การศึกษาระดับเมทิเลชันของไลน์-1 ในมะเร็งต่อมน้ำลายชนิดมิวโคเอ็พพิเดอร์มอยด์คาร์ซิโนมา

นางสาวพรทิพา ศิริวนิชสุนทร

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STUDY OF LINE-1 METHYLATION LEVELS IN SALIVARY MUCOEPIDERMOID CARCINOMA

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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

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พรทิพา ศิริวนิชสุนทร : การศึกษาระดับเมทิเลชันของไลน์-1 ในมะเร็งต่อมน้ำลายชนิดมิวโคเอ็พพิเดอร์มอยด์คาร์ซิโนมา. (STUDY OF LINE-1 METHYLATION LEVELS IN SALIVARY MUCOEPIDERMOID CARCINOMA) อ. ที่ปรึกษาวิทยานิพนธ์หลัก: อ.ทพญ.ดร. เกศกัญญา สัพพะเลข, อ. ที่ปรึกษาวิทยานิพนธ์ร่วม: ศ.นพ.ดร. อภิวัฒน์ มุทิรางกูร, ผศ.ทพ.ดร. นครินทร์ กิตกำธร, 82 หน้า.

มะเร็งต่อมน้ำลายชนิดมิวโคเอ็พพิเดอร์มอยด์คาร์ซิโนมา (mucoepidermoid carcinoma) ได้รับการจำแนกความรุนแรงตามลักษณะทางจุลพยาธิวิทยาโดยใช้สัดส่วนของเซลล์ที่เป็นองค์ประกอบหลักในรอยโรค หรือการมีถุงน้ำในรอยโรค การรุกรานเส้นประสาท การตายเฉพาะส่วน ลักษณะการแบ่งตัว และความผิดปกติในการแบ่งตัวของเซลล์ อย่างไรก็ตามเกณฑ์ที่ใช้ในการจำแนกความรุนแรงของโรคในปัจจุบันยังมีความหลากหลายและไม่มีเกณฑ์ใดเป็นที่ยอมรับโดยสากล เมทิเลชัน (methylation) ของไลน์- 1 (LINE-1) และอลู (Alu element) มีระดับที่ต่ำลงในเนื้อเยื่อที่เป็นมะเร็ง และมีความสัมพันธ์กับระดับความรุนแรงของมะเร็งบางประเภท แต่ยังไม่มีผู้ใดศึกษาเมทิเลชันของไลน์-1และอลูในมะเร็งชนิดนี้ ดังนั้นการศึกษานี้มีวัตถุประสงค์เพื่อศึกษาระดับ (level) และรูปแบบ (pattern) ต่างๆของเมทิเลชันของไลน์-1 และอลูในมะเร็งต่อมน้ำลายชนิดมิวโคเอ็พพิเดอร์มอยด์คาร์ซิโนมา โดยใช้วิธีการ combine bisulfite restriction analysis

จากการศึกษาพบว่ามะเร็งต่อมน้ำลายชนิดมิวโคเอ็พพิเดอร์มอยด์คาร์ซิโนมา มีระดับเมทิเลชันของไลน์-1และอลูต่ำกว่าต่อมน้ำลายปกติอย่างมีนัยสำคัญ เมื่อเปรียบเทียบระดับเมทิเลชัน ของไลน์-1 และอลูระหว่างเซลล์ที่เป็นองค์ประกอบหลักในรอยโรครวมไปถึงเซลล์ต่อมน้ำลายปกติข้างเคียง พบว่ามีความแตกต่างกันอย่างมีนัยสำคัญ โดยระดับเมทิเลชันของไลน์-1และอลูค่อยๆลดต่ำลงจากเซลล์ต่อมน้ำลายปกติที่อยู่ข้างเคียงรอยโรค, เซลล์อินเทอร์มีเดียท, เซลล์มิวคัส และเซลล์สความัส ตามลำดับ นอกจากนี้รูปแบบของไลน์-1ที่มีเมทิเลชันสูงกว่าปกติและรูปแบบของอลูที่มีเมทิเลชันต่ำกว่าปกติยังมีความแตกต่างกันในเนื้อเยื่อมะเร็งต่อมน้ำลายชนิดมิวโคเอ็พพิเดอร์มอยด์คาร์ซิโนมาและเนื้อเยื่อต่อมน้ำลายปกติ นอกจากนี้ยังพบว่าระดับเมทิเลชันของไลน์-1มีความสัมพันธ์กับระดับความรุนแรงของโรค ดังนั้นสัดส่วนของเซลล์ที่เป็นองค์ประกอบหลักในรอยโรค อาจเป็นเกณฑ์ที่สำคัญประการหนึ่งในการใช้จำแนกระดับความรุนแรงของโรค ระดับและรูปแบบของเมทิเลชันของไลน์-1และอลูน่าจะมีบทบาทในการวินิจฉัยและการพยากรณ์โรคให้มีความถูกต้องและแม่นยำยิ่งขึ้น ซึ่งจะช่วยเพิ่มอัตราการรอดชีวิตและเพิ่มคุณภาพชีวิตของผู้ป่วยได้

ภาควิชา ศัลยศาสตร์ ลายมือชื่อนิสิต

สาขาวิชา ศัลยศาสตร์ช่องปากและแม็กซิลโลเฟเชียล ลายมือชื่อ อ.ที่ปรึกษาวิทยานิพนธ์หลัก

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5276117032 : MAJOR ORAL AND MAXILLOFACIAL SURGERY
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PORNTIPA SIRIVANICHSUNTORN : STUDY OF LINE-1 METHYLATION LEVELS IN SALIVARY MUCOEPIDERMOID CARCINOMA. ADVISOR : KESKANYA SUBBALEKHA, D.D.S., Ph.D., CO-ADVISOR : PROF. APIWAT MUTIRANGURA, M.D., Ph.D., ASST. PROF. NAKARIN KITKUMTHORN, D.D.S., Ph.D., 82 pp.

Mucoepidermoid carcinoma (MEC), malignancy of salivary gland, can be classified into low-, intermediate-, and high-grade tumors based on its histological features. The proportion of cellular components or the presence of a cystic component, neural invasion, necrosis, mitotic activity and anaplasia are used. However, none of the system has not been universally accepted. Methylation levels of long interspersed nuclear element-1 (LINE-1) and Alu elements reduce in cancerous tissue and relate to the severity of some cancers. However, there has been no study of LINE-1 and Alu methylation in MEC tissues. This study investigated LINE-1 and Alu element methylation in MEC by using combined bisulfite restriction analysis.

MEC tissue showed a significantly lower level of LINE-1 and Alu element methylation overall compared to the normal salivary gland tissue. These levels were also significantly different between cell types and showed a stepwise decrease from the adjacent normal salivary gland to the intermediate, mucous and squamous cells. In addition, the percentage of hypermethylation pattern of LINE-1 and hypomethylation pattern of Alu were significantly difference between MEC and normal salivary gland tissue. Moreover, the reduced methylation levels of LINE-1 were correlated with poorer histological grades.

The results suggested that the proportion of cells may be important for MEC classification. LINE-1 and Alu elememt methylation levels and patterns may be useful for accurate MEC diagnosis and prognostic prediction, which can improve survival rates and quality of life of patients.

Department : Oral and Maxillofacial Surgery	Student's Signature .....
Field of Study : Oral and Maxillofacial Surgery	Advisor's Signature .....
Academic Year : 2011	Co-advisor's Signature .....

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LIST OF ABBREVIATIONS

MEC	Mucoepidermoid carcinoma
COBRA	combined bisulfite restriction analysis
LINE-1	long intersperse nuclear element-1
COBRALINE-1	COBRA of genome-wide LINE-1
ANOVA	analysis of variance
DNA	deoxyribonucleic acid
CpG	dinucleotide containing cytosine and guanine

CHAPTER I

INTRODUCTION

Background and Rationale

Mucoepidermoid carcinoma (MEC) is the most common malignant neoplasm of all carcinomas developing in the major and minor salivary glands. This tumor was first recognized by Masson and Berger in 1924. Since its first description, several studies have attempted to identify the MEC well-known variable biological behavior. This neoplasm can be highly aggressive; however, it shows sometimes a slow growth resembling a benign lesion. Histologically, MEC compose of variable cell types including squamous, mucous and intermediate cells that can be arranged in solid nests or cystic structures.

Although there is an advance in medical technology at the present time, the treatments of the progressive stage of MEC are still complicated and leave distressed disabilities. The 5-year survival rate of high-grade MEC is only 27.8-67% while that of low-grade MEC is 85-100% (1-3). Several factors relating to biological behavior and treatment outcome of MEC include anatomical site, clinical stage, invasion of adjacent structures, presence of distant metastasis and also the grading of malignancy. At present, the classification of MEC is varied, mostly depends on histological features. Some of the systems place an important on the proportion of cell types (4, 5) while others ignore cell types (6-9). However, none of the system has been universally accepted (10). Moreover, using different criteria gave various results in MEC grading (10).

Nowadays, it is accepted that epigenetic is one of the important features of development and possibly related to many diseases especially cancers. The epigenetic alteration, such as DNA methylation and histone acetylation, are considered to be important for human carcinogenesis. Unfortunately, there are few studies reported about

genetic and epigenetic alteration in MECs. Alteration in the p53 tumor suppressor gene does not seem to play a critical role in the course of tumorigenesis in adenoid cystic carcinomas and MEC (11). Besides, inactivation of the p16INK4a gene by homozygous deletion or gene methylation is probably the most common molecular event in MEC, and might be crucial for carcinogenesis in salivary glands (12).

In recent years, DNA methylation has become intensive investigation of epigenetic event in cancers. Many kinds of cancer were reported about the global hypomethylation. Surprisingly, both global hypomethylation and local hypermethylation of certain genes were found in the same kind of cancer such as hepatocellular carcinoma and urothelial carcinoma and local hypermethylation of certain genes (13-15). Methylation in promoter can inactivate tumor suppressor genes (16). DNA hypomethylation can trigger genomic instability and bring progression to malignant transformation (17).

Long interspersed nuclear element-1s (LINE-1s) are highly repetitive mobile DNA sequences which distribute randomly across the entire genome (18). They possess many methylated CpG dinucleotides. Recent study demonstrated that LINE-1 hypomethylation related to genome hypomethylation (19-21). Hypomethylation of LINE-1s has been reported in several malignancies, including oral cancer (14, 15, 20, 22). Also, hypomethylation levels of LINE-1s can be used as a prognostic marker for epithelial ovarian cancers (23), hepatocellular carcinoma (14) and cervical cancers (24). This data implies that methylation levels may be important for cellular function.

According to the difference of survival rates between high-grade and low-grade MEC, it may assume that histological grading influences on the prognosis of this tumours. Although many systems that using various criteria for MEC classification were proposed, none of the system has been universally accepted. Nowadays, the knowledge to clarify the carcinogenesis of MEC is still needed. Also, less data revealed

the genetic and epigenetic event that might be related to the cause of this disease. In addition, there is no study verifying LINE-1 methylation levels in MEC. Thus, the goal of this study was to investigate LINE-1 methylation in MECs by comparing with normal salivary gland. Moreover, the relationship with histological grading in MEC was studied in order to provide data for a better understanding biological behavior of this tumor. Furthermore, the distinction of LINE-1 methylation levels of different cell types in MEC and also the adjacent normal salivary gland cell was analyzed.

Research questions

1. Does MEC of salivary glands possess genome-wide LINE-1 hypomethylation?

2. Whether the genome-wide LINE-1 hypomethylation correlates with clinico-pathological feature of MECs.

3. Do methylation levels of LINE-1 differ among squamous cells, mucous cells, intermediate cells and adjacent normal salivary gland cells?

4. Does LINE-1 methylation level of squamous cells, mucous cells, intermediate cells and adjacent normal salivary gland cells correlate with clinico-pathological character of MECs?

Objectives

1. To investigate genome-wide LINE-1 methylation levels in normal salivary gland and MECs.

2. To explore relationship of genome-wide LINE-1 methylation levels in various clinico-pathological features of MECs.

3. To investigate genome-wide LINE-1 methylation levels in squamous cells, mucous cells and intermediate cells collected from microdissected tissue of MECs.

4. To clarify the association between methylation level of LINE-1s in squamous cells, mucous cells and intermediate cells with pathologic grading of MECs.

Hypothesis

1. MEC of salivary glands possesses different levels of LINE-1 methylation from normal salivary gland.

2. Genome-wide LINE-1 hypomethylation in MECs correlates with the clinico-pathological characters.

3. LINE-1 methylation levels are different among cell types of MEC, including squamous cells, mucous cells and intermediate cells.

4. There are some association between methylation level of LINE-1s in various cell types with pathological grading of MECs.

Keywords

Mucoepidermoid carcinoma (MECs), methylation, LINE-1s, squamous cell, mucous cell, intermediate cell

Types of Research

Analytical cross-sectional research, Translational research

Expected Benefits

The relationship of LINE-1 methylation and clinico-pathological characters of MECs may be used as an additional tool for MECs diagnosis. Moreover, the better understanding of the molecular pothogenesis of MECs may leads to more accurate diagnosis and effective treatment of MECs.

Remark

While doing this dissertation, many literatures mentioned about the methylation of Alu in various type of cancers. Thus, it was interesting to study methylation of Alu together with LINE-1. Therefore, this research was included an investigation of Alu methytion levels and patterns as a supplementary study.

CHAPTER II

LITERATURE REVIEW

Salivary Gland Cancers and Mucoepidermoid Carcinoma

Mucoepidermoid carcinoma (MEC) is a common malignant tumour in salivary glands. According to WHO classification, MEC is graded as low-grade type (or well differentiated) or high-grade type (poorly differentiated). Between these two well defined types, there may exist a third group of intermediate-grade type (moderately differentiated), which has mixed features of high- and low-grade. The histopathological degree of malignancy and prognosis appear to correlate with anatomical site, clinical stage, invasion of adjacent structures, presence of distant metastasis and histologic grading of malignancy (25). As a whole, MEC is found in major and minor salivary glands without predominance (26). Most MECs occur in persons of 40–60 years old, but occasionally children are affected. MEC, especially a high-grade type is aggressive. It can invade adjacent structures, metastase to distant organs and cause morbidity and mortality (25).

Epidemiology

Malignant neoplasms of the human major salivary are uncommon (27). The annual incidence rates in the world vary between slightly less than 2 and greater than 0.05 per 100,000 (Fig. 1) (28). Incidence of malignant salivary gland tumor at older age is higher than in youth , men are predominance (Fig. 2) (28). Recently in the US, during 1974–1999, a significant increase in the incidence rate of salivary gland cancer was reported (29). In Europe, survival rate of salivary gland cancer was studied from population-based cancer registries by the EUROCARE project. Relative survival rate for adults diagnosed with salivary gland cancer was 83% at 1 year, 69% at 3 years, and

65% at 5 years, with a significant difference between men and women, 58 and 72%, respectively. Five-year relative survival rate decreased markedly with age from 87% to 59% from the youngest (15–45 years) to the oldest age group of patients (75 years and over) (30). For MEC, a 5-year survival rate are 85-100% and 27.8-67% in low-grade and high-grade, accordingly (1-3).

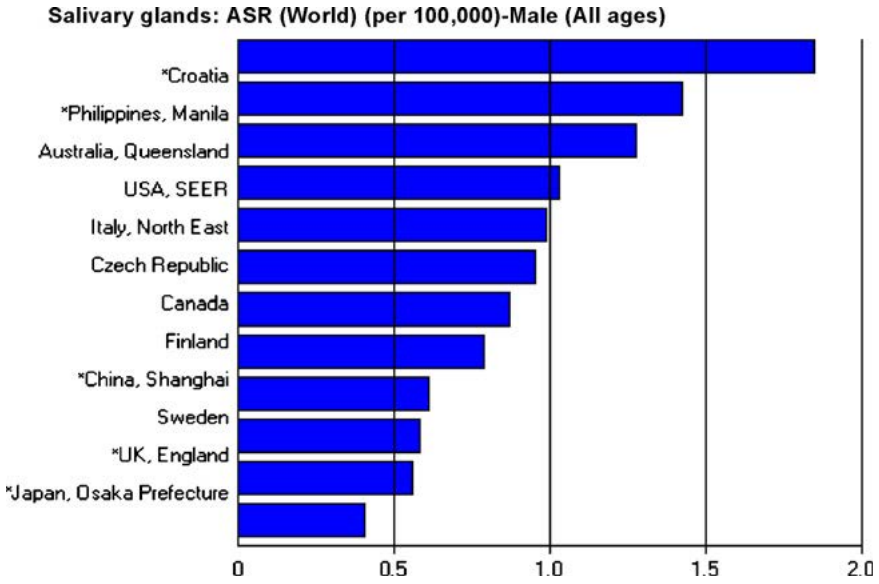

Figure 1 Annual incidence rates of salivary glands cancer in the world (28).

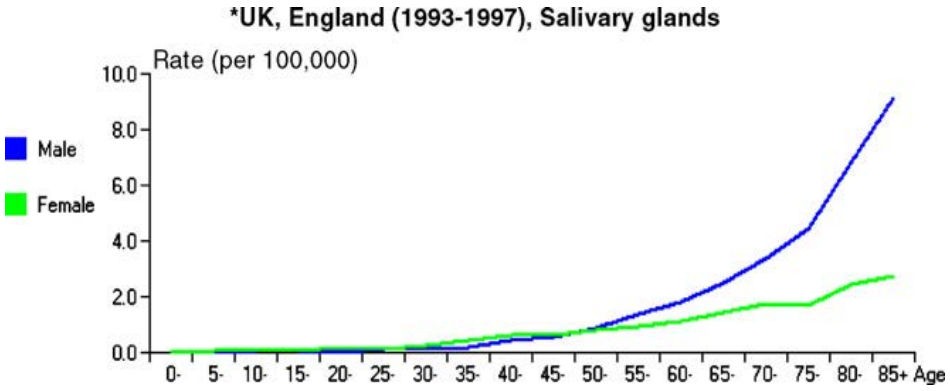

Figure 2 Incidence rate per age of salivary gland cancer (28).

Etiology and risk factor

The causes of salivary gland cancer are largely unknown. Diet may be effective in preventing salivary gland cancer, by increasing consumption of fruits and vegetables, particularly those high in vitamin C, and limiting food high in cholesterol (29). A case–control study conducted in the Chinese population revealed a significant protective effect of consumption of dark-yellow vegetables or liver, with about 70% reduced risk of salivary gland cancer among people in the highest intake group of these foods (31). Irradiation may also be a cause of malignant salivary gland tumors. This was found in Japanese survivors of the atomic bomb and in patients who received irradiation to the head and neck during childhood for benign conditions e.g. to reduce the size of the tonsils and adenoids (32). The decline in incidence under age 70 in England and Wales is consistent with the reduction of repeated ionizing radiation exposure to medical or dental X-rays (33). A history of prior cancers, especially those related with ultraviolet radiation, immunosuppression and Epstein-Barr virus, was found to be associated with salivary gland cancers in several studies. Among more than 5000 Swedish patients with Hodgkin's disease, there was an over 4-fold significant increase in cancer of the salivary glands (34). A US and Swedish study revealed an increased risk of second cancer, including salivary gland tumors in more than 1000 children with a diagnosis of medulloblastoma (35). On a total of about 70,000 Finnish patients with basal-cell carcinoma, the incidence rate to have a subsequent salivary gland carcinoma was 3.3-fold higher than in the general population (36). Patients with a histologically benign tumor (e.g. pleomorphic adenoma) which occurs at a young age, have a higher risk of developing a malignant parotid carcinoma, since these tumors have the potential for malignant transformation (3–10%)(37). In a large cohort of Southern European men with, or at high risk of, HIV infection, a very high risk to have a cancer of salivary glands was found (38). The workers in a variety of industries showed an increased incidence of

salivary gland carcinoma including rubber manufacturing, exposure to nickel compound (39) and employment at hair dresser's and beauty shops (40). Chronic inflammation of salivary glands is not clearly defined as a risk factor. Currently, no specific study devoted to etiology of MEC. Among survivors of the Hiroshima and Nagasaki atomic bomb explosion in 1945, the incidence of MEC was increased to 44% (41). In addition, MEC has been reported after radiation therapy for thyroid carcinoma or leukemia (42). It may suggest that prior exposure to ionizing radiation is a contributing factor for MEC.

Clinicopathological features

The prognosis for patients with MEC depends on both histological subtype (grade) and clinical extent (43) of tumor. MEC have been categorized into one of three histopathologic grades based on amount of cyst formation, degree of cytologic atypia and relative number of squamous, mucous and intermediate cells. Three histological grading of MEC cells are low-, intermediate-, and high-grade. Low-grade tumors show prominent cyst formation, minimal cellular atypia, and a relative high proportion of mucous cells. High-grade tumors consist of solid island of squamous and intermediate cells, which can demonstrate pleomorphism and mitotic activity. Mucous cells may be infrequent, and the tumor sometimes can be difficult to distinguish from squamous cell carcinoma. Intermediate-grade tumors show features that fall between low-grade and high-grade neoplasms. Low-grade tumors present less cystic formation, all three major cell types are present, but the intermediate cells usually predominate (44). The clinical staging of MECs is known as the TNM system (table 1) (45). T is a measure of the primary tumor size, N is an estimation of the regional lymph node metastasis, and M is a determination of distant metastases. As the clinical stage advances from I to IV, prognosis worsens.

Stage I	Stage II	Stage III	Stage IVA	Stage IVB	Stage IVC
T1N0M0	T2N0M0	T3N0M0	T1N2M0	T4bAnyNM0	AnyTAnyNM1
		T1N1M0	T2N2M0	AnyTN3M0	
		T2N1M0	T3N2M0		
		T3N1M0	T4aN0M0		
			T4aN1M0		
			T4aN2M0		

Table 1 : TNM staging system for MEC (45)

Description and abbreviations

• Primary tumor (T)

TX Primary tumor cannot be assessed

T0 No evidence of primary tumor

T1 Tumor 2 cm or less in greatest dimension without extraparenchymal extension*

T2 Tumor more than 2 cm but not more than 4 cm in greatest dimension without extraparenchymal extension*

T3 Tumor more than 4 cm and/or tumor with extraparenchymal extension*

T4a Tumor invades skin, mandible, ear canal, or facial nerve

T4b Tumor invades base of skull pterygoid plates or encases carotid artery

Note: (*) Extraparenchymal extension is clinical or macroscopic evidence of invasion of soft tissue or nerve, except those listed under T4a and T4b. Microscopic evidence alone does not constitute extraparenchymal extension for classification purposes.

• **Regional lymph nodes (N)**

NX Regional lymph nodes cannot be assessed

N0 No regional lymph node metastasis

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

N2 Metastasis as specified in N2a, 2b, 2c below

N2a Metastasis in a single ipsilateral lymph node, more than 3 cm but not more than 6 cm in greatest dimension

N2b Metastases in multiple ipsilateral lymph nodes, none more than 6 cm in greatest dimension

N2c Metastases in 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

Note: Midline nodes are considered ipsilateral nodes.

• **Distant metastases (M)**

MX Distant metastases cannot be assessed

M0 No distant metastases

M1 Distant metastases

Detection and diagnosis

Screening and case finding

Malignant salivary gland tumors are rare; therefore, no screening program has been developed. Screening for malignant salivary gland neoplasm including MEC is not recommended and clinical case finding has not been evaluated (46).

Signs and symptoms

The most common site of MEC is the parotid glands. The minor glands constitute the second most common site, especially the palate. The tumor occurs evenly over a wide age range, extending from second to seventh decades. Although it is rarely seen in the first decade, MEC is the most common malignant salivary gland tumor in children. The clinical manifestations of this lesion depend greatly on the grade of malignancy. Low-grade tumors often present with a prolonged period of painless enlargement. Within the oral cavity, MEC frequently resembles an extravasation or retention-type mucocele that sometimes may be fluctuant and have a blue or red color as a result of mucous cyst formation. High-grade MEC regularly grow rapidly and are often accompanied by pain and mucosal ulceration. Within the major salivary glands, the tumors usually appear as well-define focal nodule. In case of the lesions occur in the parotid glands, the superficial lobe is the most frequently targeted. They may be movable, which is an uncommon feature for a malignant lesion. High-grade tumors may present with evidence of facial nerve involvement or obstructive signs. Intraosseous tumors also may develop in the jaws which appear as radiolucent lesion in the maxilla or mandible (44, 47, 48)

Treatment

The treatment of MEC is predicated by the location, histopathological grade, and clinical stage of the tumor. Early-stage tumors of the parotid can be treated by subtotal parotidectomy with preservation of the facial nerve. Advanced tumors may necessitate total removal of the parotid gland, which sacrifice of the facial nerve. Submandibular gland tumors are treated by total removal of the gland. MEC of the minor glands usually is treated by assured surgical excision. For low-grade tumors, only a modest margin of surrounding normal tissue may need to be removed, but high-grade or large tumors warrant wider resection, similar to that required for squamous cell carcinomas. If there is underlying bone destruction, then the involved bone must be excised. Radical neck dissection is indicated for patients with clinical evidence of metastatic disease and also maybe considered for patients with larger or high-grade tumors. Post operative radiation therapy also may be used for more aggressive tumors.

The prognosis depends on the grade and stage of the tumor. Patient with low-grade tumor generally have a good prognosis. For most primary sites, local recurrences or regional metastasis are uncommon, and around 90% to 98% of patients are cured. The prognosis for those with intermediate-grade tumors is slightly worse than that for low-grade tumors. The outlook for patients with high-grade tumors is guarded, with only 30% to 54% of patients surviving. For unknown reasons, submandibular gland tumors are associated with a poorer outlook than those in the parotid gland. MEC of the minor glands generally have a good prognosis, probably because they are mostly low- to intermediate- grade tumors. However, tumors of the tongue and floor of the mouth are less predictable and may exhibit more aggressive behavior (44).

Genetics and epigenetics in cancers

Genetic alterations are the changes of DNA sequences which can occur in several ways such as insertion, deletion, recombination and amplification in any part of genes. For instance, transformation of proto-oncogenes to oncogenes that leads to abnormal cell growth, proliferation and differentiation in carcinogenesis. Moreover, alterations of tumor suppressor genes are also related to cancer development.

However, the transmutation from normal cells to cancerous or precancerous cells can occur without any changes in DNA sequence. This DNA modification, called the epigenetic alteration, does not involved in DNA sequence changes. The epigenetic modifications, including DNA methylation, histone deacetylation and RNA interference, naturally occur in normal human cells and influence on gene expression. Any abnormality in epigenetic alterations can affect normal gene expression and cause pathological change. Lately, it is believed that both genetic and epigenetic changes are important to the development of many diseases including cancers.

DNA methylation

DNA methylation is the addition of methyl group (CH_3) from the mythyl donor, S-adenosylmethionine (SAM), to the 5' carbon of pyrimidine ring in cytosine by enzyme DNA methyltransferase (DNMT). The products of this reaction are 5-methylcytosine (5-MeC) and S-adenolylhomocysteine (SAH) as shown in figure 3. The addition of methyl group mostly occur in "CpG dinucleotide" where cytosine (C) leads guanine (G).

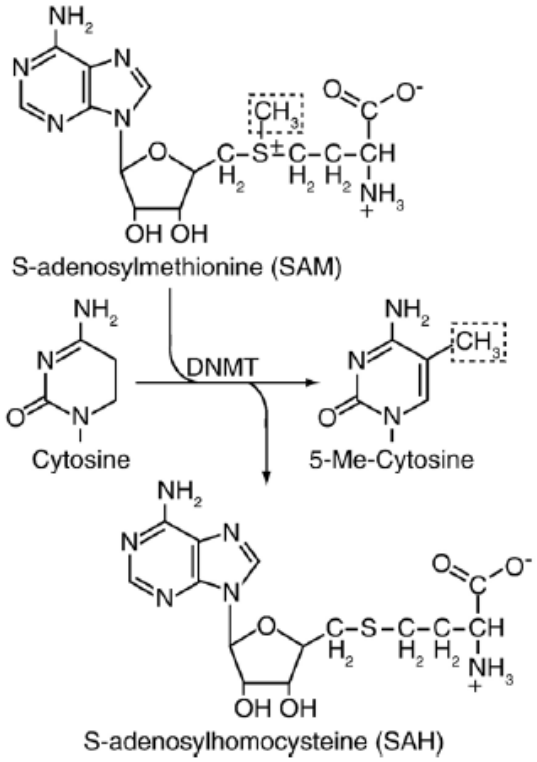

Figure 3 The enzymatic DNA methylation reaction. Methyl group (CH_3) is transfer from S- adenosylmethionine (SAM) to 5' carbon of cytosine by DNA methyltransferase (DNMT).

The DNA methyltransferase of mammalian catagorized into 3 types including DNMT1, DNMTa and DNMTb by the methylation status of the substrate in enzymatic reaction. The maintenance DNA methylation occurs by binding of DNMT1 with hemimethylated CpG dinucleotides while De Novo DNA methylation cause by binding of DNMTa and DNMTb with unmethylated CpG dinucleotides as shown in figure 4.

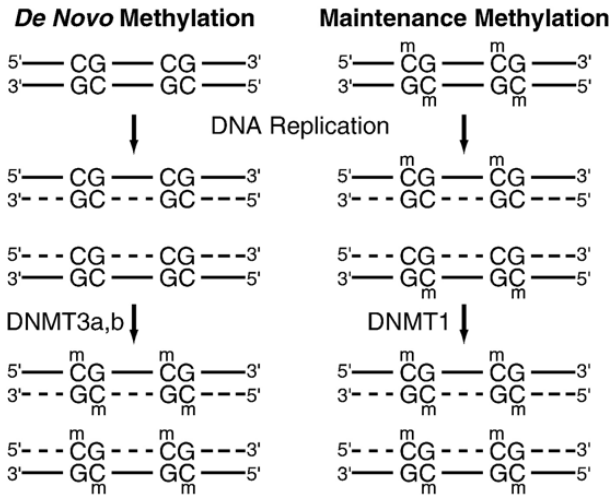

Figure 4 Maintenance DNA methylation and De Novo DNA methylation. The two patterns of DNA methylation in mammalian including maintenance DNA methylation of hemimethylated CpG by DNMT1 and De Novo DNA methylation of unmethylated CpG by DNMTa and DNMTb.

DNA methylation in cancers

Aberrant DNA methylation, including promoter hypermethylation of tumor suppressor genes and genome-wide (global) hypomethylation has been reported in many kinds of cancers including head and neck cancers (49). DNA methylation is an evolutionally conserved mechanism to regulate gene expression in mammals. In vertebrates, addition of methyl group at 5-carbon position of cytosine (50) generally occurs at the 5'cytosine in CpG dinucleotides (51, 52) (Figure 3). It has shown to be associated with transcriptional silencing of the genes in normal development (53). Explicit DNA methylation is maintained by heritability after DNA replication (54, 55). Distinct DNA methylation patterns are specific to developmentally and tissue, both in overall 5-methylcytosine content and in the sites at specific genes (56-59). Cytosine methylation has many functions, including X-chromosome inactivation, genomic

imprinting, immobilization of mammalian transposons, suppression of transcriptional noise and maintaining genomic stability (60-64).

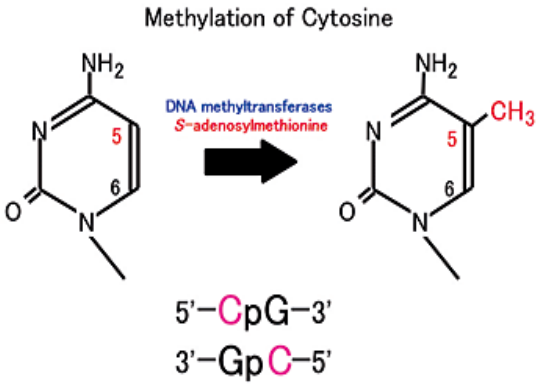

Figure 5 Methyl cytosine. Addition of methyl group at 5-carbon position of cytosine usually occurs at the 5'cytosine in CpG dinucleotides (65)

Many studies proved that epigenetic alterations involving an imbalance in cytosine methylation are found in cancers. It is well established that two kinds of changes in the DNA methylation pattern occur in many cancers, regional hypermethylation of specific genes and global hypomethylation. These imbalances can occur together in a single tumor, though the net effect is typically a decrease in total methylation levels (66-68). This paradoxical coexistence of a global decrease in methylation with regional hypermethylation suggests that independent and different processes are responsible for hypomethylation and hypermethylation. If these defects precede malignancy, signifying that they are not simply a consequence of the malignant state. In case of methylation imbalance contributes directly to tumor initiation, the alteration should occur in early stages of cancer or in premalignant cells. If it contributes directly to tumor advancement, methylation defects should increase in frequency and/or severity coordinately with advancing malignancy grades

While hypermethylation makes tumor suppressor genes inactive, global losses of methylation in cancer may lead to the alterations in the expression of proto-oncogenes critical to carcinogenesis (53, 69). It may also expedite chromosomal instability (70-72) and may activate the latent retrotransposons (15, 73-76). The extent of genome wide hypomethylation in tumors parallels closely to the degree of malignancy, though it is tumor type dependent. In breast, ovarian, cervical, brain and prostate tumors, for instance, hypomethylation increases progressively with advancing malignancy grade (67, 77, 78). Therefore hypomethylation may contributes as a biological marker with prognostic value. The human genome is not methylated uniformly and contains regions of unmethylated segments interspersed by methylated regions (79). Genome-wide hypomethylation has been demonstrated by downregulation of methylated CpG dinucleotides, which disperse throughout the whole genomes both in noncoding repetitive sequences and genes. Nevertheless, hypomethylation of the repetitive sequences, such as LINE seems to compose the major part of the global hypomethylation of the cancer genome (21, 80).

LINE-1 retrotransposons

DNA transposons and retrotransposons constitute mammalian transposable elements. DNA transposons encode a transposase activity and typically move through DNA intermediate by a cut and paste mechanism utilizing the transposase. Although roughly 3% of the human genome is consisted of DNA transposons, they are remnants or fossils of ancient elements and it is improbable that any remain transpositionally active. Retrotransposons encode a reverse transcriptase activity and move by a copy and paste process involving RNA intermediate thus the original retrotransposon is maintained *in situ* where it is transcribed. The transcript is then reverse transcribed and integrated into a new genomic location. Approximately 42% of the human genome consists of retrotransposons and even most of these elements are inactive, some withhold the ability of retrotransposition. Retrotransposable elements can be classified as autonomous retrotransposons when they encode certain proteins necessary for their mobility and nonautonomous retrotransposons such as Alu, processed pseudogenes and SVA elements which do not encode any protein. There are two classes of autonomous retrotransposons, LTR (long terminal repeat) and non-LTR retrotransposons. LINEs (long interspersed nucleotide elements) are non-LTR retrotransposons and make up 21% of the human genome. There are both inactive LINE elements such as LINE-2 and active LINE elements, such as LINE-1 (18).

LINE-1 retrotransposons, the most plenteous sequences in human genomes are self-replicating human transposable elements. Over evolutionary time, they have not only expanded greatly in number but also have other roles. Some of which are quite useful to the organisms while others are unfavorable detrimental to individual members of the species. They are approximated 600,000 copies and comprise of at least 17% of the human genomes. Some of these elements are within genes (18). Over 75% of human genes contain at least one LINE-1 insertion, usually as part of introns, 5′UTR sequences

or 3′UTR sequences (81). Most LINE-1 elements are retrotransposition defective because they are 5′ truncated; contain internal rearrangement and harbor mutations within their open reading frames (70). Full-length LINE-1s are about 2,000 copies, but only 30-60 copies may be capable for transposition (66, 82). When the full length, non-mutated LINE-1 is transcribed and then reverse transcribed, it might integrate in and disrupt important gene functions (72, 81). Germline mutations where LINE-1 retrotranspositions weaken the functional gene are known in several hereditary disorders, including the factor VIII in hemophilia A, the dystrophin gene in Duchenne muscular dystrophy, the fukutin gene in Fukuyama-type congenital muscular dystrophy, the cytochorme b_{558} heavy chain gene in X-linked chronic granulomatous disease, and the type IV collagen genes in Alpert's syndrome (83). DNA methylation at the CpG site in LINE-1 promoter is the normal mechanism for silencing of its possibly harmful retrotransposing activity in the mammalian genome (84, 85). Furthermore, hypomethylation of LINE-1 promoter can cause genome instability by inactivating the tumor suppressor genes such as *APC* in colon cancer or by activating the oncogenes such as *c-MYC* in breast cancer (86, 87). And there is also evidenced that hypomethylation of LINE-1 can cause chromosome instability (88, 89). Hypomethylation of LINE-1s has been reported in several malignancies, including neuroendocrine tumors (90), carcinoma of the breast, lung, liver, esophagus, stomach, colon, urinary bladder prostate, and head and neck (13, 15, 20, 49, 72, 90-93). Furthermore, hypomethylation levels of LINE-1s can be used as a prognostic marker for epithelial ovarian cancers (23), cervical cancers (24) and hepatocellular carcinoma (14). Full length LINE-1 is 6 kb and contains a 5′ untranslated region (5′UTR), a 1 kb ORF1 that encodes a nucleic acid binding protein, a 4 kb ORF2 which encodes a protein with endonuclease and reverse transcriptase activities, allowing their mobilization in genomes through an RNA intermediate, a 3′ untranslated region (3′UTR)cc, a poly(A) tail (Figure 5). Within LINE-1

5′UTR, they contain a sense strand promoter for their own transcription, as well as antisense promoter (ASP) (94). This ASP has been shown to provide an alternative transcription start site for a number of human genes including *c-MET*, a receptor tyrosine kinase whose activation can lead to transformation and tumorigenicity in a variety of tumors (95-97). Since LINE-1 elements are constituted most of the human genome and distributed across the entire genome, LINE-1 sequences are suitable for to study changes in genome methylation.

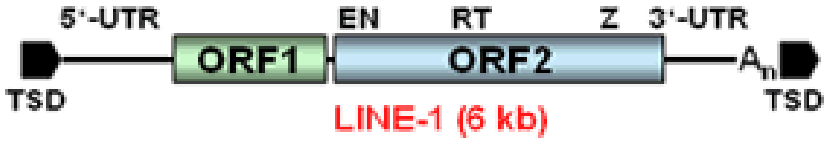

Figure 6 Full-length LINE-1 element. Full length LINE-1 is 6 kb and contains a 1) 5′ untranslated region (5'UTR), the promoter of RNA polymerase II is in this 2) 2 open reading fram (ORF1and ORF2) and 3) 3' UTR (18)

Some tissues such as thyroid and esophagus demonstrated wider ranges of the methylation levels than others (20). Furthermore, most of cancer tissues including head and neck cancers had hypomethylation of LINE-1s, comparing with their normal tissue counterparts except cancers of kidney, thyroid and lymph node (20) (Figure 7). This evidence supports that LINE-1 methylation level is specific to tissue types and the hypomethylation of LINE-1s is also specific to types of cancers. Nevertheless, the information of LINE-1 methylation in malignant salivary gland tumors including MECs have not been described. In order to study methylation levels of LINE-1s in MECs, the same type of tissues should be compared. Hence, normal tissue of salivary glands should be used.

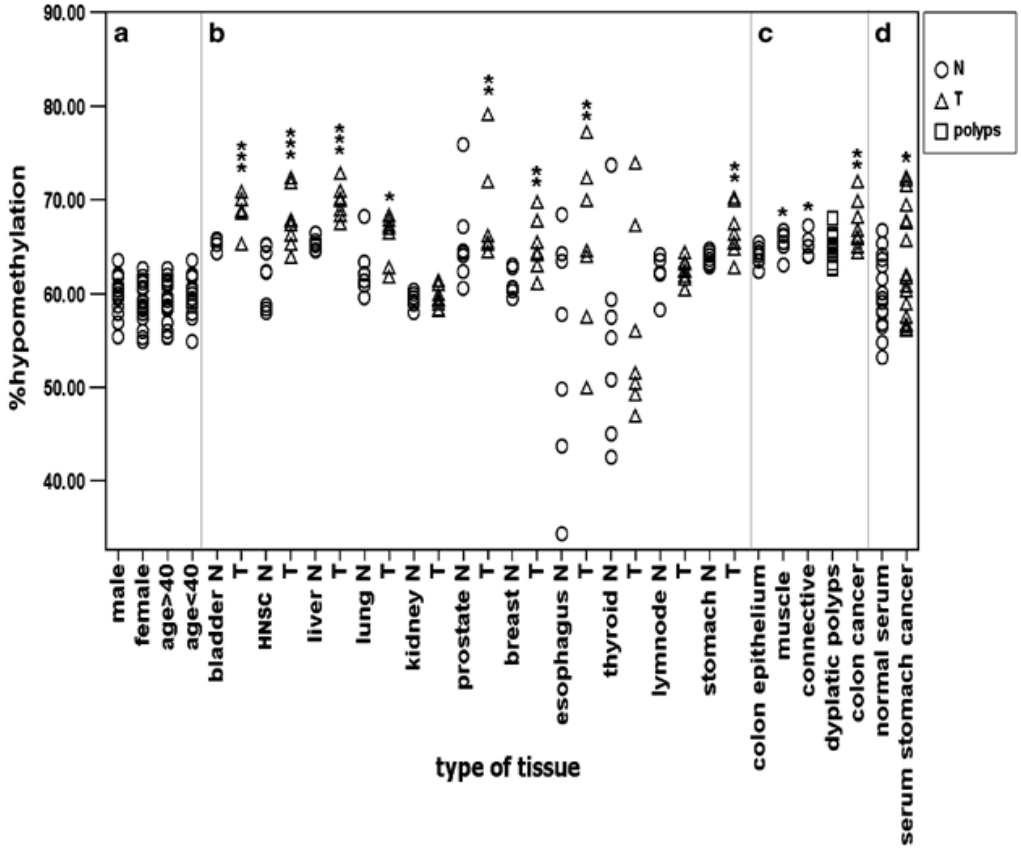

Figure 7 LINE-1 hypomethylation levels in several tissue types. Circles, triangles, and squares are levels of COBRA LINE-1 from normal, malignant, and premalignant tissues, respectively. The vertical axis displays percentage levels of LINE-1 methylation. Sample types are labeled. (a–d) are the hypomethylation levels of leukocytes, cancers, microdissected colonic tissues, and sera, respectively. Single, double, and triple asterisks indicate significant differences in hypomethylation levels between normal tissues and the tested samples at $P<0.05$, <0.01, and <0.001, respectively. HNSC stands for head and neck squamous cell. N and T are normal and malignant tissues, respectively

In previous study, combined bisulfite restriction analysis of LINE-1s (COBRALINE-1) could efficiently evaluate methylation status of genome-wide LINE-1s in genomic DNA and it also represented the whole genome methylation status (97, 98). In some event, owing to the combination of various mechanism, the measurement of level alone may not enough to detect LINE-1 methylation change. Recently, some approach can obtain the quantitative data of genomic DNA methylation such as pyrosequencing and COBRA. However, only COBRA can display the pattern information by distinguishing LINE-1 loci depending on their methylation status. By using the 2 CpG dinucleotide in each LINE-1 sequence, this technique can differentiate LINE-1 sequence in to 4 methylation status categories: hypermathelated ($^{m}C^{m}C$), hypomethylated ($^{u}C^{u}C$) and 2 forms of partial methylated loci ($^{m}C^{u}C$ and $^{u}C^{m}C$) as shown in figure 8.

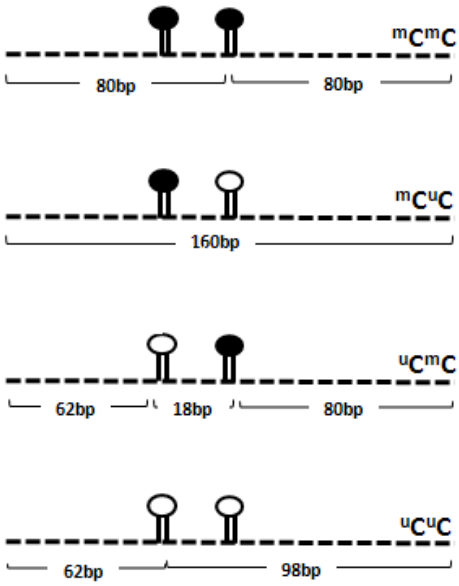

Figure 8 Diagram illustrated four methylation status categories of LINE-1. The dark circles indicated methylated CpG while the hollow circles indicated unmethylated CpG (99).

Not only LINE-1 but also Alu were suitable for represent genome-wide methylation levels (100). Alu, the most abundant short interspersed nuclear element (SINE), is another kind of repetitive sequence which comprises more than 10% of the mass of the human genome. Full-length Alu elements are ~300 bp long and are commonly found in introns, 3' untranslated regions of genes and intergenic genomic regions (Figure 9). A detailed analysis indicated that these mobile elements were present in the human genome more than one million copies which was an extremely high copy number. This makes them the most abundant of all mobile elements in the human genome (70). Because of their high copy number, methylation levels of Alu elements can also represent genome-wide methylation levels (100).

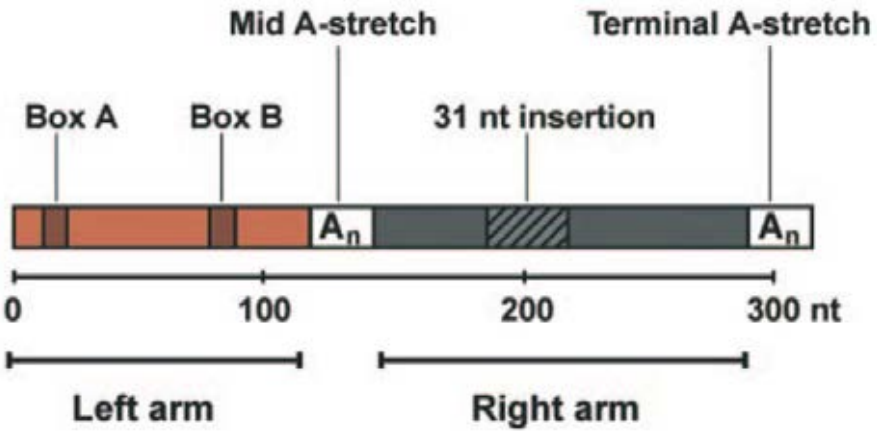

Figure 9 Architecture of Alu elements. Alu elements are about 300 nucleotides long. They have a dimeric structure composed of two related but not equivalent monomers (left and right arms). The right arm contains a 31 nt insertion as compared to the left arm. Left and right arms are separated by an A-rich region (Mid A-stretch) and followed by a short poly(A) tail (Terminal A-stretch). The left arm contains functional, but weak, A and B boxes of the RNA polymerase III internal promoter.

Resemble to LINE-1, COBRAAlu can also differentiate Alu sequence in to 4 methylation status categories: hypermathelated ($^{m}C^{m}C$), hypomethylated ($^{u}C^{u}C$) and 2 forms of partial methylated loci ($^{m}C^{u}C$ and $^{u}C^{m}C$) as shown in figure 10.

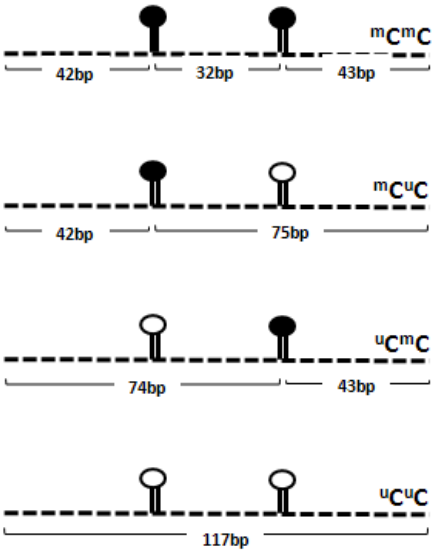

Figure 10 Diagram illustrated four methylation status categories of Alu. The dark circles indicated methylated CpG while the hollow circles indicated unmethylated CpG.

Alu element hypomethylation has also been reported in many types of cancers, such as colorectal cancer (101), gastric cancer (102) and hepatocellular carcinoma (103). Thus, we may assumed that both LINE-1 and Alu element hypomethylation plays a notable role in malignant transformation. However, the methylation of transposable elements in MEC is not clarified.

CHAPTER III

RESEARCH METHODOLOGY

Samples

Primary MEC tissues

Paraffin-embedded tissues of salivary glands from 24 MEC patients (diagnosed by histology) were obtained from

1. The Department of Pathology, Faculty of Medicine, Chulalongkorn University.
2. The Department of Pathology, Faculty of Dentistry, Chulalongkorn University.
3. The Department of Oral and Maxillofacial Pathology, Faculty of Dentistry, Mahidol University.

All MEC tissue specimens were cut into 4-μm-thick sections. After fixation, hematoxylin and eosin (H&E) staining was performed. Subsequently, the diagnosis was confirmed by two independent pathologists. These MECs were histologically graded according to the WHO diagnostic criteria. The demographic data of MEC patients was shown in table 2.

Normal salivary glands

Fourteen specimens of paraffin-embedded tissue of normal salivary glands were collected from the department of Pathology, Faculty of Medicine, Chulalongkorn University. All of these specimens were histologically confirmed to be free of tumour cell by the same pathologists. The ethical consideration of this research was approved from ethics committee, Faculty of Dentistry, Chulalongkorn University.

Microdissected MEC cell subtypes.

The squamous, mucous, intermediate cells as well as normal salivary gland cells adjacent to the MEC lesions were isolated and collected by laser microbeam

microdissection method. From 24 MEC samples, a total of 13 squamous cell samples, 16 mucous cell samples, 4 intermediate cell samples and 12 samples of adjacent normal salivary gland cells were obtained (Table 2). Approximately 1,500 cells from each specimen were used for DNA extraction to yield sufficient DNA for PCR analysis.

Sample	Sex	Age	Grade	Site	Cell type			
					N	I	M	S
MEC1	M	60	Low	Palate			√	√
MEC2	F	30	Low	Palate	√		√	
MEC3	M	35	Low	Palate		√	√	
MEC4	M	47	High	Palate	√			√
MEC5	F	38	Low	Palate	√			
MEC6	M	31	Low	Palate	√	√	√	
MEC7	F	32	Low	Palate			√	
MEC8	F	53	High	Anterior mandible				√
MEC9	M	41	Low	Palate	√		√	
MEC10	F	43	Low	Palate			√	√
MEC11	M	33	Low	Palate			√	
MEC12	F	55	Intermediate	Palate	√			√
MEC13	M	54	Low	Palate			√	√
MEC14	F	34	Intermediate	Palate				√
MEC15	M	35	Low	Palate			√	
MEC16	F	16	Intermediate	Palate	√		√	√
MEC17	F	21	Intermediate	Palate			√	√
MEC18	F	45	Intermediate	Palate		√		√
MEC19	F	51	High	Parotid gland	√			√
MEC20	M	31	Intermediate	Parotid gland	√		√	
MEC21	F	53	Intermediate	Parotid gland	√		√	√
MEC22	F	17	Low	Palate	√			
MEC23	F	41	Intermediate	Palate	√	√	√	√
MEC24	M	55	Intermediate	Palate			√	

Table 2 The demographic data of MEC patients MEC: Mucoepidermoid carcinoma, M: Male, F: Female, Low: Low-grade MEC, Intermediate: Intermediate-grade MEC, High: High-grade MEC, N: Adjacent normal salivary gland cell, I: Intermediate cell, M: Mucous cell, S: Squamous cell

Laser microbeam microdissection

Laser microbeam microdissection is a procedure for isolation of specific cells from the microscopic tissue specimens. In this study, The PALM Micro Laser Microdissection System (P.A.L.M. Micro Laser Technologies AG, Burnried, Germany) was used for collecting MEC cell subtypes including squamous, mucous, intermediate and adjacent normal salivary gland cells. The principle of the PALM system is based on a pulsed UVA laser that is focused through the microscope to allow laser ablation of cells and tissue on a tissue section.

Preparation of tissue samples

All MEC specimens were cut into 4-μm-thick sections. In each specimen, four simultaneous sections were made. All of the tissue sections were then performed hematoxylin and eosin (H&E) staining. One of the sections in each specimen was covered by cover slip while other sections were left uncovered. The histological features of uncovered sections could not be seen clearly under the microscope. Therefore, the covered sections was used as a guide map which was necessary to explicitly identify the histological characters in MEC tissues.

Laser microdissection and laser pressure catapulting

The UV laser microbeam is coupled to the epifluorescence illumination port of the microscope. A motorized controlled microscope stage is attached to the microscope (Figure 11A) and a frame grabber (Figure 11B) enables the observation of the microscopic image on a computer screen. The image is overlaid with a graphical user interface enabling the user to perform laser manipulation of tissue directly on the screen. A microfuge cap moistened with lysis buffer was mounted upside down just above the tissue section (Figure 8C). To select and isolate areas of interest, microdissection was performed by cutting with a fine focused laser beam producing a gap of 0.5 to 1.2

micron. Following isolation of cells, a high-energy pulse of the focused laser beam just below the focal plane of the tissue specimen was used to create a pressure wave separating the targeted cells and catapulting them into the microfuge cap. Approximately 1,500 cells were collected for each cell population. After that, 40 microlitres of lysis buffer was added into the microfuge tube. Then, the tube was vortexed briefly and centrifuged for 5 min to spin down the cells from the lid to later perform DNA extraction.

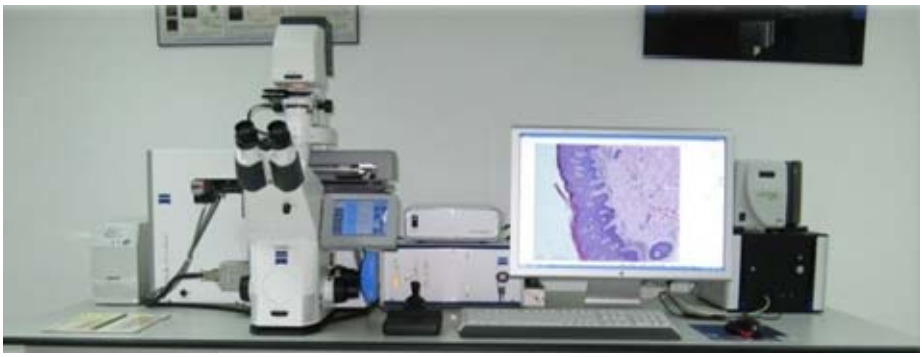

Figure 11 Laser beam microdissected machine. (A) The PALM Micro Laser Micro-dissection System (B) The frame grabber (C) The microfuge cap above the tissue section.

DNA extraction

The paraffin- embedded tissues of MEC and normal salivary gland were cut into 4-μm-thick sections then 400 μl of xylene were added for removal of paraffin. The MEC cells in xylene solution were pelleted by centrifuging at 2500g for 15 minutes at at 4°C. The supernatant was discarded and cell pellets were washed twice in ethanol. Then the cell pallets were placed in 1% SDS/proteinase K 0.5 mg/ml DNA extraction buffer and incubated at 50°C overnight. The digested pellets and fluid as well as MEC microdissected cells were subjected to phenol-chloroform extraction and ethanol precipitation. The precipitated DNA was resuspended in Tris-EDTA treated water.

Combine Bisulfite Restriction Analysis (COBRA)

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

Sodium bisulphite treatment

Principle

Bisulphite deaminates unmethylated cytosines and converts them to uracils, but leaving methylated cytosines unchanged (Figure 12,13). After bisulphite treatment, the methylated sequence can be differentiated from unmethylated sequence by further analysis, such as sequencing, methylation specific PCR, restriction enzyme analysis.

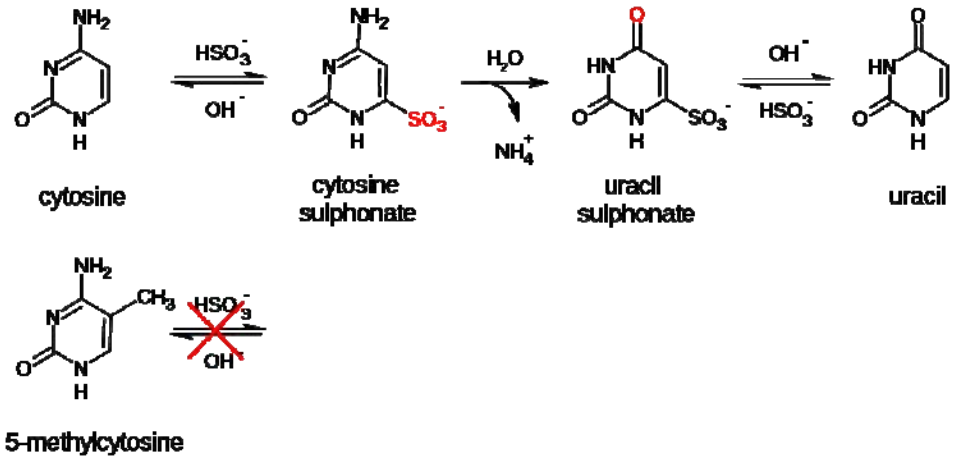

Figure 12 Sodium bisulphate conversion. The deamination of cytosine by sodium bisulphite involves the following steps: 1) addition of bisulphite to the 5-6 double bond of cytosine, 2) hydrolytic deamination of the resulting cytosine-bisulphite derivative to give a uracil-bisulphite derivative, and 3) removal of the sulphonate group by a subsequent alkaline treatment, to give uracil. This reaction was not have an affect on 5-methylcytosine (104).

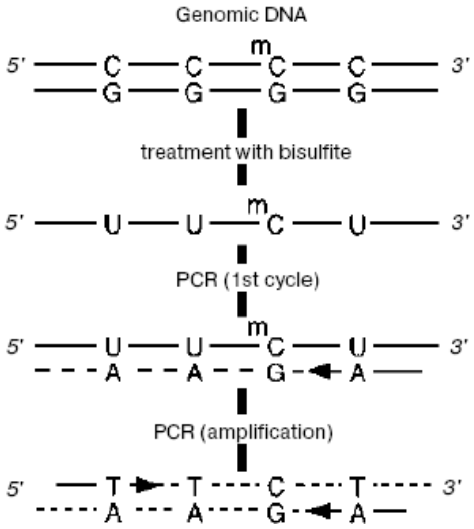

Figure 13 Complementary DNA strand after bisulfite reaction. After the bisulphite reaction, the two DNA strands are no longer complementary and therefore can be amplified independently.

Technique

Genomic DNA 2 µg in 50 µl water was denatured in 0.2 M NaOH at 37°C for 10 minutes, and then incubated with 30 µl of 10 mM hydroquinone and 520 µl of 3 M sodium bisulphite at 50°C, 16-20 hours. After that, bisulphite-treated DNA was desalted with DNA Clean-Up system (Promega, Madison, WI). Afterward, it was desulfonated by 0.3 M NaOH and precipitated with ethanol. Finally the DNA was resuspended in 20 µl of water (105).

Combine Bisulfite Restriction Analysis (COBRA) of LINE-1 and Alu element

All of the DNA samples were treated with sodium bisulfite (EZ DNA Methylation-Gold™ Kit, Zymo research). For COBRALINE-1, the bisulfate-treated DNA was subjected to 40 PCR cycles with LINE-1-F (5'-CCGTAAGGGGTTAGGGAGTTTTT-3') and LINE-1-R (5'-RTAAAACCCTCCRAACCAAATATAAA-3') primers at an annealing temperature of 50 °C. For COBRAAlu, the bisulfite-treated DNA was subjected to 40 cycles of PCR with two primers, Alu-F (5'-GGCGCGGTGGTTTACGTTTGTAA-3') and Alu-R (5'-TTAATAAAAACGAAAT TTCACCATATTAACCAAAC-3') at an annealing temperature of 53 °C. After PCR amplification, the LINE-1 amplicons (160 bp) were digested with *TaqI* and *TasI* in NEB buffer 3 (New England Biolabs, Ontario, Canada), while the Alu amplicons (117 bp) were digested with *TaqI* in *TaqI* buffer (MBI Fermentas, Burlington, Canada). Both digestion reactions were incubated at 65 °C overnight. The LINE-1 and Alu element digested products were then electrophoresed on an 8% non-denaturing polyacrylamide gel and stained with the SYBR green nucleic acid gel stain (Gelstar, Lonza, USA). Distilled water was used as negative control. All experiments were performed in duplicate.

LINE-1 methylation analysis

The intensities of the COBRALINE-1 fragments on the polyacrylamide gel were quantified using a Phosphoimager and the ImageQuant Software (Molecular-Dynamics, GE Healthcare, Slough, UK). COBRALINE-1 generated 4 products depending on the methylation state of the 2 CpG dinucleotides, as follows: partial methylation ($^{m}C^{u}C$, 160 bp), hypomethylation ($^{u}C^{u}C$, 98 bp), 1 methylated CpG (^{m}C, 80 bp) and 1 unmethylated CpG (^{u}C, 62 bp) (Figure 14A). LINE-1 methylation levels and patterns were calculated to determine the precise percentage of methylated CpG dinucleotides. The percentage was calculated as follows. First, the intensity of each band was divided by the length (bp) of the double-stranded DNA: %160/160 = A, %98/94 = B, %80/78 = C and %62/62 = D. Next, the frequency of each methylation pattern was calculated: percentage of ^{m}C = 100×(C+A)/(C+A+A+B+D), percentage of $^{m}C^{u}C$ = 100×(A)/(((C-D+B)/2)+A+D), percentage of $^{u}C^{m}C$ = 100×(D-B)/(C-D+B)/2)+A+D, percentage of hypomethylated loci ($^{u}C^{u}C$) = 100×B/(((C-D+B)/2)+A+D) and percentage of hypermethylated loci ($^{m}C^{m}C$) = 100×((C-D+B)/2)/(((C-D+B)/2)+D+A). DNA samples isolated from HeLa, Jurkat and Daudi cell lines were used as positive controls in each experiment and for interassay variation normalization (20).

Alu element methylation analysis

The ImageQuant Software (Molecular-Dynamics, GE Healthcare, Slough, UK) was used to quantify the intensities of COBRAAlu fragments on the polyacrylamide gel. COBRAAlu generated 3 bands based on the methylation status: hypomethylation ($^{u}C^{u}C$, 117 bp), partial methylation ($^{m}C^{u}C$ and $^{u}C^{m}C$, 74 and 75 bp, respectively) and methylated loci (^{m}C, 42 and 43 bp) (Figure 14B). Alu element methylation levels and patterns were calculated to determine the precise frequency of each pattern. The calculation was performed as the follows. First, the intensity of each band was divided

by the length (bp) of the double-stranded DNA: %117/117 = A, %74 and 75/74.5 = B, %42 and 43/43.5 = D, and D-B = C (C= hypermethylated loci, $^{m}C^{m}C$). Next, the frequency of each Alu element methylation pattern was calculated as follows: percentage of methylated loci (^{m}C) = 100x(2C+2B)/(2A+2B+2C) = 100x(2D)/(2A+2D), percentage of hypermethylated loci ($^{m}C^{m}C$) = 100x C/(A+B+C), percentage of partially methylated loci ($^{u}C^{m}C+^{m}C^{u}C$) = 100xB/(A+B+C) and percentage of hypomethylated loci ($^{u}C^{u}C$) = 100xA/(A+B+C). DNA samples from HeLa, Jurkat and Daudi cell lines were used as positive controls in every experiment and to standardize interassay variation (20).

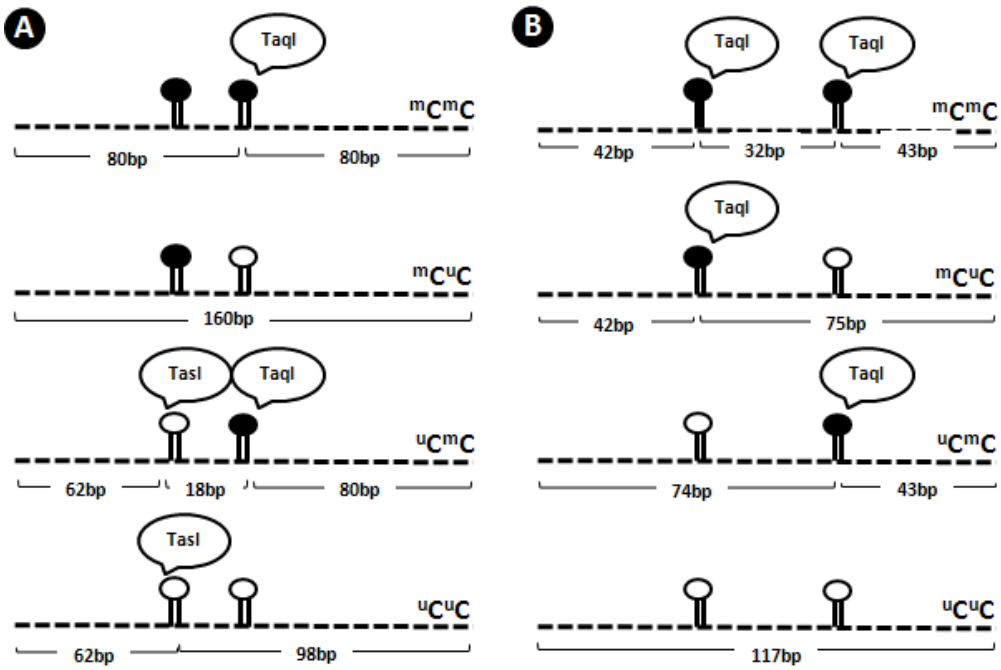

Figure 14 LINE-1 and Alu methylation patterns. The dark circles represent methylated cytosine, while the hollow circles represent unmethylated cytosine. There are four possible methylation patterns for the LINE-1 and Alu amplicons, including hypermethylated loci ($^{m}C^{m}C$), hypomethylated loci ($^{u}C^{u}C$), and 2 partially methylated loci ($^{m}C^{u}C$ and $^{u}C^{m}C$). In each model, *TaqI* specifically identified methylated cytosine, while *TasI* specifically identified unmethylated cytosine. (A) The different methylation patterns of LINE-1 resulted in four differently sized digested products of 160 bp, 98 bp, 80 bp and 62 bp. (B) The different methylation patterns of the Alu element resulted in four differently sized digested products of 117 bp, 74/75 bp, 42/43 bp and 32 bp.

Statistical Analysis

An analysis of variance (ANOVA) was used to compare methylation patterns of LINE-1 and Alu elements among squamous, mucous, intermediate and adjacent normal salivary gland cells present in MEC lesions. An independent sample *t*-test was performed to determine differences between LINE-1 and Alu element methylation patterns in total MEC tissue and normal tissue of the salivary gland. A receiver operating characteristic (ROC) analysis was performed to verify the ability of COBRALINE-1 and COBRAAlu to differentiate MEC lesions.

CHAPTER IV

RESULTS

LINE-1 methylation in microdissected MEC tissue

The percentage of each LINE-1 methylation pattern is shown in table 3. The total LINE-1 methylation level (^{m}C) decreased from the adjacent normal salivary gland cells (N) to the intermediate cells (I), mucous cells (M) and squamous cells (S). The results showed significant differences between S:M, S:I, S:N and M:N (p<0.001). However, there was no significant difference between M:I and N:I (p=1.000 and 0.138, respectively) (Figure 15A).

LINE-1 patterns	%^{m}C±SD	%$^{m}C^{m}C$±SD	%$^{m}C^{u}C$±SD	%$^{u}C^{m}C$±SD	%$^{u}C^{u}C$±SD
Adjacent normal salivary gland cell (N)	41.13±2.51	12.49±4.61	26.21±4.62	31.06±7.35	30.22±5.08
Intermediate cell (I)	37.69±0.69	7.63±3.15	27.17±0.50	32.94±5.92	32.24±3.20
Mucous cell (M)	35.84±2.24	11.98±7.93	22.90±6.43	24.80±9.75	40.30±6.92
Squamous cell (S)	31.27±3.07	8.74±5.20	24.17±4.00	20.89±8.10	46.18±4.75

Table 3 Percentage of LINE-1 methylation patterns in MEC cell subtypes.

Additionally, the percentage of unmethylated ($^{u}C^{u}C$) LINE-1s increased from N to M, I and S. Significant differences were found between S:M (p=0.048), S:I, S:N and M:N (p<0.001). However, no significant difference was found between M:I and N:I (p=0.087 and 1.000, respectively) (Figure 15B).

A significant difference in the $^{u}C^{m}C$ level of LINE-1s in N, M, I and S was found only between S:N (p=0.027). There was no significant difference between S:I (p=0.099), M:I (p=0.551), M:N (p=0.353), S:M and N:I (p=1.000). In addition, no significant difference was found in the percentage of $^{m}C^{m}C$ and $^{m}C^{u}C$ of LINE-1s between groups of microdissected cells.

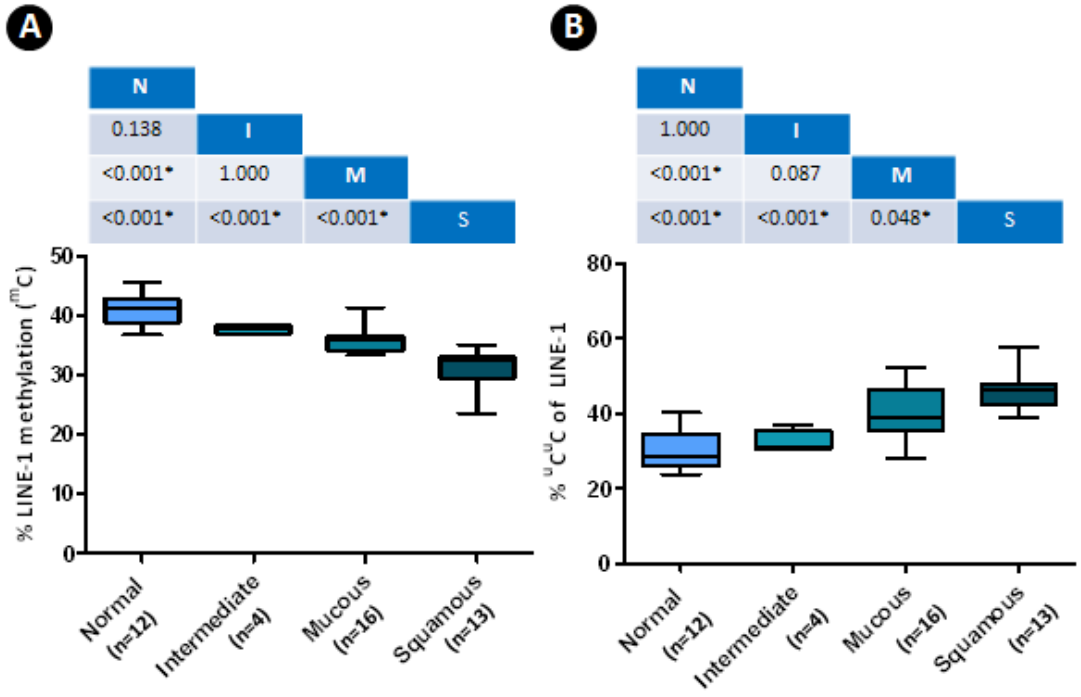

Figure 15 Comparison of the percentage of total LINE-1 methylation (^{m}C) and $^{u}C^{u}C$ of LINE-1s among MEC cell subtypes. (A) The percentage of ^{m}C of LINE-1s among MEC cell subtypes showed a stepwise decrease from normal cells (N) to intermediate cells (I), mucous cells (M) and squamous cells (S). The *p*-value between each group is shown in the table above the graph. (B) The percentage of $^{u}C^{u}C$ of LINE-1s among cell types showed a stepwise increase from normal cells to intermediate cells, mucous cells and squamous cells. The *p*-value between each group is shown in the table above the graph.

Alu element methylation in microdissected MEC tissue

The percentage of each Alu element methylation pattern is shown in table 4. Similar to LINE-1, total Alu element methylation (^{m}C) decreased from N to M, I and S. The results showed significant differences between S:M (p=0.001), S:I (p=0.002), S:N (p<0.001) and M:N (p=0.003). However, there was no significant difference between M:I and N:I (p=1.000) (Figure 16A).

Alu patterns	%^{m}C±SD	%$^{m}C^{m}C$±SD	%($^{m}C^{u}C$+$^{u}C^{m}C$)±SD	%$^{u}C^{u}C$±SD
Adjacent normal salivary gland cell (N)	65.10±2.80	23.36±6.42	41.74±4.43	34.89±2.80
Intermediate cell (I)	63.18±1.51	23.66±10.76	39.52±9.32	36.81±1.51
Mucous cell (M)	61.48±2.46	21.02±6.83	40.45±7.09	38.51±2.46
Squamous cell (S)	57.51±2.46	23.74±5.25	33.77±4.39	42.48±2.46

Table 4 Percentage of Alu element methylation patterns in MEC cell subtypes.

On the contrary, the percentage of $^{u}C^{u}C$ of Alu elements increased from N to M, I and S, respectively. A significant difference was found between S:M (p=0.001), S:I (p=0.002), S:N (p<0.001) and M:N (p=0.003). No significant difference was found between M:I and N:I (p=1.000) (Figure 16B).

A significant difference in the percentage of $^{m}C^{u}C$ + $^{u}C^{m}C$ of Alu elements was found between S:M (p=0.028) and S:N (p=0.011). However, there was no significant difference between S:I (p=0.061), M:N, M:I and N:I (p=1.000). Moreover, the percentage of $^{m}C^{m}C$ of Alu elements showed no significant difference between groups of microdissected cells.

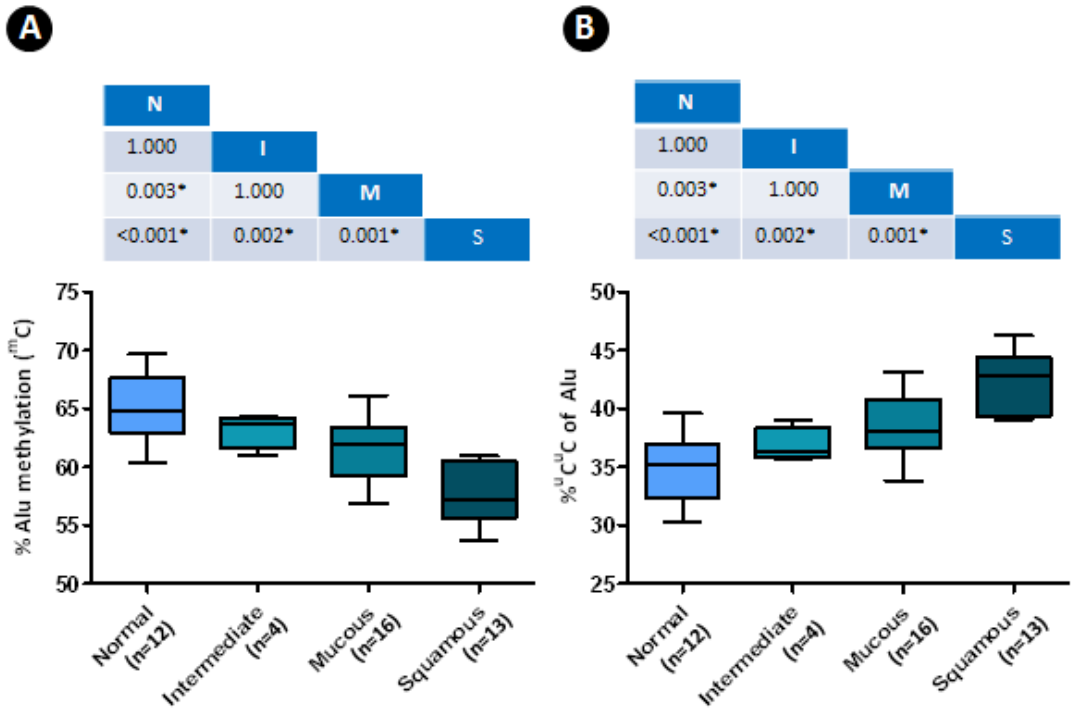

Figure 16 Comparison of the percentage of total Alu element methylation (^{m}C) and $^{u}C^{u}C$ of Alu elements among MEC cell subtypes. (A) Alu element methylation among cell types showed a stepwise decrease from normal cells to intermediate cells, mucous cells and squamous cell. The *p*-value between each group is shown in the table above the graph. (B) The percentage of $^{u}C^{u}C$ of Alu elements among cell types showed a stepwise increase from normal cells to intermediate cells, mucous cells and squamous cells. The *p*-value between each group is shown in the table above the graph.

LINE-1 and Alu element methylation in MECs of various histological grades

The total LINE-1 methylation level (^{m}C) of microdissected cells in each cell type decreased from low-grade to intermediate-grade and high-grade MEC (Figure 17A). However, total Alu element methylation (^{m}C) in microdissected cells was not related to the histological grade of the MEC (Figure 17B). Interestingly, when we compared the total LINE-1 and Alu element methylation levels of microdissected cells in each specimen, almost all of the cases (23 cases from total of 24 cases) showed decreasing levels of LINE-1 and Alu element methylation from N to I, M and S. (Figure 18A, 18B). These results demonstrate that genomic hypomethylation, and specifically LINE-1 methylation, correlates with poorer histological grade in MECs.

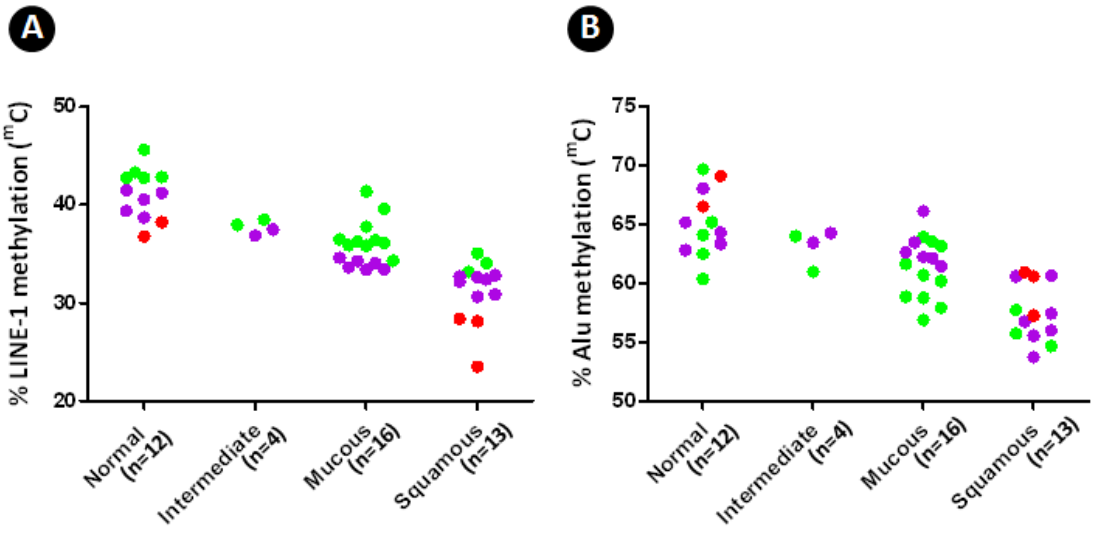

Figure 17 LINE-1 and Alu element methylation levels among MEC cell subtypes.

(A) LINE-1 methylation in MEC cell subtypes correlated with the histological grade of the MEC. (B) Alu element methylation level in MEC cell subtypes did not correlate with the histological grade of the MEC. The green circles indicated low-grade MEC while the purple and red circles indicate intermediated- and high-grade MEC, respectively.

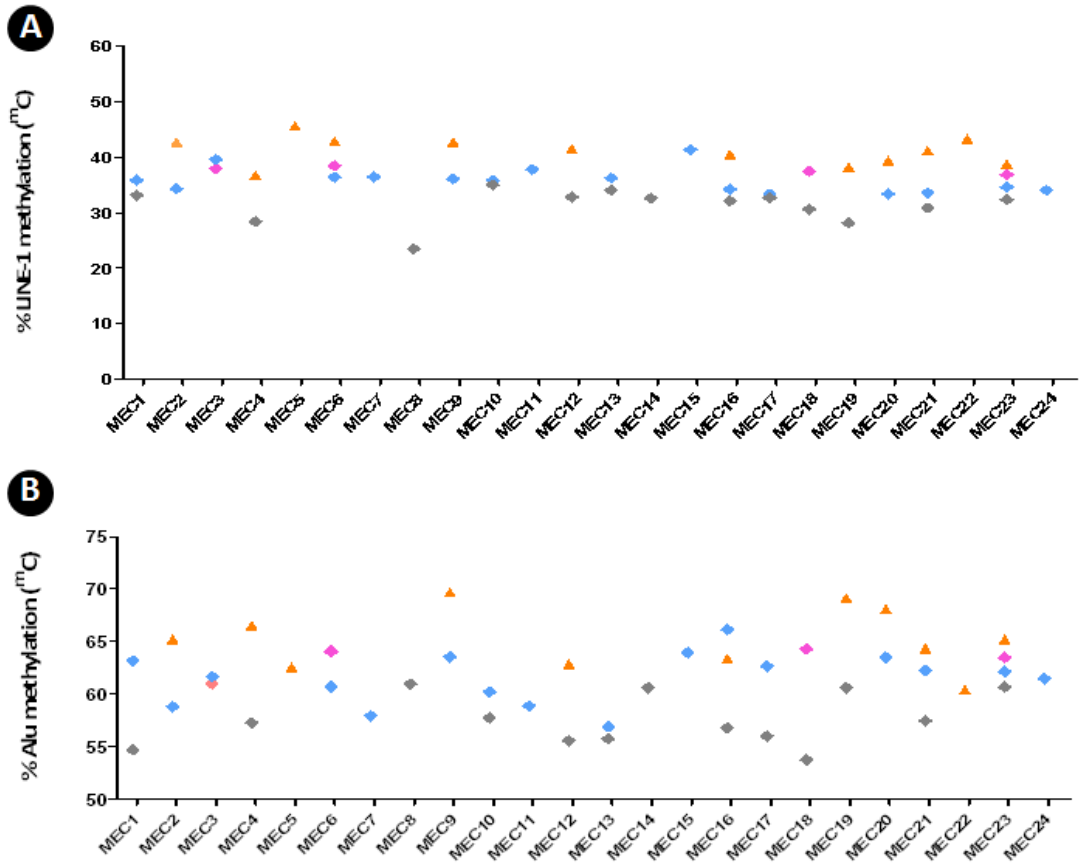

Figure 18 LINE-1 and Alu element methylation levels among each MEC specimen.

(A) LINE-1 methylation level of each microdissected MEC specimen. (D) Alu element methylation level of each microdissected MEC specimen. The orange triangles indicated adjacent normal salivary gland cells while the pink, blue and gray rhombus indicate intermediate, mucous and squamos cells, respectively.

LINE-1 and Alu element methylation in whole MEC tissue

We next asked whether these methods could be used to detect and classify MECs. To address this question, we analyzed LINE-1 and Alu element methylation in whole MEC tissue compared with normal salivary gland tissue.

The percentage of each LINE-1 methylation pattern in whole MEC tissue is shown in table 5. The percentage of ^{m}C and $^{m}C^{m}C$ LINE-1s were significantly lower in MEC tissue than in normal salivary gland tissue ($p<0.001$) (Table 5, Figure 19A and 19B). Moreover, the percentage of ^{m}C and $^{m}C^{m}C$ of LINE-1s in most of the low-grade MECs was higher than in intermediate-grade and high-grade MECs (Figure 19A and 19B).

LINE-1 patterns	%^{m}C±SD	%$^{m}C^{m}C$±SD	%$^{m}C^{u}C$±SD	%$^{u}C^{m}C$±SD	%$^{u}C^{u}C$±SD
Normal salivary gland (NG)	41.79±1.90	21.03±2.31	28.13±2.95	13.38±3.26	37.44±2.86
Whole MEC tissue (MEC)	35.69±2.23	11.52±4.71	26.64±3.20	21.69±6.96	40.13±3.71

Table 5 Percentage of LINE-1 methylation patterns in whole MEC tissues and normal salivary glands.

The percentage of each Alu element methylation pattern in whole MEC tissue is shown in table 6. Similar to LINE-1, the total Alu element methylation level (^{m}C) in MEC tissue was also significantly lower than in normal salivary gland tissue (p=0.001). In agreement with these results, the percentage of $^{u}C^{u}C$ of Alu elements in MEC tissue was significantly lower than in normal salivary gland tissue (p=0.001) (Table 6, Figure 19C and 19D). However, Alu element methylation in whole MEC tissue was not related to the histological grade of the MEC (Figure 19C and 19D).

Alu patterns	%^{m}C±SD	%$^{m}C^{m}C$±SD	%($^{m}C^{u}C$+$^{u}C^{m}C$)±SD	%$^{u}C^{u}C$±SD
Normal salivary gland (NG)	64.52±4.66	18.53±10.16	45.99±8.97	35.47±4.66
Whole MEC tissue (MEC)	57.49±5.35	22.21±5.13	35.27±5.02	42.51±5.35

Table 6 Percentage of LINE-1 methylation patterns in whole MEC tissues and normal salivary glands.

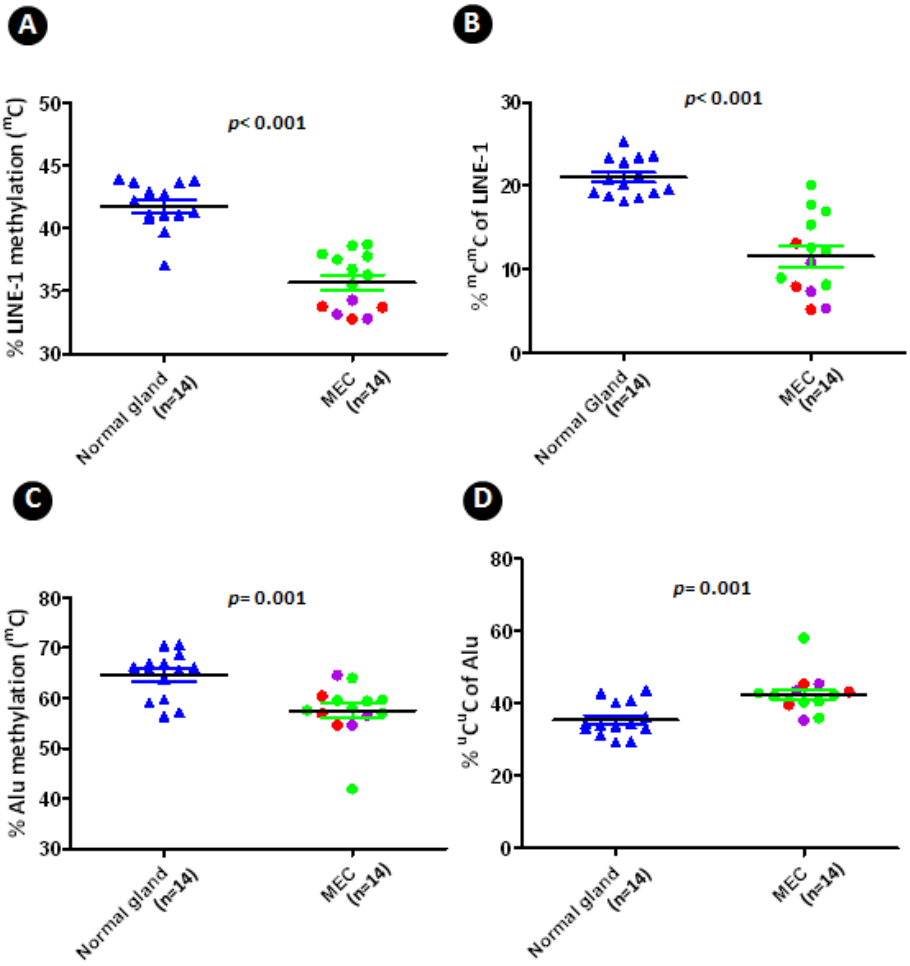

Figure 19 Comparison of total LINE-1 and Alu element methylation between normal salivary gland tissue and whole MEC tissue. (A, B) The percentage of ^{m}C and $^{m}C^{m}C$ of LINE-1 methylation in whole MEC tissue was significantly lower than in normal salivary gland tissue (p<0.001). (C) The percentage of ^{m}C of Alu elements in whole MEC tissue was significantly lower than in normal salivary gland tissue (p=0.001). (D) The percentage of $^{u}C^{u}C$ Alu elements in whole MEC tissue was significantly higher than in normal salivary gland tissue (p=0.001). The dark blue triangles indicated normal salivary gland while green, purple and red circles indicated low-, intermediated- and high-grade MEC, respectively.

Receiver operating characteristic (ROC) analysis of LINE-1 and Alu element methylation

Next, we assessed the ability of these methods to discriminate between MEC tissue and normal salivary gland tissue using an ROC analysis. Among the various patterns of LINE-1 methylation, both the ^{m}C and the $^{m}C^{m}C$ patterns yielded ROC values indicative of diagnostic reliability. For the ^{m}C pattern of LINE-1, the area under the ROC curve (AUC) value was 0.974, while the cut-off value, sensitivity and specificity were 38.73%, 100% and 92.86%, respectively (Figure 20A). The AUC value of the $^{m}C^{m}C$ pattern of LINE-1 was 0.969, while the cut-off value, sensitivity and specificity were 38.73%, 92.86% and 100%, respectively (Figure 20B).

Among the various patterns of Alu element methylation, the ^{m}C and $^{u}C^{u}C$ patterns demonstrated reasonable diagnostic values. Both the ^{m}C and the $^{u}C^{u}C$ of Alu element methylation patterns had AUC values, sensitivity and specificity of 0.847, 100% and 64.29%, respectively. The cut-off values for the ^{m}C and $^{u}C^{u}C$ of Alu element methylation patterns were 15.48% and 34.48%, respectively (Figure 20C and 20D).

These results indicate that ROC analysis of LINE-1 methylation has a better diagnostic value than analysis of Alu element methylation. This analysis is especially effective when both the ^{m}C and $^{m}C^{m}C$ patterns of LINE-1 methylation are assessed.

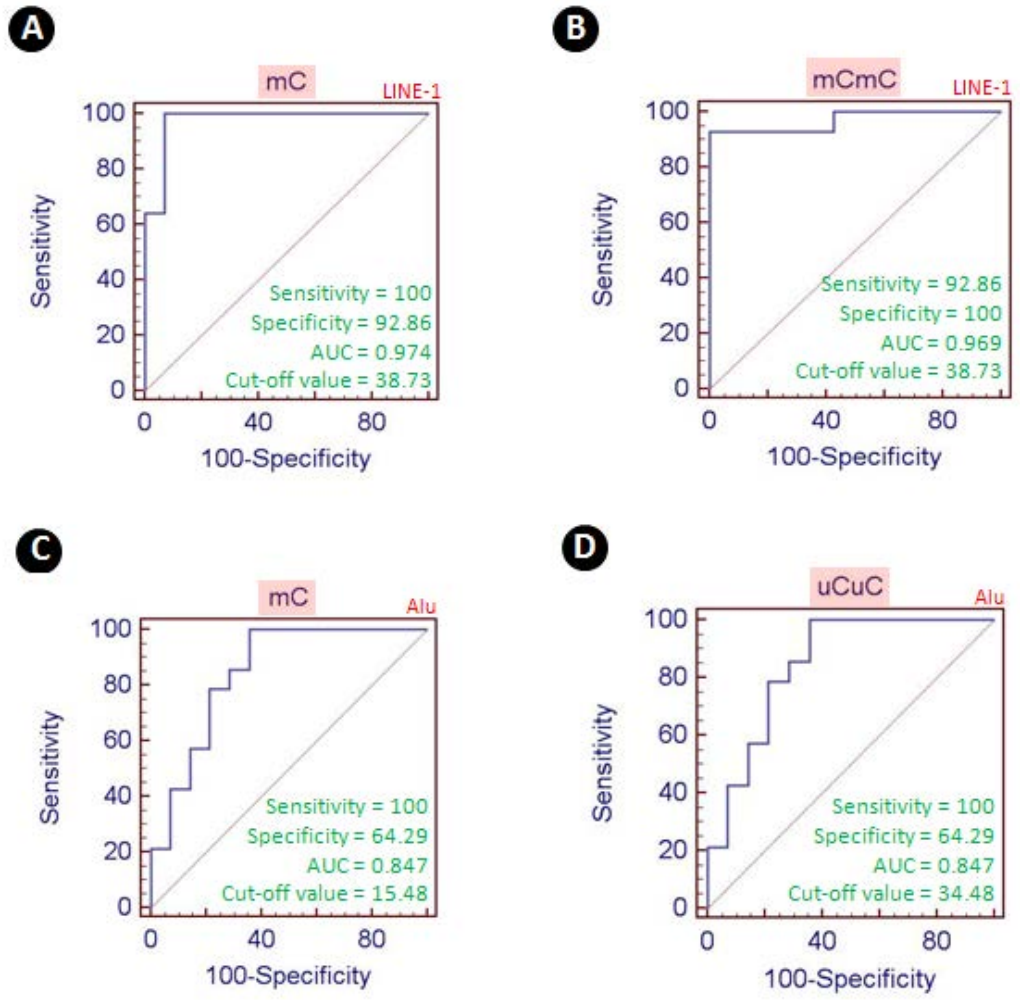

Figure 20 ROC curve analysis of LINE-1 and Alu element methylation for MEC detection. (A) The total LINE-1 methylation level (^{m}C). (B) The ^{m}C^{m}C level of LINE-1 methylation. (C) The total Alu element methylation level (^{m}C). (D) The ^{u}C^{u}C level of Alu element methylation

CHAPTER V

DISCUSSION AND CONCLUSION

To the best of our knowledge, this report represents the first epigenetic study of MEC. The aim of this study was to characterize the methylation status of the repetitive sequences in MEC. COBRALINE-1 can accurately represent the genome-wide methylation status of LINE-1s in genomic DNA(20). In this study, we used modified COBRALINE-1 methods. These methods detected 2 CpG dinucleotide sites. Thus, we could detect not only methylation level but also the methylation patterns of LINE-1.

Although MEC is the most common salivary gland cancer, malignant tumors of the salivary glands are rarely found. Hence, one of the limitations of our study was the limited number of MEC samples available for investigation. Theoretically, the parotid gland is the most common site of this tumor; however, most of MEC samples used in this study were collected from the minor salivary glands of the palate.

Laser microbeam microdissection (LMM) is a very sensitive method. In this study, we used only 1,500 microdissected cells, which provided enough DNA to be able to detect LINE-1 methylation levels and patterns. However, this method was not suitable for dissecting MEC lesions in which each cell population was not obviously separable. Therefore, another limitation of our study was that only some cell types could be collected from the MEC lesions to avoid contamination.

We observed that LINE-1 hypomethylation in adjacent normal salivary gland cells depended on the histological grade of the MEC (Figure 16). Noticeably, the $^{m}C^{m}C$ of normal salivary gland cells (N) was lower than that of normal salivary gland (NG) although the total LINE-1 methylation level was not different. This phenomenon may result from the influence of cancer cells on normal surrounding tissues. Moreover, the level of LINE-1 methylation in intermediate cells was between that of normal adjacent salivary gland cells and mucous cells. These data are consistent with a previous

hypothesis that the intermediate cells differentiate from maternal cells and transform into squamous cells or mucous cells(10).

Currently, there is no uniformly accepted criteria for classifying the histological grade of MECs. Our data showed that LINE-1 methylation patterns correlate well with WHO grading of MECs. In addition to assessing the proportion of squamous and mucous cells, we propose that this modified COBRALINE-1 method can be used as an additional tool for molecular classification of MEC. This may lead to improved diagnosis of MEC lesions.

To date, the standard method for MEC diagnosis is an assessment of pathological features with immunohistochemical staining. Our study demonstrates a possible alternative tool. We propose that this method might be useful in the case of obscure incisional biopsy results, such as those that result from some cases of fine needle aspiration (FNA). It would be informative to further determine LINE-1 methylation levels and patterns in an extensive group of samples, including various sites of the lesions and from other types of salivary gland tumors. In conclusion, our findings provide a basis for further investigations that could increase better understanding of the multistep carcinogenesis of MEC.

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APPENDIX

Statistics output

Table 1 Descriptive analysis of COBRALINE-1 products in whole tissue of MEC and normal salivary gland

Group Statistics

	status	N	Mean	Std. Deviation	Std. Error Mean
mC	Normal Gland	14	41.7907	1.90537	.50923
	MEC	14	35.6957	2.23457	.59721
mCmC	Normal Gland	14	21.0307	2.31223	.61797
	MEC	14	11.5271	4.71353	1.25974
mCuC	Normal Gland	14	28.1393	2.95750	.79043
	MEC	14	26.6479	3.20182	.85572
uCmC	Normal Gland	14	13.3821	3.26118	.87159
	MEC	14	21.6921	6.96811	1.86231
uCuC	Normal Gland	14	37.4493	2.86127	.76471
	MEC	14	40.1343	3.71202	.99208

Table 2 Independent sample test of COBRALINE-1 products in whole tissue of MEC and normal salivary gland

Independent Samples Test

		Levene's Test for Equality of Variances		t-test for Equality of Means			
						95% Confidence Interval of the Difference	
		F	Sig.	Sig. (2-tailed)	Mean Difference	Lower	Upper
mC	Equal variances assumed	1.600	.217	.000	6.09500	4.48173	7.70827
	Equal variances not assumed			.000	6.09500	4.47977	7.71023
mCmC	Equal variances assumed	7.164	.013	.000	9.50357	6.61935	12.38779
	Equal variances not assumed			.000	9.50357	6.56584	12.44131
mCuC	Equal variances assumed	.196	.661	.212	1.49143	-.90309	3.88595
	Equal variances not assumed			.212	1.49143	-.90382	3.88668
uCmC	Equal variances assumed	10.133	.004	.000	-8.31000	-12.53653	-4.08347
	Equal variances not assumed			.001	-8.31000	-12.62258	-3.99742
uCuC	Equal variances assumed	1.066	.311	.042	-2.68500	-5.25975	-.11025
	Equal variances not assumed			.042	-2.68500	-5.26789	-.10211

Table 3 Descriptive analysis of COBRALINE-1 products in MEC cell subtypes

Descriptive statistics

		N	Mean	Std. Deviation	Minimum	Maximum
mC	Normal cell	12	41.1317	2.51485	36.79	45.61
	Intermediate cell	4	37.6950	.69039	36.86	38.48
	Mucous cell	16	35.8406	2.24792	33.39	41.37
	Squamous cell	13	31.2777	3.07289	23.50	35.06
	Total	45	36.0982	4.46573	23.50	45.61
mCmC	Normal cell	12	12.4950	4.61636	5.41	19.16
	Intermediate cell	4	7.6375	3.15095	4.70	12.01
	Mucous cell	16	11.9887	7.93110	1.27	34.07
	Squamous cell	13	8.7431	5.20471	1.61	17.22
	Total	45	10.7993	6.18815	1.27	34.07
mCuC	Normal cell	12	26.2117	4.62531	18.73	35.05
	Intermediate cell	4	27.1700	.50326	26.70	27.62
	Mucous cell	16	22.9006	6.43545	2.03	30.34
	Squamous cell	13	24.1769	4.00814	18.70	30.06
	Total	45	24.5318	5.12571	2.03	35.05
uCmC	Normal cell	12	31.0633	7.35969	17.44	40.21
	Intermediate cell	4	32.9425	5.92713	24.25	36.73
	Mucous cell	16	24.8019	9.75052	6.97	42.86
	Squamous cell	13	20.8923	8.10940	7.26	32.06
	Total	45	26.0658	9.26726	6.97	42.86
uCuC	Normal cell	12	30.2283	5.08596	23.78	40.40
	Intermediate cell	4	32.2475	3.20707	30.30	37.04
	Mucous cell	16	40.3075	6.92996	28.30	52.55
	Squamous cell	13	46.1892	4.75277	39.13	57.80
	Total	45	38.6024	8.40003	23.78	57.80

Table 4 One-way ANOVA of COBRALINE-1 products in MEC cell subtypes

ANOVA

		Sum of Squares	df	Mean Square	F	Sig.
mC	Between Groups	617.375	3	205.792	32.438	.000
	Within Groups	260.108	41	6.344		
	Total	877.483	44			
mCmC	Between Groups	152.094	3	50.698	1.356	.270
	Within Groups	1532.807	41	37.386		
	Total	1684.901	44			
mCuC	Between Groups	105.913	3	35.304	1.378	.263
	Within Groups	1050.096	41	25.612		
	Total	1156.009	44			
uCmC	Between Groups	862.365	3	287.455	4.041	.013
	Within Groups	2916.445	41	71.133		
	Total	3778.811	44			
uCuC	Between Groups	1797.837	3	599.279	18.802	.000
	Within Groups	1306.824	41	31.874		
	Total	3104.661	44			

Multiple Comparisons

Dependent Variable		(I) status	(J) status	Mean Difference (I-J)	Std. Error	Sig.	95% Confidence Interval	
							Lower Bound	Upper Bound
mC	Tukey HSD	Normal cell	Intermediate cell	3.43667	1.45420	.101	-.4571	7.3305
			Mucous cell	5.29104*	.96186	.000	2.7155	7.8665
			Squamous cell	9.85397*	1.00831	.000	7.1541	12.5538
		Intermediate cell	Normal cell	-3.43667	1.45420	.101	-7.3305	.4571
			Mucous cell	1.85437	1.40802	.558	-1.9158	5.6245

		Squamous cell	6.41731*	1.44015	.000	2.5611	10.2735
	Mucous cell	Normal cell	-5.29104*	.96186	.000	-7.8665	-2.7155
		Intermediate cell	-1.85437	1.40802	.558	-5.6245	1.9158
		Squamous cell	4.56293*	.94049	.000	2.0447	7.0812
	Squamous cell	Normal cell	-9.85397*	1.00831	.000	-12.5538	-7.1541
		Intermediate cell	-6.41731*	1.44015	.000	-10.2735	-2.5611
		Mucous cell	-4.56293*	.94049	.000	-7.0812	-2.0447
Scheffe	Normal cell	Intermediate cell	3.43667	1.45420	.151	-.8026	7.6759
		Mucous cell	5.29104*	.96186	.000	2.4870	8.0950
		Squamous cell	9.85397*	1.00831	.000	6.9146	12.7934
	Intermediate cell	Normal cell	-3.43667	1.45420	.151	-7.6759	.8026
		Mucous cell	1.85437	1.40802	.633	-2.2503	5.9590
		Squamous cell	6.41731*	1.44015	.001	2.2190	10.6156
	Mucous cell	Normal cell	-5.29104*	.96186	.000	-8.0950	-2.4870
		Intermediate cell	-1.85437	1.40802	.633	-5.9590	2.2503
		Squamous cell	4.56293*	.94049	.000	1.8213	7.3046
	Squamous cell	Normal cell	-9.85397*	1.00831	.000	-12.7934	-6.9146
		Intermediate cell	-6.41731*	1.44015	.001	-10.6156	-2.2190
		Mucous cell	-4.56293*	.94049	.000	-7.3046	-1.8213
Bonferroni	Normal cell	Intermediate cell	3.43667	1.45420	.138	-.5949	7.4682
		Mucous cell	5.29104*	.96186	.000	2.6244	7.9577
		Squamous cell	9.85397*	1.00831	.000	7.0586	12.6494
	Intermediate	Normal cell	-3.43667	1.45420	.138	-7.4682	.5949

		cell	Mucous cell	1.85437	1.40802	1.000	-2.0492	5.7579
			Squamous cell	6.41731*	1.44015	.000	2.4247	10.4099
		Mucous cell	Normal cell	-5.29104*	.96186	.000	-7.9577	-2.6244
			Intermediate cell	-1.85437	1.40802	1.000	-5.7579	2.0492
			Squamous cell	4.56293*	.94049	.000	1.9556	7.1703
		Squamous cell	Normal cell	-9.85397*	1.00831	.000	-12.6494	-7.0586
			Intermediate cell	-6.41731*	1.44015	.000	-10.4099	-2.4247
			Mucous cell	-4.56293*	.94049	.000	-7.1703	-1.9556
mCmC	Tukey HSD	Normal cell	Intermediate cell	4.85750	3.53013	.521	-4.5949	14.3099
			Mucous cell	.50625	2.33496	.996	-5.7459	6.7584
			Squamous cell	3.75192	2.44771	.428	-2.8021	10.3060
		Intermediate cell	Normal cell	-4.85750	3.53013	.521	-14.3099	4.5949
			Mucous cell	-4.35125	3.41804	.585	-13.5035	4.8010
			Squamous cell	-1.10558	3.49603	.989	-10.4666	8.2555
		Mucous cell	Normal cell	-.50625	2.33496	.996	-6.7584	5.7459
			Intermediate cell	4.35125	3.41804	.585	-4.8010	13.5035
			Squamous cell	3.24567	2.28307	.493	-2.8675	9.3589
		Squamous cell	Normal cell	-3.75192	2.44771	.428	-10.3060	2.8021
			Intermediate cell	1.10558	3.49603	.989	-8.2555	10.4666
			Mucous cell	-3.24567	2.28307	.493	-9.3589	2.8675
	Scheffe	Normal cell	Intermediate cell	4.85750	3.53013	.599	-5.4335	15.1485
			Mucous cell	.50625	2.33496	.997	-6.3006	7.3131
			Squamous cell	3.75192	2.44771	.510	-3.3836	10.8874

		Intermediate cell	Normal cell	-4.85750	3.53013	.599	-15.1485	5.4335
			Mucous cell	-4.35125	3.41804	.657	-14.3154	5.6129
			Squamous cell	-1.10558	3.49603	.992	-11.2971	9.0859
		Mucous cell	Normal cell	-.50625	2.33496	.997	-7.3131	6.3006
			Intermediate cell	4.35125	3.41804	.657	-5.6129	14.3154
			Squamous cell	3.24567	2.28307	.573	-3.4099	9.9012
		Squamous cell	Normal cell	-3.75192	2.44771	.510	-10.8874	3.3836
			Intermediate cell	1.10558	3.49603	.992	-9.0859	11.2971
			Mucous cell	-3.24567	2.28307	.573	-9.9012	3.4099
	Bonferroni	Normal cell	Intermediate cell	4.85750	3.53013	1.000	-4.9293	14.6443
			Mucous cell	.50625	2.33496	1.000	-5.9671	6.9796
			Squamous cell	3.75192	2.44771	.798	-3.0340	10.5378
		Intermediate cell	Normal cell	-4.85750	3.53013	1.000	-14.6443	4.9293
			Mucous cell	-4.35125	3.41804	1.000	-13.8273	5.1248
			Squamous cell	-1.10558	3.49603	1.000	-10.7978	8.5866
		Mucous cell	Normal cell	-.50625	2.33496	1.000	-6.9796	5.9671
			Intermediate cell	4.35125	3.41804	1.000	-5.1248	13.8273
			Squamous cell	3.24567	2.28307	.976	-3.0838	9.5752
		Squamous cell	Normal cell	-3.75192	2.44771	.798	-10.5378	3.0340
			Intermediate cell	1.10558	3.49603	1.000	-8.5866	10.7978
			Mucous cell	-3.24567	2.28307	.976	-9.5752	3.0838
mCuC	Tukey HSD	Normal cell	Intermediate cell	-.95833	2.92188	.988	-8.7820	6.8653
			Mucous cell	3.31104	1.93264	.330	-1.8638	8.4859
			Squamous cell	2.03474	2.02596	.748	-3.3900	7.4595

	Intermediate cell	Normal cell	.95833	2.92188	.988	-6.8653	8.7820
		Mucous cell	4.26938	2.82910	.441	-3.3059	11.8446
		Squamous cell	2.99308	2.89365	.730	-4.7550	10.7412
	Mucous cell	Normal cell	-3.31104	1.93264	.330	-8.4859	1.8638
		Intermediate cell	-4.26938	2.82910	.441	-11.8446	3.3059
		Squamous cell	-1.27630	1.88969	.906	-6.3362	3.7836
	Squamous cell	Normal cell	-2.03474	2.02596	.748	-7.4595	3.3900
		Intermediate cell	-2.99308	2.89365	.730	-10.7412	4.7550
		Mucous cell	1.27630	1.88969	.906	-3.7836	6.3362
Scheffe	Normal cell	Intermediate cell	-.95833	2.92188	.991	-9.4761	7.5594
		Mucous cell	3.31104	1.93264	.412	-2.3229	8.9450
		Squamous cell	2.03474	2.02596	.799	-3.8713	7.9408
	Intermediate cell	Normal cell	.95833	2.92188	.991	-7.5594	9.4761
		Mucous cell	4.26938	2.82910	.524	-3.9779	12.5167
		Squamous cell	2.99308	2.89365	.785	-5.4424	11.4286
	Mucous cell	Normal cell	-3.31104	1.93264	.412	-8.9450	2.3229
		Intermediate cell	-4.26938	2.82910	.524	-12.5167	3.9779
		Squamous cell	-1.27630	1.88969	.928	-6.7851	4.2325
	Squamous cell	Normal cell	-2.03474	2.02596	.799	-7.9408	3.8713
		Intermediate cell	-2.99308	2.89365	.785	-11.4286	5.4424
		Mucous cell	1.27630	1.88969	.928	-4.2325	6.7851
Bonferroni	Normal cell	Intermediate cell	-.95833	2.92188	1.000	-9.0588	7.1421
		Mucous cell	3.31104	1.93264	.565	-2.0469	8.6690
		Squamous cell	2.03474	2.02596	1.000	-3.5819	7.6514

		Intermediate cell	Normal cell	.95833	2.92188	1.000	-7.1421	9.0588
			Mucous cell	4.26938	2.82910	.834	-3.5739	12.1126
			Squamous cell	2.99308	2.89365	1.000	-5.0291	11.0153
		Mucous cell	Normal cell	-3.31104	1.93264	.565	-8.6690	2.0469
			Intermediate cell	-4.26938	2.82910	.834	-12.1126	3.5739
			Squamous cell	-1.27630	1.88969	1.000	-6.5152	3.9626
		Squamous cell	Normal cell	-2.03474	2.02596	1.000	-7.6514	3.5819
			Intermediate cell	-2.99308	2.89365	1.000	-11.0153	5.0291
			Mucous cell	1.27630	1.88969	1.000	-3.9626	6.5152
uCmC	Tukey HSD	Normal cell	Intermediate cell	-1.87917	4.86939	.980	-14.9175	11.1592
			Mucous cell	6.26146	3.22080	.226	-2.3626	14.8855
			Squamous cell	10.17103*	3.37631	.022	1.1305	19.2115
		Intermediate cell	Normal cell	1.87917	4.86939	.980	-11.1592	14.9175
			Mucous cell	8.14062	4.71476	.323	-4.4837	20.7650
			Squamous cell	12.05019	4.82234	.075	-.8622	24.9626
		Mucous cell	Normal cell	-6.26146	3.22080	.226	-14.8855	2.3626
			Intermediate cell	-8.14062	4.71476	.323	-20.7650	4.4837
			Squamous cell	3.90957	3.14922	.605	-4.5228	12.3420
		Squamous cell	Normal cell	-10.17103*	3.37631	.022	-19.2115	-1.1305
			Intermediate cell	-12.05019	4.82234	.075	-24.9626	.8622
			Mucous cell	-3.90957	3.14922	.605	-12.3420	4.5228
	Scheffe	Normal cell	Intermediate cell	-1.87917	4.86939	.985	-16.0743	12.3159
			Mucous cell	6.26146	3.22080	.301	-3.1277	15.6506
			Squamous cell	10.17103*	3.37631	.040	.3285	20.0136

		Intermediate cell	Normal cell	1.87917	4.86939	.985	-12.3159	16.0743
			Mucous cell	8.14062	4.71476	.405	-5.6037	21.8850
			Squamous cell	12.05019	4.82234	.118	-2.0078	26.1082
		Mucous cell	Normal cell	-6.26146	3.22080	.301	-15.6506	3.1277
			Intermediate cell	-8.14062	4.71476	.405	-21.8850	5.6037
			Squamous cell	3.90957	3.14922	.675	-5.2709	13.0901
		Squamous cell	Normal cell	-10.17103*	3.37631	.040	-20.0136	-.3285
			Intermediate cell	-12.05019	4.82234	.118	-26.1082	2.0078
			Mucous cell	-3.90957	3.14922	.675	-13.0901	5.2709
	Bonferroni	Normal cell	Intermediate cell	-1.87917	4.86939	1.000	-15.3788	11.6205
			Mucous cell	6.26146	3.22080	.353	-2.6677	15.1906
			Squamous cell	10.17103*	3.37631	.027	.8107	19.5314
		Intermediate cell	Normal cell	1.87917	4.86939	1.000	-11.6205	15.3788
			Mucous cell	8.14062	4.71476	.551	-4.9304	21.2116
			Squamous cell	12.05019	4.82234	.099	-1.3190	25.4194
		Mucous cell	Normal cell	-6.26146	3.22080	.353	-15.1906	2.6677
			Intermediate cell	-8.14062	4.71476	.551	-21.2116	4.9304
			Squamous cell	3.90957	3.14922	1.000	-4.8212	12.6403
		Squamous cell	Normal cell	-10.17103*	3.37631	.027	-19.5314	-.8107
			Intermediate cell	-12.05019	4.82234	.099	-25.4194	1.3190
			Mucous cell	-3.90957	3.14922	1.000	-12.6403	4.8212
uCuC	Tukey HSD	Normal cell	Intermediate cell	-2.01917	3.25954	.925	-10.7470	6.7086
			Mucous cell	-10.07917*	2.15598	.000	-15.8521	-4.3063
			Squamous cell	-15.96090*	2.26008	.000	-22.0125	-9.9093

	Intermediate cell	Normal cell	2.01917	3.25954	.925	-6.7086	10.7470
		Mucous cell	-8.06000	3.15603	.066	-16.5107	.3907
		Squamous cell	-13.94173*	3.22804	.001	-22.5852	-5.2983
	Mucous cell	Normal cell	10.07917*	2.15598	.000	4.3063	15.8521
		Intermediate cell	8.06000	3.15603	.066	-.3907	16.5107
		Squamous cell	-5.88173*	2.10807	.038	-11.5263	-.2371
	Squamous cell	Normal cell	15.96090*	2.26008	.000	9.9093	22.0125
		Intermediate cell	13.94173*	3.22804	.001	5.2983	22.5852
		Mucous cell	5.88173*	2.10807	.038	.2371	11.5263
Scheffe	Normal cell	Intermediate cell	-2.01917	3.25954	.943	-11.5213	7.4830
		Mucous cell	-10.07917*	2.15598	.001	-16.3642	-3.7941
		Squamous cell	-15.96090*	2.26008	.000	-22.5494	-9.3724
	Intermediate cell	Normal cell	2.01917	3.25954	.943	-7.4830	11.5213
		Mucous cell	-8.06000	3.15603	.106	-17.2604	1.1404
		Squamous cell	-13.94173*	3.22804	.001	-23.3520	-4.5314
	Mucous cell	Normal cell	10.07917*	2.15598	.001	3.7941	16.3642
		Intermediate cell	8.06000	3.15603	.106	-1.1404	17.2604
		Squamous cell	-5.88173	2.10807	.065	-12.0271	.2636
	Squamous cell	Normal cell	15.96090*	2.26008	.000	9.3724	22.5494
		Intermediate cell	13.94173*	3.22804	.001	4.5314	23.3520
		Mucous cell	5.88173	2.10807	.065	-.2636	12.0271
Bonferroni	Normal cell	Intermediate cell	-2.01917	3.25954	1.000	-11.0558	7.0174
		Mucous cell	-10.07917*	2.15598	.000	-16.0563	-4.1020
		Squamous cell	-15.96090*	2.26008	.000	-22.2266	-9.6951

	Intermediate cell	Normal cell	2.01917	3.25954	1.000	-7.0174	11.0558
		Mucous cell	-8.06000	3.15603	.087	-16.8096	.6896
		Squamous cell	-13.94173*	3.22804	.001	-22.8910	-4.9925
	Mucous cell	Normal cell	10.07917*	2.15598	.000	4.1020	16.0563
		Intermediate cell	8.06000	3.15603	.087	-.6896	16.8096
		Squamous cell	-5.88173*	2.10807	.048	-11.7260	-.0374
	Squamous cell	Normal cell	15.96090*	2.26008	.000	9.6951	22.2266
		Intermediate cell	13.94173*	3.22804	.001	4.9925	22.8910
		Mucous cell	5.88173*	2.10807	.048	.0374	11.7260

*. The mean difference is significant at the 0.05 level.

Table 5 Descriptive analysis of COBRAAlu products in whole tissue of MEC and normal salivary gland

Group Statistics

	Status	N	Mean	Std. Deviation	Std. Error Mean
mC	Normal gland	14	64.5279	4.66524	1.24684
	MEC	14	57.4900	5.35862	1.43215
mCmC	Normal gland	14	18.5350	10.16252	2.71605
	MEC	14	22.2121	5.13930	1.37353
Partialmet	Normal gland	14	45.9914	8.97793	2.39945
	MEC	14	35.2771	5.02185	1.34214
uCuC	Normal gland	14	35.4721	4.66524	1.24684
	MEC	14	42.5100	5.35862	1.43215

Table 6 Independent sample test of COBRAAlu products in whole tissue of MEC and normal salivary gland

Independent Samples Test

		Levene's Test for Equality of Variances		t-test for Equality of Means				
							95% Confidence Interval of the Difference	
		F	Sig.	Sig. (2-tailed)	Mean Difference	Std. Error Difference	Lower	Upper
mC	Equal variances assumed	.130	.721	.001	7.03786	1.89886	3.13470	10.94101
	Equal variances not assumed			.001	7.03786	1.89886	3.13110	10.94462
mCmC	Equal variances assumed	6.887	.014	.238	-3.67714	3.04360	-9.93336	2.57907
	Equal variances not assumed			.242	-3.67714	3.04360	-10.04208	2.68779
Partial met	Equal variances assumed	8.118	.008	.001	10.71429	2.74931	5.06299	16.36558
	Equal variances not assumed			.001	10.71429	2.74931	4.98668	16.44189
uCuC	Equal variances assumed	.130	.721	.001	-7.03786	1.89886	-10.94101	-3.13470
	Equal variances not assumed			.001	-7.03786	1.89886	-10.94462	-3.13110

Table 7 Descriptive analysis of COBRAAlu products in MEC cell subtypes

Descriptives

		N	Mean	Std. Deviation	95% Confidence Interval for Mean	
					Lower Bound	Upper Bound
mC	Normal cell	12	65.1058	2.80705	63.3223	66.8893
	Intermediate cell	4	63.1875	1.51050	60.7839	65.5911
	Mucous cell	16	61.4850	2.46724	60.1703	62.7997
	Squamous cell	13	57.5162	2.46816	56.0247	59.0076
	Total	45	61.4553	3.78869	60.3171	62.5936
mCmC	Normal cell	12	23.3650	6.42155	19.2849	27.4451
	Intermediate cell	4	23.6625	10.76027	6.5405	40.7845
	Mucous cell	16	21.0269	6.83747	17.3834	24.6703
	Squamous cell	13	23.7415	5.25403	20.5666	26.9165
	Total	45	22.6689	6.57359	20.6940	24.6438
Partialmet	Normal cell	12	41.7408	4.43716	38.9216	44.5601
	Intermediate cell	4	39.5225	9.32302	24.6875	54.3575
	Mucous cell	16	40.4588	7.09432	36.6785	44.2390
	Squamous cell	13	33.7762	4.39819	31.1184	36.4340
	Total	45	38.7869	6.64419	36.7908	40.7830
uCuC	Normal cell	12	34.8942	2.80705	33.1107	36.6777
	Intermediate cell	4	36.8125	1.51050	34.4089	39.2161
	Mucous cell	16	38.5150	2.46724	37.2003	39.8297
	Squamous cell	13	42.4838	2.46816	40.9924	43.9753
	Total	45	38.5447	3.78869	37.4064	39.6829

Table 4 One-way ANOVA of COBRAAlu products in MEC cell subtypes

ANOVA

		Sum of Squares	df	Mean Square	F	Sig.
mC	Between Groups	373.652	3	124.551	19.798	.000
	Within Groups	257.930	41	6.291		
	Total	631.583	44			
mCmC	Between Groups	67.861	3	22.620	.506	.680
	Within Groups	1833.473	41	44.719		
	Total	1901.334	44			
Partialmet	Between Groups	477.993	3	159.331	4.461	.008
	Within Groups	1464.398	41	35.717		
	Total	1942.391	44			
uCuC	Between Groups	373.652	3	124.551	19.798	.000
	Within Groups	257.930	41	6.291		
	Total	631.583	44			

Multiple Comparisons

Dependent Variable		(I) Status	(J) Status	Mean Difference (I-J)	Std. Error	Sig.	95% Confidence Interval	
							Lower Bound	Upper Bound
mC	Tukey HSD	Normal cell	Intermediate cell	1.91833	1.44810	.553	-1.9591	5.7958
			Mucous cell	3.62083*	.95783	.003	1.0561	6.1855
			Squamous cell	7.58968*	1.00408	.000	4.9011	10.2782
		Intermediate cell	Normal cell	-1.91833	1.44810	.553	-5.7958	1.9591
			Mucous cell	1.70250	1.40212	.622	-2.0518	5.4568
			Squamous cell	5.67135*	1.43411	.002	1.8313	9.5113
		Mucous cell	Normal cell	-3.62083*	.95783	.003	-6.1855	-1.0561

		Intermediate cell	-1.70250	1.40212	.622	-5.4568	2.0518
		Squamous cell	3.96885*	.93654	.001	1.4611	6.4765
	Squamous cell	Normal cell	-7.58968*	1.00408	.000	-10.2782	-4.9011
		Intermediate cell	-5.67135*	1.43411	.002	-9.5113	-1.8313
		Mucous cell	-3.96885*	.93654	.001	-6.4765	-1.4611
Scheffe	Normal cell	Intermediate cell	1.91833	1.44810	.628	-2.3031	6.1398
		Mucous cell	3.62083*	.95783	.006	.8286	6.4131
		Squamous cell	7.58968*	1.00408	.000	4.6626	10.5167
	Intermediate cell	Normal cell	-1.91833	1.44810	.628	-6.1398	2.3031
		Mucous cell	1.70250	1.40212	.690	-2.3849	5.7899
		Squamous cell	5.67135*	1.43411	.004	1.4907	9.8520
	Mucous cell	Normal cell	-3.62083*	.95783	.006	-6.4131	-.8286
		Intermediate cell	-1.70250	1.40212	.690	-5.7899	2.3849
		Squamous cell	3.96885*	.93654	.002	1.2387	6.6990
	Squamous cell	Normal cell	-7.58968*	1.00408	.000	-10.5167	-4.6626
		Intermediate cell	-5.67135*	1.43411	.004	-9.8520	-1.4907
		Mucous cell	-3.96885*	.93654	.002	-6.6990	-1.2387
Bonferroni	Normal cell	Intermediate cell	1.91833	1.44810	1.000	-2.0963	5.9330
		Mucous cell	3.62083*	.95783	.003	.9654	6.2763
		Squamous cell	7.58968*	1.00408	.000	4.8060	10.3733
	Intermediate cell	Normal cell	-1.91833	1.44810	1.000	-5.9330	2.0963
		Mucous cell	1.70250	1.40212	1.000	-2.1847	5.5897
		Squamous cell	5.67135*	1.43411	.002	1.6955	9.6472

		Mucous cell	Normal cell	-3.62083*	.95783	.003	-6.2763	-.9654
			Intermediate cell	-1.70250	1.40212	1.000	-5.5897	2.1847
			Squamous cell	3.96885*	.93654	.001	1.3724	6.5653
		Squamous cell	Normal cell	-7.58968*	1.00408	.000	-10.3733	-4.8060
			Intermediate cell	-5.67135*	1.43411	.002	-9.6472	-1.6955
			Mucous cell	-3.96885*	.93654	.001	-6.5653	-1.3724
mCmC	Tukey HSD	Normal cell	Intermediate cell	-.29750	3.86087	1.000	-10.6354	10.0404
			Mucous cell	2.33812	2.55372	.797	-4.4998	9.1760
			Squamous cell	-.37654	2.67703	.999	-7.5446	6.7915
		Intermediate cell	Normal cell	.29750	3.86087	1.000	-10.0404	10.6354
			Mucous cell	2.63563	3.73827	.895	-7.3740	12.6453
			Squamous cell	-.07904	3.82356	1.000	-10.3171	10.1590
		Mucous cell	Normal cell	-2.33812	2.55372	.797	-9.1760	4.4998
			Intermediate cell	-2.63563	3.73827	.895	-12.6453	7.3740
			Squamous cell	-2.71466	2.49697	.699	-9.4006	3.9713
		Squamous cell	Normal cell	.37654	2.67703	.999	-6.7915	7.5446
			Intermediate cell	.07904	3.82356	1.000	-10.1590	10.3171
			Mucous cell	2.71466	2.49697	.699	-3.9713	9.4006
	Scheffe	Normal cell	Intermediate cell	-.29750	3.86087	1.000	-11.5526	10.9576
			Mucous cell	2.33812	2.55372	.840	-5.1064	9.7827
			Squamous cell	-.37654	2.67703	.999	-8.1805	7.4275
		Intermediate cell	Normal cell	.29750	3.86087	1.000	-10.9576	11.5526
			Mucous cell	2.63563	3.73827	.919	-8.2621	13.5333

			Squamous cell	-.07904	3.82356	1.000	-11.2254	11.0673
		Mucous cell	Normal cell	-2.33812	2.55372	.840	-9.7827	5.1064
			Intermediate cell	-2.63563	3.73827	.919	-13.5333	8.2621
			Squamous cell	-2.71466	2.49697	.758	-9.9938	4.5644
		Squamous cell	Normal cell	.37654	2.67703	.999	-7.4275	8.1805
			Intermediate cell	.07904	3.82356	1.000	-11.0673	11.2254
			Mucous cell	2.71466	2.49697	.758	-4.5644	9.9938
	Bonferroni	Normal cell	Intermediate cell	-.29750	3.86087	1.000	-11.0012	10.4062
			Mucous cell	2.33812	2.55372	1.000	-4.7417	9.4179
			Squamous cell	-.37654	2.67703	1.000	-7.7982	7.0451
		Intermediate cell	Normal cell	.29750	3.86087	1.000	-10.4062	11.0012
			Mucous cell	2.63563	3.73827	1.000	-7.7282	12.9994
			Squamous cell	-.07904	3.82356	1.000	-10.6793	10.5212
		Mucous cell	Normal cell	-2.33812	2.55372	1.000	-9.4179	4.7417
			Intermediate cell	-2.63563	3.73827	1.000	-12.9994	7.7282
			Squamous cell	-2.71466	2.49697	1.000	-9.6371	4.2078
		Squamous cell	Normal cell	.37654	2.67703	1.000	-7.0451	7.7982
			Intermediate cell	.07904	3.82356	1.000	-10.5212	10.6793
			Mucous cell	2.71466	2.49697	1.000	-4.2078	9.6371
Partialmet	Tukey HSD	Normal cell	Intermediate cell	2.21833	3.45046	.917	-7.0207	11.4574
			Mucous cell	1.28208	2.28226	.943	-4.8290	7.3931
			Squamous cell	7.96468*	2.39246	.010	1.5586	14.3708
		Intermediate cell	Normal cell	-2.21833	3.45046	.917	-11.4574	7.0207
			Mucous cell	-.93625	3.34089	.992	-9.8819	8.0094

		Squamous cell	5.74635	3.41712	.346	-3.4034	14.8961
	Mucous cell	Normal cell	-1.28208	2.28226	.943	-7.3931	4.8290
		Intermediate cell	.93625	3.34089	.992	-8.0094	9.8819
		Squamous cell	6.68260*	2.23154	.023	.7074	12.6578
	Squamous cell	Normal cell	-7.96468*	2.39246	.010	-14.3708	-1.5586
		Intermediate cell	-5.74635	3.41712	.346	-14.8961	3.4034
		Mucous cell	-6.68260*	2.23154	.023	-12.6578	-.7074
Scheffe	Normal cell	Intermediate cell	2.21833	3.45046	.937	-7.8404	12.2770
		Mucous cell	1.28208	2.28226	.957	-5.3711	7.9353
		Squamous cell	7.96468*	2.39246	.019	.9902	14.9391
	Intermediate cell	Normal cell	-2.21833	3.45046	.937	-12.2770	7.8404
		Mucous cell	-.93625	3.34089	.994	-10.6755	8.8030
		Squamous cell	5.74635	3.41712	.429	-4.2152	15.7079
	Mucous cell	Normal cell	-1.28208	2.28226	.957	-7.9353	5.3711
		Intermediate cell	.93625	3.34089	.994	-8.8030	10.6755
		Squamous cell	6.68260*	2.23154	.042	.1773	13.1879
	Squamous cell	Normal cell	-7.96468*	2.39246	.019	-14.9391	-.9902
		Intermediate cell	-5.74635	3.41712	.429	-15.7079	4.2152
		Mucous cell	-6.68260*	2.23154	.042	-13.1879	-.1773
Bonferroni	Normal cell	Intermediate cell	2.21833	3.45046	1.000	-7.3476	11.7842
		Mucous cell	1.28208	2.28226	1.000	-5.0452	7.6093
		Squamous cell	7.96468*	2.39246	.011	1.3319	14.5974
	Intermediate cell	Normal cell	-2.21833	3.45046	1.000	-11.7842	7.3476
		Mucous cell	-.93625	3.34089	1.000	-10.1984	8.3259

			Squamous cell	5.74635	3.41712	.601	-3.7271	15.2198
		Mucous cell	Normal cell	-1.28208	2.28226	1.000	-7.6093	5.0452
			Intermediate cell	.93625	3.34089	1.000	-8.3259	10.1984
			Squamous cell	6.68260*	2.23154	.028	.4960	12.8692
		Squamous cell	Normal cell	-7.96468*	2.39246	.011	-14.5974	-1.3319
			Intermediate cell	-5.74635	3.41712	.601	-15.2198	3.7271
			Mucous cell	-6.68260*	2.23154	.028	-12.8692	-.4960
uCuC	Tukey HSD	Normal cell	Intermediate cell	-1.91833	1.44810	.553	-5.7958	1.9591
			Mucous cell	-3.62083*	.95783	.003	-6.1855	-1.0561
			Squamous cell	-7.58968*	1.00408	.000	-10.2782	-4.9011
		Intermediate cell	Normal cell	1.91833	1.44810	.553	-1.9591	5.7958
			Mucous cell	-1.70250	1.40212	.622	-5.4568	2.0518
			Squamous cell	-5.67135*	1.43411	.002	-9.5113	-1.8313
		Mucous cell	Normal cell	3.62083*	.95783	.003	1.0561	6.1855
			Intermediate cell	1.70250	1.40212	.622	-2.0518	5.4568
			Squamous cell	-3.96885*	.93654	.001	-6.4765	-1.4611
		Squamous cell	Normal cell	7.58968*	1.00408	.000	4.9011	10.2782
			Intermediate cell	5.67135*	1.43411	.002	1.8313	9.5113
			Mucous cell	3.96885*	.93654	.001	1.4611	6.4765
	Scheffe	Normal cell	Intermediate cell	-1.91833	1.44810	.628	-6.1398	2.3031
			Mucous cell	-3.62083*	.95783	.006	-6.4131	-.8286
			Squamous cell	-7.58968*	1.00408	.000	-10.5167	-4.6626
		Intermediate cell	Normal cell	1.91833	1.44810	.628	-2.3031	6.1398
			Mucous cell	-1.70250	1.40212	.690	-5.7899	2.3849

		Squamous cell	-5.67135*	1.43411	.004	-9.8520	-1.4907
	Mucous cell	Normal cell	3.62083*	.95783	.006	.8286	6.4131
		Intermediate cell	1.70250	1.40212	.690	-2.3849	5.7899
		Squamous cell	-3.96885*	.93654	.002	-6.6990	-1.2387
	Squamous cell	Normal cell	7.58968*	1.00408	.000	4.6626	10.5167
		Intermediate cell	5.67135*	1.43411	.004	1.4907	9.8520
		Mucous cell	3.96885*	.93654	.002	1.2387	6.6990
Bonferroni	Normal cell	Intermediate cell	-1.91833	1.44810	1.000	-5.9330	2.0963
		Mucous cell	-3.62083*	.95783	.003	-6.2763	-.9654
		Squamous cell	-7.58968*	1.00408	.000	-10.3733	-4.8060
	Intermediate cell	Normal cell	1.91833	1.44810	1.000	-2.0963	5.9330
		Mucous cell	-1.70250	1.40212	1.000	-5.5897	2.1847
		Squamous cell	-5.67135*	1.43411	.002	-9.6472	-1.6955
	Mucous cell	Normal cell	3.62083*	.95783	.003	.9654	6.2763
		Intermediate cell	1.70250	1.40212	1.000	-2.1847	5.5897
		Squamous cell	-3.96885*	.93654	.001	-6.5653	-1.3724
	Squamous cell	Normal cell	7.58968*	1.00408	.000	4.8060	10.3733
		Intermediate cell	5.67135*	1.43411	.002	1.6955	9.6472
		Mucous cell	3.96885*	.93654	.001	1.3724	6.5653

*. The mean difference is significant at the 0.05 level.

VITAE

I, Miss Porntipa Sirivanichsuntorn was born on 1st October 1982 in Bangkok, Thailand. I received the degree of Doctor of Dental Surgery from Chulalongkorn University, Thailand in March, 2006. After that I worked as a dentist in Kanchanaburi province, Thailand until 2009 in which I started persuing Master Degree in Oral and Maxillofacial surgery at the Department of Oral and Maxillofacial Surgery, Faculty of Dentistry, Chulalongkorn University. My research interest was the molecular biology of 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.