Epigenetic Alterations in Endometrial Cancer and It's Precursors

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Promoter hypermethylation of Adenomatous Polyposis Coli gene in endometrial hyperplasia and

carcinoma in Proceedings of American Association for Cancer Research (94th Annual Meeting).

Washington D.C., USA. 2003.

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Microsatellite instability associated with Grading and Staging of Endometrial cancer and inversely

correlated with Methylation index in Proceedings of American Association for Cancer Research

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Lists of Abbreviations

APC – adenomatous polyposis coli

Cancerogenesis - a process of cancer formation. Can be simply explained as excessive and uncontrolled multiplication of cells.

Differentiation - a process by which cells become "specialized" for certain functions. Genetic instruction is the same in all cells in the body, but they only "read" certain "chapters". For instance, genetic material in blood cells and skin cells is the same, but different parts of genetic code are active in each cell type.

DNA – deoxyribonucleic acis

DNMT – DNA methyltransferase

dNTP – deoxynucleoside triphosphate

EAH – endometrial atypical hyperplasia

ECA – endometrial cancer

EDTA – ethylenediamine tetraacetic acid

EH – endometrial hyperplasia

Endometrium - uterine lining

FBS – fetal bovine serum

GSTP1 – glutathione S transferase P1

HDAC – histone deacetylase

HIC-1 – hypermethylated in cancer 1

Hormones - substances in the body that induce cells to behave in certain way. Each hormone makes cells behave differently.

hMLH-1 – human MutL homologue

HNPCC – hereditary non-polyposis colon cancer

kb – kilobase

LOH – loss of heterozygosity

MGMT – O6-methylguanine-DNA methyltransferase

Maturation - could be taken as a synonym for differentiation.

Metastases - cells that get detached from the main tumor may be taken to other parts of the body by lymphatic vessels or blood vessels (veins). Also, they can be scattered around the peritoneal cavity and form so called peritoneal metastases.

MSI – microsatellite instability

MSP – methylation-specific polymerase chain reaction

PBS – phosephate-buffered saline

PCR – polymerase chain reaction

PRB – progesterone receptor B

PTEN - phosphate and tensin homologue deleted on chromosome ten

TBE – tris borate EDTA

TGF-b – transforming growth factor beta

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Epigenetic Alterations in Endometrial Cancer and It's Precursors

Abstract

Endometrial cancer is a common malignancy of female genital tract. Endometrioid adenocarcinoma (ECA) is the most common type of endometrial cancer. Microsatellite instability (MSI) is observed in a subset of ECA while chromosomal abnormality or instability is believed to be uncommon event. Recently, gene promoter hypermethylation is being increasing recognized as a common and important mechanism lead to gene silencing. Information in gene promoter hypermethylation in ECA is limited. We aim to investigate methylation status of multiple genes in ECA. In addition, endometrial hyperplasia (EH) with or without atypia, which is regarded as precursor lesion of ECA, will also be examined. Furthermore, MSI status of ECA is determined, and the possible relationship with gene hypermethylation is also assessed.

Our study delineated the frequency of methylation of multiple genes in ECA. The genes being investigated include ACP, hMLH1, RASSF1A, PRB, PTEN, ATM, MGMT, HIC1, E-cad and p16. The precursor lesion, endometrial hyperplasia, further divided into endometrial hyperplasia with or without cytological atypia, and the methylation frequency of multiple genes also be determined. The methylation index (ratio or methylated gene over total numbers of gene examined) of ECA, EH with atypia and EH without atypia were 0.20, 0.29 and 0.19 respectively. Gene methylation in at least one of the genes is common. Our results indicated that gene methylation was also common in endometrial hyperplasia. It is suggested that gene methylation may be an early event in carcinogenesis of ECA. MSI status of ECA was determined and twenty-six cases (76.5%) were microsatellite stable (MSS) and 8 cases (23.5%) were MSI positive. Our study found that MSI positive cases correlate with pathological grading and staging of ECA. Interestingly, ECA cases with MSI has lower frequency of gene methylation (as assessed by methylation index) as compare to microsatellite stable (MSS) cases. The observations suggest that MSI positive and MSS cases may have different carcinogenic pathways.

摘要

子宮內膜癌是女性生殖器惡性病中普遍的一種。其中以子宮內膜腺癌最為普遍。有研究發現部份子宮內膜癌案例出現微衛星脫氧核糖核酸不穩定,而染色體不正常或不穩定是不普遍的。近來,有研究指出基因的啓動子高度甲基化是普遍而重要的機制引至基因靜止。至今,子宮內膜癌的基因啓動子高度甲基化的研究有限。故此,此研究目的是研究子宮內膜癌的多個基因的啓動子高度甲基化。另外,此研究包括子宮內膜增生及子宮內膜異常增生,它們都被視為子宮內膜癌的前期損傷。此外,子宮內膜癌的微衛星脫氧核糖核酸不穩定,以及其與基因啓動子高度甲基化會的關係會被研究。

此研究包括子宮內膜癌的多個基因的啓動子高度甲基化,研究的基因包括 ACP, hMLH1, RASSF1A, PRB, PTEN, ATM, MGMT, HIC1, E-cad 及 p16。子宮內膜癌的前期損傷,子宮內膜增生分為子宮內膜增生及子宮內膜異常增生,其基因的啓動子高度甲基化亦會被研究。子宮內膜癌,子宮內膜異常增生及子宮內膜增生的平均甲基化指數(甲基化基因的數目除以研究基因的數目)分別為 020, 0.29 及 0.19。最少一個基因出現甲基化是很普遍。結果亦指出子宮內膜增生的基因甲基化是很普遍,因此基因甲基化可能是形成子宮內膜癌的早期變異。微衛星脫氧核糖核酸不穩定在子宮內膜癌的研究顯示有廿六個案例(百份之七十六點五)是微衛星穩定,而八個(百份之廿三點五)是微衛星不穩定。此研究發現微衛星不穩定與子宮內膜腫瘤的分級及分期是有關。有趣地,根據甲基化指數的研究結果,微衛星不穩定的子宮內膜癌出現較少基因甲化,而微衛星穩定的子宮內膜癌則出現較多基因甲基化。此研究發現微衛星穩定與微衛星不穩定的子宮內膜癌可能是從不同的途徑形成。

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Chapter 1 Introduction

Endometrial cancer is a common malignancy of female genital tract. Majority of endometrial cancer are endometrioid adenocarcinoma (ECA). Endometrial hyperplasia (EH) is regarded as the precursor lesion of ECA. The association of ECA and EH with estrogen is well recognized. However, the carcinogenesis process is still poorly understood. A subset of ECA exhibits microsatellite instability (MSI). But it is uncertain if there are clinical or patho-biological important differences between ECA with and without MSI. In contrast, chromosomal abnormalities, in terms of chromosomal gains or loss appear to be uncommon event. Recently, gene promoter hypermethylation is being increasing recognized as a common and important mechanism lead to gene silencing. Information in gene promoter hypermethylation in ECA is limited. We aim to investigate methylation status of multiple genes in ECA. In addition, endometrial hyperplasia, which is regarded as precursor lesion of ECA will also be examined. Furthermore, MSI status of ECA is also determined and the possible relationship with gene hypermethylation is assessed.

Chapter 2 literature Review

2.1 Anatomy and Physiology of Endometrium

Uterus is a hollow muscular internal female reproductive organ located in pelvis behind the bladder and in front of the rectum. The uterus consists of uterine corpus and uterine cervix and both fallopian tubes are attached at the corneal region of uterus (Fig 2.1).The inner surface of the uterus is lined by endometrium which contains endometrial glands among the endometrial stroma. Beneath the endometrium is the myometrium of uterus, which consist of thick bundles of smooth muscle cells. The outer surface of the upper part of uterus is covered by serosa while the lower part is adventitial tissue.
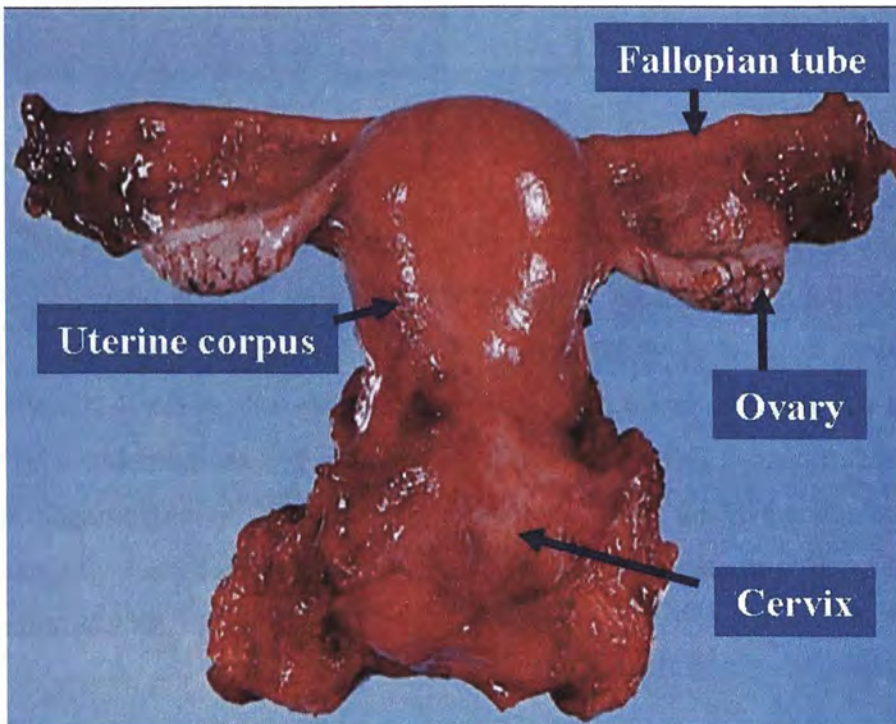
Fig.2.1. The uterus consists of uterine corpus and uterine cervix. Both fallopian tubes attached to the corneal regions of the uterus.
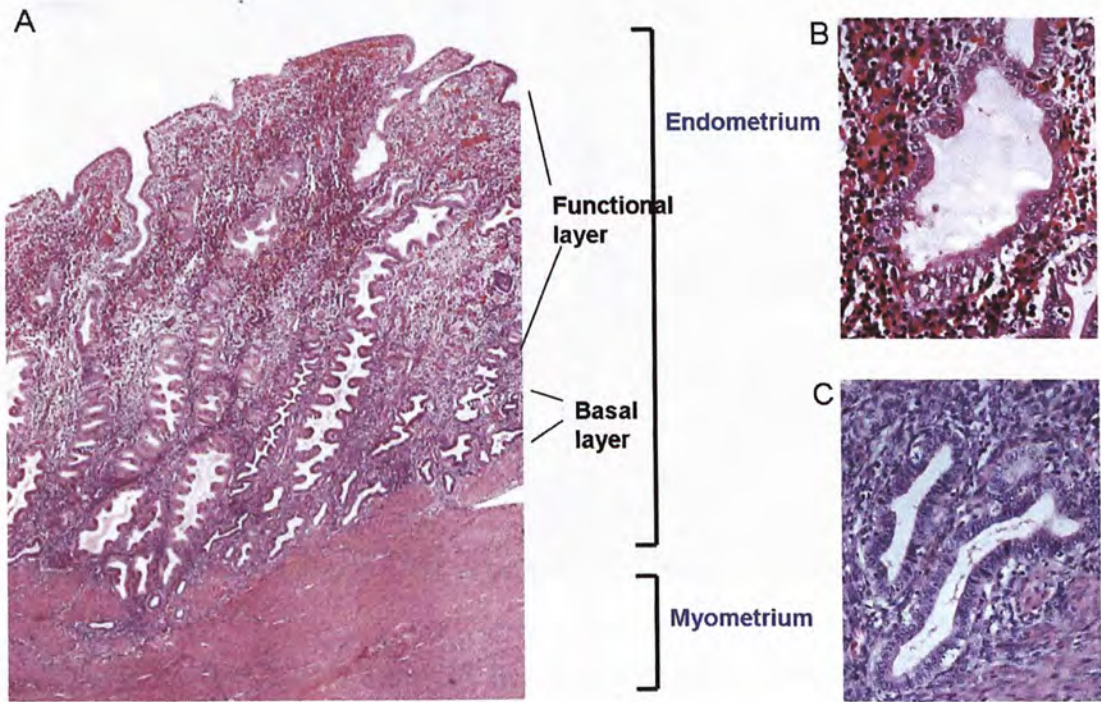
Fig.2.2 A. The endometrium is divided into the upper functional layer and the lower basal layer. The functional layer is sensitive to the hormonal changes while the basal layer is relatively in-sensitive to hormonal alterations. (Hematoxylin & eosin stain, original magnification X 40) B Higher magnification of the functional layer. In this example is secretary type endometrium. (Hematoxylin & eosin stain, original magnification X 200) C. Higher magnification of the basal layer, which consists of inactive endometrial glands and more closely packed endometrial stromal cells. (Hematoxylin & eosin stain, original magnification X 200)
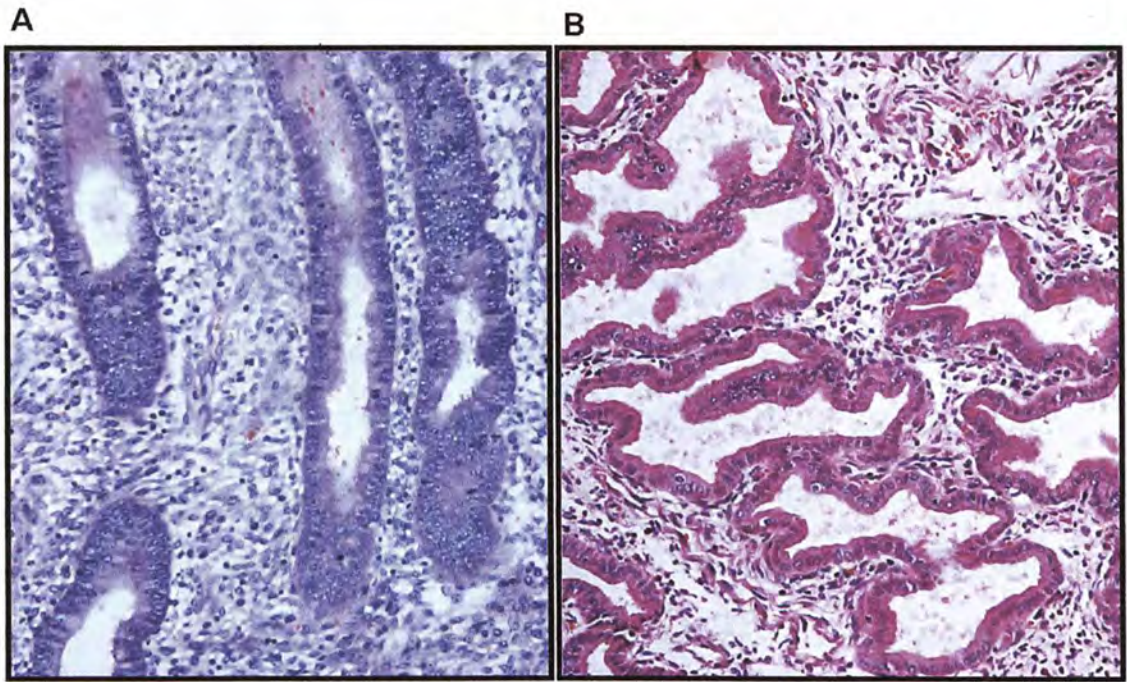
Fig.2.3. A. In the first half of the cycle, the endometrium is in proliferative phase. Both the endometrial glands and endometrial stroma are proliferative. Mitoic activity is easily identified. (Hematoxylin & eosin stain, original magnification X 200) B. After ovulation, the endometrium will enter into secretary phase. The endometrial glands become dilated and tortoused. The glandular lumen contains secretary material. (Hematoxylin & eosin stain, original magnification X 200)

Endometrium is divided into the upper portion of functional layer and lower portion of basal layer (Fig 2.2). The endometrial glands consist of single layered polygonal to columnar glandular epithelium. The endometrial stromal cells exhibit oval to spindle shape dark stained nuclei with scanty indistinct cytoplasm. In reproductive age, the functional layer is very sensitive to hormonal changes and in responds to the cyclic changes of estrogen and progesterone levels. The basal layer is relatively insensitive to hormonal changes. In the first phase of the cycle, the endometrium in responds to

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estrogen stimulation and enters into the proliferate phase. Both the endometrial gland and stroma become mitotically active and proliferate (Fig 2.3). Ovulation occurs at mid-cycle. The endometrium in response to progesterone and enter into the secretary phase. The endometrial glands are active in secretary activity and become dilated and tortured (fig 2.3). If no conception is happened, relating to the lack of hormonal support, the endometrium, especially the functional layer start to break down toward the end of the cycle and menstruation occur. The residual endometrium, mainly the basal layers will then regenerate and enter into proliferative phase of next cycle. However, if there is un-opposed estrogen stimulation, it will lead to uncontrolled proliferation of endometrial glands and resulted in endometrial hyperplasia. Endometrial hyperplasia is considered as precursor lesion of endometrioid adenocarcinoma. Endometrial hyperplasia and endometrial cancer will be discussed in the subsequent sections.

2.2 Endometrial cancer

Endometrial cancer originates in the endometrium of uterus and is one of the most common types of female genital tract malignancies. Majority of endometrial cancer are adenocarcinoma, and endometrioid adenocarcinoma (ECA) represents the most common type. Endometrial hyperplasia (EH) refers to abnormal proliferation of endometrial glands and EH is regarded as precancerous lesion of the endometrium and may progress to invasive endometrioid adenocarcinoma.

2.2.1 Epidemiology of Endometrial Cancer in Hong Kong

Endometrial cancer is one of the most common cancer of female genital tract and the 8th

most common cancer of female in Hong Kong. The crude incidence rate is about 10 per

100,000. Approximately 300 women are diagnosed with endometrial cancer each year in

Hong Kong. About 30 women die due to endometrial cancer each year. Most (~95%) of

the endometrial cancers occur in woman age 40 or older with an average age of 55-65.

(Fig.2.4).

No. of cases
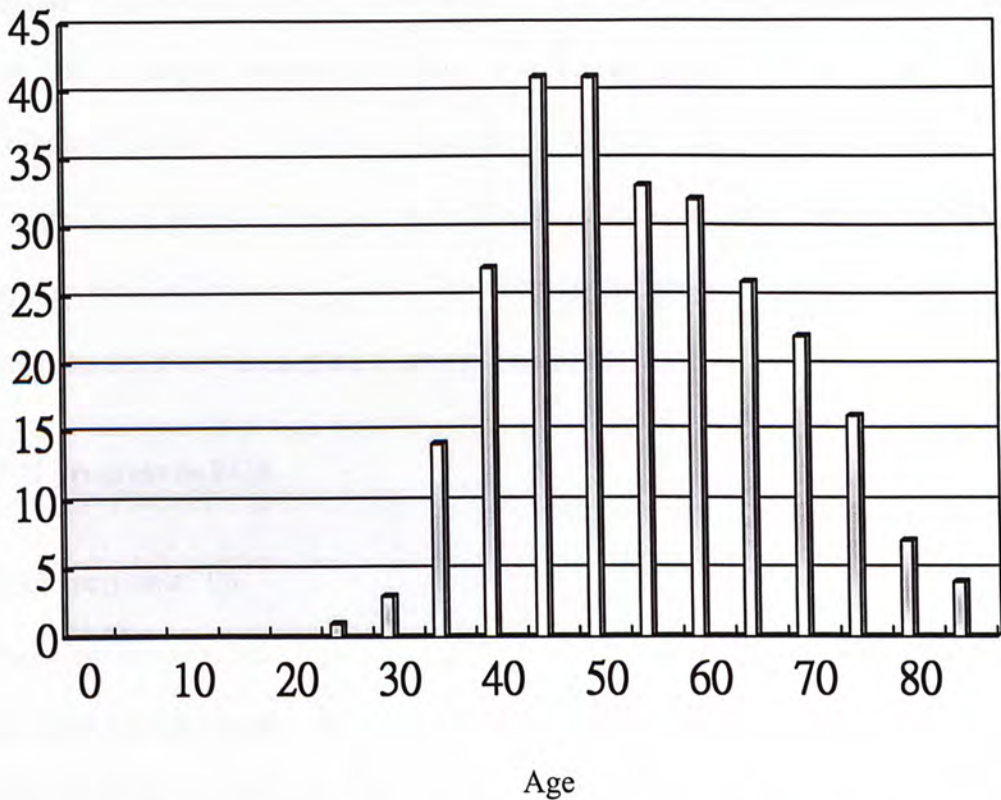
Fig.2.4. The age distribution of new cases of endometrial cancer in Hong Kong

2.2.2 Etiologies and Risk factors

For endometrioid adenocinoma (ECA), the association with unopposed estrogen, either exogenous or endogenous is well recognized, especially for those cases preceded by endometrial hyperplasia (EH). However, there are also cases of ECA without pre-existing EH. The association with estrogen with other types of endometrial cancer, which will be addressed in later section, is not well established.

With unopposed estrogen stimulation, the endometrial glands may exhibit abnormal proliferation and become hyperplastic, resulted in endometrial hyperplasia (EH). There are a spectrum of endometrial hyperplasia. According to the WHO classification, this can be divided into 1. simple hyperplasia without cytological atypia, 2. simple hyperplasia with cytological atypia, 3. complex hyperplasia without cytological atypia and 4. complex hyperplasia with cytological atypia. Simple or complex refer to the architecture of the hyperplastic endometrial glands. The risk of the progression to endometrioid adenocarcinoma does vary according to the severity of EH:

Risk of EH progress to ECA

1. Simple hyperplasia: 1%

2. Complex hyperplasia: 3%

3. Simple atypical hyperplasia: 8%

4. Complex atypical hyperplasia: 29%

Endometrial hyperplasia may regress when the unopposed estrogen stimulation is terminated or patients received progesterone treatment. However, some EH may progress even with medical treatment. The most important determination factor appears related to the presence or absence of cytological atypia (Fig 2.5).
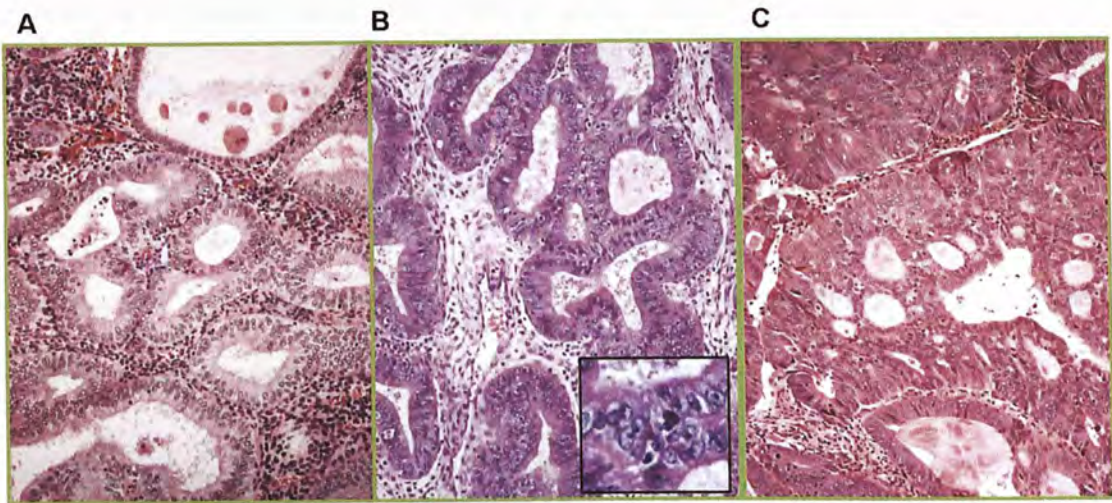
Fig.2.5. The histology of endometrial hyperplasia progress to endometrioid adenocarcinoma. A. Simple endometrial hyperplasia without cytological atypia. The endometrial glands are more closely packed as compare to normal endometrial glands. (Hematoxylin & eosin stain, original magnification X 200) B. Complex endometrial hyperplasia with cytological atypia. The glandular architecture is more complex. Cytological atypia with rounding up of nuclei, vesicular nuclei and prominent nucleoli are shown in the in-box. (Hematoxylin & eosin stain, original magnification X 200) C. Endometrioid adenocarcinoma. Complex cribriform malignant glands are formed.

Risk Factors (Oncology Channel. 2004)

Several risk factors have been identified:

Chronic Estrogen Exposure

➢ **Obesity**

Studies estimated that women with excess body weight have a 2-5x greater risk of developing endometrial cancer. This is likely due to the face that fat cells (adipocytes) produce estrogen.

➢ **Diabetes Mellitus and Hypertension**

The relationship is unclear. Women with a history of diabetes mellitus are 2x more likely to develop endometrial cancer.

➢ **Few or No Children**

Pregnancy is a period of intense progesterone stimulation by the placenta. Progesterone counterbalances the growth-stimulating effects of estrogen.

➢ **Early Menarche and Late Menopause**

Late menopause and early menarche are both associated with more estrogen exposure.

➢ **Estrogen Replacement Therapy**

It is believed that the increase in endometrial cancer in the U.S. in the 1970s was due to the introduction and widespread use of postmenopausal estrogen therapy that is used to relieve the symptoms of menopause.

➢ **Tamoxifen**

Tamoxifen is an anti-estrogenic drug and is the most widely prescribed hormonal treatment for women with breast cancer. However, Tamoxifen is weakly estrogenic and one of the side effects is that it induces uterine cancer, especially endometrial carcinoma.

➢ **Diet**

The association between diet and endometrial cancer is unclear. There is some evidence to suggest that a diet rich in animal fat and protein puts a woman at a greater risk, whereas a diet rich in vegetable, fruits, and whole-grain food reduces the risk.

Previous Cancer

Women who had history of cancer of the breast, colon, or ovary are at an increased risk for developing endometrial cancer.

Genetic Predisposition

Some women appear to have a genetic predisposition to endometrial cancer. In some families who have an inherited tendency to develop colon cancer called hereditary non-polyposis colon cancer (HNPCC). Thus this subset of endometrial carcinoma is related to microsatellite instability.

Factors associated with reduced Risk

➢ **Tobacco Smoking**

There is some evidence that tobacco smoking actually reduces the rate of endometrial cancer. This is likely due to the fact that smokers tend to have lower levels of estrogen and a lower rate of obesity.

➢ **Oral Contraceptives**

Longer use of combined oral contraceptives affords better protection from endometrial cancer. The reduced risk is presumably due to the progesterone effects of the contraceptives counterbalance estrogen's effect on endometrial cells.

2.3 Pathology

Endometrial Cancer

Majority of endometrial cancers are adenocarcinomas, cancers with differentiation toward glandular cells.

Classification of endometrial carcinoma:

- Endometrioid adenocarcinoma (about 75 percent)

- Serous adenocarcinoma (about 5 - 10 percent)

-Clear cell adenocarcinoma (less than 5%)

Others: including mucinous adenocarcinoma, Squamous cell carcinoma, Mixed carcinoma, Undifferentiated carcinoma, Malignant mixed mullerian tumors (MMMT).

There are less common uterine cancers, including:Stromal sarcomas & Leiomyosarcomas.
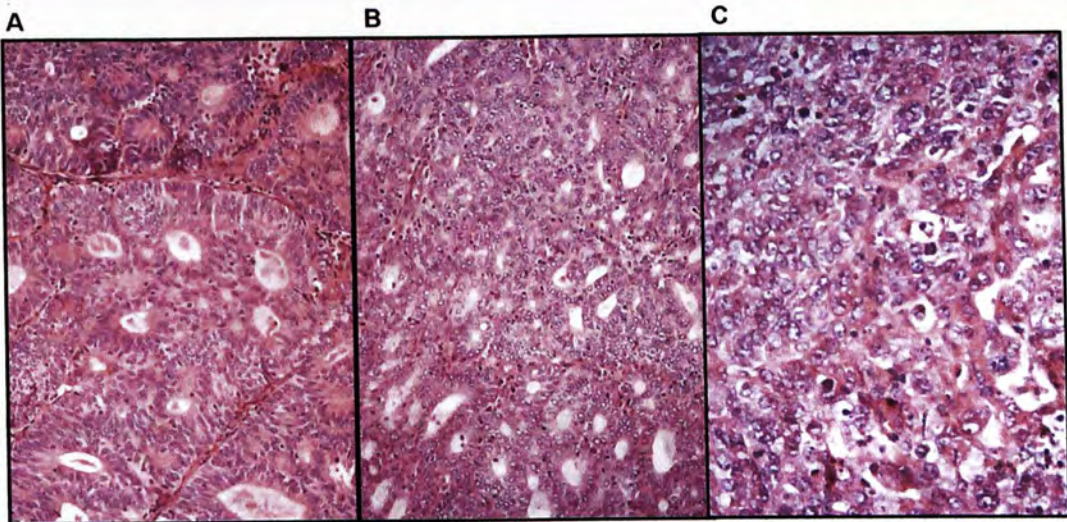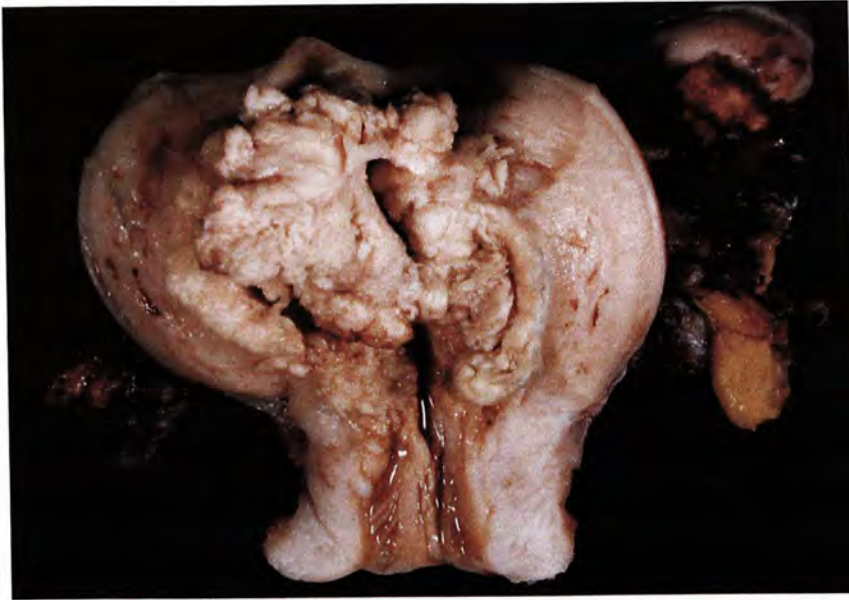
Fig.2.6. Upper panel. Gross appearance of a case of endometrial cancer. This case consists of endometrioid adenocarcinoma, forms a polypoid tumoral mass protruding into the endometrial cavity and invades into the underlying myometrium. Lower panel. FIGO grade 1 to 3 endometrioid adenocarcinoma (ECA) A. Grade 1. well differentiated ECA B. Grade 2. moderate differentiated ECA with solid area. C. Grade 3. poorly differentiated ECA with mainly solid component. (Hematoxylin & eosin stain, original magnification X 200)
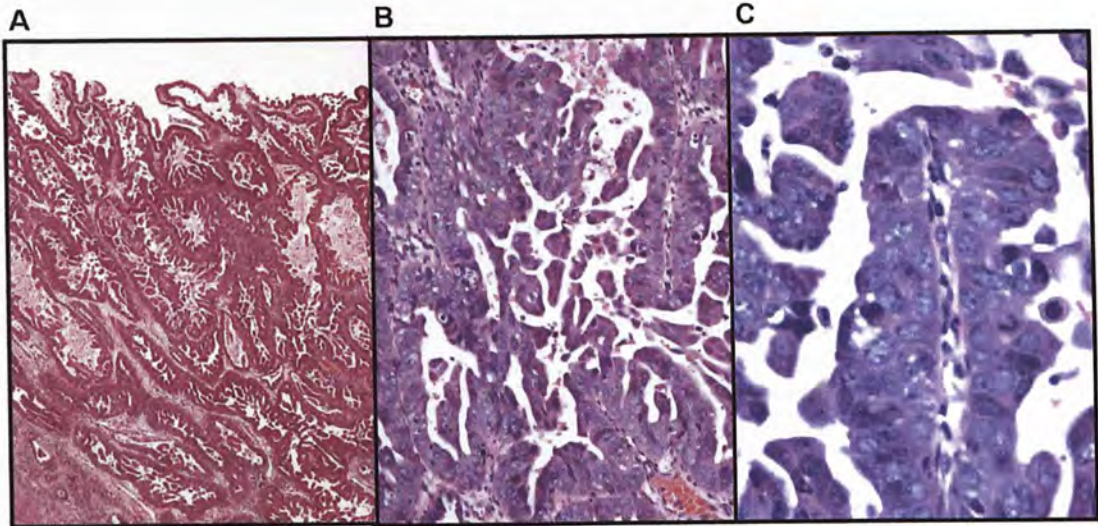
Fig.2.7. Serous adenocarcinoma. Serous adenocarcinoma is high grade adenocarcinoma and forming distinct papillary architecture with prominent nuclear atypia. A. Low magnification displaces the papillary architecture. (Hematoxylin & eosin stain, original magnification X 40) B. Higher magnification reveals the presence of small papillary tufts. (Hematoxylin & eosin stain, original magnification X 200) C. Prominent cytological atypia is evident. (Hematoxylin & eosin stain, original magnification X 400)

Endometrioid Adenocarcinoma (ECA) (Fig 2.6)

Endometrioid adenocarcinoma is the most common type of endometrial cancer and comprises about 75% of cases. The typical ECA is related to unopposed estrogenic drive and often preceded by endometrial hyperplasia. This group tends to be well differentiated and occurs in perimenopausal period. Some cases occur in old age, seemingly not related to estrogen stimulation and arising in atrophic endometrium. This group less frequently well differentiated. There are also some histological variants, including adenosqumaous carcinoma, secretary carcinoma, ciliated carcinoma, villoglandular carcinoma and sertoliform carcinoma.

Serous adenocarcinoma (Fig 2.7)

Serous adenocarcinoma, comprises 5 to 10 % of endometrial cancer. It is typically a high grade cancer and occurs in elderly. It has the propensity to have prominent myometrial and lympho-vascular invasion.

2.3.1 Grading of endometrial cancer

Grading of endometrioid adenocarcinoma is according to the International Federation of Gynecology and Obstetrics (FIGO) grading system.

- Grade 1: <5% of non-squamous, non-morular solid growth pattern.

- Grade 2 : > 5% to 50% of non-squamous, non-morular solid growth pattern.

- Grade 3: > 50% of non-squamous, non-morular solid growth pattern.

The presence of notable cytological atypia, which is more marked as compare to the grade, the grading can upgrade for one grade.

For serous adenocarcinoma is always considered as high grade cancer. The grading of other types of carcinoma is less well established.

2.3.2 Staging of endometrial cancer (Cancerlinks, 2003)

Endometrial cancers are staged according to the approved system in 1988 by the International Federation of Gynecology and Obstetrics (FIGO). Factors used to stage the disease include the depth of the myometrial invasion, whether the tumor has spread to the cervix and other nearby organs, whether it has metastasized to the lymph nodes or distant metastasis.

Stage 1:

1a - tumor is restricted to endometrium.

1b - it invades less than one half of the myometrium.

1c - it invades more than one half of the myometrium.

Stage 2:

2a – invades cervical glands.

2b – invades cervical stroma.

Stage 3:

3a - uterine serosa is affected and/or adnexa (tubes and ovaries) or there are tumor cells found in the abdomen.

3b - tumor extends to vagina, upper two thirds.

3c - lymphatic nodes are affected, paraaortal or pelvic.

Stage 4:

4a - urinary bladder and/or intestines (rectum) are affected.

4b - abdominal or inguinal lymphatic nodes are affected.
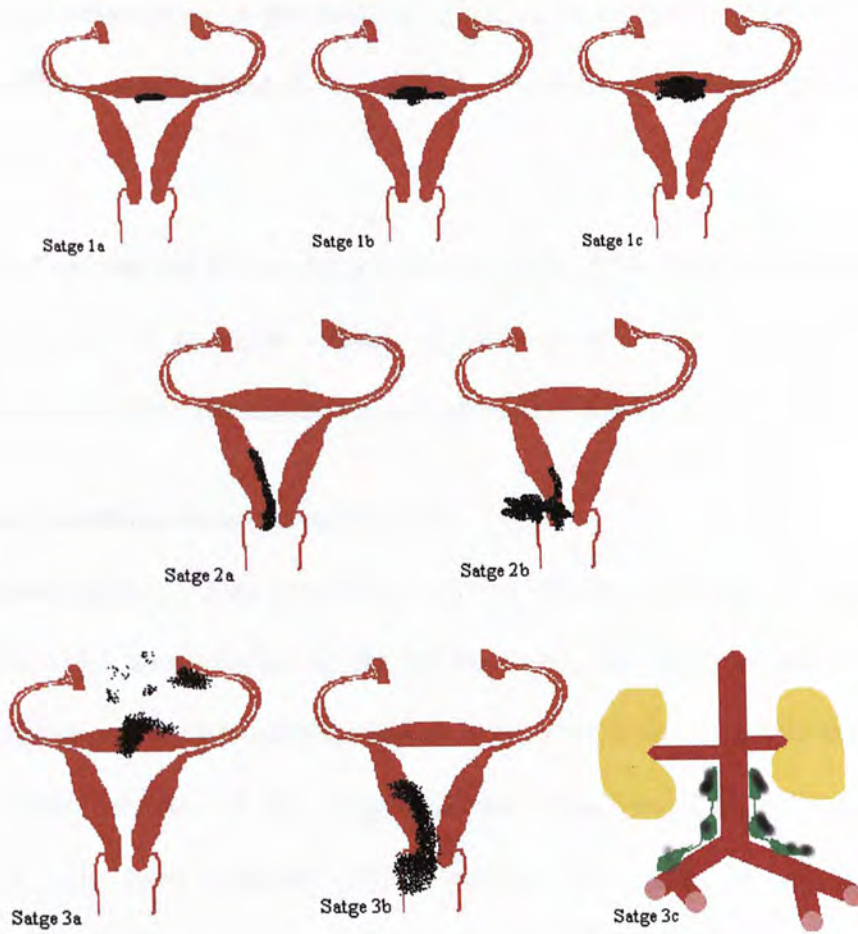
Fig.2.8. The different stages of endometrial cancer. (canerlinks, 2003)

2.4 Prevention and Treatment

The prognosis is largely depending on grading and staging of cancer. The average 5-year survival rates for endometrial cancer are 90% for Stage I, 60% for Stage II, 40% for Stage III, and 5% for Stage IV. (cancerlinks, 2003) Early detection is the best prevention from developing invasive endometrial cancer. Endometrioid adenocarcinoma may proceeded by endometrial hyperplasia. Patient with endometrial hyperplasia may present with abnormal vaginal bleeding. The diagnosis of endometrial hyperplasia may be confirmed by endometrial sampling or uterine dilation and curettage (D & C). Thus, early

treatment of the endometrial hyperplasia by progesterone containing medication or endometrial resection may prevent the progression of disease to invasive endometrioid adenocarcinoma.

The treatment of endometrial cancer depends on the stage of the disease and the general condition of patients. It includes surgical excision, hysterectomy with or without radiation and chemotherapy. Hormonal therapy may also be used.

2.5 Molecular alterations in endometrial cancer

The carcinogenesis pathway may be different among different subtypes of endometrial cancer. Endometrioid adenocarcinoma (ECA) represents the most common type of endometrial cancer and serous adenocarcinoma represents a small subset but having aggressive clinical behavior. It was suggested that these two types of endometrial carcinoma not only have different clinical behavior, but also the carcinogenetic mechanisms are also different. According to Deligdisch and Holinka, they proposed there are two types of endometrial carcinoma (Berchuck A., 1997). Type I is endometrioid adenocarcinoma (ECA) which is estrogen-dependent and appears mostly in pre- and peri-menopausal women, it is well differentiated, early stage and therefore having favorable prognosis. Most of the endometrial cancers are typical endometrioid adenocarcinomas (ECA).

Type II is non-endometrioid adenocarcinoma. In general, it is estrogen independent, diagnosed mostly in postmenopausal women. They are associated with older age, poor differentiation, greater risk for metastases, advanced stage, and hence unfavorable prognosis.

The molecular alterations associated with type 1 and type 2 endometrial carcinomas appears different (Berchuck A., 1997). These molecular features are illustrated in table 2.1.

Table 2.1. Clinical and Molecular Features of Endometrial Carcinoma

	Type I	Type II
Clinical Features		
Risk factors	Unopposed estrogen	Age
Race	White > Black	White = Black
Degree of differentiation	Well-differentiated	Poorly differentiated
Histology	Endometrioid	Nonendometrioid
Stage	I/II	III/IV
Prognosis	Favorable	Unfavorable
Molecular Features *		
Ploidy	Diploid	Aneuploid
K-ras overexpression	Yes	Yes
HER-2/neu overexpression	No	Yes
p53 overexpression	No	Yes
PTEN mutations	Yes	No
Microsatellite instability	Yes	No

2.5.1 Genetic alterations in endometrial cancer

The molecular events involved in the carcinogenesis of endometrial carcinoma are poorly defined; however, several aspects of the molecular pathology of this disease were elucidated.

2.5.1.1 Oncogene activation

Oncogene activation in endometrial cancer

HER-2/*neu*

The HER-2/*neu* gene encodes for a tyrosine kinase receptor that is activated following binding of the ligand heregulin (Berchuck A., 1997). Several studies suggest that this oncogene product is overexpressed in 10 to 15% of endometrial cancers (Lukes A.S., *et al.,* 1994; Monk B.J., *et al.,* 1994; Wang D., *et al,* 1995; Khalifa M.A., *et al.,* 1994).

Herzel and co-workers, (Hetzel D.J., *et al.,* 1992.) in an immunohistochemical analysis of 247 patients with endometrial cancer, observed 15% strong staining, 58% mild staining, and 27% no staining. The 5-year progression-free survival was 56% for the strong staining, 83% for the mild staining, and 95% for the nonstaining groups. Strong overexpression was associated with a poor (51%) overall survival.

Likewise, Lukes and co-workers (Lukes A.S., *et al.,* 1994) found high expression in 12% of patients. Overexpression was more common in stage III and IV patients (24%) than in stage I or II patients (6%) and was associated with poor progression-free survival in univariate analysis.

Ras

The *ras* family of G-proteins (N, H, K-*ras*) play a critical role in the regulation of cellular proliferation (Berchuck A., 1997). The most frequent site of mutation of the K-*ras* oncogene in endometrial carcinoma is codon 12. K-*ras* mutation is detected in

endometrial carcinoma, but this does not appear associated with survival in endometrial cancer (Enomoto T., *et al.*, 1993; Duggan B., *et al.*, 1994; Sasaki H., *et al.*, 1993).

2.5.1.2 Tumor suppressor genes inactivation

Tumor suppressor genes are inactivated by mutation, LOH or epigenetic alterations such as promoter hypermethylation.

Tumor suppressor genes may be divided into two groups:

1. Growth regulating tumor suppressor gene (regulating cell growth or apoptosis)

2. DNA repair type tumor suppressor gene (DNA damage recognition and repair)

Tumor suppressor genes (Table 2.2) such as *p53*, *APC*, and *INK4a* appear to have active roles in regulating cell growth and/or apoptosis, the DNA damage recognition and repair genes can arguably be viewed as having roles in controlling growth. Inactivation of DNA mismatch repair activity likely contributes to cancer via an increased frequency of mutations in other cellular genes, particularly genes that are rate-determining in tumor development. The DNA mismatch repair genes appear to be inactivated in a considerable subset of sporadic cancers, including roughly 10 to 20% of colorectal, endometrial, and gastric cancers.

Table 2.2. A list of tumor suppressor genes

Gene	Cancers associated	Presumed function of protein
GSTP-1	Breast, prostate cancer	It encodes the detoxification protein glutathion-S-transferase placental enzyme1
APC	Colorectal cancer, desmoid tumors	Regulates levels of β-catenin protein in cytosol binding to microtubules
MGMT	Colon, lung, pancreas cancer	O6-methylguanine-DNA methyltransferase, DNA repair enzyme
p15	Gastric cancer	An inhibitor of cyclin-dependent kinase 4, which is an important mediator of cell cycle control, especially in the pathway stimulated by TGF-β , DAPK, is involved in apoptosis.
p16	Approx. 25-30% of many different cancer types, Breast, bladder, lung cancer and leukemia	Cyclin-dependent kinase inhibitor (i.e., Cdk4 and Cdk6)
E-cad	Gastric (Diffuse type), lobular breast carcinoma, rare in other type, e.g., ovarian	Cell-cell adhesion molecule, potential invasion and metastasis suppressor
SOCS-1	Gastric cancer, hepatocellular carcinoma (HCC)	Suppressor of cytokine signaling, a component in IL-6 pathway inhibit STAT3 phosphorylation through direct interaction of Jak2
HIC-1	Breast, colon, brain cancer	Hypermethylated in cancer, a transcriptional repressor to suppress cell growth
HLTF	Colon cancer	Helicase-like transcription factor, a SWI/SNF family protein
PRB	Endometrial, breast cancer	Progesterone receptor B, response to estrogen and progesterone which has antiproliferative effect.
ATM	Breast cancer	Atactic telangiectasis mutation gene effect on apoptosis response.
PTEN	Glioma, breast, prostate, follicular thyroid carcinoma, head and neck squamous carcinoma	Phosphoinositide 3-phosphatase; protein tyrosine phosphatase. A dual-specificity phosphates that negatively regulates the phosphoinositol-3-kinase/Akt pathway and mediates cell cycle arrest and apoptosis.
TIMP-3	Colon, breast, lung cancer	Tissue inhibitors of metalloproteinases
RASSF1A	Bladder, breast, head and neck cancer	Encoding RAS-binding proteins located at 3p21.3 function to inhibit tumor growth
DAPK	Colon, lung, head and neck cancer	A pro-apoptotic calcium-regulated serine/threonine kinase, is involved in apoptosis.
hMLH1	Colorectal, gastric, endometrial	DNA mismatch repair

2.5.1.2.1 Mutation and loss of heterozygosity (LOH) of tumor suppressor genes in endometrial cancer

p53

p53 is a tumor suppressor gene that is important to control the mechanism of apoptosis when DNA is damaged (fig.2.9). Molecular data from multiple studies support the hypothesis of different genetic pathways in the development of endometrioid and serous carcinoma. p53 mutation occurs during progression of about 10-20% of endometrioid carcinomas, but not occurring in endometrial hyperplasiais. (Lax S.F., 2004) It is considered a late event. In serous carcinoma, p53 mutation is the most frequent genetic alteration, followed by inactivation of p16 and e-cadherin and amplification of her2/neu. p53 mutation occurs in endometrial intraepithelial carcinoma, the putative precursor of serous carcinoma. (Lax S.F., 2004)

Mutations in the p53 tumor suppressor gene leading to overexpression of mutant p53 protein are the most common molecular alterations described in human cancers to date. (Berchuck A., 1994) Between 4 and 49% of endometrioid carcinomas and 71.4 and 100% of serous carcinomas (Caduff R.F., 1996; Inoue M., 1994; Manek S., 1996; Tashiro H., 1997) overexpress p53 protein. Of note, p53protein overexpression has been demonstrated in 10 to 15% of early-stage disease and 40 to 50% of advanced-stage disease, (Berchuck A., 1994; Kohler M.F., 1996) but does not occur in endometrial hyperplasia (Berchuck A., 1994; Kohler M.F., 1993; Sherman M.E., 1995). This suggests that p53 mutation may be a late event in the tumorgenesis of endometrial carcinoma or

that acquisition of a p53 mutation leads to the development of a virulent endometrial cancer that does not pass through a phase of hyperplasia as postulated by Berchuck and co-workers. (Berchuck A., 1997; Lax S.F., 2004) Several studies have demonstrated a positive association between p53 overexpression and high nuclear grade and FIGO stage, though these have not been universal findings. (Save V., 1998; Yamauchi N., 1996) In addition, overexpression of p53 is independently associated with poor survival. For example, Kohler and co-workers (Kohler M.F., 1996) observed a median survival of 6.1 years in patients whose tumors did not overexpress p53 in comparison with a median survival of 1.4 years in patients whose tumors overexpressed p53.
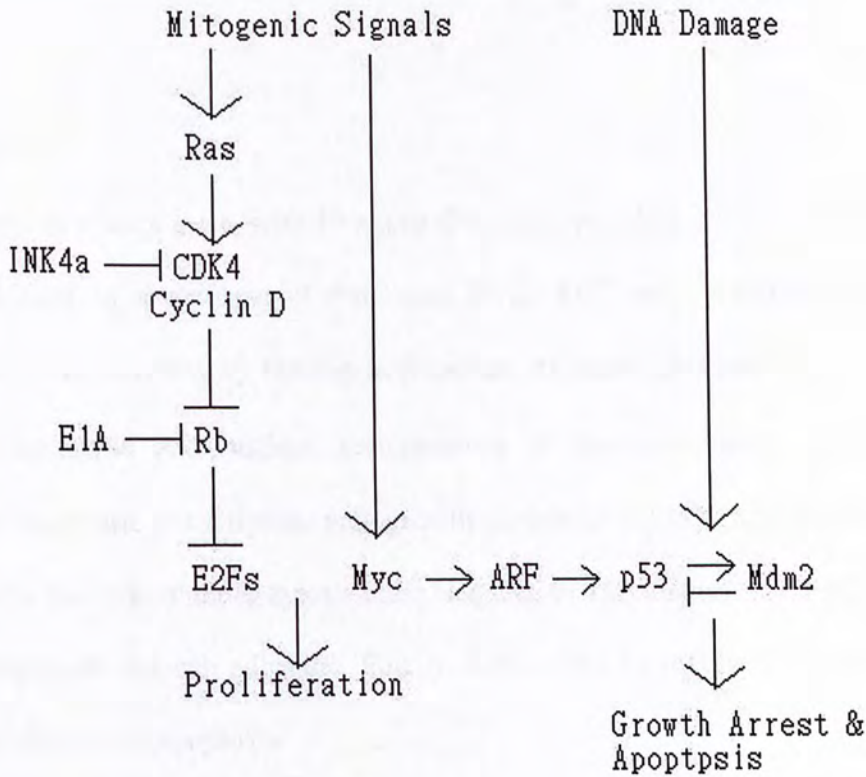
Figure 2.9. DNA damage signals (e.g., ionizing and UV radiation, hypoxic stress, etc.) activate p53 through multiple signaling pathways (Sherr CJ., 1998)

PTEN

PTEN (phosphate and tensin homologue deleted on chromosome ten) is a candidate tumor suppressor gene that has been isolated from the 10q23-24 region. (Burton J.L., 1998) It is a dual-specificity phosphates that negatively regulates the phosphoinositol-3-kinase/Akt pathway and mediates cell cycle arrest and apoptosis. Risinger and co-workers (Risinger J.I., 1997) identified *PTEN* mutations in 34% of 70 endometrial carcinoma specimens. The tumors in which *PTEN* was mutated did not have a unique clinical phenotype. Loss of heterozygosity and mutations in the PTEN (MMAC1) tumor suppressor gene are frequent in endometrial carcinoma.

APC

APC is a large gene, with 15 exons (Fig.2.10) encoding a 2843 residue large protein that appears to serve several functions. First, APC may negatively regulate the Wnt-1 signaling pathway by binding to β-catenin. Mutated APC product, unable to bind, allows cytoplasmic and nuclear accumulation of β-catenin, with persistent activation of downstream transcription and growth factors (fig.2.11). APC protein also colocalizes with the microtubule cytoskeleton, leading to speculation that APC is involved in cell migration and cell adhesion. Finally, APC might be involved in cell cycle regulation or perhaps even apoptosis.
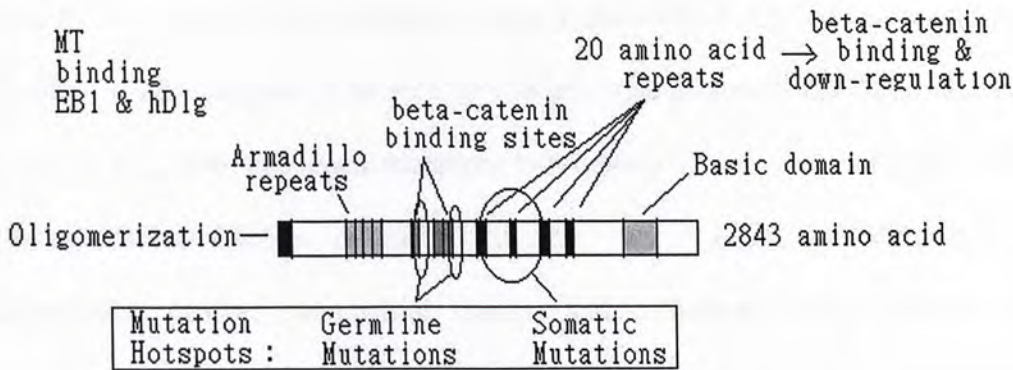
Figure 2.10. Schematic representation of APC protein domains and mutation analysis. Germline mutations in the *APC* gene (predominantly chain terminating) are dispersed throughout the 5' half of the sequence, with two apparent 'hot spots' at codons 1061 and 1309. Somatic mutations in the *APC* gene appear to cluster in a region termed the 'mutation cluster region,' and mutations at codons 1309 and 1450 are most common. (Abeloff MD)

The APC protein has been found to bind to a number of proteins, including β-catenin, γ-catenin, glycogen synthase kinase 3β (GSK3β), EB1, hDLG, microtubules, and the related proteins axin and conductin. (Kinzler K.W., 1996; Bienz M., 1999) Several lines of evidence imply that APC has a critical function in regulating β-catenin. (Bienz M., 1999; Willert K., 1998)

The truncated APC proteins present in many colorectal cancers lack some or all of the repeat motifs crucial for binding to β-catenin. APC is mutated and unable to bind and/or effectively coordinate the regulation of β-catenin, β-catenin accumulates in the cell, complexes with transcription factors of the Tcf (T-cell factor) or Lef (lymphoid enhancer

factor) family, such as Tcf-4, and translocates to the nucleus (Fig. 2.11). Though somatic

mutations in APC appear to be rare in cancers arising outside the colon and rectum,

(Kinzler K.W., 1996) oncogenic mutations in β-catenin's N-terminus have also been seen

in a significant fraction of many different cancer types, including melanoma,

hepatocellular cancer, endometrial cancer, and endometrioid-type ovarian cancer.

(Polakis P., 1999) c-MYC and cyclin D1, other Tcf/β-catenin targets with increased

expression as a result of APC or β-catenin mutations presumably promote cell growth

and/or inhibit cell death. Further work on APC function should offer crucial insights into

the development of cancers, as well as novel strategies and targets for chemotherapy and

perhaps even chemoprevention.
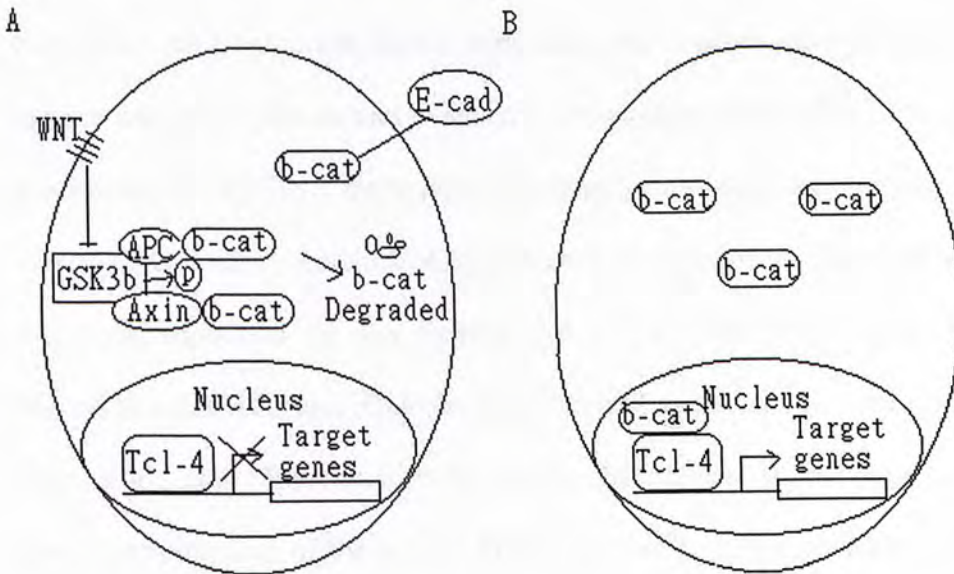
Fig.2.11. A model indicating the function of the APC in the regulation of β-catenin (β-cat)

(A) In normal cells, GSK3β, APC, and axin function to promote degradation of free

cytosolic β-cat, probably as a result of phosphorylation of the N-terminal sequences of β-

cat by GSK3β. GSK3β activity and β-cat degradation are inhibited by activation of the

wingless (Wnt) pathway, as a result of the action of the Frizzled receptor and dishevelled (DSH) signaling protein. (B) Mutation of APC in colorectal and other cancer cells results in accumulation of β-cat, binding to Tcf-4, and transcriptional activation of Tcf-4 target genes, such as *c-MYC*, *cyclin D1*, *MMP-7*, and *PPARδ*. (Fearon ER. 1997.)

In endometrial cancer study, no APC mutations were detected, but LOH at the APC locus was found in 24.3% ECs. (Moreno-Bueno G, 2002)

2.5.2 Epigenetic Alterations

DNA methylation leads to transcriptional repression. This process involved the integration of DNA methylation with chromatin organization and the regulation of histone and other protein and eventually acetylation (Baylin and Herman, 2000; Jones and Baylin, 2002). DNA methylation represses genes transcription by recruitment of the methyl-CpG-binding protein MeCP2, which in turn recruits a histone deacetylase activity. This was supported by the finding that DNMT can form complex with histone deacetylase (HDAC1 and HDAC2) and other transcriptional repressor (Fuks, et al., 2000; Fuks, et al., 2001; Robertson, et al., 2000a). HDAC can remove acetyl groups from the amino terminal core of the histone tails which are critical modulators of chromatin and rendering the sequence inaccessible to the transcriptional machinery (Jones and Baylin, 2002; Robertson, 2002).

The link between genetic changes and epigenetic alterations was found that PML-RARα (Promyelocytic leukemia-retinoic acid receptor α) fusion protein in PML cells induces

the transcriptional repression and the aberrant methylation of the promoter of a candidate RAR transcriptional target, RAR β. The PML-RAR transcriptional repression was due to temporally distinct recruitment of DNMT and HDAC. This report provided inside for the specific DNA methylation pattern observed in certain tumor types (Di Croce, et al., 2002)

The presence of DNA methylation in cancer was initially thought to be a global hypomethylation of genome that lead to massive overexpression of oncogenes whose CpG islands were normally hypermethylated (Feinberg and Vogelstein, 1983). Subsequent finding showed that DNA methylation lead to transcriptional suppression of tumor suppressor genes in cancer, thus suggesting that DNA hypermethylation of tumor suppressor gene is more important in the development of cancer (Jones and Laird, 1999). Several studies have investigated the role of DNA methylation in tumorgenesis. First, 5'methylcyctosine was subjected to spontaneous deamination to thymidine. This C to T transit mutation in CpG dinucleotides in the coding region of tumor suppressor genes are hotspots for mutation (Zingg and Jones, 1997). 24% of point mutations in p53 in human cancer are due to this phenomenon (Greenblatt, et al., 1994). Second, increased DNMTs activities may lead to cell transformation. The consequence of it was aberrant hypermethylation of promoter region of tumor suppressor gene. It was supported by the fact that dense methylation in CpG islands of the promoter in tumor tissues was absent in the corresponding normal tissues (Jones and Baylin, 2002). And methylated genes can be reactivated by demethylating agent, 5' aza-cytidine (Bender, et al., 1998). Third, the selective advantage for gene function loss is the same for promoter methylation or inactivating mutation

2.5.2.1 CpG islands methylation

In human genome, methylation takes place at the 5' position of cytosine base of a CpG dinucleotide resulting in the formation of 5-methylcytosine. It is an enzyme-mediated chemical modification that add methyl groups to the C-5 position of the cytosine rings, resulting in formation of 5-methylcytosine (5-mCyt). This unstable base is finally deaminated to Thymine (T). Between 60-90% of the cytosine at CpG dinucleotide are methylated under physiological normal condition (Bird, A.P., 1986). On the other hand, cytosines that are found in a clustering of CpG rich sequence of 0.5-4 kb in length, namely CpG islands are typically unmethylated. CpG islands are defined by having a GC content over 0.5 and frequency of occurrence of CpG site over 0.6 in a minimum of 200 bp (Gardiner-Garden and Frommer, 1987). In human, there are about 45,000 CpG islands representing 1% of the whole genome (Antequera and Bird, 1993). These areas can be found in the proximal promoter regions of about 50% of human genes and are normally protected from methylation (Antequera and Bird, 1993; Bird, 2002). On the other hand, methylated CpG islands can be found in the promoters of silenced alleles for selected imprinted autosomal genes, inactived X-chromosomes (Surani, 1991), aging tissues as well as in cancer cells (Toyota and Issa, 1999).

2.5.2.2 De novo methylation

De novo methylation of 5' CpG islands is a rare event in normal somatic tissue. In addition to imprinted genes and inactive X-chromosomal genes, methylation can be found in aging and cancer tissues which is supported by the fact that global increase in

CpG island was observed in tissue culture cells and cancer cells (Antequera, et al., 1990; Feinberg, et al.,1998; Jones and Laird, 1999).

Although the mechanism of de novo methylation is still not clearly understood, the recent discovery of DNA methyltransferases (DNMT) seems to be responsible for this process. 3 types of DNMT were identified, namely DNMT1, DNMT2 and DNMT3 (Robertson, 2002). DNMT1 was the first enzyme to be isolated as a mammalian DNA methyltransferase. Study found that modest overexpression of exogenous mouse DNMT1 in NIH3T3 cells can promote cellular transformation (Wu, et al., 1993). In another study, genetic inactivation of DNMT1 in mice decreased the intestinal tumors development (Laird, et al., 1995). These finding supported the role of DNMT1 in tumorgenesis.

DNMT2 was discovered in an attempt to identify novel putative DNMT in mammalian cells (Okano, et al., 1998b). However, no methyl-transfer activity of DNMT2 gene product was reported using in vitro assay (Okano, et al., 1998b).

The presence of de novo methyltransferase activity in DNMT1 inactivated embryonic cell supported the notion that other DNMT might exist (Lei, et al., 1996). DNMT3a and DNMT3b were identified by EST database searches using the conserved methyltransferase domain (Okano, et al., 1998a). Disruption of DNMT3a and DNMT3b genes in mice caused severe embryonic defect (Okano, et al., 1999). This model suggested that DNMT3a and DNMT3b are responsible for establishing de novo methylation that are maintained by DNMT1. Studies showed that expression of human DNMT3a and DNMT3b gene was elevated in several tumor cell lines suggesting that these 2 DNMT might be actively involved in tumorgenesis (Rhee, et al., 2002; Robertson, et al., 2002b).

2.5.2.3 Detection of gene promoter hypermethylation

In this study, methylation specific PCR (MSP) was used as the methylation analysis method. Beside MSP, gene promoter hypermethylation can be studied by other different methods. 1. combined bisulfite restriction analyses (COBRA) which constitute a specific approach releasing on the creation or modification of a target for restriction endonuclease after bisulfite treatment; 2. methylation-sensitive single nucleotide primer extension (Ms-SnuPE) which employs bisulfite/PCR combined with single-nucleotide primer extension to analyse DNA methylation status quantitatively in a particular DNA region without using restriction enzymes; 3. bisulfite sequencing which is the direct means to detect cytosine methylation in bisulfite treated DNA; 4. southern blotting analysis which employs methylation-sensitive restriction endonuclease to analyse the methylation pattern in certain restriction sites of the CpG islands. This method does not require bisulfite treatment but it require large amount of genomic DNA for analysis. Also, promoter hypermethylation can by studied by genomic approach such as methylation-sensitive arbitrarily primed PCR (MS-AP-PCR) or restriction landmark genomic scanning (RLGS). Since MSP is a sensitive, specific method and relatively simple method, we employed MSP as detection method for gene methylation in our study.

2.5.2.4 Epigenetic alterations in endometrial cancer

Cancer is often viewed as a genetic process in which the developing cancer cell acquires successive mutational lesions through the activation of proto-oncogenes or inactivation of tumor-suppressor genes. However, in the previous study, the comparative genomic hybridization results demonstrated that the number of chromosomes involved in genomic

alterations in EACs was distinctively fewer than those in other types of tumor. (Hirasawa A, 2003; Tritz D, 1997). Both frequency and patterns of LOH differed greatly between the two tumor types. Although LOH was frequently detected in the non-endometrioid adenocarinomas, it was rare in the endometrioid adenocarinomas. LOH was detected in only 8 of the 18 endometrioid adenocarinomas, with chromosomes 17, 13, and 2 being the most frequently affected (22%, 20%, and 19%, respectively). (Tritz D, 1997) In contrast, LOH was detected in all cases of non- endometrioid adenocarinomas, with chromosomes 17p, 14, and 12 showing the highest LOH (83%, 77%, and 40%, respectively). (Tritz D, 1997) These tumor types are likely caused by different tumorigenic pathways that reflect alterations of different cancer-controlling genes. (Tritz D, 1997) So carcinogenesis of endometrial cancer may be due to another mechanism rather than chromosomal mutation and deletion. Epigenetics is an alternative or complementary mechanism to mutational events in oncogenesis. Epigenetic events are heritable, non-mutational alterations in gene function that are mediated by factors other than changes in primary DNA sequence. Epigenetic mechanisms of gene silencing, including promoter hypermethylation of tumor suppressor genes, have been shown to contribute to tumorigenesis. Gene promoter hypermethylation is now the most well recognized epigenetic changes in human cancers. Therefore, epigenetic alterations were investigated in endometrial cancer in this study.

Gene promoter hypermethylation is believed to be as common as the inactivation of tumor suppressor gene by mutation and LOH (Jones and Baylin, 2002). It is also considered as the alternative mechanism in the "Knudson two-hits" hypothesis (Jones and

Laird, 1999). The list of candidate tumor suppressor genes that are silenced by promoter methylation in certain cancer is growing rapidly. In the following sections, epigenetic alteration especially DNA methylation in endometrial cancer will be reviewed.

Epigenetic mechanisms that result in aberrant gene expression are a prominent feature of many cancer types. One main epigenetic mechanism for gene silencing involves promoter hypermethylation. Type I and type II endometrial cancers exhibit differ clinical, histology and molecular genetic characteristics. We hypothesize that these differences also extend to epigenetic phenomena. Promoter methylation analysis of a panel of genes in endometrial cancers, suggested that promoter hypermethylation may occur more frequently in type 1 than type II endometrial carcinoma (Risinger JI, et al, 2003). These data tend to support the hypothesis that type I and type II endometrial cancers will exhibit distinct patterns of gene silencing based on promoter hypermethylation events.

E-cadherin

E-cadherin (E-cad) is a cell-cell adhesion molecule, potential invasion and metastasis suppressor. In a study, all normal endometrium and endometrial hyperplasia showed no methylation of the E-cadherin gene with preserved positive staining of E-cadherin. In endometrial carcinoma, methylation of E-cadherin was detected in subset of endometrial carcinoma and was associated with degree of tumor differentiation and myometrial invasion. In G1 EC, 66.7% showed positive staining and 15.6% methylation of the E-cadherin gene (fig.2.12). In G2 tumors, 19.0% showed positive staining and 50.0% methylation of the E-cadherin gene. In G3 tumors, 9.1% showed positive staining and

81.8% methylation of the E-cadherin gene. Of the samples with no-myometrial invasion, 23.1% had methylation. In those with invasion in less than half of the myometrium, 28.6% did and in those with invasion of half or more of the myometrium, 55.6% had methylation. Of samples that did not have lymph node metastasis, 33.7% had methylation, whereas of samples that had lymph node metastasis, 60.0% had methylation. (Saito T, et al, 2003)
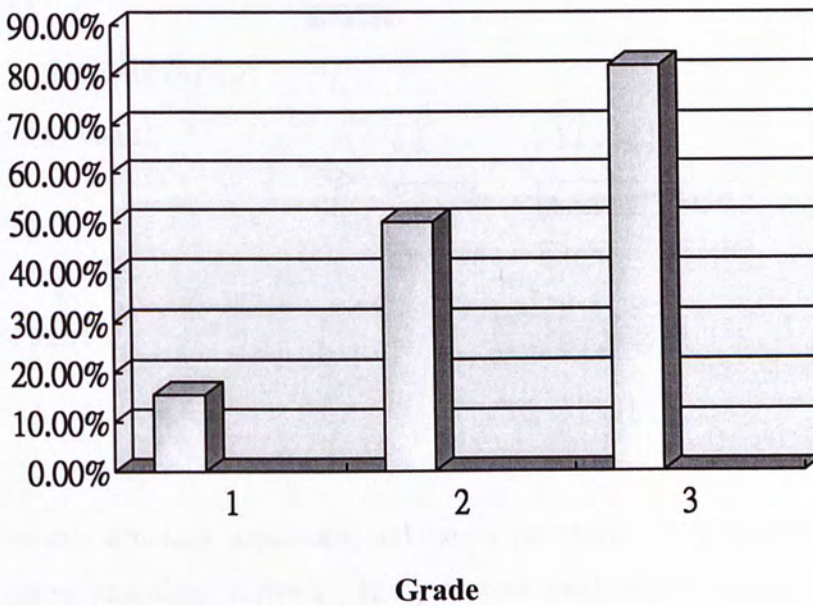
Fig. 2.12 Promoter Hypermethylation of E-cadherin in endometrial cancer. (Saito T, et al, 2003)

p16

p16 regulates Cdk4 activity which is a critical factor in regulating pRb phosphorylation. Inactivation of p16^{INK4a} results in inappropriate phosphorylation of pRb and a subsequent inability of hyperphosphorylated pRb to bind E2Fs and appropriately regulate gene expression at the G1/S transition and contribute to tumorigenesis. The p16 genomic structure and mutations is shown in fig 2.13.
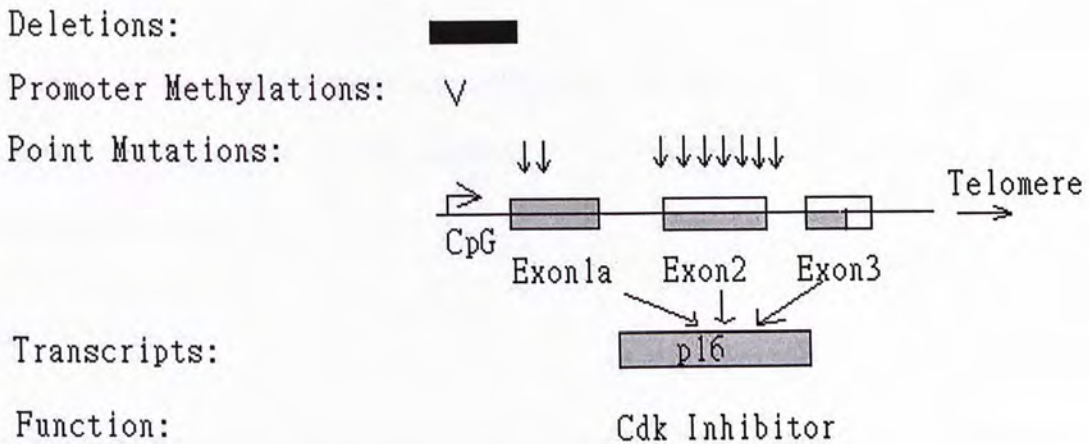
Fig2.13 Genomic structure, mutations, and transcripts of the *INK4a* (p16) locus. Genomic deletions, point mutations (arrows), and promoter methylation (arrowheads) noted in human cancers. (Haber DA, 1997; Abeloff MD)

In endometrial cancer studies, partial p16INK4A deletions were found in 8% of ECs. (Semczuk A, 2003). From 4.2% to 34% of ECs exhibited aberrant promoter methylation. (Semczuk A, 2003; Chao H, 2000). Normal endometrium(NE) displayed no methylation and showed normal expression of the p16 mRNA. 17% of atypical hyperplasia (EAH) was shown to have hypermethylation of p16 gene and p16 methylation was hypothesized to be an early event of endometrial carcinogenesis and is associated with the progression

of endometrial carcinoma (Chao H, 2000). p16INK4A alterations were generally accompanied by gene silencing, confirmed by aberrant protein immunostaining. Hypermethylation is highly correlated with inhibition of p16 gene transcription. There was a significant difference in the frequency of p16INK4A alterations between early (stage I) and advanced (stages II-IV) ECs (Semczuk A, 2003).

PTEN

Apart from mutation, PTEN promoter methylation (19%) is also relatively common in endometrial carcinoma. It is also reported to be associated with metastatic disease (Salvesen H.B., 2001)

PRB

Progesterone response is dependent on the presence of functionally intact progesterone receptor (PR). Progestin-induced activity is only initiated when the steroid is bound to the PR. An absence of hormone response will result from either the absence of the PR or its failure to bind the PR. Point mutations or total deletions in the steroid binding domain of the PR can occur results in inactive receptor. In humans, PR is composed of two progesterone-binding proteins, PRA and PRB. The expression of the human PR is reported to be controlled by two promoters that direct the synthesis of mRNA transcripts encoding the two receptor proteins. The PRA is N-terminally truncated by 164 amino acid residues and therefore is slightly smaller than PRB. The PRA and PRB co-express in

the same cells and appear to be synthesized in equal proportions. But they appear to be functionally different (E. McGowan, 1999).

In endometrial cancer study, the results suggest that only PRB is inactivated. PRB was methylated, whereas PRA was all unmethylated. In 83 cancerous and 33 normal samples, 75% ECs had only methylated alleles of PRB that was well correlated with negativity in immunohistochemical staining for PRB. Treatment with a demethylating agent, 5-aza-2'-deoxycytidine, restored PRB expression in all cell lines, suggesting that inactivation of this gene is through methylation. (Sasaki M, 2001)

APC

In endometrial cancer, APC promoter 1A hypermethylation was observed in 43% to 46.6% of ECs, and was associated with the endometrioid phenotype and microsatellite instability. (Moreno-Bueno G, 2002; Zysman M, 2002).

MSH2 and *MLH1*

The protein products of the *MSH2* (chromosome 2p) and *MLH1* (chromosome 3p) genes appear to have critical roles in the recognition and repair of DNA mismatches (Fig. 2.14). Once the cell acquires impaired mismatch repair function, for instance, as a result of inactivation of either *MSH2* or *MLH1*, hundreds of errors/mutations may arise and fail to be repaired during each cell division cycle. Because many of the mutations arise in mononucleotide, dinucleotide, and trinucleotide repeat tracts (i.e., microsatellite sequence

tracts) scattered throughout the genome, it has also been termed the microsatellite instability (MSI) phenotype.

a.

```
                                    ACAC
                                   C    A
                                    A    C
                                   TCAC  ACAG
                                   AGTGTGTGTC
```

b.

c. GTBP/MSH6 MSH2

d. PMS2 MLH1
    GTBP/MSH6 MSH2

e.
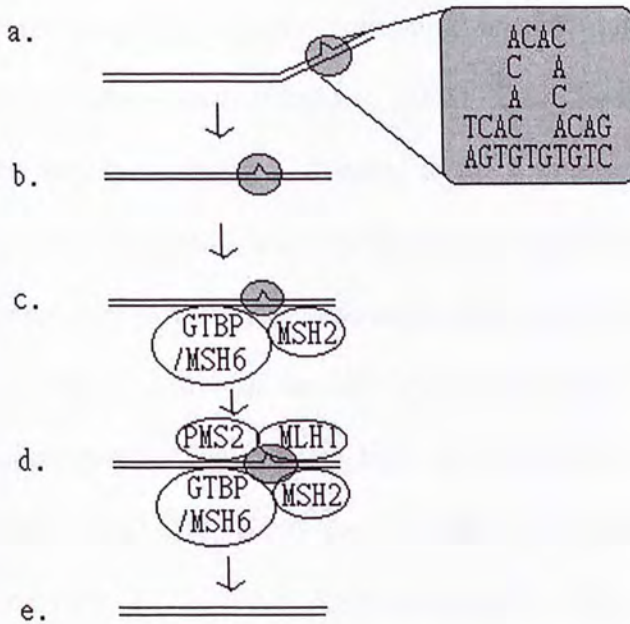
Fig2.14. Mismatch repair pathway in human cells. (a and b) During DNA replication, DNA mismatches may arise (c) The mismatch is recognized by MutS homologs, MSH2 and GTBP/MSH6, MSH3 may substitute for GTBP/MSH6 in some cases. (d and e) MutL homologs, such as MLH1 and PMS2, are recruited to the complex and the mismatch is repaired through the action of a number of proteins, including an exonuclease, helicase, DNA polymerase, and ligase (Kinzler KW, 1996)

A study investigated the region 700 bp upstream of MLH1 covering 48 CpG sites. In endometrial cancer, 29% of ECs were fully methylated (over 80% of CpGs are methylated) (Kanaya T, 2003), 25% were partially methylated (10-80%) and 46% were

not methylated (less than 10%) (Kanaya T, 2003). Among methylated cancers, 42% contained methylated promoters in their normal endometria adjacent to carcinoma with profiles similar to those of cancer lesions (Kanaya T, 2003), 7% cases of atypical endometrial hyperplasia coexisting endometrial carcinoma had hMLH1 promoter hypermethylation (Miturski R, 2003). These findings suggest that hypermethylation of the MLH1 promoter is frequent in the histologically normal endometrium adjacent to cancers, supporting the notion that hypermethylation of mismatch repair genes is the initial step that triggers various genetic events in endometrial carcinogenesis. And these were associated with the MSI phenotype (Kanaya T, 2003). 91% cases of endometrial carcinoma (EC) displaying MSI had hMLH1 promoter hypermethylation (Miturski R, 2003; Esteller M, 1999). Loss of hMLH1 and hMSH2 expression was reported in 25% and 18% of ECs, respectively (Miturski R, 2003), but it did not correlate with the known clinicopathological variables of cancer. In addition, no obvious correlation was found between methylation and the lack of hMLH1 and hMSH2 protein expression in human uterine tumors. The results showed that DNA methylation may influence tumor progression, but they are not directly associated with the inactivation of the mismatch-repair machinery in ECs. (Miturski R, 2003)

2.5.2.5 Promoter methylation of tumor suppressor genes in other cancers

RASSF1A

RASSF1A is located in 3p21. RASSF1A was isolated as a tumor suppressor gene in this

region from a two-hybrid screen (Dammann, et al., 2000). One of the slice variants of the gene RASSF1A undergoes frequent epigenetic changes in tumors of lungs, nasopharynx, breast, kidney, stomach, ovary and urinary bladder (Agathanggelou, et al., 2001; Burbee, et al, 2001; Byun, et al, 2001; Dreijerink, et al, 2001; Lee, et al, 2001; Lo, et al., 2001; Yoon, et al., 2001). Although the functions of RASSF1A are not completely understood, several studies found that it can reduce tumor growth both in vitro and in nude mice model (Burbee, et al, 2001; Dreijerink, et al., 2001; Vos, et al, 2000). Those experiments suggested that it was a candidate tumor suppressor gene. Structural analysis also found that its RAS-association domain likely in the RAS-mediated apoptotic mechanism. RASSF1A was also showed to bind with NORE1, the novel RAS effecter and MST1, the pro-apoptotic protein kinase in mediating the apoptotic effecter of RAS (Khokhlatchev, et al, 2002; Ortiz-Vega, et al, 2002).

HIC-1

In 90 Cervical cancer (CC) cases, a high frequency of promoter methylation in HIC-1 gene was found. Correlation of promoter methylation with clinical characteristics and other genetic changes revealed the following: a) overall promoter methylation was higher in more advanced stage of the disease, b) the HIC1 promoter methylation was frequently seen in association with microsatellite instability. Promoter methylation was associated with gene silencing in CC cell lines. Treatment with methylation or histone deacetylation-inhibiting agents resulted in profound reactivation of gene expression. (Narayan G, 2003)

MGMT

The DNA repair protein O^6-methylguanine DNA methyltransferase (MGMT) removes alkyl adducts from the O^6 position of guanine in DNA. Failure to remove the alkyl adducts generate G:C to A:T transition mutation and promote tumorgenesis as demonstrated (Esteller, et al., 2001). MGMT which located at 10q26, is frequently hypermethylated in human cancers resulting in loss of expression (Bae, et al, 2002, Esteller, et al, 2002, Oue, et al, 2001).

ATM

The Ataxia-telangiectasia-mutated (ATM) gene is located on chromosome 11q22-23 (Gatti R.A., 1988; Lange E., 1995; Savitsky K., 1995), it's gene product is a protein kinase belonging to the PI3 kinase family. Multiple phosphorylation substrates have been identified including p53, MDM2, and BRCA1, proteins involved in DNA repair and cell cycle checkpoint control. ATM-dependent modifications of p53 result in the induction of apoptotic mediators. ATM is a tumor suppressor that plays a role in maintenance of genomic stability.

It is believed that ATM promoter is a target for epigenetic silencing in cultured tumor cells. Aberrant methylation of the ATM promoter occurs in 25% of head and neck squamous cell carcinomas (Ai L., 2004). The presence of methylated ATM promoter shows a statistically significant correlation with an earlier age of initial diagnosis and

decreased overall survival, particularly in early-stage tumors. These findings indicate that ATM promoter hypermethylation occurs in head and neck squamous cell carcinoma, and this feature is a potentially useful prognostic marker in this tumor type.

2.5.3 Microsatellite instability (MSI)

Microsatellites are short, highly repetitive DNA tandem repeat (usually 1–6 base pair) sequences found throughout the genome. (Eshleman J.R., 1996) They are usually, but not always, noncoding sequences. Two of the most common sequences are repeats of $(A)_n$ and (C) where $n = 10$–60 distributed throughout the genome. These sequences contribute much of the polymorphisms seen in the human genome shows variation between individuals (Weber and May, 1989) and can be used as markers in genetic analysis. Despite the high degree of polymorphisms between individuals, the patterns of microsatellites within a given individual are stable.

The change of any length in these repetitive sequences due to insertion or deletion in tumor tissue as compared to normal is called microsatellite instability. Microsatellite instability can be detected by the PCR. Analysis using primers at the boundaries of known microsatellites will produce two bands on gel electrophoresis with each band representing one parental homologue in informative cases. In the replication errors phenotype, the size of the bands will be increased or decreased when compared to the bands obtained from PCR of genomic DNA from normal tissues. It is now recognized that the patterns of microsatellites are unstable in many tumors as manifested by expansions and contractions of these sequences. These sequences are believed to be error prone due to their monotonous nucleotide repeats. It is theorized that during DNA

replication, there is slippage of DNA polymerase in these repetitive sequences that leads to addition or deletions of bases that are not detected by the proofreading activity of the polymerase. These errors are detected, excised, and repaired by a complex of mismatch repair proteins.

Many studies found MSI is important and has significant role in carcinogenesis, it occurs in both hereditary and sporadic form of various organs including stomach, lung, breast and prostate (Caldes, et al., 2000; Hayden, et al., 2000; Perinchery, et al., 2000; Yamashita, et al., 2000). MSI is one of the major mechanisms of cancer susceptibility. It was first observed in hereditary nonpolyposis colorectal cancer (HNPCC) and also later observed in sporadic colorectal carcinoma (Aaltonen, et al., 1994; Liu, et al., 1996; Peltomaki, et al., 1993).and has been identified in 17 to 43% of endometrial carcinomas. (M.H. Wallace, 1998; A. Horii, 1992; H. Itoh , 1994; Y. Hattori , 1996; S. Akimoto , 1998; Taniguchi K., 1998) The sporadic endometrial carcinomas displaying microsatellite instability were mainly stage I adenocarcinomas (Taniguchi K., 1998) .In a large fraction of MSI + cancers, inactivation of the *MLH1* gene may occur via epigenetic changes, such as DNA methylation of *MLH1* transcriptional regulatory sequences. (Leung S.Y., 1999; Cunningham J.M., 1998; Herman J.G., 1998; Kane M.F., 1997; Markowitz S., 1995) MSI is showed to be related to hMLH1 methylation. (Whelan AJ, 2002) Also, MSI was correlated with histological grade, International Federation of Gynecologists and Obstetricians (FIGO) stage, myometrial invasion, and lymphonode metastasis. (Hirasawa A, 2003)

In MSI study, several MSI markers were commonly used in MSI status detection. It was a panal of 7 markers, including both mononucleotide (BAT25, BAT26, BAT40) and dinucleotide repeats (D2S123, D5S346, D17S250, TGFβ2R). This panel contained markers from the standard panel of the National Cancer Institute (NCI) recommendation (Boland at al., 1998). This panal was used in previous colorectal cancer studies and showed highly sensitive in MSI detection.

Chapter 3 Objectives of study

We aim to investigate the epigenetic alterations in endometrioid adenocarcinoma (ECA) and its' precancerous lesion endometrial hyperplasia. In addition, microsatellite status of ECA will also be determined. The possible relationship between methylation status, MSI status and clinical-pathological features of ECA will be assessed.

1. Gene promoter hypermethylation study in endometrioid adenocarcinoma

To delineated the occurrence and frequency of methylation of multiple genes in endometrioid adenocarcinoma (ECA) and endometrial hyperplasia. We examined the promoter hypermethylation of 10 candidate tumor suppressor genes in ECA. The relationship between methylation status and tumor grade and stage will be assessed.

2. Microsatellite status analysis in endometrioid adenocarcinoma.

We examined the MSI status with 7 markers in ECA. The relationship between MSI status and, tumor grade and stage will be assessed.

3. Correlation between MSI and promoter methylation analysis

To assess the possible relationship between methylation status and microsatellite instability status in ECA.

Chapter 4 Materials and Methods

4.1 Samples

4.1.1 Formalin fixed paraffin embedded tissues.

Samples are retrieved from the archival tissue bank from the Anatomical and Cellular pathology Department, CUHK. Formalin fixed, paraffin embedded tissue blocks were used. These include:

- Endometroid adenocarcinoma (ECA) samples were obtained from 34 cases of endometrioid adenocarcinoma (ECA) which included 6 cases of ECA co-existing with endometrial hyperplasia and 28 cases without co-existing endometrial hyperplasia.

- 8 cases of Serous adenocarcinomas (SCA)

- 21 cases of endometrial hyperplasia without atypia (EH)

- 21 cases of endometrial hyperplasia with atypia (EAH)

-12 Cases of normal endometrium were used as control (including 4 cases in proliferative phase, 4 cases in secretary phase and 4 cases in inactive phase)

4.1.2   Cell lines

Endometrial cancer cell lines RL-95 and KLE were obtained from American Type Culture Collection, Rockville, MD. Cell lines RL-95 was grown in 1:1 mixture of Dulbecco's modified Eagle's medium and Ham's F12 medium with 1.2g/L sodium bicarbonate and 0.005mg/ml insulin. KLE was grown in 1:1 mixture of Dulbecco's modified Eagle's medium and Ham's F12 medium with 1.17g/L sodium bicarbonate and

1.0mM sodium pyruvate. All cell lines were supplemented with 10% fetal bovine serum (FBS) (Sigma, St Louis, MO), 100U/ml penicillin and 10 μ g/ml streptomycin and maintained in log phase growth in an incubator with humidified atmosphere of 5% CO_2.

4.2 Histological grading and staging of samples

All samples were stained with hematoxylin and eosin (H&E) and the histological assessment was performed by an experienced pathologist. All ECA were FIGO staged and graded.

4.3 Microdissection on tissue sections.

Microdissection is a technique that can precisely identify and specifically select groups of the cells for molecular analysis. It has been used to separate the mixed cellular components of tumor and loci of varied differentiation in carcinomas. This is particularly useful when the cellular content in the specimen of interest is low. The ratio of tumor cells to normal contaminant is critical in molecular analysis, particularly in methylation and microsatellite studies.

15 serial 5μm sections were cut from formalin-fixed, paraffin-embedded blocks and the first was stained with hematoxylin and eosin as reference slides examined under the microscope. The tumor areas were marked with a marker pen for localizing the tumor of interest. The other slides were placed one by one beneath the reference slides, and the areas of interests were similarly marked. The slides were kept moist all the time and the

tumor tissues were scraped out under a microscope using fine needles. Normal endometrium tissues were excluded.

4.4 Extraction of nucleic acid

4.4.1 Extraction of DNA from paraffin embedded tissues

The dissected tissue pellet was resuspended in 200μl tissue lysis buffer and 40μl of 20μg/ml proteinase K and incubated at 55℃ until complete digestion of the samples.

DNA was extracted from endometrial cancer microdissected paraffin tissues by High Pure PCR Template Preparation Kit (Boehringer Mannheim, Indianapolis, IN) according to the manufacturer protocol. 200μl of binding buffer was added and incubated at 72℃ for 10 minutes. The reaction mixture was then mixed with 100μl isopropanol. The reaction mixture was transferred to a High Pure Filter column and centrifuged at 8000 rpm for 1 minute. The flow through was discarded and 500μl washing buffer was added to the column followed by centrifugation at 8000rpm for 1min. this procedure was repeated. Finally the column was combined to a new 1.5ml tube and DNA was eluted with 200μl MilliQ water. The concentration of DNA was measured by a UV-spectrophotometer (Pharmacia) and stored in -20℃.

4.4.2　Extraction of DNA from cell lines

All cell lines were grown in 90mm tissue culture plate with 10 ml medium. For DNA extraction, to obtain cell pellet, medium was discarded and cells were washed 3 times by cold 1x PBS in the culture plate. Tissue pellet was resuspended and DNA was extracted by High Pure PCR Template Preparation Kit (Boehringer Mannheim, Indianapolis, IN) as above.

4.5　DNA methylation analysis

The technique of methylation specific PCR (MSP) was used to detect methylated DNA.

4.5.1　Overview of Methylation-Specific PCR (MSP)

MSP is a breakthrough in speed and sensitivity for gene methylation analysis. The procedure takes advantages of bisulfite-mediated chemical conversion of cytosine to uracil, followed by PCR using primers designed to distinguish methylated from unmethylated DNA. Advantages of MSP are that it avoids the use of restriction enzymes and problems of incomplete enzymetic digestion; it is very sensitive, the sensitivity is up to 0.1% methylated alleles of a given CpG island locus, permitting the analysis of small and heterogeneous samples, it requires only small amount of DNA; it is specific for relevant CpG sites; it is not prone to false-positive results and it can be performed on paraffin-embedded samples.

4.5.2 Bisulfite modification of DNA

Bisulfite modification allows for let methylated and unmethylated primer designed for MSP distinguish between methylated and unmethylated DNA. DNA from paraffin tissues and cell lines was modified using CpGenome DNA modification kit (Intergen Company, Purchase, NY). Sodium bisulfite converts all unmethylated cytosine residues to uracil while methylated cytosine residues are unaltered in this process. After sequence conversion, methylation specific PCR is employed to detect these using primers specific for any gene of interest.

DNA modification was carried out by adding 1μg DNA in 99μl MilliQ. DNA was denatured using 0.2M NaOH at 37℃ for 10 minutes. 550ml of DNA modification reagent 1 (Intergen Company) was added to denatured DNA incubated at 50℃ for 20 minutes. The modified DNA was purified by incubating with 750μl and 5ml DNA modification reagent 2 and 3 (Intergen), respectively, at room temperature for 10 minutes. The reaction mixture was spun at 5000rpm for 1minute followed by discarding the supernatant. The pellet was washed with 1.0ml 70%ethanol, vortexed and spun at 5000 rpm for 10 minutes. The supernatant was removed. The washing steps were repeated for 3 times. Modification was completed by incubated with 50μl of 20mM NaOH with 90%ethanol for 5 mins followed by centrifugation at 5000 rpm for 1 min. the modified DNA was precipitated with 1 ml 90% ethanol and centrifuged at 5000 rpm for 10 mins. The modified DNA was stored at -20℃ for future use.

4.5.3 Methylation-Specific PCR (MSP)

Two µl of bisulfite modified DNA were amplified in a total volume of 25µl containing 1x PCR buffer2 (Applied Biosystems), 2mM Mg Cl_2, 0.25mM dNTP, 5µM of each primer and 1U of AmpliTag Gold polymerase (Applied Biosystems) at 95℃ for 10 mins, 35 cycles of 95℃ for 45 sec, the specific annealing temperature for 45 sec and 72℃ for 45 sec, followed by a final extension of 72℃ for 10 mins. In-vitro methylated DNA (IVD) (Intergen, purchase, NY) was used as a positive control for methylation and water was used as a negative control. 10µl of PCR products were loaded onto nondenaturing 10% polyacrylamide gels. Gels were stained with ethidium bromide, and visualized under UV illumination.

In this part of study, we have examined the promoter hypermethylation of 10 candidate tumor suppressor genes in endometrial cancer (ECA) and its precursor lesions, endometrial hyperplasia with atypia and without atypia, by MSP. Table 4.1 summarized the function of these 10 tumor suppressor genes.

Table 4.1 Function of 10 tumor suppressor genes investigated.

Gene	Cancers associated	Presumed function of protein
APC	Colorectal cancer	Regulates levels of β-catenin protein in cytosol binding to microtubules
MGMT	Colon, lung, pancreas cancer	O6-methylguanine-DNA methyltransferase, DNA repair enzyme
hMLH1	Colorectal, gastric, endometrial	DNA mismatch repair
p16	Approx. 25-30% of many different cancer types, Breast, bladder, lung cancer and leukemia	Cyclin-dependent kinase inhibitor (i.e., Cdk4 and Cdk6)
E-cad	Gastric (Diffuse type), lobular breast carcinoma, rare in other type, e.g., ovarian	Cell-cell adhesion molecule, potential invasion and metastasis suppressor
HIC-1	Breast, colon, brain cancer	Hypermethylated in cancer, a transcriptional repressor to suppress cell growth
PRB	Endometrial, breast cancer	Progesterone receptor B, response to estrogen and progesterone which has antiproliferative effect.
ATM	Breast cancer	Atactic telangiectasis mutation gene effect on apoptosis response.
PTEN	Glioma, breast, prostate, follicular thyroid carcinoma, head and neck squamous carcinoma	Phosphoinositide 3-phosphatase; protein tyrosine phosphatase. A dual-specificity phosphates that negatively regulates the phosphoinositol-3-kinase/Akt pathway and mediates cell cycle arrest and apoptosis.
RASSF1A	Bladder, breast, head and neck cancer	Encoding RAS-binding proteins located at 3p21.3 function to inhibit tumor growth

All the primer sequences and annealing temperatures were listed in table 4.2

Table4.2 Primer sequences and MSP conditions.

Marker		Primer Sequence	Temp.(°C)
E-cadherin (Herman, et al, 1996)	M	F: TTAGGTTAGAGGGTTATCGCGT R: TAACTAAAAATTCACCTACCGAC	57
	U	F: TAATTTTAGGTTAGAGGGTTATTGT R: CACAACCAATCAACAACACA	53
p16 (Herman, et al, 1996)	M	F: TTATTAGAGGGTGGGGCGGATCGC R: GACCCCGAACCGCGACCGTAA	60
	U	F: TTATTAGAGGGTGGGGTGGATTGT R: CAACCCCAAACCACAACCATAA	60
hMLH1 (Fleisher, et al, 1999)	M	F: ACGTAGACG TTT TATTAGGGTCGC R: CCTCATCGTAACTACCCGCG	58
	U	F: TTTTGATGTAGATGTTTTATTAGGGTTGT R: ACCACCTCATCATAACTACCCACA	58
RASSF1A (Lo, et al, 2001)	M	F: GTGTTAACGCGTTGCGTATC R: AACCCCGCGAACTAAAAACGA	60
	U	F: TTTGGTTGGAGTGTGTTAATGTG R:AAACCCCACAAACTAAAAACAA	60
MGMT (Esteller, et al, 1999a)	M	F: TTTCGACGTTCGTAGGTTTTCCG R: GCACTCTTCCGAAAAACGAAACG	60
	U	F: TTTGTGTTTTGATGTTTGTAGGTTTTTGT R: AACTCCACACTCTTCCAAAAACAAAACA	60
APC (Esteller, et al, 2000)	M	F: TAT TGC GGA GTG CGG GTC R: TCGACGAACTCCCGACGA	60
	U	F: GTG TTT TAT TGT GGA GTG TGG GTT R: CCAATCAACAAACTCCCAACAA	60
PRB (Sasaki, M., 2001)	M:	F: TGA TTG TCG TTC GTA GTA CG R: CGA CAA TTT AAT AAC ACG CG	55
	U:	F: TGA TTG TTG TTT GTA GTA TG R: CAA CAA TTT AAT AAC ACA CA	55
PTEN (Kiyoshi Sato, 2002)	M:	F: TTTAGCGTTTGTGAGTAGTCGC R: CCTTCCCTTTCAAAAAAAACCG	56
	U	F: GTTTAGGTGAGGGAGATGAGAGAT R: CACCTTCCCTTTCAAAAAAAAACCA	56
ATM (Designed by Lo, K.W., CUHK)	M:	F: TCC AAT ATC ACG CGA TCT CC R: CGA AGG GCG AGT CGT AAA C	62
	U:	F: TCC AAT ATC ACA CAA TCT CCA CA R: GGA GTT TGA GTT GAA GGG TGA G	62
HIC-1 (Strathdee, et al, 2001)	M:	F: TTCGGGTTAGGGTCGTAGTC R: CTAACGGAAAACTAT	60

4.6 Microsatellite Analysis

Microsatellites (Tautz, 1989) are highly polymorphic DNA markers that include small arrays of di-, tri- and tetra-nucleotide tandem repeats, e.g., (CA)n/(TG)n, with repeat size of 1-6 base pairs and are interspersed throughout the genome (Weber and May, 1989).

The size of microsatellite is highly variable or polymorphic, ranging around a mean of 100 base pairs. These sequences are not transcribed into RNA, the function and formation mechanism of these repeats are unclear.

PCR-based methods can detect allelic abnormalities in the genome of cancer patients, such as loss of heterozygosity deletion, microsatellite instability and gene loss in cancer. This approach relies on an individual being heterozygous at a specific genetic locus, such as at a microsatellite, where the number of tandem repeats is often polymorphic. A heterozygous individual will have one number of repeats at the locus on one chromosome, and a different number at the same locus on the other chromosome

Tumor tissue from a cancer patient who is heterozygous at a particular locus (termed as informative individual) can be analyzed using PCR, and is compared with normal tissue that has been analyzed in the same way. If one of the alleles that is present in the normal DNA of the heterozygous individual is missing from the DNA of the tumor calls, the tumor is said to have undergone a reduction to homozygosity or loss of heterozygosity whereas subtle alterations in number of nucleotide repeats within microsatellites in tumor cell compared to normal one results in microsatellite instability.

34 Cases of paired normal and tumor tissues were used to determine the microsatellite instability (MSI) status. A panal of 7 markers, including both mononucleotide (BAT25, BAT26, BAT40) and dinucleotide repeats (D2S123, D5S346, D17S250, TGFβ2R), was examined. The microsatellite markers are fluorescent based. This panel contained markers from the standard panel of the National Cancer Institute (NCI) recommendation (Boland at al., 1998). The primer sequences, annelealing temperatures and estimated length of PCR products for each locus was obtained from Genome Data D Base. The

primers were labeled with florescence and analyzed on urea-polyacryamide gel according to standard protocol (Leung et al., 2000). Microsatellite instability (MSI) was demonstrated by the presence of new fragments of variable size in tumor DNA. The tumors were classified as high-level MSI (MSI-H) if >30% of loci displayed instability, low level MSI (MSI-L) if <30% of loci displayed MSI, or microsatellite stable (MSS) if no loci displayed MSI (Boland at al., 1998)

PCR or multiplex PCR examinatioin was performed in reaction volumn 25μl containing 1x PCR buffer2 (Applied Biosystems), 2mM Mg Cl$_2$, 0.25mM dNTP, 5μM of each primer and 1U of AmpliTag Gold polymerase (Applied Biosystems) at 95℃ for 10 mins, 35 cycles of 95℃ for 45 sec, the specific annealing temperature for 45 sec (table 4.3) and 72℃ for 45 sec, followed by a final extension of 72℃ for 10 mins. PCR products were separated by electrophoresis in 4% polyacrylamide gell with 6M urea on an ABI PRISM 377 automated DNA sequencer. Paired up normal and tumor samples were loaded into consecutive lane which contained overlapping alleles of the same lane. Two fluorescent-labeled dyes, FAM and HEX were displayed on the ABI PRISM 377 as blue and green colour respectively. The data collected was analyzed using GeneScan analysis software v3.1 (Applied Biosystems).

Table 4.3 Primer sequences and PCR conditions for MSI analysis

Primer	Primer sequence (5'-3')	Temperature (°C)
BAT-25	Hex-TCG CCT CCA AGA ATG TAA GT TCT GCA TTT TAA CTA TGG CTC	50°C Touch Down
BAT-26	Hex-TGA CTA CTT TTG ACT TCA GCC AAC CAT TCA ACA TTT TTA ACC C	50°C
D5S346	Fam-ACT CAC TCT AGT GAT AAA TCG GG AGC AGA TAA GAC AGT ATT ACT AGT T	55°C
D2S123	Fam-AAA CAG GAT GCC TGC CTT TA GGA CTT TCC ACC TAT GGG AC	55°C
D17S250	Fam-GGA AGA ATC AAA TAG ACA AT GCT GGC CAT ATA TAT ATT TAA ACC	54°C Touch Down
BAT-40	Fam-ATT AAC TTC CTA CAC CAC AAC GTA GAG CAA GAC CAC CTT G	54°C Touch Down
TGFβ2R	Fam-CTT TAT TCT GGA AGA TGC TGC GAA GAA AGT CTC ACC AGG C	50°C

4.7 Statistical analysis

The significance of the correlation between MSI, staging and grading were tested by Spearman test, and that of the correlation between MSI and methylation index was tested by Mann-Whitney test. All statistical analysis was performed by SPSS software, version 10.0 (SPSS, Inc). A two side P value of less than 0.05 was considered statistically significant.

Chapter 5 Results

5.1 Clinical-pathological features of endometrioid adenocarcinoma.

The mean age of patients was 55.7 +/- 10.6 years old (range from 38 to 78). All ECA were FIGO staged and graded and the histological assessment was performed by an experienced pathologist. Among the 34 cases, 52.9% were FIGO grade 1, 26.5% grade 2 and 20.6% grade 3; 61.8% were FIGO stage I, 11.8% stage II and 26.5% stage III. Tumor grade correlate with stage. (Spearman p=0.002) The correlation between grading and staging of patients were shown in table 5.1 .

Table 5.1. The relationship between grading and staging of patients (Spearman p=0.002)

		Stage			Total
		I	II	III	
Grade	1	15	1	2	18
	2	5	1	3	9
	3	1	2	4	7
Total		21	4	9	34

5.2 Promoter hypermethylation in endometrial cancer

Ten tumor-suppressor genes were analyzed in 34 ECA which include 6 cases of ECA coexisting with hyperplasia, 8 SCA, 21 EAH, 21 EH and 12 normal endometrium by MSP. These include p16, hMLH1, E-Cadherin (E-Cad), RASSF1A, MGMT, ATM, PTEN, PRB, APC, HIC1. The MSP results were shown in 10% acrylamide gel stained with ethidium bromide (Fig.5.1). In ECA samples, the methylation frequency is 44% in hMLH1, 35% in RASSF1A, 26% in E-Cad, 21% in MGMT, 15% in PRB and ATM, 12% in APC, 9% in p16 and HIC1, and no methylation was detected in PTEN. The

methylation frequency in individual cases of ECA, SCA, EAH and EH were shown in table 5.2 and table 5.3. The methylation frequency of each gene were summarized in table 5.4. We compared the methylation pattern of these genes between endometrial adenocarcinoma (ECA) and its precancerous lesions (EH and EAH) and serous adenocarcinoma (SCA) (fig. 5.2), and 2 groups of ECA were compared and analyzed (fig 5.3). The differential increase in methylation frequencies from hyperplasia to cancer was found in E-cad, APC and MGMT, while interestingly, PTEN had differential decrease in methylation frequency. In contrast, hMLH1 had comparable methylation frequencies in cancer and hyperplasia. Concurrent methylation of multiple genes appears common in both hyperplasia and cancer. The methylation index in EH, EAH and ECA were 0.19, 0.29 and 0.20, respectively. It indicated that gene methylation occurs in endometrial hyperplasia and may be an early event in carcinogenesis of ECA. Twelve normal endometrium controls did not show any aberrant hypermethylation in all these genes by MSP.

By comparing 2 groups of ECA, ECA co-existing with hyperplasia and ECA without co-existing with hyperplasia showed difference in methylation frequency. However, the distinct difference between 2 groups of ECA in pattern of methylation frequency cannot be found and not significant.

By comparing 2 types of ECs. Interestingly, for the Serous adenocarcinoma (SCA) which is non-endometrioid adenocarcinoma, no methylation was detected. However, methylation is common in endometrioid adenocarcinoma (ECA).

The correlation between promoter hypermethylation and clinicopathologic parameters was investigated. The distribution of gene promoter hypermethylation and staging and

grading of ECA were shown in table 5.5 and table 5.6 respectively. There was no statistically significant association was found between the methylation status of the genes and the clinical pathological parameters.

Table 5.2. The methylation of individual markers and the methylation index (MI) in individual cases of ECA. For the first 6 cases, they are endometrioid adenocarcinoma coexisting with hyperplasia. (+ methylation; - no methylation. For cases of endometrioid adenocarcinoma with co-exist endometrial hyperplasia, the results were listed as carcinoma / hyperplasia.)

Stage	Grade	CA+H	Ecad	p16	hMLH1	rassf1a	APC	PRB	PTEN	HICI1	ATM	MGMT	MI
1B	1	1	+/-	-/+	-/+	-/+	-/+	-/-	-/-	-/+	+/-	+/-	0.3/0.5
1A	1	2	+/-	-/-	+/-	+/-	-/-	-/-	-/-	-/-	-/-	-/-	0.3/0
1B	1	3	-/-	-/-	+/-	-/-	+/-	-/-	-/-	-/-	-/-	-/-	0.2/0
3A	1	4	+/-	-/+	-/-	-/-	+/-	-/-	-/-	-/-	-/-	-/-	0.2/0.1
1C	1	5	-/-	-/-	+/+	-/-	-/+	-/-	-/+	-/-	+/-	-/+	0.2/0.4
1B	1	6	-/-	-/-	+/-	-/+	-/-	-/-	-/-	-/-	-/-	-/-	0.1/0.1
Stage	Grade	CA	Ecad	p16	hMLH1	rassf1a	APC	PRB	PTEN	HICI1	ATM	MGMT	MI
1B	1	7	-	-	-	+	-	+	-	-	-	-	0.2
1C	1	8	-	-	-	-	-	-	-	+	-	-	0.1
1B	1	9	-	-	-	-	-	-	-	-	-	+	0.1
1B	1	10	-	-	-	-	-	-	-	-	-	+	0.1
1B	1	11	-	+	-	-	-	-	-	-	-	-	0.1
1B	1	12	-	-	+	+	-	+	-	-	-	-	0.3
1B	1	13	-	+	-	-	-	-	-	-	-	+	0.2
1A	1	14	-	+	+	-	-	-	-	-	+	-	0.3
1B	1	15	-	-	+	+	-	-	-	+	+	-	0.4
2B	1	16	-	-	-	-	-	-	-	-	+	-	0.1
3C	1	17	-	-	+	+	-	-	-	+	-	-	0.3
2B	1	18	+	-	-	+	-	-	-	-	-	-	0.2
1A	1	19	+	-	-	-	-	-	-	-	-	+	0.2
3C	2	20	-	-	-	+	-	-	-	-	-	-	0.1
1B	2	21	-	-	-	-	-	-	-	-	-	-	0
1B	2	22	+	-	-	-	+	-	-	-	-	-	0.2
3C	2	23	+	-	+	-	-	-	-	-	-	+	0.3
1C	2	24	-	-	-	-	-	-	-	-	-	-	0
1B	2	25	-	-	+	+	-	-	-	-	-	+	0.3
3C	2	26	-	-	+	-	-	+	-	-	-	-	0.2
2B	2	27	+	-	-	+	-	-	-	-	-	-	0.2
2B	3	28	-	-	+	-	-	-	-	-	-	-	0.1
3A	3	29	-	-	+	+	-	+	-	-	-	-	0.3
3C	3	30	-	-	+	+	+	-	-	-	-	-	0.3
1B	3	31	-	-	+	-	-	+	-	-	-	-	0.2
3A	3	32	+	-	-	-	-	-	-	-	-	-	0.1
2B	3	33	-	-	-	-	-	-	-	-	-	-	0
3A	3	34	-	-	-	+	-	-	-	-	-	-	0.1

Table 5.3. The methylation of individual markers and the methylation index (MI) in individual cases of SCA, EAH and EH. +methylation; - no methylation.

SCA	Ecad	p16	hMLH1	rassf1a	APC	PRB	PTEN	HICI1	ATM	MGMT	MI
1	-	-	-	-	-	-	-	-	-	-	0
2	-	-	-	-	-	-	-	-	-	-	0
3	-	-	-	-	-	-	-	-	-	-	0
4	-	-	-	-	-	-	-	-	-	-	0
5	-	-	-	-	-	-	-	-	-	-	0
6	-	-	-	-	-	-	-	-	-	-	0
7	-	-	-	-	-	-	-	-	-	-	0
8	-	-	-	-	-	-	-	-	-	-	0
AH	Ecad	p16	hMLH1	rassf1a	APC	PRB	PTEN	HICI1	ATM	MGMT	MI
1	-	-	-	-	+	+	+	-	-	-	0.3
2	-	-	-	+	-	+	-	-	-	+	0.3
3	-	-	-	+	-	-	+	-	-	-	0.2
4	-	-	+	+	-	-	-	-	-	-	0.2
5	-	-	-	-	-	-	-	-	-	-	0
6	+	-	+	+	-	-	+	-	-	-	0.4
7	+	-	-	-	-	-	-	+	-	-	0.2
8	-	-	+	+	-	+	-	+	-	+	0.5
9	+	-	-	-	-	-	-	-	+	-	0.2
10	+	-	-	+	-	+	-	+	+	-	0.5
11	-	-	-	+	-	-	-	-	+	-	0.2
12	-	-	-	+	-	+	-	+	-	-	0.3
13	-	-	-	+	-	-	-	-	+	-	0.2
14	-	-	+	-	-	-	-	+	+	-	0.3
15	+	-	-	+	-	-	-	+	-	+	0.4
16	-	-	+	+	-	-	-	+	+	-	0.4
17	-	-	-	+	-	-	-	+	+	-	0.3
18	-	-	-	-	-	+	-	-	+	+	0.3
19	-	-	-	-	-	-	-	-	+	-	0.1
20	-	-	+	+	-	-	-	-	+	-	0.3
21	-	-	+	+	+	+	-	-	+	-	0.5
H	Ecad	p16	hMLH1	rassf1a	APC	PRB	PTEN	HICI1	ATM	MGMT	MI
1	-	-	+	+	-	-	+	-	-	-	0.3
2	+	+	-	-	-	-	+	-	-	-	0.3
3	-	-	+	+	-	-	+	-	-	-	0.3
4	-	+	-	-	-	-	+	-	-	-	0.2
5	-	-	+	-	-	-	-	-	-	-	0.1
6	-	-	+	-	-	-	+	-	-	-	0.2
7	-	-	-	-	-	-	-	-	-	-	0
8	-	-	-	+	-	-	-	-	-	-	0.1
9	-	-	+	-	-	-	+	-	-	-	0.2
10	-	-	+	+	-	-	-	-	-	-	0.2
11	-	-	+	+	-	-	-	-	-	+	0.3
12	-	+	+	+	-	-	+	-	-	-	0.4
13	-	-	+	-	+	-	+	-	-	-	0.3
14	-	+	-	-	-	-	-	-	-	-	0.1
15	-	-	-	-	-	-	-	-	-	-	0
16	+	-	-	-	-	+	+	-	-	-	0.3
17	+	-	+	-	-	+	-	-	-	+	0.4
18	+	-	-	-	-	-	-	-	-	-	0.1
19	-	-	-	-	-	-	-	-	-	-	0
20	-	-	-	-	-	-	-	-	-	-	0
21	-	-	-	-	-	-	-	-	-	+	0.1

APC
M U M U M U M U M U M U+ - -
ECA22 ECA/H3 EAH2 EH1 N

P16
M U M U M U M U M UMU + - -
ECA11 ECA/H2 EAH1 AH1 N

PRB
M U M U M U U M U M M U + - -
ECA1 ECA/H8 EAH3 EH1 N

hMLH-1
M U M U M U M U M U M U + - -
ECA12 ECA/H4 EAH4 EH2 N

PTEN
M U M U M U M U M U M U + - -
ECA7 ECA/H5 EAH2 EH5 N

E-cad
M U M U M U M U M U M U + - -
ECA7 ECA/H1 EAH6 EH1 N

MGMT
M U M U M U M U M U M U + - -
ECA7 ECA/H5 EAH1 EH11 N

RASSF1A
M U M U M U M U M U M U + --
ECA8 ECA/H3 EAH1 EH1 N

ATM
M U M U M U M U M U M U + - -
ECA14 ECA/H2 EAH1 EH2 N

HIC-1
M M M M M M + -
ECA8 EAH1 N
ECA/H2 EH1

Fig.5.1. MSP products were shown in gel under UV illumination. 10 markers, APC p16, PRB, hMLH-1, PTEN, E-cad, MGMT, RASSF1A, ATM and HIC-1, were investigated. In each marker, 6 cases were shown, there are ECA (Endometrioid adenocarcinoma), ECA/H (Endometrioid adenocarcinoma coexisting with hyperplasia and hyperplasia with ECA), EAH (Endometrial atypical hyperplasia), EH (Endometrial simplex hyperplasia). And the case number also shown. U is amplified with unmethylation primers, whereas M is amplified with methylation primers, M bands show methylation. + is positive control, IVD was used as template, - is negative control, water was used.

Table 5.4. The frequency of methylation in normal endometrium, SCA, EH, EAH and
ECA, including 6 cases of ECA coexisting with hyperplasia (ECA+H).

	EH (n=21)	EAH (n=21)	ECA (n=34)	ECA (n=28)	ECA+H (n=6)	SCA (n=8)
E-cad	19%	24%	26%	21%	50%	0%
p16	19%	0%	9%	11%	0%	0%
hMLH1	48%	33%	44%	39%	67%	0%
RASSF1A	29%	67%	35%	39%	17%	0%
APC	5%	10%	12%	7%	33%	0%
PRB	10%	33%	15%	18%	0%	0%
PTEN	43%	14%	0%	0%	0%	0%
HIC1	0%	38%	9%	11%	0%	0%
ATM	0%	52%	15%	11%	33%	0%
MGMT	14%	19%	21%	21%	17%	0%
MI	0.19	0.29	0.20	0.19	0.22	0
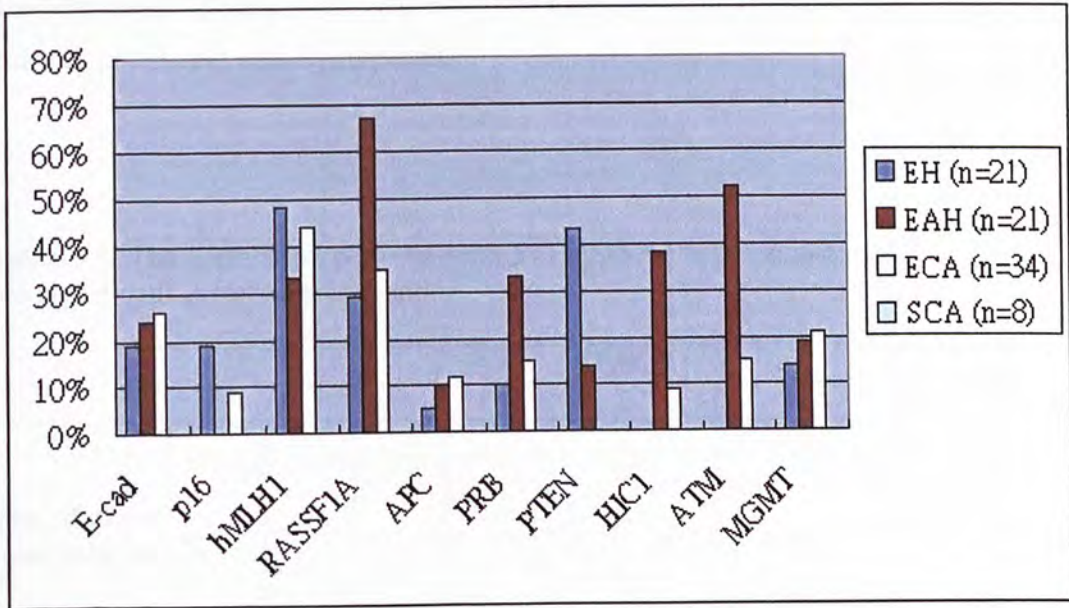
Fig. 5.2. Summery of methylation of tumor suppressor genes in ECA, SCA, EAH and EH
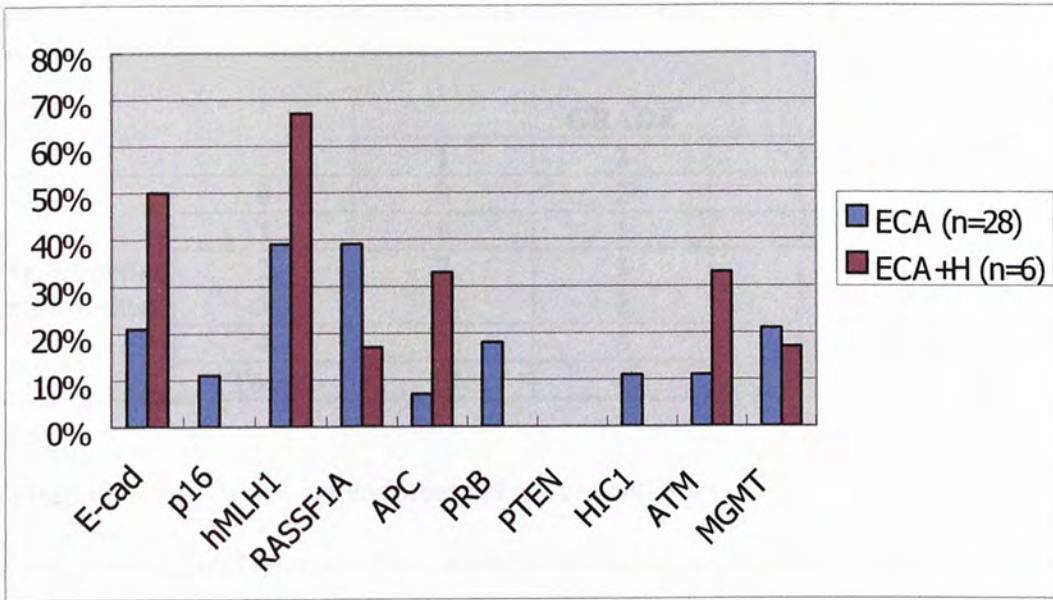
Fig. 5.3. Summery of methylation of tumor suppressor genes in ECA which can be divided into 2 groups. They are ECA coexisting with hyperplasia (ECA+H) and ECA without coexisting with hyperplasia.

Table 5.5. The correlation between promoter hypermethylation and staging.
No significant correlation is found.

		STAGE			Total
		1	2	3	
	0	2	1	0	3
	1	5	2	3	10
No. of genes methylated	2	7	2	2	11
	3	5	0	4	9
	4	1	0	0	1
	Total	20	5	9	34

Table 5.6. The correlation between promoter hypermethylation and grading.
No significant correlation is found.

| | | GRADE | | | Total |
		1	2	3	
No. of genes methylated	0	0	2	1	3
	1	6	1	3	10
	2	7	3	1	11
	3	5	2	2	9
	4	1	0	0	1
	Total	19	8	7	34

Promoter methylation on endometrial cancer cell lines

Gene promoter methylation of ten candidate tumor suppressor genes were investigated in endometrial cell lines RL-95 and KLE. The results were shown in table 5.7. Promoter methylation is frequent in endometrial cancer cell lines.

Table 5.7 Methylation data on endometrial cell lines. +Partial Methylation; ++Complete Methylation; -No methylation.

Cell lines	Ecad	p16	hMLH1	RASSF1A	APC	PRB	PTEN	HICI1	ATM	MGMT	MI
RL-95	-	+	+	++	+	+	+	-	-	+	0.7
KLE	-	-	+	++	-	-	-	-	-	+	0.3

5.3 MSI analysis:

ECA was classified as MSI positive (MSI +ve) when at least 1 of the 7 markers exhibit instability. In our study, the markers are **BAT-25**(4q12), **BAT-26**(2p16), **BAT-40**, **D2S123**(2p16-p21), **D5S346**(5q21-q22), **D17S250**(17q11.2-q12), **TGFb2R**. MSI+ were divided into MSI-High and MSI-Low. When 2 of the 7 markers or above show instability,

it is MSI-High. If only 1 marker show instability, it is MSI-Low. The MSI results were shown in fig.5.4, and the MSI status of individual markers in individual cases were shown in table 5.8.

In 34 cases of endometrial cancer, twenty-six cases (76.5%) were microsatellite stable (MSS), 8 cases (23.5%) were MSI positive. Among the MSI positive cases, 4 cases were MSI-low and 4 cases were MSI-high. The presence of MSI was correlated with higher grade (spearman p=0.014) (table 5.9) and higher stage (spearman p=0.010) (table 5.10).

Interestingly, the MSI status does not correlate with the methylation status of hMLH-1 in this series. The hMLH-1 methylation status, methylation index (MI) and the MSI status of individual cases were shown in table 5.12. The mean MI was 0.21 for MSS cases and 0.11 for MSI positive cases. MSS cases has significantly higher MI than MSI positive cases (Mann-Whitney U p=0.028). (Table 5.11)
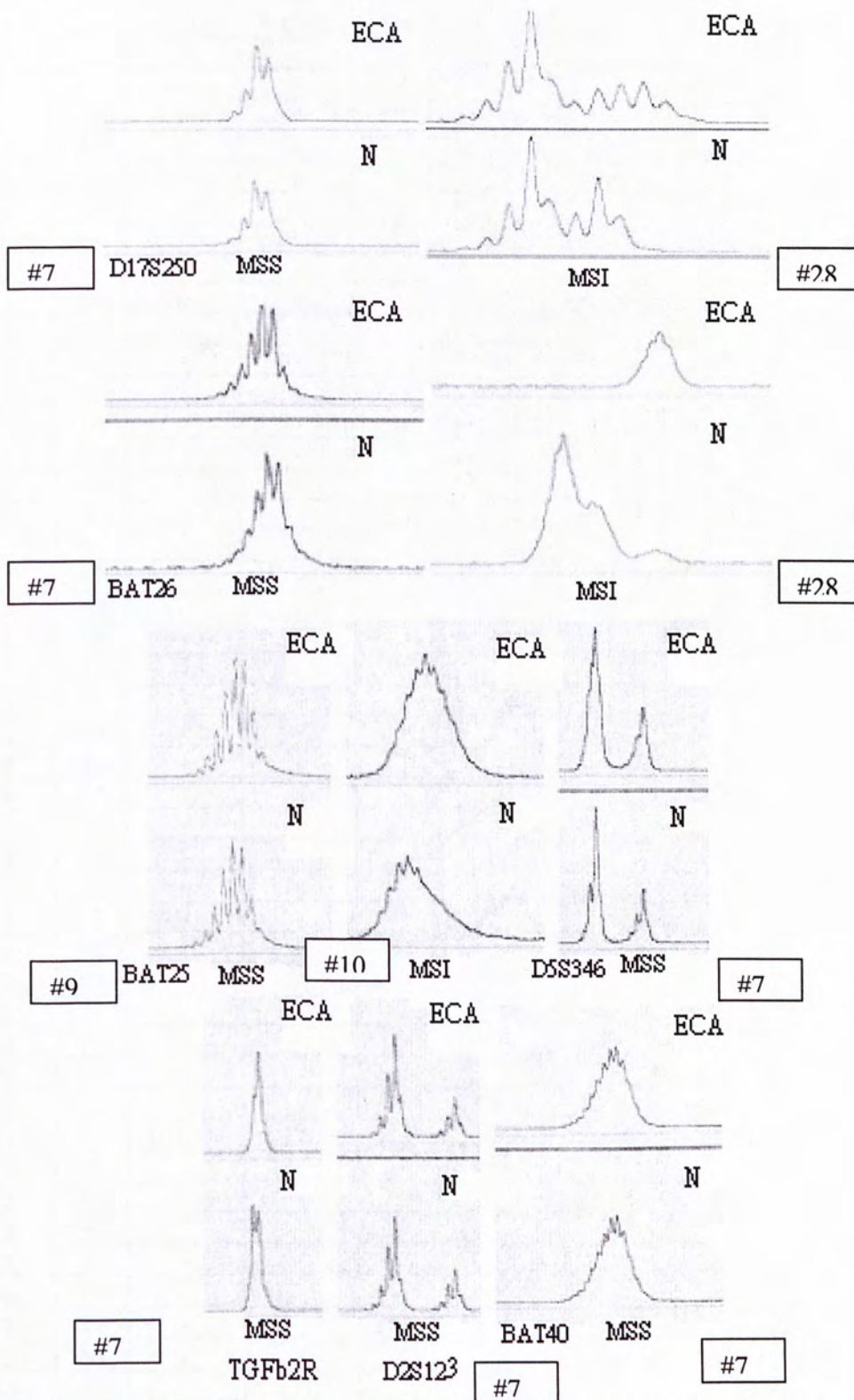
Fig.5.4. The analysis of MSI. MSI- microsatellite instability+; MSS- microsatellite stable. Example case number shown in the boxes.

Table 5.8. The MSI of individual marker in individual patient. +MSI+; - MSS.

CA+H	Grade	Age	D5S346	D2S123	BAT40	BAT25	BAT26	D17S250	TGFb2R	M+
6	1	55	-/-	-/-	-/-	-/-	-/-	-/-	-/-	0/0
1	1	38	-/-	-/-	-/-	-/-	-/-	-/-	-/-	0/0
2	1	47	-/-	-/-	-/-	-/-	-/-	-/-	-/-	0/0
3	1	47	-/-	-/-	-/-	-/-	-/-	-/-	-/-	0/0
4	1	39	-/-	-/-	-/-	-/-	-/-	-/-	+/-	1/0
5	1	44	-/-	-/-	-/-	-/-	-/-	-/-	-/-	0/0
CA	Grade	Age	D5S346	D2S123	BAT40	BAT25	BAT26	D17S250	TGFb2R	M+
7	1	62	-	-	-	-	-	-	-	0
8	1	59	-	-	-	-	-	-	-	0
9	1	75	-	-	-	-	-	-	-	0
10	1	68	-	-	-	+	-	-	-	1
11	1	65	-	-	-	-	-	-	-	0
12	1	70	-	-	-	-	-	-	-	0
13	1	53	-	-	-	-	-	-	-	0
14	1	48	-	-	-	-	-	-	-	0
15	1	52	-	-	-	-	-	-	-	0
16	1	50	-	-	-	-	-	-	-	0
17	1	41	-	-	-	-	-	-	-	0
19	1	49	-	-	-	-	-	-	-	0
18	1	32	-	-	-	-	-	-	-	0
25	2	47	-	-	-	-	-	-	-	0
26	2	53	-	-	-	-	-	-	-	0
27	2	49	-	-	-	-	-	-	-	0
20	2	62	+	-	+	-	-	-	+	3
21	2	52	-	-	-	-	-	-	+	1
22	2	51	-	-	-	-	-	-	-	0
23	2	62	-	-	-	-	-	-	-	0
24	2	55	-	-	-	-	-	-	-	0
29	3	76	-	-	+	+	-	-	+	3
30	3	52	-	-	-	-	-	-	-	0
31	3	53	-	-	-	-	-	-	-	0
32	3	54	-	-	+	+	-	-	-	2
33	3	71	-	-	-	-	-	-	+	1
34	3	67	-	-	-	-	-	-	-	0
28	3	49	-	-	-	-	+	+	-	2
SCA	Grade	Age	D5S346	D2S123	BAT40	BAT25	BAT26	D17S250	TGFb2R	M+
1	3	58	-	-	-	-	-	-	-	0
2	3	56	-	-	-	-	-	-	-	0
3	3	52	-	-	-	-	-	-	-	0
4	3	66	-	-	-	-	-	-	-	0
5	3	70	-	-	-	-	-	-	-	0
6	3	72	-	-	-	-	-	-	-	0
7	3	69	-	-	-	-	-	-	-	0
8	3	59	-	-	-	-	-	-	-	0

Table 5.9. Correlation between MSI and grading of EAC. (Spearman p=0.014)

		Grade			Total
		1	2	3	
	MSS	16	7	3	26
MSI status	MSI-low	2	1	1	4
	MSI-high	0	1	3	4
Total		18	9	7	34

Table5.10. Correlation between MSI and staging of EAC. (Spearman p=0.010)

		Stage			Total
		1	2	3	
	MSS	19	2	5	26
MSI status	MSI-low	2	1	1	4
	MSI-high	0	1	3	4
Total		21	4	9	34

Table5.11. Correlation between MSI and methylation index (MI). (Mann-Whitney U p=0.028)

	MI
MSS	0.21
MSI	0.11

Table 5.12. Summery of methylation index (MI) calculated from 10 markers, hMLH-1 methylation status and MSI status. For cases of endometrioid adenocarcinoma with co-exist endometrial hyperplasia, both ECA and EH were investigated the results were listed as carcinoma / hyperplasia.(hMLH1:-unmethylated, +methylated; MSI: - MSS, +MSI)

Stage	Grade	CA+H	MI	hMLH-1	MSI
1B	1	1	0.3/0.5	-/+	-/-
1B	1	6	0.1/0.1	+/-	-/-
1A	1	2	0.3/0	+/-	-/-
1B	1	3	0.2/0	+/-	-/-
3A	1	4	0.2/0.1	-/-	+/-
1C	1	5	0.2/0.4	+/+	-/-
Stage	Grade	CA	MI	hMLH1	MSI
1B	1	7	0.2	-	-
1C	1	8	0.1	-	-
1B	1	9	0.1	-	-
1B	1	10	0.1	-	-
1B	1	11	0.1	-	-
1B	1	12	0.3	+	-
1B	1	13	0.2	-	-
2B	1	18	0.2	-	-
1A	1	14	0.3	+	-
1B	1	15	0.4	+	-
2B	1	16	0.1	-	-
3C	1	17	0.3	+	-
1A	1	19	0.2	-	-
3C	2	20	0.1	-	+
1B	2	21	0	-	+
1B	2	22	0.2	-	-
3C	2	23	0.3	+	-
1C	2	24	0	-	-
1B	2	25	0.3	+	-
3C	2	26	0.2	+	-
2B	2	27	0.2	-	-
2B	3	28	0.1	+	-
3A	3	29	0.3	+	+
3C	3	30	0.3	+	-
1B	3	31	0.2	+	-
3A	3	32	0.1	-	-
2B	3	33	0	-	+
3A	3	34	0.1	-	-
Stage	Grade	SCA	MI	hMLH1	MSI
2B	3	1	0.1	-	-
3C	3	2	0.2	+	-
2A	3	3	0.2	-	-
3A	3	4	0.2	-	-
SC	3	5	0.2	+	-
2A	3	6	0.3	+	-
2B	3	7	0.2	-	-
3C	3	8	0.2	+	-

Chapter 6 Discussion

6.1 Promoter hypermethylation in endometrial cancer

DNA methylation is an epigenetic event that is regarded as an alternative mechanism for inactivation of tumor related genes. Promoter hypermethylation had been discovered in multiple tumor suppressor genes in different malignancies and considered playing an important role in carcinogenesis (Baylin and Herman, 2000). In this part of study, we have examined the promoter hypermethylation of 10 candidate tumor suppressor genes in endometrial cancer (ECA) and its precursor lesions, endometrial hyperplasia with atypia and without atypia. The study provides comprehensive analysis of gene promoter methylation profiles in endometrioid adenocarcinoma and its precancerous lesion.

In this study, ten markers were selected in methylation detection. They are E-cad, p16, MGMT, APC, hMLH-1, ATM, HIC-1, RASSF1A, PRB and PTEN. They are tumor suppressor genes and they are commonly used as markers in methylation analysis because they are frequently methylated in different tissue cancer types. Tumor suppressor genes play active roles in apoptosis, cell cycle regulation or DNA mismatch repair. All these function is important, tumor suppressor gene inactivation is important in carcinogenesis in many different types of cancer.

In this study, methylation-specific PCR (MSP) was selected to detect the methylation in tumor samples. The reasons we choose MSP is that MSP is very specific and sensitive method and it is relatively simple. Comparing to other methods such as combined bisulfite restriction analyses (COBRA) which is commonly used, COBRA need restriction enzyme, its creation and modification is critical in the experiment. However, MSP do not need restriction enzyme, so these problem do not occur, also the incomplete

digestion do not appear. On the other hand, other methods such as bisulfite sequencing and southern blotting analysis were not selected in our study. Since our EC samples are paraffin-embedded tissue, DNA extracted from paraffin-embedded tissue is just small amount and the quality is not good enough to employ bisulfite sequencing and southern blotting analysis. Therefore, we employed MSP as detection in our study. MSP can be performed with small amount of DNA, and DNA extracted from paraffin-embedded tissue is good enough to perform MSP because MSP is highly sensitive and specific.

6.1.1 Concurrent hypermethylation of multiple genes in endometrioid adenocarcinoma and its precursor lesions.

In this study, promoter hypermethylation of tumor suppressor genes is frequently detected in endometrioid adenocarcinoma. Thirty four cases of endometrioid adenocarcinoma (ECA) were examined, 91% of ECA demonstrated gene methylation in at least one of the 10 candidate tumor suppressor genes. 59% of ECA demonstrated hypermethylation in more than one genes and 26% of ECA demonstrated hypermethylation in 3 or above genes. Methylation indexes (MI) were calculated and the mean MI of 10 genes in ECA is 0.19. None of the normal control had hypermethylation detected. Thus, gene promoter hypermethylation appears to be common event in endometrioid adenocarcinoma.

Endometrial hyperplasia is considered as pre-cancerous lesion of ECA. In our study, we examined both endometrial hyperplasia with and without cytological atypia. It appears that gene methylation is also common in endometrial hyperplasia. The methylation index

for endometrial hyperplasia without atypia (EH) and with atypia (EAH) are 0.20 and 0.29 respectively. There are no statistical significant difference in promoter hypermethylation among ECA, EAH and EH. In this study, both precancerous lesions and carcinomas show promoter methylation. This finding suggested that gene promoter hypermethylation are more likely to be involved in the early events in development of ECA.

6.1.1.1 Promoter hypermethylation of E-cadherin

Methylation of E-cadherin (E-cad) gene has been widely reported in different tumors (Bornman, et al, 2001; kanai, et al., 1997; Nass, et al., 2000). E-cadherin belongs to a class of cell adhesion molecules (CAM) and is important for tissue integrity, cell movement and morphogenesis (Gumbiner, 1996). Loss of function of E-cad was strongly associated with tumor invasion (Birchmeier and Behrens, 1994). Saito T, et al. demonstrated that gene promoter hypermethylation of E-cad is associated with grading and the metastasis level of endometrial carcinoma (Saito T, et al, 2003). In this study, the methylation frequency of E-cad in EH, EAH and ECA are 19%, 24% and 26% respectively, but not detected in normal endometrium. Frequent methylation of E-cad in ECA was demonstrated, and it is consistent with others' previous studies.

6.1.1.2 Promoter hypermethylation of APC

Adenomatous polyposis coli (APC) gene is located on chromosome 5q21. APC is a tumor suppressor gene to form complex with Axin and GSK3 in the Wnt signaling

pathway to inactivate β-catenine by promoting its degradation. Loss of function of APC protein resulted in stabilization of β-catenine within the cell. Accumulation of β-catenine eventually leaded to the formation of complexes with DNA binding proteins T-cell factor (Tcf) and lymphoid enhancer-binding factor (Lef-1) which translocated into the nucleus and activated specific gene. Loss of function of APC by genetic mechanism was common in cancer (Fodde, et al., 2001). Epigenetic mechanism was also found to involve in the gene inactivation in many cancers (Brabender, et al., 2001, Maruyama, et al, 2001; Virmani, et al, 2001; Zysman, et al, 2002). Promoter hypermethylation methylation can silence gene as same as the results of loss of function mutation, however, this gene is not permanent silence. In this study, the methylation frequency of APC in EH, EAH and ECA are 5%, 10% and 12% respectively. However, previous studies showed that methylation frequency of endometrial cancer is from 43% to 46.6%, and the methylation frequency of endometrial hyperplasia is about 17%. Comparing to others' previous studies, the methylation frequency detected in this series of endometrial cancer and its precursor lesions are lower than that found in others' studies. Nevertheless, APC promoter hypermethylation occur in both ECA and its precancerous lesions, endometrial hyperplasia and endometrial atypical hyperplasia. APC promoter methylation in endometrial cancer appears to be an early event in carcinogenesis of endometrial cancer.

6.1.1.3 Promoter hypermethylation of MGMT

The DNA repair protein O^6-methylguanine DNA methyltransferase (MGMT) removes alkyl adducts from the O^6 position of guanine in DNA. Failure to remove the alkyl adducts generate G:C to A:T transition mutation and promote tumorgenesis as

demonstrated (Esteller, et al., 2001). MGMT which located at 10q26, is frequently hypermethylated in human cancers resulting in loss of expression (Bae, et al, 2002, Esteller, et al, 2002, Oue, et al, 2001). There is no previous study of endometrial cancer investigated for MGMT gene promoter methylation. In this study, we investigated the promoter methylation of MGMT as a new marker in endometrial cancer, the promoter methylation frequency of MGMT detected in EH, EAH and ECA are 14%, 19% and 21% respectively. It is a new finding indicated that MGMT gene promoter methylation is common in endometrial cancer, and it may be an early event in endometrial cancer development. The significance of the biological effect needs further investigation.

6.1.1.4 Promoter hypermethylation of RASSF1A

RASSF1A is located in 3p21. RASSF1A was isolated as a tumor suppressor gene in this region from a two-hybrid screen (Dammann, et al., 2000). One of the slice variants of the gene RASSF1A undergoes frequent epigenetic changes in tumors of lungs, nasopharynx, breast, kidney, stomach, ovary and urinary bladder (Agathanggelou, et al., 2001; Burbee, et al, 2001; Byun, et al, 2001; Dreijerink, et al, 2001; Lee, et al, 2001; Lo, et al., 2001; Yoon, et al., 2001). Although the functions of RASSF1A are not completely understood, several studies found that it can reduce tumor growth both in vitro and in nude mice model (Burbee, et al, 2001; Dreijerink, et al., 2001; Vos, et al, 2000). Those experiments suggested that it was a candidate tumor suppressor gene. Structural analysis also found that its RAS-association domain likely in the RAS-mediated apoptotic mechanism. RASSF1A was also showed to bind with NORE1, the novel RAS effecter and MST1, the pro-apoptotic protein kinase in mediating the apoptotic effecter of RAS (Khokhlatchev, et

al, 2002; Ortiz-Vega, et al, 2002). There is no previous data on the methylation status of RASSF1A in endometrial cancer. In this study, we investigated the promoter methylation of RASSF1A as a new marker in endometrial cancer. Promoter hypermethylation was detected in EH, EAH and ECA in 29%, 67% and 35% respectively, but not in normal endometrium. High methylation frequencies were detected in RASSF1A in both cancer and its precursor lesions, endometrial hyperplasia were demonstrated. Also, complete promoter hypermethylation was detected in both 2 endometrial cancer cell lines, RL-95 and KLE. This new findings indicated that RASSF1A promoter methylation is common in endometrial cancer and its precursor lesions. Promoter hypermethylation are more likely to be involved in an early event in cancer development in endometrial cancer.

6.1.1.5 Promoter hypermethylation of hMLH-1

hMLH-1 is a DNA mismatch repair gene located on human chromosome 3p21. In previous study in endometrial cancer, 54% cases of ECs showed promoter methylation. Among methylated ECs, 42% contained methylated promoters in adjacent normal. In this study, promoter hypermethylation of hMLH-1 was detected in EH, EAH and ECA in 48%, 33% and 44% respectively. High methylation frequencies were detected in hMLH1 in both carcinoma and its precursor lesions, hyperplasia. hMLH-1 promoter hypermethylation is common in endometrial cancer and its precursor lesions. It is more likely to be involved in an early event in cancer development in endometrial cancer. This results is consistent with others' previous studies.

6.1.1.6 Promoter hypermethylation in ECA coexisting with hyperplasia and not coexisting with hyperplasia

By comparing the ECA coexisting with hyperplasia and not coexisting with hyperplasia, the methylation status of individual genes and overall methylation index appear different, and the significant differences in methylation pattern detected in current study cannot be found. Thus, there is no significant differences found in this series of ECAs.

6.1.2 Promoter hypermethylation in serous adenocarcinoma (SCA)

Interestingly, none of the serous adenocarcinoma demonstrated any gene methylation in our study. Although the numbers of serous adenocarcinoma is small, the results suggest that gene methylation is rare in serous adenocarcinoma.

In previous studies, results showed there are differences in genetic and molecular alterations between these 2 types of carcinoma, endometrioid and non-endometrioid adenocarcinoma. The carcinogenic pathway of these 2 types of carcinomas seen to be different. (D Tritz, 1997; Berchuck A., 1997)

In addition, previous study showed that both frequency and patterns of LOH differed greatly between the two tumor types. Loss of heterzygosity (LOH) was frequently detected in the non-endometrioid adenocarinomas, but it was rare in the endometrioid adenocarinomas. (D Tritz, 1997) Therefore, it is hypothesis that carcinogenesis of ECA is due to epigenetic alterations such as gene promoter hypermethylation, but it is not due to genetic changes. However, SCA is non-endometrioid adenocarcinoma, the carcinogenesis

of SCA is seem to be due to the genetic alterations such as LOH and deletion, but it is not due to methylation. All these findings supported the hypothesis that carcinogenetic pathways are distinct between endometrioid and serous adenocarcinoma. (D Tritz, 1997; Berchuck A., 1997) In our study, the results is consistent with others' previous studies.

6.2 Microsatellite status analysis

Microsatellites are DNA tandem repeat sequences found throughout the genome. (Eshleman J.R., 1996) These sequences show variation between individuals (Weber and May, 1989) and can be used as markers in genetic analysis. Microsatellite instability (MSI) can be detected by the change of any length in these repetitive sequences due to insertion or deletion in tumor tissue as compared to that in normal tissue. It was recognized that the patterns of microsatellites are unstable in certain tumors.

6.2.1 MSI in endometrial cancer

Many studies found MSI has significant role in carcinogenesis in stomach, colon, lung, breast and prostate (Caldes, et al., 2000; Hayden, et al., 2000; Perinchery, et al., 2000; Yamashita, et al., 2000). It was also identified in 17 to 43% of endometrial carcinomas. (Wallace, 1998; Horii, 1992; Itoh, 1994; Hattori, 1996; Akimoto, 1998; Taniguchi, 1998) In this study, MSI positive was detected in 23.5% of ECA, while 76.5% of ECA were microsatellite stable (MSS). This result is consistent with the previous studies. Microsatellite instability is quite common in endometrial cancer.

In our study, the presence of MSI was higher in higher grade ECA (Spearman p=0.014) and higher stage ECA (Spearman p=0.010). This result demonstrated that the MSI positive cases correlate with pathological grading and staging of ECA. It is consistent with previous that MSI was correlated with histological grade, International Federation of Gynecologists and Obstetricians (FIGO) stage, and also myometrial invasion, and lymphonode metastasis. (Hirasawa A, 2003) The prognostic implications of MSI status in patients with ECA deserve further investigation. However, it is different from other tissue type of cancers such as colorectal cancer. Previous studies showed that MSI+ sporadic colorectal cancers were associated with poor differentiation and less frequent systemic metastasis than MSI- tumors. MSI+ status (p=0.038) were independent favorable prognostic factors for survival in sporadic colorectal cancer patients. (Lim SB, 2004) So the results detected in this study in endometrial cancer inverse with previous colorectal cancers studies, it is unexpected. Therefore, different tissue types of cancers show different molecular characteristics, and different types of cancer seem to be due to different carcinogenic pathways. So prognostic implications of MSI status deserve further investigation.

6.2.2 MSI and concurrent promoter hypermethylation

Methylation index (MI) was calculated in relation to the MSI status. The mean MI of ECA was 0.21 for MSS cases and 0.11 for MSI positive cases. MSS cases had significantly higher MI than MSI positive cases (Mann-Whitney U p=0.028). This finding suggests that MSI positive and MSS cases may have different distinct

carcinogenic pathways in ECA. The possible underlying explanation is unknown and deserve for further investigation.

6.2.3 MSI and promoter hypermethylation of hMLH-1

In previous study, inactivation of the *MLH1* gene may occur via epigenetic changes in a large fraction of MSI + cancers, such as DNA methylation of *MLH1* transcriptional regulatory sequences. (Leung S.Y., 1999; Cunningham J.M., 1998; Herman J.G., 1998; Kane M.F., 1997; Markowitz S., 1995) However, MSI status was not always correlated with hMLH1 gene promoter hypermethylation. (Poala Baldinu, Cancer, 2002; Whelan AJ, 2002) It is consistent with our study that MSI is not correlated with hMLH-1 promoter methylation. Thus, there may also be other mechanism(s) that associated with microsatellite instability.

Chapter 7 Conclusion

1. Our study demonstrated gene promoter methylation is a common event in endometrioid adenocarcinoma but not in serous adenocarcinoma. Relatively high frequency of gene promoter methylation was detected in hMLH1, RASSF1A, E-cadherin and MGMT genes. The frequent methylation of RASSF1A and MGMT genes represent new observations and deserve for further investigation.

2. Gene promoter methylation in multiple genes also commonly observed in endometrial hyperplasia. The findings suggested that gene methylation may be an early event in carcinogenesis of endometrioid adenocarcinoma.

3. Microsatellite instability (MSI) is detected in a subset of endometrial adenocarcinoma. The MSI status is correlated with tumor grade and stage.

4. Interestingly, MSI is inversely correlated with frequency of gene promoter methylation. Microsatellite stable (MSS) cases had significantly higher MI than MSI positive cases. The observations suggested that ECA with or without MSI have distinct patho-biological features. The findings may have implications in understanding of carcinogenic pathways in endometrial cancer.

Further studies

Our study indicated that gene promoter methylation is common in both endometrioid adenocarcinoma and the precancerous lesion, endometrial hyperplasia. Other epigenetic alterations, like histone acetylation may also be involved. Thus, future histone acetylation analysis may need to be investigated. We observed the frequent RASSF1A and MGMT gene methylation in our study. The cellular pathways that may be disrupted are of interest and deserve further investigations. Since gene methylation is commonly detected in ECA and endometrial hyperplasia, it opens the possibility of using gene methylation as markers for endometrial pathology. Our findings also support the prognostic significance of MSI status in ECA. However, the clinical usefulness of MSI status as prognostic marker required a larger scale study to determine. Moreover, microsatellite stable (MSS) cases had significantly higher MI than MSI positive cases. The observations suggested that ECA with or without MSI have distinct patho-biological features. However, the underlying mechanism that link up the MSI and methylation status remained unexplained. Further studies along this line may provide more insight in carcinogenesis of endometrial cancer.

Reference

Aaltonen,LA, Peltonamki,P, Mecklin,JP, Jarvinen,H, Jass,JR, Green,JS, Lynch, HT, Watson,P, Tallqvist,G, and Juhola,M, Replication errors in benign and malignant tumors from hereditary nonpolyposis colorectal cancer patients. *Cancer Research*, 54: 1645-8, 1994

Abeloff MD, Armitage JO, Lichter AS, Niederhuber JE. *Clinical Oncology 2nd Ed.*, pp. 77-118.

Agathanggelou,A., Honorio,S, Macartney,DP, Martinez,A, Dollol,A, Rader,J, Fullwood,P, Chauhan,A, Walker,R, Shaw,JA, Hosoe, S, Lerman,MI, Minna,JD, Maher, ER, and Latif,F. Methylation associated inactivation of RASSF1A from region 3p21.3 in lung, breast and overian tumors. *Oncogene*,20: 1509-1518, 2001

Ai L, Vo QN, Zuo C, Li L, Ling W, Suen JY, Hanna E, Brown KD, Fan CY. Ataxia-telangiectasia-mutated (ATM) gene in head and neck squamous cell carcinoma: promoter hypermethylation with clinical correlation in 100 cases.*Cancer Epidemiol Biomarkers Prev.*;13(1):150-6. 2004

Akimoto S., Ochiai A., Inomata M., and Hirohashi S.. Expression of cadherin-catenin cell adhesion molecules, phosphorylated tyrosine residues and growth factor receptor-tyrosine kinases in gastric cancers *Jpn J Cancer Res*. 89: 829-836. 1998

Antequera, F., Boyes, J., and Bird, A. High levels of de novo methylation and altered chromatin structure at CpG islands in cell lines. *Cell,* 62:503-514, 1990.

Antequera, F. and Bird, A. Number of CpG islands and genes in human and mouse. *Proc Natl Acad Sci USA*, 90:11995-11999, 1993.

Bae, SI, Lee, HS, Kin, SH, and Kin, WH. Inactivation of O6-methylguanine-DNA methyltransferasen by promoter CpG island hypermethylation in gastric cancers. *Br J Cancer*. 86: 1888-1892, 2002

Baylin, S.B., and Herman, J.G. DNA hypermethylation in tumorgenesis: epigenetics joins genetics. *Trends Genet*, 16:168-174, 2000.

Bender, C.M., Pao, M.M., and Jones, P.A. Inhibition of DNA methylation by 5-aza-2'-deoxycytidine suppresses the growth of human tumor cell lines. *Cancer Res,* 58:95-101, 1998.

Berchuck A., Maxwell G.L., and Risinger J.. Genetic alterations in endometrial cancer *Hung J Gynecol Oncol*. 2: 153-157. 1997

Berchuck A., Kohler M.F., and Marks J.R., *et al*. The p53 tumor-suppressor gene frequently is altered in gynecologic cancers *Am J Obstet Gynecol*. 170: 246-252. 1994

Bienz M. APC: the plot thickens *Curr Opin Genet Dev* 9: 595-603. 1999.

Birchmeier, W abd Behrens, J. Cadherin expression in carcinomas: role in the formation of cell junctions and the prevention of invasiveness. *Biochim Biophys Acta*, 1198:11-26, 1994

Bird, A. DNA methylation patterns and epigenetic memory. *Genes dev*, 16:6-21, 2002.

Bird, A.P. CpG-rich islands and the function of DNA methylation. *Nature*, 321:209-213, 1986 .

Boland, CR, Thibodeau, SN, Hamilton, SR, Sidransky, D, Eshleman, JR, Burt, RW, Meltzer, SJ, Rodriguez-Bigas, MA, Fodde, R, Ranzani, GN and Srivastava, S, A National Cancer Institute Workshop on Microsatellite Instability for cancer detection and familial predisposition: development of international criteria for the deamination of microsatellite instability in colorectal cancer. *Cancer Res,* 58, 5248-57, 1998

Bornman, DM, Mathew, S, Alsruhe, J, Herman, JG and Gabrielson, E. Methylation of the E-cadherin gene in bladder neoplasia and in normal urothelial epithelium from elderly individuals. *Am J Pathol*, 159:831-835, 2001

Brabender, J, Usadel, H, Danenberg, MJ, Emi, M, Fujiwara, Y, Kyprianou, N, Jacobs, SC, Robinson, JC, Epstein, JI, Walsh, PC, and et al. Homozygous deletion and frequent allelic loss of chromosome 8p22 loci in human prostate cancer. *Cancer Res*, 53: 3869-3873, 1993

Burbee, DG, Forgacs, E, Zochbauer-Muller, S, Shivakumar, L, Fong,K, Gao,B, Randle,D, Kondo, M, Virmani,A, Bader,S, Sekido, Y, Latif, F, Milchgrub, S, Toyooka, S, Gazdar, AF, Lerman, MI, Zabarovsky, E, White, M, and Minna, JD. Epigenetic inactivation of RASSF1A in lung and breast cancers and malignant phenotypr suppression. J Natl Cancer Inst, 93: 691-699, 2001

Burton J.L. and Wells M.. Recent advances in the histopathology and molecular pathology of carcinoma of the endometrium *Histopathology*. 33: 297-303. 1998

Byun, DS, Lee, MG, Chae, KS, Ryu, BG, and Chi, SG. Frequent epigenetic inactivation of RASSF1A by aberrant promoter hypermethylation in human gastric adenocarcinoma. *Cancer Res*, 61:7034-7038, 2001

Caduff R.F., Johnston C.M., and Svoboda -Newman S.M., *et al.* Clinical and pathologic significance of microsatellite instability in sporadic endometrial carcinoma *Am J Pathol* 148: 1671-1678.1996.

Caldes, T, Perez-Segura, P, Tosar, A, De La Hoya, M, and Diaz-Rubio, E. Low frequency of microsatellite instability in sporadic breast cancer. *Int J oncol*, 16: 1235-1242, 2000

Cancerlinks. http://www.cancerlinks.org/Endometrial/index.html#introduction, 2003

Chao H, Sun J, Lu S.Methylation and expression of the p16 gene in endometrial carcinoma. *Zhonghua Zhong Liu Za Zhi.* 2000

Cunningham J.M.,. Christensen, E.R, and Tester D.J., *et al.* Hypermethylation of the hMLH1 promoter in colon cancer with microsatellite instability *Cancer Res.* 58: 3455-3460. 1998

Dammann R, Li, C, Yoon, JH, Chin PL, Bates, S, and Pfeifer, GP. Epigenetic inactivation of a RAS association domain family protein from the lung tumor suppressor locus 3p21.3. *Nat Genet,* 25: 315-319, 2000

Department of Health, HKSAR, 2003, http://www.info.gov.hk/dh/, 2003

Di Croce, L., Raker, V.A., Corasro, M., Fazi, F., Fanelli, M., Fuks, F., Lo Coco, F.,

Dreijerink, K, Braga, E, Kuzmin, I, Geil, L, Duh, FM, Angeloni, D, Zbar, B, Lerman, MI, Stanbridge, EJ, Minna, JD, Protopopoy, A, Li, J, Kashuba, V, Klein, G, and Zabarovsky, ER. The candidate tumor suppressor gene, RASSf1A, from human chromosome 3p21.3 is involved in kidney tumorgenesis. *Proc Natl Acad Sci USA*, 98: 7504-7509, 2001

Duggan B., Felix J., and Muderspach L., *et al.* Early mutational activation of the c-Ki-ras oncogene in endometrial carcinoma, *Cancer Res,* 1994.

Enomoto T., Fujita M., and Inoue M., *et al.* Alteration of the p53 tumor-suppressor gene and its association with activation of the c-K-ras-2 proto-oncogene in premalignant and malignant lesions of the human uterine endometrium, *Cancer Res,* 1993.

Eshleman J.R. and Markowitz S.D. . Mismatch repair defects in human carcinogenesis. *Hum Mol Genet*5: 1489-1494 .1996.

Esteller, M, Gaidano, G, Goodman, SN, Zagonel, V, Capello, D, Botto, B, Rossi, D, Gloghini, A, Vitolo, U, Carbon, A, Baylin, SB, and Herman JG. Hypermehtylation of the DNA repair gene O6-methylguanine DNA methyltransferase and survival of patients with diffuse large B-cell lymphoma. *J Natl Cancer Inst*, 94: 26-32, 2001

Esteller, M, Hamilton, SR, Burger, PC, Baylin, SB, and Herman, JP. Inactivation of the DNA repair gene O6-methylguanine-DNA methyltransferase by promoter hypermethylation is a common event in primary human neoplasia. *Cancer Res*, 59: 793-797, 1999a

Esteller M, Catasus L, Matias-Guiu X, Mutter GL, Prat J, Baylin SB, Herman hMLH1 promoter hypermethylation is an early event in human endometrial tumorigenesis. *Am J Pathol* 155(5):1767-72. 1999b

Esteller M, Risques, RA, Toyota, M, Capella, G, Moreno, V, Peinado, MA, Baylin, SB, and Herman, JG. Promoter hypermehtylation of DNA repair gene O6-methylguanine-DNA methyltransferase is associated with the presence of G:C to A:T transition mutations in p53 in human colorectal tumorgenesis. *Cancer Res*, 61: 4689-4692, 2001

Fearon ER. Human cancer syndromes: clues to the origin and nature of cancer. *Science* 278:1043-1048, 1997

Feinberg, A. P., Gehrke, C. W., Kuo, K. C., and Ehrlich, M. Reduced genomic 5-methylcytocine content in human colonic neoplasia. *Cancer Res,* 48: 1159-1161, 1998.

Feinberg, A.P. and Vogelstein, B. Hypomethylation distinguishes genes of some human cancers from their normal counterparts. *Nature,* 301:89-92, 1983.

Fodde, R, Smits, R, and Clevers, H. APC, signal transduction and genetic instability in colorectal cancer. *Nature Rev Cancer*, 1: 55-67, 2001

Fuks, F., Burgers, W.A., Brehm, A., Hughes-Davies, L., and Kouzarides, T. DNA methyltransferase Dnmt1 associates with histone deacetylase activity. *Nat Genet,* 24:88-91, 2000.

Fuks, F., Burgers, W.A., Godin, N., Kasai, M., and Kouzarides, T. Dnmt3a binds deactylases and is recruited by a sequence-specific repressor to silence transcription. *Embo J*, 20: 2536-2544, 2001.

Gardiner-Garden, M. and Frommer, M. CpG islands in vertebrate genomes. *J Mol Biol*, 196:261-282

Gatti R.A., Berkel I., and Boder E., *et al.* Localization of an ataxia-telangiectasia gene to chromosome 11q22-23 *Nature.* 336: 577-580. 1988

Greenblatt, M.S., Bennett, W.P., Hollsterin, M., and Harris, C.C. Mutations in the p53 tumor suppressor gene: clues to cancer etiology and molecular pathogenesis. *Cancer Res,* 54:4855-4878, 1994.

Gumbiner, BW. Cell adhesion: the molecular basis of tissue architecture and morphogenesis. *Cell*, 84:345-357, 1996

Haber DA. Splicing into senescence: the curious case of p16 and p19[ARF]. Cell 91:555-558, 1997.

Hattori Y., Itoh H., and Uchino S., *et al.* Immunohistochemical detection of K-sam protein in stomach cancer. *Clin Cancer Res* 2: 1373-1381. 1996.

Hayden, JD, Cawkwell, L, Dixon, MF, Pardal, F, Murgatroyd, H, Gray, S, Quirke, P, and martin, IG. A comparison of microsatellite instability in early onset gastric carcinoma from relatively low and high incidence European populations. *Int J Cancer*, 85: 189-191, 2000

Herman J.G., Umar A., and Polyak K., *et al.* Incidence and functional consequences of hMLH1 promoter hypermethylation in colorectal carcinoma *Proc Natl Acad Sci U S A* 95: 6870-6875. 1998.

Hetzel D.J., Wilson T.O., and Keeny G.L., *et al.* Her-2/neu expression: a major prognostic factor in endometrial cancer, *Gynecol Oncol,* 1992.

Hirasawa A, Aoki D, Inoue J, Imoto I, Susumu N, Sugano K, Nozawa S, Inazawa J, Unfavorable prognostic factors associated with high frequency of microsatellite instability and comparative genomic hybridization analysis in endometrial cancer. Clinical Cancer Research Vol. 9, 5675-5682, 2003

Horii A., Nakatsuru S., and Miyoshi Y., *et al.* The APC gene, responsible for familial adenomatous polyposis, is mutated in human gastric cancer *Cancer Res.* 52: 3231-3233. 1992

Inoue M., Fujita M., and Enomoto T., *et al.* Immunohistochemical analysis of p53 in gynecologic tumors *Am J Clin Pathol* 102: 665-670. 1994.

Ito K., Watanabe K., and Nasim S., *et al.* Prognostic significance of p53 overexpression in endometrial cancer *Cancer Res* 54: 4667-4670. 1994.

Jones, P.A., and Baylin, S.B. The fubdamental role of epigenetic events in cancer. *Nat Res Genet,* 3:415-428, 2002.

Jones, P.A. and Laird, P.W. Cancer epigenetics comes of age. *Nat Genet,* 21:163-167, 1999.

Kanai, Y, Ushijima, S, Hui, AM, Ochiai, A, Tsuda, H, Sakamoto, M, and Hirohashi, S. The E-cadherin gene is silenced by CpG methylation in human hepatocellular carcinoma. *Int J Cancer*, 71:335-359, 1997

Kanaya T, Kyo S, Maida Y, Yatabe N, Tanaka M, Nakamura M, Inoue M Frequent hypermethylation of MLH1 promoter in normal endometrium of patients with endometrial cancers. Oncogene. 2003

Kane M.F., Loda M., and Gaida G.M., *et al.* Methylation of the hMLH1 promoter correlates with lack of expression of hMLH1 in sporadic colon tumors and mismatch repair-defective human tumor cell lines *Cancer Res.* 57: 808-811. 1997

Khalifa M.A., Mannel R.S., and Haraway S.D., *et al.* Expression of EGFR, HER-2/neu, p53, and PCNA in endometrioid, serous papillary, and clear cell endometrial adenocarcinomas, *Gynecol Oncol,* 1994.

Khokhlatchev, A, Rabizadeh, S, Xavier, R, Nedwidek, M, Chen, T, Zhang, XF, Seed, B, and Avruch, J. Identification of a novel Ras-regulated proapoptotic pathway. *Curr Biol,* 12: 253-265, 2002

Kinzler KW, Vogelstein B. Lessons from hereditary colorectal cancer. Cell 87:159-170, 1996.

Kohler M.F., Nishii H., and Humphrey P.A., *et al.* Mutation of p53 tumor-suppressor gene is not a feature of endometrial hyperplasia *Am J Obstet Gynecol.* 169: 690-694. 1993

Kohler M.F., Carney P., and Dodge R., *et al.* p53 overexpression in advanced-stage endometrial adenocarcinoma *Am J Obstet Gynecol.* 175: 1246-1252. 1996

Laird, P.W., Jackson-Grusby,L., Fazeli,A., Dickinson, S.L., Jung, W.E., Li,E., Weinberg, R.A., and Jaenisch, R. Suppression of intestinal neoplasia by DNA hypomethylation. *Cell,* 81:197-205, 1995.

Lange E., Borresen A.-L., and Chen X., *et al.* Localization of an ataxia-telangiectasia gene to a ~500-kb interval on chromosome 11q23.1: linkage analysis of 176 families in an international consortium *Am J Hum Genet.* 57: 112-119. 1995

Lax S.F. Molecular genetic pathways in various types of endometrial carcinoma: from a phenotypical to a molecular-based classification. *Virchows Arch.* 444(3):213-23. 2004

Lee, MG, Kim, HY, Byun, DS, Lee, SJ, Lee, CH, Kim, JI, Chang, SG, Chi, SG. Frequent epigenetic inactivation of RASSF1A in human bladder carcinoma. *Cancer Res,* 61: 6688-6692, 2001

Lei, H., Oh, S.P., Okano, M., Juttermann, R., Goss, K.A., Jaenisch, R., and Li, E. De novo DNA cytosine methyltransferase activities in mouse embryonic stem cells. *Development,* 122:3195-3205, 1996.

Leung S.Y., Yuen S.T., and Chung L.P., *et al.* hMLH1 promoter methylation and lack of hMLH1 expression in sporadic gastric carcinomas with high-frequency microsatellite instability *Cancer Res.* 59: 159-164. 1999

Leung,WK, Kim, JJ, Jim,JG, Graham, DY, and Sepulveda, AR, Microsatellite instability in gastric intestinal metaplasia in patients with and without gastric cancer. *Am J Pathol,* 156, 537-43, 2000

Lim SB, Jeong SY, Lee MR, Ku JL, Shin YK, Kim WH, Park JG. Prognostic significance of microsatellite instability in sporadic colorectal cancer. *Int J Colorectal Dis.* 2004

Liu,B, Parsons,R, Papadopoulos,N, Nicolaides,NC, Lynch,HT, Watson,P, Jass,JR, Dunlop,M, Wyllie,A, Peltomaki,P, de la Chapelle,A, Hamilton,SR, Vogelstein,B, and Kinzler,KW, Analysis of mismatch repair genes in hereditary non-polyposis colorectal cancer patients. *Nature Medicine,* 2, 169-74, 1996

Lo, KW, Kwong, J, Hui, AB, Chan, SY, To, KF, Chan, AS, Chow, LS, Teo, PM, Johnson, PJ, and Huang, DP. High frequency of promoter hypermethylation of RASSF1A in nasopharyngeal carcinoma. *Cancer Res,* 61:3877-3881, 2001

Lukes A.S., Kohler M.F., and Pieper C.F., *et al.* Multivariable analysis of DNA ploidy, p53, and HER-2/neu as prognostic factors in endometrial cancer, *Cancer,* 1994.

Manek S. and Wells M.. The significance of alterations in p53 expression in gynecologic neoplasms *Curr Opin Obstet Gynecol* 8: 52-55. 1996.

Markowitz S., Wang J., and Myeroff L., *et al.* Inactivation of the type II TGF-beta receptor in colon cancer cells with microsatellite instability *Science.* 268: 1336-1338. 1995

Maruyama, R, Toyooka, KO, Harada, K, Virmani, AK, Zochbauer-Muller,S, Farinas, AJ, Vakar-Lopez, F, Minna, JD, Sagalowsky, A, Czerniak, A, Gazdar, AF. Abberent promoter methylation profile of bladder cancer and its relationship to clinicopathological features. *Cancer Res,* 61: 8659-8663, 2001

McGowan E. and Clarke C.. Effect of overexpression of progesterone receptor A on endogenous progestin-sensitive endpoints in breast cancer cells *Mol Endocrinol.* 13: 1657-1671. 1999

Miturski R, Postawski K, Semczuk A, Bogusiewicz M, Baranowski W, Jakowicki JA, Keith G.Global DNA methylation in relation to hMLH1 and hMSH2 protein immunoreactivity in sporadic human endometrial carcinomas.*Int J Mol Med.* 11(5):569-74. 2003

Monk B.J., Chapman J.A., and Johnson G.A., *et al.* Correlation of c-myc and HER-2/neu amplification and expression with histopathologic variables in uterine corpus cancer, *Am J Obstet Gynecol,* 1994.

Moreno-Bueno G, Guo M, Herman JG, Matias-Guiu X, Esteller M, Palacios Abnormalities of the APC/beta-catenin pathway in endometrial cancer. *Oncogene.* 14;21(52):7981-90. 2002

Nass, SJ, Herman, JG, Gabrielson, E, Iversen, PW, Parl, FF, Davidson, NE, and Graff, JR. Abberrant methylation of the estrogen receptor and E-cadherin 5' CpG islands increases with malignant progression in human breast cancer. *Cancer Res,* 60:4346-4348, 2000

Narayan G, Arias-Pulido H, Koul S, Vargas H, Zhang FF, Villella J, Schneider A, Terry MB, Mansukhani M, Murty VV.Frequent Promoter Methylation of CDH1, DAPK, RARB, and HIC1 Genes in Carcinoma of Cervix Uteri: Its Relationship to Clinical Outcome. *Mol Cancer.* 13;2(1):24 2003

Okano, M., Xie, S., and Li, E. Cloning and characterization of a family of novel mammalian DNA (cytosine-5) methyltransferases. *Nat Genet,* 19:219-220, 1998a.

Okano,M., Xie, S., and Li, E. Dnmt2 is not required for de novo and maintenance methylation of viral DNA in embryonic stem cells. *Nucleic Acids Res,* 26:2536-2540, 1998b.

Oncology Channel. http://oncologychannel.com/endometrialcancer, 2004

Ortiz-Vega, S, Khokhlatchev, A, nedwidek, M, Zhang, XF, Dammann, R, Pfeifer, GP, and Avruch, J. The putative tumor suppressor RASSF1A homodimerizes and heterodimerizes with the Ras-GTP binding protein Nore1. *Oncogene,* 21: 1381-1390, 2002

Oue, N, Shigeishi, H, Kuniyasu, H, Yokozaki, H, Kuraoka, K, Ito, R, and Yasui, W. Promoter hypermethylation of MGMT is associated with protein loss in gastric carcinoma. *Int J Cancer,* 93:805-809, 2001

Polakis P. The oncogenic activation of beta-catenin *Curr Opin Genet Dev.* 9: 15-21.1999

Peltomaki,P, Lothe,RA, Aaltonen,LA, Pylkkanen, L, Nystrom-Lahti,M, Seruca,R, David,L, Holm, R, Ryberg,D, and Hauhen,A, Microsatellite instability is associated with tumor that characterize the hereditary non-polyposis colorectal carcinoma syndrome. *Cancer Res,* 53, 5853-5, 1993

Perinchery, G, Nojima, D, Goharderakhshan, R, Tanaka, Y, Alonzo, J, and Dahiya, R. Microsatellite instability of dinucleotide tandem repeat sequences is higher than trinucleotide, tetranucleotide and pentanucleotide repeat sequences in prostate cancer. *Int J Oncol,* 16: 1203-1209, 2000

Rhee, I., Bachman, K.E., Park, B.H., Jair, K.W., Schuebel, K.E., Cui, H., Feinberg, A.P., Lengauer, C., Kinzler, K.W., Baylin, S.B., and Vogelstein, B. DNMT1 and DNMT3b cooperate to silence genes in human cancer cells. *Nature,* 416:552-556, 2002.

Risinger J.I., Hayes A.K., Berchuck A., and Barrett J.C.. PTEN/MMAC1 mutations in endometrial cancers *Cancer Res.* 57: 4736-4738. 1997

Risinger JI, Maxwell GL, Berchuck A, Barrett JC, Promoter hypermethylation as an

epigenetic component in Type I and Type II endometrial cancers, *Ann N Y Acad Sci*, 2003 .

Robertson, K.D., Ait-Si-Ali, S., Yokochi, T., Wade, P.A., Jones, P.L., and Wolffe, A.P. DNMT1 forms a complex with Rb, E2F1 and HDAC1 and represses transcription from E2F-responsive promoters. *Nat Genet*, 25:338-342, 2000.

Robertson, K.D. DNA methylation and chromatin – unraveling the tangled web. *Oncogene*, 21:5361-5379, 2002.

Saito T, Nishimura M, Yamasaki H, Kudo R. Hypermethylation in promoter region of E-cadherin gene is associated with tumor dedifferention and myometrial invasion in endometrial carcinoma. *Cancer*. 15;97. 2003

Salvesen HB, MacDonald N, Ryan A, Jacobs IJ, Lynch ED, Akslen LA, Das S. PTEN methylation is associated with advanced stage and microsatellite instability in endometrial carcinoma. *Int J Cancer*. 91(1):22-6, 2001

Savitsky K., Bar-Shira A., and Gilad S., *et al.* A single ataxia telangiectasia gene with a product similar to PI-3 kinase *Science*. 268: 1749-1753. 1995

Sasaki H., Nishii H., and Tada A., *et al.* Mutation of the Ki-ras proto-oncogene in human endometrial hyperplasia and carcinoma *Cancer, Res,* 1993.

Sasaki M, Dharia A, Oh BR, Tanaka Y, Fujimoto S, Dahiya R. Progesterone receptor B gene inactivation and CpG hypermethylation in human uterine endometrial cancer. Cancer Res. 61(1):97-102. 2001

Save V., Sylander K., and Hall P.A.. Why is p53 protein stabilized in neoplasia? Some answers but many more questions *J Pathol* 184: 348-350. 1998.

Semczuk A, Boltze C, Marzec B, Szczygielska A, Roessner A, Schneider-Stock R. p16INK4A alterations are accompanied by aberrant protein immunostaining in endometrial carcinomas.*J Cancer Res Clin Oncol.* 129(10):589-96. 2003

Sherman M.E., Bur M.E., and Kurman R.J.. P53 in endometrial cancer and its putative precursors: evidence for diverse pathways of tumorigenesis *Hum Pathol*. 26: 1268-1274. 1995

Sherr CJ. Tumor surveillance via the ARF-p53 pathway. *Genes & Development* 12:2984-2991, 1998

Surani, M.A. Genomic imprinting: development significance and molecular mechanism. *Curr Opin Genet Dev*, 1:241-246, 1991.

Taniguchi K., Yonemura Y., and Nojima N., *et al.* The relation between the growth patterns of gastric carcinoma and the expression of hepatocyte growth factor receptor (c-met), autocrine motility factor receptor, and urokinase-type plasminogen activator receptor *Cancer* 82: 2112-2122.1998.

Tashiro H., Isacson I., and Levine R., *et al.* p53 gene mutations are common in uterine serous carcinoma and occur early in their pathogenesis *Am J Pathol* 150: 177-185. 1997.

Tautz,D, Hypervariability of simple sequences as a general source for polymorphic DNA markers. *Nuclic Acids Res*, 17:6463-71, 1989.

Toyota, M. and Issa, J. P. CpG island methylator phenotypes in aging and cancer. *Semin Cancer Biol,* 9:349-357, 1999.

Tritz D, Pieretti M, Turner S, Powell D. Loss of heterozygosity in usual and special variant carcinomas of the endometrium. *Hum Pathol.* 28(5):607-12. 1997.

Virmani, AK, Rathi, A, Zochbauer-Muller, S, Sacchi, N, Fukuyama, Y, Bryant, D, Maitra, A, Heda, S, Fong, KM, Thunnissen, F, Minna, JD, and Gazdar, AF. Promoter methylation and silencing of the retinoic acid receptor-beta gene in lung carcinomas. *J Natl Cancer Inst,* 92: 1303-1307, 2000

Vos, MD, Ellis, CA, Bell, A, Birrer, MJ, and Clark, GJ. Ras uses the novel tumor suppressor RASSF1A as an effector to mediate apoptosis. *J Biol Chem*, 275: 35669-35672, 2000

Wallace M.H. and Pphillips R.K. . Upper gastrointestinal disease in patients with familial adenomatous polyposis *Br J Surg* 85: 742-750. 1998.

Wang D., Konishi I., and Koshiyama M., *et al.* Expression of c-erbB-2 protein and epidermal growth factor receptor in endometrial carcinomas, *Cancer,* 1995.

Weber, JL, and May, PE. Abundant class of human DNA polymorphophisms which can be typed using polymerase chain reaction. American Journal of Human Genetics, 44: 388-96, 1989

Willert K. and Nusse R. Beta-catenin: a key mediator of Wnt signaling *Curr Opin Genet Dev* 8: 95-102. 1998.

Whelan AJ, Babb S, Mutch DG, Rader J, Herzog TJ, Todd C, Ivanovich JL, Goodfellow PJ.MSI in endometrial carcinoma: absence of MLH1 promoter methylation is associated with increased familial risk for cancers. Int J Cancer. 10;99(5):697-704. 2002

Wu, J., Issa, J.P., Herman, J., Bassett, D.E., Jr., Nelkin, S.B. Expression of an exogeneous eukaryotic DNA methyltransferase gene induces tranformation of NIH 3T3 cells. *Proc Natl Acad Sci USA*, 90:8891-8895, 1993

Yamashita, K, Arimura, Y, Kurokawa, S, Itoh, F, Endo, T, Hirata, K, Imamura, A, Kondo, M, Sato, T, and Imai, K. Microsatellite instability in patients with multiple primary cancers of the gastrointestinal tract. *Gut,* 46, 790-794, 2000

Yamauchi N., Sakamoto A., and Uozaki H., *et al.* Immunohistochemical analysis of endometrial adenocarcinoma for bcl-2 and p53 in relation to expression of sex steroid receptor and proliferative activity *Int J Gynecol Pathol* 15: 202-208. 1996.

Yoon, JH, Dammann, R, and Pfeifer, GP. Hypermethylation of the CpG island of the RASSF1A gene in overian and renal cell carcinomas. *Int J Cancer*, 94: 212-217, 2001

Zheng W., Cao P., and Zheng M., *et al.* p53 overexpression and bcl-2 persistence in endometrial carcinoma: comparison of papillary serous and endometrial subtypes *Gynecol Oncol.* 61: 167-174. 1996

Zingg, J.M. and Jones, P.A. Genetic and epigenetic aspects of DNA methylation on genome expression, evolution, mutation and acrcinogenesis. *Carcinogenesis,* 18: 869-882, 1997.

Zysman M, Saka A, Millar A, Knight J, Chapman W, Bapat B. Methylation of adenomatous polyposis coli in endometrial cancer occurs more frequently in tumors with microsatellite instability phenotype. Cancer Res. 1;62(13):3663-6. 2002