Prevalence of K-*ras* Mutation in Endometrial Endometrioid Adenocarcinoma

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ชื่อวิทยานิพนธ์ การศึกษาความชุกของการกลายพันธุ์ของยีน K-*ras* ในมะเร็งเยื่อบุโพรงมดลูกชนิดเอนโดเมทริออยด์

ผู้เขียน นางสาวปพิชญา วัชรานุรักษ์

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บทคัดย่อ

มะเร็งเยื่อบุโพรงมดลูกพบมากเป็นอันดับสามของมะเร็งในระบบอวัยวะสืบพันธุ์สตรีในประเทศไทย ความชุกของโรคมะเร็งเยื่อบุโพรงมดลูกมีแนวโน้มที่จะเพิ่มขึ้นในผู้ป่วยไทยที่มีอายุน้อยเฉลี่ยกว่า 40 ปี และมีปัจจัยเสี่ยงของโรคอ้วน การศึกษาในระดับอณูชีวโมเลกุลพบว่ายีน K-*ras* มีความสัมพันธ์กับกลไกของการเกิดมะเร็งเยื่อบุโพรงมดลูกโดยเฉพาะชนิดเอนโดเมทริออยด์ มีรายงานการศึกษาพบ K-*ras* mutation ร้อยละ 10 ถึง 30 ในมะเร็งเยื่อบุโพรงมดลูก อย่างไรก็ตาม การศึกษาความสัมพันธ์ของการเกิด K-*ras* mutation กับปัจจัยทางคลินิกและพยาธิวิทยายังไม่ชัดเจน การวิจัยครั้งนี้จึงมีวัตถุประสงค์เพื่อหาความชุกของการเกิด K-*ras* mutation ในมะเร็งเยื่อบุโพรง-มดลูกชนิดเอ็นโดเมทริออยด์ในผู้ป่วยชาวไทย และศึกษาความสัมพันธ์ของการเกิด K-*ras* mutation กับปัจจัยต่างๆทางคลินิกและพยาธิวิทยา รวมถึงการตรวจคัดกรอง K-*ras* mutation ด้วยเทคนิค reverse dot blot hybridization ตัวอย่างการศึกษาเป็นกลุ่มผู้ป่วยมะเร็งเยื่อบุโพรงมดลูกชนิดเอนโด-เมทริออยด์ จำนวน 190 ราย ทำการวิเคราะห์หา K-*ras* mutation บริเวณ exon2 ด้วยวิธี direct sequencing และวิเคราะห์ความสัมพันธ์ทางสถิติของการเกิด K-*ras* mutation กับปัจจัยทางคลินิกและพยาธิวิทยาและทำการศึกษาตัวอย่างจำนวน 30 ราย โดยนำมาตรวจสอบการเกิด K-*ras* mutation ด้วยเทคนิค reverse dot blot hybridization ผลการศึกษาตรวจพบ K-*ras* mutation ในผู้ป่วย 37 ราย จาก 190 ราย คิดเป็นร้อยละ 19.5 โดย 33 ราย เกิดแบบ missense mutation และอีก 4 ราย เกิดแบบ silent change ตำแหน่งของการเกิด mutation ที่พบได้บ่อยคือตำแหน่ง codon 12 และ codon 13 นอกจากนี้ยังตรวจพบการเกิด missense mutation ตรงตำแหน่งอื่นๆ ของบริเวณ exon 2 ได้แก่ codon 3, 14, 15, 27, 29, 33 และ codon 34 การเกิด K-*ras* mutation มีแนวโน้มพบได้บ่อยในผู้ป่วย grade 1-2 และ stage I-II แต่ไม่มีความสัมพันธ์อย่างมีนัยสำคัญ สำหรับปัจจัยอื่น ๆ ทางคลินิกและ พยาธิวิทยา ได้แก่ อายุ, ค่า BMI, การมีบุตร, depth of myometrial invasion, LVSI, uterine cervical involvement, lymph node involvement และ synchronous ovarian cancer/ovarian metastasis พบว่าไม่มี

ความสัมพันธ์กับ K-*ras* mutation อย่างมีนัยสำคัญ การศึกษาตัวอย่างจำนวน 30 ราย ด้วยวิธี reverse dot blot hybridization ให้ผลสอดคล้องทุกรายกับการตรวจด้วยวิธี direct sequencing กล่าวโดยสรุป การศึกษานี้แสดงให้เห็นว่าการเกิด K-*ras* mutation น่าจะมีความเกี่ยวข้องกับกระบวนการเกิดมะเร็งเยื่อบุโพรงมดลูกในกลุ่มผู้ป่วยไทยจำนวนหนึ่ง แต่ไม่มีผลต่อการพยากรณ์โรค สำหรับวิธีทางเลือกที่นำมาประยุกต์ใช้ในการตรวจ K-*ras* mutation เมื่อเปรียบเทียบกับวิธี direct sequencing การตรวจด้วยวิธี reverse dot blot hybridization ถือเป็นวิธีที่มีประสิทธิภาพและเชื่อถือได้ในการตรวจคัดกรอง K-*ras* mutation ในชิ้นเนื้อมะเร็งที่เป็นชนิดฝังพาราฟิน (formalin-fixed, paraffin-embedded tissue)

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ABSTRACT

Endometrial carcinoma is the third most common gynecologic malignancy in Thailand. The prevalence of endometrial cancer seems to be increasing in young Thai patients with mean age less than of 40 years and with risk factor of obesity. K-*ras* oncogene is one of the genetic alterations and plays an important role in the mechanism of carcinogenesis in endometrial cancer, especially endometrioid type. K-*ras* mutations are found in 10% to 30% of endometrial carcinomas; however, the relationship between K-*ras* mutations and clinicopathological variables remains unclear. The aims of this study were to examine the prevalence of K-*ras* mutations in Thai patients with endometrial endometrioid adenocarcinoma, to investigate the correlation between the mutations and clinicopathogical variables, and to screen K-*ras* mutations using reverse dot blot hybridization technique. A total of 190 patients with endometrioid adenocarcinoma were analyzed for K-*ras* exon2 mutation using direct sequencing. Thirty selected samples were tested using reverse dot blot hybridization assay. Statistical correlation of K-*ras* mutation with clinicopathogical variables was evaluated. K-*ras* mutations were detected in 37 of 190 cases (19.5%). Thirty three (17.4%) of 190cases were missense mutation, whereas 4 cases(2.1%) showed silent change. The frequent mutations were detected in codon 12 and 13.There were 9 cases of missense mutations detected at other sites of exon2, including codon 3, 14, 15, 27, 29, 33, and 34. Trend of K-*ras* mutations was found in patients with grade1-2 and stage I-II, but none of significant correlation. There was no significant relationship between the presence of K-*ras* mutations and clinicopathological variables

(age, BMI, parity, depth of myometrial invasion, LVSI, uterine cervical and lymph node involvement, and synchronous ovarian cancer/ovarian metastasis). Detecting all of K-*ras* mutations in 30 selected cases, both direct sequencing and reverse dot blot hybridization assay were found to have similar results. In conclusion, this study suggests that the presence of K-*ras* mutations may be a relatively common event in endometrial carcinogenesis in subgroup of Thai patients, but their prognostic value is limited. With an alternative tool applicability comparing to direct sequencing, reverse dot blot hybridization is an effective and reliable assay for screening of K-*ras* point mutations in formalin-fixed paraffin-embedded tissues.

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LIST OF ABBREVIATIONS AND SYMBOLS

AJCC	American Joint Committee on Cancers
°C	Degree celsius
DNA	Dideoxyribonucleic acid
dNTPs	Deoxyribonucleic acid
EGFR	Epidermal growth factor receptor
EIC	Endometrial intraepithelial carcinoma
FFPE	Formalin-fixed, paraffin-embeded tissue
FIGO	Internal Federation of Gynecology and Obsterics system
GAPs	GTPase activating proteins
GDP	Guanosine diphosphate
GEFs	Guanine nucleotide exchange factors
GTP	Guanosine triphosphate
KRAS	Kirsten rat sarcoma viral oncogene homolog
LVSI	Lymphovascular space invasion
mg	Milligram
min	Minute
ml	Milliliter
mM	Milimolar
mol	Mole
mRNA	Messenger RNA
MSI	Microsatellite instability
MW	Molecular weight
p	*p*-value

PI3K	Phosphatidylinositol 3-kinases
PCR	Polymerase chain reaction
PTEN	Phosphatase Tensin Homologue Delete on Chromosome 10
RDB	Reverse dot blot hybridization
RFLP	Restriction length polymorphism
sec	Second
SSCP	Single stranded conformation polymorphism
TBS	Tris Buffer Solution
µl	Microlititre
UV	Ultraviolet
V	Volt
WHO	World Health Organization

CHAPTER 1

INTRODUCTIONS

BACKGROUND AND RATIONAL

Endometrial carcinoma is the third most common gynecologic malignancy in Thailand with the incidence rate of 4/100,000/years (Khunhaprema et al., 2013). Most of patients are menopause with the average age of diagnosis around 60 years. The prevalence of endometrial cancer seems to be increasing in young Thai patients with mean age of 40 years and have risk factor of obesity (Hanprasertpong et al., 2008; Manchana et al., 2008). The major type of endometrial carcinomas is endometrioid adenocarcinoma (Amant et al., 2005). This endometrioid type not only relates to endogenous or exogenous hyperestrogenic stimulation, but also relates to obesity, diabetes, hypertension, nulliparity, and chronic anovulation (Dahlgren et al., 1991; Doll et al., 2008). The carcinogenesis of endometrioid type involves a wide variety of molecular alterations, consisting of microsatellite instability, PTEN mutation, K-*ras* mutation, and β-catenin mutation (Bansal et al., 2009).

Alterations of K-*ras* gene are considered to be one of many molecular factors detected in several human cancers, including endometrial cancer. The RAS family of oncogenes is constituted of three members, known as K-*ras*, H-*ras*, and N-*ras*. The K-*ras* oncogene encodes a 21 kDa guanine nucleotide-binding protein (G-protein) that has a central role in the regulation of cell proliferation and differentiation (Friday and Adjei, 2005). Mutations of K-*ras* gene result in constitutive signal transduction pathway and subsequently effect on upregulated cell proliferation, cell death, and cancer progression (Kiaris and Spandidos, 1995).

Previous studies have shown that K-*ras* gene mutations were identified in 10% to 30% of endometrial carcinomas (Sasaki et al., 1993; Duggan et al., 1994; Lax et al., 2000). The point mutations were predominantly found in codons 12 and 13 (Mammas et al., 2005). K-*ras* mutation has been documented that it associates with

submicroscopic myometrial invasion and can be used to assess the depth myometrial invasion in stage I endometrial cancer (Alexander-Sefre et al., 2003). Furthermore, some studies have identified the mutations in endometrial hyperplasia, suggesting that K-*ras* mutation may be an early event in the pathogenesis of endometrium (Sasaki et al. 1993; Dobrzycka et al., 2009).

Nowadays, there are several methods to detect K-*ras* point mutations. Although DNA sequencing remains the gold standard for detecting the K-*ras* mutation, DNA extracted from FFPE tissues is poor quality and it is short DNA fragmentations for use as DNA template. Thus, reverse dot blot hybridization assay is an alternative method to be applied in detection of K-*ras* mutation in FFPE samples.

The detection of K-*ras* mutations in endometrial carcinoma Thai patients has not yet been investigated. Therefore, the aims of this study were to examine the prevalence of K-*ras* mutations in Thai patients with endometrial endometrioid adenocarcinoma, to investigate the correlation between the mutations and clinicopathogical variables, and to screen K-*ras* mutations using reverse dot blot hybridization technique.

REVIEW OF LITERATURE

Incidence of Endometrial Carcinoma

Endometrial carcinoma is the sixth most common malignancy worldwide, accounting for an estimated 287,100 new cases and 74,000 death cases diagnosed in 2008 (Jemal et al., 2011). The age-standardized incidence rates and age-standardised mortality rate are 8.2 and 2.0 per 100,000, respectively (Ferlay et al., 2010). Endometrial carcinoma is the most common cancer among women in America and Europe while its incidence is low among women in Africa and Asia. The incidence of endometrial carcinoma is higher in developed countries (5.5%) than in developing countries (4.2%) (Jemal et al., 2011). Endometrial carcinoma is the third most common gynecologic malignancy in Thai population after cervical cancer and ovarian cancer with relative frequency of 3.6% (Attasara et al., 2011). The estimated incidence rate of endometrial carcinoma is 4.0 per 100,000 female populations (Khunhaprema et al., 2013).

Epidemiology and Risk Factors of Endometrial Carcinoma

The majority cases of endometrial carcinoma are usually found in women after menopause. The mean age at diagnosis is 60 years. It has been reported to occur 20–25% in women with premenopause and approximately 5% in women with age of 40 years or younger (Garg et al., 2009). Abnormal vaginal bleeding is the most common symptom in this cancer. Most endometrial carcinomas are adenocarcinomas (cancers of the cells that form glands in the endometrium) and the most common type is endometrioid adenocarcinoma (Amant et al., 2005).

Increased or long-term estrogen stimulation of endogenous and exogenous estrogen is the main cause for developing endometrial carcinoma with other risk factors. The risk factors that associated with endometrial carcinoma include obesity, diabetes mellitus, anovulation, nulliparity, polycystic ovarian disease, and estrogen replacement therapy (Kaaks et al., 2002).

The Normal Endometrium

The uterus is a pear-shaped, hollow, and muscular organ. It is composed of three tissue layers: the outer layer covering the uterus (perimetrium), the middle thick muscular layer (myometrium), and the inner layer mucous membrane (endometrium) (Figure 1) (Ross et al., 2006). The endometrium is composed of glands, stroma, and blood vessels. Throughout female reproductive life, the endometrium undergoes cyclic changes each month. Hormone changes, particular estrogen and progesterone, during a woman's menstrual cycle cause the endometrium to change (Robboy, 2002). Among normal menstrual cycle, endometrium consists of the proliferative phase, the secretory phase, and the menstrual phase. In cases of fertilization, the endometrium becomes thick and enriches with blood vessels. If fertilization does not occur, the endometrium is shedded and menstruation begins.

Pathogenesis of Endometrial Carcinoma

The dualistic model of endometrial carcinogenesis has been described in Figure 2. Type I endometrial carcinoma is derived from a setting of endometrial hyperplasia and the molecular alteration is associated with mutations in PTEN, K-*ras*, and β-catenin gene and microsatellite instability. In contrast, type II endometrial carcinoma usually arises in the setting of atrophic endometrium and involves with p53 mutation and aneuploidy (Doll et al., 2008). In this model, different types of precursor lesion leading to the development in two types of endometrial carcinoma are atypical hyperplasia and endometrial intraepithelial carcinoma (EIC). Atypical hyperplasia is accepted as a precursor lesion for endometrioid carcinoma, while endometrial intraepithelial carcinoma is a precursor lesion in non-endometrioid carcinoma.

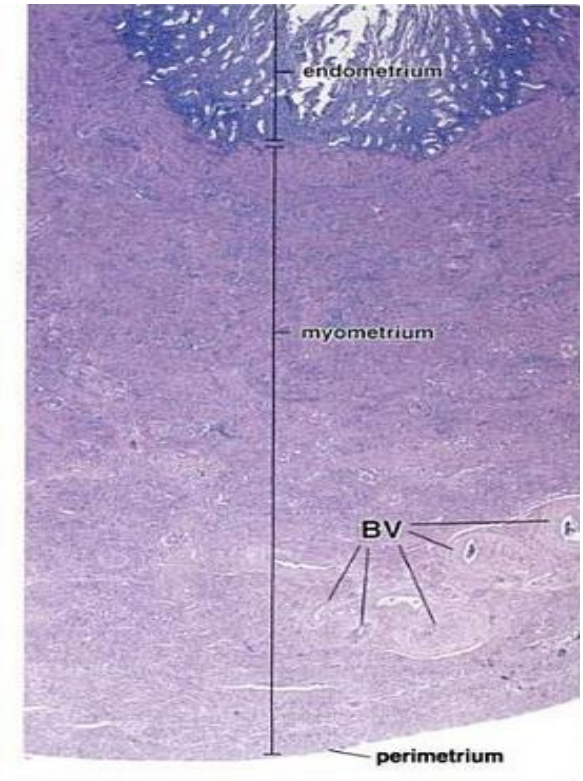

Figure 1. Photomicrograph of a sagittal section of a human uterus. This section shows three tissue layers of the uterine wall: the endometrium, the inner layer mucous membrane; the myometrium, the middle thick muscular layer; and the perimetrium, the outer layer covering the uterus. The deep portion of the myometrium contains the larger blood vessels (BV) that supply the uterus (Ross et al., 2006)

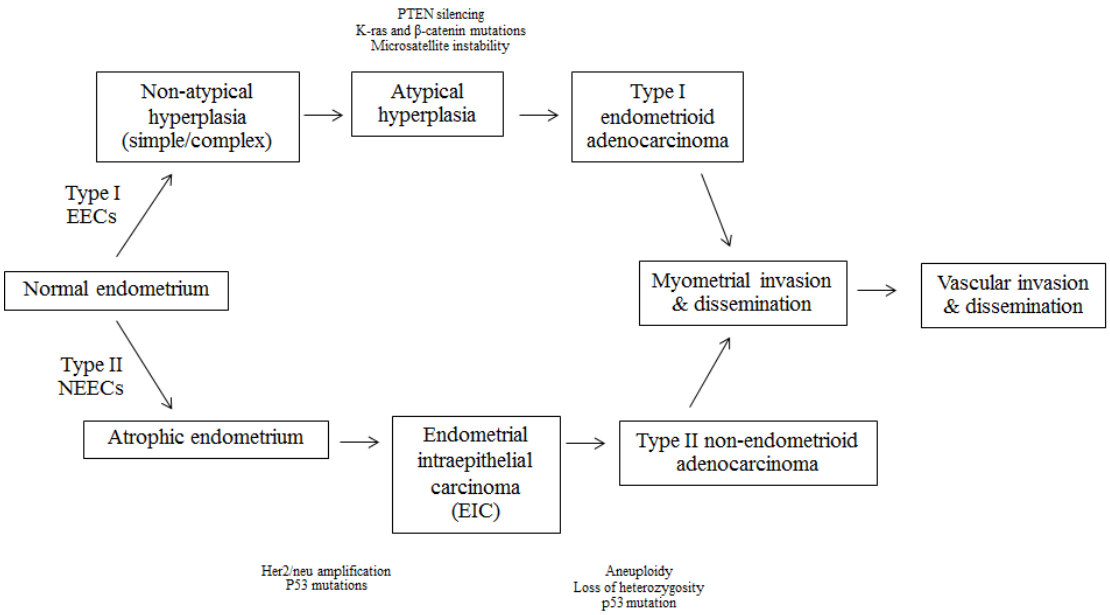

Figure 2. A dualistic model of endometrial carcinogenesis (adapted from Doll et al., 2008)

WHO histological classification

Endometrial carcinoma, the cancer in the cells that form the inner lining of the uterus is the most common type of uterine cancer. The histological classification of tumors of the uterine corpus according to the World Health Organization (WHO) is shown in Table 1 (Silverberg et al., 2003).

Type of Endometrial Carcinoma

Ninety percent of endometrial carcinomas are sporadic and 10% are hereditary carcinoma which are associated with hereditary non-polyposis colorectal cancer (HNPCC) (Doll et al., 2008).Sporadic endometrial carcinomas can be divided into two types based on clinicopathological characteristic and molecular alteration. Type I or endometrioid endometrial carcinomas are estrogen related tumors. They are the major type of endometrial carcinomas consisting of approximately 80% of sporadic cancers. This type occurs in pre/perimenopausal women and frequently develops on a background of atypical hyperplasia. The histology is endometrioid type and low grade differentiation. In contrast, Type II or non-endometrioid endometrial carcinomas are less common, accounting for 10–20% of endometrial carcinoma. This type mainly occurs in older women and unrelated to estrogen stimulation. They typically arise on a background of atrophic endometrium. They are commonly high grade tumors with serous or clear-cell histology. Clinically, type II carcinomas are more aggressive than type I carcinomas and usually have poor prognosis. The histopathological and clinical features of the two types of endometrial carcinomas are summarized in Table 2 (Amant et al., 2005; Di Cristofano et al., 2007; Liu, 2007; Doll et al., 2008).

The prognosis factors involve in endometrial carcinomas including age, parity, histological type, histological grading, stage, depth of myometrial invasion, lymphovascular space invasion (LVSI), cervical involvement, and lymph node metastasis etc. (Uharček, 2008;Prat, 2004).

Table 1. WHO histological classification of tumors of the uterine corpus (Silverberg et al., 2003)

Type
Epithelial tumors and related lesions
Endometrial carcinoma
Endometrioid adenocarcinoma
Variant with squamous differentiation
Villoglandular variant
Secretory variant
Ciliated cell variant
Mucinous adenocarcinoma
Serous adenocarcinoma
Clear cell adenocarcinoma
Mixed cell adenocarcinoma
Squamous cell carcinoma
Transition cell carcinoma
Small cell carcinoma
Undifferntiated carcinoma
Others
Endometrial hyperplasia
Nonatypical hyperplasia
Simple
Complex (adenomatous)
Atypical hyperplasia
Simple
complex
Endometrial polyp
Tamoxifen-related lesions
Mesenchymal tumors
Mixed epithelial and mesenchymal tumors
Gestational trophoblastic disease
Miscellaneous tumors
Lymphoid and haematopoetic tumors
Secondary tumors

Table 2. Clinicopathologic characteristic of endometrial carcinomas (Liu, 2007).

Characteristics	**Type I**	**Type II**
Incidence	80%	≤ 20%
Age	Pre/perimenopause	> 60 yr
Histology	Endometrioid	Serous, clear
Cell differentiation	Low grade	High grade
Precursor lesion	Endometrial hyperplasia	Atropic endometrium
Estrogen stimulation	Related	Unrelated
Clinical behavior	Indolent	Aggressive

Stage of Endometrial Carcinoma

The staging systems for endometrial carcinoma are the International Federation of Gynecology and Obstetrics system (FIGO) and the American Joint Committee on Cancers (AJCC) TNM classification. Both systems are basically the same. The TNM staging system is based on the size of the primary tumor (T), spreading to nearby lymph nodes (N) and distant metastasis (M). The staging of endometrial carcinoma is shown in Table 3 (Silverberg et al., 2003).

Grade of Endometrial Carcinoma

The histological grade of endometrial carcinoma refers to how the cancer cells look different from normal under a microscope. Type I endometrial carcinomas is endometrioid histology (its histology similarity to normal endometrial glands). This tumor is graded according to the degree of morphological differentiation by FIGO; grade 1 (well differentiated) consisting of well-formed gland, absence of intervening stroma between the irregularly shaped glands, and with less than 5% solid growth, grade 2 (moderately differentiated) consisting of glandular patterns mixed with less than 50% solid areas of malignant cells, and grade 3 (poorly differentiated) consisting of predominant solid growth pattern of malignant cells (Figure3) (Kumar et al., 2010).

Molecular Alterations in Endometrial Carcinoma

Type I and Type II endometrial carcinomas are characterized by difference types of molecular genetic alterations. Type I carcinomas involve common mutations in PTEN, K-*ras*, and β-catenin, as well as microsatellite instability (MSI). In contrast, Type II carcinomas are associated with p53 mutations, Her2/neu overexpression, p16 inactivation, and low E-cadherin expression. The genetic alterations of Type I and Type II endometrial carcinomas are shown in Table 4 (Bansal et al., 2009; Llauradó et al., 2012).

Table 3. FIGO and TNM staging of tumors of the uterine corpus (Silverberg et al., 2003)

FIGO stage	TNM categories	
0	Tis	Carcinoma in situ
I	T1	Tumor confined to the corpus uteri
IA	T1a	Tumor limited to endometrium
IB	T1b	Tumor invades less than one half of myometrium
IC	T1c	Tumor invades one half or more of myometrium
II	T2	Tumor invades cervix but does not extend beyond uterus
IIA	T2a	Endocervical glandular involvement only
IIB	T2b	Cervical stromal invasion
III	T3 and/or N1	Local and/or regional spread as specified in T3a, b, N1, and FIGO IIIA, B, C
IIIA	T3a	Tumor involves serosa and/or adnexa (direct extension or metastasis) and/or cancer cell in ascites or peritoneal washing
IIIB	T3b	Vaginal involvement (direct extension or metastasis)
IIIC	N1	Metastasis to pelvic and/or para-aortic lymph nodes
IVA	T4	Tumor invades bladder mucosa and/or bowel mucosa
IVB	M1	Distant metastasis (excluding metastasis to vagina, pelvic serosa, or adnexa)

T = Primary tumor

N = Regional Lymph Node (N0 = No regional lymph node metastasis, N1 = Regional lymph node metastasis)

M =Distant metastasis (M0 = No distant metastasis, M1 = Distant metastasis)

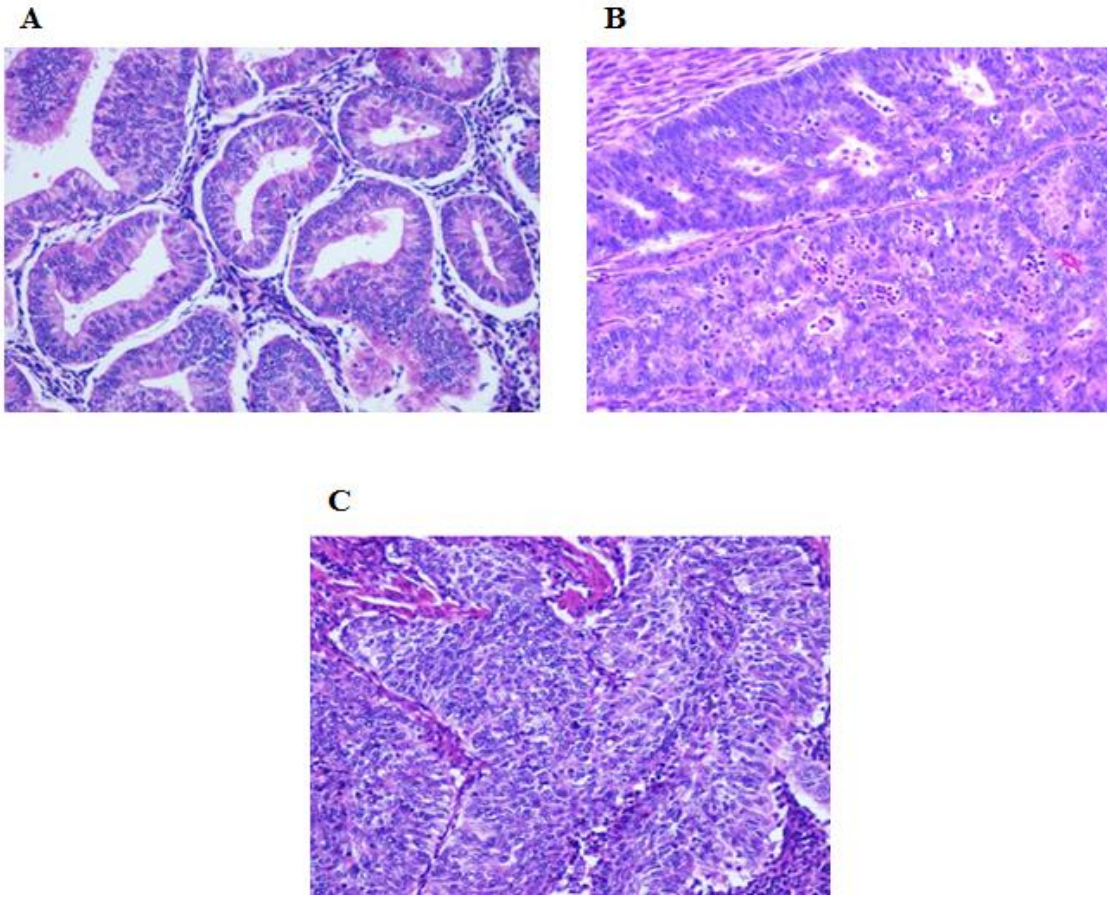

Figure 3. Histological grading of endometrioid adenocarcinoma:

A. Grade 1 showing recognizable glandular patterns, but lacking intervening stroma

B. Grade 2 showing glandular patterns mixed with solid areas of malignant cells

C. Grade 3 showing predominantly solid growths of malignant cell.

Table 4. Genetic alterations associated with Type I and Type II endometrial carcinomas (Bansal et al., 2009).

Genetic alteration	**Type I (%)**	**Type II (%)**
PTEN inactivation	50-80	10
K-ras mutation	15-30	0-5
β-catenin mutation	20-40	0-3
Microsatellite instability	20-40	0-5
p53 mutation	10-20	80-90
Her2/neu	10-30	40-80
p16 inactivation	10	40
E-cadherin	10-20	60-90

Molecular Alterations in Endometrioid Endometrial Carcinogenesis

Molecular events involved in endometrial carcinoma pathogenesis and progression has been investigated. Endometrioid adenocarcinoma develops through a multistep process characterized by a variety of genetic alterations involving tumor suppressor genes, proto-oncogenes, and mismatch repair genes (Lax, 2004).

PTEN (phosphatase and tensin homologue deleted from chromosome 10) is a tumor suppressor gene, which locates on chromosome 10q23.3. It encodes a protein and lipid phosphatase and play a role mainly in cell survival and apoptosis (Ma et al., 2014). PTEN is the most frequent genetic alteration in endometrial carcinogenesis, occurring in 32–50% of endometrioid adenocarcinoma cases (Tashiro et al., 1997; Risinger et al., 1998). Mutations in PTEN have also been reported in endometrial hyperplasia with and withoutatypia (Maxwell et al., 1998; Mutter et al., 2000) and have been detected frequently in MSI positive tumors (Bussaglia et al., 2000). These findings suggested that PTEN mutation is an early event in the development of endometrial carcinoma (Maxwell et al, 1998; Mutter et al., 2000). For the clinic-pathological features, PTEN mutations is associated with favorable clinical and pathological characteristic in endometrial carcinoma (Risinger et al., 1998)

Microsatellite instability (MSI) has been reported in 75% of endometrial carcinoma and it involved in hereditary non-polyposis colon cancer (HNPCC). In sporadic endometrial carcinoma, MSI has been found predominantly in endometrioid types with the frequency of 45% (MacDonald et al., 2000). MSI is due to the inactivation of the DNA mismatch repair genes; MLH1, MSH2 and MSH6 (Jacob and Praz, 2002). MLH-1 promoter hypermethylation seem to be the most common in endometrioid carcinoma (Esteller et al, 1998; Esteller et al, 1999). MSI has been detected in 20-45% endometrioid endometrial carcinomaand it is associated with favorable outcome (MacDonald et al., 2000; Maxwell et al., 2001).

PI3K (Phosphatidylinositol 3-kinases) mutations occur in about 24-39% of endometrial tumors and coexist frequently with PTEN mutations (Oda et al., 2005). The mutations are common found in the helical (exon 9) and kinase (exon 20) domains. PIK3CA mutations, especially in exon 20, have been associated with adverse prognostic factors such as high histological grade and myometrial invasion (Catasus et al., 2007).

The Beta-catenin gene (CTNNB1) is located on chromosome 3p21. Beta-catenin is a component of the E-cadherin–catenin unit, which control cell differentiation and maintenance of normal tissue architecture. In addition, Beta-catenin also plays an important role in Wnt signal transduction pathway (Komiya and Habas, 2008). The common mutation is in exon 3 of beta-catenin gene and lead to nuclear accumulation. The frequency of CTNNB1 mutations have been reported in 14-44% of endometrioid endometrial carcinomas and there are no correlation between Beta-catenin mutations and the presence of MSI, K-ras, and PTEN mutations (Matias-guiu et al., 2001; Llobet et al., 2009).

Activating mutations of K-*ras* have been identified in 10-30% of endometrioid carcinoma (Lax et al., 2000). The K-*ras* gene encoding the GTPase functions as a molecular switch in intracellular transduction pathway and the mutation in the gene has been associated with tumor growth and differentiation. Mutations are predominantly found in codon 12 and 13 of exon 2 (Kiaris and Spandidos, 1995) and rarely in codon 61 (Semczuk et al., 2001). Moreover, K-*ras* mutations also detected in endometrial hyperplasia which suggest that mutation in the K-*ras* gene appears an early event in the development of endometrial carcinoma (Sasaki et al., 1993).

Kirsten rat sarcoma viral oncogenes homolog (K-*ras*)

K-*ras* gene

K-*ras* is a proto-oncogene that belongs to ras gene family. The *ras* family genes consist of three homologue genes (H-*ras*, K-*ras*, and N-*ras*). The K-*ras* gene is located on the short (p) arm of chromosome 12 at position 12.1. It encodes a 21 kDa guanine nucleotide binding protein (G protein) (Jancík et al., 2010). This gene consists of 6 exons, one 5'non-coding exon and five coding exons (exons 1 to 4A/4B), which exon 4 has 2 alternative spice variants, including K-*ras*4A and K-*ras*4B. The K-*ras*4A isoform includes all coding exons in the mRNA transcript, while K-*ras*4B excludes exon 4a. Approximately 99% of all K-*ras* proteins are in the K-*ras*4B form. The structure of the K-*ras* gene and its splice variants is shown in Figure 4 (McGrath et al., 1983).

K-ras protein

The ras proteins contain 188 amino acids in length for H-ras, N-ras, and K-ras4A, while K-ras4B contains 189 amino acids residues. The structure of ras protein (Figure 5) consists of conserved domain and hypervariable region. The four ras proteins are highly conserved at amino acids 1-165 and differed 25 amino acids at carboxy-terminal hypervariable domain. In the conserved domain, there are several motifs important for protein function including GTP binding, effector binding, switch I, and switch II loop. Switch I region interact with GTPase activating proteins (GAPs) and switch II interact with guanine nucleotide exchange factors (GEFs). The C-terminal hypervariable region involve with membrane localization of the protein. This region contains sequences important for post-translation modification, including CAAX box (C represents cysteine; A represents an aliphatic amino acid (leucine, isoleucine or valine); and X represents methionine, serine, leucine or glutamate) responsible for targeting lipid modification. K-ras4A and K-ras4B differ in this region; K-ras4A has the palmitoylation process while K-ras4B has positive charged lysine residues which result in localization of the K-ras4B at the plasma membrane (Ellis and Clark, 2000; Friday and Adjei, 2005).

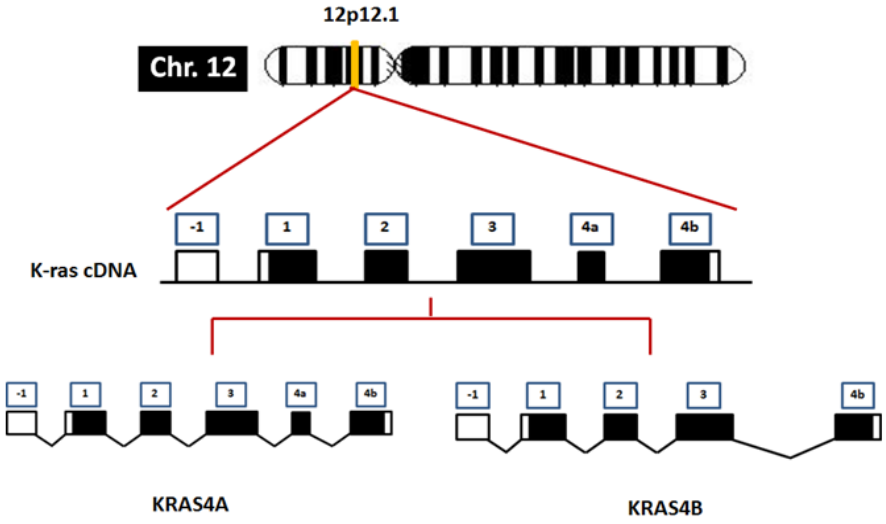

Figure 4. The structure of the K-*ras* gene and its splice variants. Exons that encode protein are shown as black boxes and non-coding exons as white boxes (Adapted from McGrath et al., 1983).

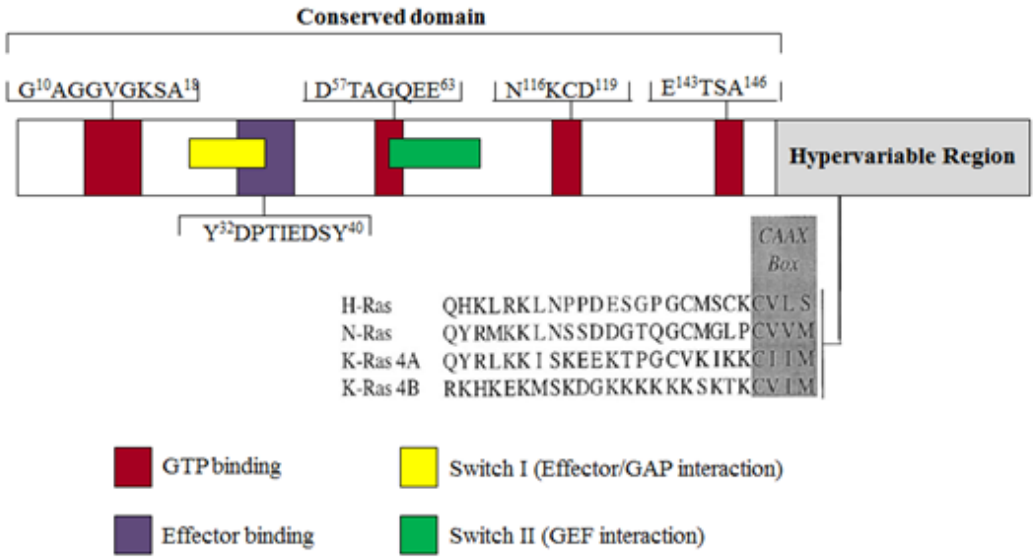

Figure 5. Structure of K-*ras* protein (modified from Ellis and Clark, 2000; Friday and Adjei, 2005)

K-ras Functions and Signaling Pathway

K-ras controls cell proliferation, differentiation, and apoptosis through intracellular signaling transduction by interacting with multiple effectors. K-ras has a GTPase activity and functions as a molecular switch during cell signaling. The GTPase is cycle between active state (ras-GTP) and inactive state (ras-GDP) at the plasma membrane (Figure 6A) (Jancík et al., 2010). This cycle is regulated by guanine nucleotide exchange factors (GEFs) and GTPase activating proteins (GAPs) molecules. K-*ras* play an important role in RAS-RAF-MEK-ERK signaling pathway, its activation begins when epidermal growth factors (EGF) bind to epidermal growth factor receptor (EGFR) at the cell surface then lead to autophosphorylation of receptors tyrosine kinase. Adaptor proteins such as growth factor receptor bound protein 2 (Grb2) interact with receptor phosphorylated tyrosine, and recruited GEFs such as Son of Sevenless (SOS) to plasma membrane which promote the exchange of GDP to GTP by adding phosphate group to target molecules. And then send the signal through Raf, MEK, and ERK signaling cascade. The ERK molecules stimulate the transcription factors in the nucleus in order to regulate proliferation, survival, and metastasis. The signal is inactivated by GAPs molecules that stimulate hydrolysis of the GTP to GDP (Figure 7) (Friday and Adjei, 2005; Mammas et al., 2005). Oncogenic K-ras mutants are resistant to GAPs stimulation. Therefore, the proteins accumulate in the active GTP bound state and constitutively activated (Figure 6B) (Jancík et al., 2010).

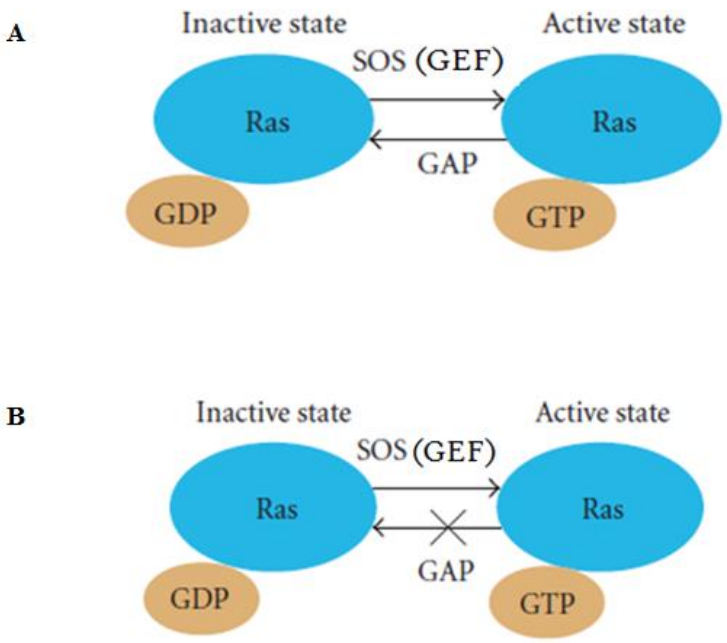

Figure 6. K-ras activation/inactivation, normal activation (6A) and mutation activation (6B) (modified from Jancík et al., 2010)

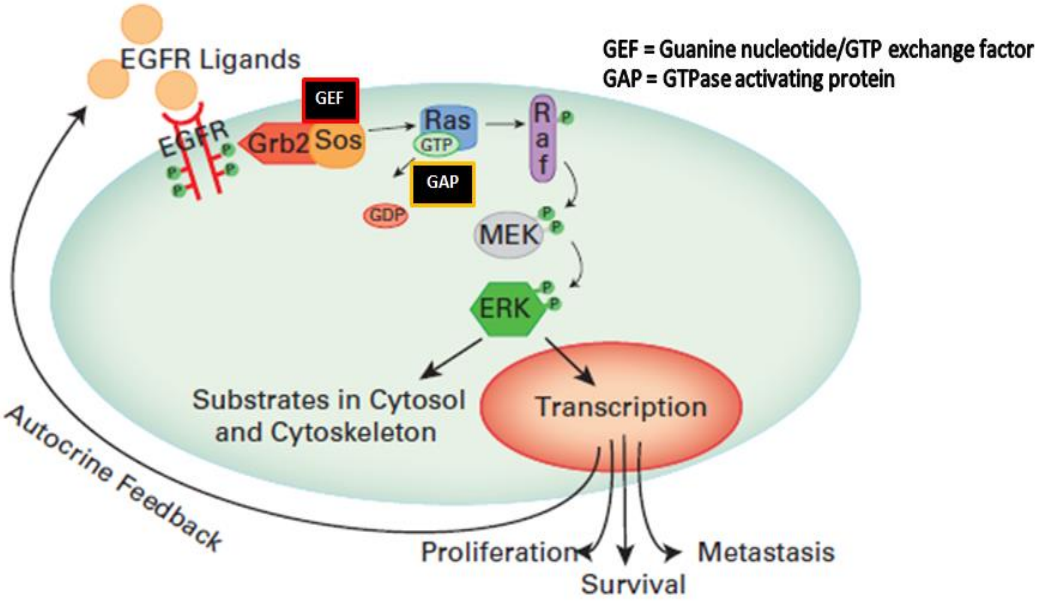

Figure 7. K-ras signaling pathway (modified from Roberts et al., 2010)

K-*ras* in Human Cancer

Genetic alterations of the *ras* genes are the most common somatic gain-of-function mutations in human malignancies. Among *ras* genes, K-*ras* is the most common mutations and has been detected in many types of tumors, approximately 30% of human cancers. It suggests that K-*ras* plays an important role in cancer development. K-*ras* mutations are more frequently found in adenocarcinomas and solid tumors, especially in tumors of pancreas, colorectal, and lung (Fernández-Medarde and Santos, 2011; Kiaris and Spandidos, 1995). They also were found in gynecologic malignancies including ovarian, endometrial, and cervical cancers (Mammas et al., 2005) (Table 5).

Several mutations have been reported in the K-*ras* gene, however the hotspot codon are codons 12 and 13 of exon 2 which participate in the GTP binding domain of the protein. The alterations in these codons cause their protein impaired to the GTPase activity. Therefore, they persist into an active state and continuously send signals for cell proliferation and differentiation leading to cancer development (Pajkos et al., 2000)

Mutations of the K-*ras* gene involve in the regulation of tumor growth, cell death, and cancer progression that provide the important fundamental basis for the development of molecular targeted therapy (Allgayer and Fulda, 2008). In addition, detection of the K-*ras* mutations is relevant for molecular diagnosis and patient prognosis (Tada et al., 1991; Huncharek et al., 1999).Currently, K-*ras* mutation analysis is used as a predictive marker for EGFR-targeted therapies in both colorectal cancer (CRC) and non-small cell lung cancer (NSCLC) patients. Because activating mutations in this gene at codons 12 and 13 have been associated with lack of response to anti-EGFR therapies. Mutant K-*ras* affected to treatment with cetuximab and panitumumab in metastasis CRC patients (Lievre et al., 2006; Amado et al., 2008) and Lonarfarnib and Tipifarnib in NSCLC patients (Sun et al., 2007).

K-*ras* Mutation in Endometrial Carcinoma

K-*ras* proto-oncogene is the most frequently activated member of the *ras* family in endometrial carcinoma (Mammas et al., 2005). Activation of the K-*ras* mutations results in abnormal proliferation and differentiation of the endometrium. The ras proteins are expressed in normal endometrium, and levels may be upregulated in some endometrial carcinomas (Scambia et al., 1993). The most frequent mutations of K-*ras* are found in codons 12 and 13 (Mizuuchi et al., 1992; Duggan et al., 1994; Semczuk et al., 1998) and less frequent mutation occurs in codons 61 (Semczuk et al., 2001).

The frequency of somatic K-*ras* mutation in endometrial carcinoma ranges between 10 and 30% (Kiaris and Spandidos, 1995; Jeyarajah et al., 1996). The majority of K-*ras* mutations are detected in endometrioid types and rarely identified in serous or clear-cell adenocarcinoma (Duggan et al., 1994; Caduff et al., 1995; Lax et al., 2000; Lagarda et al., 2001). The frequency of K-*ras* mutation in two types of endometrial carcinoma is difference suggesting that the genetic pathway that involve in the carcinogenesis is distinction.

Mutations of the K-*ras* gene have been detected by several investigators in endometrial carcinoma as shown in Table 6. The difference in the frequency of K-*ras* alterations among populations introduced to the different epidemiological and geographical characteristics. Caduff et al (1995) have identified the K-*ras* mutation at codon 12 in the United States population using amplified created restriction sites (ACRS) method. The mutation was observed in 13 cases (11.6%), consisting of 11 endometrioid carcinomas, one undifferentiated carcinoma, and one carcinosarcoma. The prevalence in this study suggested that K-*ras* mutations in carcinoma occurred in American less frequency than in Japanese (Caduff et al., 1995). Enomoto et al (1995) have shown a significant difference (p=0.02) in the prevalence of the K-*ras* gene activation between the European/American and Japanese populations. This study suggested that the existence of demographic and/or dietary factors play a role in frequency of the K-*ras* mutation.

The correlation between the presence of K-*ras* mutations and clinicopathological parameters has been reported from many studies. However, the prognostic significance of K-*ras* mutation in endometrial carcinoma is controversial. Qurratulain and Shakoori (2013) have identified the K-*ras* mutation using single stranded conformation polymorphism (SSCP) analysis. The frequency of K-*ras* mutation was seen in 34% (70 cases) of endometrioid carcinoma in Pakistani population with age less than 50 years. Well differentiated histological grade was present in 91.7% of mutant samples. Fujimoto et al (1993) have shown that ten of 45 endometrial carcinomas cases were presented K-*ras* point mutation at codon 12 (22.2%). There was no correlation between the mutation and clinical prognosis, such as clinical stage, histological type, histological grade, depth of myometrial invasion, and ascitic cytology. However, the positive rates of lymph node metastasis tended to be high in the cases with K-*ras* point mutation. This result is corresponding with the study of Ito et al (1996) who demonstrated the significantly association of K-*ras* mutations and the presence of lymph node metastasis ($p \leq 0.04$). In addition, they also evaluated the effect of K-*ras* mutation on outcome in patients with different age categories: premenopausal (<53 years), perimenopausal (54-59 years), and postmenopausal (>60 years). K-*ras* mutation has been found more aggressive clinical behavior in postmenopausal than in premenopausal patients. Alexander-Sefre et al (2003) showed molecular assessment of depth of myometrial invasion basis on K-*ras* mutation. In contrast, most studies have not found the significant association between K-*ras* mutations and clinicpathological parameters including age, histological grade, clinical stage, depth myometrial invasion, and lymph node metastasis (Sato et al., 1991; Ignar-Trowbridge et al., 1992; Mizuuchi et al., 1992; Caduff et al., 1995). Mizuuchiet al (1992) found no relationship of K-*ras* mutation and age of patients, clinical stage, and depth of myometrial invasion. This result indicated that K-*ras*activation is an independent risk factor in endometrial carcinoma.

Esteller et al (1997) examined the prevalence and clinicopathological significance of K-*ras* mutation and gene amplification in endometrial carcinoma using polymerase chain reaction associated with restriction fragment length polymorphism (PCR-RFLP) and genomic differential polymerase chain reaction. K-*ras* point mutations at codon 12 were detected in 8 of 55 (14.5%) endometrial carcinoma

specimens and none of K-*ras* gene amplification in tumor samples was detectable. No correlation was found between K-*ras* gene mutation and age, histological subtype, grade, clinical stage or current patient status. They concluded that K-*ras* mutation is a relatively common event in endometrial carcinomas with no clear prognostic value.

Somatic K-*ras* mutations have been presented in endometrial hyperplasia which is a precursor lesion to the endometrial carcinoma. Sasaki et al (1993) investigated the incidence of K-*ras* mutation in endometrial hyperplasia and tumors samples. They found the percentage of K-*ras* mutation in hyperplasia (16%) was similar to the total percentage of carcinomas (18%). It has also reported that K-*ras* mutation was detected in 2/11 (18.2%) of complex atypical hyperplasia and 7/39 (14.3%) of tumors specimens (Dobrzycka et al., 2009). These findings suggested that K-*ras* mutations may represent an early event in endometrial carcinogenesis.

Table 5. Frequency of K-*ras* mutation in human cancers (modified from Kiaris and Spandidos, 1995)

Tumor site	**Frequency range (%)**
Pancreas	80-90
Colon and rectum	30-60
Lung	27-60
Liver	12-26
Ovary	0-48
Cervix	20
Endometrium	10-40
Breast	0-12
Kidney	0-50

Table 6. Frequency of K-*ras* gene mutation in endometrial carcinoma

Author	**Country**	**No. of cases**	**Codon**	**Histologic type**	**Frequency (%)**
Semczuk et al., 1997	Poland	13	12, 13	endometrioid	2/13 (15)
Semczuk et al., 1998	Poland	57	12, 13	endometrioid	8/57 (14)
Shakoori, 2013	Pakistan	70	12, 13	endometrioid	24/70 (34)
Ito et al., 1996	Japan	221	12, 13	endometrioid	41/221 (18.6)
Mizuuchi et al., 1992	Japan	49	12, 13	endometrioid	5/49 (10.2)
				clear cell	1/49 (2)
Sato et al., 1991	Japan	21	12	endometrioid	3/21 (14.3)
Dobryzycka et al., 2009	Poland	49	12	endometrioid	7/49 (14.3)
Enomoto et al., 1995	Japan	38	12	endometrioid	4/38 (11)
Lagarda et al., 2001	Spain	58	12	endometrioid	11/58 (18.9)
Caduff et al., 1995	USA	112	12	endometrioid,	11/112 (10.2)
				undifferentiated	1/112 (0.9)
				carcinosarcoma	1/112 (0.9)

OBJECTIVES

The objectives of our study were as follows;

1. To examine the prevalence of K-*ras* mutations in Thai patients with endometrial endometrioid adenocarcinoma.

2. To investigate the correlation between the K-*ras* mutations and clinicopathogical variables of endometrial endometrioid adenocarcinoma.

3. To screen K-*ras* mutations using reverse dot blot hybridization technique.

CHAPTER 2

RESEARCH METHODOLOGY

MATERIALS

1. Samples

1.1 Patients and tissue samples

A total of 190 women who underwent hysterectomy for endometrial cancer between 2008-2012 were assembled with approved from the Hospital Ethics Committee of Faculty of Medicine, Prince of Songkla University. All patients were diagnosed as endometrioid adenocarcinoma of uterus

Inclusion criteria of selected samples, all patients are Thai. Clinical and pathological data are completely recorded. Tumor tissues are enough quantity to extract DNA in the concentration of 50-500 ng/µl

.

1.2 Clinical data

Clinical and phathological data for cohort, including age, parity, Body Mass Index (BMI), diabetes mellitus, hypertension, grade, stage, myometrial invasion, lymphovascular invasion, lymph node metastasis, uterine cervical involvement, and synchronous ovarian cancer were collected from the patient's hospital records.

1.3 Sample size calculation

Formula (Naing et al., 2006)

$$N = \frac{(Z_{\alpha/2})^2 p(1-p)}{d^2}$$

$$N = \frac{1.96^2\ (0.1)(0.9)}{0.05^2} = 138.3 = 139 \text{ cases}$$

N = Sample size

α = Level of Significant = 0.05 (95% confidence interval)

$Z_{\alpha/2}$ = Value of 95% confidence interval = 1.96

p = expected prevalence or proportion
(expected prevalence of 10%, p = 0.1)

d = Error of estimation
(if error of estimation of 5%, d = 0.05)

2. Reagents

Agarose powder
5-bromo-4-chloro-3-indolyphosphatase (BCIP)
Bigdye sequencing buffer (5X)
Bigdye terminator V 1.1 (Applied Biosystems)
Biotinylated primer
DNA marker
dNTP Mix: dATP, dCTP, dGTP, dTTP
1-ethyl-3-(3-dimethyl- aminopropyl) carbodiimide (EDAC)
70%, 95% Ethanol
Hidiformamide
PCR buffer (1X) with $MgCl_2$
Prehybridization buffer (2XSSPE/0.1%SDS)
Proteinase K (20 mg/ml)
Nitro blue tetrazolium (NBT)
Sodium acetate (NaOAc)
Sodiumbicarbonate buffer, pH 8.4
Sodiumhydroxide (NaOH)
Streptavidin-alkaline phosphatase
Detection buffer, pH 9.5 (0.1M Tris-HCL, 0.1M NaCl)
Taq DNA polymerase (Invitrogen)
Tris-acetate-EDTA (TAE) buffer
Tris buffer, pH 7.5
Tween 20

3. Scientific instruments

Auto pipette
Automate sequencer, ABI PRISM 310
Biodyne C membrane
Centrifuge
Filter paper 3M
Gel documentation
Heat block (BIOSAN)
Horizontal electrophoresis
Hot air oven
Incubator
Microcentrifuge tubes
Microtips
Nanodrop 2000 Spectrophotometer (Thermo scientific)
PCR Thermal cycler (Biorad)
Slide warmer
Spin down
Sequencing tray
Hybridization tray
Vertical electrophoresis
Vortex
Shaking water bath
96-well plate

METHODS

1. DNA extraction

Sections (5μm) from archival formalin-fixed paraffin-embedded tissue blocks were placed in 1.5 ml microcentrifuge tube and warmed at 60°C for 30 minutes. Xylene (1 ml) was added into the tube twice, each time for 5 minutes with centrifuged at 13,000 rpm. Then 1 ml of absolute ethanol was added twice, each time for 5 minutes with centrifuged at 13,000 rpm. After removing ethanol, the tube was dried at 37 ºC for 30 minutes to evaporate the remaining ethanol.

Total DNA from tumor tissues were extracted using QIAamp Tissue Kit (Qiagen, Hilden, Germany) according to the manufacturer's instructions. The tissue samples were mixed with 200 µl of buffer ATL and heated at 95ºC for 10 minutes. Subsequently, enzymatic digestion was performed by adding 25 µl of Proteinase K. The samples were incubated at 56 ºC for 2 hours. To aid with enzymatic digestion, the samples were vortexed for 4 seconds at 30-minute intervals during this incubation period. Then 200 µl of buffer AL was added and the mixture was incubated at 70 ºC for 10 minutes. After precipitation with adding 200 µl of absolute ethanol to the samples, the entire mixture was transferred to the QIAampMinElute column and centrifuged at 10,000 rpm for 1 minute. The column was placed in a new collection tube and the collection tube containing the flow-through was discarded. Five-hundred µl of buffer AW1 and AW2 was added to the column, respectively, each time for 1 minute with centrifuged at 10,000 rpm. The column was removed to a 1.5 ml microcentrifuge tube and the collection tube containing the flow-through was discarded. Finally, elution was performed by adding 20-35 µl of buffer ATE and then left at room temperature for 1 minute and centrifuged at 10,000 rpm for 1 minute. The genomic DNA was stored at -20 ºC.

2. DNA direct sequencing

2.1 PCR Amplification

PCR K-*ras* exon 2 were amplified using the primers shown in Table 7. All samples were screened for the first PCR amplification with F1/R1 primer pair. DNA samples that were negative by primers F1/R1 were re-amplified using 2 µl of the first PCR product as a template for the nested PCR. The nested PCR was performed with primers F2/R2. The quality of DNA was amplified with primer of beta globin gene. Each PCR reaction was performed in a 25 µl of total volume containing 1x PCR buffer with 2 mM $MgCl_2$, 200 µM of dNTPs, 0.2 µM of each primer, 1 unit of FastStartTaq DNA polymerase (Roche, Germany), 50-500 ng of genomic DNA, and distilled water. The PCR reaction was amplified with initial denaturation at 94°C for 4 minutes, followed by 40 cycles of denaturation 94°C for 45 seconds, annealing at 51 °C (K-*ras*), and 55 °C (beta-globin) for 45 seconds, and extension at 72 °C for 1 minute and with final extension at 72 °C for 7 minutes. The PCR products were run on a 2% agarose gel electrophoresis using 0.5 TAE buffer at 100 voltages for 30 minutes. The DNA was stained with ethidium bromide and visualized in UV light for size verification.

Table 7. The primer sequences of K-*ras* gene and beta globin gene

Gene	Primer	Nuclotide (5'-3')	Product size (bp)	Reference
K-ras	F1	GTACTGGTGGAGTATTTGAT	291	Thomas et al., 1999
	R1	ACTCATGAAAATGGTCAGAG		
	F2	CTTATGTGTGACATGTTCT	214	
	R2	AGAATGGTCCTGCACCAGTA		
Beta-globin	F	CAACTTCATCCACGTTCACC	268	Saiki et al., 1985
	R	GAAGAGCCAAGGACAGGTAC		

2.2 PCR product purification

PCR products were purified using Purelink PCR Purification kit (Invitrogen, Germany) according to the manufacturer's instructions. Briefly, binding buffer was added to the PCR products, then the entire mixture was transferred to the column and centrifuged at 10,000 rpm for 1 minute. After washing with wash buffer (AW), the mixture was centrifuged at 10,000 rpm for 1 minute and the purified PCR products were eluted in a 15 µl of elution buffer. DNA concentration was assessed at 260/280 nm using nanodrop spectrophotometer.

2.3 BigDye sequencing PCR

Purified PCR product was used as template in cycle sequencing with the BigDye Terminator v1.1 kit (Applied Biosystems, USA). The reaction was performed in a volume of 10 µl containing 1X sequencing buffer, 1µ of BigDye terminator V 1.1, 3 pmole of forward or reverse primer, distilled water, and 30 ng of purified PCR products. The PCR reactions were run according to the following protocol; one cycle of 96 ºCfor 1 minute; 25 cycles of 96ºC10 seconds, 50ºCfor 5 seconds, and 60ºCfor 4 minutes.

2.4 DNA precipitation and sequencing

The sequencing PCR product was precipitated using ethanol. Fifty µl of 95% ethanol and 2 µl of 3M sodium acetate pH 4.6 were added, the product was vortexed and left on ice for 10 minutes. Then, the mixture was centrifuged at 14,000 rpm for 15 min at 4 ºC and the supernatant was discarded. The DNA pellet was washed with 70% ethanol and centrifuged at 14,000 rpm for 10 min at 4 ºC. After removing the supernatant, the pellet was dried at 80 ºC for 2 seconds and resuspended in 10µl of Hi-Di Formamide. The sample was transferred to sequencing tray. The tray was loaded to ABI PRISM 310 sequencer (Applied Biosystems). Sequencing data was analyzed using sequencing analysis 3.7 software. Electropherogram data were evaluated.

3. Reverse dot blot hybridization

3.1 Preparation probe membrane strip

Amino modified oligonucleotides were synthesized as probes targeting 7 mutations in codon 12, 13, and 33 of the K-ras gene. An additional oligonucleotide of wild type in codon 12 and 13 was included. All probes are listed in Table 8. Biodyne C membrane (Pall life Sciences, Mexico) was activated with 16% 1-ethyl-3-(3-dimethyl- aminopropyl) carbodiimide (EDAC) for 15 minutes on a shaker at room temperature. After washing with distilled water, the membrane was dried with filter paper. Subsequently, 1 µl of each 2 pmole oligonucleotide probes diluted in 0.5M sodiumbicarbonate buffer, pH 8.4 was spotted onto a membrane strip and let the membrane dry.The membrane strip was soaked in 0.1 M NaOH for 5 minutes, rinsed in distilled water, and air dried. The strip was stored at room temperature.

3.2 PCR amplification

Genomic DNA was amplified using primers K-*ras* F: 5'GGCCTGCTG AAAATGACTGAA-3', and K-*ras* R1: 5'-biotin-TTCGTCCACAAAATGATTGTA A-3' for codons 12 and 13, and K-rasR2: 5'-biotin-GTTGGATCATATTCGTCCAC-3' for codon 33. The reaction mixture is performed in a 25 µl volume containing 1X PCR buffer-2 mM $MgCl_2$, 200 µM of dNTPs, 0.2 µM of each primer, and 1 unit of FastTaq DNA polymerase. The PCR reaction was performed following conditions: initial denaturation at 94^{o}C for 5 minutes, followed by 40 cycles of denaturation 94^{o}C for 30 seconds, annealing at 50^{o}C for 45 seconds, and extension at 72^{o}C for 50 seconds, and final extension at 72 ^{o}C for 7 minutes. The PCR products were run on a 2% agarose gel electrophoresis and stained with ethidium bromide for size verification.

3.3 Hybridization

The membrane strips were placed into individual well of the typing tray and covered with 2 ml of hybridization buffer (2X SSPE, 0.1%SDS) prewarmed to 48 °C for 15 minutes in shaker water bath. The biotinylated PCR products were denatured at 95 °C for 7 minutes, immediately placed on ice, and then added to each well. The typing tray was incubated in a shallow, shaking water bath at 48 °C for 45 minutes. Following hybridization, tray was removed from the water bath, and hybridization solution was removed by pipetting. The strips were rinsed in the tray with wash buffer (2X SSPE, 0.1% SDS) and incubated in a shaking water bath at 56°C for 20 minutes. After wash buffer was removed, 2 ml of streptavidin-alkaline phosphatase conjugated (1:1000 in TBS, 0.05%Tween 20) was added to each well. The tray was placed on a rotating platform at room temperature for 30 min with shaking at 70 rpm. Unbound conjugate was removed by wash buffer (TBS, 0.3% Tween 20) for 10 minutes. The strips were briefly rinsed in detection buffer, color development reaction was performed by incubation in a mixture of nitro blue tetrazolium (NBT) and 5-bromo-4-chloro-3-indolyphosphatase (BCIP) according to protocol. The strips were rinsed several times with distilled water. The presence of blue staining in a clear background was interpreted as positive.

4. Statistical analysis

Statistical analysis was performed using R program. The relationship between clinicopathological variables and the presence of K-*ras* mutation was determined using χ^2 (chi-square) test. Statistically significant was set at $P< 0.05$.

Table 8. Primers and probes of K-*ras* gene using reverse dot blot hybridization

Probe	Sequence 5'-3'	Reference
Primer-Ras F	5-GGCCTGCTGAAAATGACTGAA-3	Aush et al., 2009
Primer-RasR	5-biotin-TCAGAATCATTTTG TGGACGAA 3	
Probe-Ras-w	5-NH2-GGA GCT GGT GGC GTA GGC-3	
Probe-Asp 12	5-NH2-GGA GCT GAT GGC GTA GGC-3	
Probe-Val 12	5-NH2-GGA GCT GTT GGC GTA GGC-3	
Probe-Cys 12	5-NH2-GGA GCT TGT GGC GTA GGC-3	
Probe-Ser 12	5-NH2-GGA GCT AGT GGC GTA GGC-3	
Probe-Asp13	5-NH2-GGA GCT GAT GAC GTA GGC-3	
Probe-Cys 13	5-NH2-GGA GCT GAT TGC GTA GGC-3	
Probe-Gly 33	5-NH2-ACG AAT ATG GTC CAA CA-3	

CHAPTER 3

RESULTS

Mutation analysis of exon2 of the K-*ras* gene was performed on genomic DNA from primary endometrioid adenocarcinoma of 190 patients using direct sequencing and reverse dot blot hybridization. Of the 190 patients, the ages ranged from 26 to 86 years (median age = 57 years). Sixteen patients (8.4%) were age ≤ 40 years. One hundred and ten (57.9%) were age > 40- ≤ 60 years. Sixty four patients (33.7%) were in postmenopausal period (> 60 years). Sixty patients (31.6%) were nulliparous while 130 patients (68.4%) were multiparous. Of all patients investigated, the BMI ranged from 17 to 51 kg/m^2 (median BMI=27.4 kg/m^2).Seventy six patients (40%) were overweight (BMI =25-30 kg/m^2), and 46 patients (24.2%) met the criteria of obesity (BMI> 30 kg/m^2). Fifty two patients (27.4%) had diabetes mellitus and 87 patients (45.8%) had hypertension. One hundred fifty seven patients (82.6%) had grade I-II tumors while 33 (17.4%) had grade III tumor. The distribution by FIGO stage was: stage I, 138 (72.6%); II, 19(10%); III, 30 (15.8%); and IV, 3 (1.6%). One hundred seventy eight patients (93.7%) had undergone lymph node dissection or sampling, and 21 patients (11.1%) showed lymph node involvement. Synchronous ovarian cancer/ovarian metastasis was found in 9 patients (4.7%). Clinicopathological characteristics of the patients are summarized in Table 9.

Table 9. Clinical and pathological characteristics of 190 endometrioidadenocarcinoma patients

Characteristic		No. of patients (%)
Age (years)		
	≤40	16 (8.4)
	>40 - ≤60	110 (57.9)
	>60	64 (33.7)
BMI (kg/m^2)		
	<25	68 (35.8)
	≥25 - 30	76 (40)
	>30	46 (24.2)
Parity		
	Nulliparous	60 (31.6)
	Multiparous	130 (68.4)
DM		
	No	138 (72.6)
	Yes	52 (27.4)
HT		
	No	103 (54.2)
	Yes	87 (45.8)
Tumor grade		
	Grade I	98 (51.6)
	Grade II	59 (31)
	Grade III	33 (17.4)
Stage		
	I	138 (72.6)
	II	19 (10)
	III	30 (15.8)
	IV	3 (1.6)
Myometrial invasion		
	No	25 (13.1)
	<50%	94 (49.5)
	≥50%	71 (37.4)
LVSI		
	No	140 (73.7)
	Yes	50 (26.3)
Uterine cervical involvement		
	No	175 (92.1)
	Yes	15 (7.9)
Lymph node involvement		
	No	157 (82.63)
	Yes	21 (11.1)
	*none	12 (6.31)
Synchronous Ovarian cancer/ Ovarian metastasis		
	No	181 (95.3)
	Yes	9 (4.7)

*none= no lymph node

K-*ras* mutations by direct sequencing

PCR products of exon2 of K-*ras* gene analyzed with agarose gel electrophoresis showed the presence of a 214 bp and the amplification of the DNA internal control with beta-globin primers was present of a 268 bp (Figure 8). Of all cases investigated, K-*ras* mutations were detected in 37 of 190 cases (19.5%) is shown in Table 10. In particular, 33 of the 190 cases (17.4%) were amino acid substitutions (missense mutation), whereas 4 of the 190 cases (2.1%) showed silent change. All missense mutation, 57.6% (19 of 33) were found in codon 12 and 18.2% (6 of 33) in codon 13 (19 of 33 tumors presented single point mutations, and 5 of 33 presented double point mutations in codon 12 with other codons: 2, 6, 7, and 13). There were 9 cases of missense mutations detected at other sites of exon2, namely codon 3, 14, 15, 27, 29, 33, and 34. All 9 cases showed single point mutations.

The most frequent mutation in codon 12 was glycine to aspartate (GGT to GAT), which was detected in 11 of 33 cases (33.3%). The other mutations observed in codon 12 resulted in replacement of glycine with serine (GGT to AGT; 1 case, 3%), cysteine (GGT to TGT; 4 cases, 12.1%), and valine (GGT to GTT; 3 cases, 9.1%). The most frequent mutation in codon 13 was glycine to aspartate (GGC to GAC), which was present in 5 of 33 cases (15.2%). The other mutation was glycine to cysteine (GGC to TGC) in one of 33 cases (3%). The frequency of missense mutations identified in endometrioid adenocarcinoma is summarized in Table 10. Overall, the type of mutation was G:C to A:T transitions (29 of 33, 87.9%) at high frequency than G:T transversions (8 of 33, 24.2%).

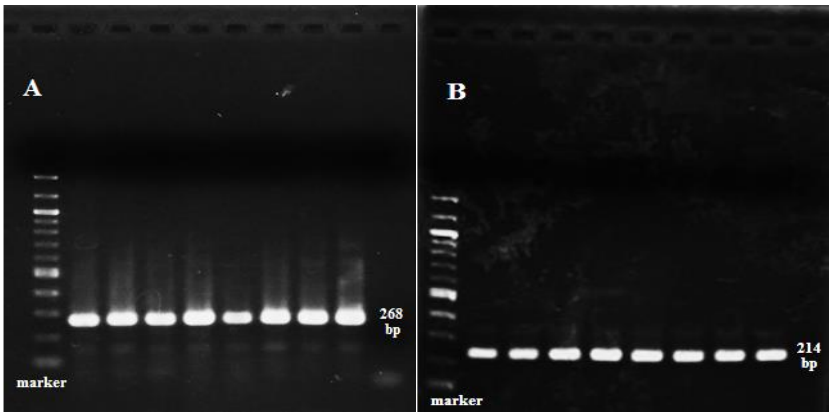

Figure 8. Representative agarose gel of PCR products; beta globin gene with size 268 bp (A), and K-*ras*gene with size 214 bp (B)

Table 10. Pattern of K-*ras* mutation status in exon 2 detected in 37 patients with endometrioid adenocarcinoma

K-*ras* Codon	Mutation type	Nucleotide alterations	Amino acid alterations	Frequency (%)
Hotspots				
12	Missense	GGT > AGT (12-1-A)	Gly>Ser	1
12	Missense	GGT > TGT (12-1-T)	Gly>Cys	2
12	Missense	GGT > GAT (12-2-A)	Gly> Asp	8
12	Missense	GGT > GTT (12-2-T)	Gly> Val	3
			Total	14 (37.9)
13	Missense	GGC > TGC (13-1-T)	Gly>Cys	1
13	Missense	GGC > GAC (13-2-A)	Gly> Asp	4
			Total	5 (13.5)
12&2	Missense	GGT > GAT (12-2-A)	Gly> Asp	1
	Missense	ACT > ATT (2-2-T)	Thr> Ile	
12&6	Missense	GGT > TGT (12-1-T)	Gly>Cys	1
	Missense	TTT > CTT (6-1-C)	Phe>Leu	
12&7	Missense	GGT > GAT (12-2-A)	Gly> Asp	1
	Missense	GTG > ATG (7-1-G)	Val > Met	
12&13	Missense	GGT > TGT (12-1-T)	Gly>Cys	1
	Missense	GGC > GAC (13-2-A)	Gly> Asp	
12&13	Missense	GGT > GAT (12-2-A)	Gly> Asp	1
	Silent	GGC > GGT (13-3-T)	Gly>Gly	
			Total	5 (13.5)
Others				
3	Missense	GGA > AAA (3-1-A)	Glu> Lys	1
3	Missense	GAA > GGA (3-2-G)	Glu>Gly	1
11	Silent	GCT > GCC (11-3-C)	Ala>Ala	1
14	Missense	GTA > ATA (14-1-A)	Val > Ile	1
15	Missense	GGC > AGC (15-1-A)	Gly>Ser	1
16	Silent	AAG > AAA (16-3-A)	Lys > Lys	1
25	Silent	CAG > CAA (25-3-A)	Gln>Gln	1
27	Missense	CAT > TAT (27-1-T)	His > Tyr	1
29	Missense	GGA > GAA (29-2-A)	Gly>Glu	1
33	Missense	GAT > GGT (33-2-G)	Asp >Gly	2
34	Missense	CCA > TCA (34-1-T)	Pro >Ser	1
19&22	Silent	TTG > TTA (19-3-A)	Leu>Leu	1
	Silent	CAG > CAA (22-3-A)	Gln>Gln	
			Total	13 (35.1)

The correlation between K-*ras* mutation and clinicopathological parameters

No significant relationship was seen between the presence of K-*ras* mutations and clinicopathological variables (age, BMI, parity, grade, stage and depth of myometrial invasion, LVSI, uterine cervical and lymph node involvement, and synchronous ovarian cancer/ovarian metastasis) analyzed as shown in Table 11. With respect of prognosis, 3 out of 33 patients with K-*ras* missense mutations died of cancer, whereas, 10 out of 153 patients without K-*ras* mutation died of cancer (data not shown).

Table 11. Correlation between clinicopathological features and K-*ras* mutation status (missense type) in 190 endometrioid adenocarcinomas

	Total (%)	Mutant K-*ras* n (%)	Wild type K-*ras* n (%)	P-value
All patients	190 (100)	33 (17.4)	157 (82.6)	
Age (years)				
Range	26-86	27-86	26-79	
Average age	56.1	56.4	56.1	
≤60	126	23 (18.3)	103 (5.1)	0.49
>60	64	10 (15.6)	54 (34.4)	
BMI (kg/m^2)				
<25	68	14 (20.6)	54 (79.4)	0.827
≥25 – 30	78	13 (16.7)	65 (83.33)	
>30	44	6 (13.6)	38 (86.4)	
Parity				
Nulliparous	60	11 (18.3)	49 (81.7)	0.596
Multiparous	130	22 (16.9)	108 (83.1)	
DM				
No	138	26 (18.8)	112 (81.2)	0.167
Yes	52	7 (13.5)	45 (86.5)	
HT				
No	103	18 (17.5)	85 (82.5)	0.61
Yes	87	15 (17.2)	71 (82.8)	
Tumor grade				
Grade I	98	19 (19.4)	79 (80.6)	0.226
Grade II	59	12 (20.3)	59 (79.7)	
Grade III	33	2 (6.1)	31 (93.9)	
Stage				
I	138	29 (21)	109 (79)	0.171
II	19	2 (10.5)	17 (89.5)	
III+IV	33	2 (6.1)	31 (80.3)	
Myometrial invasion				
No	25	4 (16)	21 (84)	0.952
<50%	94	15 (16)	79 (84)	
≥50%	71	14 (19.7)	57 (80.3)	
LVSI				
No	140	24 (17.1)	116 (82.9)	1.0
Yes	50	9 (18)	41 (82)	
Uterine cervical involvement				
No	175	31 (17.7)	144 (82.3)	0.501
Yes	15	2 (13.3)	13 (86.7)	
Lymph node involvement				
No	157	31 (19.7)	126 (80.3)	0.495
Yes	21	2 (9.5)	19 (90.5)	
*none	12	0	12 (100)	
Synchronous Ovarian cancer/Ovarian metastasis				
No	181	32 (17.7)	149 (82.3)	0.821
Yes	9	1 (11.1)	8 (88.9)	

*none = no lymph node

Reverse dot blot hybridization

Reverse dot blot hybridization was tested in 30 selected samples. Four of the 30 were interpreted as K-*ras* wild type, whereas 26 of the 30 showed K-*ras* mutations. The mutations were reported on codon 12, namely Gly12Asp (11 cases, 42.3%), Gly12Cys (4 cases, 15.4%), Gly12Ser (1 case, 3.8%), and Gly12Val (3 cases, 11.5%); on codon13, namely Gly13Asp (4 cases, 15.4%), and Gly13Cys (1 case, 3.8%); only 2 mutations (7.7%) were reported on codon33, both Asp33Gly (Figure 9).

Concordance of reverse dot blot hybridization and direct sequencing

Detecting all of K-*ras* mutations, the 2 methods were found to have the same results (Table 12, Figure 10A-D).

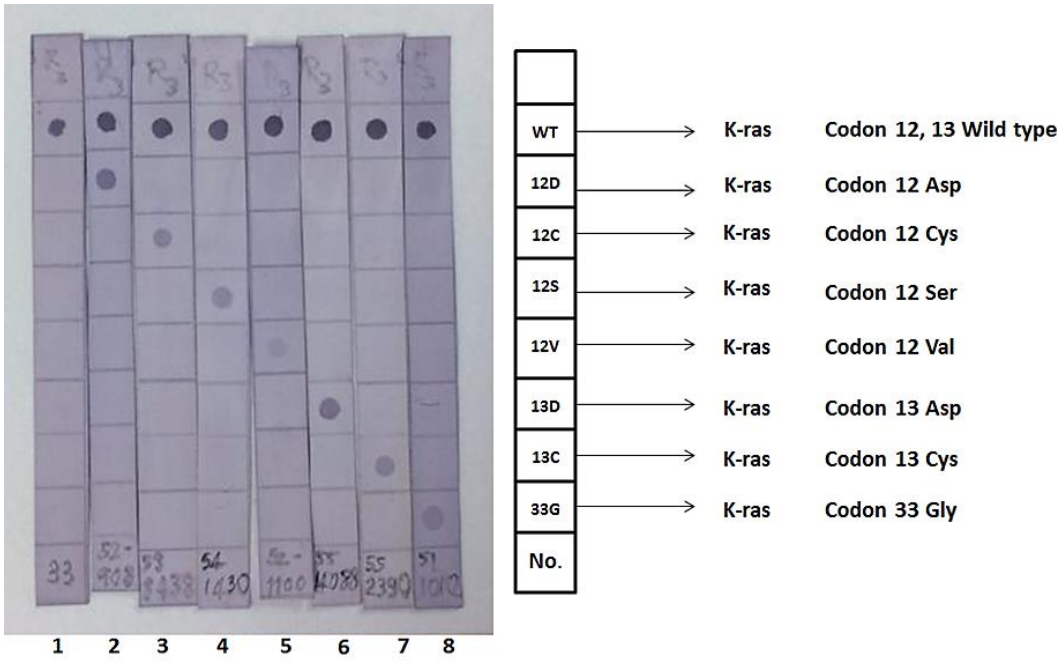

Figure 9. Representative strip of reverse dot blot hybridization analysis from tumor samples. Strip 1-8 show wild type on codons 12 and 13 of K-*ras* gene, mutation on codon 12 (Gly>Asp), mutation on codon 12 (Gly>Cys), mutation on codon 12 (Gly>Ser), mutation on codon 12 (Gly>Val), mutation on codon 13 (Gly>Cys), mutation on codon 13 (Gly>Asp), and mutation on codon 33 (Asp>Gly), respectively.

Table 12**.** Comparison between reverse dot blot hybridization and direct sequencing

No.	Reverse dot blot hybridization	Direct sequencing
1	G12D	G12D
2	G12V	G12V
3	G12V	G12V
4	G13D	G13D
5	G12D	G12D
6	G13D	G13D
7	G12D	G12D
8	G12D	G12D, G13G
9	G12D	G12D
10	G12D	G12D
11	G12C	G12C
12	G12S	G12S
13	G12C	G12C
14	G12C, G13D	G12C, G13D
15	G12D	G12D
16	G12D	G12D
17	G13D	G13D
18	G12V	G12V
19	G13C	G13C
20	G12D	G12D, V7M
21	G12D	G12D
22	G13D	G13D
23	G12D	G12D
24	G12C	G12C, F6L
25	D33G	D33G
26	D33G	D33G
27	WT	WT
28	WT	WT
29	WT	WT
30	WT	WT

WT = Wild Type

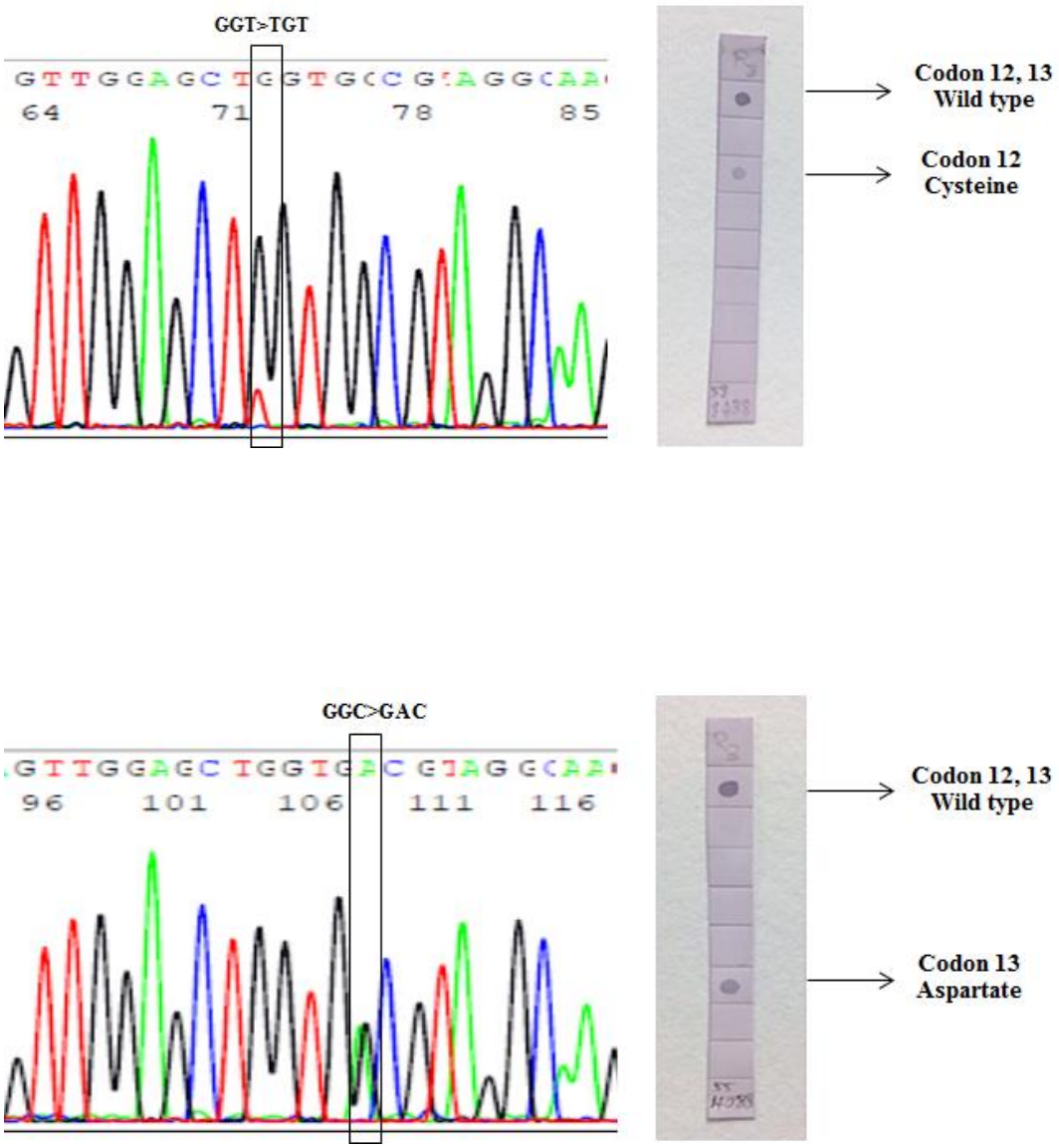

Figure 10. Concordance of direct sequencing and reverse dot blot hybridization analysis. A shows mutation at codon 12 (GGT>TGT, Gly>Cys). B shows mutation at codon 13 (GGC>GAC, Gly>Asp).

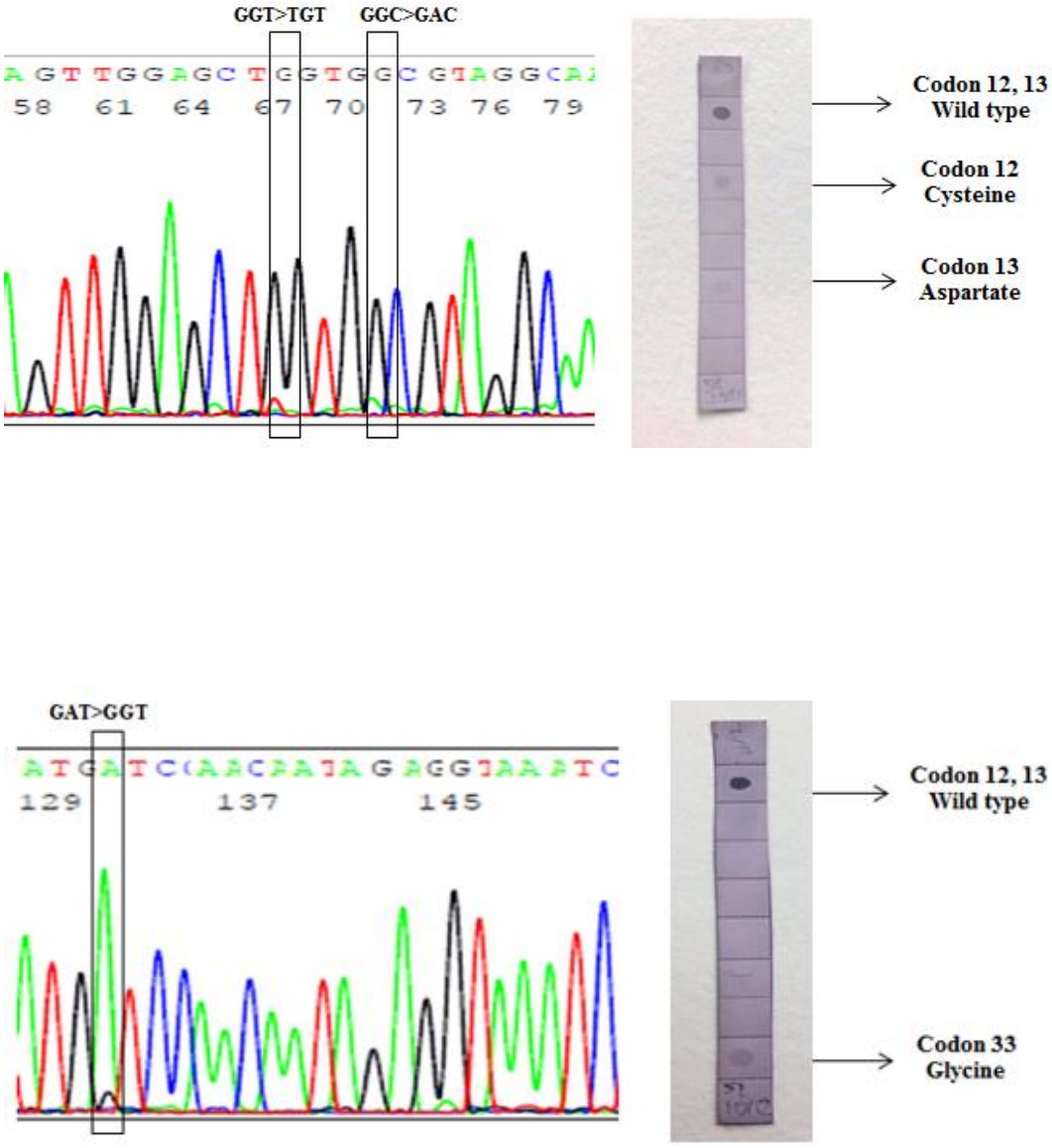

Figure 10. Concordance of direct sequencing and reverse dot blot hybridization analysis. C shows mutation at both codons 12 (GGT>TGT) and 13 (GGC>GAC). D shows mutation at codon 33 (GAT>GGT, Asp>Gly).

CHAPTER 4

DISCUSSIONS

Endometrial cancer is the third most common gynecologic cancers in Thailand. The majority of tumors are histolocially diagnosed as the endometrioid type. It has been reported that obesity was only the independent factor associated with endometrial cancer in Thai patients aged 45 years or younger (Hanprasetpong et al., 2008; Manchana and Khemapech, 2008). The molecular basis of carcinogenesis in endometrial cancer Thai patient was still little studied. Several reports have demonstrated a number of molecular alterations in endometrial cancer, such as PTEN inactivation, loss of FOXO1 expression, amplification and overexpression of HER-2, overexpression of p53, and mutation in K-*ras* (Ellis et al., 2010). The K-*ras* proto-oncogene is associated with changes increased cell proliferation and inhibition of apoptosis. Mutations of K-*ras* gene have been detected in several types of tumors including carcinomas of pancreas, colon, lung, and endometrium. Our study showed that the frequency of mutations in exon 2 of K-*ras* gene in endometrioid adenocarcinoma was 17.4% (33/190). This finding is in line with previous studies, in which the frequency ranges from 10 to 30% (Mizuuchi et al., 1992; Llobet et al., 2009; Dobrzycka et al., 2009). The most common location of point mutations occurred in K-*ras* codon 12 rather than codon 13 (Ito et al., 1996; Semczuk et al., 1998). Our results showed K-*ras* mutation in exon 2 located at codon 12 (10%), codon13 (3.2%), and other sites (4.7%). K-*ras* codons 12 and 13 coding for two adjacent glycines are the most common sites of oncogenic activation (Edkins et al., 2006). Any mutation resulting in amino acid alterations at these codons, which encodes amino acids adjacent to the GDP/GTP binding pocket, reduces or abolishes GTPase activity of K-*ras* after GAP binding and locks the protein in an active, GTP-bound state (Seeburg et al., 1984). In accordance with our data, previous studies have usually identified the incorporation of other amino acids, most commonly aspartate and valine at codon 12 and aspartate at codon 13 (Andreyev et al., 1998; Neumann et al., 2009; Byron et al., 2012; Xiong et

al., 2013). These amino acids alterations cause projection of larger amino acid side chains into the GDP/GTP binding pocket of the protein that interfere with geometry of the transition state in which GTP hydrolysis is catalysed (Malumbres and Barbacid, 2003).

Interestingly, among 19 missense mutations of K-*ras* gene located at codon12 in our study, the presence of double mutations was detected in 5 samples, recoding K-*ras* codons 12 and 2 (G12D/T2I); codons 12 and 6 (G12C/F6L); codons 12 and 7 (G12D/E7M); and codons 12 and 13 (G12C/G13D). These double mutations of K-*ras* codon 12 and codons 2, 6, and 7, respectively, have not been previously reported. Additionally, the K-*ras* mutation at codons 12 and 22 occurring in the same allele was identified in a case of colon cancer (Miyakura et al., 2002). It has been reported a double mutation of K-*ras* codon 19 (leucine to phenylalanine, L19F) and codon 20 (threonine to alanine, T20A) in a colorectal cancer (Naguib et al., 2011). This co-mutation of the K-*ras* L19F/T20A was shown to the synergistic effects on transformation in vitro.

Our present study showed the K-*ras* point mutation sites outside of codons 12 and 13. They were located at codons 3, 14, 15, 27, 29, 33, and 34. Previous studies have reported that the mutational sites were not only located on hot spot codons 12 and 13, but they were also located at codons 15, 18, 20, 27, 30, and 31 in colorectal cancerous tissues and adrenal tumors (Lin et al., 1998; Wang et al., 2003; Yen et al., 2010). The mutation of K-*ras* at codon 15 decreased the binding ability of the mutant K-*ras* protein to GTPase activation proteins. However, the exact mechanism of any K-*ras* mutation outside codon hot spots in cancerous tissues remains largely unknown.

Point mutations in K-*ras* gene are relatively found in atypical endometrial hyperplasia, suggesting they are early events in endometrial carcinogenesis (Sasaki et al., 1993; Dobrzycka et al., 2009). Additionally, the frequency of K-*ras* mutations has been reported in endometrial carcinomas with various histological types (Sasaki et al., 1993; Pappa et al., 2006; Alexander-Sefre et al., 2003; Xiong et al., 2013). The prognostic impact of K-*ras* mutational status in endometrial cancers has been inconsistent. Ito et al (1996) showed the presence of K-*ras* mutation in endometrioid cancers was significantly associated with lymph node metastases and poor survival among patients in ages above 60 years. A borderline significant correlation between presence of submicroscopic myometrial invasion and depth of myometrial invasion was

observed in endometrial cancer patients with stage I (p=0.053) (Alexander-Sefre et al., 2003). The presence of K-*ras* mutations was significantly associated with endometrial subtype, low grade and obesity, but was not correlated with metastatic lesions and clinical outcome (Birkeland et al., 2012). Our findings showed that K-*ras* mutations occurred mostly in patients with grade I- II and stage I-II, however, no significant correlation was noted. In agreement with previous studies, K-*ras* mutations were not related to any pathological, histological, or clinical parameters (Ignar-Trowbridge et al., 1992; Semczuk et al., 1998; Pappa et al., 2006).

Comparing between direct sequencing and reverse dot blot hybridization, the concordance of mutation detection was 100%. Although DNA sequencing remains the gold standard for identification of specific mutations, reverse dot blot hybridization assay described here is effective and sensitive technique for detection of mutations in codons 12, 13, and 33 of K-*ras* gene. The procedure seems to be fast and easy to follow a simple protocol using ready to use test strips and visible color detection. Moreover, this assay also has the advantage of using short DNA fragment (less than 104 bp) isolated from samples of FFPE tissues to be amplified by PCR.

In conclusion, this study provides preliminary evidence that the presence of K-*ras* mutations may be a relatively common event in endometrial carcinogenesis in subgroup of Thai patients, but their prognostic value is limited. With an alternative tool applicability comparing to direct sequencing, reverse dot blot hybridization in our study is an effective and reliable assay for screening of K-*ras* point mutations in FFPE tissues.

CHAPTER 5

CONCLUSIONS

This research provides preliminary evidence to report the prevalence of K-*ras* mutations and their correlation with the clinicopathological parameters in Thai patients with endometrial endometrioid adenocarcinoma. In addition, with alternative tool applicability, reverse dot blot hybridization technique is an effective and reliable to identify the K-*ras* point mutation in FFPE tissues.

The main findings of this study are concluded as follows;

1. The prevalence of K-*ras* mutations in Thai patients with endometrial endometrioid adenocarcinoma was 19% (37 of 190) and the mutations were predominantly found in codons 12 and 13 of the K-*ras* exon2.

2. The novel mutational sites of K-*ras* exon2 were reported in this study, namely codons 3, 14, 27, 29, 33, and codon 34.

3. K-*ras* gene mutations were associated with the development of malignancy in subgroup of Thai endometrial cancer patients but their prognostic value was limited.

4. Reverse dot blot hybridization assay appears to be effective and reliable method for detecting K-*ras* mutation in DNA isolated from FFPE tissue samples.

Prevalence and status of K-*ras* mutations might be useful for management in diagnosis, treatment, and prognosis. However, further studies need to test on other genes which may be associated with endometrial carcinogenesis. Concerning clinical screening, reverse dot blot hybridization technique could be applied in routine laboratory for identifying the K-*ras* mutations, especially in FFPE tissues.

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APPENDIX

APPENDIX A

Nucleotide sequence of K-ras gene exon 2 fragment

Description of symbols

Red alphabet = Exon

10381 attgaattttgtaaggtattttgaaataatttttcatataaaggtgagtttgtattaaaa

10441 ggtactggtggagtatttgatagtgtattaaccttatgtgtgacatgttctaatatagtc

10501 acattttcattatttttattataaggcctgctgaaaatgactgaatataaacttgtggta

10561 gttggagctggtggcgtaggcaagagtgccttgacgatacagctaattcagaatcatttt

10621 gtggacgaatatgatccaacaatagaggtaaatcttgttttaatatgcatattactggtg

10681 caggaccattctttgatacagataaaggtttctctgaccattttcatgagtacttattac

Nucleotide sequence of K-ras primer

Description of symbols

Yellow band = Outer primer for sequencing

Pink band = Inner primer for sequencing

Gray band = Primer for reverse dot blot hybridization

10381 attgaattttgtaaggtattttgaaataatttttcatataaaggtgagtttgtattaaaa

10441 ggtactggtggagtatttgatagtgtattaaccttatgtgtgacatgttctaatatagtc

10501 acattttcattatttttattataaggcctgctgaaaatgactgaatataaacttgtggta

10561 gttggagctggtggcgtaggcaagagtgccttgacgatacagctaattcagaatcatttt

10621 gtggacgaatatgatccaacaatagaggtaaatcttgttttaatatgcatattactggtg

10681 caggaccattcttgatacagataaaggtttctctgaccattttcatgagtacttattac

APPENDIX B

0.5 Sodium bicarbonate buffer, pH 8.4 (100 ml)

$NaHCO_3$	4.2	g
Distilled water	100	ml
Adjust to pH 8.4 using Na_2CO_3 solution (5.28 g in DW 1000 ml)		

20X SSPE, pH 7.4 (500 ml)

NaCl	87.65	g
$Na_2HPO_42H_2O$	13.8	g
EDTA	3.7	g
Distilled water	500	ml
Adjust to pH 7.4 using NaOH powder		

Detection buffer, pH 9.5 (500 ml)

Tris (MW 121.14 g/mol)	6.06	g
$MgCl_26H_2O$ (MW 203.3 g/mol)	0.508	g
NaCl	2.922	g
Distilled water	500	ml
Adjust to pH 9.5 using 1N HCL		
5X Tris Buffer Solution, pH 7.5 (500 ml)		
Tris (MW 121.14 g/mol)	6.06	g
NaCl	11.68	g
Distilled water	500	ml
Adjust to pH 7.5 using 1N HCL		

Prehybrihybridization buffer (40 ml)

2XSSPE	4	ml
0.1%SDS	0.4	ml
Distilled water	35.6	ml

1X Tris Buffer Solution, pH 7.5 (40 ml)

5X Tris Buffer Solution	8	ml
Distilled water	32	ml

0.3% Tween 20 (20 ml)

Tween 20	60	µl
1X Tris Buffer Solution	20	ml

0.05% Tween 20(Blocking), (20 ml)

Tween 20	10	µl
1X Tris Buffer Solution	20	ml

Streptavidin alkaline phosphatase (1:5000 in blocking), (20 ml)

Streptavidin alkaline phosphatase	4	µl
0.05% Tween 20	20	ml

NBT solution (250 µl)

NBT powder	0.125	g
70% DMF	250	µl

BCIPsolution (250 µl)

BCIP powder	0.125	g
100% DMF	250	µl