35 OSCC patients for intra-individual comparison

	type	N	Mean	Std. Deviation	Minimum	Maximum
OR 98bp	normal	44	14.4105	1.17221	12.61	17.68
	OSCC	35	15.5018	1.40315	11.48	17.77
BC 98bp	normal	44	14.6357	2.10709	8.31	20.30
	OSCC	35	15.7193	1.04639	13.66	17.77
PM 98bp	normal	44	14.8364	1.94753	10.59	20.78
	OSCC	35	16.0743	1.39305	12.74	19.93

BIOGRAPHY

I, Miss Tuangtong Pobsook was born on August 16, 1982 in Bangkok, Thailand. I received bachelor degree of Doctor of Dental Surgery (D.D.S.) from Chulalongkorn University in March, 2006. After that, I began my practice at several private dental clinics in Bangkok until 2008 in which I started pursuing Master Degree in Oral and Maxillofacial Surgery at the Department of Oral Surgery, Faculty of Dentistry, Chulalongkorn University. My research interest was molecular marker of oral cancer and my base was at the Center of Excellence in Molecular Genetics of Cancer and Human Diseases, Department of Anatomy, Faculty of Medicine, Chulalongkorn University. During these academic years, I was accepted in three international conferences. The first event was “International Summer Program: Advances in Cancer Research” for selected Asian participants at Tokyo Medical and Dental University (TMDU), Japan in September, 2008. Joining as poster presenter, the next one was “International Association of Dental Research (IADR) General Session” in Barcelona, Spain during July, 2010. I participated in my last program, “the 70th Anniversary Celebration 2010 of Faculty of Dentistry, also as a poster presenter, in August. Currently, a part of this study was published in Clinical Chemistry Acta, 2011 in the title of “Improved measurement of LINE-1 sequence methylation for cancer detection”.